THE CELLULAR AND CLINICAL PATHOLOGY OF CLOSTRIDIUM PERFRINGENS TYPE D ENTEROTOXAEMIA

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The Cellular and Clinical Pathology of Cl. perfringens type D Enterotoxaemia.

Abstract.

Enterotoxaemia of sheep is caused by the absorption of the epsilon toxin of Cl. perfringens type D. In preliminary experiments it was found that the sex and nutritional status of mice could influence the outcome of experiments in which this toxin was used. Female mice tended to survive longer than males and food deprivation caused the accumulation of free lipid in the proximal tubules of the kidneys of control, as well as intoxicated, mice.

It appears that there may be species differences in the morphological changes which epsilon toxin induces in the intestinal mucosa. Severe inflammatory necrosis occurred in the mucosa of toxin-containing loops of rabbit intestine but was absent from similar loops of lamb intestine from cases of experimentally induced enterotoxaemia.

While the toxin itself is fairly stable *in vitro* it may deteriorate during storage unless held at low temperatures. The addition of chloroform, as a preservative for the toxin in intestinal contents, does not increase the persistence of the toxin to a significant extent.

The pattern of absorption of epsilon toxin from the intestine into the bloodstream during the course of experimental enterotoxaemia was followed by using a radioactive tracer (I_{125}Polyvinylpyrroldine). It was found that, as with the protein tracers used by other workers, the values of I_{125}PVP in studies of this nature is limited. However, it did reveal that there is a loss of high molecular weight substances from the bloodstream into the extravascular tissues during this intoxication.

Studies of the ultrastructural changes which occur in the tissues of intoxicated animals have revealed that there is a severe generalised vascular endothelial damage, both when epsilon toxin is administered intravenously and when it is absorbed from the intestine. There is no histochemical or histological evidence to suggest that epsilon toxin produces primary morphological changes in tissues, other than endothelium,

although secondary effects are detectable in other organs. These secondary changes include the development of protein-containing effusions in serous cavities, and oedema in a number of tissues.

In the brain, fluid accumulation is primarily intracellular and is confined to the protoplasmic astrocytes. This results in swelling of astrocyte processes in the grey matter so that the increase in fluid content can be detected by electron microscopy as well as quantitatively. The changes in the astrocytes form the basis for early brain lesions which are detectable by light microscopy e.g. vacuolation in the cerebellar granular layer. Associated with the intracellular fluid accumulation there is an extravasation of protein into the extracellular spaces of the brain and this is detectable in mice by using exogenous peroxidase as a tracer.

Fluid loss from the bloodstream is also prominent in the heart and the lungs. In the former tissue there is severe myocardial oedema and fluid accumulation within the cardiac muscle cells, which may explain the electrocardiographic abnormalities that develop during the course of intoxication. Although lung oedema is not a consistent feature of the disease, and may be a reflection of damage caused by high concentrations of epsilon toxin entering the pulmonary circulation, it can be extremely severe with accumulation of fluid in the alveoli and interstitium.

Although it is possible to relate these changes to the vascular damage, no possible mechanism for the action of the toxin on the endothelium has been established. The use of red cell stroma and fluorescent antibody tests has failed to provide any evidence that the toxin is bound to cell membranes either <u>in vitro</u> or <u>in vivo</u>. Warburg respirometry did not reveal any reduction in the metabolic efficiency of tissue slices that were under the influence of epsilon toxin.

The overall loss of fluid into the tissues of intoxicated animals results in a severe haemoconcentration which, because of the severity of the endothelial damage, is not associated with any increase in levels of plasma proteins or inorganic ions such as sodium, potassium or chloride.

One of the most prominent of the biochemical changes that occur in enterotoxaemia is a severe progressive hyperglycaemia which appears to result from the rapid mobilisation of hepatic glycogen. The reduction in the glycogen content of the hepatic tissue can be detected histochemically. A further finding which suggests that hepatic glycogenolysis forms the basis for the blood sugar changes is that the hyperglycaemic response was suppressed in animals in which hepatic glycogen stores had been depleted prior to the administration of epsilon toxin.

Associated with the rise in blood glucose there are also increased amounts of lactate, pyruvate and alphaketoglutarate in the blood. These changes are considered to be a reflection of increased metabolic activity due to the increased amounts of available glucose. The build-up of intermediate substances reflects normal rate-limiting steps in aerobic glycolysis rather than any direct interference with a particular biochemical pathway by the toxin.

The high levels of lactate in the blood cause a severe metabolic acidosis in intoxicated animals which may sometimes be masked by alterations in blood pH associated with deficient respiratory exchange and elevated values of blood pCO_2 caused by the pulmonary oedema.

The importance of the morphological, biochemical, haematological and physiological changes, which have been found in the present investigation, in increasing our understanding of the pathogenesis of enterotoxaemia are discussed. In addition the relevance of these findings to the broader fields of experimental pathology and to the inter-relationships which exist between the cellular and clinical pathology of disease states are described.

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LIST OF ILLUSTRATIONS

Chapter 1: INTRODUCTION

A disease syndrome, known as Pulpy Kidney Disease, was first described in New Zealand lambs in the early part of this century. The aetiology of the condition was unknown and the most notable clinical features were the sudden onset of convulsions followed rapidly by the death of the animal. Because the clinical phase of the disease was generally very short, the majority of affected lambs were found dead without evidence of prior illness. A small proportion of the affected animals survived for longer periods and exhibited signs of central nervous system derangement.

Post mortem examination of lambs which had died of 'Pulpy Kidney' generally revealed the following features:

1. The carcases were in good physical condition.
2. Excess fluid, containing fibrin clots, was present in the pericardial sac.
3. The lungs were congested and rather oedematous.
4. If the carcases were examined a short time after death had occurred the kidneys were enlarged and congested. If necropsy was deferred, until several hours after the death of the animal, the kidneys had degenerated to a soft pulpy mass.
5. Providing autolysis was not too advanced, light microscopic examination of these kidneys showed necrosis of the epithelium of the proximal convoluted tubules and interstitial haemorrhage.
6. A toxin was present in the intestinal contents of affected animals.
7. Animals that survived, but exhibited signs of neurological damage, did not show the above changes. Visual examination of their brains revealed bilaterally symmetrical haemorrhagic or malacic areas in the thalamus, mid-brain and cerebellar peduncles. The latter condition is known as Focal Symmetrical Encephalomalacia and is considered to be a subacute or chronic form of the disease.

In 1932, Australian workers confirmed that Pulpy Kidney Disease and Infectious Enterotoxaemia were identical and showed that the toxin, which

was present in the intestinal contents of animals affected with either condition, was neutralised by antisera prepared against Clostridium perfringens type D.

Although enterotoxaemia is a general term that describes any intoxication resulting from the absorption of toxins produced in the intestine, its use in Australian and New Zealand veterinary literature generally refers only to the disease caused by Cl. perfringens type D. In the present thesis the term will be restricted to refer only to the latter condition. Similarly, synonyms for the causative organism, e.g. Bacillus ovitoxicus (Bennetts), Cl. welchii type D, Bacillus agni, will not be used except when referring to the published literature.

Cl. perfringens type D appears to be part of the normal enteric flora of sheep and enterotoxaemia only occurs when environmental conditions in the intestine favour its rapid multiplication and the production of large amounts of epilon toxin. This usually occurs when excessive quantities of readily fermentable nutrient, such as starch, enter the intestine. It has also been found that other factors may help to determine whether or not the disease will occur in a given animal. For example, high concentrations of epsilon toxin must also be present in the intestine for some time before the permeability of the mucosa is increased sufficiently to allow lethal amounts of toxin to enter the bloodstream. In addition, some flocks of unvaccinated lambs may contain a number of animals which have antitoxic antibodies (antitoxins) against epsilon toxin in their sera and these antitoxins provide protection against enterotoxaemia.

Because antitoxic immunity provides good protection against the disease under field conditions, the use of vaccination as a prophylactic measure has become widespread. The research which has been necessary for the development of efficient vaccines has defined the optimum environmental conditions for the growth of Cl. perfringens type D and the production of epsilon toxin in vitro. It has also elucidated some features of the structure of the toxin itself.

Studies on the morphological damage which occurs in intoxicated animals have been largely confined to the sequence of changes in the brains

and kidneys which can be detected by light microscopy. While there is some evidence that vascular damage occurs in the tissues of intoxicated animals the ultrastructure of blood vessels of these animals does not appear to have been investigated. Careful investigation of early cellular changes of this type is necessary to extend our understanding of the pathological changes which occur in enterotoxaemia.

A considerable amount of data has been published on the effect of epsilon toxin and enterotoxaemia on the physiological, haematological and biochemical parameters of living animals but there are still some notable gaps in our knowledge of the 'clinical' pathology of the disease. Similarly, although some studies have also been made on the physiological responses of isolated organs and tissues to the action of epsilon toxin, no attempts appear to have been made to examine the metabolic efficiency of tissues from intoxicated animals.

These deficiencies in the information which is available on the various pathological aspects of enterotoxaemia are particularly unfortunate as enterotoxaemia is not only an economically important disease in its own right, but has also been implicated as a possible complicating factor in other diseases such as ruminal bloat in cattle on pasture and 'overeating' disease in stall fed animals. Both these latter conditions are serious problems where intensive forms of animal production are employed. Until such time as the exact nature of the pathological changes which occur in enterotoxaemia is known it will not be possible to ascertain what part, if any, it plays in these other disease syndromes.

Apart from these economic aspects of enterotoxaemia, an investigation of its cellular and clinical pathology is of considerable interest on a broader basis since it is a disease in which both morphological and biochemical changes are prominent. It is therefore possible that it could be useful as an experimental model for studying the relationship between morphological and functional changes in tissue and also between the cellular and clinical pathology of disease conditions. This field of research is becoming more important as the demand for more precise means for investigating animal disease problems increases.

Information obtained from a condition such as enterotoxaemia may therefore assist in increasing our understanding of the changes which occur in other diseases of man and animals.

The basic aims of the present study will therefore be to carry out an intensive investigation of the morphological changes which occur in enterotoxaemia, especially at the ultrastructural level. In parallel with this, additional information will be gathered on the biochemical and physiological status of intoxicated animals. An attempt will then be made to utilise the information obtained to increase our understanding of the pathogenesis of the disease.

Chapter 2: A REVIEW OF THE LITERATURE ON CLOSTRIDIUM PERFRINGENS TYPE D ENTEROTOXAEMIA

The salient features of the aetiology, epidemiology, pathogenesis and prophylaxis of enterotoxaemia are well covered in textbooks of veterinary medicine and veterinary pathology. Nevertheless a preliminary review of some of the important investigations which have been made in the past is of interest as this serves to emphasise the rather complex nature of the disease.

The occurrence of enterotoxaemia in New Zealand was originally described by Gilruth (1907), Bull (1924), Gill (1927 & 1929) and Hopkirk (1928). Bull and Hopkirk both reported that the intestinal contents, of animals which died of this disease, contained very large numbers of toxigenic '*Cl. welchii-type*' organisms. At this time it was also postulated by Gill and Bull that the disease might be the result of the absorption of a toxin produced by organisms, which were normal bowel inhabitants, multiplying under abnormal environmental conditions in the intestine. This hypothesis was subsequently shown to be essentially correct although the pathogenesis of enterotoxaemia was not fully elucidated until the late 1950's.

Gill (1931) demonstrated the presence of a toxin in bacteria-free filtrates of the intestinal contents from affected sheep and Bennetts (1932a) showed that this toxin was neutralised by antisera prepared against *Bacillus ovitoxicus* (Bennetts). The latter organism was reclassified as *Cl. welchii (perfringens) type D* by Wilsdon (1932). Although *Cl. perfringens type A* is widely distributed in Australian and New Zealand soils (Seddon & Edgar, 1932 and Bacon, 1953), it appears that some of the other types of this organism, including *Cl. perfringens type D*, may be intestinal commensals. Smith (1957) states that both Bennetts (1932a) and Taylor & Gordon (1940) have produced some evidence that the latter organism can only persist in soil for a limited period and Bullen (1952) has confirmed that it is present in the intestinal contents of a proportion of normal sheep.

Both Bennetts (1932a) and Oxer (1932) produced the clinical signs and gross lesions of enterotoxaemia in lambs by the intravenous adminis-

tration of bacteria-free filtrates of Cl. perfringens type D. Bennetts also succeeded in initiating the disease in lambs by the oral administration of cultures of the organism to animals which had previously been given powdered opium to reduce intestinal motility. Oxer was unable to produce the disease in this way and later workers also experienced difficulty in producing the disease by the oral or intraduodenal administration of cultures of Cl. perfringens type D. It will be seen, later in this chapter, that it was research into this problem which led to the elucidation of some important features of the pathogenesis of enterotoxaemia.

Most strains of Cl. perfringens produce a number of potent exotoxins which result in fairly specific disease syndromes in animals and man. They also provide a basis for the classification of the individual strains or types of the organism and it is therefore necessary to outline briefly the relationships of the various toxins to the different strains of Cl. perfringens before proceeding to a more detailed consideration of Cl. perfringens type D, epsilon toxin and enterotoxaemia. One of the first detailed classifications of the types of Cl. perfringens, based primarily on the toxins which they produce, was published by Oakley & Warrack (1953) although the major types of this organism had been described, prior to Wilsdon's (1932) reclassification of Bacillus ovitoxicus (Bennetts) as Cl. perfringens type D. A number of simplified versions of Oakley & Warrack's classification, which are suitable for routine use, have been published e.g. Warrack (1963).

Table 2.1, which is based on a table from Smith (1957), illustrates the interrelationship between the toxins and the classification of the types of Cl. perfringens.

Table 2:1 The Major Toxins of the Important Types of Clostridium perfringens.

Type	Major Toxins			
	alpha	beta	epsilon	iota
A	+	-	-	-
B	+	+	+	-
C	+	+	-	-
D	+	-	+	-
E	+	-	-	+

+ = present - = absent

The table shows that, apart from the alpha toxin which is produced to some extent by all of these strains, the major toxin of Cl. perfringens type D is the epsilon toxin. However it can be seen that epsilon toxin is also produced by Cl. perfringens type B. In the absence of this information some early confusion over the classification of the causative agent of enterotoxaemia had arisen since Bennetts (quoted by Oxer 1932) had shown that the toxicity of intestinal contents from cases of enterotoxaemia could be neutralised by Lamb Dysentry antiserum which had been prepared against Cl. perfringens type B, although antisera prepared against Bacillus ovitoxicus (Bennetts) (Cl. perfringens type D) would not protect animals from the effect of the toxins of Cl. perfringens type B.

Some emphasis has been placed upon the similarity between these two organisms because, although beta and epsilon toxins are both produced in, and absorbed from, the intestine the disease syndrome produced by Cl. perfringens type B is very different from that produced by Cl. perfringens type D. The most probable explanation for some of these differences provides a valuable insight into how host factors may on occasions profoundly influence the nature of the disease syndrome produced by a particular organism.

The economically important disease problems of lambs which are associated with Cl. perfringens types B and C are due to the action of the beta toxin, and occur in the first few days of life while enterotoxaemia, which results from the absorption of epsilon toxin, tends to occur in somewhat older lambs. Griner (1963) has suggested that the difference in age incidence may be associated with the different responses of the beta and epsilon toxins to the action of trypsin. He postulated that intoxications due to types B and C tend to occur in the first few days of life because the trypsin-labile beta toxin is protected by the presence, in the intestinal contents, of colostrum- derived trypsin inhibitors. At the same time, epsilon toxin, which is produced by the organism as an inert prototoxin requiring tryptic activation (Turner & Rodwell, 1943) would not be toxic under these conditions. On the other hand, in older animals epsilon toxin would be activated by trypsin while beta toxin would be destroyed.

There is a considerable amount of published information on the structure and characteristics of epsilon toxin although the data is not always consistent. Orlans et al (1960) stated that the toxin and prototoxin were immunologically, electrophoretically and chemically identical. They stated that the molecule was a globulin-type protein with a molecular weight of 38,000 and considered that toxicity was associated with steric factors in the toxin molecule, with activation of the prototoxin being due to removal of a small peptide by trypsin. Thomson (1963) considered that activation might be a stepwise process involving the removal of basic amino acids or small peptides from the prototoxin molecule. On the basis of his data the toxin molecule was a prolate elipsoid with a molecular weight of 40,500. Subsequently, Habeeb (1964) gave a considerably lower figure of 23,000 to 25,000 for the molecular weight of the toxin and has stated that its optical density characteristics are similar to those of nucleoproteins although the molecule does not appear to contain substantial amounts of either phosphorus or pentose sugars, which are normally present in nucleoproteins. Both Thomson and Habeeb gave the amino acid composition of the toxin.

From the time of Gill's (1933) comprehensive investigation of the epidemiology, pathology and control of 'Pulpy Kidney Disease' in New Zealand

and his confirmation of its relationship to Oxer's 'Infectious Enterotoxaemia' in Australia, one of the basic aims of the research into this disease has been to develop methods for protecting animals from it. Gill described attempts to provide protection by vaccination and the use of hyperimmune antisera although at that time he considered that management procedures such as yarding of the flock overnight at weekly intervals were more economical and practical methods for controlling enterotoxaemia. Since that time vaccination and the use of antisera have both come into widespread use as prophylactic measures against its occurrence and have proved to be very successful for this purpose in many countries.

Epsilon toxin is a potent antigen and the antitoxins which animals produce against it provide good protection against the disease. A great deal of information is available on the optimal conditions for the growth of Cl. perfringens type D and the production of epsilon toxin in vitro as these are important in the commercial production of vaccines against enterotoxaemia (Gilroy, 1967 and Hauschild, 1965a & b). Similarly, methods for increasing the antigenicity of vaccines have been described by Buddle (1941), Thompson & Batty (1967) and others. Considerable field experience of the efficacy of vaccines and antisera in the control of enterotoxaemia has been accumulated and reviews of this aspect of their use have been published by workers including Smith (1957) and Jansen (1967).

Probably the most important single problem that was encountered in enterotoxaemia research carried out prior to 1953 was the difficulty which was experienced in regularly reproducing the disease in normal lambs. Culture filtrates of Cl. perfringens type D which had been shown to be toxic to mice were without effect when given orally or intraduodenally to lambs and, on occasions, even intravenous administration of filtrates which were known to be toxic failed to produce intoxication in lambs.

Bullen et al (1953) and Bullen & Batty (1957a) provided a partial explanation for this phenomenon when they found that a proportion of their experimental sheep developed detectable levels of epsilon antitoxin in their sera although they had not been exposed to the toxin or the organism in experimental procedures. Later investigators have shown that the incidence of protective levels of epsilon antitoxin in animals in

unvaccinated flocks of sheep can be quite high, probably as a result of the animals absorbing sublethal amounts of epsilon toxin produced by Cl. perfringens type D normally present in the intestine. Smith (1957) in his general review of the clostridial diseases stated that 90% of an unvaccinated flock of sheep could be shown to possess detectable levels of epsilon antitoxin and Griner (1961c) subsequently showed that 32.8% of a flock of unvaccinated lambs had developed at least 0.1 units of epsilon antitoxin/ml. of serum before they reached 7 months of age. Levels of 0.1 - 0.2 units of epsilon antitoxin per ml. of serum are regarded as being sufficient to protect sheep against enterotoxaemia under field conditions (Wallace,1963,1964;Jansen,1967). The unit in these instances tended to be rather variable, depending on the standard used but was probably similar to the current international unit defined in the British Veterinary Codex (1965).

Since maternal antibodies are readily transferred to the newborn ruminant in the dam's colostrum, flocks of young lambs which are unlikely to have a high incidence of active immunity to the disease may, on occasions, have a high incidence of protective levels of epsilon toxin in their sera. For example, Cooper (pers. comm.) encountered a flock of 100 lambs from unvaccinated ewes in which the incidence of levels of epsilon antitoxin of 0.2 units/ml. or more was 47% in lambs less than 6 weeks of age.

However, the presence of circulating antitoxin was not the only factor which limited the success of attempts to produce enterotoxaemia experimentally. While non-immune sheep were susceptible to intravenously administered culture filtrates of Cl. perfringens type D or partially purified epsilon toxin, the oral or intraduodenal administration of individual doses of epsilon toxin or Cl. perfringens type D cultures seldom resulted in clinical evidence of intoxication. This was not altogether surprising since, as in other animals, the intestinal mucosa of lambs becomes practically impermeable to intact protein molecules after the first few days of life. Nevertheless the mere fact that enterotoxaemia occurs implies that under some circumstances epsilon toxin must be absorbed from the intestine. The explanation was provided by Bullen & Batty (1956 and 1957a) when they showed that high concentrations of epsilon toxin, infused continuously into the duodenum of mice or sheep, caused an increase in the

permeability of the intestinal mucosa to globulin type molecules. They found that the concentration of the toxin and the time that it was in contact with the mucosa were important factors in altering permeability. Subsequently Todorov & Trifinov (1962) showed that epsilon toxin produced damage to the intestinal mucosa and was itself absorbed into the bloodstream when the permeability of the intestine was increased. However, they also found that, unless toxin was infused continuously, the increased peristaltic activity which occurred in response to the mucosal irritation led to rapid dilution and removal of the toxin from the intestine.

These findings no doubt help to explain the success which accompanied Bennetts (1932a) administration of Cl. perfringens type D culture after intestinal motility had been reduced by opium. They also form the basis for the method used by Stoyanov & Nedyalkov (1964) for producing enterotoxaemia. These workers infused Cl. perfringens type D cultures into the duodenum of animals in which intestinal motility had been reduced as a result of reflex inhibition of peristalsis induced by ruminal distension. This had been produced by the inflation of an intraruminal balloon. Nedyalkov & Stoyanov (1962) had previously shown that reflex slowing, produced in this manner, favoured the multiplication of the anaerobic component of the intestinal microflora. It is possible that these findings may be relevant to investigations into the pathogenesis of ruminal bloat in pasture fed cattle since Mumford (1961) has suggested that the latter condition may be associated with, or complicated by, enterotoxaemia in Australia. Rumen distension may also occur in some lambs which develop enterotoxaemia since it is frequently the well fed, rather 'greedy' lambs which are affected. A degree of ruminal bloat could therefore play a part in increasing the susceptibility of the animal to the development of enterotoxaemia. It appears however that a different aspect of the availability of abundant or excessive food may be important in precipitating the disease in lambs under field conditions. Bullen and Scarisbrick (1957) found that the intraduodenal administration of cultures of Cl. perfringens type D to animals which had been subjected to a sudden change of diet, to one high in carbohydrate, usually produced enterotoxaemia in susceptible sheep, and Bullen & Batty (1957b) showed that undigested starch was present in the intestinal contents of some field cases of enterotoxaemia. It is now believed that it is the presence of readily fermentable carbo-

hydrate in the intestine which allows the rapid multiplication of Cl.perfringens type D to occur and thus provides conditions which are suitable for the continuous production of high levels of epsilon toxin.

As a result of these findings, the method which is now commonly used for the successful experimental production of enterotoxaemia involves the infusion of Cl.perfringens type D culture plus a readily fermentable carbohydrate, such as starch or dextrin, into the duodenum of lambs which have been shown to be substantially free of epsilon antitoxin (Jansen, 1963 and Niilo, 1963).

One of the first lesions which was described as occurring in the carcases of animals which died of enterotoxaemia was the softening of the kidneys which gave the condition its popular New Zealand name of 'Pulpy Kidney' disease. Bennetts (1932b) however, proved that this gross softening was not present at the time of death of the animal and that it developed some hours post-mortem. On the other hand Gill (1933) considered that necrosis of the renal proximal tubular epithelium was present at the time of death and also published photomicrographs of the renal lesions. A description of these changes can be conveniently deferred until Chapter 10 but reference to Figure 10.1 in that chapter will indicate the type of change which is seen in kidneys from many field cases of enterotoxaemia. Although similar renal lesions can be produced experimentally with alloxan (Innes, 1950) and mercury (Gritzka, & Trump, 1968), under field conditions this type of change in ruminant kidneys is regarded as being almost pathognomonic of enterotoxaemia. It is possible that the change in the physical consistency of the kidneys of intoxicated animals may be associated with alterations in the solubility of the protein components of these organs (Sotirov 1965c.).

In flocks which were experiencing outbreaks of acute enterotoxaemia Gill (1927) found that there was also a small proportion of animals which survived but exhibited clinical signs of central nervous system derangement. At necropsy, the brains of these animals were found to contain bilaterally symmetrical, focal, haemorrhagic and malacic lesions in the brain stem. No further reports of the association between this type of lesion and enterotoxaemia were published until Hartley (1956) described

the gross and microscopic appearance of the lesions of a focal symmetrical encephalomalacia (F.S.E.) of lambs. This description was followed by Griner's (1961a) experimental production of similar brain lesions in both mice and sheep by the parenteral administration of Cl. perfringens type D epsilon toxin. He employed levels of toxin which were close to the minimal lethal dose for the particular species and provided detailed descriptions of the gross and light microscopic appearance of the brain lesions. The lesions in lambs were essentially similar to those which had been found in the field cases of F.S.E. described by Hartley.

Griner described alterations in the microscopic architecture of the vasculature of the brain in intoxicated animals and in an associated paper (Griner, 1961b) he demonstrated that the permeability of the brain capillaries was increased in intoxicated animals. He suggested that the increased permeability of these vessels might lead to brain oedema and presented some evidence to support this hypothesis. Studies on the vasculature of intoxicated animals do not appear to have been extended to include tissues other than brain. This is rather surprising in view of the fact that pericardial effusions, pulmonary oedema and interstitial haemorrhage in the kidney have all been described as being features of enterotoxaemia. Further, Matthes (1965) has shown that, of all the major Cl. perfringens toxins, epsilon toxin is the most potent in altering the permeability of skin capillaries when injected intradermally into rabbits.

It appears that a variable proportion of the epsilon toxin which is absorbed from the intestine may be removed from the bloodstream by the liver since Trifinov & Todorov (1964 and 1965) have shown that, following the intravenous administration of epsilon toxin, it is removed as it passes through the liver and that there is also biochemical evidence of hepatic damage under these conditions. It should be noted however, that morphologically detectable liver damage is not a prominent feature of either natural or experimental enterotoxaemia.

Lambs suffering from experimental enterotoxaemia, or which have been given epsilon toxin parenterally, exhibit biochemical changes which require special mention as they are almost exclusively associated with enterotoxaemia in sheep. A severe progressive hyperglycaemia develops

during the course of intoxication and this may be associated with glycosuria. These changes do not occur in ruminants following the ingestion of excessive amounts of carbohydrate (Bullen & Scarisbrick, 1957), nor do they occur following the parenteral administration of Cl. perfringens alpha or beta toxins to sheep (Bullen & Batty, 1957b). Similarly, a severe haemoconcentration which follows the administration of epsilon toxin is also absent in animals intoxicated by alpha and beta toxins (Gordon, et al 1940).

Previous investigators, including Gordon et al (1940), Kellaway et al (1940) and Sotirov (1962 a & b, 1964 a & b), have also described other biochemical and physiological alterations in intoxicated animals which are of considerable interest but these changes are best considered in relation to the data which was obtained in the present investigation and are therefore discussed in the relevant chapters later in this thesis (Chapters 12-22).

Chapter 3: RATIONALE OF THE EXPERIMENTAL METHODS USED IN THE PRESENT INVESTIGATIONS INTO THE PATHOGENESIS OF ENTEROTOXAEMIA

The foregoing survey of the literature on enterotoxaemia has shown that there is no lack of references to morphological and biochemical changes in animals during this intoxication. Unfortunately most investigations have concentrated on a particular aspect of the disease and there has been little attempt to examine the pathogenesis of the disease as a whole. As a result the existing information is fragmentary and rather limited in scope. Recent developments in the instruments and techniques that are available for the investigation of many morphological and biochemical phenomena have opened up the possibility of gathering information about the changes that may occur during the course of intoxication which could not have been obtained in earlier investigations.

Materials and Methods to be Employed in Producing Intoxication

Many of the factors which are necessary for the occurrence of enterotoxaemia in the field are difficult to control under experimental conditions. While the influence of some, such as circulating antitoxin, can be excluded by appropriate experimental design, there are others, like a favourable intestinal environment, which are difficult to maintain in a state which will ensure the production and absorption of lethal quantities of epsilon toxin.

Epsilon Toxin of Cl. perfringens type D:

Although Cl. perfringens type D produces a number of toxins, enterotoxaemia results almost entirely from the action of the epsilon toxin. It was therefore decided to carry out the major part of the experimental work using the epsilon toxin alone, administered under defined conditions. This technique has been employed extensively by other workers studying enterotoxaemia and therefore comparisons could be made with the large volume of data which has already been accumulated by using this approach.

Standardisation of the epsilon toxin would be necessary and the dose that would be required to produce intoxication in experimental animals

had to be established. The preparations of toxin used were required to be reasonably stable to ensure reproducible results over a period of time. Problems associated with lack of stability of epsilon toxin have been encountered by other workers and there appeared to be some confusion over the optimal storage conditions for the toxin.

Laboratory Animals:

Mice were used in a number of the experiments because many of the previous investigations into the pathogenesis of enterotoxaemia have been made, at least in part, by studying the effect of epsilon toxin on this species. Results from the present project could therefore be compared with published information.

Although it is possible for genetic strain, age, sex and nutrition to influence the outcome of experiments in which mice are used, they do tend to be more uniform in their characteristics and responses than domestic animals. It is therefore preferable to use mice wherever possible in studies on toxic agents such as epsilon toxin and to overcome the influence of the factors mentioned above by appropriate experimental design.

Domestic Animals:

Although laboratory animals can provide useful information in experimental pathology it is unwise to assume that results obtained in one species will necessarily apply to another. For this reason most previous workers have extended their studies to the natural host. In the present work sheep were used, not only for the above reason, but also because they had distinct advantages over mice in experiments where repetitive blood samples were needed and where clinical data such as heart rate and electrocardiograms had to be recorded.

There are, however, some disadvantages associated with using sheep in studies on enterotoxaemia. For example the digestive system of the lamb develops from a basically simple stomached form at birth to the ruminant state of the adult. During this transition there is a marked change in the manner in which the animal deals with ingested carbohydrate

and alterations in blood glucose levels and ionic concentrations in the urine also occur. The more serious limitation of the use of lambs in experimental studies on enterotoxaemia is associated with the presence of variable levels of antitoxin in normal unvaccinated lambs. This can make the experimental induction of intoxication difficult or impossible. (Griner, 1961a).

While the parenteral administration of epsilon toxin to lambs is a convenient means of studying the effect of the toxin on the animal, it is very different from the natural situation where the toxin is absorbed from the intestine and passes through the liver before entering the systemic circulation. Further, there is some evidence that the rate of parenteral administration of toxin may influence the course of intoxication (Trifinov & Todorov, 1964).

Thus, before detailed studies on the pathology of enterotoxaemia in lambs could be undertaken it was necessary to determine what influence the above factors might have on experimentally induced intoxication in this species.

Information required on the Pathogenesis of Enterotoxaemia

Morphological Damage:

It is in the field of the changes induced in tissue morphology, more than any other, that the existing knowledge about the action of epsilon toxin is deficient. All the information to date has been obtained by gross visual examination and light microscopy. Even at this level the available data is fragmentary. Alterations in renal architecture and intestinal damage have been recorded and the changes which occur in the brains of intoxicated animals over a period of time have been described, but there have been no reported studies on the action of the toxin on a comprehensive range of tissues. Even the feature from which the disease takes it popular name of 'Pulpy Kidney' is poorly understood. The pulpiness of the kidneys has been conclusively shown to develop some hours after death but the proximal tubular necrosis and certain histochemical changes have been regarded as being present at the time of death. There does not appear to be any clear-cut evidence to substantiate this

belief, nor has any attempt been made to explain the pathogenesis of the haemorrhage in this organ.

No ultrastructural studies of enterotoxaemia have been made by other workers and it appeared likely that such studies would be of value. Griner (1961a,b) has produced some evidence that vascular damage occurs in the brain as a result of the action of epsilon toxin. The occurrence of pericardial effusions and pulmonary oedema suggests that vascular damage could be an important lesion in other tissues also. Because of the attenuated morphology of vascular endothelial cells these can be studied most effectively with the electron microscope. A further advantage of employing electron microscopy for studying the morphological changes induced by the toxin is that the alterations in tissue architecture which occur early in the course of intoxication can be studied. It appeared that early changes in organs such as liver, pancreas and kidneys might provide an explanation for some of the biochemical features such as hyperglycaemia and glycosuria which are so prominent in this disease.

Alteration in Tissue Function:

It was also necessary to consider the possibility that morphological damage might not be a major feature in the early stages of the disease since many toxic agents can produce severe disease or death without observable morphological change, even at the ultrastructural level. Therefore, although it is beyond the scope of the present project to determine any precise site of action of the toxin, it is necessary to decide whether the toxin can affect the functional integrity of tissues by binding to reactive sites on the cell membranes or interfering directly with metabolic processes.

Biochemical Changes:

Considerable information is available on the pattern of changes which the toxin induces in blood glucose levels and, to a lesser extent, on some of the factors which influence it. Nevertheless, there does not appear to be any published data on alterations in the levels of the intermediate compounds of glucose metabolism present in the bloodstream, nor of the effect of nutritional status or exogenous insulin on the

development of hyperglycaemia. All these factors are of prime importance in any attempt to understand the underlying pathogenesis of the blood glucose changes which occur in enterotoxaemia.

A variety of other biochemical and haematological parameters have been determined in previous investigations but, in some instances, these appear to have been gathered at random. For example, a severe haemoconcentration has been recorded and it has been shown that exogenous protein can leave the cerebral circulation, yet there is no published information on possible changes in plasma proteins during experimental intoxication. A similar situation exists in the field of physiological measurements where some information has been published on the changes which occur in the electrocardiograms of vagotomised and decerebrate animals which have been given epsilon toxin intravenously, yet the sequence of changes in the electrocardiogram of intact animals during the course of intoxication has been only briefly mentioned. The present investigation will attempt to fill some of these obvious gaps in our knowledge and will also re-examine some of the existing information on levels of components such as blood gases, plasma ions and enzymatic components in intoxicated animals in the light of modern knowledge and techniques.

Relationship Between the Experimental and the Natural Disease:

In some disease conditions a comparison between the natural and the experimentally produced disease could be extremely important but, in the case of enterotoxaemia, the experimental disease produced by the intraduodenal infusion of Cl. perfringens type D plus starch so closely resembles the natural condition that it is extremely unlikely that significant differences would be encountered between the two situations. The very short clinical course of acute enterotoxaemia in the field and the widespread vaccination against it currently carried out in New Zealand results in few field cases of this disease being seen while the animal is alive. Nevertheless it appeared to be worthwhile to examine any available field cases of the disease in the same way as the experimental ones to enable comparisons to be made between the natural and the experimental disease.

Chapter 4: CHARACTERISTICS OF THE EPSILON TOXIN USED IN THE EXPERIMENTAL PROCEDURES AND THE STABILITY OF EPSILON TOXIN IN VITRO.

Vaccines and antisera prepared against the toxins of Cl. perfringens type D are produced on a very large scale for the protection of animals against enterotoxaemia and most countries have legislative requirements regarding the quality of these products. In general the activity of the vaccines and antisera is compared directly or indirectly with reference sera in tests similar to those described in the British Veterinary Codex (1965). For convenience, most of the firms producing vaccines manufacture partially purified toxin which is periodically checked against the reference standard and is itself used as a standard in routine testing. Reasonable quantities of these toxin preparations are usually held and samples of this material were available for experimental use in the present project.

Because these materials are routinely checked by the manufacturers, exhaustive preliminary testing was unnecessary in the present investigation although it was essential to confirm the identity of individual batches of toxin and to ascertain their potency under existing experimental conditions.

The form in which preparations of epsilon toxin are held by the manufacturing companies varies. Some consider that the toxin is quite stable in solution while others prefer to retain it in a lyophilised, dessicated state *in vacuo* to avoid any spontaneous inactivation. Because the stability of the toxin *in vitro* is important, not only in the present study to ensure reproducible results, but also in the submission of samples of intestinal contents to the laboratory from suspected field cases of enterotoxaemia, a close watch was kept on the stability of the batches of epsilon toxin which were used. In addition experiments were set up to investigate the stability of epsilon toxin in intestinal contents under commonly encountered storage conditions.

All the major toxins of Cl. perfringens strains are lethal to animals when given intravenously and have a necrotising action when given intradermally, actions which are specifically neutralised by antisera

prepared against the individual toxins. These characteristics are used for identifying the toxins (Oakley, 1943), and the lethal action also forms the basis for the units used to describe the potency of toxin preparations. Tests based on both the lethal and the necrotising action of epsilon toxin were employed in the present investigation to establish the specificity of the toxin batches and to determine a suitable dose level for use in subsequent experiments.

Determination of the Lethal Levels of the Epsilon Toxin Preparations for Mice.

Materials & Methods:

Two batches of epsilon toxin were obtained. One, batch ANZD, a liquid preparation containing 0.5% phenol as a preservative was provided by Burroughs Wellcome (N.Z.) Ltd. who stated that this batch of toxin had retained its full potency of 325Lf units for two years at 4°C. The Lf unit is based on an *in vitro* flocculation test, a point which will be referred to again in the discussion section of this chapter. The other toxin preparation used, batch CWD was a freeze dried (lyophilised) preparation supplied by Glaxo Laboratories who recommended that it be stored *in vacuo* over a dessicant at room temperature. For use this batch was prepared as a concentrated solution by dissolving 5 mg. of the powdered toxin in 5 ml. of sterile bacteriological nutrient broth. Direct solution in 0.85% saline was difficult due to the tendency for the powder to form a fine particulate suspension. The concentrate was made up as required and stored at 4°C. It was not held for more than 3 days.

Intravenous injections were made, using a 1 ml. tuberculin syringe with a ½" 26swg needle, into the lateral tail veins of warmed mice held in a holder of the type described by Salisbury and Hamlin (1968).

On the basis of data supplied with the batches of toxin, a preliminary experiment was carried out in which 1:100, 1:200 and 1:400 dilutions of each toxin concentrate were made in saline and 0.2 ml. aliquots of each dilution were injected intravenously into pairs of 6 week old male mice which weighed between 21 and 27 grams. Animals receiving the first two dilutions died while those receiving the 1:400 dilution survived.

Further 1:200 dilutions of the two batches were therefore prepared and given to similar mice on a bodyweight basis. For batch ANZD 5 mice per group received toxin doses ranging from 0.020 - 0.010 Lf units/gram(g) bodyweight (B.W.), while for batch CWD 4 mice per group received doses of toxin ranging from 0.02 - 0.10 ug/g B.W.

Results:

The lethal dose of batch ANZD for mice was found to be 0.012 LF units/g B.W. while that for batch CWD was between 0.02 and 0.05 ug/g B.W. The clinical syndrome induced by the toxin was the same with both batches of toxin and will be described in Chapter 7.

The experimental results are shown in Table 4:1.

Table 4:1 The Lethal Dose of Epsilon Toxin Preparations ANZD and CWD for Mice.

Batch ANZD	Dose (Lf/gram Bodyweight)				
n= 5 per group	0.015	0.014	0.013	0.012	0.010
Mice surviving at 24 hrs. post inoculation	0	1	1	4*	4
Batch CWD	**Dose (ug/gram Bodyweight)**				
n= 4 per group	0.08		0.05		0.02
Mice surviving at 24 hrs. post inoculation	0		1		3

* All showed depression and residual signs of brain damage.

Confirmation of the Necrotising Action of the Epsilon Toxin Preparations on Guinea Pig Skin

Materials & Methods:

Serial ten fold dilutions of both batches of toxin were prepared. The skin of the lateral abdominal wall of two adult male guinea pigs was depilated by clipping with hair clippers, then rubbing on a paste consisting

of 1 part Barium Sulphide and 1 part powder detergent ('Surf') in glycerol. After the paste had been in contact with the skin for 10 minutes the animal was washed in warm soapy water, rinsed and dried. It was left overnight and on the following morning, 0.1 ml. of each dilution of toxin was injected intradermally, into the lateral abdominal wall avoiding the areas 2cm on either side of the dorsal and ventral midlines as recommended by Craig & Miles (1961). The animals were left for 12 hours then 1.2 ml. of a 2.5% solution of Evans Blue in saline was injected intravenously to 'blue' the animal and thus outline the areas of necrosis and increased capillary permeability more clearly.

Results:

The minimum necrotising dose of batch ANZD was 0.325 Lf units and that of batch CWD was 1 ug.

The type of response obtained is shown in the top row of lesions in Figure 4.1.

Neutralisation of the Toxicity of the Epsilon Toxin Preparations with Type Specific Antisera.

i Lethal Action

Materials & Methods:

To confirm that the toxicity of both batches of toxin was neutralised by homologous antiserum the following tests were carried out.

Serial tenfold dilutions of 'Wellcome' brand Cl. perfringens type D diagnostic antiserum, (Burroughs Wellcome Ltd.) ranging from 1:10 to 1:10,000, were prepared in 0.85% saline. To 1 ml. aliquots of each dilution of the antiserum, 1 ml. of each toxin batch, diluted to contain 20 ug/ml. or 6.5 Lf units/ml,, was added. The toxin/antitoxin mixtures were held at room temperature for 30 minutes then 0.2 ml. of each dilution was given intravenously to each of a pair of 6 week old male mice. To ensure that no other Cl. perfringens toxins were present at detectable levels, the same quantities of each diluted toxin batch were mixed with an equal volume of undiluted commercial Cl. perfringens types A,B,C, & E

Figure 4.1: The Necrotising Action of Cl. perfringens type D Epsilon Toxin and its Neutralisation by Type-specific Antitoxin.

This photograph is of the lateral abdominal wall of a 'blued' guinea pig into which a series of toxin dilutions and toxin/antitoxin dilutions, similar to those employed in the titration experiments, were injected intradermally for illustrative purposes. Black spots of alcoholic nigrosin have been placed on the skin to show the points of inoculation.

Top Row - Left to Right: Lesions caused by 100; 10; 1; 0.1; and 0.01ug of batch CWD epsilon toxin.

Bottom Row - Left to Right: Lesions caused by 5ug of batch CWD epsilon toxin after 30 minutes incubation with Cl. perfringens type D diagnostic anitserum diluted 1:2; 1:20; 1:200; 1:2,000; and 1:20,000.

antisera and treated in the same way as described for the homologous serum.

Results:

Two mouse lethal doses of batch ANZD were neutralised by homologous antiserum diluted 1:2,000 but not when diluted 1:20,000, while two mouse lethal doses of batch CWD were neutralised by homologous antiserum diluted 1:20,000. There was no detectable neutralisation of the toxicity of either batch of toxin by Cl. perfringens types A,C & E antisera which contain predominantly alpha, beta and iota antitoxins respectively. Neutralisation occurred with Cl. perfringens type B antiserum because this contains epsilon as well as beta antitoxin.

These results are summarised in Table 4:2.

Table 4:2 Neutralisation of the Lethal Action of the Epsilon Toxin Preparations ANZD and CWD with Clostridial Type-Specific Antisera.

Antiserum type		A	B	C	E	D	D	D	D	D
Major antitoxins		α	$\beta\varepsilon$	β	ι	ε	ε	ε	ε	ε
Final dilution of antiserum	1:	2	2	2	2	2	20	200	2,000	20,000
Mice surviving at 24 hours post inoculation	Batch ANZD	0	2	0	0	2	2	2	2	2
Mice surviving at 24 hours post inoculation	Batch CWD	0	2	0	0	2	2	2	2	2

n= 2 mice per dilution.

Absence of Detectable Alpha Toxin in the Epsilon Toxin Preparations.

Materials & Methods:

A sample of 5 ml. of heparinised sheep blood was obtained from a sheep with less than 0.08 units of epsilon antitoxin/ml. of serum. The blood was centrifuged to separate the erythrocytes. The plasma was discarded and the erythrocytes washed three times with saline. A 2% sus-

pension of these cells in Krebs-Ringer-Phosphate solution was prepared. An equal volume of the cell suspension was added to 1 ml. of each of the epsilon toxin concentrates. The resulting suspension was held at room temperature for 30 minutes then centrifuged to deposit the erythrocytes and the supernatant examined visually for the presence of haemolysis.

Results:

No visible haemolysis occurred with batch CWD toxin and a very slight haemolysis was detectable in the batch ANZD supernatant.

Neutralisation of the Toxicity of the Epsilon Toxin Preparations with Type Specific Antiserum.

ii Necrotising Action

Materials & Methods:

Two sets of serial tenfold dilutions of commercial Cl. perfringens type D antiserum in saline ranging from 1:10 to 1:10,000, were prepared . Aliquots of 0.5 ml. of toxin solutions containing either 50 ug of batch CWD or 16 Lf units of batch ANZD epsilon toxin were added to 0.5 ml. of each dilution of antiserum. After standing at room temperature for 30 minutes, 0.1 ml. of each mixture was injected into the depilated abdominal wall of a guinea pig, as described previously.

Results:

Five necrotising doses of both toxin batches were neutralised by the homologous antiserum diluted 1:200. The type of pattern obtained is shown in the lower row of lesions in Figure 4.1.

The Stability of the Samples of Epsilon Toxin used in Experimental Procedures.

Materials & Methods:

Both batches of epsilon toxin were stored according to the manufacturers recommendations i.e. batch ANZD at $4^{o}C$ in a domestic type refrigerator and batch CWD in an evacuated dessicator over anhydrous calcium chloride at room temperature. Since both batches were in constant

use at levels of the order of 1 mouse lethal dose, based on the original titrations, any loss of toxicity would have been rapidly detected.

Results:

Batch CWD toxin retained its original potency throughout the entire course of the experimental work. Batch ANZD on the other hand proved satisfactory for 5 months but thereafter there was a marked drop in potency and the material was discarded.

The Stability of Epsilon Toxin in Intestinal Contents Under Different in vitro Storage Conditions

Because delays and unfavourable storage conditions may be encountered in the transmission of specimens from field cases of enterotoxaemia to the laboratory, 0.5% chloroform is frequently added to samples of intestinal contents as a preservative, and it was desirable to determine whether the toxin is unstable in intestinal contents under normal environmental conditions and also whether chloroform assists in preserving toxicity.

Materials & Methods:

Two milligrams of batch CWD epsilon toxin was added to 100 ml. of intestinal contents from a normal sheep and the contents divided into three aliquots. One was held at $4^{\circ}C$, one at room temperature (N.Z. summer) and the third at room temperature with 0.5% chloroform added. In addition a sample of intestinal contents containing 1,000 mouse lethal doses of toxin/ml. which had been obtained from an experimental case of enterotoxaemia, was divided into three aliquots which were stored under the conditions described above.

Periodically both sets of intestinal content samples were checked for toxicity by centrifuging 1 ml. aliquots at 1800G for 20 minutes and injecting 0.2 ml. of the supernatant intravenously into each of two mice. At intervals the specificity of the toxicity of the samples was also confirmed by setting up neutralisation tests in which 0.8 ml. of the centrifuged supernatant from each sample was mixed with 0.2 ml of commercial Cl. perfringens type D antiserum, stood at room temperature for 30

minutes, then 0.3 ml. of the mixture injected intravenously into each of a pair of mice.

Results:

In the experiments in which batch CWD epsilon toxin was added to intestinal contents the sample held at 4°C was toxic at 18 weeks but had lost its toxicity at 21 weeks. In contrast the sample held at room temperature, without chloroform, while toxic at 3 weeks had lost its toxicity at 5 weeks. When chloroform was added, toxicity was detected at 6 weeks but not at 9 weeks.

A similar situation occurred in the samples from the case of experimental enterotoxaemia. In this instance the sample held at 4°C retained its toxicity for at least 48 weeks while the room temperature samples with and without chloroform retained their toxicity for 12 weeks but had both lost it when retested at 15 weeks.

There was no evidence of the development of non-specific toxicity in any of the samples.

The results of these experiments are illustrated in Figure 4.2a & b.

Discussion

It appears from the foregoing results that both batches of toxin behave in a similar manner in in vitro tests. The approximate LD_{50} of batch CWD at 0.02 - 0.05 ug/g B.W., i.e. 0.5 - 1.0 ug/25g mouse, is of the same order as that found by Griner (1961a) for one of his batches of toxin where the figure was 0.8 - 1.0 ug/mouse.

Despite the fact that the lethal action of the toxin to mice is widely used for potency testing, some workers consider that the severity and appearance of the lesions caused by the necrotising action of the major Cl. perfringens toxins are more useful criteria for detection and

Figure 4.2a: The Persistence of Epsilon Toxin in Intestinal Contents under Different Storage Conditions

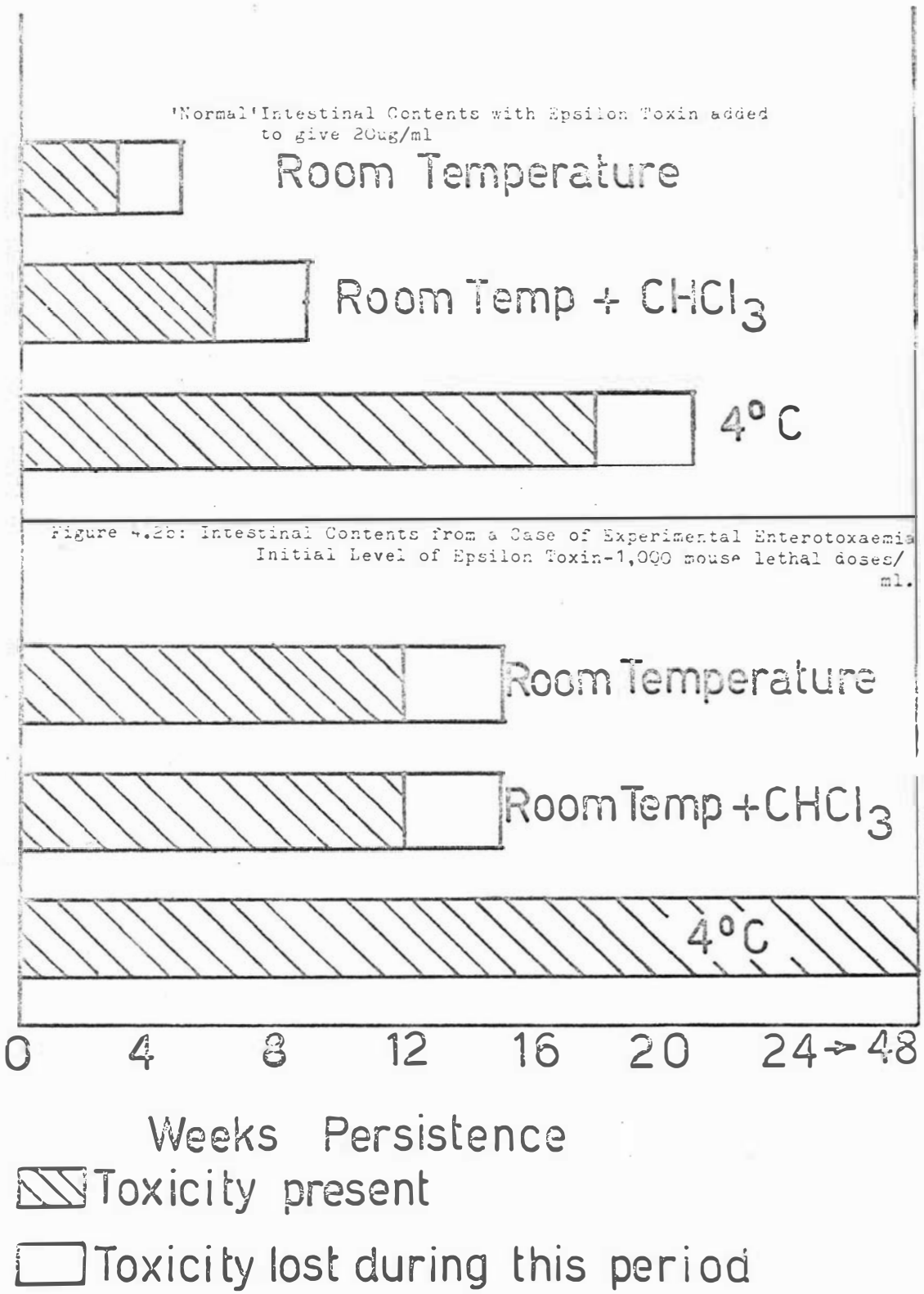


Figure 4.2b: Intestinal Contents from a Case of Experimental Enterotoxaemia Initial Level of Epsilon Toxin-1,000 mouse lethal doses/ml.

identification. Warrack (1963) has recommended that the typing of strains be based on the appearance of the lesions produced in guinea pig skin. Matthes (1965) has described the lesions produced in rabbit skin by these toxins but Oakley (1968) considers that the guinea pig is superior to the rabbit for demonstrating the necrotising action of the Cl. perfringens toxins. He has published coloured photographs of the skin lesions produced by the various toxins in the former species and those produced by epsilon toxin are similar to the lesions produced in the present study.

It should be mentioned in respect to the skin tests in guinea pigs that other workers have used Pontamine Sky Blue 6XB to 'blue' their animals (Elder & Miles, 1957; Craig & Miles, 1961), but at the level of 1.2 ml. of a 5% solution/kg B.W. recommended by these workers the batches of this dye available here proved to be toxic to guinea pigs, causing severe depression and death.

One feature which must be borne in mind throughout the present investigation is that the so-called epsilon toxin used in these investigations is not a highly purified substance and could contain other toxic substances produced by the organism. However, in commercial laboratories Cl. perfringens type D is usually grown under conditions which will produce maximal quantities of epsilon toxin and these conditions tend to be less favourable for the production of some of the other toxic products. Similarly, since the commercial antisera are prepared against the whole gamut of antigenic substances passed into the culture medium by the particular strain of the organism, their use in neutralisation tests only determines whether the bulk of toxicity is due to a particular major toxin. In actual fact these considerations are of rather academic interest in the present study which is concerned with the pathogenesis of enterotoxaemia rather than the specific actions of pure epsilon toxin. Nevertheless, although the organism will produce a variety of toxic substances in the intestine, the pattern of their absorption may be very different from that of epsilon toxin. The use of impure preparations parenterally could therefore produce non-specific effects such as those described by Kellaway (1940) and Bullen & Batty (1956).

One of the most important contaminants which could have been

present in the present batches of toxin would have been alpha toxin. This toxin is produced by Cl. perfringens type D, but since it is a potent lecithinase which causes lysis of erythrocytes, the virtual absence of haemolysis when the batches of toxin were mixed with sheep erythrocytes suggests that alpha toxin was absent in batch CWD and only present in trace amounts in batch ANZD.

Some of the results obtained in the experiments on the stability of the toxin in vitro were very interesting and have considerable relevance to existing laboratory procedures for the diagnosis of enterotoxaemia and the testing of commercial vaccines and antisera. Firstly the loss of toxicity in the sample of liquid toxin, which was stated to have retained its potency for two years prior to the present study, is surprising. While this could have been due to storage conditions such an explanation appears to be unlikely as it was stored at 4°C at all times. However, the potency of the preparation was given in Lf units which, as mentioned earlier, are used in reporting results of in vitro flocculation tests. These tests will detect both active and inactive forms of the toxin, i.e. prototoxin, toxin and toxoid and are therefore not directly related to the toxicity of the preparation. It would therefore be quite possible if, for example, spontaneous toxoiding occurred in the preparation, for the material to retain its potency in the flocculation test and at the same time progressively lose its toxicity. This type of phenomenon could be of considerable importance in potency testing of vaccines and antisera if appropriate precautions were neglected.

When considering the results of the studies on the persistence of toxin in intestinal contents it is interesting to look at the history behind currently adopted practices such as the addition of chloroform to intestinal contents as a preservative. In the early work of Oxer (1932), chloroform was added to intestinal contents to ensure that there was no increase in toxicity of the sample in vitro due to continued multiplication of the causative organism. He also showed that there was a loss of toxicity in samples of intestinal contents held at room temperature whereas toxicity was retained for considerable periods in samples held at 4°C.

Jansen (1960) (in Niilo, 1965) found that the contents of an unopened loop of intestine held in glycerol overnight lost its toxicity, but that intestinal contents removed from a loop of intestine and treated with 0.5% chloroform retained their toxicity for a month. They do not mention having held untreated intestinal contents and, since glycerol can cause toxoiding of some materials, the rapid loss of potency of the sample held in this material is not surprising. Chloroform itself may be toxic to laboratory animals and could therefore interfere with biological tests for enterotoxaemia (Bennetts, 1961).

Since the time of Oxer's work it has been the practice of laboratories dealing with samples of intestinal contents from suspected cases of enterotoxaemia to recommend the addition of chloroform to the samples as a preservative and it is only recently that this recommendation has been questioned. Niilo (1965) found that cooling was the most important factor in retaining epsilon toxin in an active state in intestinal contents and was unable to show any beneficial effect from the addition of chloroform although he suggested that it reduced bacterial multiplication.

The results of the present experiments emphasise the importance of cooling for maintaining toxicity. They also suggest that since epsilon toxin will persist in untreated intestinal contents, even in the absence of cooling, for a much longer period than would be necessary to transport samples to a laboratory, the addition of a preservative is unnecessary. Further, since chloroform is of no practical value for this purpose, and may interfere with other examinations which may be required, its continued use is undesirable.

Conclusions

1. The approximate LD_{50} for mice of batch ANZD epsilon toxin was 0.325 Lf units and that of batch CWD epsilon toxin was 0.5 - 1.0 ug. The latter figure is comparable to values obtained by other workers using partially purified epsilon toxin.

2. The minimum necrotising dose of batch ANZD for guinea pig skin was 0.325 Lf units and of batch CWD was 1 ug. The lesions produced resemble those described by other workers as being typical of the effect of Cl. perfringens type D epsilon toxin.

3. Some batches of Pontamine Sky Blue can be toxic to guinea pigs when administered intravenously at the dose rate recommended by other workers and Evan's Blue is preferable for 'blueing' this species.

4. The lethal and the necrotising actions of both preparations of epsilon toxin were neutralised by commercial Cl. perfringens type-specific antisera which contain epsilon antitoxin but not by those antisera in which this component was absent.

5. No detectable haemolysis of sheep erythrocytes was produced by batch CWD epsilon toxin and only minimal amounts by batch ANZD epsilon toxin. This suggests that these samples contain little or no contaminating alpha toxin.

6. Loss of potency can occur in liquid preparations of epsilon toxin held at $4^{\circ}C$ but there was no detectable loss of potency in samples of dessicated lyophilised epsilon toxin.

7. Low temperature appears to be one of the most important factors for maintaining the toxicity of epsilon toxin in intestinal contents during storage.

8. Non-specific toxicity did not develop in intestinal contents stored at room temperature or at $4^{\circ}C$.

9. Chloroform is not an effective preservative for intestinal contents suspected to contain epsilon toxin.

Chapter 5: FACTORS AFFECTING THE ACTION OF EPSILON TOXIN ON LABORATORY ANIMALS

Considerable information is available to show that although laboratory animals are very useful for experimental purposes, the outcome of experiments in which they are used can be markedly influenced by host factors unless care is exercised. References to the effect of such factors as age, sex, nutritional and genetic status on the results of a variety of investigations can be found in textbooks such as Green's "Biology of the Laboratory Mouse' and Cotchin and Roe's "Pathology of Laboratory Rats and Mice".

While it is usually possible to eliminate some of the host variables from experiments it is seldom possible to remove them all. In these circumstances it is generally advisable to ascertain the influence which the irremediable factors may have and to be aware that unexpected effects may be encountered.

Although some workers recommend that inbred strains of laboratory animals should be used in all experimental procedures to ensure uniformity of response, it was not considered necessary in the present instance where adequate control animals could be employed, nor were specific pathogen free animals required for an investigation of this nature. It was possible to obtain animals of similar age and genetic status but, because of the number of animals involved, both sexes of mice were used.

Because sex influences not only morphological factors such as brain: bodyweight ratio and the distribution of alkaline phosphatase in the kidney, but also the animal's susceptibility to some toxic agents such as chloroform, it was essential to determine what effect this factor would have on the outcome of experiments. If such an influence were shown to occur it would be desirable to know whether this was the result of the hormonal status of the animal since sex hormones have been shown to influence a number of metabolic processes. In this way

information on the possible mode of action of epsilon toxin might be obtained.

Similarly, although animals could be maintained under reasonably constant environmental conditions and adequate nutrition could be ensured, there was no guarantee that an intoxicated animal which survived for any length of time would continue to eat. Under these circumstances, changes unrelated to the direct action of the toxin could be encountered in a small animal in which the metabolic rate is high. Apart from these general nutritional considerations it was realised that the lesions which have been described in epsilon toxin intoxication resemble those occuring in certain deficiency states or conditions in which vitamins or cofactors have been shown to have an ameliorative effect. It therefore appeared to be desirable to determine whether some of these factors could affect the outcome of any of the present investigations.

It was with these factors in mind that the series of experiments described in this chapter was planned and the experimental mice selected.

General Description of the Mice Used in Experimental Work

The mice used in all the experiments reported in the present investigation were a non-inbred strain of conventional laboratory white mice obtained from the Small Animal Production Unit of Massey University. The colony has been maintained for several years and was originally started from stock from a number of different laboratory animal colonies within New Zealand. All animals were housed under identical conditions and given the same pelletted ration and water *ad lib*. Animals were used at between 6 and 12 weeks of age and the ages of the animals used in any experiment where this may be relevant are recorded.

The Effect of Toxin Dose on the Survival Time of Intoxicated Mice

Materials & Methods

The data recorded here was obtained in the course of establishing an approximate LD_{50} for the toxin batches and from other experiments where the survival times of the animals and the dose of toxin used were both recorded. In all instances the toxin was administered intravenously in a volume of 0.2ml over a period of 10-15 seconds.

Results

A fairly wide range of survival times was encountered in animals given approximately one lethal dose of toxin. Many animals died approximately 3 hours after inoculation while a few survived for 24 hours or more. These latter animals often showed signs of central nervous system damage. The pattern of survival time was not markedly affected by increasing the inoculum to approximately two lethal doses although the number of survivors was reduced. A summary of the survival time data derived from these experiments is shown in Figure 5.1. Above 2 lethal doses the survival time was reduced considerably and with very high doses, e.g. 1,000 mouse lethal doses, death was practically instantaneous.

The Influence of Sex on Survival Time in Intoxicated Mice

Materials & Methods

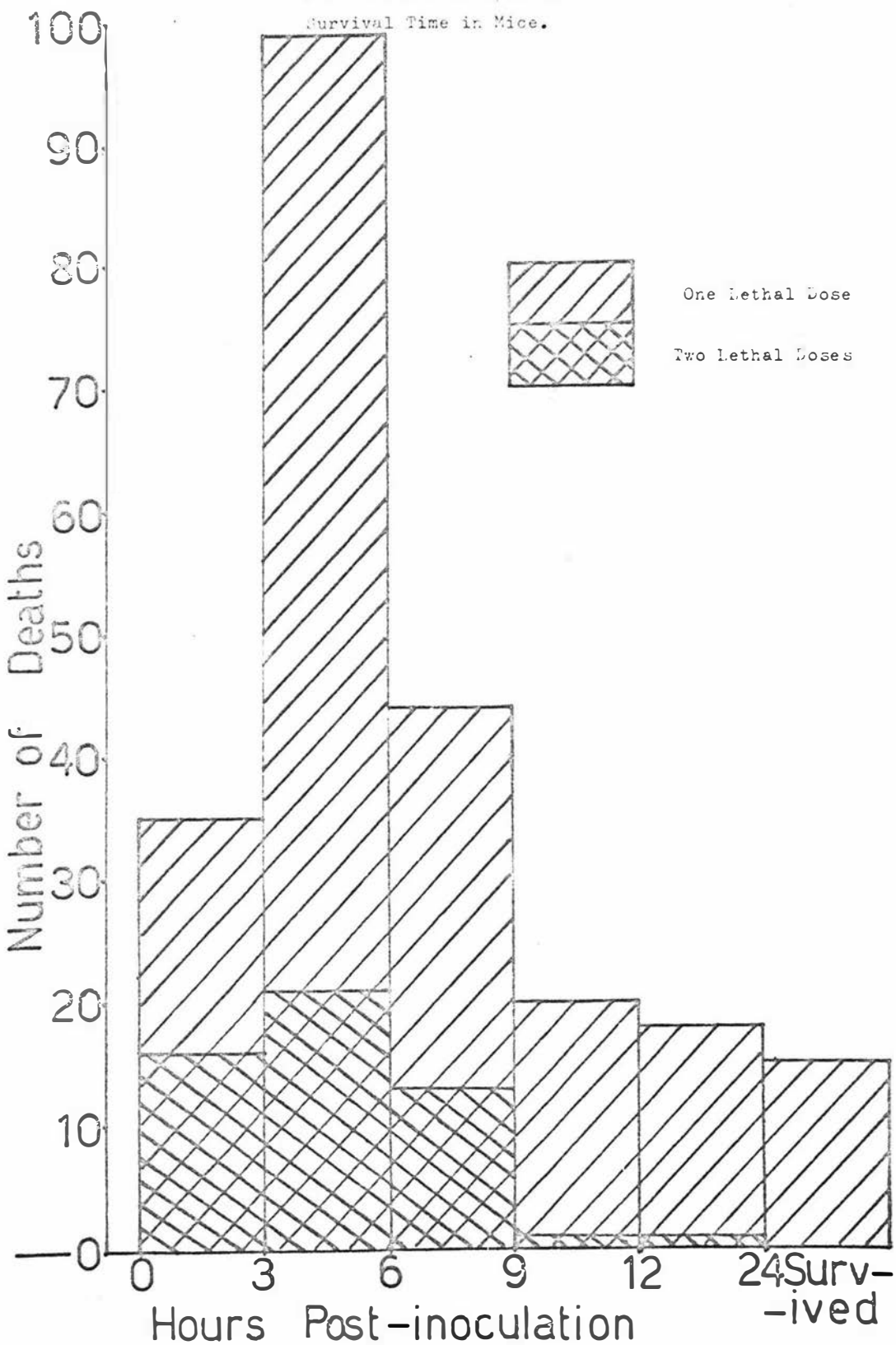
Forty male and forty female 8 eight week old mice were weighed and batch CWD epsilon toxin administered at a dose of 0.04ug/g bodyweight intravenously.

Results

Two of the female mice showed evidence of intercurrent infection at necropsy and were removed from the experiment.

The pattern of survival in the two sexes was different.

Figure 5.1: The Influence of the Administration of One or Two Lethal Doses of Epsilon Toxin on the Pattern of Survival Time in Mice.



Deaths occurred fairly regularly over the period from 2 hours until 12 hours post-inoculation in the females while, in the males there was a preponderance of early deaths. This resulted in the mean survival time of the male animals being reduced. Mean survival time for the male mice was 5.13 ± 2.84 hours and for the females 7.08 ± 2.77 hours. The difference between these two values is highly significant (t test: $p < .005$). The data from these experiments are recorded in Appendix 2, and the different pattern of survival in the two sexes is illustrated by the histograms in Figure 5.2.

The Possible Hormonal Basis for the Sex Differences In the Survival Time Pattern

Because a sex difference was encountered in the experiments just described, a further experiment was carried out to examine the influence of sex hormones on the patterns of survival time.

Materials & Methods

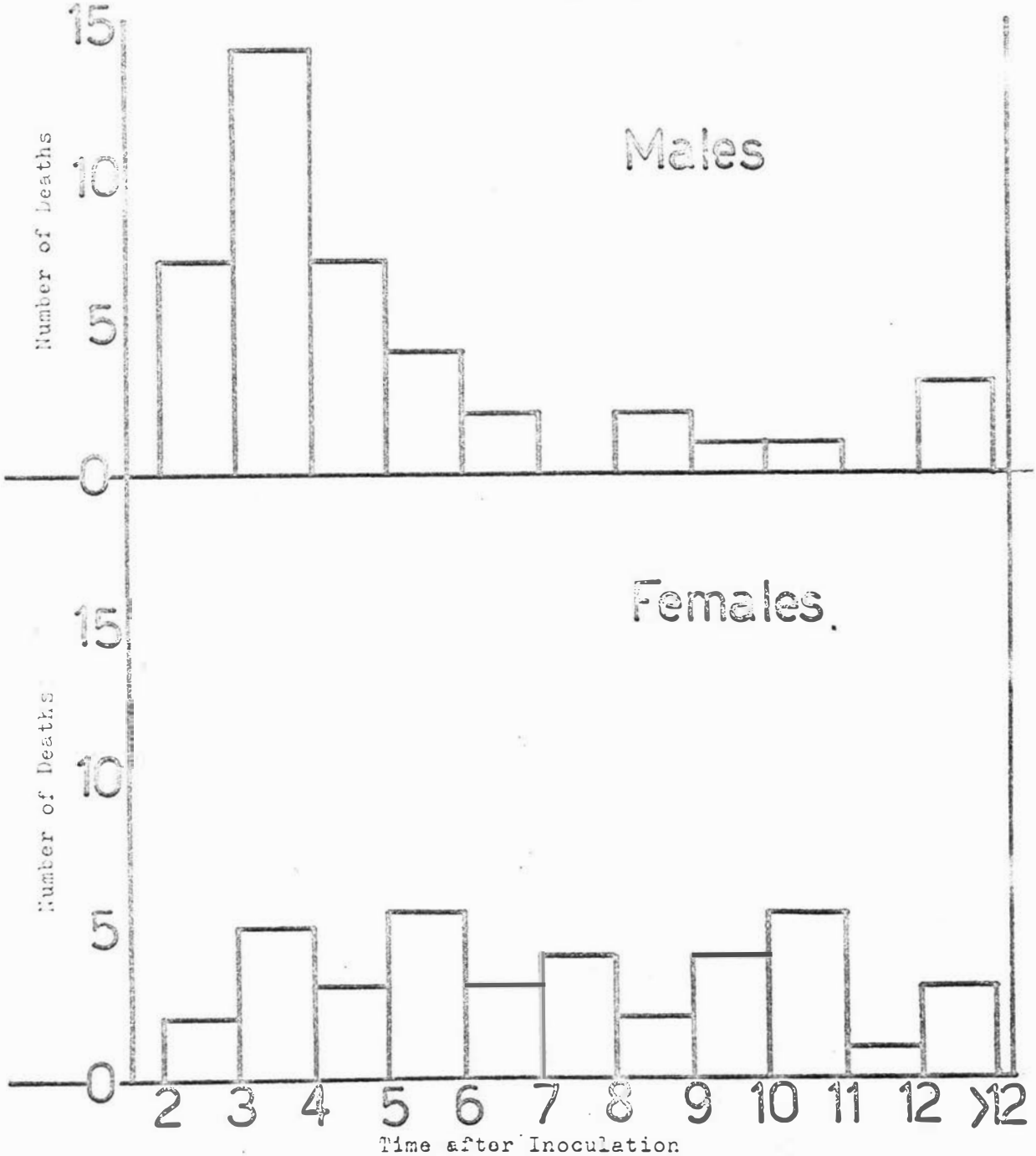
Groups of 25 males and 25 female mice were castrated at one month of age using techniques which are described in Appendix 1. Further groups of 25 male and 25 female mice of similar age and breeding were given 3 subcutaneous doses of 0.5 mg of oestradiol in peanut oil and 1 ug of testosterone ('Sustanon' - Organon Laboratories - London, England) at 2 day intervals respectively. The last injection of the hormones was given on the day before toxin was administered. A third untreated group of 25 normal male and 25 normal female mice, similar to the above was also used. All these animals were held under identical conditions until they were given toxin at 6 weeks of age, i.e. 14 days after castration of that particular batch of animals.

At this time all animals were given 1 ug of batch CWD epsilon toxin intravenously and the survival times were recorded.

Results

The results in this experiment were not as clearcut as those obtained in the previous section. In general the survival pattern

Figure 5.2 The Influence of Sex on Survival Time in Mice



of the castrated males and females and testosterone treated females was similar to that of the entire females, while oestrogen-treated males and normal males had a 'male-type' survival pattern. The mean survival times for the different groups were as follows:

Entire Males:	5.25 ± 2.47 hours
Entire Females	6.46 ± 3.26 "
Castrated Males	6.03 ± 3.19 "
Castrated Females	5.98 ± 2.35 "
Oestrogen Males	5.61 ± 2.37 "
Testosterone Females	5.52 ± 2.12 "

(t test on mean values for entire males and entire females: p between .200 and .100.)

The results are shown in graph form in Figure 5.3 and recorded in Appendix 3.

The Effect of Nutritional Factors on the Action of Epsilon Toxin in Mice

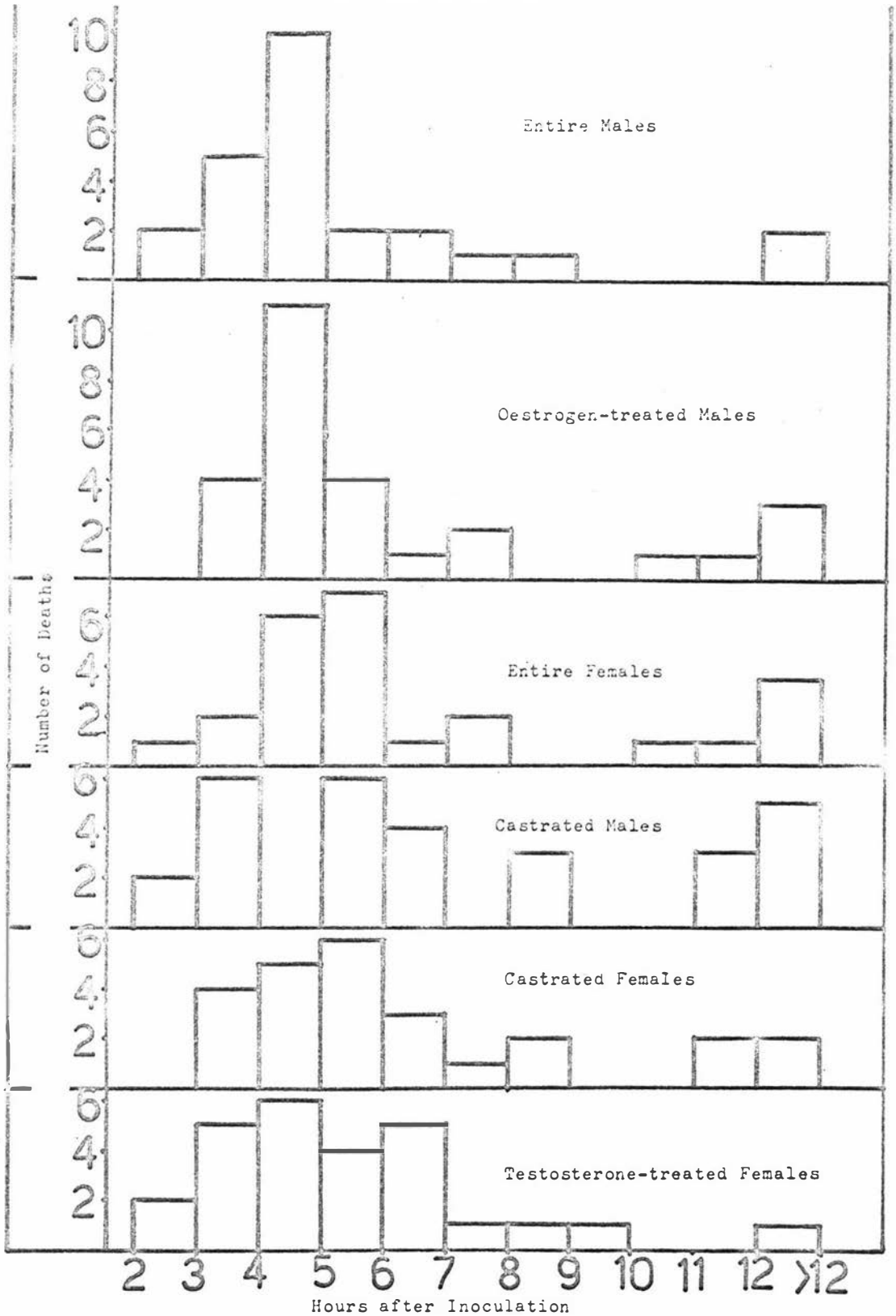
Uncomplicated starvation and its influence on the lesions produced in the course of epsilon toxin intoxication in mice will be discussed separately in Chapter 10.

Materials & Methods

Ten groups of five 6 week old female mice were prepared by administering one of the following compounds:

Adenosine triphosphate 0.5 mg intravenously.
Adenosine monophosphate 2 mg intravenously.
Thiamine pyrophosphate 0.5 mg intravenously (3 animals only)
Lipoic acid 100 ug intraperitoneally and 50 ug subcutaneously.
Coenzyme A 2 mg intravenously.
Nicotinamide 2.5 mg intravenously.
Pantothenic acid 0.7 mg intravenously.
Diphosphopyridine nucleotide (DPN) 2 mg intravenously.
Diphosphopyridine nucleotide (reduced) (DPNH) 2 mg intravenously
Thiamine hydrochloride 2 mg intravenously.

Figure 5.3: The Effect of Hormonal Status on Survival Time in Intoxicated Mice.



Five untreated animals were retained as controls.

Animals which received intraperitoneal or subcutaneous injections were held overnight before use while those receiving the compound intravenously were used immediately. Following the administration of the test substances all the animals were given 2 ug of batch CWD epsilon toxin intravenously.

Results

All mice died after toxin administration with the exception of the groups of animals which had received DPNH or coenzyme A. There was no difference in the survival times of the animals within the various groups where deaths occurred. Three animals survived in the group of animals which received DPNH and the experiment was repeated, using four mice and the same dose of nucleotide, but increasing the dose of epsilon toxin to 4 ug. On this occasion also, there were three animals which survived.

An interesting situation arose in the case of coenzyme A. The initial experiment had been carried out using the tri-lithium salt of coenzyme A supplied by Koch-Light Laboratories Ltd (Colnbrook England). When the experiment was repeated using the same doses of coenzyme and toxin there were no survivors from a group of five mice. On this occasion coenzyme A supplied by Sigma Chemical Co. (St. Louis, U.S.A.) had been used. In view of this anomalous result, the experiment was again repeated. Two groups of four female mice were given either 2 mg of Koch-Light Coenzyme A or 2 mg of Sigma Coenzyme A, followed by 4 ug of batch CWD epsilon toxin intravenously and again the animals which received the Koch-light compound survived while those receiving the Sigma compound succumbed.

The results of the experiments using DPNH and Coenzyme A summarised in Table 5.1.

Table 5.1. The Protective Effect of the Reduced Form of Diphosphopyridine Nucleotide (DPNH) and Coenzyme A Against the Action of Epsilon Toxin in Mice.

Protective Agent	Experiment	Dose of Epsilon Toxin	Number of Animals per Group	Number of Surviving Animals, 24 hours Post-Inoculation
DPNH	Initial	2ug	5	3
DPNH	Repeat	4ug	4	3
Coenzyme A 2mg (Sigma)	Initial	2ug	5	0
Coenzyme A 2mg (Sigma)	Repeat	4ug	4	0
Coenzyme A 2mg (Koch-Light)	Initial	2ug	5	5
Coenzyme A 2mg (Koch-Light)	Repeat	4ug	4	4

Discussion

Levels of toxin above one or two lethal doses were only employed in exceptional circumstances in the present investigation as, for example, in the assay of toxin levels in the intestinal contents of lambs. It was considered to be desirable, in the experiments where parenteral administration of toxin was used, to keep the dose as low as possible to avoid non-specific effects and to reduce the influence of any toxic agents, apart from epsilon toxin, which may have been present in the samples of toxin which were employed. (see Chapter 4.)

The preponderance of early deaths in the male mice appeared to be due to the sudden onset of violent convulsions with a fatal outcome, a pattern which was more common in the males than in the females.

In view of the fact that the survival time patterns of entire males and oestrogen-treated males were similar, while that of castrated males resembled the pattern for female animals, it appears that testosterone may be an important factor influencing the pattern of survival times in intoxicated mice. This is not surprising since Dunn, in Cotchin & Roe (1967), refers to the fact that sex differences in some of the morphological features in the kidneys of mice are dependent on the presence of testosterone. It is possible that, in the present instance, it is a modification of the internal environment induced by this hormone which shortens the survival time of male animals after toxin administration. However, testosterone per se does not appear to be the only factor involved as there was no marked change in the survival pattern of female mice which had received this hormone. Ideally this work would have been extended to include studies in which the effect of the different hormones on castrated animals was examined and the lethal dose of toxin for animals of different hormonal status established but this appeared, at the time, to be a divergence from the main aim of the project and was not carried out.

There are many difficulties in studying the effects of vitamins and co-factors in an acute intoxication and the absence of a significant result does not exclude the possibility that the toxin affects steps in which the particular substances are involved. In many instances, for example nicotinamide and pantothenic acid, the co-factor is only part of a complex and in some cases the intact complex must be synthesised within the cell as many of these compounds, in their active state, cannot pass across cell membranes.

In the present instance the apparent protective effect obtained with DPNH and coenzyme A is difficult to explain. In the case of DPNH any protection provided by the reduced nucleotide should have

been associated with a similar effect from the oxidised nuclectide, since it is the latter form which is the more active in energy metabolism. As in the case of DPNH the protective effect of coenzyme A was unlikely to be associated with any action of the coenzyme itself as only one brand of the substance was effective.

It appears from the foregoing considerations that the protection may have been of an indirect nature or due to some extraneous factor and, while more extensive investigations of this phenomenon appear to be warranted they were considered to be beyond the scope of the present investigation.

Conclusions

1. The survival times of intoxicated mice are not dose-dependent when levels of epsilon toxin of the order of one to two lethal doses are employed.

2. The survival time pattern differs in intoxicated male and female mice with a preponderance of acute early deaths in the male animals.

3. The pattern of survival time in intoxicated mice is influenced by the hormonal status of the animal, being determined to some extent, by the presence or absence of testosterone.

4. No protective effect, against the action of epsilon toxin was provided by the prior administration of adenosine triphosphate or monophosphate, thiamine pyrophosphate or hydrochloride, lipoic acid, oxidised diphosphopyridine nucleotide, pantothenic acid or nicotinamide. Apparent protection was provided by reduced diphosphopyridine nucleotide and one brand of coenzyme A but not by coenzyme A from another commercial source. The precise action of these protective agents was not determined.

Chapter 6: SOME OF THE FACTORS WHICH INFLUENCE THE ACTION OF EPSILON TOXIN ON LAMBS.

Previous workers have found that host factors can have a considerable influence on the outcome of attempts to produce enterotoxaemia in lambs. For instance, an important prerequisite for the successful production of the disease has been found to be the absence of appreciable levels of circulating epsilon antitoxin and this can sometimes present problems since it has been shown that epsilon antitoxin is produced in a proportion of lambs when they begin to graze pasture. In addition, in contrast to Lamb Dysentery, enterotoxaemia is rare in the first few days of life, suggesting that it may be preferable to avoid using newborn lambs in investigations into the action of epsilon toxin or enterotoxaemia.

Parenteral administration of epsilon toxin is a convenient means of studying enterotoxaemia but there is some evidence that the results obtained are not always typical of the natural disease. Trifinov & Todorov (1964) have shown that the rate of intravenous administration of the toxin can affect the survival time of rabbits and, in experiments where the toxin has been given rapidly to lambs, a severe pulmonary oedema has been described. (Kellaway et al., 1940). This is not a prominent feature of the natural disease, nor has it been described when the toxin has been infused slowly by the intravenous route (Griner, 1961a).

While it is possible to study the action of epsilon toxin by infusing sterile culture filtrates or partially purified toxin into the duodenum, the method has serious limitations. Surgical cannulation of the animals is required and in addition, the toxin must be held at a high concentration in the intestinal contents for some time before intoxication can be induced. During this time the toxin is being continually removed by peristaltic activity, which makes the quantity of toxin required too great to recommend the technique for experimental use. Other workers have therefore turned to the use of a continuous intraduodenal infusion of cultures of Cl. perfringens type D plus a carbohydrate such as starch or dextrin.

It was decided to study the effect of parenterally administered

toxin on lambs in the first instance, keeping the limitations of the method in mind and trying, wherever possible, to obtain information on the influence which factors such as rate of administration might have on the outcome of the experiments.

Because of the possible shortcomings of parenteral administration of epsilon toxin as a model for studying enterotoxaemia it was also necessary to produce the disease by intraduodenal infusion of Cl. perfringens type D culture, plus carbohydrate so that appropriate comparisons could be made.

In the latter animals, any change in intestinal permeability which occurred when high levels of epsilon toxin developed in the intestine would be useful as a reference point to which clinical evidence of intoxication could be related. Bullen & Batty (1957) did show a difference between the rate of absorption of a protein tracer between control and intoxicated animals but there was no obvious point of inflection in the absorption pattern in intoxicated animals nor did the absorption of the tracer appear to be closely related to the development of high levels of epsilon toxin in the intestine of these animals.

The tracers employed by these workers had certain disadvantages, the most important being that, because they were protein, they were liable to be broken down in the intestine. The ideal marker to use in the present instance would appear to be the epsilon toxin itself, but detection techniques are not particularly sensitive. A further problem is that, according to Trifinov & Todorov (1964), a variable proportion of the absorbed toxin is removed by the liver. Direct sampling from the mesenteric veins would therefore be required and the latter technique would not fit in with other aspects of the present study.

A tracer was required which was readily detectable, stable in the intestine and not subject to metabolic breakdown or binding within the body. One substance which fulfills these criteria is radioiodinated polyvinylpyrrolidone (PVP) which is currently used in human medicine for detecting loss of protein from the bloodstream into the intestine in certain enteric disorders, and it was decided to use this agent in

experiments designed to indicate the time at which absorption of toxin from the intestine was maximal.

The Influence of Different Rates and Routes of Administration of Epsilon Toxin Upon the Pathological Changes Produced in Lambs.

Materials & Methods:

The lambs available for these experiments were the progeny of Southdown rams mated to Romney ewes which had not received Pulpy Kidney vaccine during this pregnancy. Their previous vaccination history was uncertain. All the available lambs were female and this precluded any study of possible sex differences in the action of epsilon toxin in this species. Lambs were used at between two and three weeks of age when their bodyweights were between 6.5 and 13.5 kg (mean 9.5 kg.). A total of 43 lambs were obtained over a period of 5 weeks and animals which were shown at necropsy to have intercurrent infections were removed from the experiments.

Because of farm management considerations the epsilon antitoxin status of these animals could not be determined before experimental procedures were undertaken. It was however determined retrospectively on serum samples which had been taken from the animals before any toxin was administered. Levels of epsilon antitoxin were estimated within broad limits by mouse protection tests as described in the British Veterinary Codex (1965).

Of the available lambs, 10 were retained as uninoculated controls for the biochemical and morphological studies. The remainder were divided into two groups. One group of 18 lambs was given individual doses of 0.15 to 0.25 mg. of batch CWD epsilon toxin intravenously into the jugular vein at 30 minutes intervals until the onset of clinical signs of intoxication. If clinical intoxication did not develop after four injections, the dose of toxin was increased first to 0.5 mg. and finally to 1 mg. The other group of 13 lambs received the toxin (0.1 mg./ml.) by slow intravenous infusion from a disposable intravenous drip set at a rate of 100 ml per hour. One further animal was given 6 mg. of epsilon toxin intraperitoneally in two equal doses 2 hours apart.

Results:

The intraperitoneal injection of the toxin was non-lethal and produced a severe fibrinous peritonitis. This route was thus unsuitable for the experimental induction of intoxication.

In both groups of lambs which received intravenous toxin, the amount needed to produce intoxication varied considerably and ranged from 0.25 to 25.0 mg. In one animal with 4 - 8 units epsilon antitoxin/ml. of serum intoxication was not induced by the administration of 100 mg. of toxin. The doses of toxin administered are summarised in Figure 6.1 where they are related to the level of circulating antitoxin in the animals.

In all instances where the toxin was lethal, the animals died within an hour of the onset of clinical signs. The clinical signs and gross pathology which developed in both these groups of animals will be described in detail in Chapter 7, but it is appropriate to mention here that, with the exception of one animal, all animals which received the toxin in individual doses developed a most severe pulmonary oedema and a massive effusion of pale straw-coloured fluid into the pleural cavity. On the other hand eight of the animals which received the toxin by continuous infusion did not develop any pleural effusion and showed little or no lung oedema. (Figure 6.2a & b). The data from these experiments are given in Appendix 5.

The Effect of the Infusion of Cl. perfringens Type D Culture plus Carbohydrate into the Duodenum of Lambs.

Materials & Methods:

Most of the animals which were available for this aspect of the work were 8 month old male Romney lambs from a group of 20 which had been screened for epsilon antitoxin. Six animals with antitoxin levels less than 0.08 units per ml of serum were selected for cannulation. Of these, two died from intercurrent infection prior to cannulation.

Figure 6.1 The Influence of Circulating Epsilon Antitoxin on the Dose of Epsilon Toxin required to produce Intoxication in Lambs

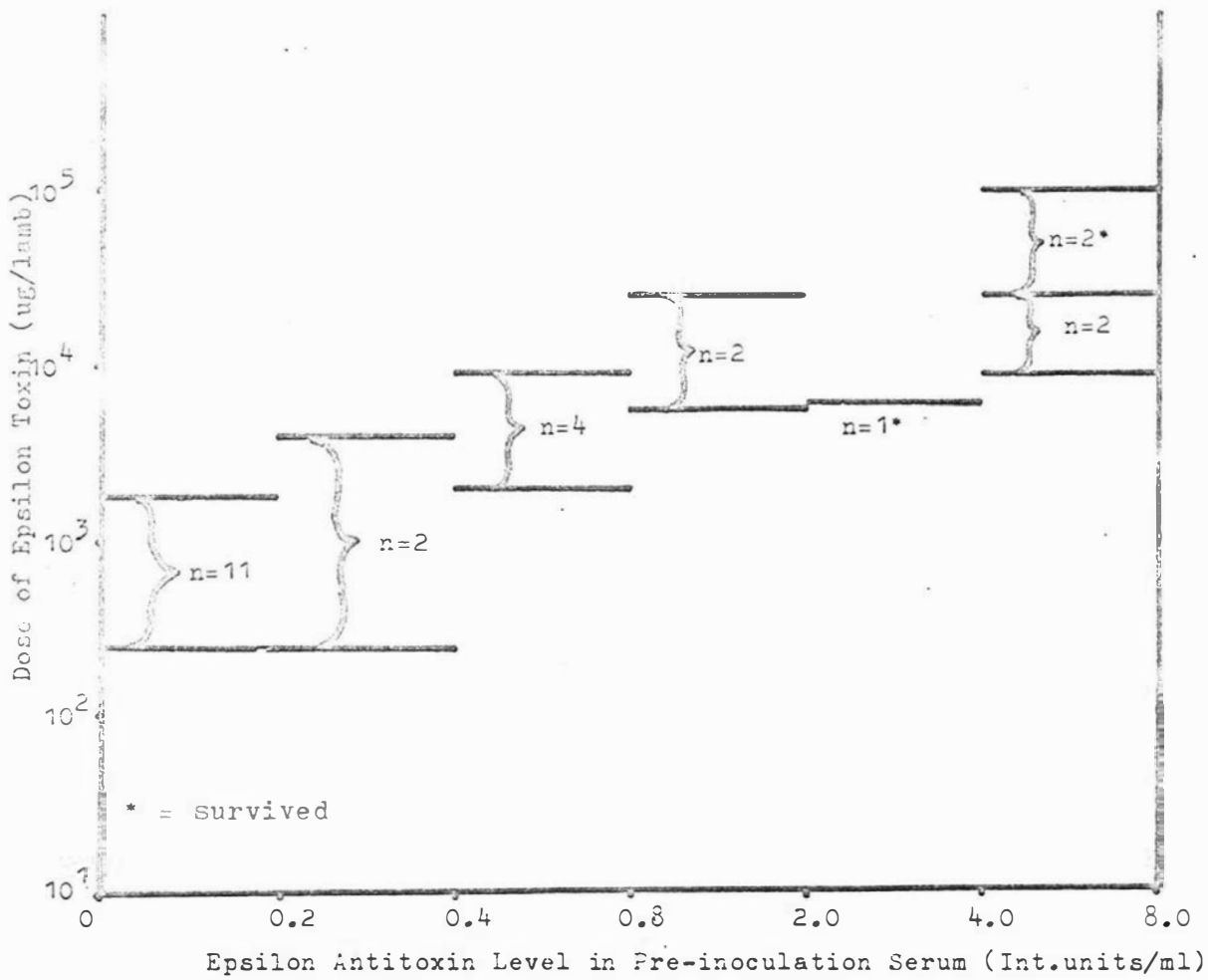


Figure 6.2a: The Effect of Mode of Administration of Epsilon Toxin on the Development of Pulmonary Oedema in Lambs

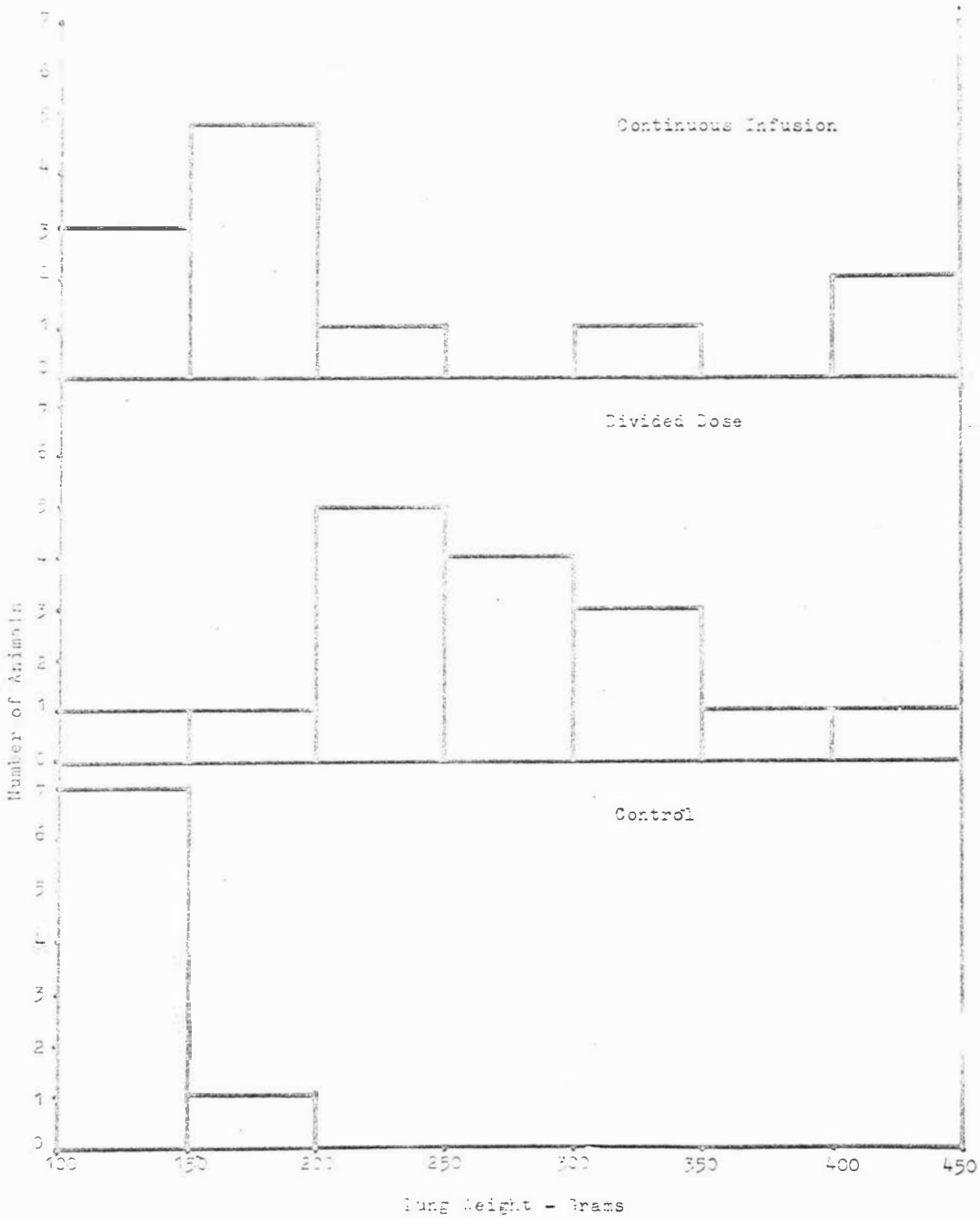


Figure 6.3b: The Effect of Mode of Administration of Epsilon Toxin on the Development of Pleural Effusions in Lambs.

A young female lamb of similar age and breeding to those receiving the parenteral toxin was also cannulated.

The surgical techniques employed for inserting the cannulae into these animals are described in Appendix 4.

Cultures of Cl. perfringens type D were grown in Cooked Meat Medium at 37°C for 18 hours. After shaking the cultures and allowing the larger meat particles to settle, 500 to 700 ml. of the supernatant was decanted into a 1 litre bottle and either 100 grams of cornflour starch or 50 grams of dextrin were added. This mixture was infused into the duodenum of the animals via an intravenous drip set at 100 ml./hour until the onset of clinical signs of intoxication. Frequent agitation of the bottle was necessary to hold the carbohydrate in suspension.

The toxin level in the intestinal contents of these animals at the time of death was ascertained as follows:

A sample of the intestinal contents was centrifuged at 1800G for 20 minutes and the supernatant retained. In samples where the material was too thick to centrifuge it was weighed and mixed with an equal quantity of saline before centrifugation. The supernatant was prepared in the following series of dilutions in saline viz. 1:5, 10, 20, 50, 100, 200, 500, 1,000, 2,000. Aliquots of 0.2 ml of each dilution were injected intravenously into each of two mice. In addition 0.8 ml. samples of the undiluted supernatants were mixed with 0.2 ml. of either commercial Cl. perfringens type D or Cl. perfringens type C antisera and held at room temperature for 30 minutes. Aliquots of 0.3 ml. of the intestinal contents-serum mixture were injected intravenously into pairs of mice to confirm that the toxicity was due to the action of the toxins of the former organism.

The pattern of the development of toxin levels was also studied in the animal in which an additional ileal cannula had been inserted. Samples of the ileal contents were removed at intervals from the start of infusion until death occurred and the level of toxin in each sample was established as described above.

Results:

With one exception the animals developed clinical signs of intoxication and died with post mortem lesions consistent with enterotoxaemia. These findings will be discussed in detail in later chapters and it is sufficient to note here that pleural effusions were absent and pulmonary oedema minimal or absent in all animals.

The level of epsilon toxin in samples of the intestinal contents at the time of death was high in all intoxicated animals and ranged from 500 to 5,000 mouse lethal doses/ml. It is interesting to note that a sample of faeces passed by the young female lamb, during a period of severe diarrhoea, 8 hours after the onset of infusion and 15 minutes before death, also had 1,000 mouse lethal doses of toxin per gram of faeces. In the animal in which the pattern of development of toxin was studied there was an initial lag of $2\frac{1}{2}$ hours from the start of infusion until detectable levels of toxin were present in the ileum. Thereafter the level of toxin rose rapidly to over 1,000 mouse lethal doses/ml. and remained above this level until death occurred $4\frac{1}{2}$ hours later (Figure 6.3).

The Pattern of Absorption of Radioiodinated Polyvinylpyrrolidone (I_{125} PVP) from the Intestine of Lambs.

Two separate experiments were carried out using I_{125}PVP as a tracer.

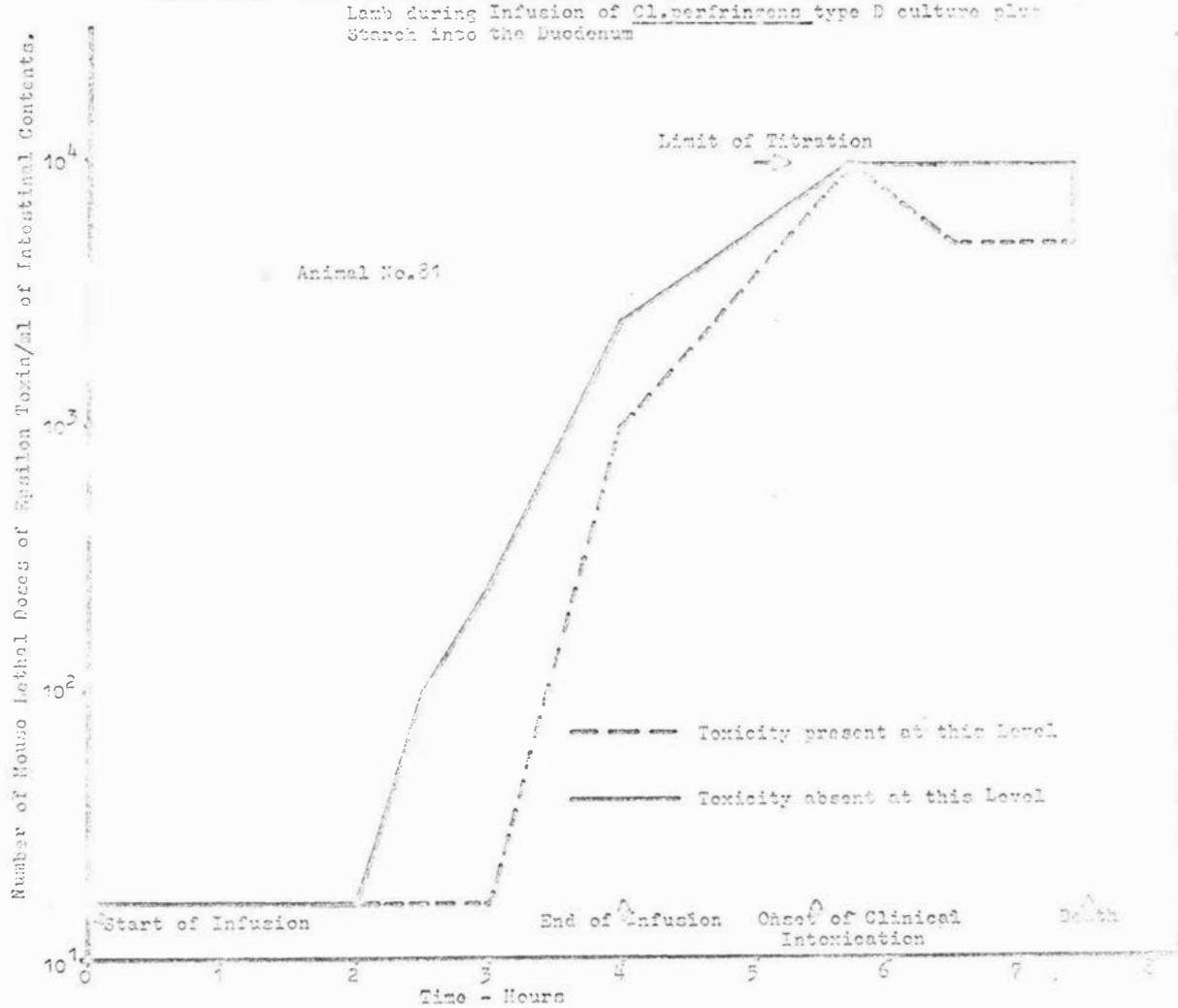
Materials & Methods:

PVP labelled with the I_{125} isotope was used in these experiments as it has a longer half life than I_{131} and is therefore more convenient.

In the first experiment the tracer was infused into the duodenum of two separate animals. One animal (No. 77) received 1.75 ml. of the I_{125}PVP concentrate containing 5.5 mg. PVP/ml. in 700 ml. of the Cl. perfringens type D/dextrin mixture over a period of five hours. The other animal (No. 78) received 1.9 ml. of the same I_{125}PVP concentrate in 750 ml. of saline intraduodenally over a period of six hours to act as a control.

In the second experiment one animal (No. 81) was used and was intended to act as its own control. On the first day of the experiment

Figure 6.3: The Pattern of Development of Epsilon Toxin in the Intestine of a Lamb during Infusion of Cl.perfringens type D culture plus Starch into the Duodenum



1.9 ml. of I_{125}PVP (2.5 mg.PVP/ml.) was infused intraduodenally in 500 ml. of saline over a period of 6½ hours. The animal was left overnight and on the following day 1.8 ml. of the same I_{125}PVP sample was infused in 500 ml of culture/dextrin mixture over a period of 4 hours.

Heparinised blood samples were taken from the jugular vein of these animals with "Vacutainers" at intervals throughout the experiments and, after centrifugal separation, aliquots of the plasma were taken and the radioactivity measured. This was done by counting each sample for 10 minutes in a Picker "Autowell" gamma emission counter (Picker Xray corp., White Plains, N.Y.). In the first experiment 1.5 ml. aliquots of plasma were used and in the second experiment the volume was reduced to 1.0 ml. The radioactivity of similar aliquots of each of the mixtures infused intraduodenally was established in the same way and the results of the gamma emission counts were converted to nanograms of PVP/ml. of plasma.

Results:

In the first experiment the absorption of the tracer was greater and increased more rapidly in the animal which received the culture plus dextrin. These results are shown in graph form in Figure 6.4a. In the second experiment however, the carryover of circulating tracer from the first to the second day made it impossible to establish whether there was any alteration in the rate of absorption of the tracer. There was however a precipitous drop in the radioactivity of the two blood samples taken just before the death of the animal. The pattern obtained in this animal is illustrated in Figure 6.4b. The data from these experiments is given in Appendix 6.

Discussion

Although it was realised that a proportion of the lambs used in these experiments were liable to receive passive antibodies from their dams in the colostrum, no attempt was made to rear them artificially as farm management factors precluded obtaining many of the lambs before they had had at least one feed from their mothers. In addition, colostrum-deprived animals are susceptible to enteric disorders and these could have adversely affected the experimental investigations.

...ts of ... from the Intestine
... Toxicated Lambs.

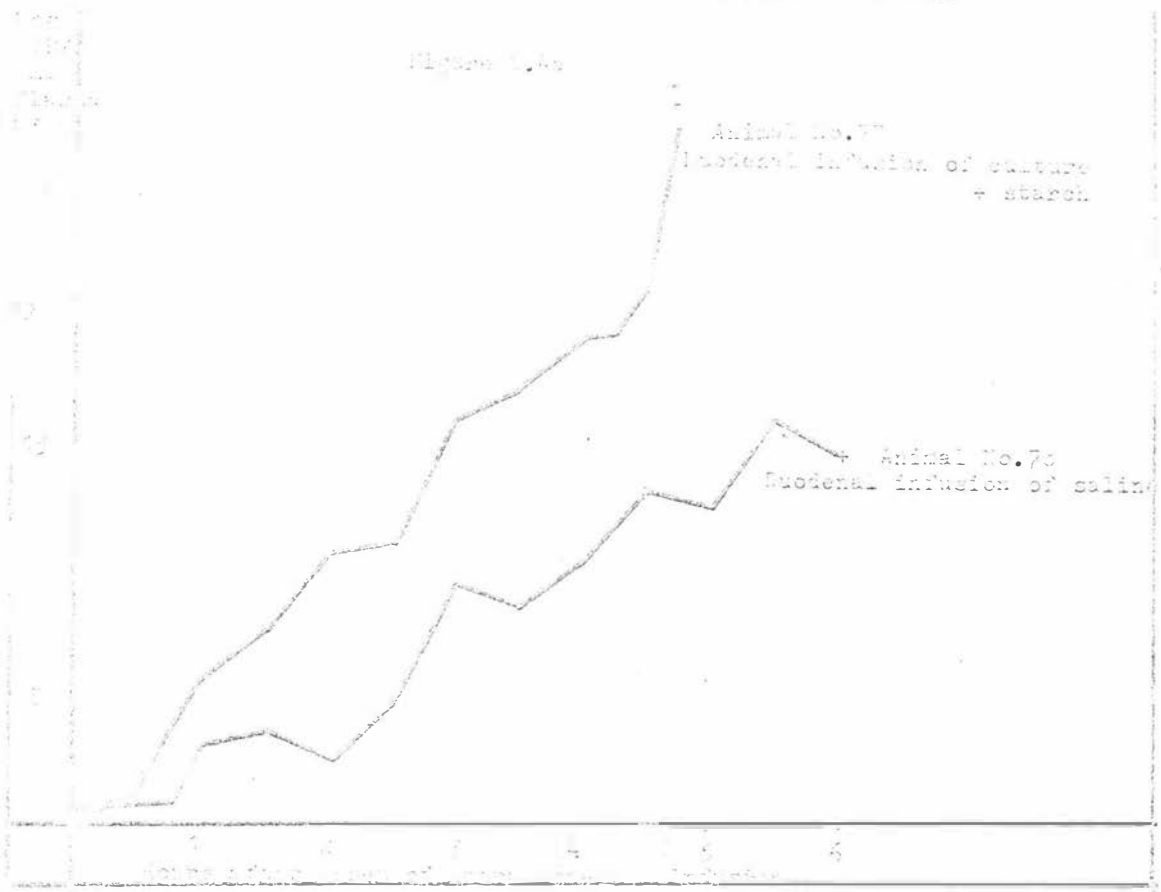
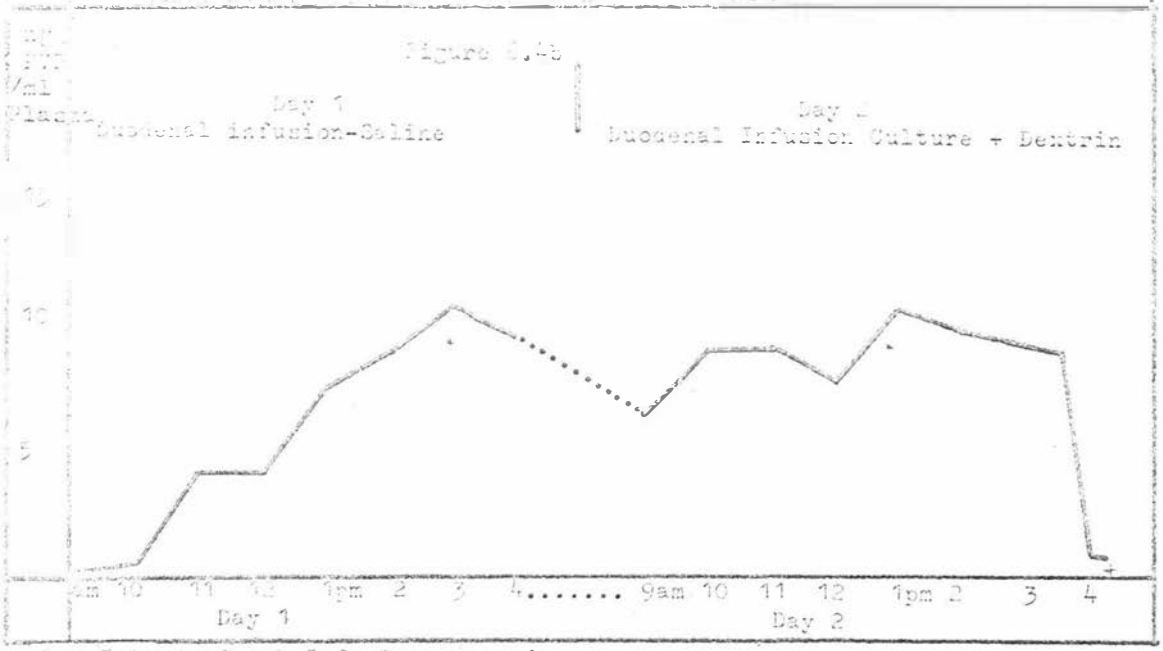


Figure 4.4b



* = Intraduodenal Infusion stopped
+ = Time of Death

Intoxication could be induced in susceptible animals in the present experiments with 25 ug epsilon toxin/kg. B.W. or slightly less. This figure is comparable with Griner's (1964) figure of 18 - 36 ug/kg. The presence of approximately 1 int. unit of antitoxin/ ml. of serum increased the dose of toxin required to the order of 1000 ug/kg. B.W.

One of the prominent features of this series of experiments is the marked influence that the concentration and rate of administration of the toxin had upon the lesions which were produced. The rapidity of onset and severity of the pulmonary oedema when the animal received concentrated toxin over a short period of time was remarkable and could have profoundly influenced many of the parameters associated with respiratory exchange and fluid balance (See Chapters 16 and 17). The development of pulmonary oedema in a proportion of the animals which received the continuous infusion was possibly associated with the high concentration of the toxin which was used, 100 ug/ml. compared with Griner's 10 ug/ml., because of the unknown antitoxin status of the present series of animals at the time when the toxin was administered. The possible basis for the variable occurrence of pulmonary oedema will be considered again in Chapter 9.

No explanation can be advanced for the animal which survived the intraduodenal infusion of culture and starch. It had received a continuous infusion of 100 ml/hour for 8 hours, but, apart from mucoid diarrhoea 6 hours after the onset of infusion, it was unaffected and remained clinically normal for a further 24 hours at which time it was destroyed. The antitoxin titre of this animal, at the time the infusion was started, had not changed from the preliminary screening value of 0.08 units/ml.

The levels of toxin and the pattern of its development in the intestinal contents of the other animals which received intraduodenal infusions was broadly similar to that found by previous workers.

The pattern of absorption of the PVP tracer during the course of intoxication was also much the same as that obtained by Bullen & Batty with their protein tracers. No inflection was detectable in the curves from the intoxicated animals. On the other hand it must be realised that, 3 hours after the onset of infusion the levels of tracer in the plasma had

reached 50% of its final value in both the intoxicated and control animal and since, in a different animal, toxin was only just becoming detectable in the ileum at this time it may be that the marker does not truly reflect the pattern of absorption of the toxin. It would be possible to confirm these results therefore, only by carrying out further work on the relationship between the levels of toxin at a number of different points along the length of the small intestine and the pattern of absorption of different tracer substances. Because these factors are only indirectly associated with the biochemical, physiological and morphological changes induced in the animal as a whole during intoxication they were not pursued further in this study.

It is unfortunate that the experiment in which the animal was used as its own control was not satisfactory for demonstrating changes in intestinal permeability, but the sudden fall in the level of circulating tracer in the terminal blood samples is of some interest. It can be seen from Figure 6.4b that the decrease in circulating tracer after the cessation of the infusion on the first, control, day was very slight and in fact the tracer was at much the same level on the following morning. Similarly, on the second day, the initial decrease after stopping the infusion was gradual and the precipitous fall did not occur until clinical intoxication was at an advanced stage. This suggests that the tracer was being lost from the circulation at this time. Since Griner, Carlson (1961b) have shown that radioiodinated albumin escapes from the circulation into the tissues of the central nervous system in intoxicated animals it is possible that the PVP was also being lost in a similar manner into nervous and other tissues.

This possibility will be mentioned again in Chapters 8 and 16.

Conclusions

1. The intraperitoneal injection of epsilon toxin into lambs produced a severe fibrinous peritonitis and was an unsuitable route for the administration of toxin.

2. The dose of intravenously administered toxin required to produce intoxication was influenced by the level of naturally acquired specific antitoxin in the recipient lambs.

3. The rate of administration of toxin modified the pathological effect of intravenously administered toxin. Rapid administration of the toxin was more likely to produce pulmonary oedema than administration over a longer period of time.

4. Intraduodenal infusion of Cl. perfringens type D culture plus starch or dextrin into lambs produced a clinical and pathological syndrome which closely resembled natural cases of enterotoxaemia, with high levels of epsilon toxin developing in the intestinal contents of affected animals.

5. PVP labelled with I_{125} was no more effective than the protein tracers used by other workers for demonstrating altered intestinal permeability during intoxication.

6. The sharp fall in the levels of circulating PVP tracer in the terminal blood samples from an intoxicated animal suggest that it is lost from the bloodstream into the tissues in the late stages of intoxication.

Chapter 7: THE CLINICAL SIGNS AND GROSS PATHOLOGICAL CHANGES ASSOCIATED WITH CL. PERFRINGENS TYPE D EPSILON TOXIN INTOXICATION.

Because enterotoxaemia generally has a very short clinical course, few field cases are seen alive. Where natural or experimental cases are seen antemortem nervous signs are prominent and may include rigidity, opisthotonus and convulsions (Gill, 1933 and Griner, 1961a). On occasions the clinical syndrome in these animals superficially resembles tetanus in lambs, although the rigidity is generally not as marked (Gill, 1933). The present study provided a good opportunity to observe the range of clinical signs and gross pathological changes which can be encountered in intoxicated mice and lambs. It is appropriate to discuss these before embarking on a detailed description of the biochemical, haematological and morphological changes induced by the action of the toxin.

As stated earlier, there have been some investigations into the physiological responses of the animal to the action of epsilon toxin. In particular, Kellaway et al. (1940) studies on the changes which occur in blood pressure and the electrocardiogram (ECG) during the course of intoxication are noteworthy. Unfortunately their work was confined entirely to the action of parenterally administered partially purified toxin and many of the experiments were carried out on surgically modified animals e.g. decerebrate preparations. In the present study it was possible to study the change produced in physiological parameters such as the electrocardiogram both when the toxin was administered parenterally and when it was absorbed from the intestine, under conditions as nearly approaching the natural situation as possible. Information could therefore be obtained on the overall pattern of changes which could be expected to be present in the natural disease as well as that produced by parenterally administered toxin. At the same time it was possible to relate these changes to the occurrence of gross lesions at necropsy and to structural alterations in the tissues.

The Clinical Signs and Gross Pathology of Epsilon Toxin Intoxication in Mice.

The pattern of development of clinical signs was broadly similar in all mice which received doses of epsilon toxin of the order of one lethal dose in the various experiments undertaken in the course of this study. Animals remained normal for from 5 to 30 minutes. Thereafter they became depressed and sat hunched up with fur erect and eyes closed. If disturbed at this stage they would move sluggishly. After an hour or more, usually in response to a sudden stimulus such as light, sound or movement, a proportion of these animals would suddenly exhibit violent convulsions, initiated by running, leaping or rolling. The convulsions usually terminated in a spastic episode with the neck ventriflexed, and fore and hind limbs extended rigidly backward. If the animal recovered from this convulsion the episode would be repeated after an interval of time had elapsed and this pattern would continue until death supervened. No gross lesions which could be associated with the action of the toxin were seen in any of these animals.

In animals given high concentrations of epsilon toxin e.g. in assays of lamb intestinal contents or where tissues were required for fluorescence microscopy (see Chapter 14), death occurred rapidly. Violent convulsions occurred as soon as the toxin was administered and the animal generally died within 30 minutes of inoculation. In these mice there was severe pulmonary oedema.

The Clinical Signs and Gross Pathology of Epsilon Toxin Intoxication in Lambs.

With one exception the susceptible lambs which received parenterally administered toxin in divided doses developed depression within a few minutes of the administration of the final dose of toxin. This was followed by dyspnoea with rapid forced respiration and a double expiratory effort. The animals then became cyanosed and recumbent and thereafter the syndrome progressed rapidly to coma and death, often with a few terminal convulsive movements. In all instances the animals died within an hour of the administration of the final dose of toxin and these were the lambs which showed severe pulmonary oedema. A similar pattern of

clinical signs also occurred in those animals which developed pulmonary oedema after receiving continuous intravenous infusions of toxin.

On the other hand there was no evidence of respiratory distress in the lamb which received epsilon toxin in divided doses but did not develop pulmonary oedema. The first clinical signs of intoxication in this animal consisted of a mild ataxia which developed 30 minutes after the last injection of toxin. This progressed, over the next 30 minutes, to depression, nystagmus and fine lip tremor, and was rapidly followed by prostration, 'star-gazing', rolling and running convulsions and opisthotonus. This pattern continued with only occasional short relaxed periods until death occurred 2 hours after the final inoculation of toxin. A similar syndrome was seen in two of the lambs which received an intraduodenal infusion of Cl. perfringens type D culture plus carbohydrate.

There was no evidence of respiratory distress in the lambs which did not develop pulmonary oedema after receiving continuous intravenous infusions of toxin. As soon as these animals became depressed during the course of infusion, the administration of toxin was discontinued. The depression was accompanied by rapid shallow respiration but there was no dyspnoea. From depression, the clinical signs progressed rapidly to coma. The animals died quietly or else with just one or two terminal convulsive movements. This type of syndrome was also seen in two of the lambs which received the intraduodenal infusions of Cl. perfringens type D culture plus carbohydrate.

Some aspects of the gross pathology of intoxicated lambs are described elsewhere e.g. the pulmonary oedema and pleural effusions in Chapter 6 and the renal changes in Chapter 10. Apart from these changes the only gross lesions regularly associated with intoxication were pericardial effusions and subepicardial and subendocardial haemorrhages. In animals which received toxin intravenously the pericardial effusions varied considerably in quantity. Pericardial fluid was often absent or present in very small amounts, in animals which died shortly after the onset of clinical signs of intoxication. In other animals, including all those in which the toxin was of enteric origin, 20 - 50 ml. of clear straw-coloured fluid, which clotted after being exposed to air, was present in the peri-

cardial sac. Subendocardial haemorrhages were usually present in the hearts of intoxicated animals and often consisted of extensive ecchymoses in the walls of the ventricles, both ventricles usually being affected to some extent in all animals. On the other hand subepicardial haemorrhages were variable in occurrence and were usually petechial in nature and most noticeable over the fat deposits in the coronary grooves. The only other gross lesion of note was petechial haemorrhages in the thymus in a proportion of the intoxicated animals.

The Electrocardiographic Changes which Occur in Lambs Receiving Epsilon Toxin

Materials & Methods:

Electrocardiograms (ECG's) were recorded from 11 of the 2 - 3 week old female lambs which received parenteral epsilon toxin by continuous intravenous infusion and also from 3 of the 8 month old male lambs which received Cl. perfringens type D culture plus carbohydrate. Recordings of the standard bipolar and unipolar limb leads (I, II, III, aVR, aVL, aVF) were made with a 'Cardiofax' portable electrocardiograph using 25swg subcutaneous needle electrodes and a chart speed of 25mm/sec. Duplicate recordings were also taken at a chart speed of 50mm/sec. if this proved necessary.

Because the position of the animal may affect the electrocardiogram, pre and post-inoculation recordings were made with the animals restrained in identical postures. Recordings from the lambs which received parenteral toxin were taken with the animal held lying quietly in right lateral recumbency on a table covered with a $\frac{1}{4}$" thick rubber mat over an earthed grid. The left forelimb was held anterior to the right in all animals. Further recordings were taken under the same conditions when clinical signs of intoxication were detectable and, if possible, again just before the death of the animal occurred.

Of necessity the ECG's of the animals which received intraduodenal infusions were recorded with the animal standing in its crate and sling (see Appendix 4) and in this instance the animal stood on the rubber mat and the crate itself was earthed. Preinoculation and serial post-

inoculation recordings were taken as before with needle electrodes. During the later stages of intoxication the position of the animals was maintained as nearly as possible to the preinoculation posture by use of the sling.

Results:

Parenteral Toxin Administration:

The ECG's taken after the onset of clinical signs of intoxication developed differed markedly from the preinoculation recordings in all the animals examined. The most consistent changes were as follows:

i. A decrease in heart rate, with an associated increase in P-R interval.

ii. A decrease in the amplitude of the QRS complex and marked alterations in the pattern of this complex.

iii. A decrease in the duration of the S-T segment and a displacement of this segment from the isoelectric line.

iv. A tendency for the T wave to have an increased amplitude with changes in the polarity of this wave in some animals.

The cardiac rhythm of most animals remained normal after administration of toxin. In some instances however there were very severe alterations in rhythm consisting of extreme bradycardia, sinus arrythmia, incomplete heart block, electrical alternans, atrial fibrillation and ventricular fibrillation. These changes occurred singly or in combination in any given animal.

The normal electrocardiographic pattern and the changes encountered following the parenteral administration of epsilon toxin are summarised in Table 7.1a, and illustrated in Figure 7.1.

Enteric Origin Toxin:

The electrocardiographic changes were not as prominent in these animals as in the animals which received parenteral toxin and the situation was further complicated by the fact that one animal (No. 81) had a pre-existing electrocardiographic defect in the form of S-T segment displacement. Further, for technical reasons the pre- and post-inoculation recordings from this animal were made with different instruments. Although

Figure 7.1: Electrocardiograms from Lambs which received Parenterally Administered Bacillus Toxin

Lead I - 50mm/sec

Pre-inoculation

ANIMAL No. 62 (Parenteral)

Post-inoculation.

S-T segment elevation

Lead aVR - 50mm/sec

Pre-inoculation

ANIMAL No.70 (Parenteral)

Post-inoculation

S-T segment elevation

Lead III - 50mm/sec

Pre-inoculation

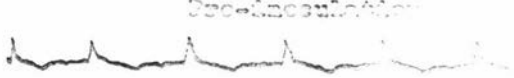
ANIMAL No 70 (Parenteral)

Post-inoculation

S-T segment depression

Lead I - 50mm/sec

Pre-inoculation



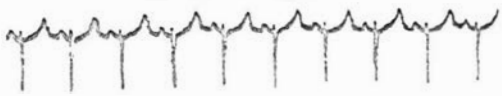
ANIMAL No. 72 (Parenteral)

Post-inoculation

DB = Dropped Beat

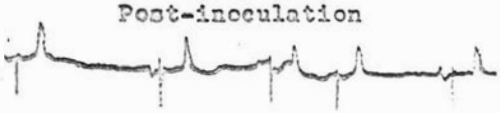
Figure 7.1(cont'd): Electrocardiograms from lambs which received Parenterally Administered Epsilon Toxin.

Lead III - 25mm/sec
Pre-inoculation



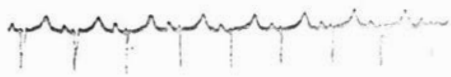
ANIMAL No.64(Parenteral)

Post-inoculation



Severe sinus arrhythmia

Lead aVF - 25mm/sec
Pre-inoculation

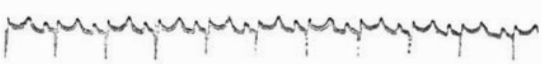


ANIMAL No. 64 (Parenteral)

Post-inoculation

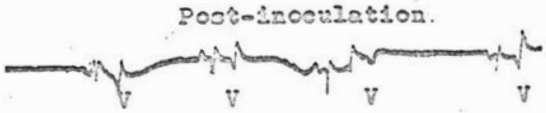
A = Atrial fibrillation

Lead II - 25mm/sec
Pre-inoculation



ANIMAL No. 64 (Parenteral)

Post-inoculation.



V = Ventricular extrasystoles

Lead II - 50mm/sec
Pre-inoculation

ANIMAL No. 72 (Parenteral)

Post-inoculation

Electrical alternans

Table 7.1a: The Effect of Parenterally Administered Cl. Perfringens Type D Epsilon Toxin on the Electrocardiogram of Lambs.

Animal	Rate Beats/min	Rhythm	P Wave Size	P Wave Form	P-R Interval	QRS Complex Amplitude	QRS Complex Form	S-T Segment Duration	S-T Segment Position	T Wave Size	T Wave Polarity
Summary of Preinoculation values of lambs which received Parenterally Administered Toxin											
n = 11	165-250	Regular	.1-.2mV .02-.04sec	+ve in I,II, III,aVF -ve in aVR, aVL	.04 sec	.3-.8mV 0.02-0.04 sec	I and aVL qRs or QRs II qrS IIIxaVF qrS or qRS aVR qRs or qRS	.08-.10sec	Isoelectric	.1-.3mV .04sec	+ve in II,
Post Intoxication Alterations in the ECG of Individual Animals											
62	Decrease From To 214 201	No change	Increased Duration (slight) II,III,aVF	No change	Increase .02sec I,II,aVR	Decrease all leads	Altered I,II, aVR,aVL	Decrease .02-.04 sec all leads	Elevated .1mV I,II Depressed .1mV in aVR	Increase .1-.2mV .02sec II,III,aVR,aVF	Inverted I,II,aVR
73	Decrease From To 201 188	No change	slightly Increased Duration I,II,III	No change	Increase .02sec III,aVL aVF	Decrease I,II,III aVR,aVL	Altered all leads	Decrease I,II,III aVR,aVF	Sl.elevation in I	Increase .05-.4mV .02sec I,II,III, aVR,aVF	No change
74	Decrease From To 167 125	Incomplete Heart Block & Bradycardia to65/min aVF	Increased Duration .04sec all leads	"	Increase .02-.08 I,II,III aVL,aVF	Increase all leads	Altered aVR,aVL	Decrease .04sec all leads except aVL	Elevated .15-.2mV II,III,aVF Depressed aVR	Increase .2-.5mV .04sec all leads	Inverted III,aVR,aVF
70	Decrease From To 214 167	No change	Increased Duration .02-.04sec I,II,aVR	"	Increase .01 sec aVR	Increase .2-.5mV II,III, aVR,aVF Decrease .3mV I	Altered II,aVR aVF	No change	Elevated .1-.15mV aVR,aVL Depressed .2-.3mV II,III,aVF	Increase .1-.3mV .02sec II,III,aVR,aVF	No change
63 20min. Intox	No change	"	Sl.Increased Duration I Sl.Decreased Duration III,aVF	"	No change	Increase .1mV I Decrease .2-.6mV II,III, aVL,aVF	No change	"	Elevated 1.-.15mV in I,II Depressed .1mV aVR	Decrease III,aVF	"
63 45min Intox	"	"	Increased Duration .05mV III	"	Increase .02sec aVL	Decrease .1-.2mV I,II,III aVR,aVF	Altered I	Decrease .02sec I,II	No change	Decrease .1-.3mV II,III,aVF	"
72	Decrease From To 214 167	Intermittent type II incomplete Heart Block & Sinus arrhythmia I,III,aVR,aVL,aVF Electrical Alterations II	No change	"	Increase .01-.04 sec II,III, aVR,aVL, aVF	Increase .2-.4mV in III, aVR,aVL Decrease .1mV I,aVF	Altered II,aVF	No change	Elevated .1mV aVR Depressed .1mV II & III	Increase .1-.2mV I,III,aVR,aVL, aVF	"
64	Decrease From To 214 75-100	Severe Sinus arrhythmia all leads Atrial Fibrillation aVF Ventricular Extrasystoles I	Increased Duration I,II,aVR,aVL aVF	Biphasic aVR,aVF Fibrillation aVF	Increase .02sec I Decrease .02-.04 sec. aVR,aVF	Decrease .2-.4mV all leads	Altered aVR ventricular Extrasystoles I	Decrease .02.04 sec. I, aVR,aVF Increase .04sec III	Elevated .1mV aVL,aVF	Increase .1-.3mV all leads	Biphasic II,aVR
75	Decrease From To 214 150	No change	No change	No change	Decrease .04sec I Increase .02sec II	Decrease .1-.2mV I,II,III aVF	Altered II	Decrease .04sec aVR,aVL Increase .04sec I	Depressed .1mV II	Increase .2mV II,III,aVR, aVL,aVF	No change
66	Decrease From To 232 201	"	Increased Duration .04sec III,aVF	Biphasic aVR Bifid III,aVL	Increase .04sec aVR,aVL	Decrease .4-.8mV II,III, aVR,aVF Increase .1mV I	Altered I,II aVR,aVF	Slight Increase II Slight Decreaee aVR	Slight Depression II	Increase .1-.2mV II,aVR,aVF	"
71	Increase From To 188 214	"	Increased Duration .04sec aVR	No change	No change	Decrease .1-.2mV I,II,aVR Increase .2mV III	Altered I,aVR aVL	Decrease .08-12 all leads	Elevated .1-.2mV I,II,III,aVF Depressed aVR	Increase .1-.3mV .08-.12sec all leads	"
61	Decrease From To 201 68	Severe Bradycardia	Increase .1mV .04sec II	"	Increase .02sec III	Decrease .2mV I,II,III aVL,aVF Increase .1mV aVR	Altered I,aVL	Increase .04-.08 sec. II,III, aVR,aVF	No change	Decrease .2mV .04-.08sec III,aVR	Biphasic II,aVR

ECG's recorded with lambs in right lateral recumbency.

the results obtained from this animal therefore need to be interpreted with caution, the overall pattern was similar to that obtained from the other two animals.

The notable alterations in the postinoculation tracings of these animals were as follows:

i. In contrast to the animals receiving parenterally administered toxin, the heart rate of the animals receiving enteric origin toxin increased.

ii. The changes in the P-R interval were inconclusive. It was decreased in the recordings from one animal, unchanged in one and increased in the recordings from animal 81.

iii. The form of the QRS complex was altered in one or more leads from all animals although the changes in amplitude of the complex varied considerably.

iv. The S-T segment was decreased in duration in all animals and showed marked displacement from the preinoculation level in two animals.

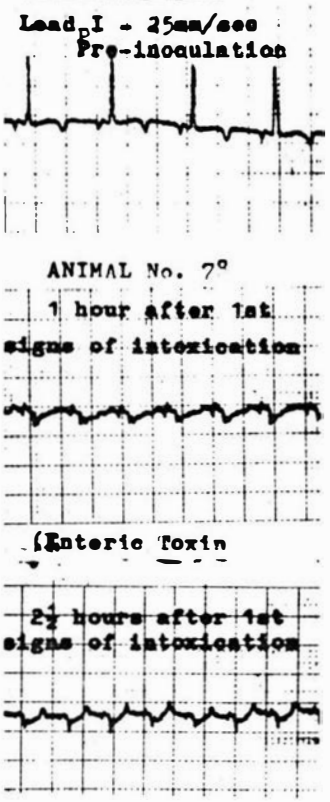
v. The T wave was increased in amplitude and showed an alteration in polarity in several leads in the latter two animals.

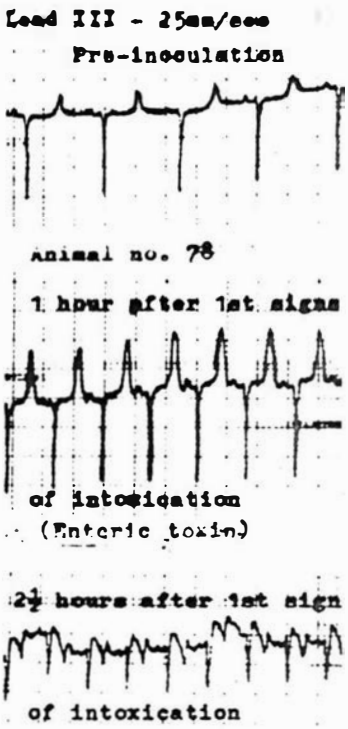
The changes in this group of animals are summarised in Table 7.1b and illustrated in Figure 7.2.

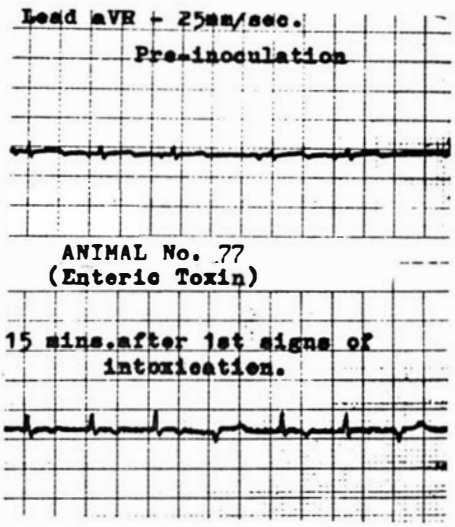
Discussion

It is interesting to note that mice, in contrast to lambs, do not usually show evidence of pulmonary oedema when lethal levels of epsilon toxin are administered intravenously over a short period of time. However, the presence of pulmonary oedema in mice which had received high concentrations of toxin implies that the lung capillaries of this species are not entirely resistant to this action of the toxin. In addition it can be seen from Chapters 5 and 6, that the lethal dose of toxin of 0.04 ug/Gm bodyweight for mice is approximately double the dose for susceptible lambs which, when calculated on a bodyweight basis is of the order of 0.02 ug/Gm B.W. It appears probable therefore that the difference between mice and lambs in the development of lung oedema may be the result of a species difference in the susceptibility of the lung capillaries to the action of epsilon toxin. The extent of the capillary endothelial damage in these two

Figure 7.2:


Lead I - 25mm/sec
Pre-inoculation
ANIMAL No. 76
1 hour after 1st signs of intoxication
(Enteric Toxin
2½ hours after 1st signs of intoxication


Lead III - 25mm/sec
Pre-inoculation
Animal no. 78
1 hour after 1st signs
of intoxication
(Enteric toxin)
2½ hours after 1st sign
of intoxication


Lead aVR - 25mm/sec.
Pre-inoculation
ANIMAL No. 77
(Enteric Toxin)
15 mins.after 1st signs of intoxication.

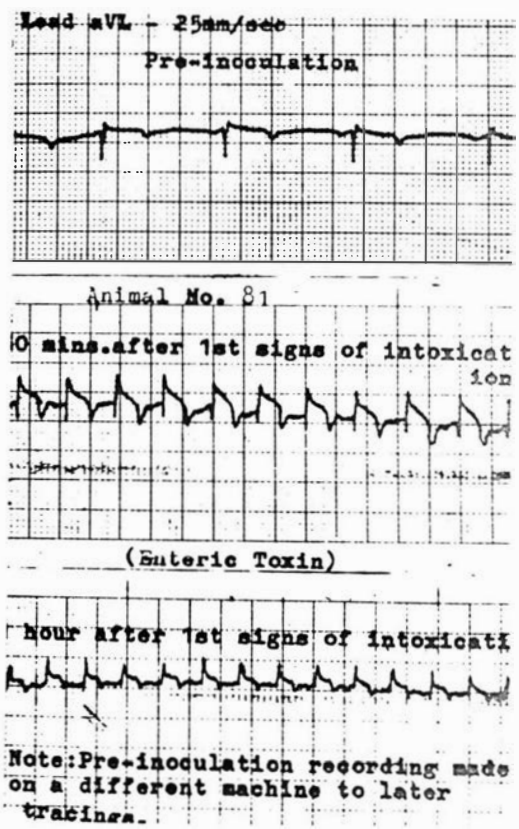

Lead aVL - 25mm/sec
Pre-inoculation
Animal No. 81
0 mins.after 1st signs of intoxicat
ion
(Enteric Toxin)
hour after 1st signs of intoxicati
Note:Pre-inoculation recording made on a different machine to later tracings.

Table 7.1b: The Effect of Experimental Cl. Perfringens Type D Enterotoxaemia on the Electrocardiogram of Lambs.

Animal	Rate	Rhythm	P Wave			QRS Complex		S-T Segment		T Wave	
	Beats/min		Size	Form	P-R Interval	Amplitude	Form	Duration	Position	Size	Polarity
Summary of Preinoculation values from lambs receiving Toxin of Enteric Origin											
n = 3	100-136	Regular or mild Sinus Arrhythmia	.05-.3mV .02-.04sec	+ve in III aVF variable in I,II,aVR aVL	.03-.06 sec	.3-1.4mV .04sec	I qrs,qRs or QRs II & III qrS or qRS aVR,qrS,qRS or QRe aVL,qRs,qrS aVF,qrS or qRS	.10-.20sec	Isoelectric (2) Depressed .1mV II,III aVF Lamb 81 Elevated .1mV aVR,aVL	.1-.3mV .04sec	+ve in II, III,aVF
Post Intoxication Alterations in ECG's of Individual Animals											
78 1 Hr. post intox	Increase From To 115 188	No change	No change	Biphasic II aVR	Decrease .02sec II	Decrease .6-1.0mV I, aVL Increase .2-1.0mV II,III, aVR,aVF	Altered I, aVR Bizarre Bifid Complex in I	Decrease .04-.12 sec all leads	Elevated .1mV in aVR	Increase .1-.7mV II,III,aVR, aVL,aVF	No change
78 2¼hrs post intox	Increase From To 115 214	"	"	No change	Decrease .02sec all leads	Decrease .8-.9mV I,aVL Increase .3-.7mV II,III aVR,aVF	Altered I,aVR	Decrease .08-.12 all leads	Elevated .2-.3mV II,III,aVF Depressed .2mV aVL	Increased	Inverted I,III,aVL Biphasic aVR,aVF
77	Almost unchanged 100-150	Sinus Arrhythmia in both pure and post intox ECG's	No detectable change	No detectable change (animal shivering)	No detectable change	Increase .1-.6mV all leads	Altered with interspersed low voltage complexes all leads	Decrease .04-.08 sec. all leads	No detectable change	No change	Unreadable
81 30min post intox	Increase From To 136 188	No change	Increased Duration .02sec I,II,III,aVR	No change	Increase .02sec aVL	Increase .1-.4mV I,aVR, aVF	Altered aVL	Decrease .04sec all leads	Elevated .1-.3mV in I,aVR,aVL Depressed .3mV II,III,aVF *	Increase .1-.8mV II,III,aVR, aVL,aVF	Inverted I,II,aVR
81 1hr. post	Increase From To 136 250	No change	Increased amplitude I,II,aVF	No change	Increase .02sec I	Decrease .2-.4mV II,aVL aVF	Altered I,II,III aVL,aVF	Decrease .04sec I,II,III aVR	Elevated .1mV I,aVL *	Increase .2mV aVF	Inverted I,II,aVR

* Relative to preinoculation level - not isoelectric line

ECG recorded with animals standing supported in sling.

species will be discussed more fully, along with other ultrastructural changes which occur during intoxication, in Chapters 8 - 12. The morphological basis for the clinical signs of neurological damage in mice will also be discussed in Chapter 8.

The absence of prominent clinical signs of neurological damage in many of the present animals is interesting, since convulsions etc. are usually prominent in the few field cases of enterotoxaemia which are seen alive. However, Griner (1961a) and Kellaway (1940) have reported that some of their lambs which received parenteral epsilon toxin died quietly. The fact that, in the present study, some animals receiving toxin of enteric origin also died quietly implies that clinical signs of neurological damage may not always occur in enterotoxaemia in the field.

While the presence of a fibrin-containing pericardial effusion is by no means pathognomonic of enterotoxaemia caused by Cl. perfringens type D its presence in some animals a relatively short time after toxin administration, implies that the extravasation of fluid must occur fairly rapidly. There must be damage to the vasculature of the serosal surface for fluid loss to occur and the presence of protein in the exudate suggests that this damage must be severe. Since haemorrhage is not a prominent feature of the gross lesions of enterotoxaemia and the effusions in the natural and experimental disease are very seldom blood stained, it appears that there is no complete loss of vascular integrity.

The presence of subendocardial haemorrhage does not contradict this statement as it occurs in a wide variety of conditions unassociated with vascular damage e.g. hypomagnesaemia of ruminants, and when depolarising muscle relaxants are used to immobilise horses. It is generally considered to be an agonal change unassociated with any specific action of the causative agent. It could be however that there is in fact a common underlying factor and that subendocardial haemorrhage is associated with abnormal myocardial function induced by the pre-existing condition. Thus in hypomagnesaemia of cattle and the use of some muscle relaxants in horses, electrocardiographic evidence of severe interference with myocardial function has been described. In this respect it is interesting to note that the electrocardiographic changes encountered in the present study

were considerably more prominent than those encountered by Kellaway et al. (1940) when using parenterally administered epsilon toxin. The main electrocardiographic changes which they described in intact sheep were bradycardia and T wave inversion. In rabbits they also recorded S-T segment displacement. The presence of a small but fairly consistent fall in heart rate in the animals receiving parenterally administered toxin implies that there is some increase in vagal tone under these conditions. On the other hand the fact that the heart rate increased markedly in the animals which received toxin of enteric origin implies that the effect on heart rate of parenterally administered toxin may, as Kellaway et al. (1940) suggest, be the result of impurities in the toxin preparation, and that these are either not absorbed from the intestine under normal circumstances, or else are removed from the blood as it passes through the liver.

Nevertheless epsilon toxin does appear to have a pronounced direct effect upon the heart in all intoxicated animals. The alterations in the S-T segment and T wave of the ECG's are of particular interest as these portions of the cycle are considered to be associated with the repolarisation of the myocardial fibre; but it is more convenient to defer any further discussion of these aspects of the electrocardiograms until the morphological changes which occur in the myocardium have been described in Chapter 9.

Before leaving the present chapter however it is of interest to compare the ECG patterns obtained in the animals which received parenterally administered toxin with the gross lesions which have been discussed so far. Of the four animals in which there was evidence of severe bradycardia or incomplete heart block, three showed severe pulmonary oedema with the lungs weighing 380, 420 and 410 grams respectively. Of the animals in which changes in cardiac rhythm were absent six did not have obvious lung oedema, the lungs weighing 220 grams or less in all instances. These relationships are not absolute however since one animal with severe lung oedema did not show any evidence of conduction disturbance and one animal with no lung oedema showed severe heart block.

Conclusions

1. The most prominent clinical signs of epsilon toxin intoxication in mice are neurological disturbances including severe depression and convulsions.

2. No grossly detectable lesions occur in mice which receive low lethal levels of toxin but pulmonary oedema occurs when high concentrations of toxin are given.

3. Differences in the susceptibility of mice and lambs to the toxin on the basis of absolute dosage and susceptibility to the development of pulmonary oedema imply that there may be a species difference in the susceptibility of the capillary endothelium to the action of epsilon toxin.

4. Some of the clinical signs of intoxication in lambs depend upon the type of change produced in the tissues. Dyspnoea only occurs if pulmonary oedema develops. Signs of neurological damage were present in some animals only, suggesting that not all field cases of enterotoxaemia show these signs.

5. In the absence of pulmonary oedema the most notable gross lesions in intoxicated lambs, at the time of death, were fluid and fibrin in the pericardial sac and subendocardial haemorrhages. The former may be associated with vascular damage in the serosal vessels and the latter with disturbances in myocardial function.

6. Distinct alterations occur in the electrocardiograms. The bradycardia which occurs when toxin is administered parenterally may be due to substances other than epsilon toxin. Arrhythmias occur in a proportion of the animals but the most consistent changes are alterations in the depolarisation and repolarisation patterns of the ventricular myocardium.

7. The electrocardiographic changes are only broadly related to the pattern of development of pulmonary oedema.

CHAPTER 8: THE MORPHOLOGICAL CHANGES PRODUCED IN THE CENTRAL NERVOUS SYSTEM DURING CL.PERFRINGENS TYPE D EPSILON TOXIN INTOXICATION

Many of the clinical signs of enterotoxaemia are referable to the central nervous system and it is now appropriate to proceed to a closer study of it, since previous workers have described morphological changes in the brains of intoxicated animals.

Comprehensive studies on the progression of the lesions which occur in the brains of intoxicated mice and lambs have been published by Griner (1961a). On the basis of these studies, and the results of experiments where radioactive tracers were used to detect altered vascular permeability, (Griner 1961b) he suggested that the early changes in the brain are due to brain oedema following vascular damage produced by epsilon toxin. One of the criteria used for judging the presence of oedema was total brain wet weight. However, in five of the six pairs of intoxicated and control lambs, which he used, the animal receiving the toxin was also heavier on a body-weight basis. While Green (1966) states that the brain wet weight and total bodyweights of adult mice are uncorrelated, brain weight in this species is highly heritable and varies between strains. The brains of female animals are heavier in relation to bodyweight than those of males and the water content of the brain is also stated to be age dependent although the decrease with increasing age is small. It was therefore considered to be advisable to determine the actual water content of samples of brain tissue in all quantitative experiments on brain oedema.

Although Griner's light microscopic studies have provided useful information on the development of the lesions in the brain during intoxication, the finer morphological aspects of brain oedema are best examined with the electron microscope. Much of the confusion about brain oedema as it occurs experimentally and in man, has been clarified by ultrastructural studies of the condition. For example Wechsler*etal* in Klatzo and Seitelberger (1967), have shown that, in grey matter, excess fluid accumulates intracellularly within the astrocytes. By using electron histochemical techniques

employing exogenous peroxidase as a tracer

it has also been shown that there is a true extracellular space in the brain and that there are complete 'tight junctions' between adjacent endothelial cells which probably contribute to the so-called 'Blood-Brain Barrier'.

Three papers which describe recent advances in this field, derived from a symposium on the blood-brain barrier held in New York in 1966, are those of Hirano et al (1970), Brightman et al (1970) and Pappas (1970).

The relevance of these factors to the changes which occur in enterotoxaemia will be examined in the present study as it appears probable that the accumulation of fluid in the 'Space of His' described by Griner is in reality a swelling of astrocyte foot processes around blood vessels, the 'Space of His' now being regarded as an artefact. Damage to vascular endothelium by the toxin could also have a profound influence on the permeability of the 'Blood-Brain Barrier' and account for the loss of tracer substances from the bloodstream into the tissues in this region.

Griner did not consider that tissue hypoxia was a major factor in the production of the cerebral lesions, mainly because of the absence of cortical lesions and haemorrhage which are common in so-called anoxic damage. There is however gross focal haemorrhage in the brain stem in most cases of focal symmetrical encephalomalacia (Hartley 1956). It is also appropriate to observe at this stage that the brain oedema which develops in accidents during anaesthesia in man is now considered to result from a combination of transient anoxia and hypercapnia, brought about by hypoxia and ischaemia, rather than being due to hypoxia alone (Bakay in Klatzo and Seitelberger, 1967). Despite these considerations it was felt that prolonged critical hypoxia might produce morphological damage in the brain which could be compared with the action of epsilon toxin.

Because vascular damage could lead to localised ischaemia and anoxia in the brain, and Becker (1961) has shown that the histochemical staining affinity of nervous tissue for a number of

enzymes is reduced under these conditions, it was decided to study the histochemical activity of several enzymes in the brains of intoxicated animals.

Quantitative Aspects of Alterations in the Fluid Balance in the Brains of Intoxicated Mice

Preliminary Experiment

Materials & Methods:

Thirty six 6-8 week old female mice were divided into two groups. One of these groups received 0.2ml of batch ANZD toxin diluted 1:200 with saline (equivalent to 1ug batch CWD toxin) intravenously, and the other (control) group received 0.2ml of nutrient broth containing 0.5% phenol diluted 1:200 with saline by the same route.

One hour after inoculation, all the control mice were killed by neck dislocation and the brains removed. Brains of the intoxicated animals were removed as soon as the animal died. Each brain was divided in half longitudinally and one half was placed on a preweighed 4cm. diameter plastic petri dish, weighed and freeze-dried for 24 hours in a 'Speedivac' centrifugal freeze drier model 5PS (Edwards High Vacuum Ltd., Crawley, England) with phosphorus pentoxide as dessicant. The sample was then re-weighed and the water content of the tissue calculated and expressed on a dry matter basis.

Results:

The mean water content of the brains of intoxicated mice was higher than that of control mice and the water content in the samples from the former animals also increased with increasing survival time. The data from this experiment is tabulated in Appendix 7 and illustrated by the histograms in Figure 8.1a.

Main Experiment

Materials & Methods

Sixty 6-8 week old male mice and sixty 6-8 week old female mice were each divided into two equal groups. One group of each sex received phenol broth saline and the other group received batch ANZD toxin at the same dose rate and by the same route as in the preliminary experiment. In addition five animals of each sex were given 5ml of distilled water intraperitoneally to produce a known brain oedema (Zeman & Kalsbeck in Klatzo & Seitelberger, 1967).

A control animal of the same sex was sacrificed whenever one of the epsilon toxin intoxicated animals died. All brains were removed and treated in the same manner as in the preliminary experiment.

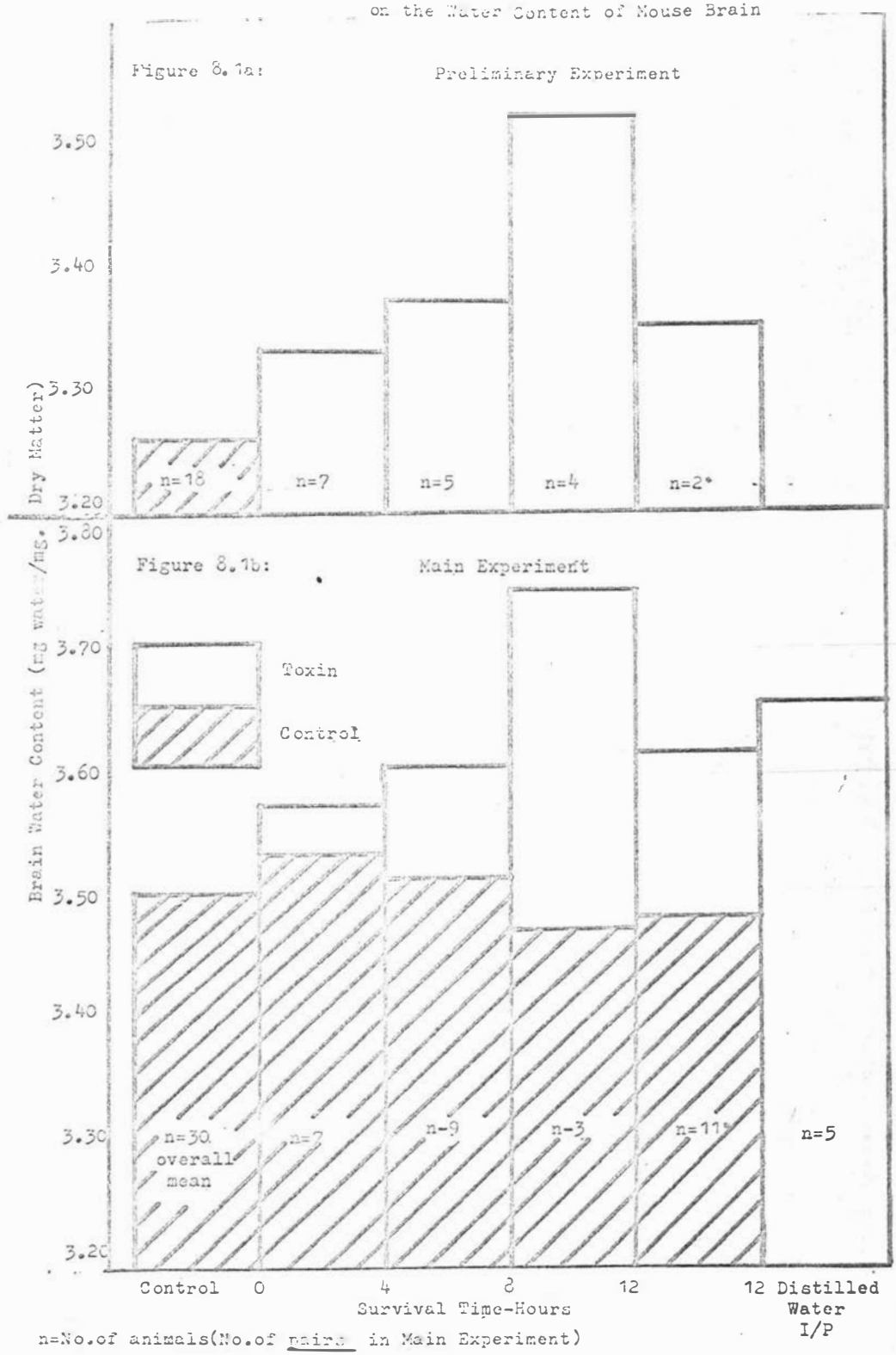
Results:

All mice which had received the intraperitoneal injections of water died within one hour of inoculation. The mean water content of the brains of these animals was greater than that of the control animals of the same sex. Intoxicated female mice also had a higher mean water content than the control females and, as in the preliminary experiment, this increased with increasing survival time.

On the other hand, the pattern in the male mice was not as clear cut. Although there was a progressive increase in water content of the brains of intoxicated animals with increasing survival time, it was not until more than 8 hours had elapsed after inoculation that the mean water content of the brains from the intoxicated group exceeded the paired control value. By that time the number of surviving animals was very small and the results are not statistically significant.

Data from this experiment is given in Appendix 7 and the pattern of change in water content in the brains of the female mice is illustrated by the histograms in Figure 8.1b.

Figure 8.1: The Effect of Epsilon Toxin Intoxication on the Water Content of Mouse Brain



n=No.of animals(No.of pairs in Main Experiment)

* Includes some animals which survived for more than 24 hours and were destroyed.

Quantitative Aspects of Alterations in the Fluid Balance in the Brains of Intoxicated Lambs.

Materials & Methods:

A similar procedure to that described for mice was used to establish the water content of the brains of control lambs which had received parenterally administered or enteric-origin epsilon toxin (see Chapter 6). In this instance however, due to the size of the brains, individual samples weighing approximately 0.5gram were taken from the same area of the cerebral cortex, thalamus and cerebellum of each animal. Because water content of brain tissue decreases with age, the water content of these areas of the brains from 5 normal 8 month old lambs was established and used as control data for the three 8 month old animals which had absorbed enteric-origin toxin, and succumbed.

Results:

The mean water content of all areas of the brains of the 8 month old lambs was lower than the value for the same area of the 2-3 week old lambs.

No significant increase in mean water content could be detected in any of the areas of brain from lambs which had received parenterally administered or enteric-origin toxin, when compared with values for similar control animals.

The lamb data from these experiments are given in Appendix 8a & b and summarised in Table 8.1.

Table 8.1:

The Mean Water Content of Different Areas of the Brains of Control and Intoxicated Lambs

Age of Lambs: 2-3 weeks	Region of Brain		
	Cerebral Cortex	Thalamus	Cerebellum
	Mean Water Content (mg H_2O/mg dry matter) ± 1std deviation		
Control Lambs (10)	4.43 ± 0.13	4.10 ± 0.33	4.11 ± 0.33
Intoxicated lambs (31) (parenterally administered)	4.33 ± 0.08	4.05 ± 0.08	3.94 ± 0.14
Age of lambs: 8 months			
Control lambs (5)	3.83 ± 0.55	2.08 ± 0.37*	3.72 ± 0.18
Intoxicated lambs (3) (Enteric-origin)	3.39 ± 0.28	3.53 ± 0.34*	3.64 ± 0.30

* t test - p = < .005 - the control value may be anomalous - see Appendix 8b

The Morphological Changes seen in the Brains of Intoxicated Animals During Electron Microscopic Examination.

Materials & Methods:

Sections of cerebral cortex, thalamus and cerebellum were taken from mice which survived for different periods of time in the foregoing experiments. Small ($\frac{1}{2}$-1mm) cubes of these tissues were placed in cold 3% glutaraldehyde in 0.1M phosphate buffer at pH7.2 and held at 4°C overnight. The tissues were then post fixed in buffered 1% osmium tetroxide at 4°C, dehydrated in an ethanol series and infiltrated and embedded in epoxy resin (Fluka ACM) via a propylene oxide series as recommended by Luft (1961). Sections were cut with glass knives on an LKB ultramicrotome and stained with aqueous uranyl nitrate and lead citrate, (Reynolds, 1963). General descriptions of these embedding and staining techniques are given by Glauert in Kay (1965).

The sections were then mounted unsupported on copper grids and examined in a Philips EM200 electron microscope using an anode voltage of 60Kv. The electron photomicrographs were taken on Kodak FRP426 Finegrain positive film and developed and printed by conventional photographic techniques. Similar material was taken from representative control and intoxicated lambs and processed in the same way.

Results:

In mice, the earliest changes detectable by electron microscopy occurred in the vascular endothelium and the astrocytic 'foot' processes surrounding the capillaries. Swelling of the foot processes was detectable in some cases as early as three hours post-inoculation and became progressively more severe as time of survival increased (Figure 8.2.) The swelling of the astrocytes was not confined to the foot processes alone. Figure 8.3, which is series of low magnification electron photomicrographs of the thalamic neuropil, from control mice and those surviving for increasing periods of time, clearly demonstrates the progressive swelling of astrocytic processes in this region. In the cerebellum the swelling of the astrocytes served to differentiate them from other cell types which were unaffected by the toxin (Figure 8.4). There was marked swelling of astrocytic processes around neurones as well as around the capillaries in intoxicated animals (Figure 8.5).

Although the changes described above are the most prominent, there is also evidence of severe vascular endothelial damage (Figure 8.6). The lesions in the endothelium were more easily seen in the tissues from intoxicated lambs however and will therefore be described shortly.

Both of the above types of change seen in mice, i.e. astrocytic swelling and endothelial damage, were also seen in the lambs which received toxin by parenteral or enteric routes. The close resemblence between the lesions in this species and those described above, can be clearly seen by comparing Figure 8.7a, which shows the lesions in lamb thalumus with the lesions in the same area

of mouse brain illustrated in Figures 8.2, 8.3 and 8.6. The extremely severe damage which occurred in the capillary endothelium of intoxicated lambs can be seen in Figure 8.7b. In intoxicated animals the endothelium can be seen to be reduced to a thin, electron dense band in which no subcellular architecture or structures such as mitochondria, ribosomes etc., can be detected. There were also discontinuities in the endothelial lining of vessels suggesting that the intercellular junctions had separated. There were no detectable alterations in the capillary basement membranes. In both mice and lambs the neurones of intoxicated animals were unaffected.

The Use of Horse Radish Peroxidase as an Indicator of Altered Capillary Permeability in Intoxicated Animals.

Materials & Methods:

Three mice were given 2 ug of batch CWD epsilon toxin followed by 10mg of horse radish peroxidase (100-150 purpurogallin units/mg - Sigma Chemical Co., St. Louis, U.S.A.) in saline intravenously. A similar group of control mice were given the same dose of peroxidase only. The control mice were killed when the intoxicated mice died 1, 3 and 5 hours after inoculation.

In addition, two 3 week old female lambs, with less than 0.08 units of epsilon antitoxin/ml of serum, were each given 1mg of batch CWD epsilon toxin followed by 100mg of peroxidase intravenously and two similar lambs received the same dose of peroxidase only. The latter pair of lambs and a lamb which had not received either toxin or peroxidase were killed with intravenous 'Euthatal' (May & Baker, Dagenham, England) when the 'toxin' lambs died, 1-2 hours after inoculation.

Very thin (0.1mm) slices of cerebral cortex, thalamus and cerebellum from all these animals were fixed in cold 3% glutaraldehyde solution overnight then stained histochemically by the method of Karnovsky (1967) to demonstrate peroxidase. Sectioning and uranyl nitrate/lead citrate staining were carried out

Figure 8.2: Swelling of Astrocyte Foot Processes around Capillaries in the Thalamic Region of the Brain in Intoxicated Mice.

A B

C D

A. Control:

The capillary endothelium (En) appears normal and the astrocyte foot processes (AFP) are not prominent.

B. Three Hours Post-inoculation:

The capillary endothelium is reduced in thickness and there is some cytoplasmic vacuolation. There is little change in the astrocyte foot processes.

C. Six Hours Post-inoculation:

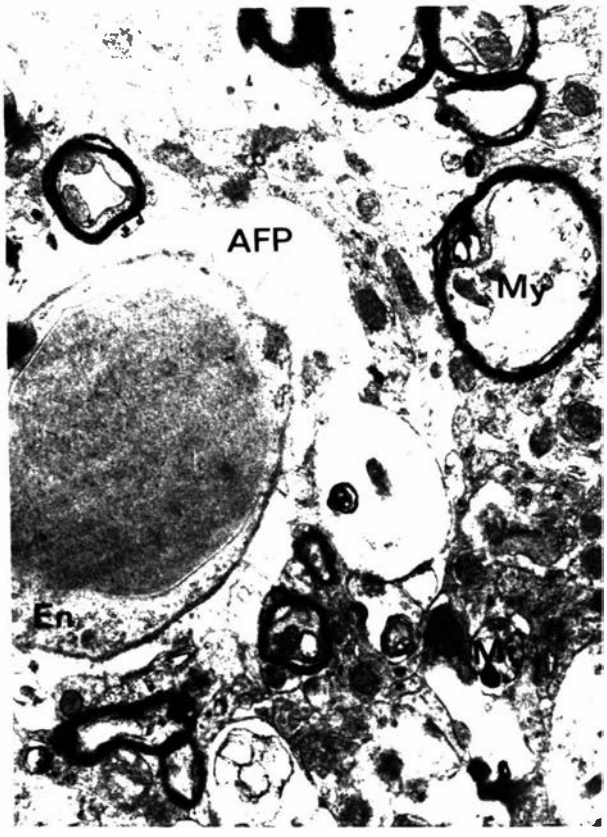
Extremely severe capillary endothelial damage is present. The endothelium has been reduced to a thin, electron-dense band. The astrocyte foot processes are very distended.

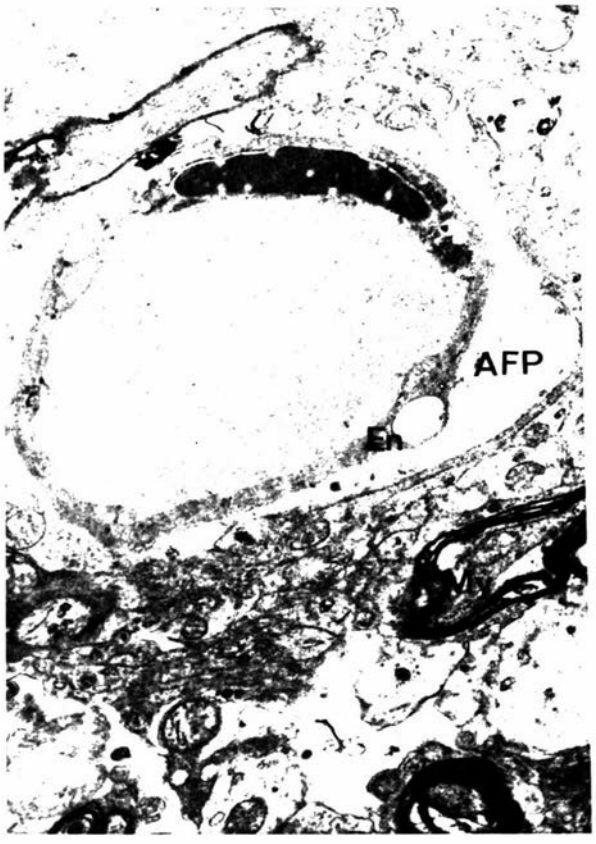
D. Twelve Hours Post-inoculation:

The changes are similar to those in C with further swelling of the astrocyte foot processes.

My = myelinated nerve fibres
Mi = mitochondria
N = nucleus of endothelial cell

Magnification 11,200


AFP
My
En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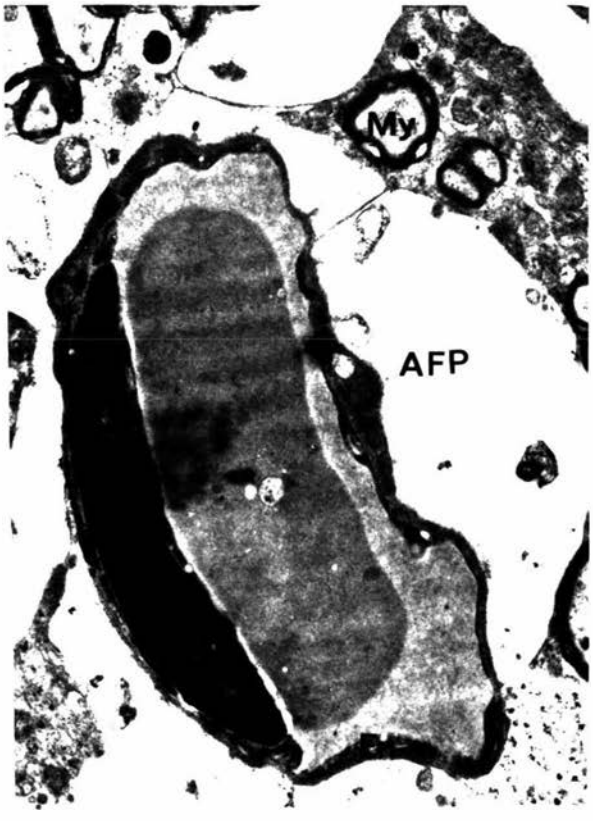

My
AFP

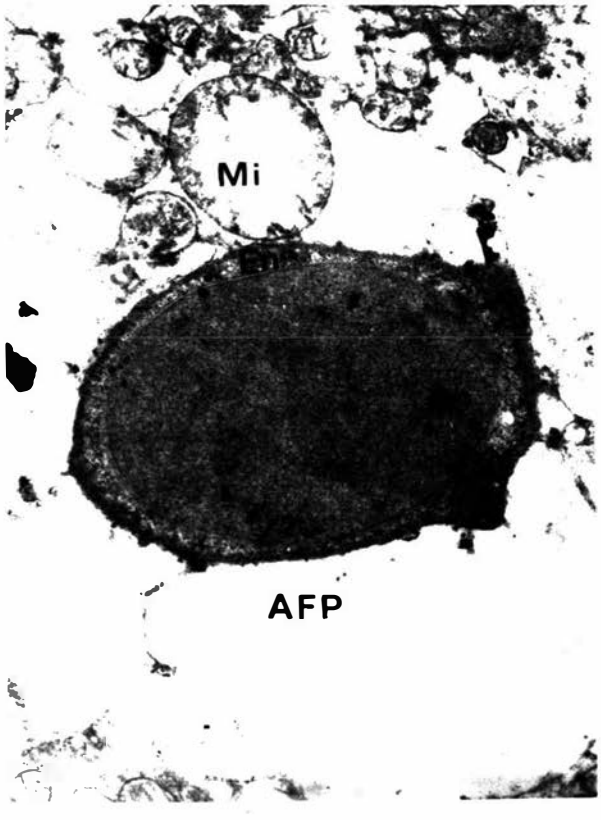

Mi
AFP

Figure 8.3: Swelling of Astrocyte Processes in the Thalamic Neuropil of Intoxicated Mice.

A B

C D

A. Control:

This is an area of normal neuropil from a control mouse showing the mass of glial and nerve cell processes which constitute the neuropil.

N = glial cell nucleus. The cell is probably protoplasmic astrocyte.

B. Six Hours Post-inoculation:

Swelling of astrocyte processes (AP) has developed, giving the neuropil a lacy appearance.

C. Twelve Hours Post-inoculation:

The swelling of astrocyte processes has become more pronounced.

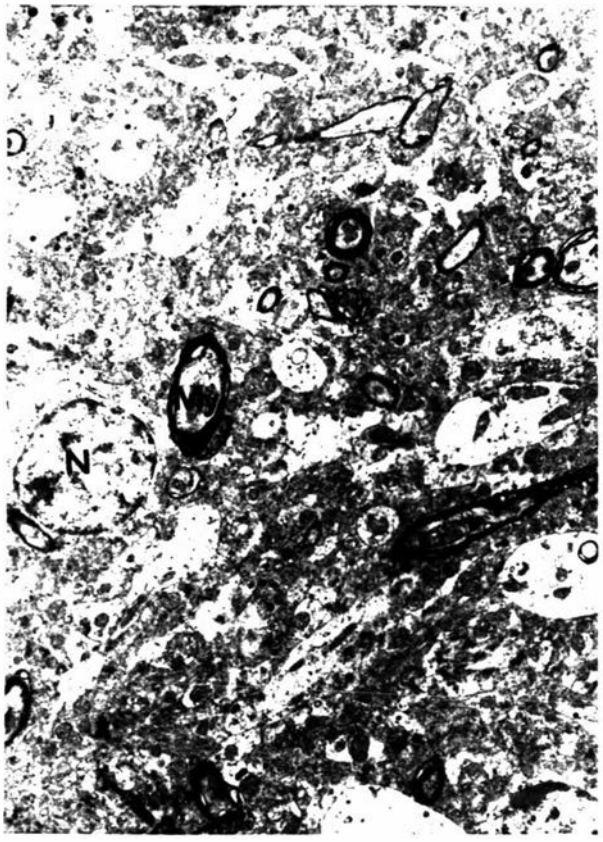
D. Eighteen Hours Post-inoculation:

The swollen astrocyte processes occupy almost the entire neuropil in this field.

Ne = portion of a neurone.

My = myelinated nerve fibres.

Magnification 4,450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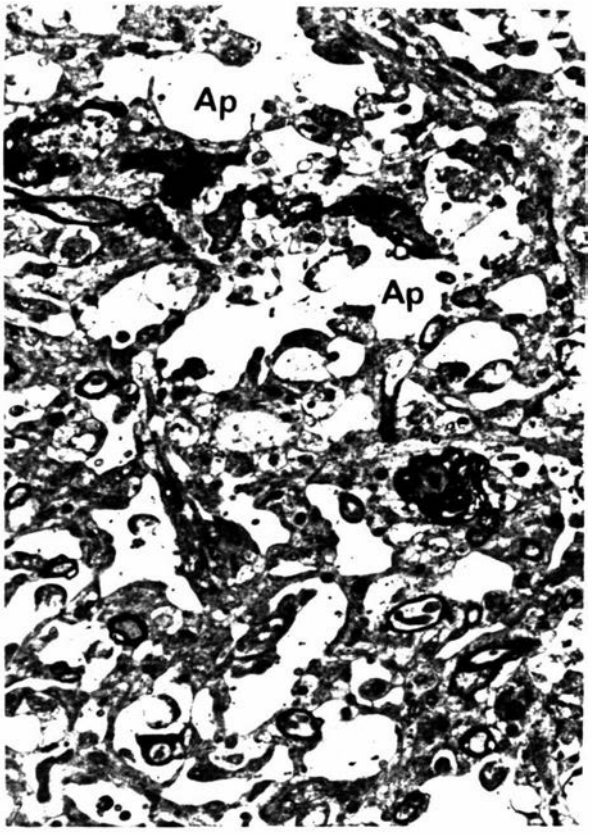
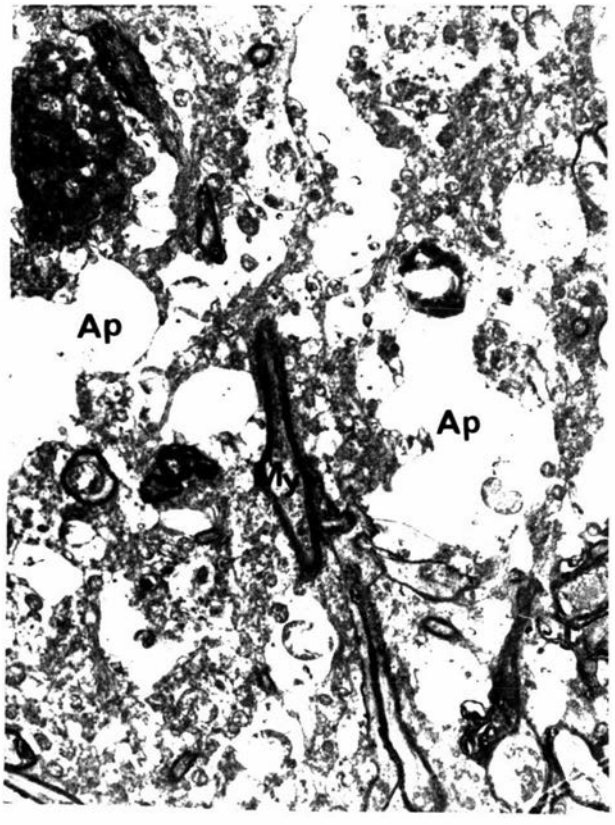

Ap
Ap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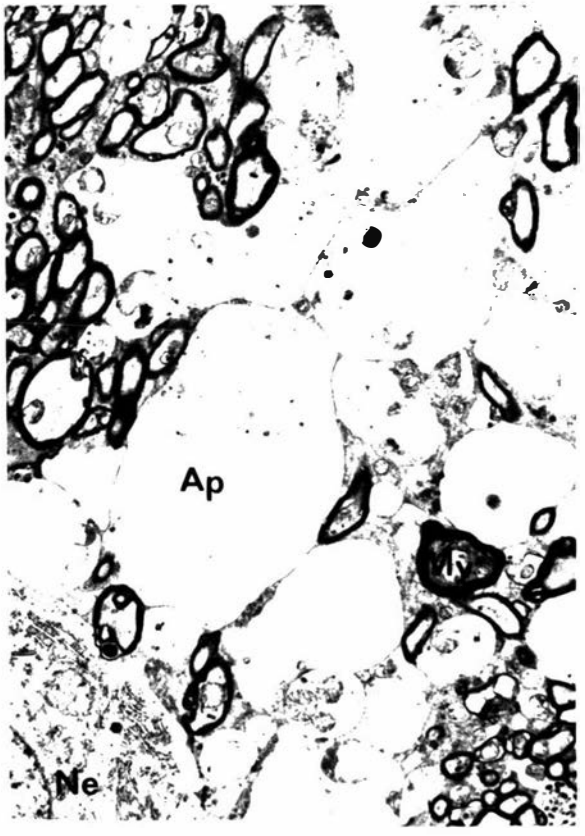

Ap
Ne

Figure 8.4: Differential Swelling of Protoplasmic Astrocytes in the Cerebellar Granular Layer of Intoxicated Mice:

A

B

A. Portion of the Granular Layer of the Cerebellum from a Normal Mouse:

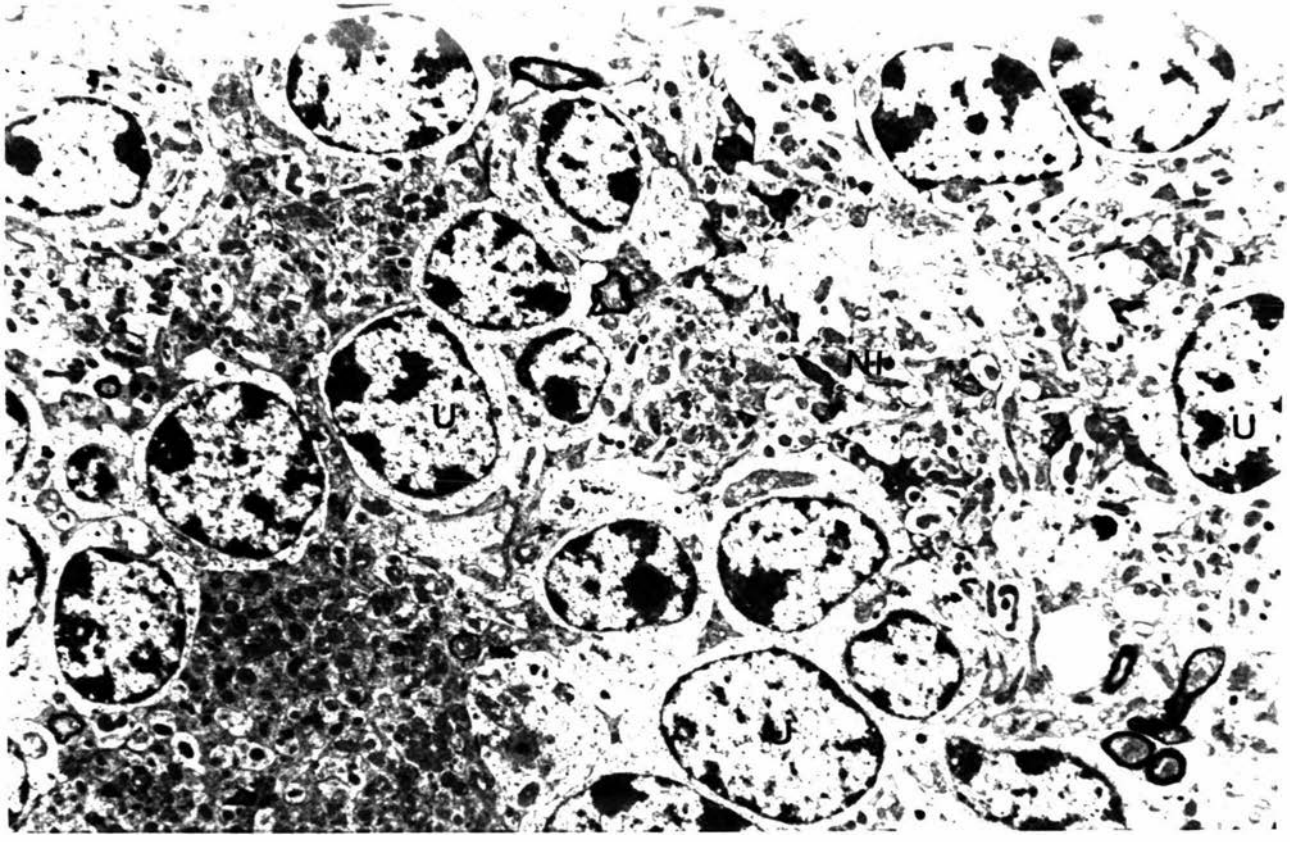
Nl = neuropil

U = unidentified cells of the cerebellar granular layer

B. Portion of the Cerebellar Granular Layer of a Mouse which died 6⅓ Hours after the Parenteral Administration of Epsilon Toxin:

There is marked swelling and probable early degeneration of a protoplasmic astrocyte (As) and also swelling of astrocyte processes (AP) in the neuropil.

Magnification 4,250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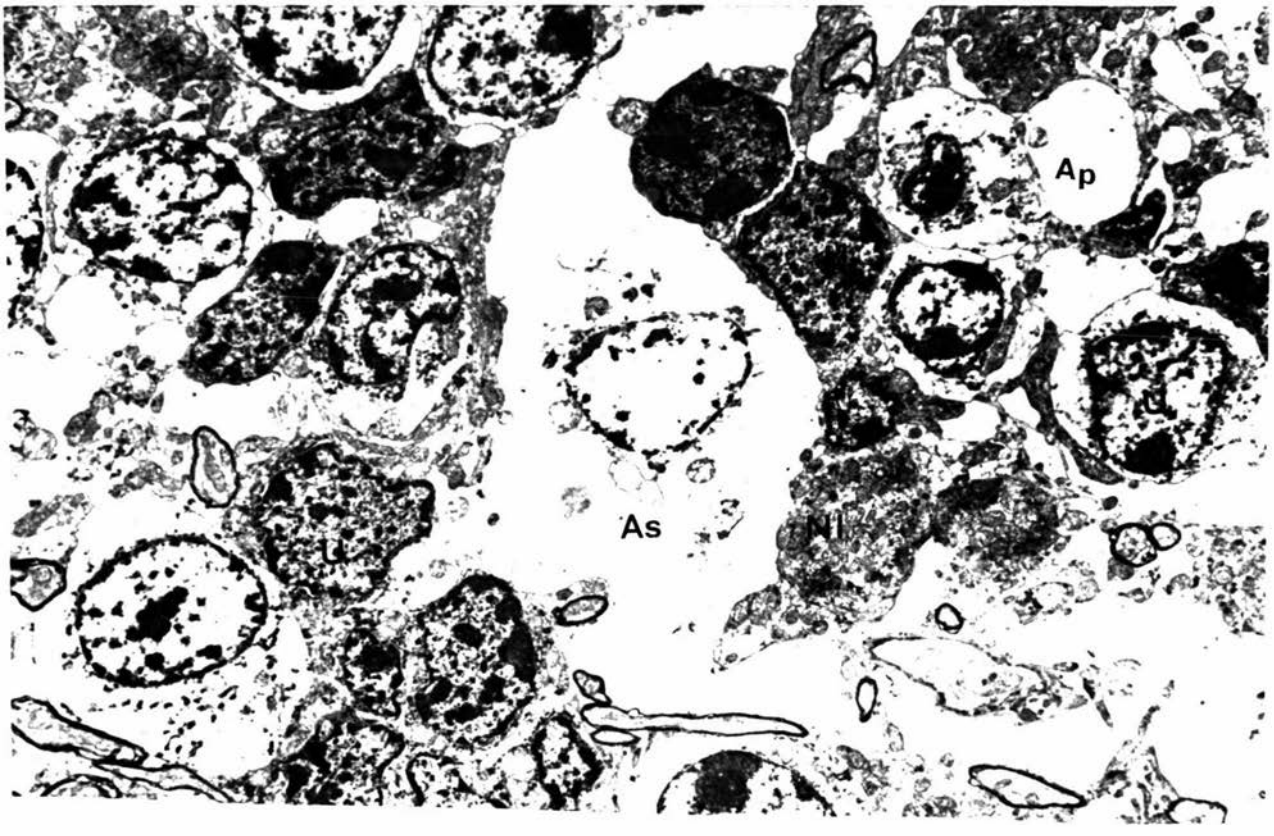

Ap
As

Figure 8.5: Swelling of Astrocyte Processes around Neurones in the Thalamic Neuropil of Intoxicated Mice.

A B

C D

A. Control:

Portion of a large normal neurone (Ne) and the adjacent neuropil.

No = nucleolus

N = nucleus

ER = endoplasmic reticulum

Gc = golgi cisternae

Mi = mitochondria

Ly = lysosomes

V = vacuoles

My = myelinated nerve fibres

B. Three hours Post-inoculation:

Very little change from the normal appearance but probable early swelling of astrocyte processes (AP) adjacent to the neurone.

C. Six Hours Post-inoculation:

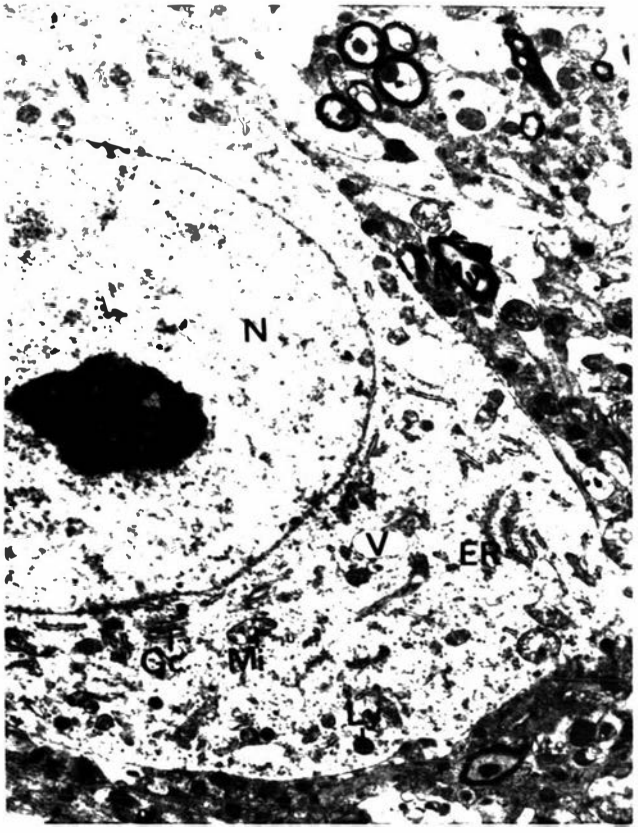
Very marked swelling of astrocyte processes adjacent to a neurone as well as in the neuropil. Note the close proximity of the plasmalemma of these processes to that of the neurone.

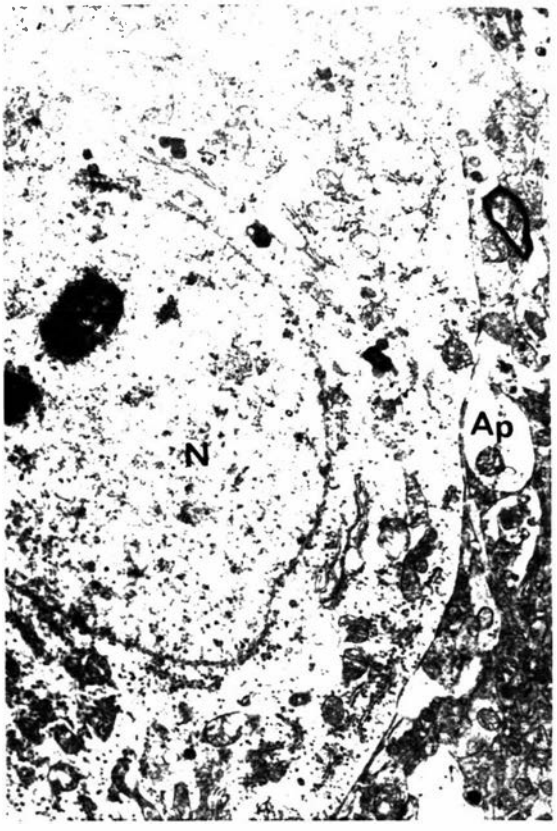
S = Satellite cell

D. Eighteen Hours Post-inoculation:

Most severe swelling of astrocyte processes adjacent to a neurone. There is little or no evidence of degenerative change in the latter cell.

Magnification 4,700


N
V
ER
G
M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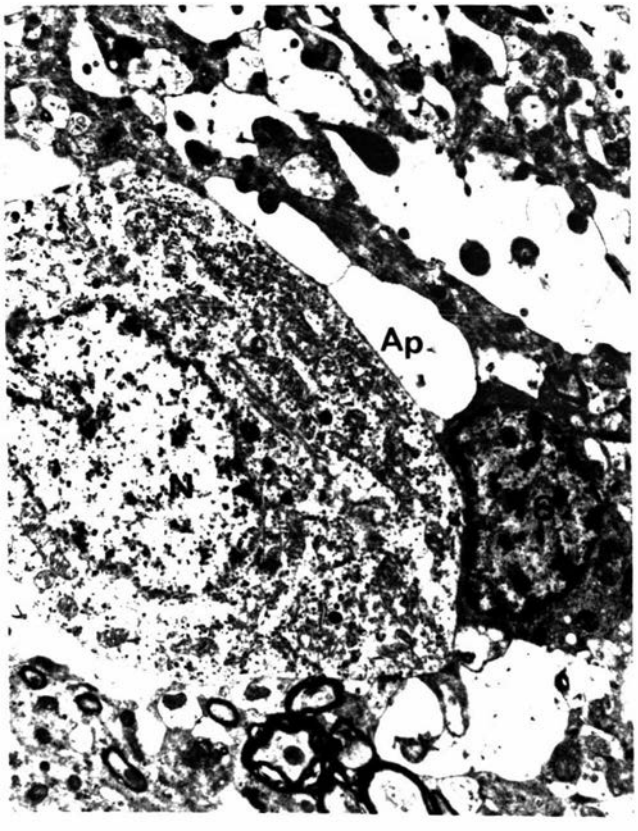
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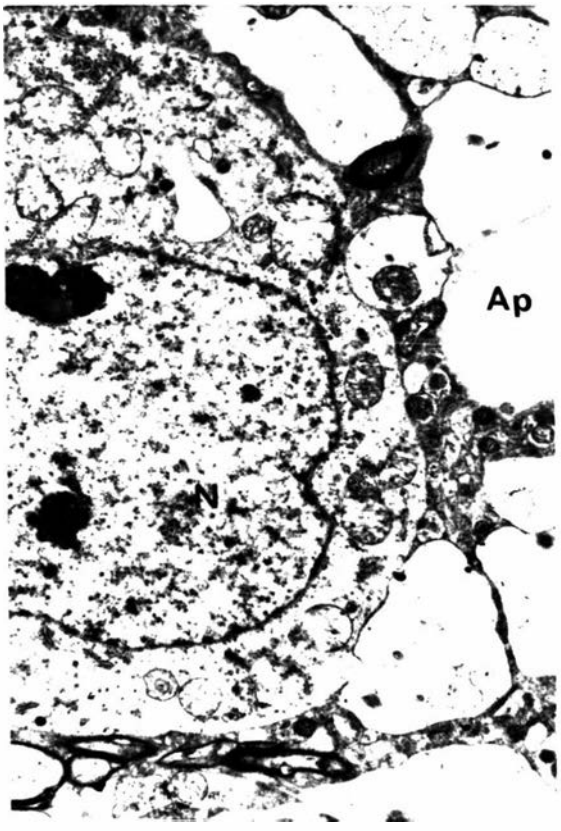

Ap
N

Figure 8.6: Vascular Endothelial Damage in the Thalamic Region of the Brains of Intoxicated Mice.

A B

C D

A. Control:

The appearance of a normal capillary in this region of the brain. The endothelial cytoplasm (En) contains numerous ribosomes and occasional mitochondria, vacuoles and lysosomes.

BM = basement membrane

E = erythrocyte

AFP = astrocyte foot processes

B. Capillary from Intoxicated Mouse Brain:

The cytoplasm of the capillary endothelium has become reduced to a thin, electron-dense band containing occasional cytoplasmic organelles. The surrounding astrocyte foot processes are very swollen.

C. Control:

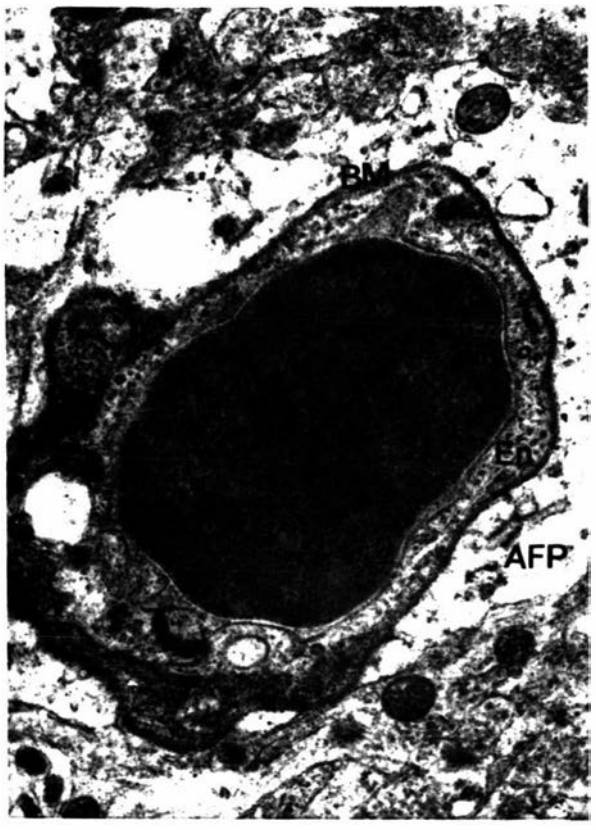
Higher magnification showing the cytoplasm of the capillary endothelium in greater detail and illustrating a 'tight' junction (Tj) between two adjacent endothelial cells.

D. Capillary from Intoxicated Mouse Brain:

This is an area of capillary at the same magnification as C. There is almost complete loss of recognisable endothelial cytoplasm.

Magnification A & B 18,000

C & D 24,000


BM
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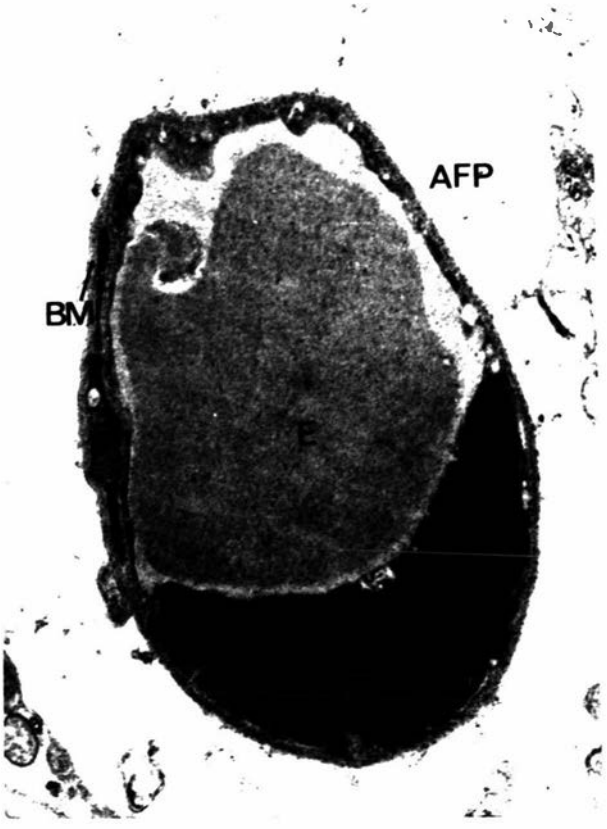
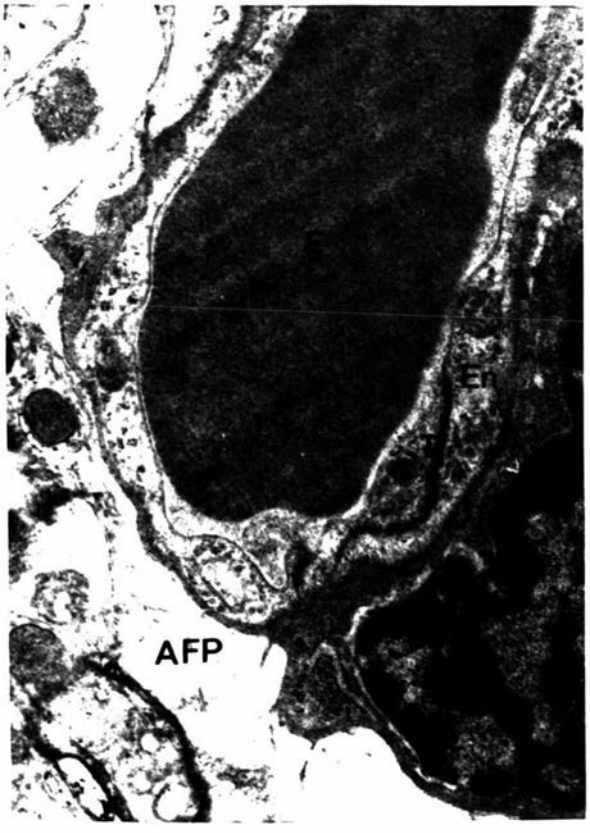

AFP
BM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AFP

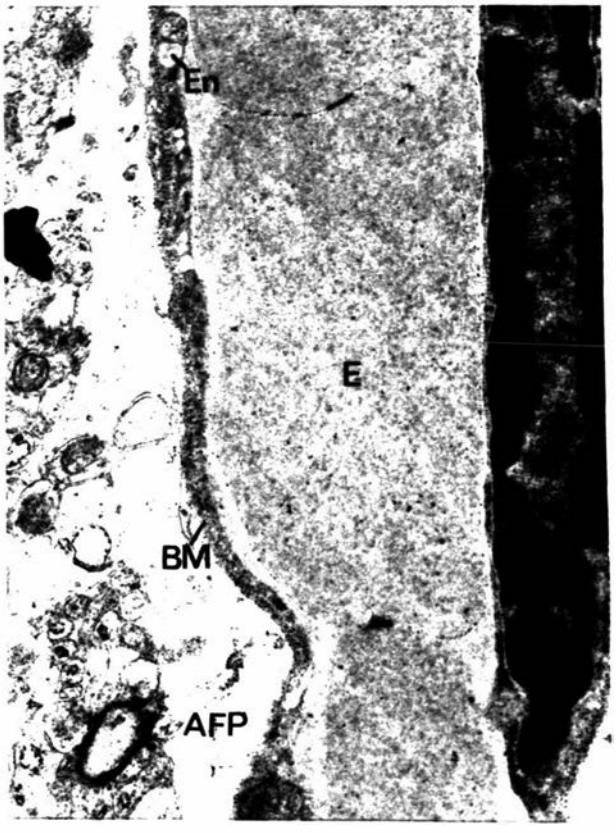

En
E
BM
AFP

Figure 8.7a: The Development of Brain Oedema in Intoxicated Lambs

A B

C D

A. Control Capillary:

A normal capillary and adjacent neuropil in the thalamus of a lamb.

En = endothelial cytoplasm

N = nucleus

BM = basement membrane

E = erythrocyte

B. Capillary from Intoxicated Lamb:- Parenteral Toxin

There is severe capillary endothelial damage and swelling of adjacent astrocyte foot processes (AFP).

C. Control Neuropil:

The appearance of the thalamic neuropil in a normal lamb is similar to that of mice (see Figure 8.3).

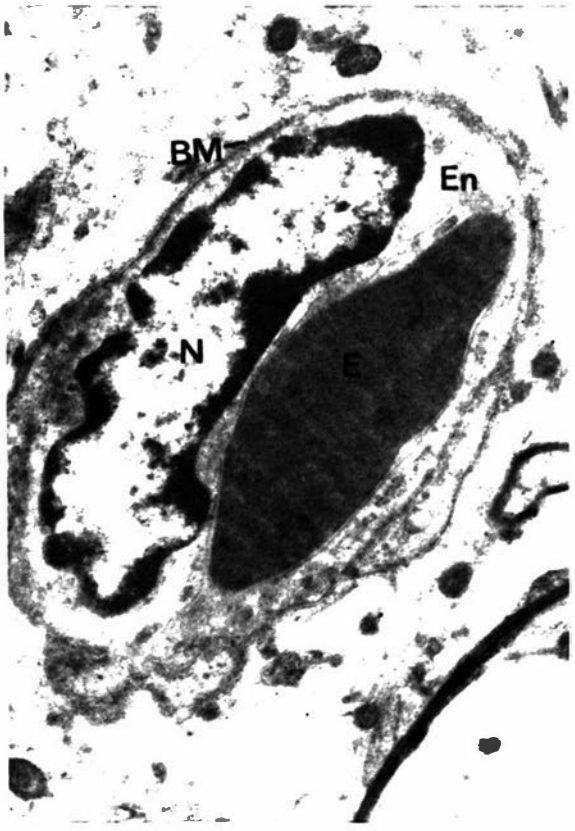
BV = Small capillary with normal endothelium.

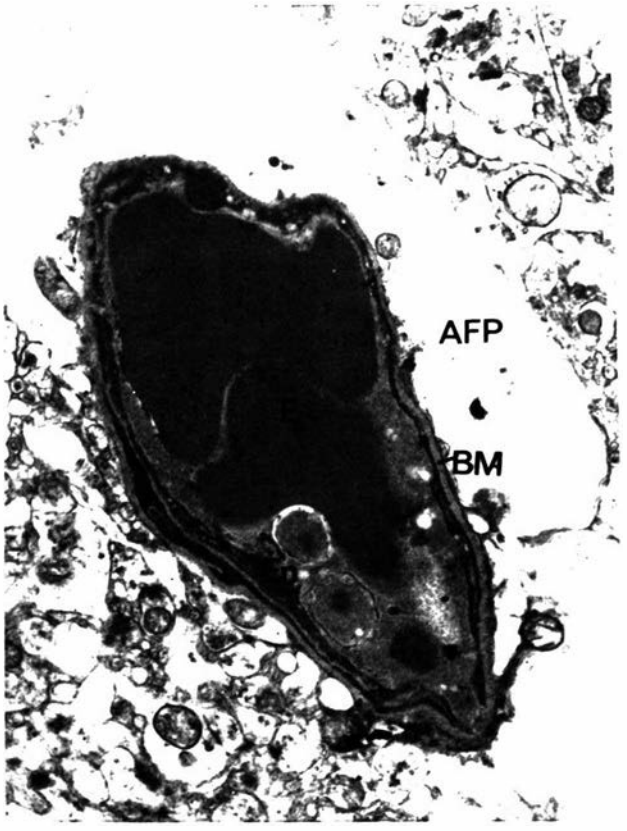
D. Thalamic neuropil of an Intoxicated Lamb:- Enteric Toxin

Swelling of the astrocyte processes (AP) has given the neuropil a lacy appearance. The endothelium of the small capillary which is visible in this figure is damaged.

Magnification A & B 9,500

C & D 3,950


BM
En
N
E


AFP
BM

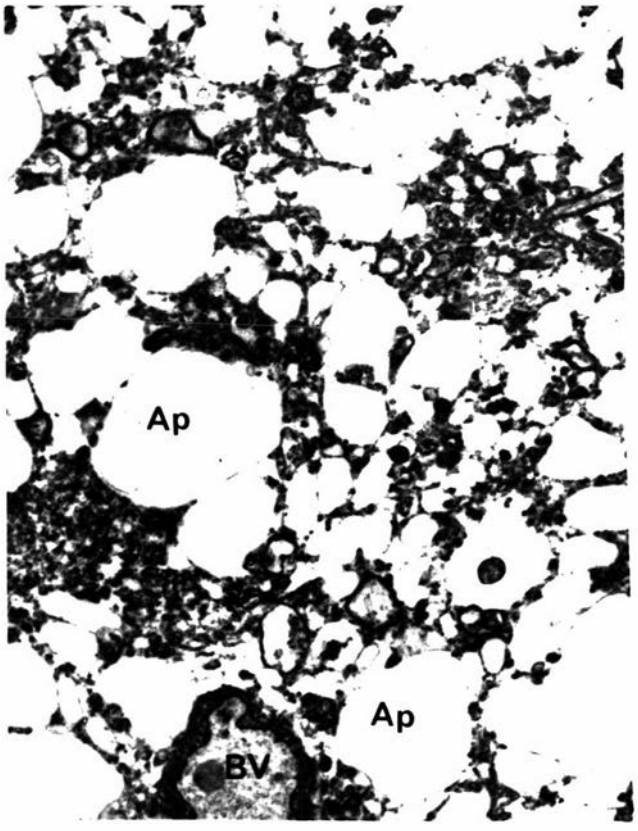

Ap
Ap
BV

Figure 8.7b: Vascular Endothelial Damage in the Brain of Intoxicated Lambs.

A

B

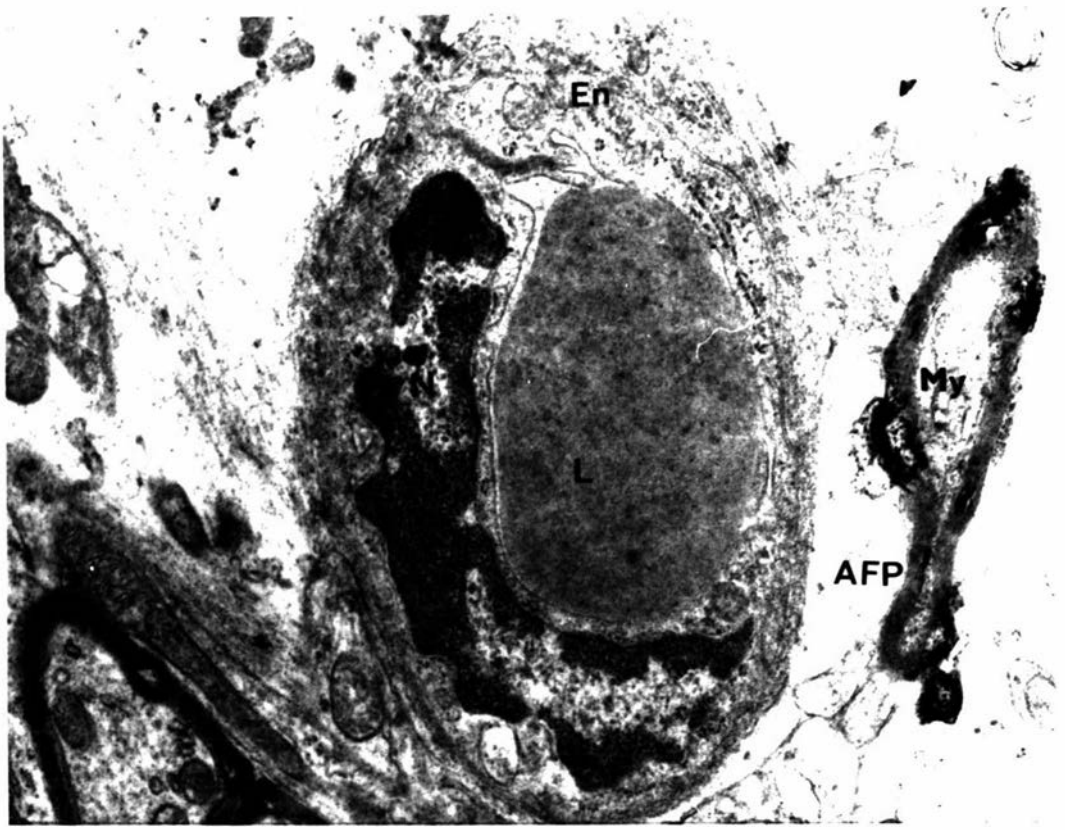
A. Normal Capillary from the Cerebral Cortex of a Control Lamb:

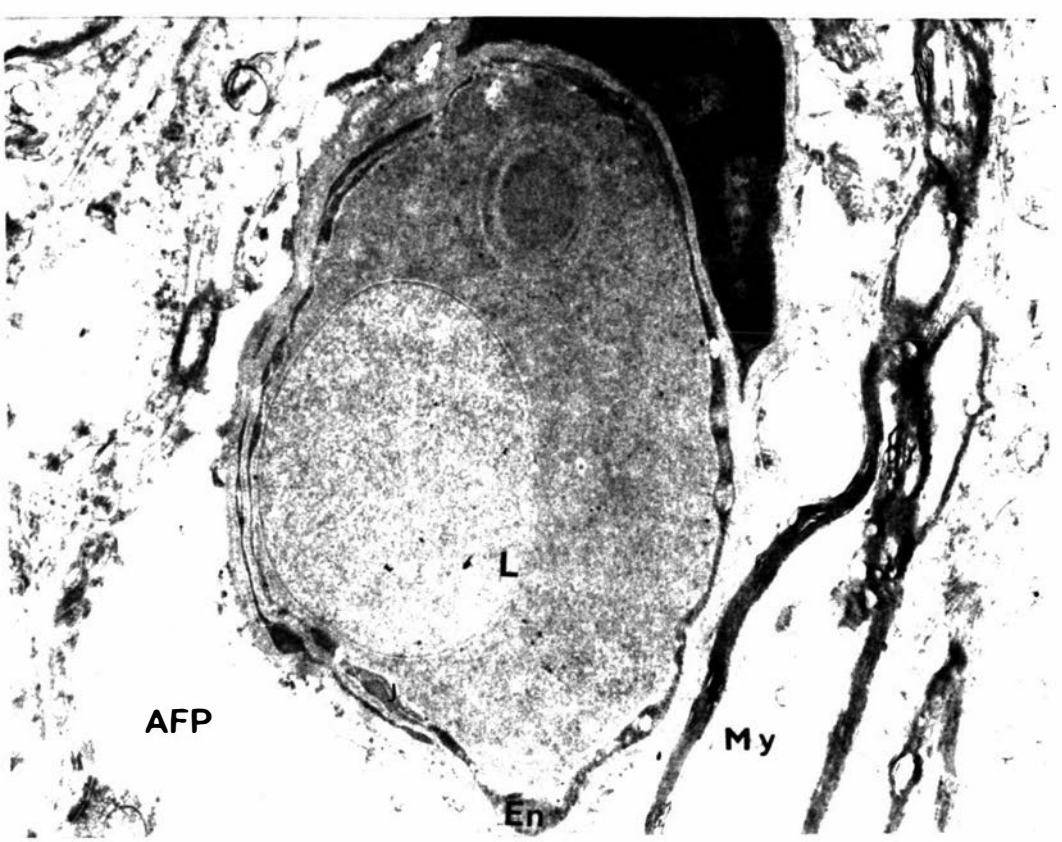
En = endothelial cytoplasm
L = capillary lumen
AFP = Astrocyte Foot Processes
My = Myelinated Nerve Fibre

B. Capillary from the Cerebral Cortex of a Lamb which had received Parenterally Administered Epsilon Toxin:

The endothelial cytoplasm is severely damaged and reduced to a thin, electron-dense band. There does not appear to be any marked separation of the cell junctions (J).

Magnification 22,000


En
My
L
AFP


L
AFP
My
En

in the usual manner.

Results:

In the experiments in which peroxidase was given to mice it was confined to the vascular lumen for at least three hours in the control animals. In the intoxicated animals it was found to be extensively distributed throughout the extracellular space of the neuropil as well as being trapped in the region of the capillary basement membranes (Figure 8.8). This extravascular distribution of the peroxidase occurred even in the animals which died within an hour of the administration of the toxin and tracer.

The peroxidase tracer in intoxicated animals was confined to the limited extracellular space which is present between the glial processes in the brain and did not enter the swollen astrocytic processes (Figure 8.9).

The level of horse radish peroxidase used in the lamb experiments was not detectable by the histochemical methods used. The erythrocytes, which have a high level of endogenous peroxidase activity (Strum & Karnovsky 1970) were clearly stained in all animals, including the animal which had not received any exogenous peroxidase. Where this protein tracer had been administered there was no detectable exogenous peroxidase activity in the capillary lumina or elsewhere in either the control or the intoxicated animals.

Morphological and Histochemical Changes Detectable by Light Microscopy in the Brains of Intoxicated Animals.

Materials & Methods:

The brain tissue for light microscope examination was obtained from the cerebral cortex, thalamus, mid-brain, cerebellum and medulla of the same mice and lambs that were used to obtain the tissues for electron microscopy.

Duplicate $\frac{1}{2}$cm thick pieces of these tissues were fixed in either 10% Formol saline or Bouin's fixative, dehydrated through grades of ethanol and embedded in paraffin wax. Sections between

Figure 8.8: The Distribution of Horse Radish Peroxidase in the Brains of Control and Intoxicated Mice.

A

B

A. Capillary and Thalamic Neuropil from a Control Mouse which had received Peroxidase Intravenously:

The peroxidase (P) is confined to the capillary lumen. Note that there has been some artefactual shrinkage of the main body of the peroxidase away from the margins in this section. The capillary wall is free of peroxidase.

Nl = neuropil

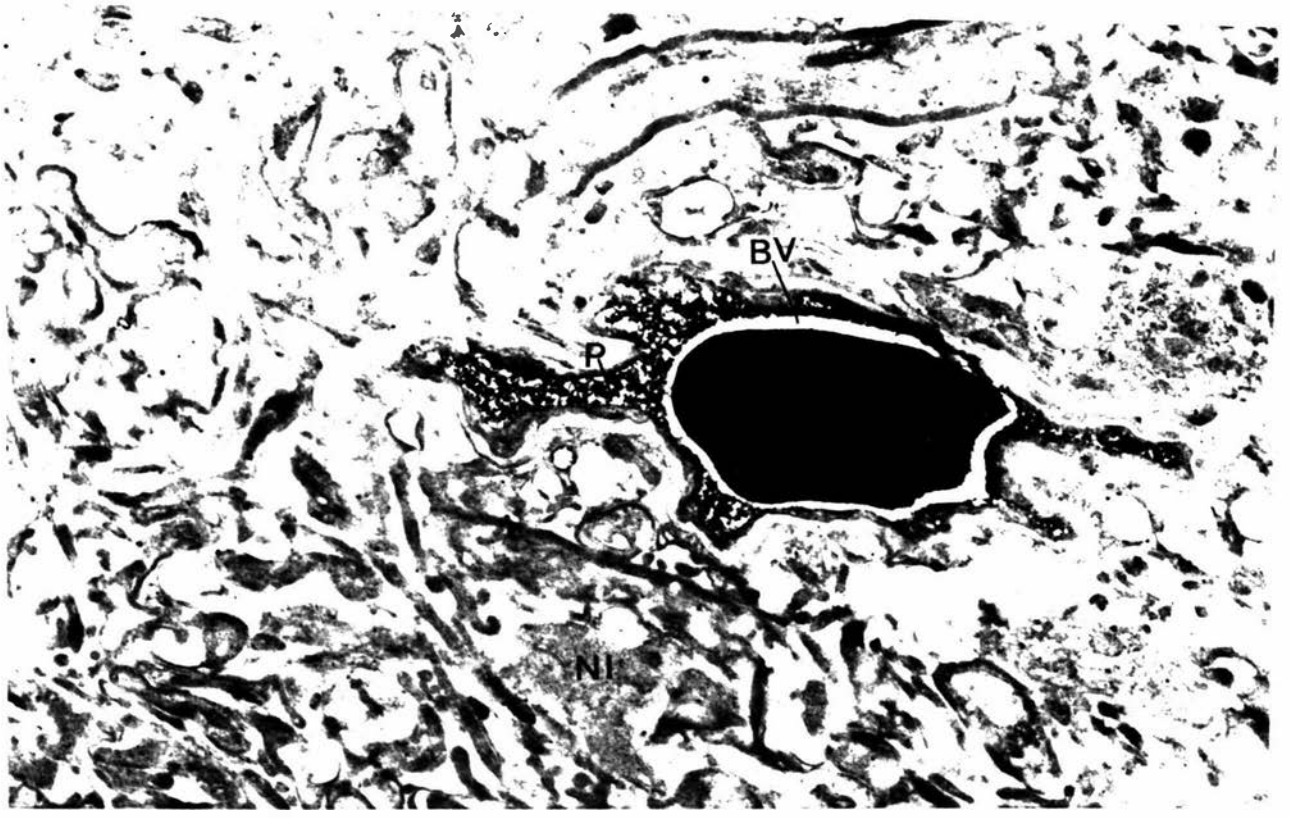
BV = Blood Vessel

B. Capillary and Thalamic Neuropil from a Mouse which had received Epsilon Toxin and Peroxidase Intravenously:

The peroxidase has escaped from the capillary lumen and has been extensively trapped in the capillary endothelium and basement membrane. In addition it has spread extensively through the tenuous extracellular spaces (ES) of the neuropil.

AS = Astrocyte

Magnification 10,600


BV
P
NI

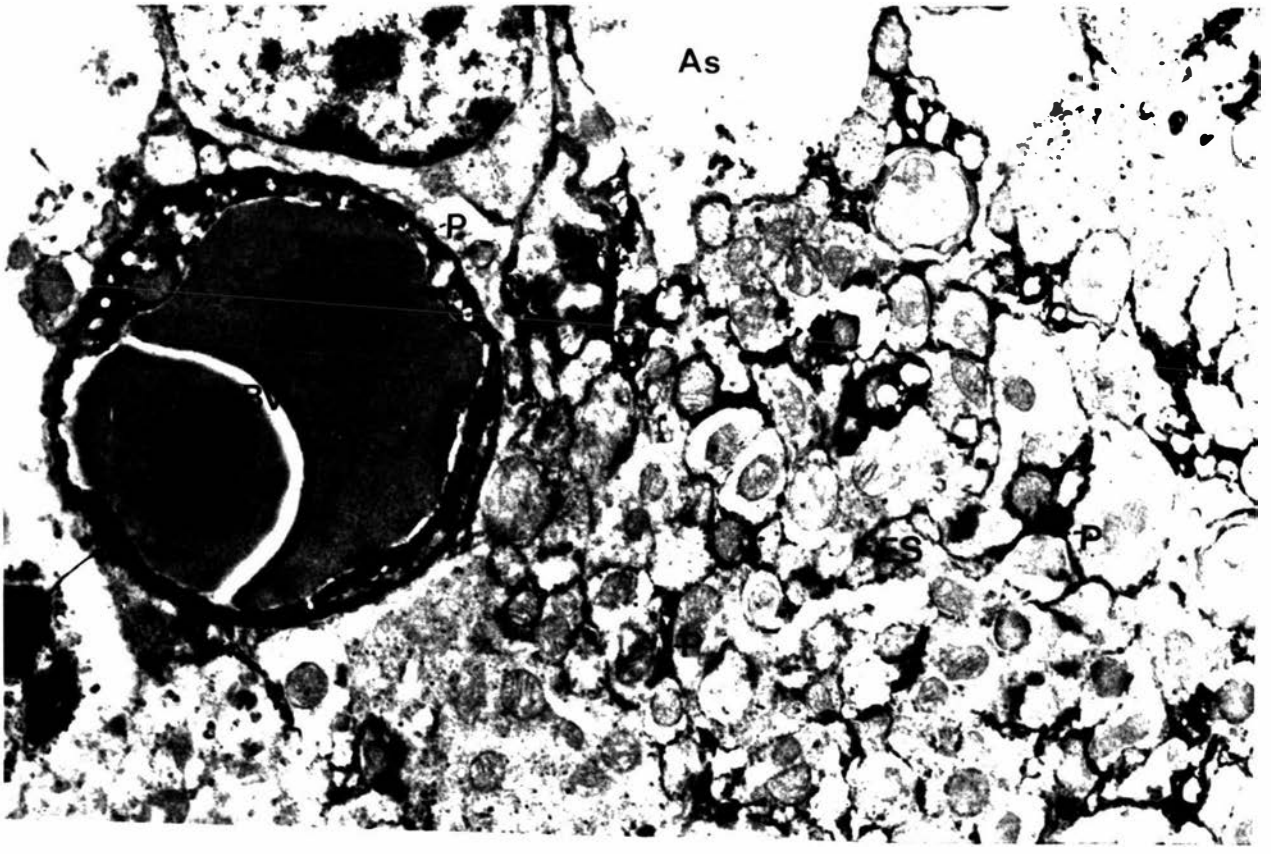
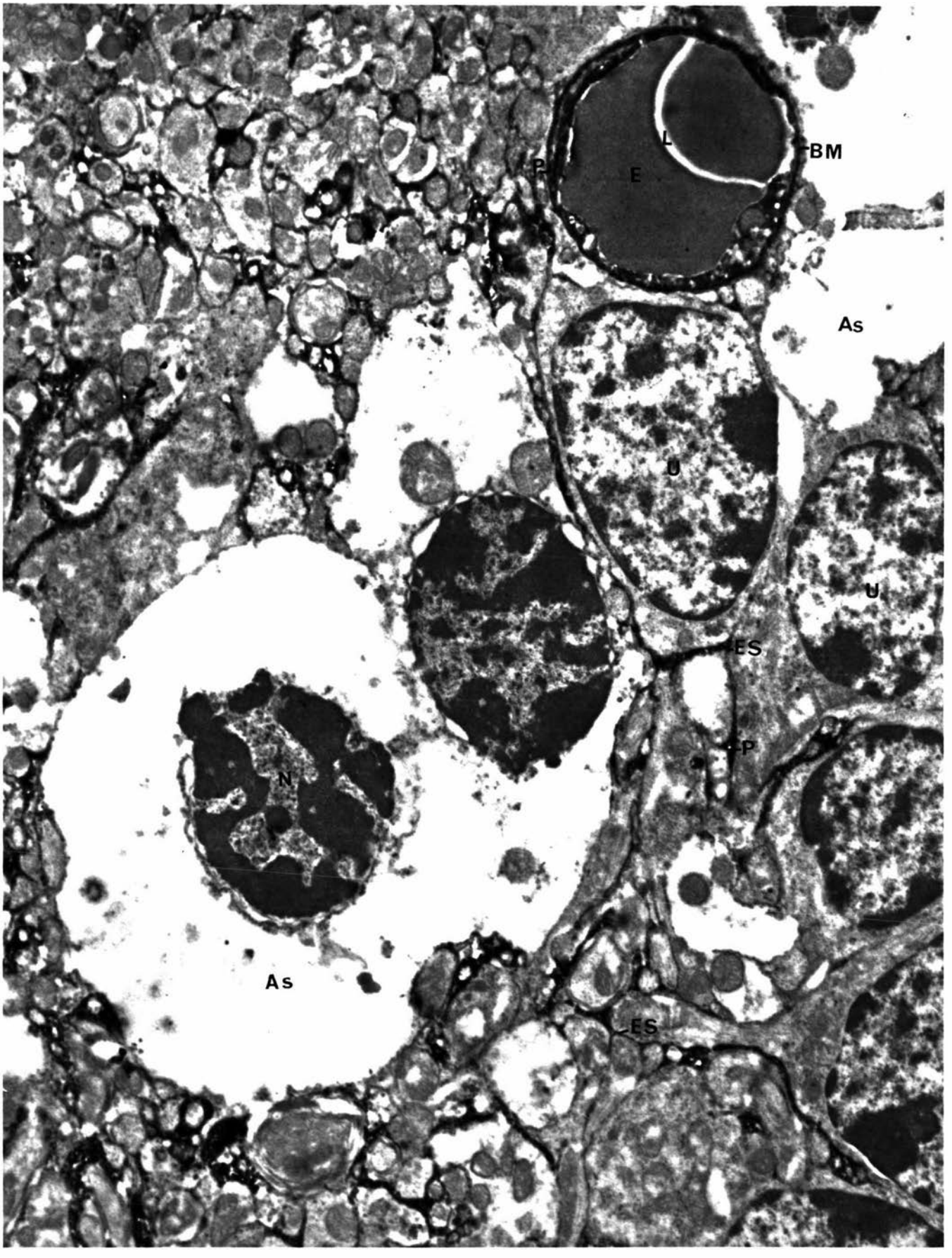

As
P
P
P

Figure 8.9: The Distribution of Horse Radish Peroxidase in the Cerebellar Granular of Intoxicated Mice.

Peroxidase (P) has left the vascular lumen (L) and has been trapped in the capillary wall, including the basement membrane (BM). In addition it is extensively distributed throughout the tenuous extracellular space (ES) in the neuropil. It should be noted that, although there is obvious swelling of protoplasmic astrocytes (As), none of the peroxidase appears to have entered these cells.

E = erythrocyte
N = nucleus
U = unidentified cells of the granular layer

Magnification 8,500


L
BM
P
E
As
U
U
ES
P
N
As
ES

2 and 5u in thickness were cut and stained with Erlich's Haematoxylin and Eosin (H&E) and by the Periodic Acid Schiff (PAS) method.

Further similar blocks from the brains of the mice were quenched on dry ice, while the lamb material was fixed overnight in cold (4°C) 10% formol calcium then infiltrated with the 0.88M sucrose/1%Gum Acacia solution recommended by Pearse (1968) for certain histochemical procedures. Frozen sections of these tissues were cut at 8u on a Lipshaw 'Cryotome' (Lipshaw Man'f. Co., Detroit, U.S.A.), and the techniques described in Thompson (1966) used to demonstrate acid and alkaline phosphatase (azodye method) and DPN and TPN diaphorase (nitro-blue tetrazolium method) activity respectively.

Results:

There were no consistent qualitative differences in the levels of either the phosphatases or the diaphorases in the brains of control and intoxicated mice and lambs.

No lesions were detectable in the H&E and PAS stained sections of brains from intoxicated animals which survived for less than 6 hours. At 6 hours, foci of apparent vacuolation were seen in the cerebellar granular layer. These areas consisted of dense basophilic nucleii surrounded by a clear zone of cytoplasm. Initially the foci only appeared to involve a few cells but as time progressed the lesions became more extensive and the white matter underlying these lesions showed focal pale areas, probably the result of oedematous swelling. The sequence of changes is illustrated in Figure 8.10 . By 24 hours the type of lesion described by Griner (1961a), and seen in early field cases of F.S.E. in lambs by Hartley (1956), was present. There were no alterations in the capillary endothelium, fixed by either formol saline or Bouins fluid, detectable by light microscopy.

There were no detectable lesions in the brains of lambs which received either parenteral or enteric-origin toxin.

Figure 8.10: The Sequence of Changes seen by Light Microscopy in the Cerebellum of Intoxicated Mice.

A B

C D

A. Control:

The normal appearance of portion of a cerebellar folium.

P = pia mater

M_L = Molecular layer

G_L = Granular layer

WM = cerebellar white matter

B. Cerebellum from a Mouse which died 6 hours after Inoculation with Epsilon Toxin:

The granular layer contains scattered foci (X) in which the cells are swollen and have pyknotic nuclei and clear cytoplasm.

C. Cerebellum 6 Hours after Inoculation:

This is a montage of a similar area to B showing the changes in more detail. The swollen cells are almost certainly the protoplasmic astrocytes seen by electron microscopy.

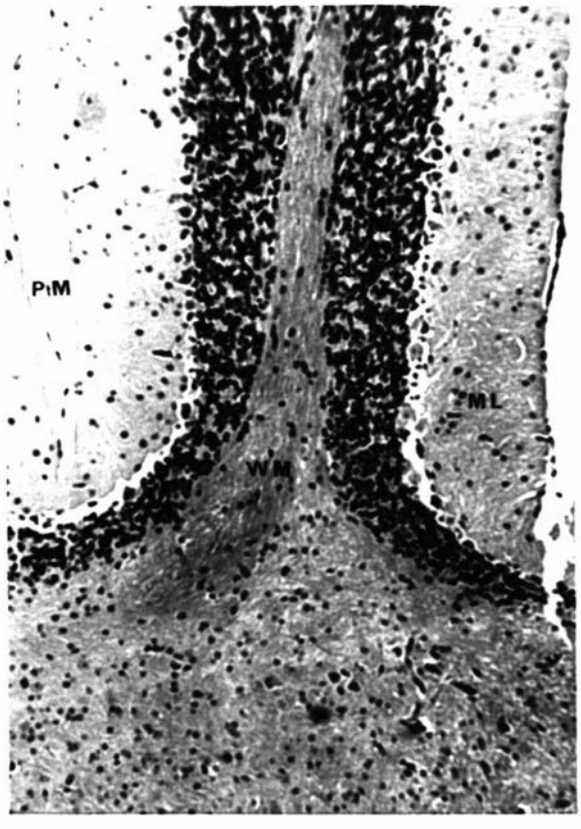
PC = Purkinje cells

D. Cerebellum 12 Hours after Inoculation:

In addition to the focal areas in the granular layer which have been described above, there is also focal rarefaction (Y) in the white matter in sections taken at this time.

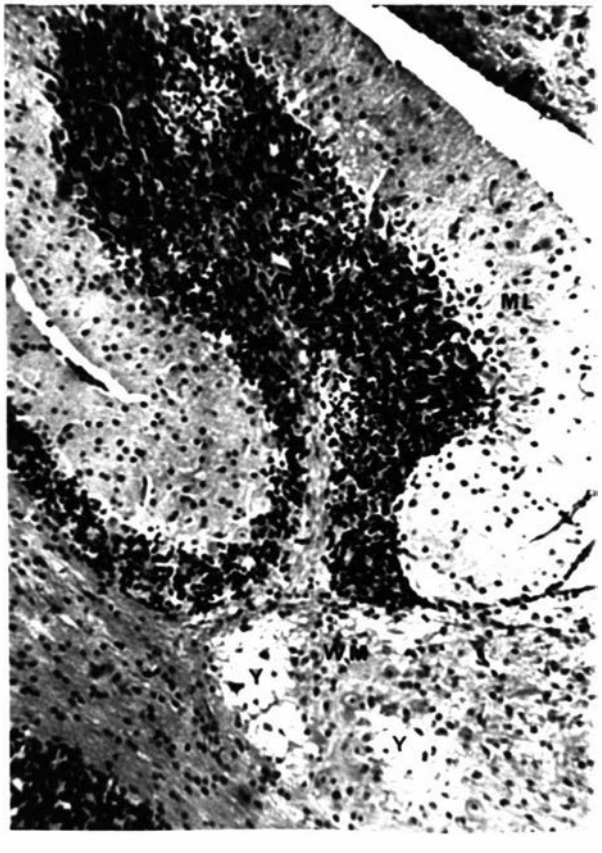
Magnification A, B & D 70

C 170


PiM
ML
WM


RM
WM
M-L


ML
WM


ML
WM

The Effect of Critical Hypoxia on the Morphology and Fluid Balance in Mouse Brain

Materials & Methods:

A series of fourteen 8 week-old female mice were subjected to critical hypoxia by means of a Boyles Anaesthetic apparatus (British Oxygen Co., England) delivering 8% oxygen (O_2) in nitrous oxide (N_2O) at 2.16 litres per minute through water to a stainless steel chamber 25cm x 45cm x 25cm with bottom entry and top exit ports. The chamber was initially flushed with pure oxygen for 5 minutes at a flow rate of 8 litres per minute and the flow rate and partial pressure of oxygen were gradually reduced to the final figure over 15 minutes. A control group of four similar mice were killed by neck dislocation at the start of the experiment and in addition four further mice were maintained in the chamber for 24 hours in an atmosphere of 20% O_2 in N_2O delivered in the same manner as previously. Two of these latter animals were killed after 12 hours in the chamber and the remaining two after 23 hours. Brain water content was measured and sections prepared for microscopic examination as described previously.

Results:

After an initial period of excitement which occurred when nitrous oxide was first introduced into the chamber, all mice became very depressed in the hypoxic atmosphere. Animals generally remained conscious for some time but became comatose and died after varying periods of survival. All except two of the mice died within 24 hours of the onset of hypoxia and the two surviving animals were *in extremis* at that time and were killed by neck dislocation.

There was no significant increase in the water content of the brains of these animals (Table 8.2).

Table 8.2:

The Water Content of the Brains of Normal and Hypoxic Mice.

Control Normal Air	Control 20%O_2 in N_2O		Hypoxia 8%O_2 in N_2O			
	Survival Time (Hours)					
	12	24	0 - 4	5 - 8	9 - 12	13 - 24
	Water Content (mg H_2O per mg. Dry Matter)					
3.37	3.48	3.44	3.48	3.48	3.39	3.37
3.32	3.37	3.32	3.57	3.39	3.39	3.55
3.29					3.46	3.28
3.45					3.40	3.21
					3.42	
					3.36	
Mean 3.36 ..	3.40		3.53	3.43	3.40	3.35

Note: 1 brain sample from hypoxic group lost in processing.

Mice which died after surviving for up to 23 hours in the hypoxic atmosphere did not exhibit vascular endothelial damage or astrocytic swelling. In addition there was clear evidence of neuronal damage in the hypoxic animals (Figure 8.11) which was not present in epsilon toxin intoxicated animals. There was no evidence that the use of N_2O as a carrier gas, instead of nitrogen, had any effect on the outcome of the hypoxia experiments since the animals which were held in N_2O plus 20%O_2 did not show any detectable evidence of nervous system damage either clinically or at the ultrastructural level.

Discussion

Before entering into a discussion of the results obtained, certain other aspects of this chapter must be mentioned. For example, freeze-drying has not been widely employed in the past as a means of determining the dry matter content of tissues. It has however been employed by Quadbeck (in Klatzo & Seitelberger 1967) in his quantitative studies on brain oedema. On theoretical

Figure 8.11: A / B — Neuronal Damage in Mice resulting from Prolonged Critical Hypoxia.

A. Neurone from a Mouse which died after 12 hours in an Hypoxic Atmosphere 8% O_2 in N_2O

The neurone (Ne) is shrunken and the mitochondria (Mi) are closely aggregated and possibly swollen.

AFP = Astrocyte Foot Process

N = nucleus

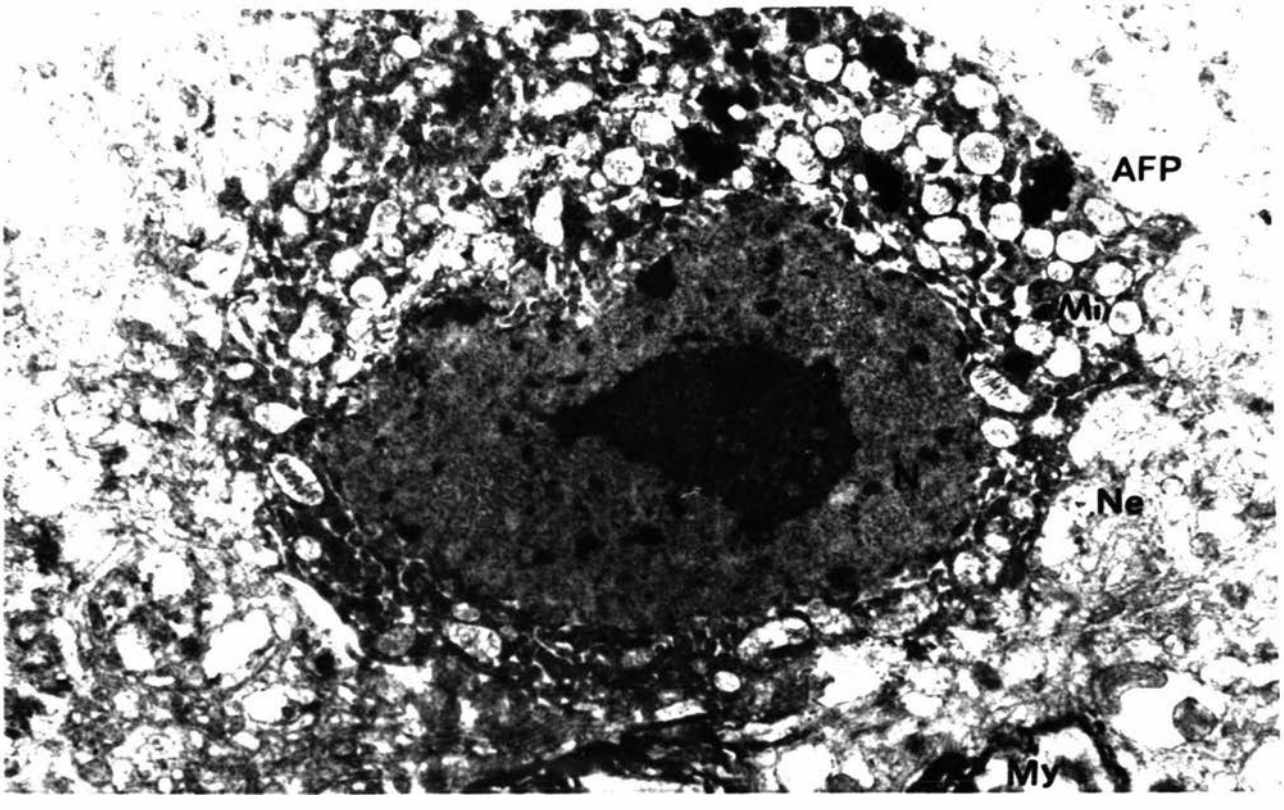
B. Neurone from a Mouse which died after 23 hours in a Hypoxic Atmosphere:

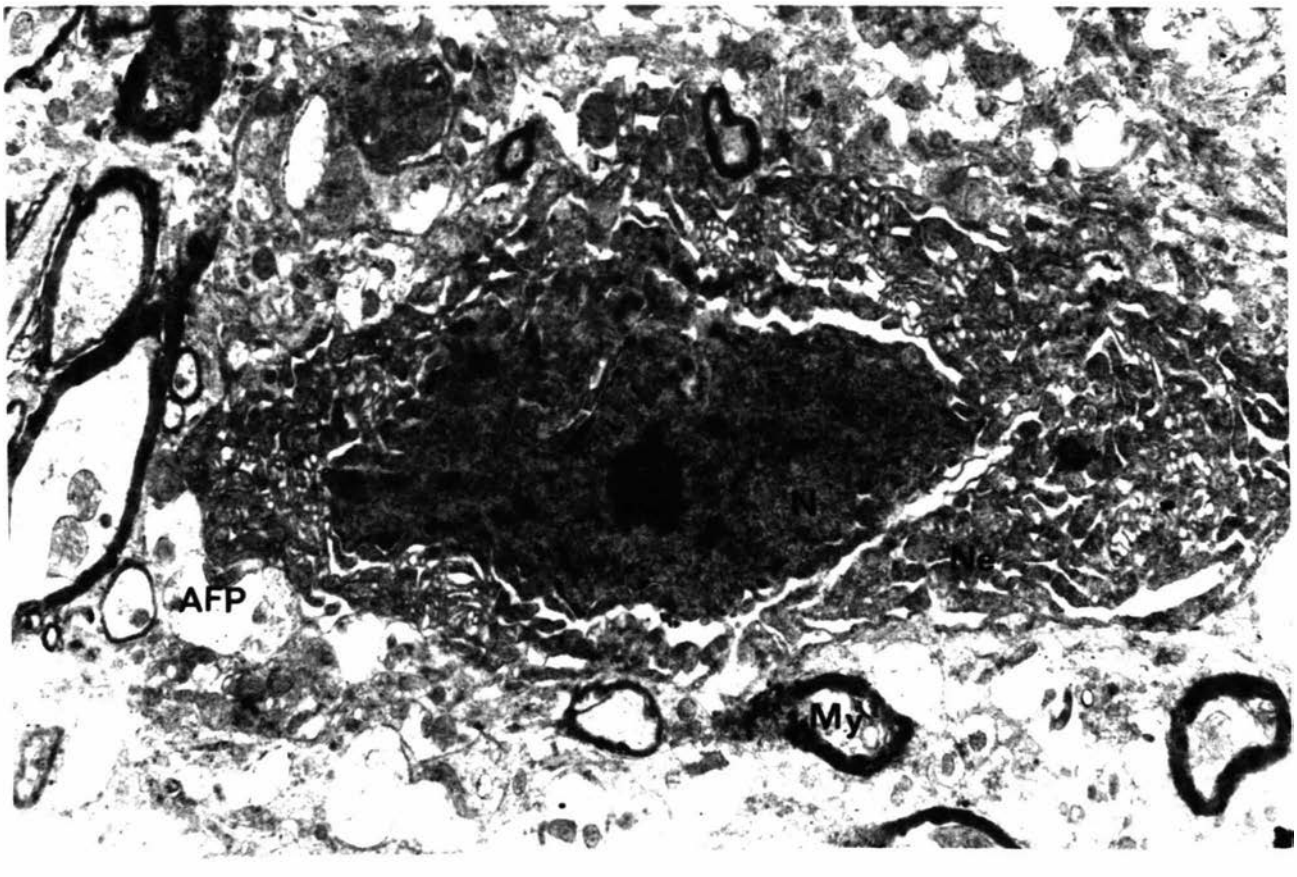
The neurone is shrunken and there is practically no recognisable cytoplasmic architecture.

My = myelinated nerve fibres

These neurones should be compared with those, from control and intoxicated mice, shown in Figure 8.5.

Magnification 8,000


AFP
Mi
N
Ne
My


N
Ne
AFP
My

grounds this method would appear to be superior to oven drying methods where the result obtained is dependent upon the temperature used.

Cold formol-calcium fixation followed by sucrose-acacia infiltration gave excellent preservation of tissue architecture and histochemical localisation in the lamb tissues and gave results which were superior to those obtained in experimental mice in which the tissues were used fresh and quenched on dry ice.

Although it was mentioned that the water content of the brain tissue of mice is age dependent, the decrease in water content between 3.5 and 10.5 weeks of age is only 1.7% (Fuller & Wimer, in Green 1966) and all animals in the present experiments were between 6 and 8 weeks of age and divided equally between intoxicated and control groups so that any age influence in these experiments would have been negligible.

Because the accumulation of fluid in grey matter is largely intracellular within the astrocytes the condition is not an oedema in the strict sense of the word, but this is a semantic problem usually disregarded by workers in the field of brain oedema. On the other hand, the Space of His is now definitely considered to be an artefact, but since the astrocyte foot processes are readily influenced by osmotic effects it is probable that they could develop artefactual swelling during fixation and appear as spaces when seen by light microscopy. Any pathological swelling of these processes, such as that which has been shown to occur during epsilon toxin intoxication, would result in an apparent increase in the size of this 'space' and would account for Griner's description of fluid accumulation in the Space of His.

While on the subject of the astrocytic swelling there are a number of points which require consideration. Firstly the problem of the definition of an astrocyte at the ultrastructural level arises. Although the broad groupings of the 'non-neuronal' cells have been worked out, with astrocytes acting as supporting and possibly transport cells, oligodendrocytes producing myelin, and microglia in a scavenging role, the identification and differentiation of cells

into these groups can be difficult. This is particularly true of the protoplasmic astrocyte in which fibre formation is practically absent.

In the present study, the morphological features of the cells which undergo swelling appear to be similar, whatever their situation within the brain, and since many of them have foot processes around blood vessels and resemble the cells which other workers have called protoplasmic astrocytes, they will all be so classified. It has been suggested that the transport of metabolites to and from the neurone may be an active process across the astrocyte, which is in close contact with both the capillary and the neurone. This was held to be a possible explanation for the blood-brain barrier, but since the discovery of the true extracellular space within the neuropil and the contribution of tight junctions between endothelial cells to the barrier, these theories are once more open to reconsideration. However, the rapid and almost specific swelling of astrocytes in a number of intoxications and anoxic states and the profound neurological disturbances which occur under these conditions, where there is minimal detectable involvement of the neurones, suggest that the astrocyte has a major role in maintaining the functional integrity of the central nervous system. The other point worthy of mention in this regard is the fact that, as can be seen in Figure 8.12, there is marked swelling of cells which are closely associated with the Purkinje neurones of the cerebellum. These cells presumably correspond to the Bergmann glial cells of light microscopy which are considered to be of oligodendroglial origin. At the ultrastructural level however the swollen cells closely resemble protoplasmic astrocytes (Fananas cells - Peters et al 1970). No doubt the exact relationship of Bergman glial cells to Fananas cells will be clarified in due course.

Although the astrocyte changes are the most prominent, they are of secondary importance to the changes in the vascular endothelium. The changes in this tissue are extremely severe and the rapidity of onset of the damage is remarkable, as can be judged by the fact that the lesions in Figure 8.8 are from a lamb which received toxin only 45 minutes before death. Griner (1961a) described alterations in brain capillaries and their endothelium

Figure 8.12: The Differential Swelling of Cells resembling Bergman Glial Cells in the Cerebellum of Intoxicated Mice.

A

B

A. Portion of the Cerebellar Granular Layer of a Normal Mouse:

PC = Purkinje neurone

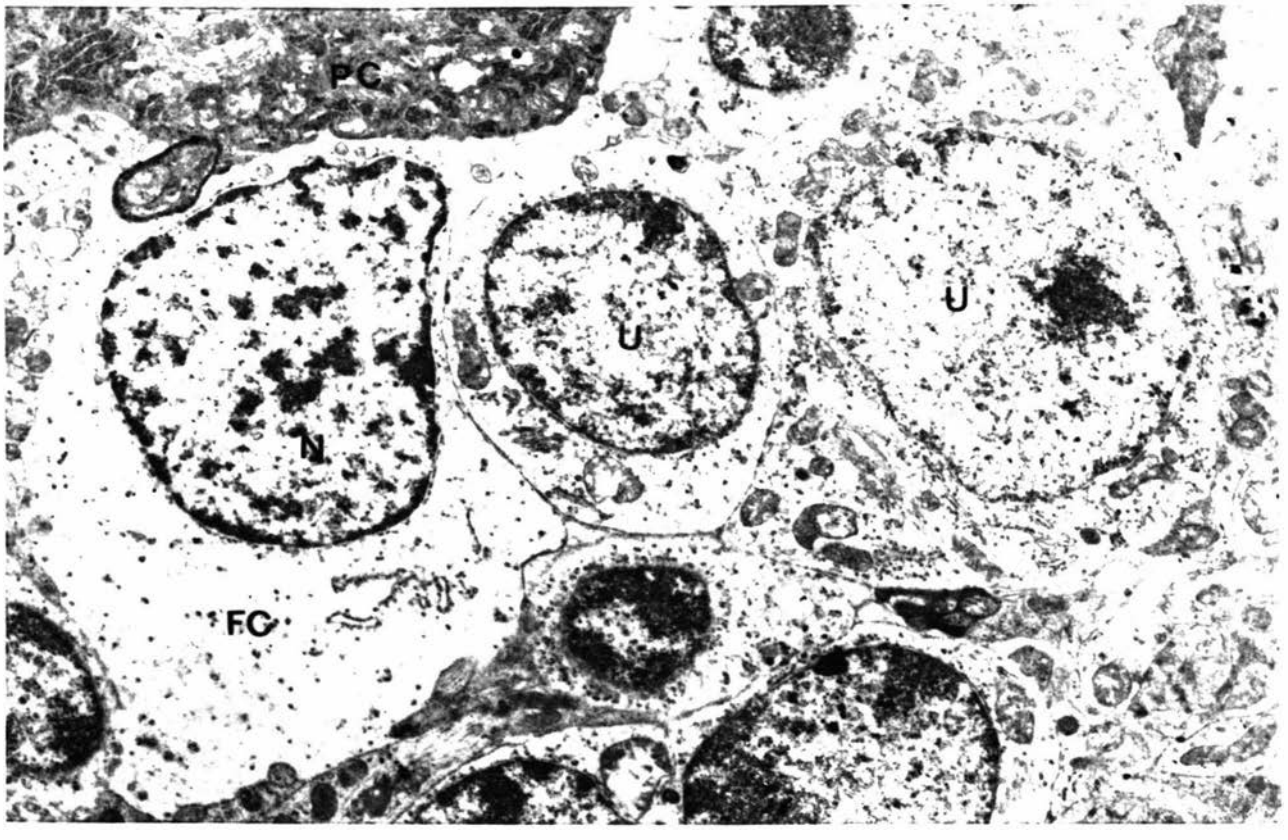
U = unidentified cells of the cerebellar granular layer

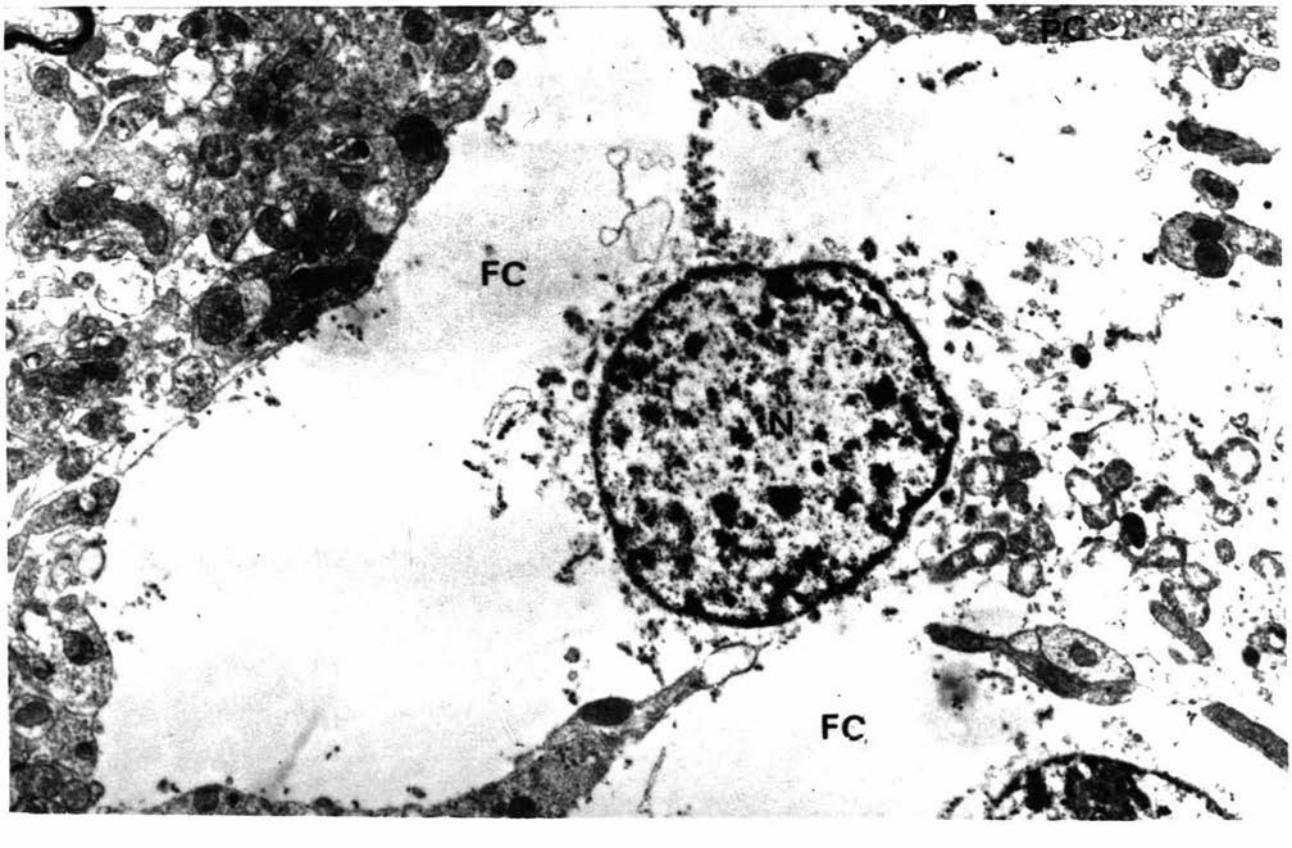
F C = probable Fananas glial cells

B. Portion of the Cerebellar Granular Layer of an Intoxicated Mouse:

There is extensive swelling of the Faranas cells, adjacent to the Purkinje neurones, which presumably correspond to the Bergman glial cells seen by light microscopy.

Magnification


PC
U
U
N
FC


PC
FC
N
FC

but his descriptions of lesions, seen by light microscopy, are rather difficult to reconcile with the ultrastructural findings in the present study. In his acute cases he described "... walls of the more severely affected arterioles had a homogeneous eosinophilic appearance.....swollen hypochromatic endothelial cells were occasionally observed." In his subacute cases he found "....capillary walls appeared to be hyalinised and the endothelial cells were swollen and hypochromatic." The present study did not reveal any detectable swelling of endothelium when it was examined with either the light or the electron microscope and there was no obvious involvement of the basement membranes.

Woodbury et al (1956) found that 70% of a group of mice died when subjected to an atmosphere in which the partial pressure of oxygen had been reduced to 76mmHg ie 10% oxygen. It should be noted that it is extremely difficult to maintain experimental animals in an atmosphere with a reduced oxygen tension, when the carrier gas is nitrogen, as the animals go into acute convulsions and die. This is a problem which has been encountered by workers investigating the effect of hypoxia on brain oedema (Levine 1960). Nitrous oxide appears to be a much less critical carrier gas than nitrogen and from the evidence of the present study it does not appear to produce any morphological changes in the brain when it is used with a partial pressure of oxygen approaching that in the normal atmosphere.

In view of the severity of the capillary endothelial damage it is not surprising that the horse radish peroxidase, which is a small protein with a molecular weight of 40,000, could escape from the bloodstream into the extracellular spaces within the neuropil, and its use has provided visual confirmation of Griner's (1961b) finding that proteins escape into the brain substance in intoxicated animals. The distribution of the tracer is similar to that found in experimental brain oedema produced by other workers (Hirano et al 1970).

This finding is of importance when the status of plasma proteins in intoxicated animals is considered and will be discussed

more fully in Chapter 16. It is interesting to note that, although the peroxidase escaped into, and was confined to, the tenuous extracellular spaces between the cell processes of the neuropil, there was no evidence of excess fluid in this region and the fluid accumulation which occured during intoxication appeared to be almost entirely intracellular. It is unfortunate that the level of peroxidase used in the lambs was not detectable. The result was not altogether surprising however, since the dose rate is considerably below that recommended for mice and rats on a body-weight basis. To provide an equivalent amount for lambs would necessitate using a dose of the order of 4 grams or more. Although fluorescein-conjugated proteins have been used in permeability studies they cannot be employed for ultrastructural visualisation and to date no techniques which are entirely satisfactory for use in larger animals have been described.

The earliest lesions detectable in the cerebellar granular layer by light microscopy are directly referable to the changes seen by electron microscopic examination. The focal areas of rarefaction are almost certainly areas in which the swelling of the astrocytes is most marked. The very focal nature of these lesions and the lesions which develop in the white matter made it difficult to relate them directly to the ultrastructural changes. The sequence of changes which develop over a period of time in the brains of intoxicated animals, have been adequately described by Griner (1961a). They need not concern us here since they result from tissue damage subsequent to the action of epsilon toxin rather than from any direct effect of the toxin itself.

The histochemical findings must be mentioned briefly. Becker, Barron (1961, 62) have shown that reductions in the levels of acid phosphatase, as well as other enzymes, occur in animals in which anoxic-ischaemic encephalopathy has been induced. The earliest time at which these changes were detectable was 6-12 hours and it is obvious that, because of the acute nature of epsilon toxin intoxication in lambs, few histochemical changes could have been expected.

Conclusions

1. In mice, epsilon toxin produces an increase in brain water content which becomes greater with increasing survival time. Similar changes could not be demonstrated in lambs as the survival time was short in intoxicated animals.

2. The increase in brain water content in intoxicated mice was associated with morphological evidence of oedema in the grey matter of the brain, where swelling of astrocytes and astrocyte processes was prominent. These changes were also present in the intoxicated lambs.

3. Extensive destruction of vascular endothelium occurred in all intoxicated animals and resulted in the loss of fluid and protein into the brain tissue.

4. The use of horse radish peroxidase as a tracer confirmed the loss of protein from the blood stream of intoxicated mice and showed that it was confined to the extracellular space within the neuropil and did not enter the swollen astrocyte processes. Peroxidase given at a dose of 100mg intravenously was not detectable in either control or intoxicated lambs.

5. The changes induced by epsilon toxin were not the result of tissue hypoxia since animals which died from critical hypoxia showed no vascular damage or swelling of astrocytes and had neuronal changes which were not present in intoxicated animals.

6. Nitrous oxide is a more suitable carrier gas than nitrogen for studies of the effects of hypoxia on mice and does not produce clinical disturbance or morphological change in the absence of hypoxia.

7. The earliest brain lesions, detectable by light microscopy, occurred in the cerebellar granular layer where focal cytoplasmic clearing and nuclear pyknosis occurred. These changes were probably the result of the astrocytic changes seen at the ultrastructural level.

8. No alterations in the levels of acid or alkaline

phosphatase, DPN or TPN diaphorase were detectable histochemically in intoxicated animals.

9. The histochemical localisation of these components and the preservation of architecture in lamb brain was improved by cold formol calcium fixation and sucrose/acacia infiltration when the results obtained with the use of these agents were compared with those obtained by quenching fresh mouse brain in dry ice.

Chapter 9: THE MORPHOLOGICAL ASPECTS OF THE PULMONARY AND MYOCARDIAL OEDEMA WHICH OCCURS IN EXPERIMENTAL ENTEROTOXAEMIA.

One of the prominent features of the gross pathological findings in intoxicated animals is the pulmonary oedema mentioned in Chapters 6 and 7. It was obvious that pulmonary oedema was not consistently present in experimentally intoxicated animals, nor was it a prominent feature of the natural disease in sheep.

In view of the vascular endothelial damage in both the brain and kidney it appeared probable that this was a universal phenomenon which occurred throughout the body and that the presence or absence of oedema of the lungs was dependent upon the severity of the vascular damage in those organs. It was therefore of interest to determine whether endothelial damage did in fact occur in the lungs of intoxicated animals and what other changes were present in lung tissue. Any damage to pulmonary architecture could affect biochemical parameters associated with respiratory exchange, but these will be discussed in Chapter 17.

The presence of pericardial effusions and electrocardiographic evidence of myocardial damage suggested that the heart had not escaped from the effects of the toxin. Any attempt to interpret electrocardiographic changes on a morphological basis is difficult since the electrical changes may be transient as exemplified by the regression in the S-T segment changes in the second post-intoxication ECG tracing taken from Lamb no. 63 in Table 7.1a, Chapter 7. Further, in many instances ECG alterations are not associated with detectable morphological damage. However the presence of evidence of a repolarisation defect in many of the ECG's suggested that there may be myocardial injury or interference with ionic or metabolite transfer between the intra and extracellular compartments of the myocardium in intoxicated animals. If a morphological basis for the electrocardiographic changes could be established this would not only provide a useful clinicopathological relationship but would also assist in increasing the available information on electrocardiographic changes in acute myocardial damage.

The Histopathological Changes in the Lungs of Epsilon Toxin Intoxicated Lambs

Materials & Methods

Lung sections for light and electron microscopic examination were prepared from the lungs of the control and intoxicated lambs described in Chapter 6 In addition lung sections for electron microscopy were taken from control mice and intoxicated mice which survived for 3 and 9 hours in the experiments described in Chapter 8. The only modification to the preparative procedures already described was that the tissue required for electron microscopy was taken from an area where a small quantity of buffered 3% glutaraldehyde had been injected into the substance of the lung a few moments before the fragment of tissue was removed for immersion fixation. This prevented tissue collapse.

Sections were stained with H & E and PAS for light microscopy and uranyl nitrate/lead citrate for electron microscopy as described in Chapter 8.

Results

The most obvious feature of intoxicated lamb lungs was oedema of the interlobular septae and, to a lesser extent, of the perivascular connective tissue. There was no evidence of fluid accumulation in the alveoli (Figure 9.1) and the alveolar walls, vasculature and bronchiolar epithelium all appeared normal, when examined by light microscopy.

Electron microscopic examination, however, revealed extremely severe capillary endothelial damage and Figure 9.2, of affected lung capillaries from one of these lambs, illustrates the extent of this damage which included discontinuities in the capillary endothelium with adhesion of thrombocytes to the damaged endothelium and areas where the endothelium had been lost. In some areas there was mild swelling of the alveolar epithelial cells, fluid accumulation in the interstitial connective tissue and protein containing fluid in the alveoli (Figure 9.3).

In contrast to these findings in intoxicated lambs, the ultrastructural changes in the lungs of mice, which received low lethal doses of

Figure 9.1: Pulmonary Oedema in intoxicated Lambs - Light Microscopic Appearance

A

B

A. Normal Lamb Lung:

This is a section of lung from a control lamb for comparison with B below.

P = pleura

Al = alveoli

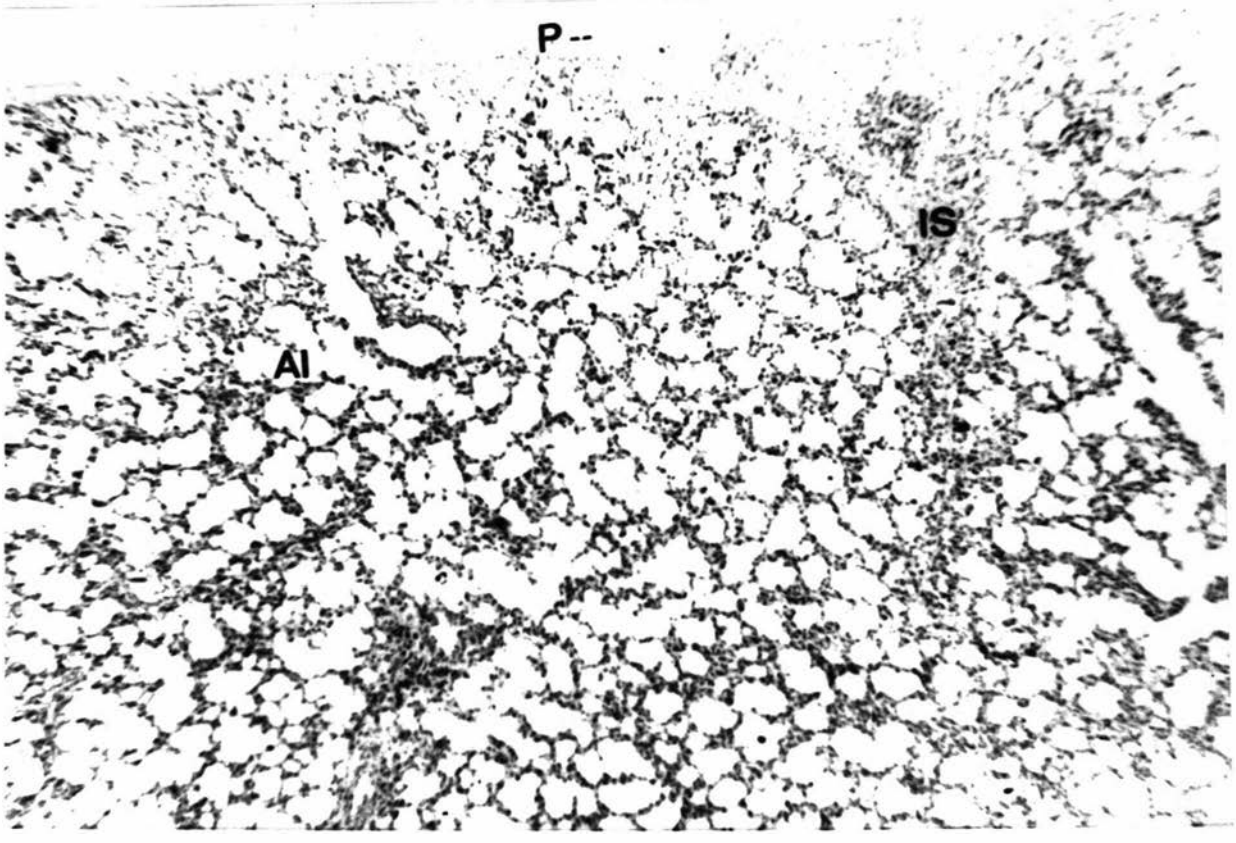
IS = interlobular septa

B. Lung - Intoxicated Lamb:

The subpleural tissues and the interlobular septa are oedematous (Oe). There is no evidence of fluid accumulation in the alveoli.

Stain PAS

Magnification 70


P
IS
Al

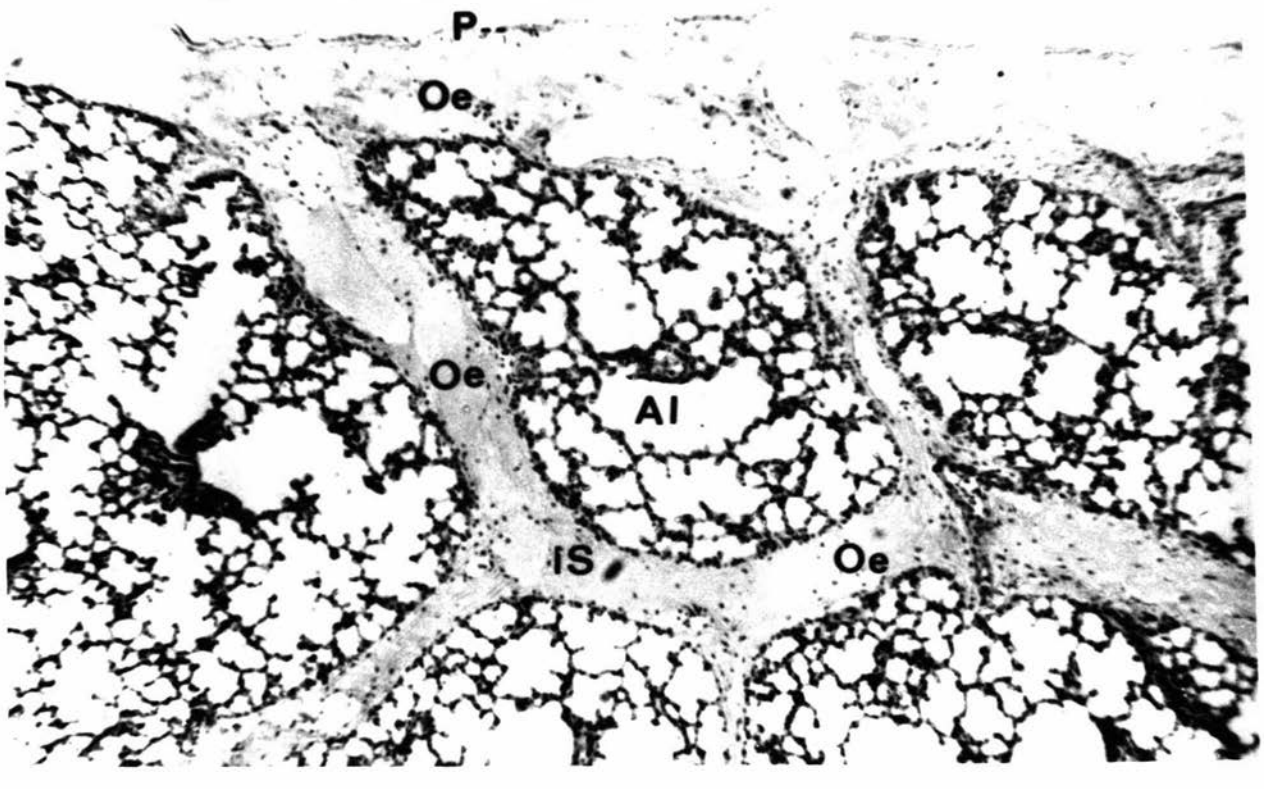

P
Oe
Oe
Al
IS
Oe

Figure 9.2: Vascular Damage in the Lungs of Lambs after the Parenteral Administration of Epsilon Toxin.

A B

C D

A. Control:

The junction between two adjacent endothelial cells in a normal lung capillary.

L = capillary lumen
En = endothelial cytoplasm
BM = basement membrane
Ep = alveolar epithelium
J = cell junction
TJ = 'tight' junction

B. Capillary from an Intoxicated Lamb Lung:

There is a large area over which the endothelium appears to have been completely lost and this is covered by two adherent thrombocytes (T).

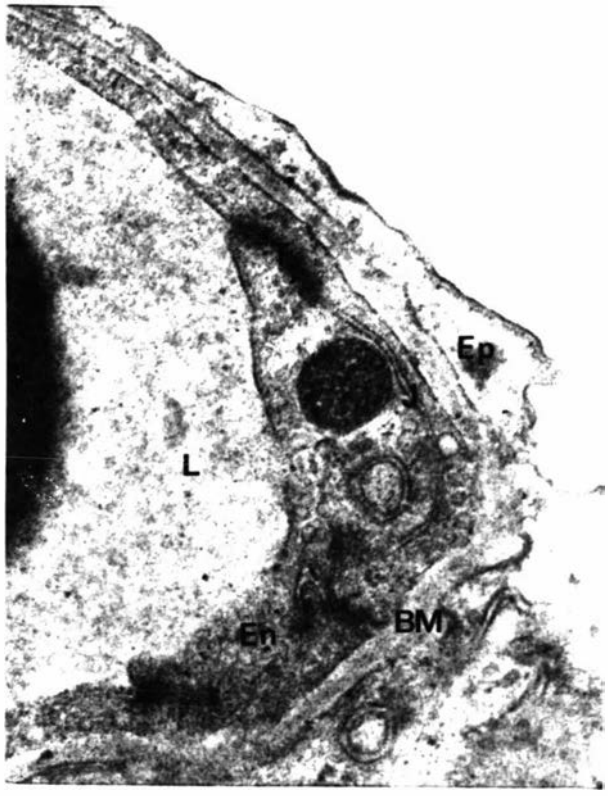
C. Alveolar wall from Intoxicated Lamb Lung:

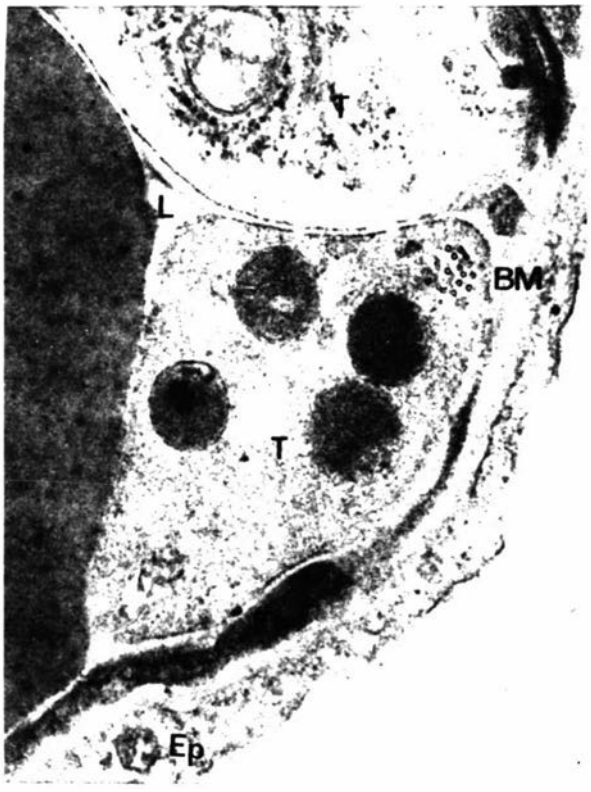
There is fluid accumulation (F) in the interstitial tissue of the lung. In some areas there are discontinuities (Di) in the damaged capillaries.

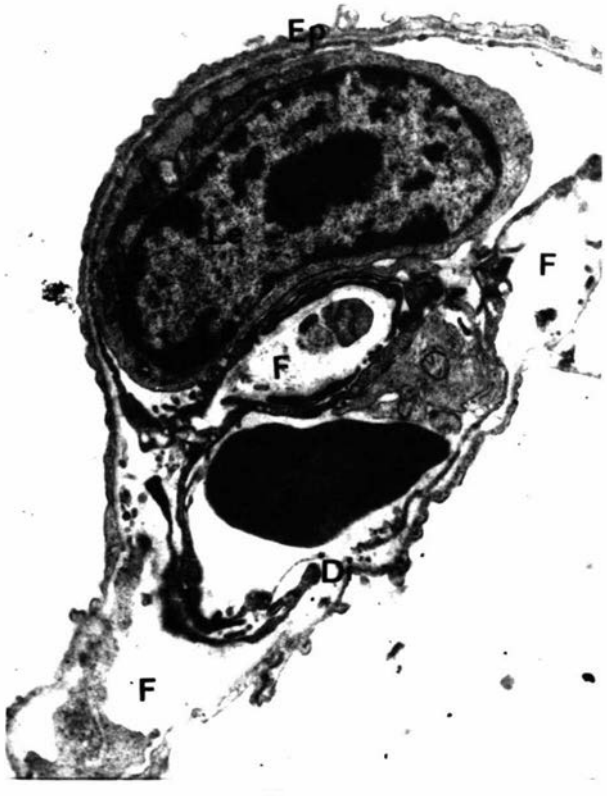
D. Alveolar Wall from an Intoxicated Lamb Lung:

Similar changes to those seen in C are present in this section. In addition there are areas where thrombocytes (T) are covering the discontinuities in the capillary wall.

Magnification A & B 34,500
C & D 8,750


Ep
L
En
BM


T
L
BM
T
Ep


Ep
F
F
Di
F

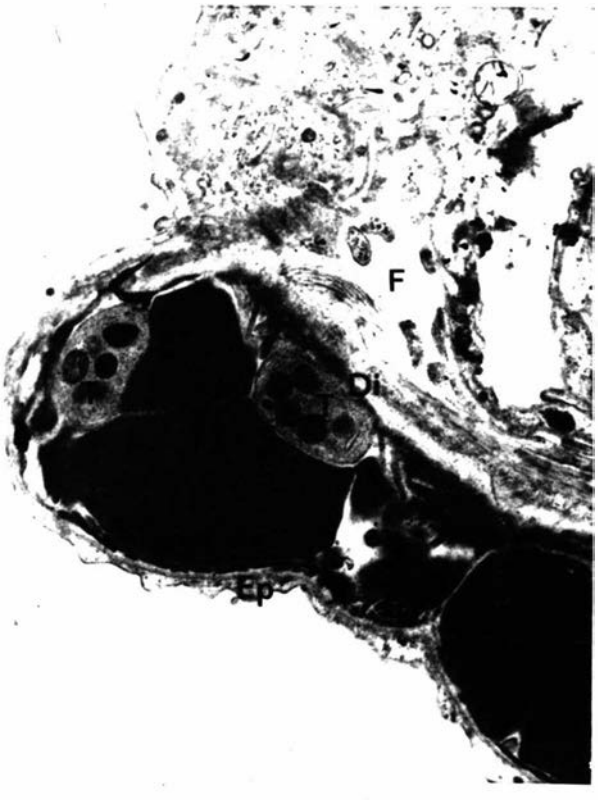

F
Di
Ep

Figure 9.3: The Development of Pulmonary Oedema in Lambs after the Parenteral Administration of Epsilon Toxin

A B

C D

A. Control:

Portion of the normal alveolus showing the alveolar space (AS), the tenuous alveolar epithelium (Ep) and the capillaries with their endothelial nuclei (N).

E = erythrocytes

B. Lung from Intoxicated Lamb:

Moderately electron dense fluid (F) is present in the alveolar space. Thrombocytes (T) can be seen adhering to the capillary walls.

C. Control:

This is a higher magnification of a portion of the normal alveolar wall for comparison with D. The appearance of the alveolar epithelium and the capillary endothelium (En) should be noted.

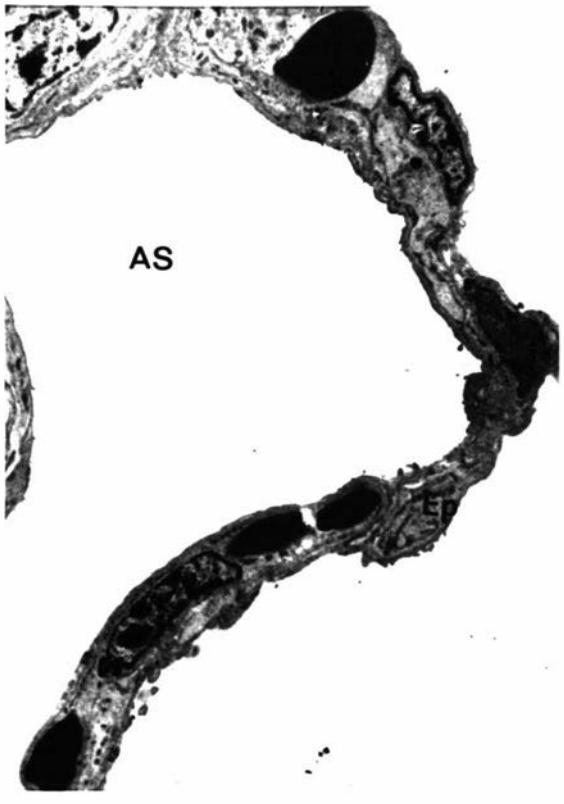
D. Lung from Intoxicated Lamb:

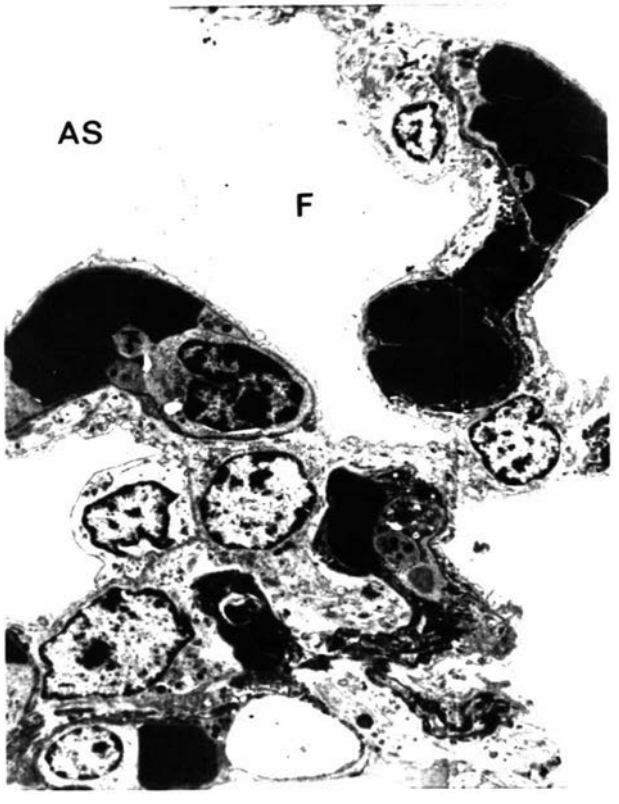
Electron dense fluid is present in the alveolar space. In addition there is swelling of the cytoplasm of the alveolar epithelial cells. There is also oedema of the interstitium (I). Thrombocytes are adherent to the severely damaged capillary endothelium.

G = Great alveolar cell which can be identified by its microvilli (Mv).

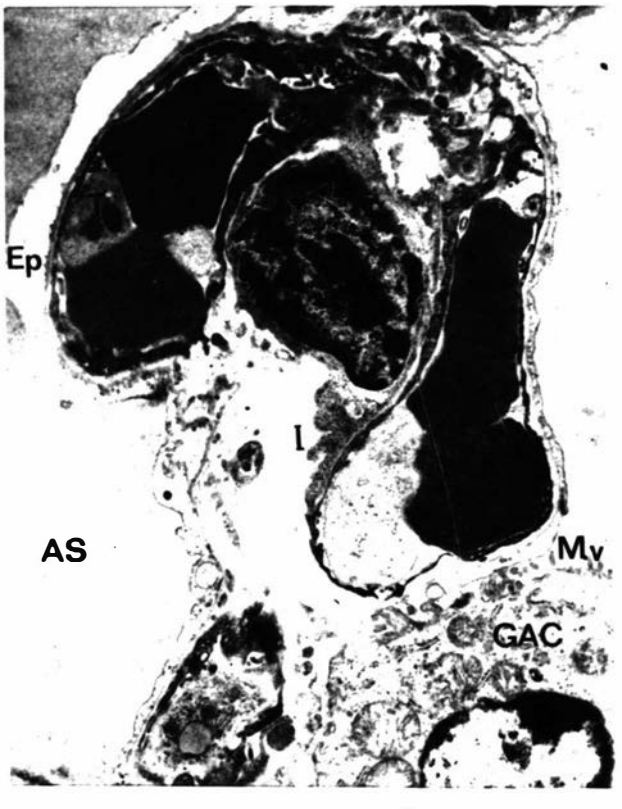
Magnification A & B 2,950

C & D 5,900


AS
Ep


AS
F


Ep
AS
En


Ep
L
AS
Mv
GAC

toxin, were not prominent. There was no evidence of fluid accumulation in the alveoli or interstitial tissues and the capillary endothelium was not markedly altered in the lungs of these animals.

Quantitative and Morphological Evidence of the Presence of Myocardial Oedema in Intoxicated Lambs.

A detailed examination of the electrocardiograms from the lambs which had received parenteral toxin was deferred until the conclusion of the experiments and for this reason no quantitative measurements of the water content of the hearts of these lambs were made. However, in view of the electrocardiographic findings in these animals, the water content of the hearts of the lambs which received toxin of enteric origin was assessed.

Materials & Methods:

Heart material was taken from the four male lambs which received enteric origin toxin and also from 5 normal 8 - 10 month old Romney cross lambs which had not received toxin.

Pieces of myocardium weighing approximately 1 gram were removed from the centre of the interventricular septum of the heart, chopped up finely with a scalpel, weighed, freeze-dried and reweighed to establish the water content as described in Chapter 8.

Myocardial sections for light and electron microscopy were taken from the control lambs and the lambs which received parenteral and enteric origin toxin described in Chapter 6. In addition myocardial sections for electron microscopy were taken from control mice and intoxicated mice which survived for 3 and 9 hours in the experiments described in Chapter 8.

Results:

There was a quantitative increase in the water content of myocardium from the lambs which had received enteric origin toxin, when figures obtained from these animals were compared with those obtained from the normal control lambs. The $\overset{mean}{\wedge}$ water content of the heart muscle from the intoxicated animals was $4.46 \pm 0.49 mgH_2O/mg$ D.M., while that of the controls

was $3.68 \pm 0.19 mgH_2O/mg$ D.M.. The difference between these figures is statistically significant (p between 0.05 and 0.025). Data from this experiment is included in Appendix 8b.

Severe endothelial damage, similar to that already described in other tissues, was present in the myocardium from intoxicated lambs, (Figure 9.4) but in this instance it was overshadowed by changes in the myocardium itself. At the ultrastructural level there was extensive fluid accumulation in the interstitial tissues, particularly around blood vessels. In addition the myocardial cells themselves showed evidence of an increase in intracellular fluid. The sarcolemma bulged in the areas between the 'Z' discs and there was also separation of the bundles of myofibrils and mitochondria by accumulated fluid (Figures 9.5, 9.6). At a higher magnification it was possible to demonstrate swelling of the mitochondrial cristae compared with these structures in normal heart (Figure 9.7).

These lesions were also present in the hearts of lambs which had received enteric origin toxin but there was no detectable fluid accumulation in the hearts of intoxicated mice, nor was capillary endothelial damage prominent in the latter animals.

In some, but not all, of the lambs there was also light microscopic evidence of fluid accumulation in the myocardium. The interstitial tissues were oedematous, particularly around the Purkinje Fibres and blood vessels, with proteinaceous material in the fluid in some areas (Figure 9.8). No Purkinje fibres were sectioned in any of the material taken for electron microscopic examination and it is not possible to say with any certainty whether the fibres themselves were affected by the fluid accumulation. There was evidence of very recent focal subendocardial haemorrhage in formalin fixed material from hearts in which this lesion had been seen grossly.

Discussion

The extremely severe endothelial damage seen in the lung capillaries of lambs which had received parenterally administered toxin is almost certainly due to the fact that the pulmonary capillary network is the first such network encountered by the toxin following intravenous administration.

Figure 9.4a: Vascular Endothelial Damage in Lamb Heart Following the Parenteral Administration of Epsilon Toxin.

The cytoplasm of the endothelium (En) is almost entirely absent and there is focal complete loss of continuity (X) in the capillary wall with an adjacent aggregation of thrombocytes (T).

N = nucleus of endothelial cell
E = erythrocytes
F = fluid in interstitial tissue
PM = sarcolemma of cardiac muscle cell
My = myofibrils

Magnification 30,000

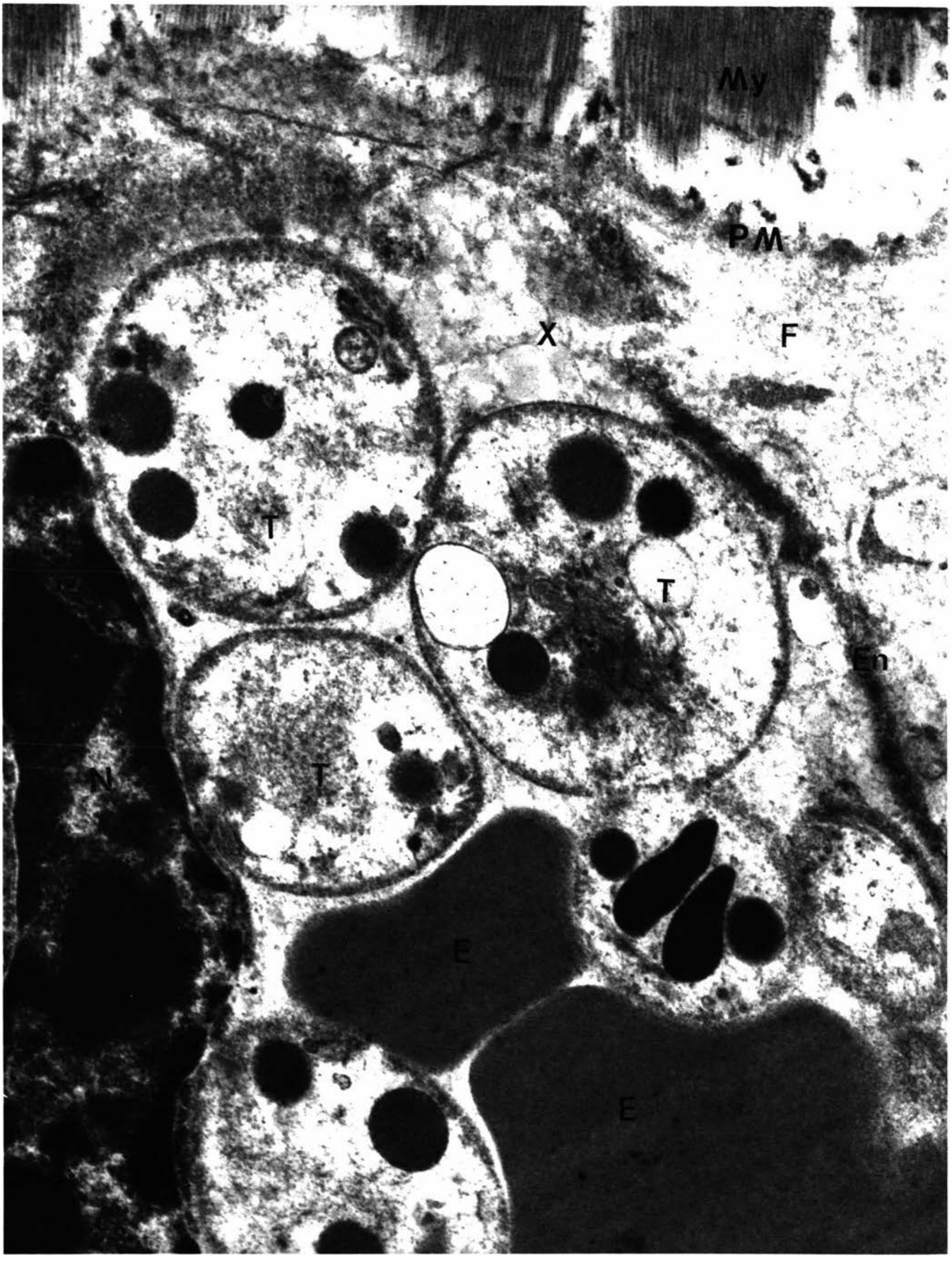

My
PM
X
F
T
T
T
En
N
E
E

Figure 9.4b: Vascular Endothelial Damage in the Myocardium of Intoxicated Lambs.

A

B

A. Normal Capillary in the Heart of a Control Lamb:

N	=	nucleus of endothelial cell
En	=	endothelial cytoplasm
E	=	erythrocyte
Tj	=	'tight' junction
PM	=	Plasma membrane
Z	=	respective band in the myofibrils (My).

B. Capillary from the Heart of a Lamb which had received Parenterally Administered Epsilon Toxin:

The endothelium is severely damaged and the cytoplasm has become reduced to a thin, electron-dense band which is extremely tenuous in some areas. A thrombocyte (T) is adherent to the endothelium adjacent to the nucleus. There is marked oedema (Oe) of the interstitium and the heart muscle cells are swollen, resulting in bulging of the sarcolemma (PM) between the 'Z' bands.

Magnification 22,000


My
N
En
PM

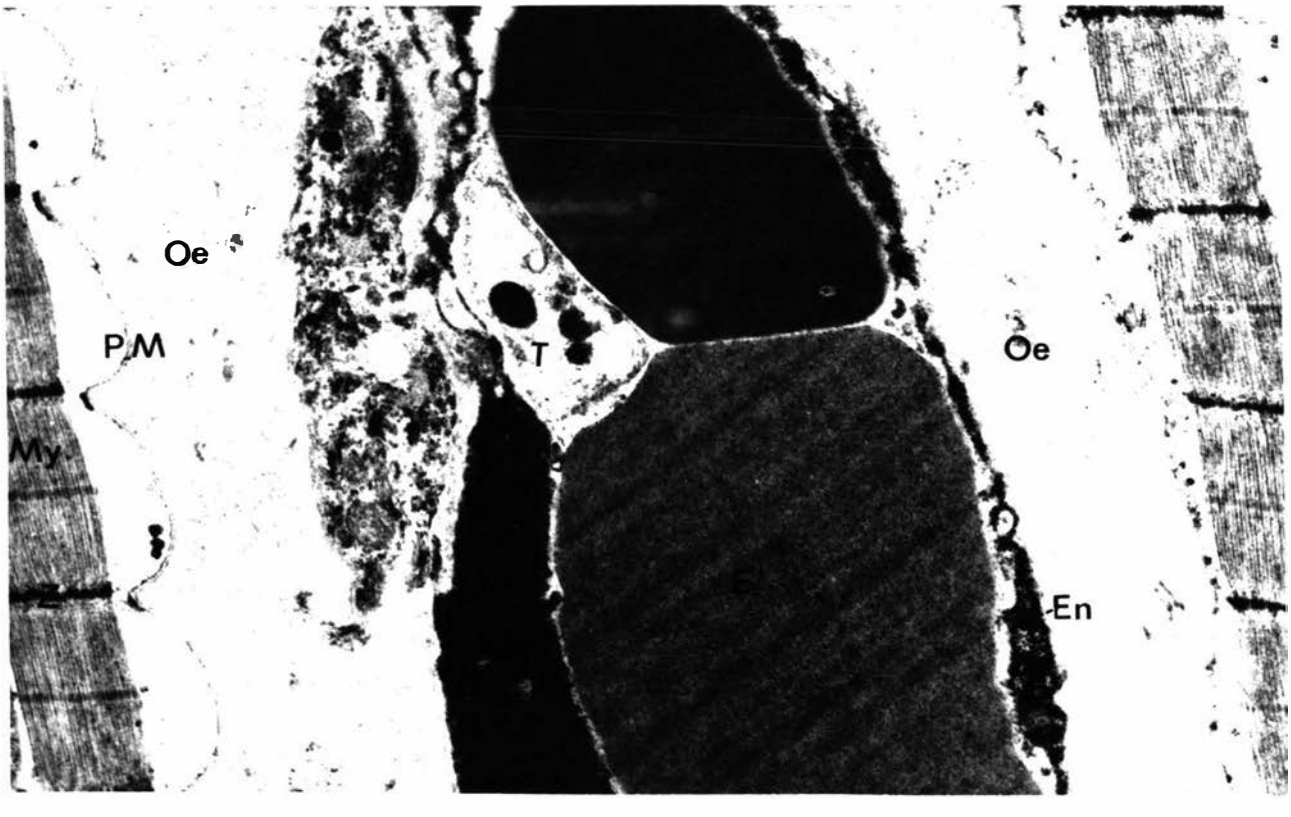

Oe
PM
My
T
Oe
En

Figure 9.5: Myocardial Oedema in Intoxicated Lambs.

A

B

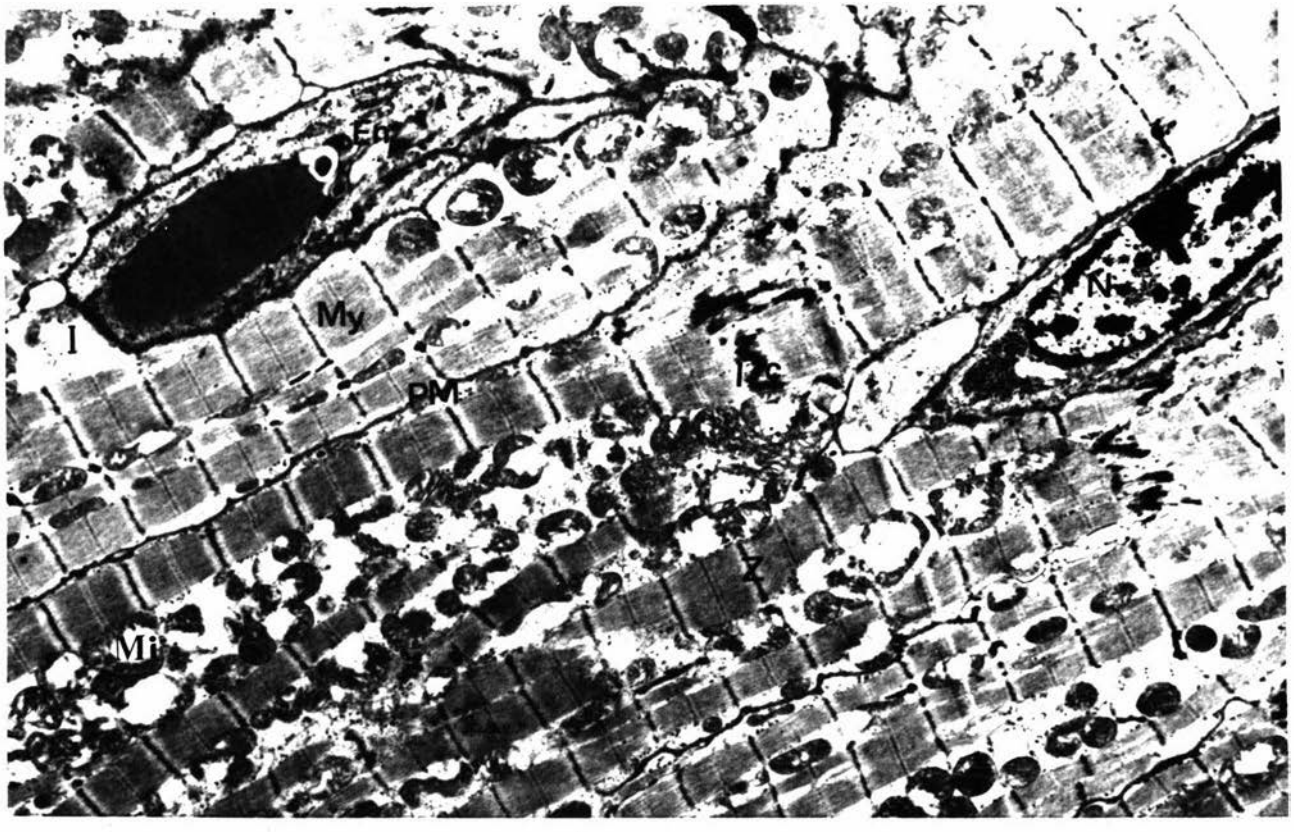
A. Normal Myocardium from Control Lamb:

En	=	normal capillary endothelium
Mi	=	mitochondria
Mf	=	myofibrils
Ic	=	intercalated discs
PM	=	Plasma membrane (sarcolemma of cardiac muscle cells)
Z	=	'Z' bands in the myofibrils

B. Myocardium from a Lamb which had received Parenterally Administered Epsilon Toxin:

There is evidence of capillary endothelial damage which is most prominent in the capillary on the left of this electron photomicrograph. Extensive oedema (Oe) of the interstitium (I) is also present. In addition the cardiac muscle cells are swollen and it can be seen that there is separation of the myofibrils and mitochondria, plus bulging of the sarcolemma between the 'Z' bands.

Magnification 6,200


En
My
I
PM
Ki
Mi
A
Z

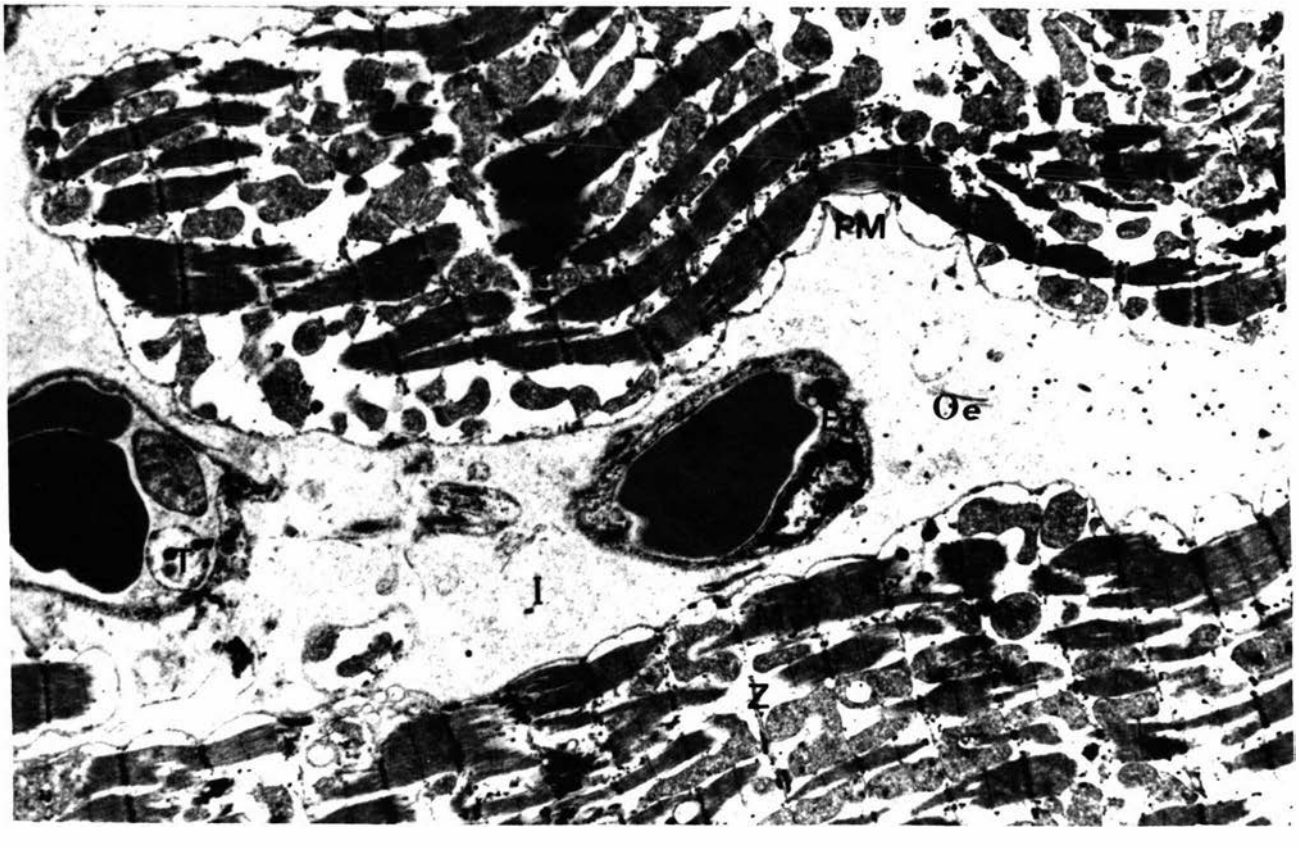

PM
Oe
T
I
Z

Figure 9.6: Myocardial Oedema in Epsilon Toxin Intoxication and Experimental Enterotoxaemia.

A

B

C

A. Normal Myocardium from a Control Lamb:

The cardiac muscle cells are closely applied to one another and also to the capillary (BV) in this section. The capillary endothelium is normal.

N = nucleus of cardiac muscle cell.

PM = Sarcolemma (plasma membrane)

My = Myofibrils with electron-dense 'Z' bands

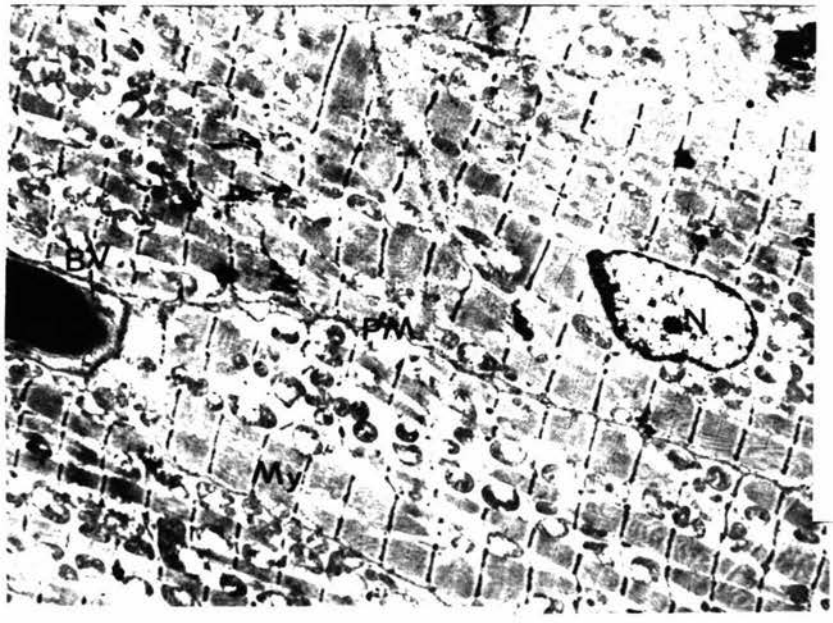
B. Myocardium from a Lamb which had received Parenterally Administered Epsilon Toxin:

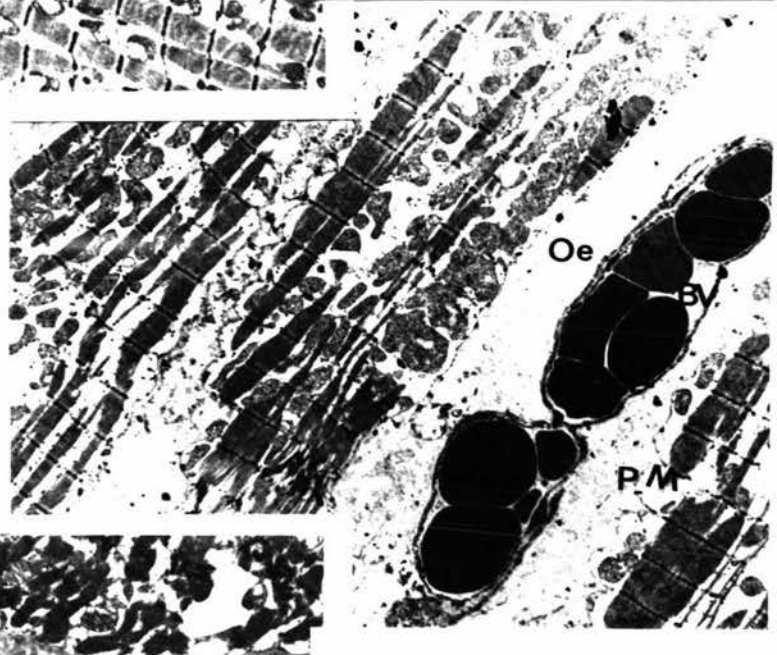
There is extensive oedema (Oe) of the interstitium and, in addition, the cardiac muscle cells are swollen and the sarcolemma (plasma membrane - PM) bulges between the 'Z' bands. The endothelium of the capillary is damaged.

C. Myocardium from a Case of Experimental Enterotoxaemia in a Lamb:

Extremely severe oedema of the interstitium is present in this section and, as in B, the cardiac muscle cells are swollen.

Magnification 3,000


BV
PM
N
My


Oe
BV
PM

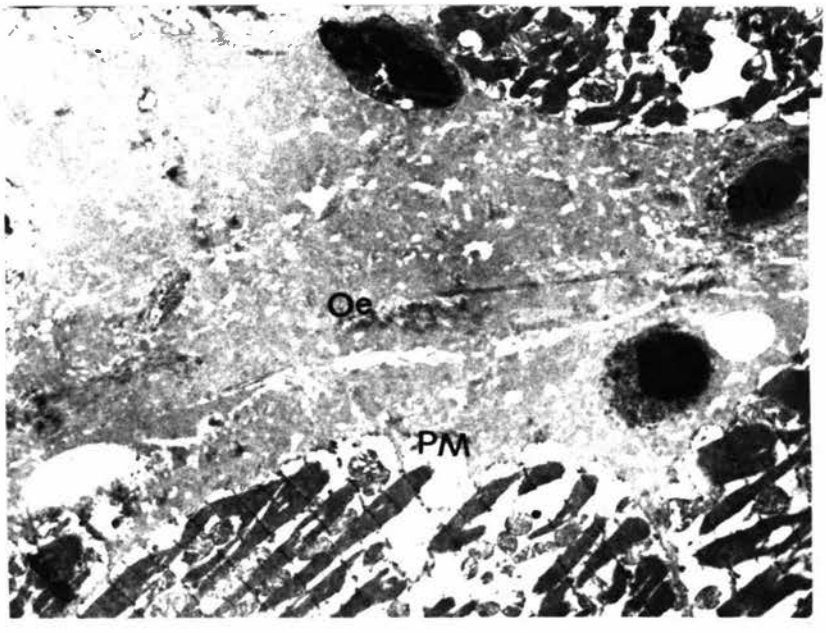

Oe
PM

Figure 9.7: A / B — Swelling of Mitochondrial Cristae in the Myocardium of Intoxicated Lambs.

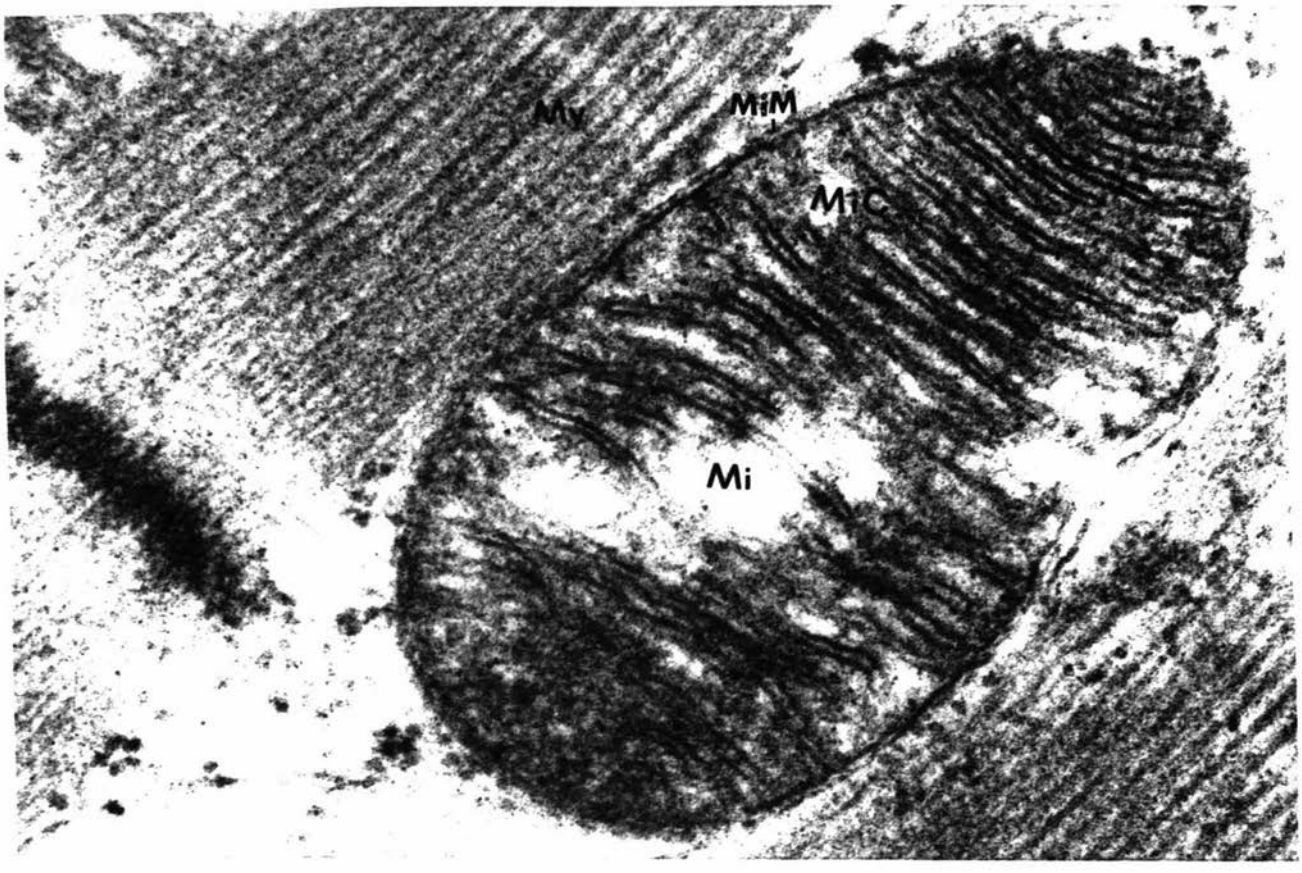
A. Normal Mitochondrion of Cardiac Muscle from a Control Lamb:

Mi = mitochondrion
MiC = mitochondrial cristae
MiM = outer and inner mitochondrial membrane. The inner membrane is continuous with the cristae.
My = myofibrils
Z = 'Z' lines

B. Mitochondrion from Cardiac Muscle Cell of a Lamb which had Received Parenterally Administered Epsilon Toxin:

The mitochondrial cristae are swollen and the mitochondrion is surrounded by a clear space, indicating intracellular fluid accumulation.

Magnification 106,000


My
MiM
MiC
Mi


MiC
Mi

Figure 9.8: Myocardial Oedema in Intoxicated Lambs - Light Microscopy.

A

B

A. Normal Lamb Myocardium:

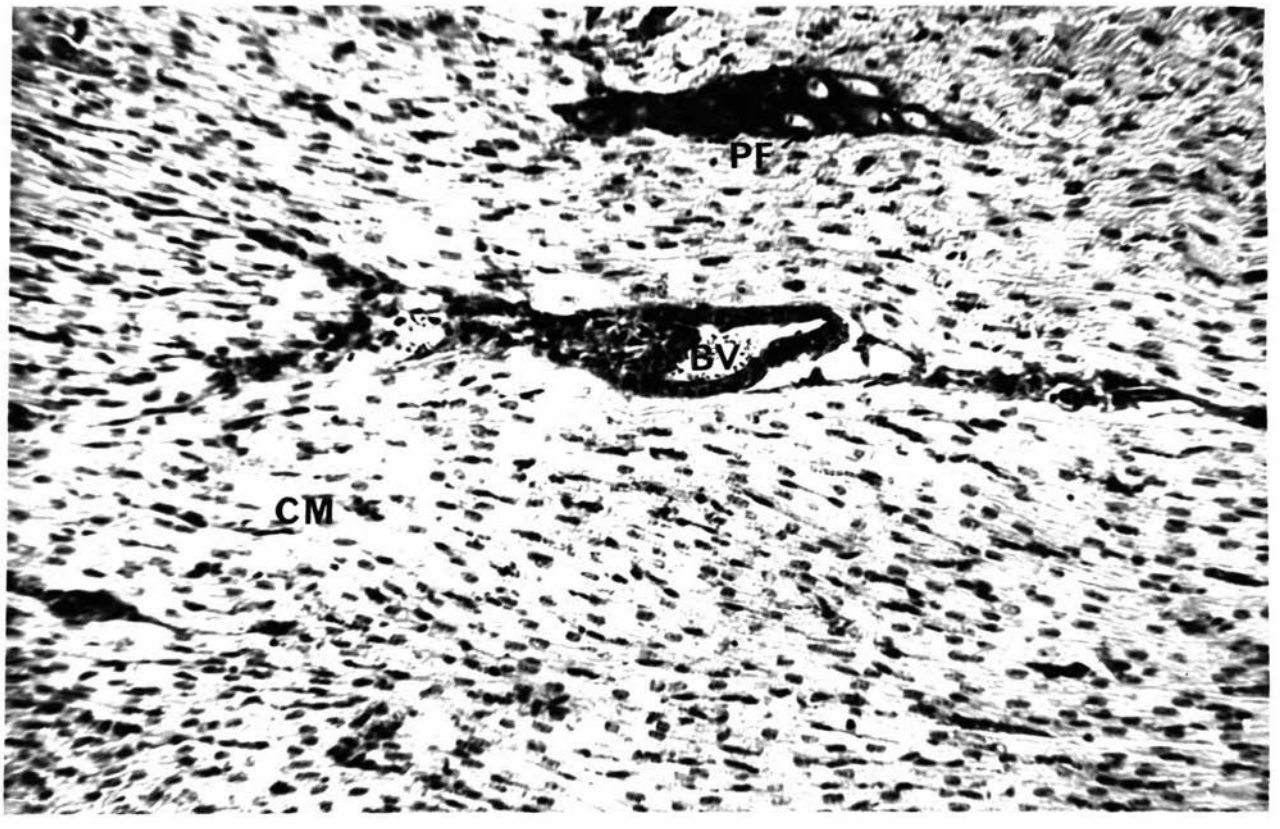
Section of heart from a control lamb for comparison with B below.

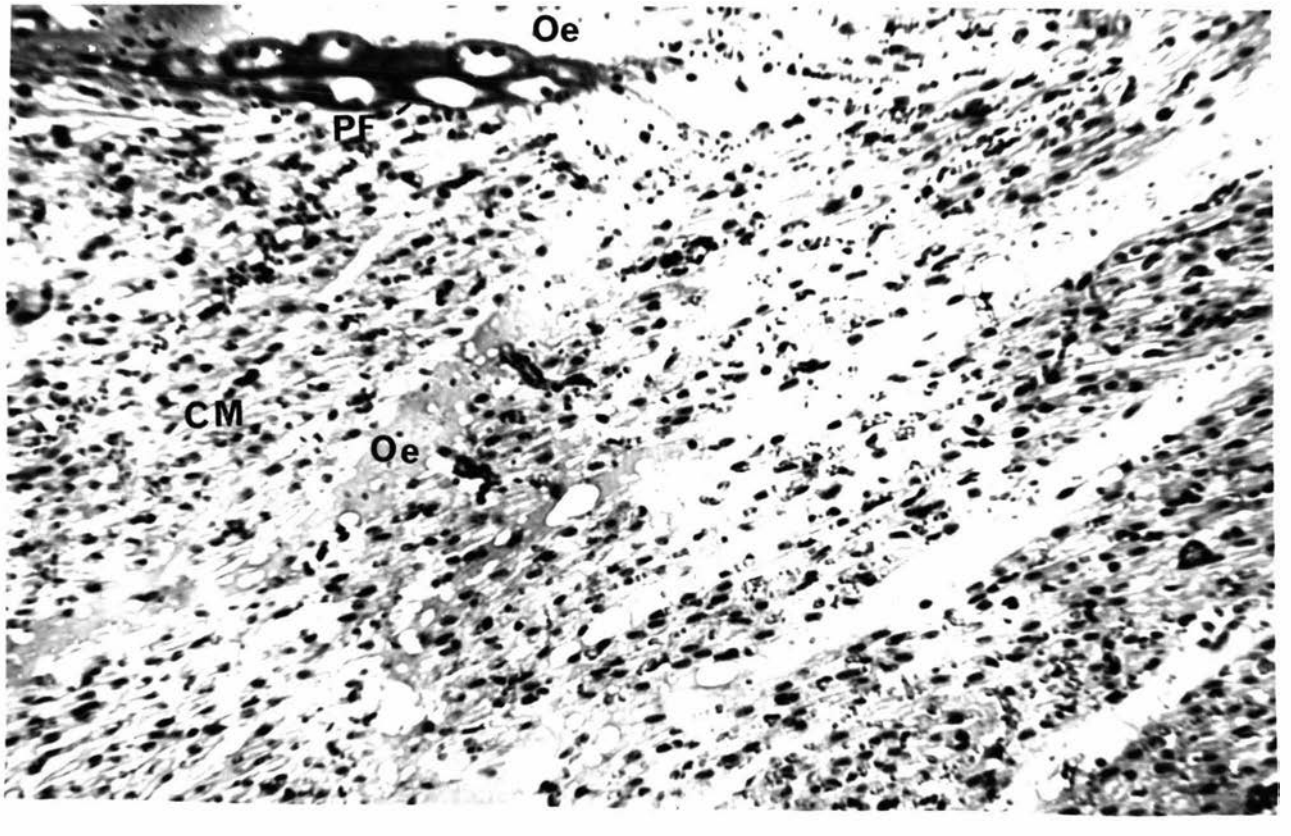
CM = cardiac muscle fibres.
BV = blood vessel
PF = Purkinje Fibre

B. Myocardium - Intoxicated Lamb:

Severe interstitial oedema (Oe) has resulted in separation of the bundles of cardiac muscle fibres. It is particularly noticeable around the Purkinje fibres.

Stain PAS
Magnification 180


PF
BV
CM


Oe
PF
CM
Oe

In contrast, when the toxin is absorbed from the intestine, it first encounters the sinusoidal network of the liver and the damage to lung capillaries would be similar to that in the systemic circulation under these circumstances. It is also possible that the liver may remove some other toxic components which could be present in parenterally administered toxin as discussed earlier when considering electrocardiographic alterations in Chapter 7. If pulmonary vascular damage were due to impurities in the parenterally administered toxin preparations the lesion could be expected to occur consistently when the material was administered at a dose great enough to produce lung oedema in a susceptible animal, but this was not the case. The fact that pulmonary oedema did not occur when toxin was administered by continuous infusion suggests that the most severe capillary endothelial damage occurs when high concentrations of toxin enter the capillary network, as would occur when toxin was administered in divided doses. This is further born out by the fact that pulmonary oedema only occurred in mice when very high levels of toxin were administered intravenously as described in Chapter 7.

From the comparative studies on the action of the toxin on the capillaries in lungs and hearts from intoxicated lambs and mice it also appears that, as postulated in Chapter 7, there may be a very marked species difference in the susceptibility of capillary endothelium to the action of epsilon toxin.

Although there is obvious fluid accumulation in the interstitial tissue around Purkinje fibres in the heart detectable by a light microscopy, it appears that the fibres themselves are not affected functionally since any disturbance in their activity could be expected to result in a prolonged atrio-ventricular conduction time with a concommittent increase in duration of the P-R interval and QRS complex of the ECG's. This was not a prominent feature of the ECG's of intoxicated animals in the present study (Chapter7.)

Turning now to the ultrastructural changes in the cardiac muscula-
ture itself, these too are associated directly or indirectly with capillary endothelial damage and extracellular fluid accumulation. This would cause considerable interference with the transfer of nutrients to, and waste products from the cardiac muscle cells and therefore the changes in the myocardial fibres

themselves require consideration in the light of existing knowledge of their metabolism, structure and function.

As in other cells, the electrical potentials of the healthy resting myocardium are maintained by a difference in ionic concentration across the plasma membrane, with high concentrations of potassium normally being maintained within the cells, by exclusion or removal of sodium ions. The maintainance of the ionic gradients is an energy dependent process with active transfer of sodium ions against the tendency to establish ionic equilibrium. This is the so-called 'sodium pump'. It is generally considered that the transfer of water across cell membranes is also intimately associated with ionic transfer. Depolarisation of the fibre, following the arrival of an impulse via the conducting system, results in an inflow of sodium ions with a resultant change in electrical potential. This is followed by an energy dependent recovery phase during which the original ionic gradient is re-established. The rapidity of the spread of depolarisation and repolarisation of the myocardium is associated with the presence within the muscle cells of a complex system of microtubules which are continuous with the extracellular space through pores which open through the sarcolemma in the region of the 'Z' lines (Page, 1968). The high energy metabolism of the cells is emphasised by the large numbers of mitochondria interspersed between the myofibrils which form the contractile portion of the cell.

In the intoxicated lambs in the present study the increased amount of intracellular fluid in the cardiac muscle cells suggests that there is an impairment of water and ionic homeostasis. The swelling of mitochondrial cristae suggests that their function may also be impaired.

At this stage it is possible to consider the electrocardiographic changes described in Chapter 7 in relation to the morphological changes described in the present chapter. In view of the vascular damage and pericardial effusions which occur in intoxicated animals the changes in the S-T segment of the ECG could be due to damage to the subepicardial myocardium. since S-T segment displacement also occurs in pericarditis and is believed to result from associated involvement of the superficial myocardium. This is unlikely to provide a complete explanation in the

present instance as there is no morphological evidence to suggest that there is selective damage to this region of the myocardium in intoxicated animals. On the other hand, there is every reason to regard the ECG changes which occur in intoxicated animals as being associated with myocardial ischaemia and injury. The vascular damage and myocardial oedema, produced by epsilon toxin, would tend to limit the transfer of nutrients to the cardiac muscle cells and, as a result, available energy would be reduced. This could interfere with the maintenance of ionic concentrations and polarity of the plasma membrane and would be reflected as reduced voltage in the QRS complex, S-T segment deviations and T wave alterations in the ECG.

Conclusions

1. There is severe capillary endothelial damage in the lungs of intoxicated lambs and this results in pulmonary oedema in some animals.

2. The development of pulmonary oedema is dependent upon the severity of the capillary endothelial damage and this is directly dependent upon the concentration of toxin reaching the lung capillaries. High concentrations of epsilon toxin are most likely to enter the pulmonary circulation when the toxin is administered intravenously in divided doses.

3. There is a possible species variation, between mice and lambs, in the susceptibility of vascular endothelium to the action of epsilon toxin. This results in pulmonary oedema being rare in the former species unless high concentrations of toxin are administered. It may also account in part for the relatively higher dose of toxin required to produce intoxication in mice.

4. The capillary endothelium in the hearts of intoxicated lambs is severely damaged by epsilon toxin and this leads to the development of a severe myocardial oedema, with both extra- and intracellular accumulations of fluid.

5. The vascular damage and extravasation of fluid may be associated with defective transfer of essential nutrients from the blood stream to

the heart muscle. This could reduce available energy and interfere with the maintainance of ionic homeostasis, resulting in intracellular fluid accumulation within cardiac muscle cells.

6. Acute capillary endothelial damage and oedema in the myocardium can produce changes in the electrocardiographic pattern which are similar to those which occur in myocardial ischaemia or injury. It is possible that the ECG changes may be due to depressed energy metabolism and thus inability to establish and maintain the normal ionic gradients within the cardiac muscle fibres.

CHAPTER 10: THE DEVELOPMENT OF RENAL LESIONS IN ENTEROTOXAEMIA.

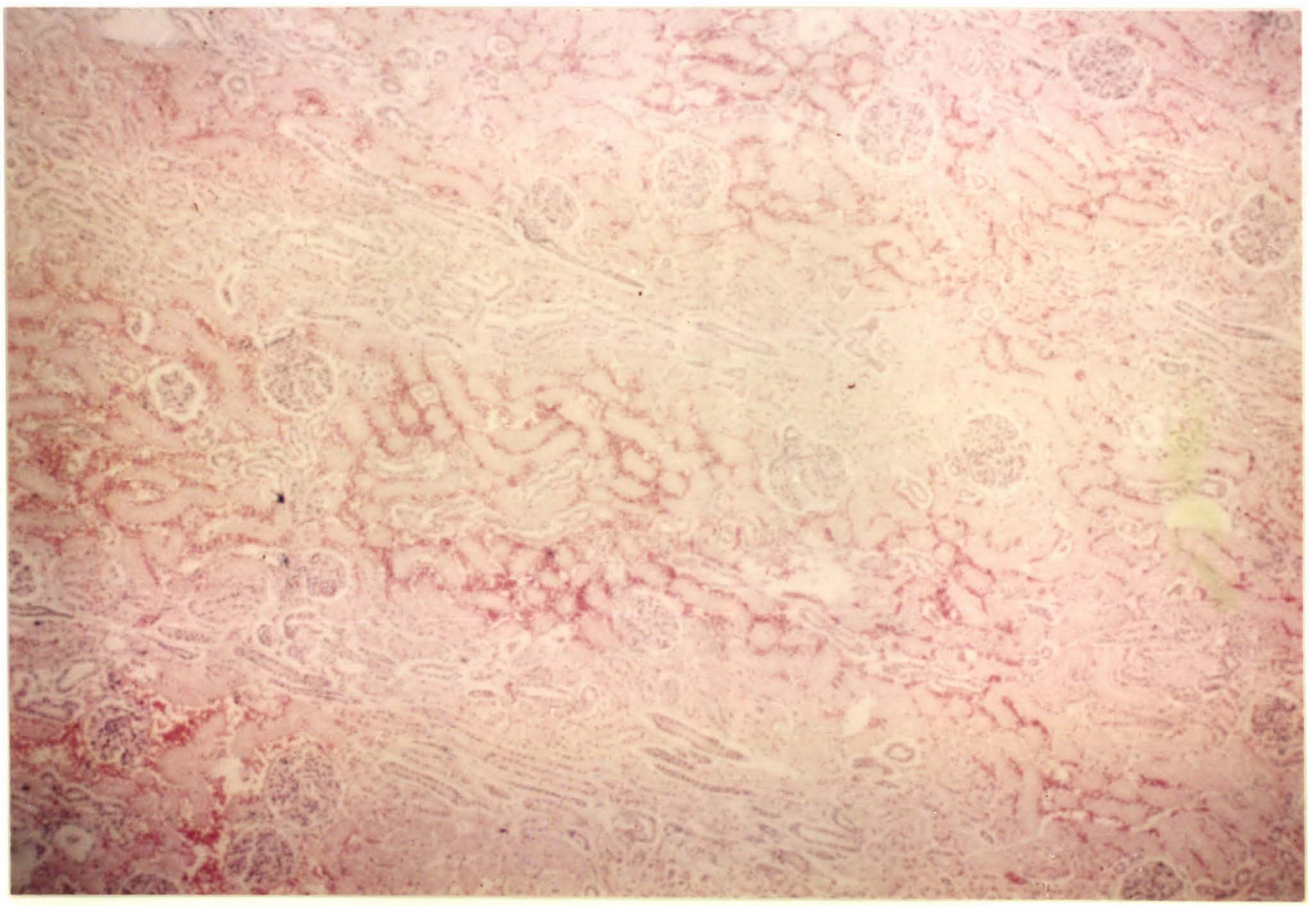
Although it is generally agreed that the dissolution of the kidneys which occurs in enterotoxaemia does not develop until several hours have elapsed after death of the animal, a number of changes such as proximal tubular necrosis (Gill, 1933), alterations in the solubility of the renal proteins (Sotirov, 1965c) and reduction in the alkaline phosphatase activity of the brush border of the proximal tubular epithelium (Sotirov & Bozhkov, 1965d) have all been recorded as being present at the time of death. The exact time at which samples were taken in relation to death has not been recorded in many of these reports and it is often difficult to ascertain whether autolytic changes played a part in the development of the lesions that were described.

The features of the renal histopathology which are regarded in N.Z. as being suggestive of enterotoxaemia are necrosis of the renal proximal tubular epithelium and interstitial haemorrhage between these tubules, as illustrated in Figure 10.1. Both Gill (1933) and Bennetts (1932b) have mentioned the rapid development of degenerative changes in the renal proximal tubular epithelium of intoxicated lambs after death when compared with normal control lambs, and have suggested that the tubular necrosis is present at the time of death although no conclusive evidence has been presented. Further, while Gill (1933) and Niilo (1963) have referred to the interstitial haemorrhage, there does not appear to be any published work on the possible pathogenesis of this lesion or whether it develops before or after death.

The lesions which have been described in the lung, heart and brain suggest that the renal haemorrhage might be associated with the antemortem vascular damage. Therefore the sequence of changes which occurred in the kidneys of intoxicated animals up to the time of death and for several hours thereafter were investigated in an attempt to elucidate the pathogenesis of the renal changes.

Because a number of factors unassociated with the toxin can influence the results of a study of this nature, certain control experiments had to be carried out in association with the studies on the effect of the toxin. For example it was mentioned earlier that free

Figure 10.1: The Light Microscopic Appearance of the Renal Changes in a Field Case of Cl. perfringens type D Enterotoxaemia



This photomicrograph illustrates the typical 'necrosis' of the proximal tubular epithelium and the interstitial 'haemorrhage' which are seen in sections of kidneys from many of the cases of enterotoxaemia which are submitted for laboratory examination.

Stain H & E

Magnification 80

lipid accumulates in the cells of the proximal tubular epithelium of the kidneys of mice which are deprived of food for a period of time. Intoxicated mice which survive for any length of time are extremely depressed and do not eat. It was therefore necessary to compare the renal lesions which developed in intoxicated animals which survived for different lengths of time after inoculation, with those which occurred in normal mice which had been deprived of food for similar periods. While on this subject it is appropriate to mention that, although free lipid has been shown to be present in the tubular epithelium of mice after 24 hours starvation (Mowry, 1965 quoted by Dunn in Cotchin & Roe, 1967), there does not appear to be any published description of the time sequence of its appearance, nor has the ultrastructural site of accumulation of the lipid been described. It proved possible to elucidate both these features in the course of the experiments to be described in this chapter. Because uncomplicated autolysis can cause degenerative changes in the renal tubular epithelium and a reduction in the alkaline phosphatase activity of the brush border of the proximal tubular epithelium, it was necessary to compare the sequence of renal changes which developed in intoxicated animals after death with autolytic changes which occurred in normal animals after a similar time had elapsed post mortem. In this respect it should be borne in mind that the temperature of intoxicated animals rises terminally associated with convulsive episodes and the carcase temperature will be considerably higher than that of the environment or of a normal animal which has been killed with an anaesthetic overdose. Autolysis is therefore likely to develop more rapidly in the carcase of the intoxicated animal. In a good-condition, woolly lamb in a warm environment the carcase temperature is liable to remain high for some hours after death, resulting in extremely rapid autolysis. Thus, in the present investigation the renal changes in intoxicated lambs were compared with those in a similar normal lamb and, in addition, the renal changes in intoxicated mice held at 37°C were compared with those in normal mice held under the same conditions to compensate for differences in carcase temperature.

The Lesions which Occur in the Kidneys of Intoxicated Animals at the Time of Death

Materials & Methods:

Along with brain and a range of other tissues, kidneys were taken, at the time of death, from all the control lambs and lambs which received parenterally administered or enteric origin epsilon toxin. Kidneys were also available from control and intoxicated mice used in the experiments described in Chapter 8. Sections for light and electron microscopy were prepared as described in that chapter.

Results:

There were no gross differences between the appearance of kidneys taken from control mice and those taken from intoxicated mice, at the time of death.

There were no prominent gross lesions in the kidneys removed from the carcases of intoxicated lambs at the time of death. The kidneys of these animals were congested and blood oozed freely from the cut surface, but the consistency and gross appearance of the cortex and medulla appeared to be quite normal.

No marked changes were detectable by light microscopy in H&E or PAS stained sections of control or intoxicated mouse or lamb kidneys. Bouins fluid gave excellent preservation of the fine structure of the kidney and features such as mitochondria, and the brush border. Even the outline of the tubular margin of the rather vacuolated cytoplasm of the cells of the distal convoluted tubules could be seen when this fixative was used. On the other hand the erythrocytes were almost completely lysed and appeared as faint ghosts in Bouin's fluid fixed material. Thus, in these sections it was not possible to detect interstitial haemorrhage and formalin fixed material had to be used for this purpose.

The PAS staining affinity of the brush border of the proximal convoluted tubules and the basement membranes was prominent and of

similar intensity in kidney sections from control and intoxicated animals. In formalin fixed H&E stained material, particular attention was paid to the appearance of the proximal tubular epithelium and these sections were also examined carefully for the presence of interstitial haemorrhage. There was no evidence of proximal tubular necrosis or interstitial haemorrhage in any of the kidney sections from intoxicated mice or lambs.

Severe vascular endothelial damage, detectable by electron microscopic examination, was present in the capillaries between the renal tubules in the kidneys from intoxicated lambs (Figure 10.2). This was essentially similar to the changes which were seen in the capillaries in the brain, lung and heart of intoxicated animals. These changes have already been covered in Chapters 8 and 9 and need not be described in detail here. Vascular endothelial damage was also detectable in the intertubular capillaries of the kidneys of intoxicated mice and here again the changes were similar to the vascular lesions in other organs of intoxicated mice. It should be noted that the specialised arterial capillaries in the glomerular capillary tuft were not affected by epsilon toxin in either mice or lambs, nor was the glomerular architecture affected. The proximal tubular epithelium of kidneys from intoxicated mice and lambs was also unaffected by the toxin. There was accumulation of free lipid in this region in some sections from the latter species but this finding will be discussed more fully later in the present chapter.

The Influence of Epsilon Toxin Upon the Histochemical Activity of the Kidney

Alterations in the activity of a large number of histochemically demonstrable enzymes in the kidney have been described in a wide variety of conditions in which renal damage occurs. However it is still questionable whether histochemical changes are demonstrable before there is evidence of renal damage detectable by conventional histological stains such as H&E and PAS in many of these conditions (Wachstein in Becker 1968).

Figure 10.2: Vascular Endothelial Damage in the Renal Intertubular Capillaries of Intoxicated Lambs.

A

B

A. Normal Intertubular Capillary from a Control Lamb:

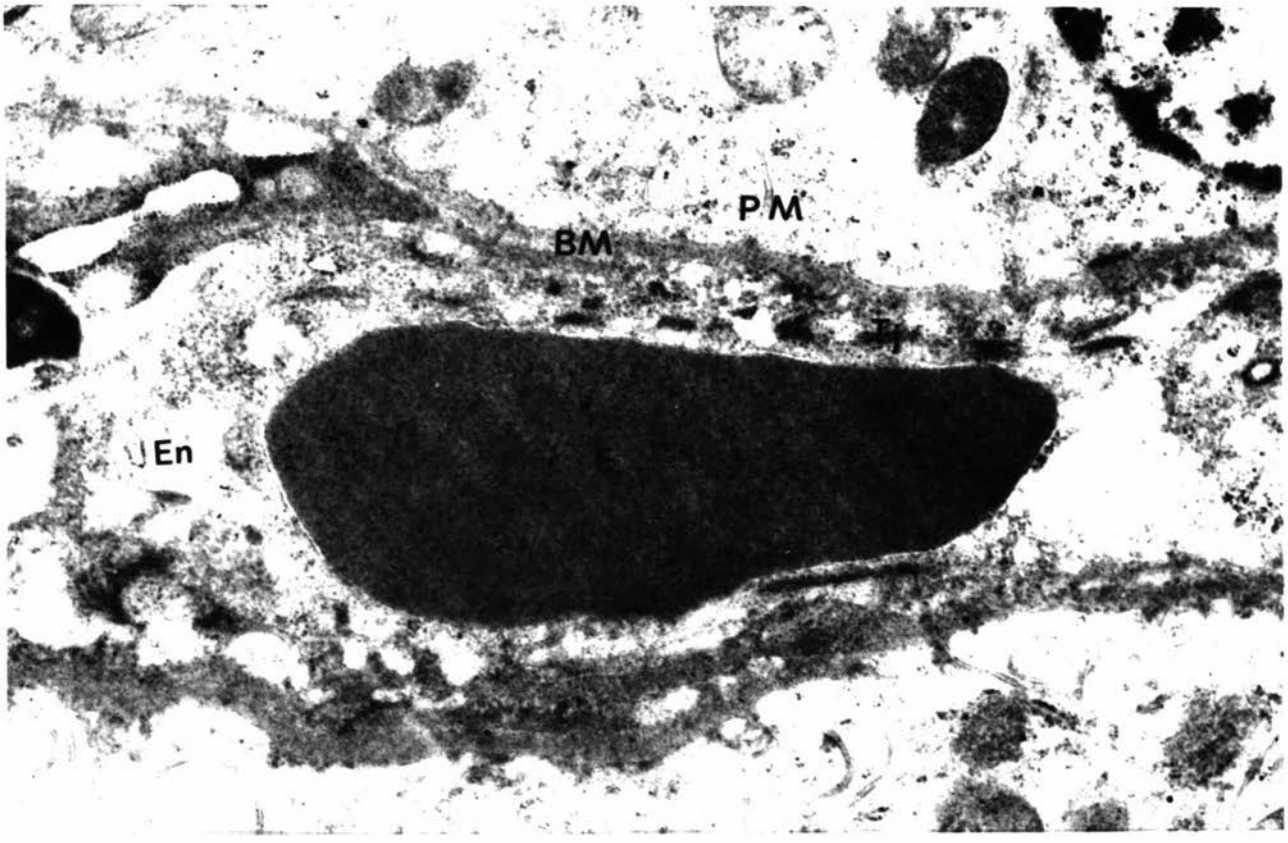
En = endothelial cytoplasm
E = erythrocyte
BM = basement membrane
PM = folded plasma membrane of the proximal tubular epithelium.

B. Portion of a Capillary from a Lamb which had received Parenterally Administered Epsilon Toxin:

The endothelial cytoplasm is severely damaged and there are two adherent thrombocytes (T) adjacent to the endothelial nucleus (N).

Mi = mitochondria

Magnification 23,000


PM
BM
En


E
T
T
BM
PM

Since it has been suggested that there is a reduction in alkaline phosphatase activity of the brush border of the proximal tubular epithelium in experimental epsilon toxin intoxication it was of interest to determine whether any reduction in the activity of this enzyme or some of the other enzymes which are abundant in kidney could be detected in the absence of morphological damage detectable with H&E or PAS staining.

Materials & Methods:

Frozen sections of the kidneys of the mice and lambs described in Chapter 8 were prepared. Because the distribution of histochemically demonstrable alkaline phosphatase in the kidneys of mice can be markedly influenced by the sex of the animal, (Dunn in Cotchin & Roe, 1967) sections from paired male and female mice from the intoxicated and control groups were examined in all the histochemical studies. Lamb tissues were fixed in cold formal calcium and infiltrated with sucrose-acacia prior to sectioning while the mouse tissues were used fresh and quenched on solid carbon dioxide ('Dry Ice') for sectioning. The histochemical procedures described by Thompson (1966) were employed to demonstrate the following enzymes:

Extramitochondrial Adenosine triphosphatase (ATPase), Glucose 6 phosphatase ($G6PO_4$ase), Succinic dehydrogenase, Triphosphopyridine diaphorase and Diphosphopyridine diaphorase (TPN and DPN diaphorase), Acid and Alkaline phosphatases. Trehalase was demonstrated by the method of Grossman & Sackter (1968).

Results:

There was no detectable difference between the activity of any of these enzymes in sections of kidneys taken from control and intoxicated animals at the time of death.

The Influence of Starvation Upon the Renal Lesions which Occur During Epsilon Toxin Intoxication in Mice

Materials & Methods:

The kidneys of intoxicated mice were obtained from animals which

survived for 3,6,9,12 or 18 hours in the experiments which were described in Chapter 8. The control animals for this experiment consisted of a similar group of male and female mice which were deprived of food and killed in pairs 0,3,6,9,12 or 18 hours after food and litter had been removed from the cages. Tissue samples were removed from the carcases of all animals and placed in Bouin's fluid, formol saline or glutaraldehyde for fixation. Some of the formalin fixed material was removed from the fixative after 24 hours and infiltrated with sucrose-acacia. Sections for light microscopy were prepared and stained with H&E and PAS as described in Chapter 8. In addition, frozen sections were cut from the sucrose-acacia infiltrated material and stained to demonstrate free lipid with Sudan 7B and Oil Red O fat stains. Sections for electron microscopy were prepared and stained as described forbrain in Chapter 8.

Results:

Light Microscopy:

There were no marked changes in H&E and PAS stained sections of kidneys from these animals at any of survival times examined.

Both Sudan 7B and Oil Red O fat stains gave essentially similar results but the definition and localisation of the lipid appeared to be superior with Sudan 7B and the changes seen in sections stained with this stain will be described.

No free fat could be demonstrated in the tubular epithelium of animals which were killed 3 hours after the administration of toxin or removal of the food supply. After 6 hours survival, the staining affinity of the epithelium for the fat stains was increased compared with that of kidney sections from animals with shorter survival times. In the kidneys of animals which had survived for nine hours small fat droplets were clearly visible in the tubular epithelium and the droplets became progressively larger and more numerous as the survival time increased to 12 and 24 hours. At no time was there any detectable difference between the kidneys of control and intoxicated animals.

Electron Microscopy:

An essentially similar situation to that described above was seen when sections from the kidneys of these animals were examined with the electron microscope and it was obvious with this instrument that the lipid accumulation began to occur at about the same time that it could be detected by fat stains and light microscopy. Again there was no detectable difference in the time of onset of lipid accumulation between control and intoxicated animals. The lipid was first seen in the kidneys of animals which survived for six hours and at this time consisted of small, amorphous, rather electron dense vacuoles scattered among the mitochondria in the deeper areas of the cells. These vacuoles became progressively larger and more abundant as survival time increased but in general they tended to be more numerous in the deeper parts of the cell and did not accumulate in the region below the microvilli which constitute the brush border at the ultrastructural level. (Figure 10.3). It should be noted that, with the exception of the 'control' sections electron photomicrographs are of the kidneys of intoxicated animals and it can be seen that epithelial changes, apart from lipid accumulation, were minimal when the kidneys were removed from the carcase at the time of death.

The lipid droplets themselves appear to be surrounded by a single membrane and can thus be described as true vacuoles. These vacuoles are morphologically distinct from the very dense [illegible] cytosomes with their 'myelin figures' which tend to be more numerous below the microvilli than in the deeper parts of the cell.

The Influence of Autolysis on the Development of Renal Lesions in Epsilon Toxin Intoxication and Enterotoxaemia

Experiment 1:

Materials & Methods:

In a preliminary experiment to ascertain whether autolysis affected the type of lesion seen, the carcase of one of the lambs which

Figure 10.3: Accumulation of Demonstrable Lipids in the Kidneys of Starved and Intoxicated Mice

A B

C D

A. Proximal Tubular Epithelium from a Mouse which died 3 hours after the Administration of Epsilon Toxin:

As in the control mouse (not shown) there is no detectable lipid present in this kidney.

N = nucleus
Mv = microvilli of the brush border
BM = basement membrane
PM = infoldings of the basal plasma membrane
Mi = mitochondria
Cy = cytosomes
V = vacuoles
Tj = 'tight' junctions between cells

B. Six hours after Toxin Administration:

Scattered small lipid-containing vacuoles (Lv) are present in the deeper regions of the cell adjacent to the basement membrane.

C. Twelve Hours after Toxin Administration:

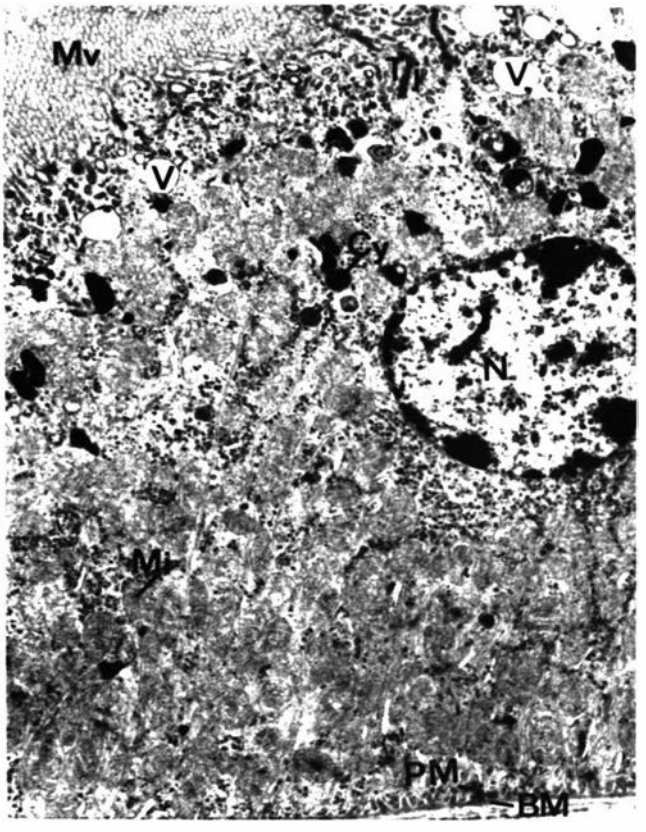
The lipid-containing vacuoles have become larger and more numerous.

D. Eighteen Hours after Toxin Administration:

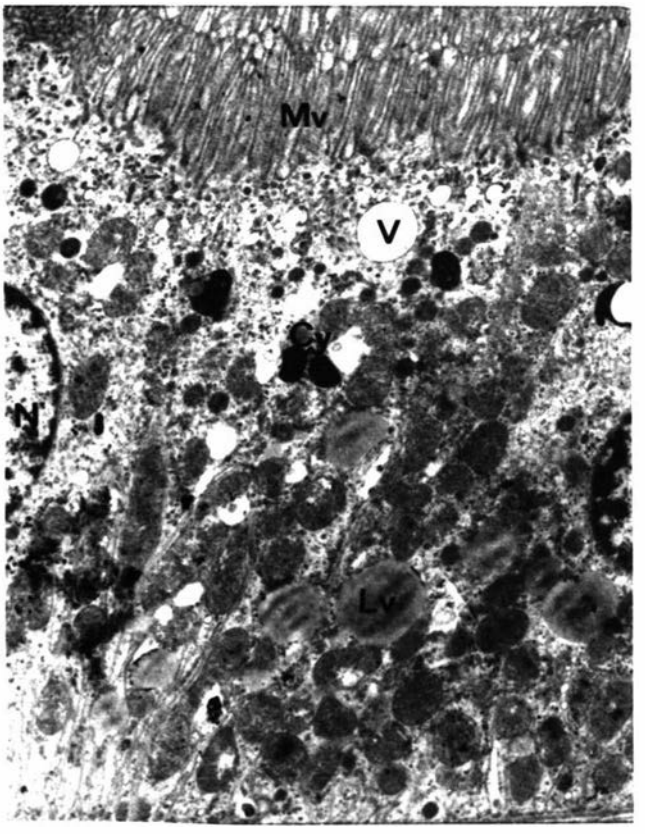
The appearance of the section of tubular epithelium is essentially similar to that seen at twelve hours. Fat stains and light microscopy suggest that in fact the amount of lipid increases during this period.

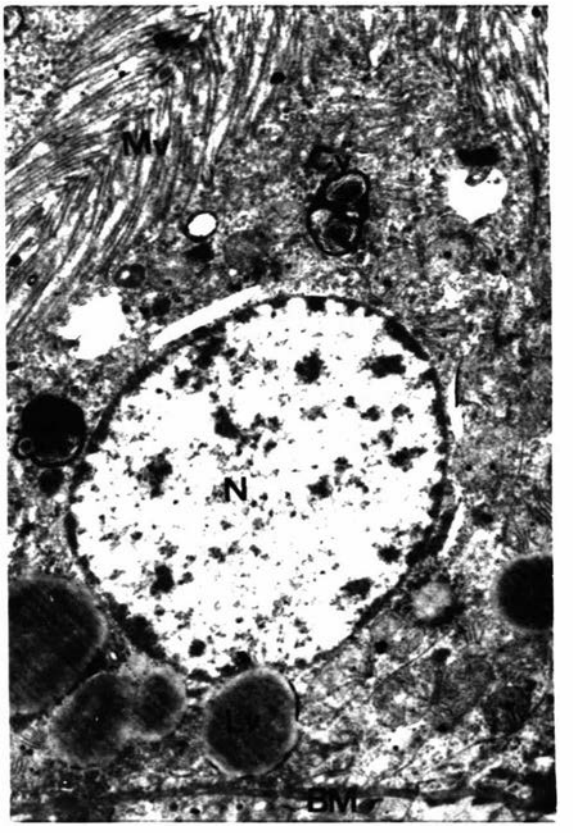
All these electron photomicrographs are of kidneys from intoxicated mice. The sequence of changes in starved animals was indistinguishable from those shown here.

Magnification 7,000


Mv
V
V
N
Mi
PM
BM


Mv
V
Mi
N
Lv
BM


Mv
V
N
Lv


Mv
N
BM

received parenterally administered toxin was reclosed immediately after removal of one kidney at the time of death. The carcase was held in a warm room for 6 hours then the second kidney was removed and treated in the same manner as the one obtained at the time of death. However, no material was taken from the second kidney for electron microscopic examination and the formalin fixed material was all used for alkaline phosphatase histochemistry.

Results:

The changes detectable in the kidney removed from the carcase 6 hours post mortem were as follows:

Grossly the kidney was firm, but pale fawn in colour and the cortex had an amorphous appearance. There was a marked reduction in the alkaline phosphatase activity of the brush border of the proximal tubular epithelium and a loss of PAS staining affinity of this region. This was associated with evidence of epithelial degeneration in the form of karyolysis. Due to the absence of H&E stained sections of formalin fixed material it was not possible to determine whether interstitial haemorrhage was present in this kidney.

Comment:

Since the histochemical and histological changes in the above kidney were similar to those described in field and experimental cases of enterotoxaemia, while detectable lesions had been absent from the kidney of this animal at the time of death, it was decided to investigate further the sequence of changes which occur post mortem in kidneys.

Experiment 2:

Materials & Methods:

Sixteen male and 16 female mice were divided into two equal groups and one group of each sex was given 2ug of batch CWD epsilon toxin. When an intoxicated mice died a similar control mouse was killed

by neck dislocation. Kidneys were removed from one pair of control and intoxicated male and female mice as soon as death occurred. The carcases of the remaining animals were held in petri dishes at 37°C in an incubator and the kidneys were removed from similar pairs of animals at 1,2,4 and 6 hours post mortem.

All samples of the kidneys were fixed, the alkaline phosphatase activity assessed histochemically and sections prepared and stained for light and electron microscopy as described previously.

Results:

As in previous mice, there were no differences between the microscopic appearance of the kidneys of control animals and those of intoxicated animals at the time of death, nor were any gross changes seen after the carcases had been held for up to 6 hours at 37°C.

The distribution and amount of histochemically demonstrable alkaline phosphatase in the kidneys of all animals altered as autolysis progressed. The reaction was intense in the brush border of the proximal convoluted tubules in both groups of animals at the time of death. There was an apparent reduction in the alkaline phosphatase activity of the brush border of the kidneys of intoxicated animals which were necropsied 4 hours after death. This change was prominent at 6 hours post mortem and at the same time the formazan pigment tended to become distributed throughout the tubular epithelium. In control animals the reduction in activity was first detectable in kidneys which had been removed from the carcase 6 hours post mortem.

In both intoxicated and control animals, the first renal changes that were detectable in H&E and PAS stained sections of kidneys was karyolysis in the proximal convoluted tubular epithelium which was seen in sections of kidney taken 6 hours after the death of the animal. At no time was there any detectable difference in the appearance of H&E and PAS stained

sections of kidney from control and intoxicated mice. There was no evidence of interstitial haemorrhage in the sections of formalin fixed kidney from these animals.

The electron microscopic appearance of the autolytic changes which developed in the kidneys was essentially similar in control and intoxicated mice, with the exception that the vascular endothelium of control animals tended to become swollen and vacuolated as autolysis progressed. The endothelium was still intact in these animals 6 hours after death, while it was almost completely absent in intoxicated animals at all times. There was however no apparent dissolation of the capillaries with release of enythroytes into the interstitium in the latter mice. The post mortem sequence of autolytic changes in the kidneys of control and intoxicated mice is shown in Figures 10.4a-b.

Experiment 3:

Materials & Methods:

Two of the 8 month old lambs which had received enteric origin toxin and two control sheep, which had been destroyed by the intravenous administration of 'Euthatal' (May & Baker, Dagenham, England) were placed in left lateral recumbency as soon as death occurred. An incision was immediately made in the abdominal wall parallel to, and just below the lumbar musculature. A piece of both the upper and lower kidney was removed from each animal and the carcases closed and left at room temperature for 10 hours. During this time the incision was opened at 2 hourly intervals and further pieces of kidney removed. The kidney material was treated as described in Experiment 2.

Results:

The renal changes in intoxicated lambs at the time of death were confined to the vascular endothelium and were only detectable at the ultra-structural level. There were no gross alterations

Figure 10.4a: The Sequence of Autolytic Changes in the Kidneys of Normal Mice-Electron Microscopy

A B
C D

A Time of Death:

The endothelium (En) of the blood vessels and the tubular epithelium (Ep) are normal.

E = erythrocyte.

B. 2 Hours Post Mortem:

The endothelium is vacuolated and the mitochondria of the tubular epithelium are swollen.

C. 4 Hours Post Mortem:

The changes in this section are more advanced. The endothelium is extremely vacuolated and swollen.

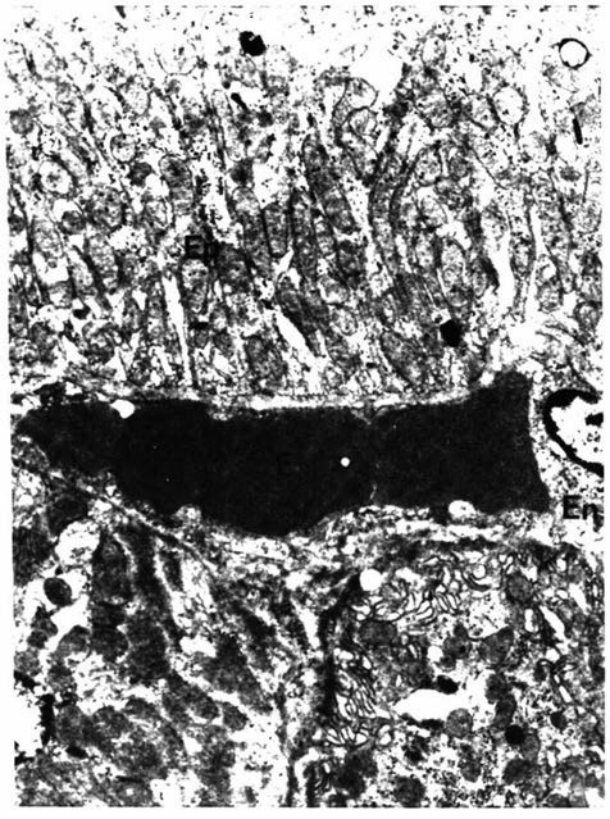
D. 6 Hours Post Mortem:

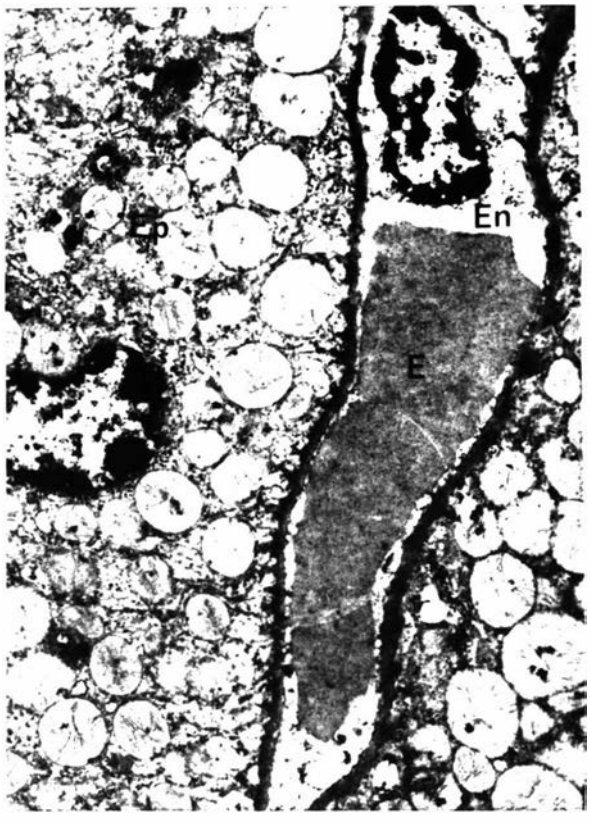
There has been little further detectable degeneration when this section is compared with C.

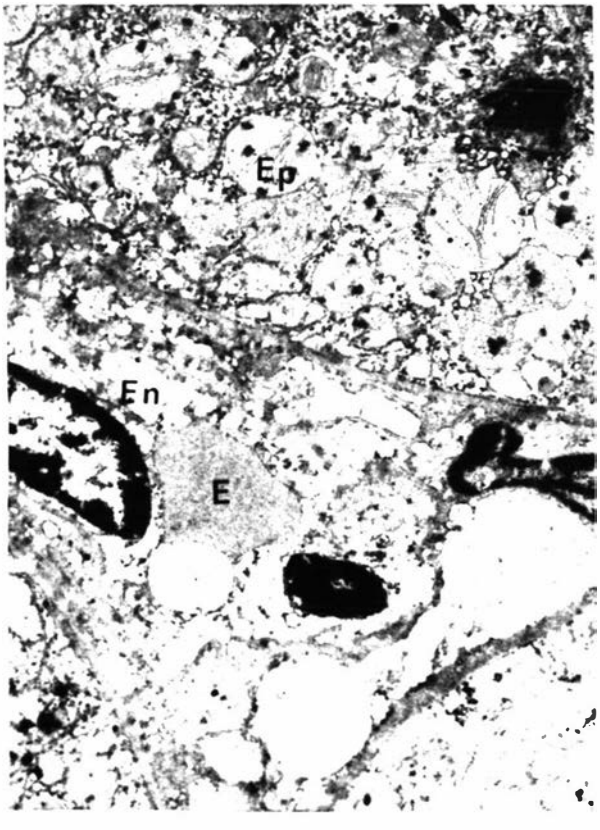
These sections should be compared with those in Figure 10.4b.

Note: The carcases of these animals were held at 37°C.

Magnification 5,500


Ep
En


Ep
En
E


Ep
En
E

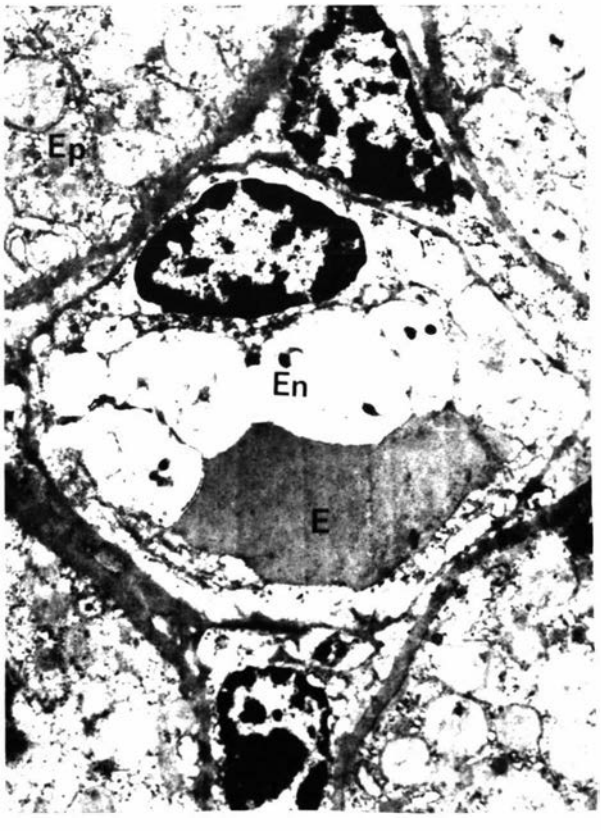
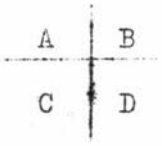

Ep
En
E

Figure 10.4b: The Sequence of Autolytic Changes in the Kidneys of Intoxicated Mice Electron Microscopy.



A. Time of Death:

The endothelium (En) of the blood vessel is vacuolated but the tubular epithelium (Ep) appears normal.

E = Erythrocyte.

B. 2 Hours Post Mortem:

The endothelium in this section has degenerated with only a few vacuolated remnants recognisable. There is no loosening of the interstitium.

C. 4 Hours Post Mortem:

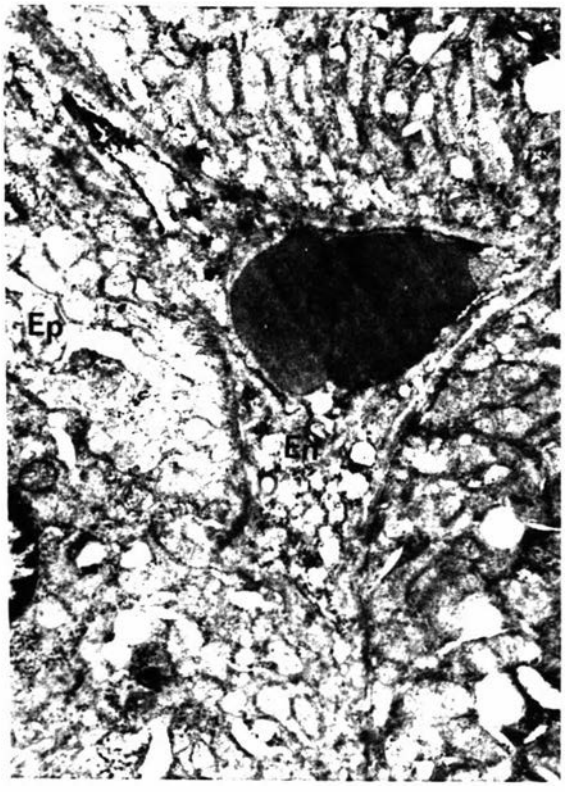
Despite the obvious endothelial damage there is no tendency for erythrocytes to escape into the interstitium and the tubules are closely apposed.

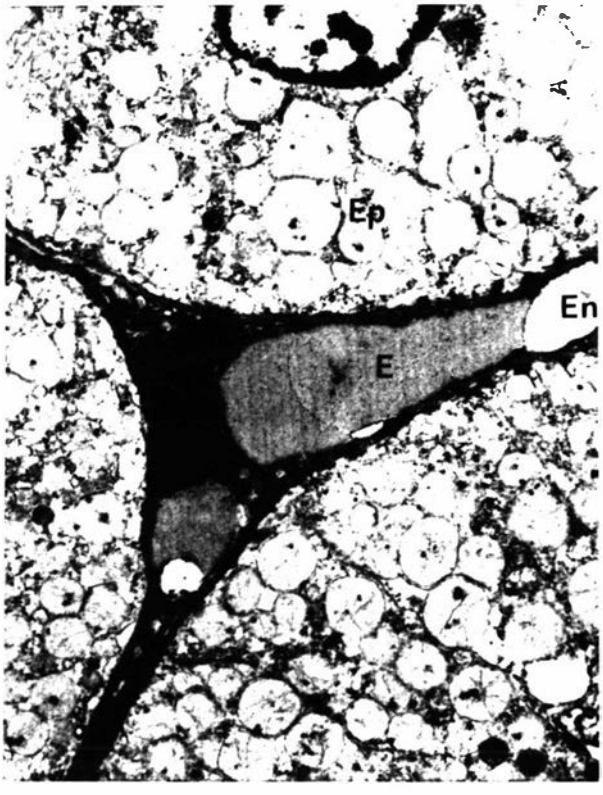
D. 6 Hours Post Mortem:

There is little difference between the changes in this section and those in C. Components such as mitochondria are still recognisable in the tubular epithelium.

These sections should be compared with Figure 10.4a.

Magnification 5,500


Ep
En


Ep
En
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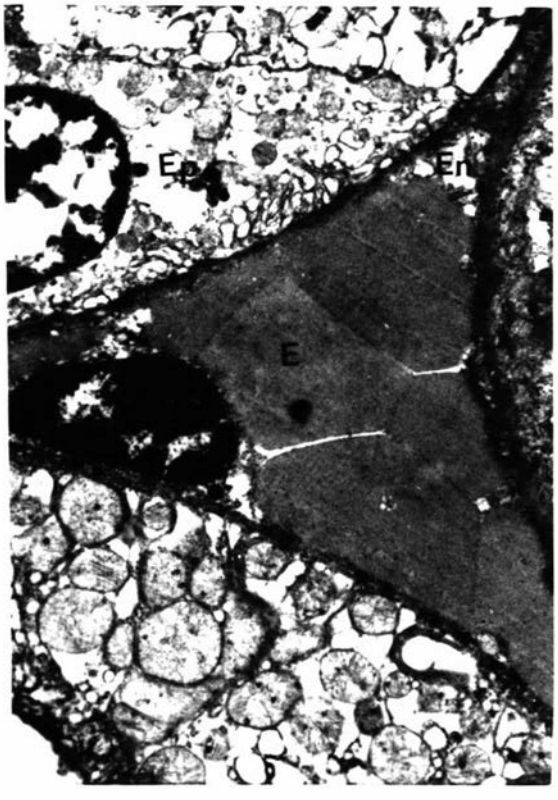
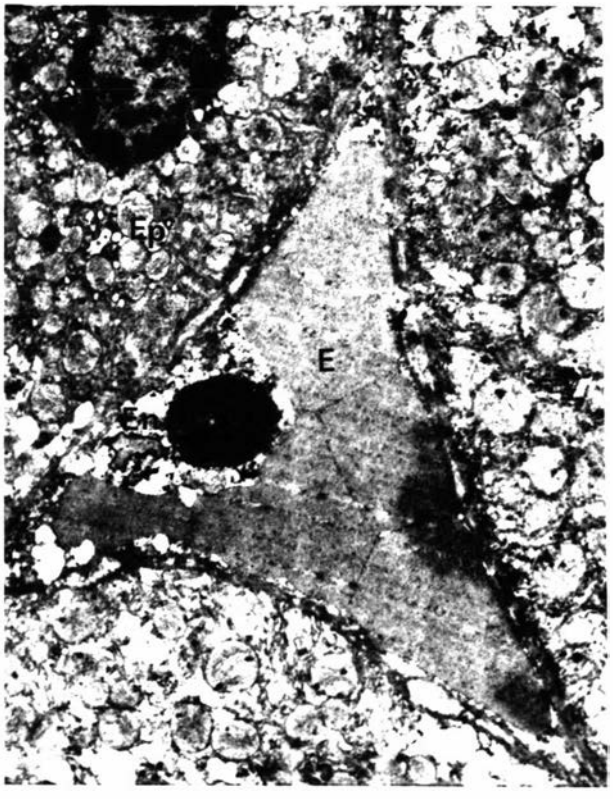

Ep
En
E


Ep
E

in consistency or evidence of tubular necrosis or interstitial haemorrhage at this time.

Grossly, the first lesion which was detectable in the lambs which received enteric origin toxin occurred 2 hours post mortem when the kidneys were still congested but had also developed areas beneath the capsule where there was apparent capillary dilatation, giving an appearance similar to the cutaneous 'spider naevus' of man. Tissue was removed from these areas at the time of sampling. The areas of 'capillary dilatation' remained obvious and appeared to become more extensive at later samplings and at the same time the kidneys became less firm in consistency and began to soften. By the time 10 hours had elapsed after the death of the animals the kidneys were extremely mushy, and a clean knife cut could no longer be made. In contrast, the kidneys of the control lambs remained quite firm in consistency for the whole 10 hours after death although by the end of that period the superficial 1-2mm of renal cortex was greenish black due to decomposition. The latter change had first become visible at 6 hours post mortem and there were no other changes such as those seen in the intoxicated animals.

Lesions detectable by light microscopy first became visible in the kidneys of the intoxicated lambs 2 hours after death. At this time there were focal areas in the renal cortex where the alkaline phosphatase activity of the brush border of the proximal tubular epithelium was reduced. This was associated with a loss of the PAS staining affinity of the region and there was also evidence of nuclear degeneration in the form of karyolysis in the epithelial cells in these areas. By the time 4 hours had elapsed post mortem, interstitial 'haemorrhage' between the tubules became obvious in the formalin fixed H&E stained sections of the kidney samples and the degenerative changes in proximal tubules was almost universal. The time sequence of these changes can be seen in Figure 10.5. There was no difference in the sequence of events in the upper and lower kidney of these animals.

The sequence of changes which occurred in the kidneys of the control sheep after death differed from those described above.

Figure 10.5: The Sequence of Autolytic Changes in the Kidneys of Intoxicated Lambs Light Microscopy

A | B
C | D

A. Time of Death:

The proximal tubular epithelium (PrT) appears to be normal and there is no evidence of interstitial haemorrhage.

G = glomerulus

B. 2 Hours Post Mortem:

Degenerative changes, in the form of karylosis, are beginning to appear in the proximal tubular epithelium and there is early dissemination of erythrocytes (E) between the tubules in some areas.

C. 4 Hours Post Mortem:

Extensive karyolysis is present in the proximal tubular epithelium and erythrocytes are lying free in the interstitium.

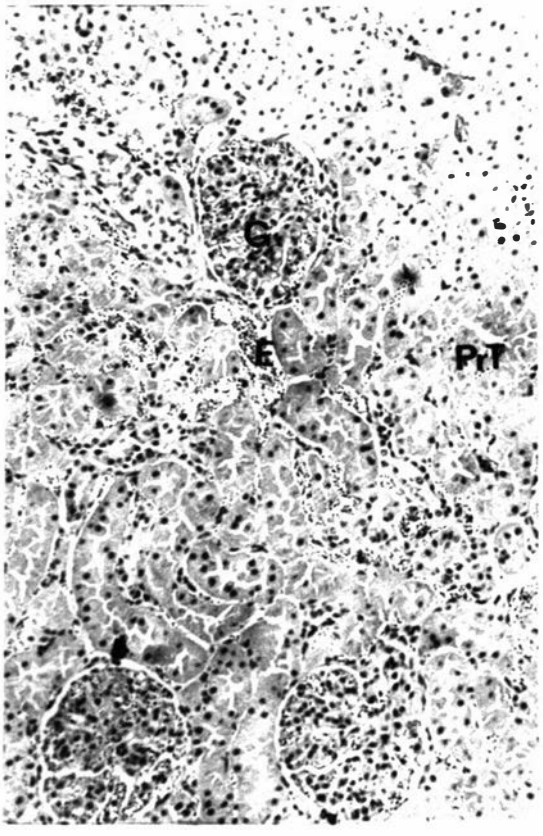
D. 8 Hours Post Mortem:

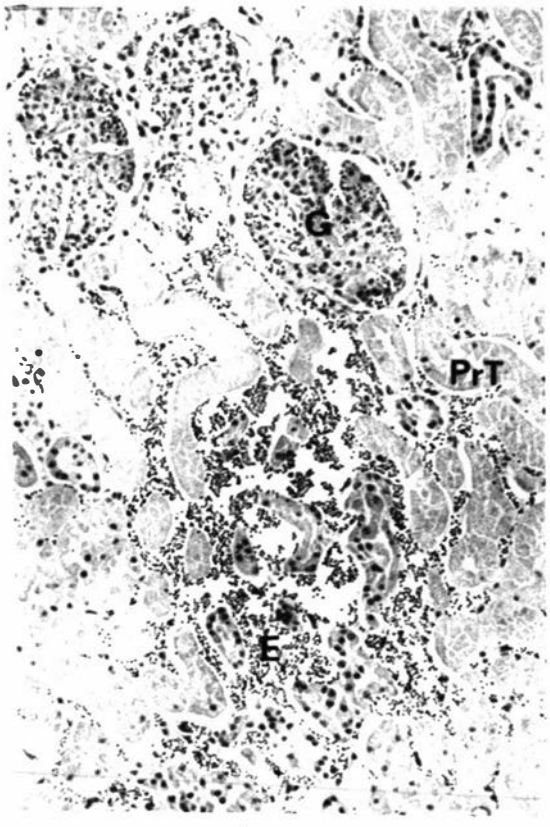
The renal changes are essentially similar to those shown in Figure 10.1 of the field case of enterotoxaemia. The widespread distribution of the erythrocytes between the tubules resembles interstitial haemorrhage and the degenerative changes in the tubules have given them a necrotic appearance.

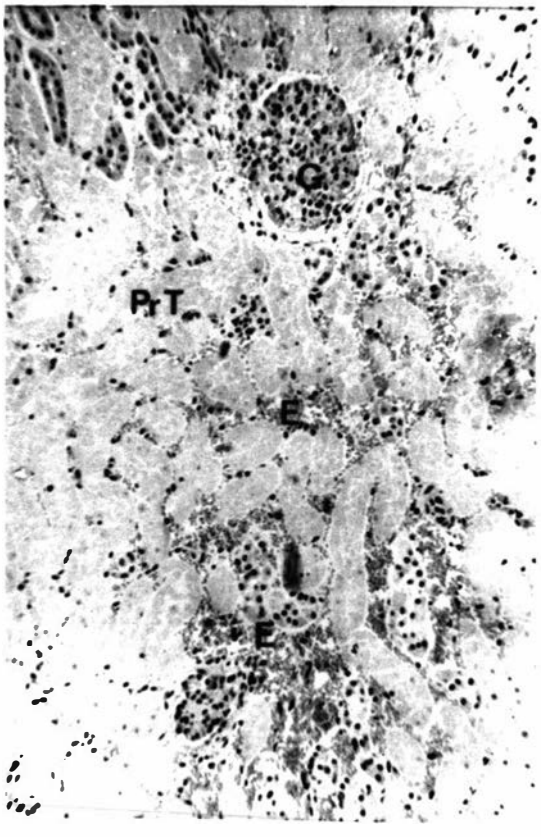
These sections should be compared with those in Figure 10.6.

Stain H & E
Magnification 120


PrT


G
E
PrT


G
PrT
E


G
PrT
E
E

There was also a reduction in the alkaline phosphatase activity of the brush border of the proximal tubular epithelium and this tended to be associated with a loss of PAS staining affinity of this region. The changes however were not marked until between 4 and 6 hours after death. At this time there was evidence of degenerative changes in the proximal tubular epithelium in the form of karyolysis but this was not as marked as in the intoxicated lambs and even at 8 hours, when there was obvious post-mortem decomposition of the superficial layers of the kidney, the tubular changes had not progressed as far as they had done in the intoxicated lambs. The outstanding difference between the two groups however was the fact that, in the control animals there was no evidence of interstitial haemorrhage at any stage. Figure 10.6 illustrates the sequence of changes which occurred in the kidney of control lamb.

It is now appropriate to examine the ultrastructural changes which occurred in the kidneys of these two groups.

By the time two hours had elapsed after the death of the intoxicated animal there was electron microscopic evidence of degenerative changes in the proximal tubular epithelium of the kidney. There was evidence of loss of microvilli in the brush border, random distribution of the normally polarised mitochondria and cellular material escaping into the lumen of the tubules. The tubules were still closely apposed at this time and the capillaries were still intact although the endothelium was extensively damaged. From 4 hours post mortem onward there was a progressive dissolution of the already damaged capillaries between the tubules with the release of intact erythrocytes into the interstitium. At the same time there appeared to be a general widening of the spaces between tubules which allowed the erythrocytes to spread between them giving the appearance of interstitial haemorrhage. The epithelium of the proximal tubules from 4 hours post mortem onward had almost entirely degenerated and appeared to be 'necrotic'. By the time 10 hours had elapsed post mortem the architecture of the kidney was almost completely lost at the ultrastructural level.

The sequence of ultrastructural changes which occurred in the kidneys of the intoxicated animal after death are illustrated in

Figure 10.6: The Sequence of Autolytic Changes in the Kidneys of Normal Sheep. Light microscopy

A B

C D

A. Time of Death:

The appearance of formalin fixed, H&E stained sections of normal kidney, removed from the carcase at the time of death.

G = glomerulus

PrT = Proximal Tubular Epithelium

B. 2 Hours Post Mortem:

There has been practically no detectable change in the renal architecture during this time.

C. 4 Hours Post Mortem:

Early degenerative changes in the form of karyolysis can be seen to be present in the proximal tubular epithelium. There is no evidence of release of erythrocytes from the blood vessels.

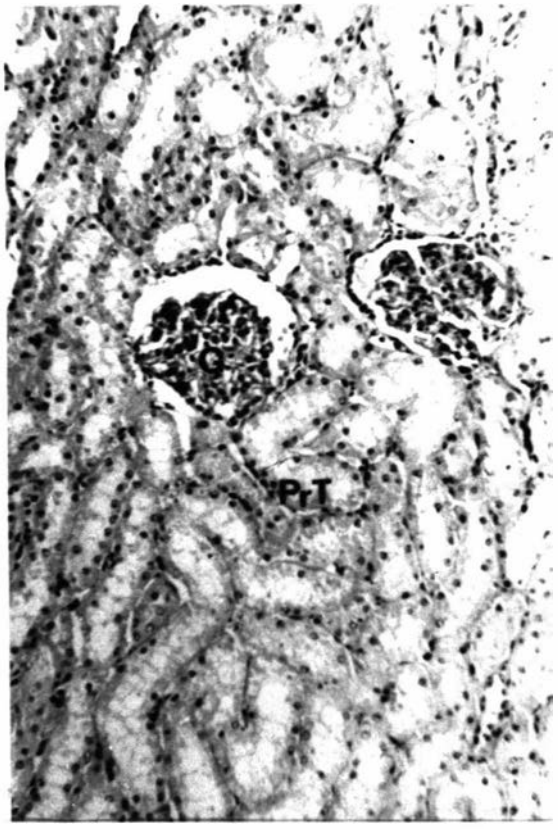
D. 8 Hours Post Mortem:

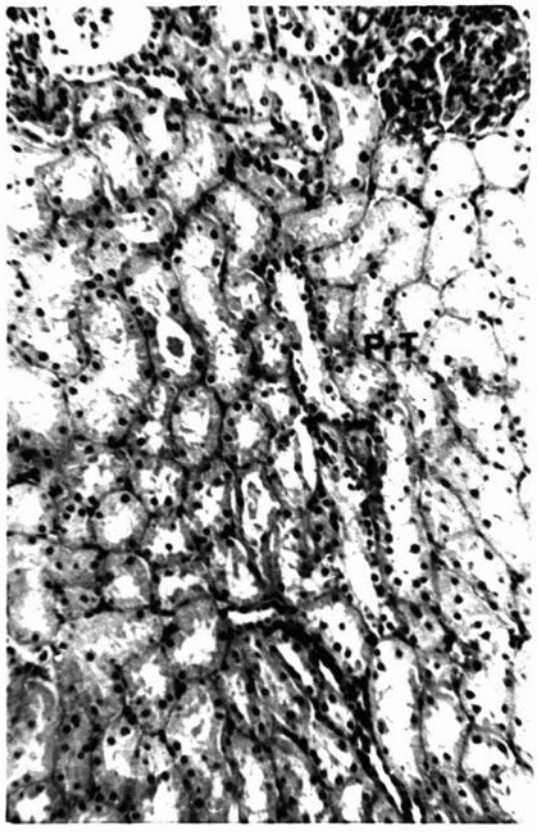
Although tubular degeneration is present at this time, it is not as obvious as that which occurred in intoxicated animals. There are no free erythrocytes in the interstitium and the tubules are still closely apposed.

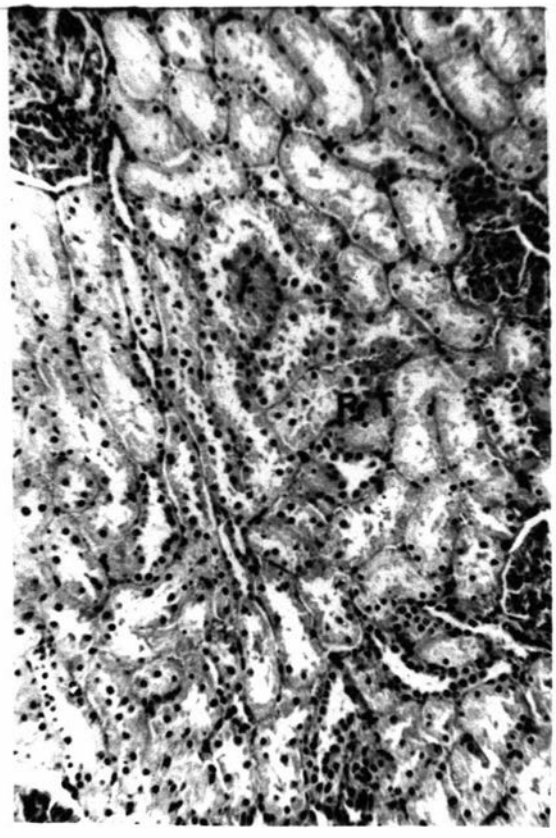
These changes should be compared with those in Figure 10.5.

Stain H & E

Magnification 120


PrT


PrT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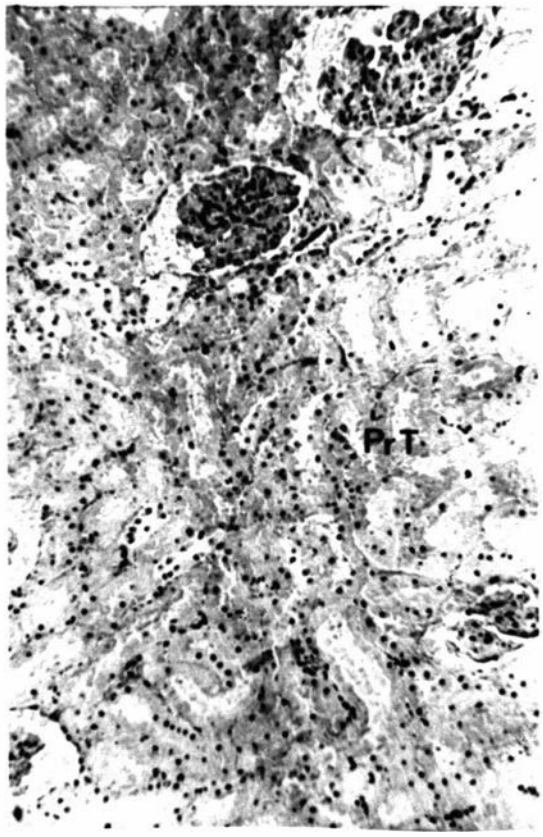

PrT

Figures 10.7 and 10.8.

In the control animal the changes in the epithelium of the tubules were essentially similar to those which were seen in the tubules of intoxicated lambs. Obvious degenerative changes were present in the tubular epithelium four hours post mortem. Nuclear degeneration in these cells had begun to occur at that time but there were still a number of intact nucleii and recognisable cells present in sections from kidney removed from the carcase 6 hours after death of the animal. There was no loosening of the interstitium between the tubules in the control animals at any time and the capillary endothelium was still intact 10 hours post mortem although, as in mice, swelling and vacuolation occurred. No release of erythrocytes from these vessels occurred at any time. The ultrastructural changes associated with autolysis in a normal lamb kidney are illustrated in Figure 10.9.

Discussion.

In view of the confusion in the literature over whether or not renal lesions are present at the time of death it is interesting to note that, in the present study where animals were necropsied as soon as death occurred, there were no lesions in the kidneys detectable by visual inspection or light microscopic examination.

This was the case in both mice and lambs and also, in the latter species, when toxin was either parenterally administered or absorbed from the intestine following the infusion of Cl.perfringens type D plus carbohydrate. These findings imply that the lesions seen in field cases of enterotoxaemia are largely the result of post mortem change in the intoxicated animals.

The lack of any qualitative change in the histochemical activity of the various enzymes in the kidneys of intoxicated animals at the time of death is also noteworthy. By the time alterations in alkaline phosphatase activity were demonstrable histochemically there were usually other degenerative changes in

Figure 10.7: The Sequence of Autolytic Changes which occur in the Kidneys of Intoxicated Lambs after Death.

A B

C D

A. Kidney, removed at the Time of Death, from a Case of Experimental Enterotoxaemia in a Lamb:

The tubular epithelium (Ep) appears to be normal although there is some debris in the tubular lumen (L). The basement membranes (BM) of adjacent tubules are in close proximity and the interstitium (I) is limited in extent. The endothelium of the blood vessels (BV) is damaged.

N = nucleii

Mv = microvilli

E = erythrocytes

B. Kidney removed from the same carcase as A, 2 Hours after Death of the Animal:

The tubular epithelium has lost much of its cytoplasmic architecture and the microvilli have been lost. The appearance of the vasculature and interstitium is similar to A.

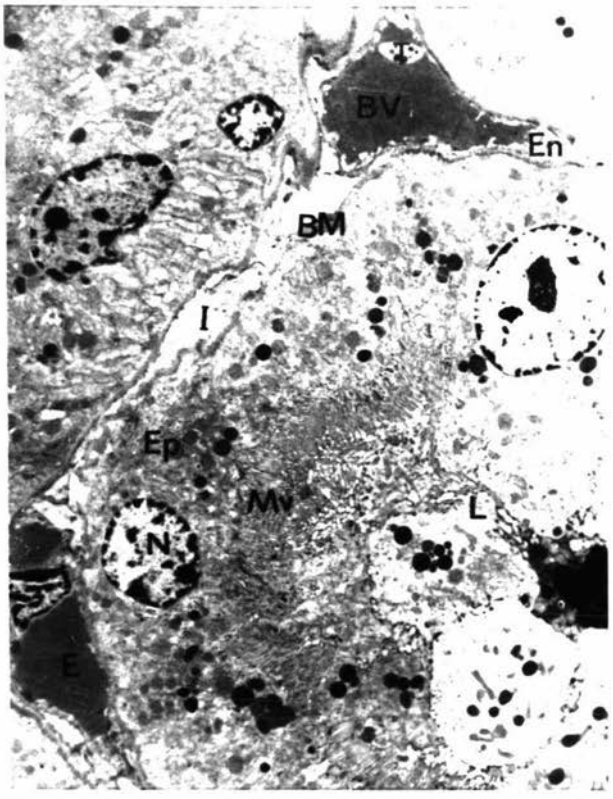
C. Kidney removed from the same carcase as A, 4 Hours after Death:

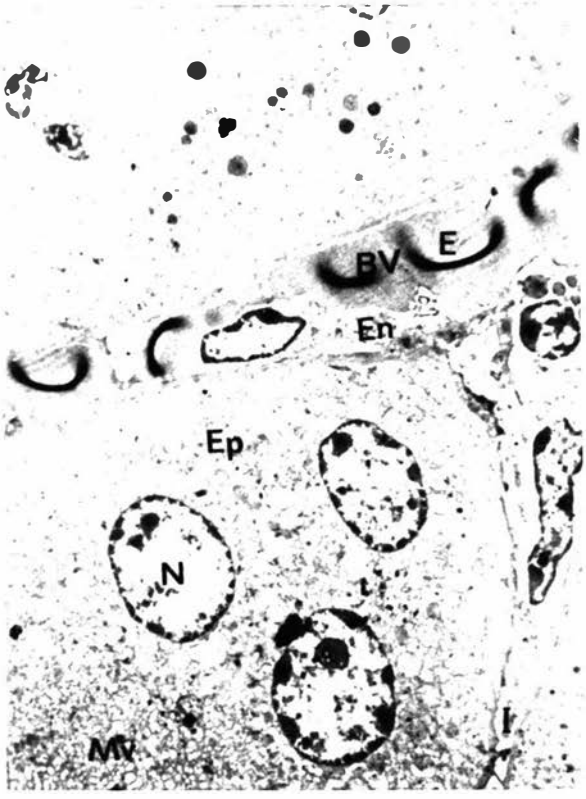
The tubular epithelium is almost entirely degenerated. Similarly the walls of the blood vessels has almost completely disappeared, although the erythrocytes have not been released into the loosened interstitium.

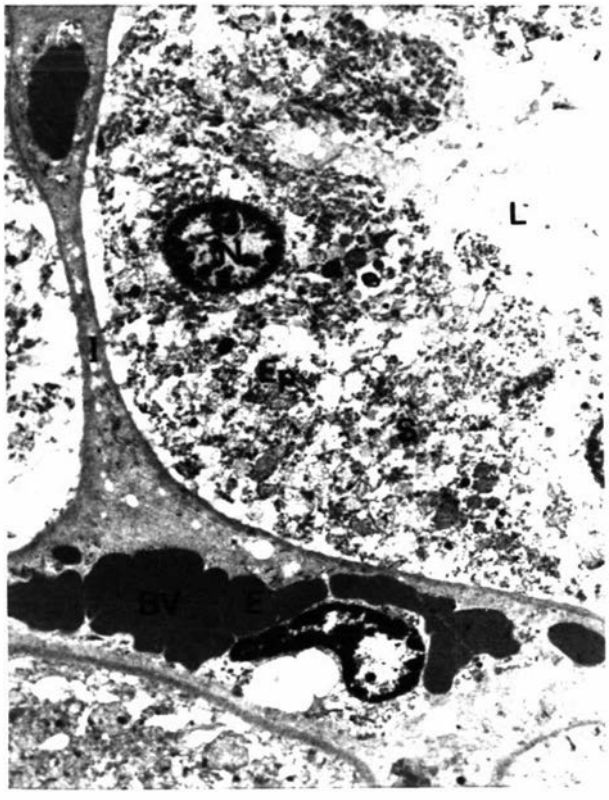
D. Kidney removed from the same carcase as A, 6 Hours after Death:

There has been almost complete loss of vascular and tubular architecture and erythrocytes are free in the loosened interstitium.

Magnification 2,650


BV
En
BM
I
Ep
Mv
L
N
E


BV
E
En
Ep
N
Mv
I


L
N
I
Ep
BV
E

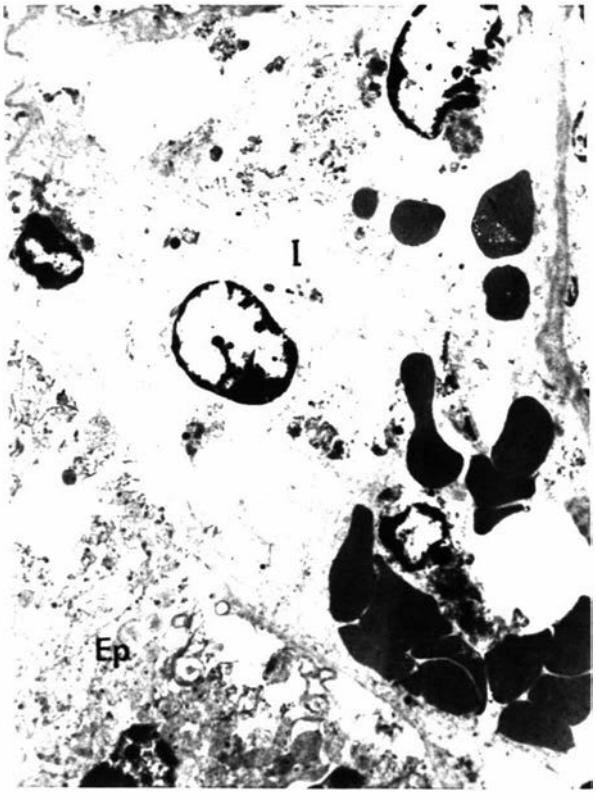

I
Ep

Figure 10.8: Vascular Changes which Developed 4 Hours After Death in a Case of Experimental Enterotoxaemia in a Lamb.

This section is a more highly magnified view of an area of the same kidney that was used to prepare the sections shown in Figure 10.7.

Fragments of the damaged capillary endothelium (En) and a thrombocyte (T) are still recognisable although the greater part of the capillary wall has disappeared. Several erythrocytes (E) have escaped from the blood vessels and are lying free in the loosened interstitium (I).

Ep = tubular epithelium
BM = basement membrane
Le = degenerated lymphocyte

Magnification 14,000

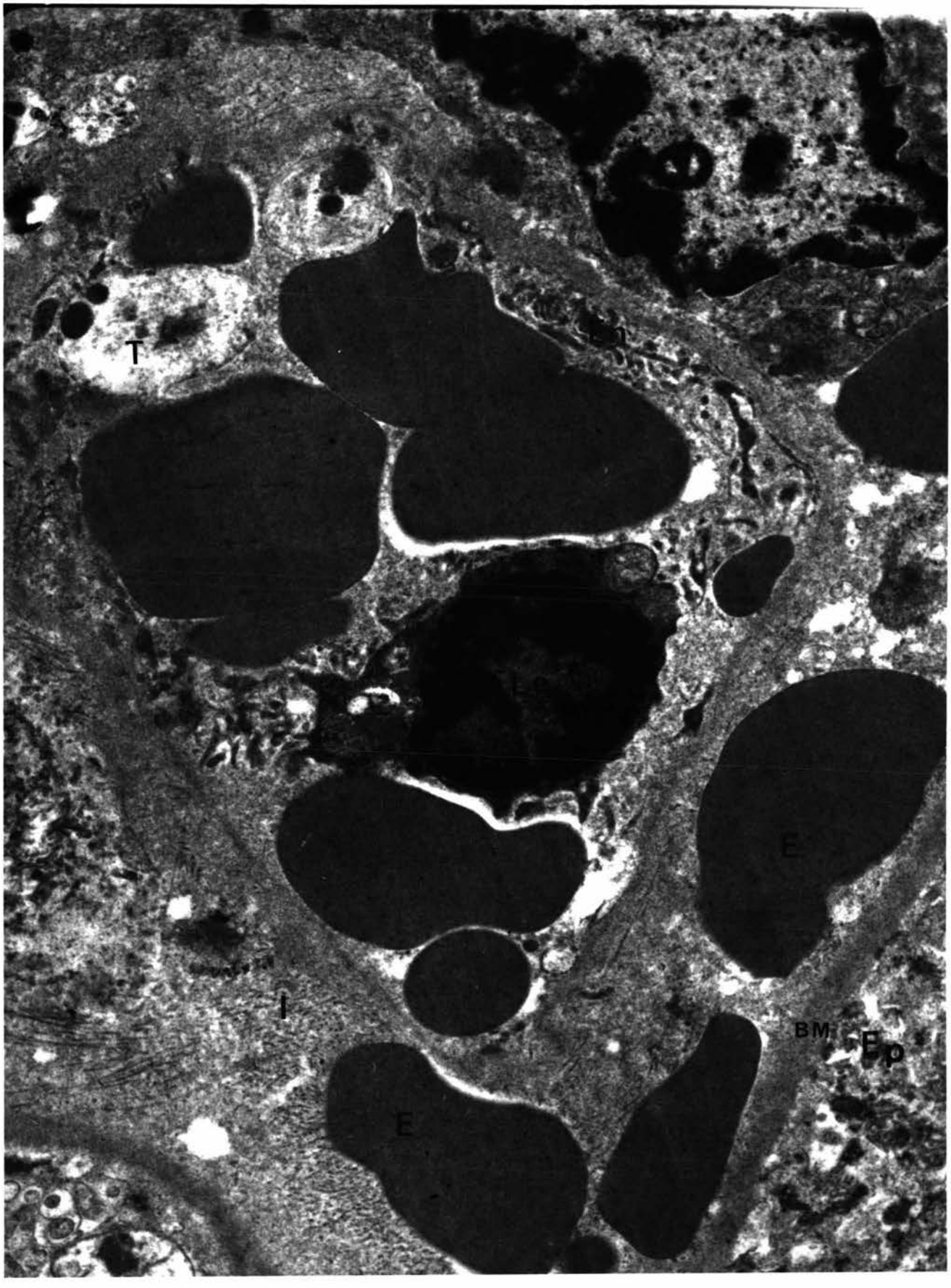

T
E
I
BM
Ep
E

Figure 10.9: The Sequence of Autolytic Changes in the Kidneys of Normal Lambs. Electron Microscopy.

A B

C D

A. Time of Death:

The endothelium (En) of the blood vessel (BV) is intact and the interstitium (I) between the tubules appears normal. The microvilli (Mv) of the tubular epithelium (Ep) are intact.

B. 2 Hours Post Mortem:

The endothelium of the blood vessel is still intact but there are degenerative changes in the microvilli of the tubular epithelium. The interstitium is unchanged.

C. 4 Hours Post Mortem:

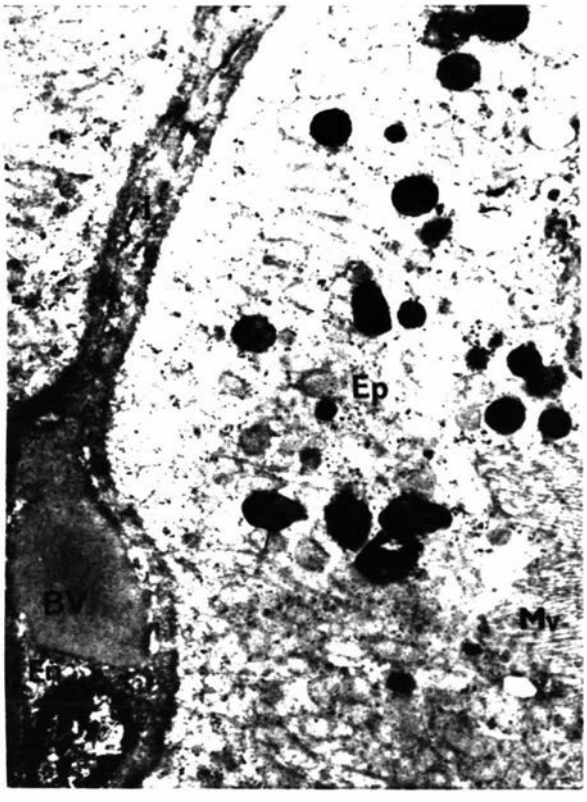
The appearance of this section is essentially similar to that of B with no evidence of loosening of the interstitium or breakdown of the endothelium. Degeneration of the epithelium is more advanced.

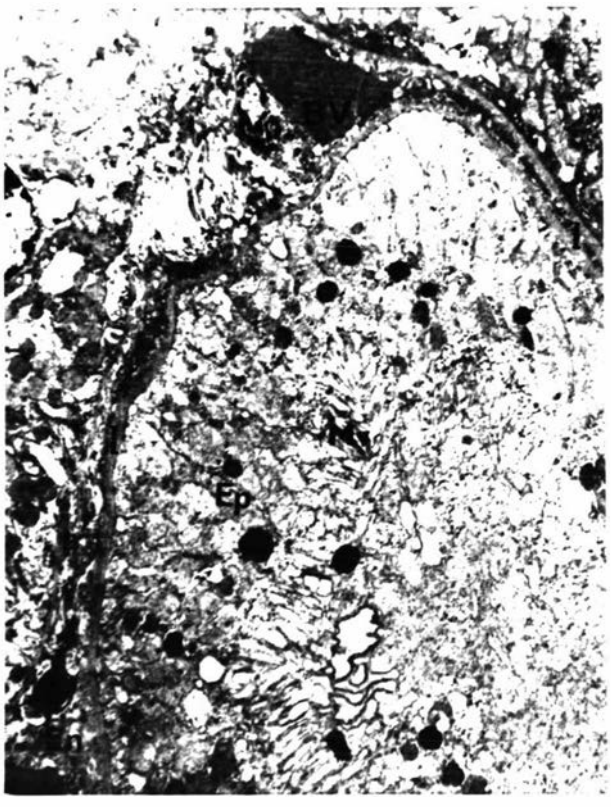
D. 8 Hours Post Mortem:

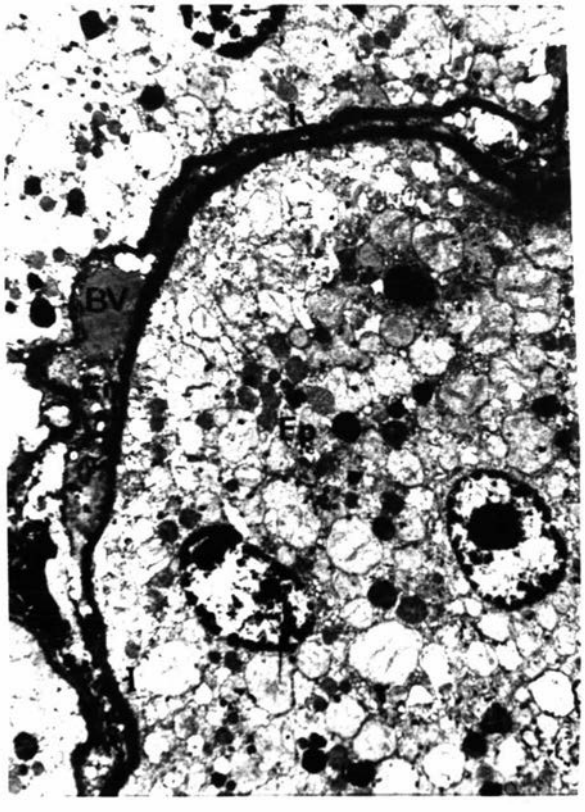
Even at this time the vascular endothelium is still easily recognisable although the architecture of the tubular epithelium has been largely lost, apart from the nuclei.

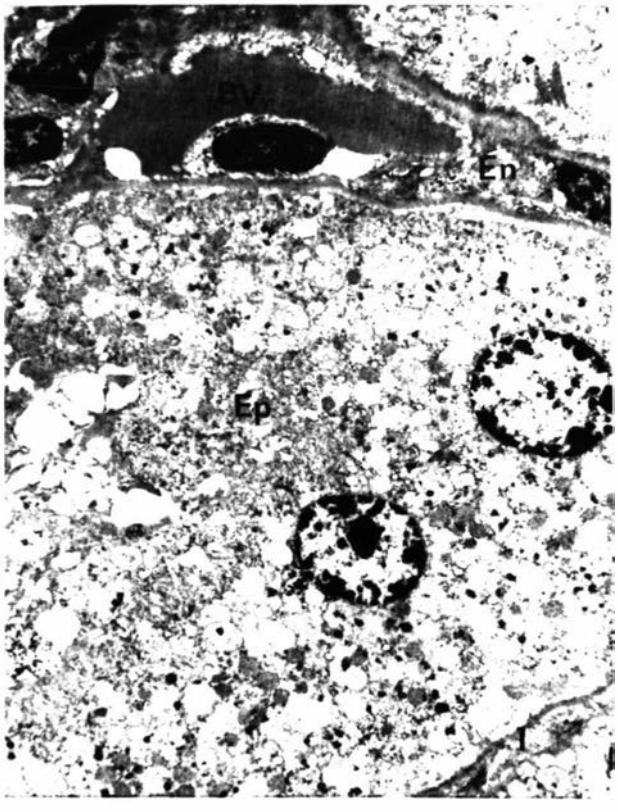
The sequence of changes in these sections should be compared with those in Figures 10.7 and 10.8.

Magnification 2,800


Ep
BV
Mv


Ep


BV
Ep


BV
Ep
Ep

the proximal tubular epithelium detectable by routine histological stains. This adds support to Wachstein's (in Becker 1968) suggestion that histochemical changes in the kidney do not necessarily precede other evidence of structural damage, nor do they indicate the functional efficiency of the cells. The lack of change in renal alkaline phosphatase activity in the intoxicated animals in the present experiments is in direct conflict with the results obtained by Sotirov & Bozkhov (1965) who used the Gomori method to demonstrate alkaline phosphatase activity in the renal tubules of their intoxicated animals.

Sex differences in the distribution of alkaline phosphatase activity in the brush border of the proximal tubules in different regions of the renal cortex were not particularly noticeable in the mice used in the present experiments and it may be that this reflects the fact that the mice used were of a different strain to those used by Dunn (in Cornelius & Rae 1967) in which she described these differences. Nevertheless, the possibility that such changes can occur stresses the importance of selecting appropriate control animals. The progressive accumulation of lipid in the proximal tubular epithelium in intoxicated animals and also in starved animals emphasises the fact that, on occasions, results can be obtained which are only indirectly due to the experimental procedure which was employed. This also underlines the importance of maintaining control animals under exactly similar conditions as those affecting the test animals.

Some reference must be made to the basis for the selection of the particular group of enzymes it was decided to examine histochemically in the kidney, although the limitations inherent in enzymatic analyses will be deferred until Chapter 21 where the estimation of serum enzymes is discussed. In the present instance, because glycosuria is a prominent feature of enterotoxaemia, particular attention was paid to the enzymes which have been suspected of being involved in the resorption of glucose from the glomerular filtrate by the proximal tubules. This is an energy

dependent process, so that enzymes which have not been suspected of being directly associated with glucose metabolism but which are important in energy yielding processes e.g. succinic dehydrogenase, ATPase and the diaphorases, were included in the present study. Some of these enzymes have been shown to be decreased in other intoxications where glycosuria and/or renal damage occur although, as mentioned earlier, it appears unlikely that this is a specific effect of the toxic agent and is more likely to be associated with cell death. It should be noted in passing that the exact role of enzymes such as the phosphatases and trehalase in glucose transport in the kidney is as yet unknown and even as recently as 1969 there were conflicting reports, such as those of Grossman & Sackter (1968), Van Handel (1969) and Semenza & Rihova (1969) on the importance or otherwise of trehalase in renal glucose transport.

From the fact that similar lesions developed in the kidneys of control and intoxicated mice when the carcases were held at a constant temperature it would appear that the changes in the proximal tubular epithelium may have been due to the effect of autolysis and it could be that the differences that were seen between control and intoxicated lambs were the result of a higher body temperature in the intoxicated animals at the time of death. This is unlikely to be the whole explanation as it does not take into account the gross alterations in the consistency of the kidney and it is possible that the changes in the solubility pattern of renal proteins described by Sotirov (1965c) is associated with a specific action of the toxin upon the tubular epithelium or interstitium.

The finding, in the present investigation, that the interstitial haemorrhage in the kidney in enterotoxaemia is in fact a post mortem change is intriguing and its mode of development may be unique. The fact that in this disease autolysis accelerates the breakdown of blood vessels which have been severely damaged by the action of a toxic agent, thus allowing the release of erythrocytes, has not been described previously in this or other intoxications. It would be interesting to know whether a similar situation occurs in other conditions where interstitial haemorrhage in the renal cortex has been described following the administration

of toxic agents e.g. mercuric chloride and Cl.perfringens type A alpha toxin which damage vascular structures.

While on the subject of the development of the interstitial haemorrhage two other points should be mentioned. The first is that the upper and lower kidneys were sampled from these animals to eliminate the rather unlikely possibility that the renal lesions were influenced by gravitational or hydrostatic effects produced by the position of the carcase, as occurs in post mortem hypostatic congestion. The second point concerns the fixatives which were employed in the preparation of material for light microscopic examination. While Bouin's fluid was definitely superior to formol saline in preserving the fine structure of the kidney the fact that the erythrocytes were almost completely lysed and appeared as faint ghosts, or not at all, in Bouin's fixed material suggests that, where haemorrhagic states may be encountered, this fixative is definitely contraindicated despite its superiority as a cytological fixative.

Conclusions.

1. Free lipid accumulated in the cells of the proximal convoluted tubules of the kidneys of mice deprived of food and the appearance of free lipid in this region of the kidneys was only an indirect effect of epsilon toxin, being brought about by the depression and anorexia in intoxicated animals.

2. Lipid accumulation in the proximal convoluted tubular epithelium of the kidney, first becomes detectable in mice which have been deprived of food for six hours and becomes progressively more severe as time increases.

3. At the time of death there were no detectable histochemical or morphological alterations in the kidneys of intoxicated mice or lambs. This is in contrast to published descriptions of the renal changes in enterotoxaemia.

4. The reduction in histochemically demonstrable alkaline phosphatase and PAS staining affinity in the brush border

of the renal proximal tubular epithelium in intoxicated animals was associated with nuclear degeneration and did not occur until some time had elapsed after death. It was therefore an autolytic lesion rather than a direct effect of epsilon toxin.

5. Interstitial haemorrhage in the renal cortex of intoxicated animals is also a post mortem change but is associated with the antemortem vascular damage. Degeneration of the already damaged capillaries allows the release of intact erythrocytes into the loosened interstitial tissue and it is therefore probable that this 'lesion' is almost pathognomonic of enterotoxaemia of sheep when it occurs.

6. The rapidity of the onset of degenerative changes in the kidneys of intoxicated animals after death may be due to a combination of high body temperature, resulting from antemortem convulsions, and a direct effect of epsilon toxin upon renal tubular epithelium or the interstitium.

7. Alterations in the histochemical affinity of enzymes in the kidney did not occur in the absence of degenerative changes detectable by conventional H&E and PAS staining methods.

8. Bouins fluid is not a satisfactory fixative for studying haemorrhagic lesions due to its action in lysing erythrocytes. It is however an excellent fixative for retaining cytological architecture in the kidney.

Chapter 11 THE MORPHOLOGICAL CHANGES PRODUCED IN THE INTESTINE BY CL.PERFRINGENS TYPE D EPSILON TOXIN

As an alteration in intestinal permeability is one of the prerequisites for the absorption of the protein molecule of epsilon toxin, and thus for the production of enterotoxaemia, it was of interest to determine whether the changes in permeability induced by the toxin itself are associated with evidence of morphological damage to the intestinal mucosa. Todorov and Trifonov (1962) have stated that following contact with epsilon toxin the change in permeability in the intestine is associated with an inflammatory necrosis of the mucosa in rabbits and kids. They did not describe the lesions which occur in detail nor do they appear to have examined the intestinal mucosa from cases of experimentally induced exterotoxaemia in sheep. In the latter instance the presence of toxic substances in addition to epsilon toxin, produced during the multiplication of Cl. perfringens type D. in the intestine, may influence the severity or type of lesion which develops.

In an attempt to obtain more information on the morphological changes in the intestine produced by epsilon toxin it was decided to study the action of the toxin invivo on the mucosa of isolated loops of rabbit and lamb intestine. In experiments of this type adequate controls are essential as interference with either the intestinal blood supply or the movement of ingesta through the intestine can rapidly have adverse effects upon the animals well-being. It was therefore necessary to compare the effect of the toxin on ligated intestinal loops with the changes which occurred in adjacent ligated loops which did not contain toxin.

Subsequently, the changes produced in isolated loops of lamb intestine could be compared with any lesions in intestine taken from lambs in which enterotoxaemia had been induced by the infusion of Cl. perfringens type D culture plus carbohydrate.

The Effect of Epsilon Toxin upon Ligated Intestinal Loops in the Rabbit

Materials & Methods

Two pairs of rabbits were anaesthetised and intestinal loops, with their blood supply intact, were isolated by ligation. The surgical procedures employed are described in Appendix 1. In one pair of animals, 2mg (2,000 mouse lethal doses approx.) of batch CWD epsilon toxin in 1 ml of nutrient broth was introduced into one of the intestinal loops and 1 ml of nutrient broth was introduced into the adjacent loop. The other pair of rabbits was treated similarly with the exception that the level of epsilon toxin employed was reduced to 0.25 mg/ml (250 mouse lethal doses/ml approx.)

As soon as death occurred the segment of intestine containing the loops was removed from the animal, photographed and pieces of each of the loops were then fixed and prepared for light and electron microscopy as described for other tissues.

Results

All the animals recovered rapidly from the anaesthetic and laparotomy. The two animals that received the higher dose of toxin remained normal for 2-2½ hours then became depressed and died in terminal convulsions 3 hours after inoculation. The two animals which received the lower dose of toxin remained clinically normal for 7 hours after inoculation. They were destroyed at that time by neck dislocation.

The gross appearance of the intestine was similar in all animals. The broth containing loops were flaccid and empty while the toxin containing loops were distended with fluid. These changes are shown in Figure 11.1. The difference between the toxin-containing and control loops was greatest in the animals which had received the lower dose of toxin and had survived longest. These animals also showed early fibrinous adhesions around both loops of intestine.

Figure 11.1: The Effect of Epsilon Toxin on the Intestine of the Rabbit.

A

B C

A. Adjacent Broth-containing and Toxin-containing Intestinal Loops:

The loop on the left was inoculated with 1 ml. of sterile nutrient broth and appears normal, while the distended loop on the right was inoculated with 1 ml. of a solution containing 1,000 mouse lethal doses of epsilon toxin/ml. Note the largely independent blood supplies of the two loops.

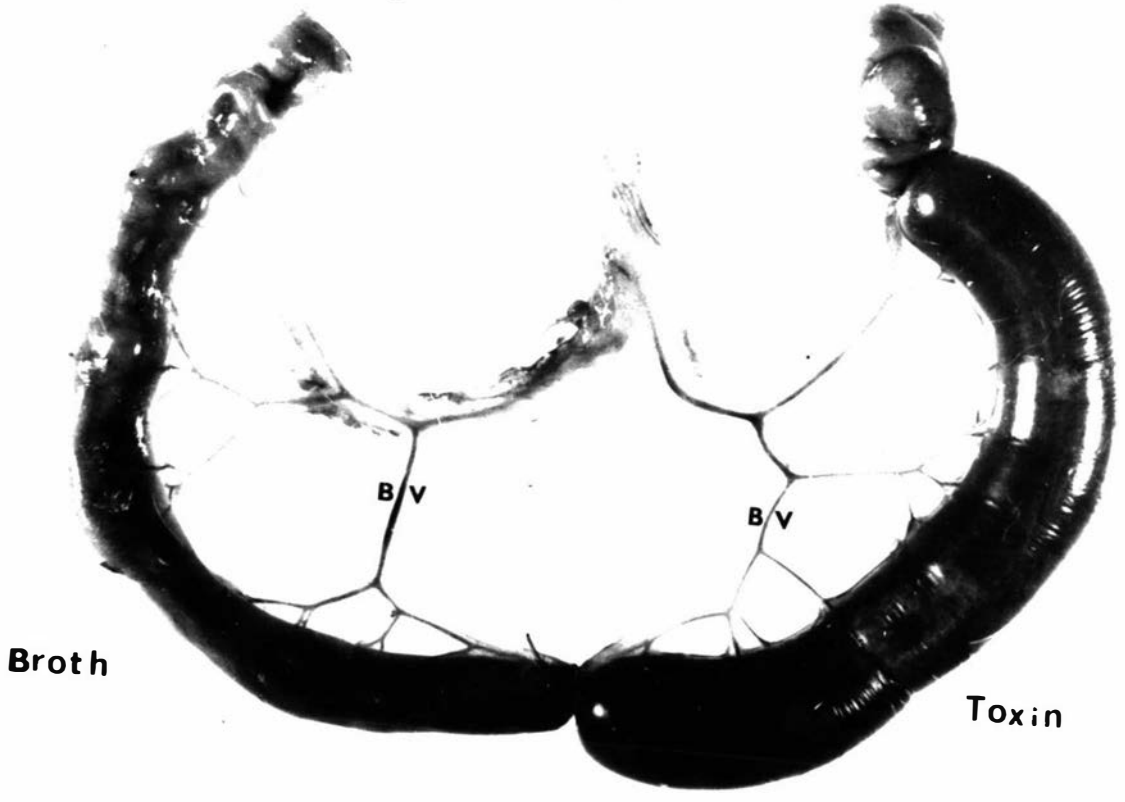
B. Semi-macrophotographs of Stained Sections of the Broth-containing Loop:

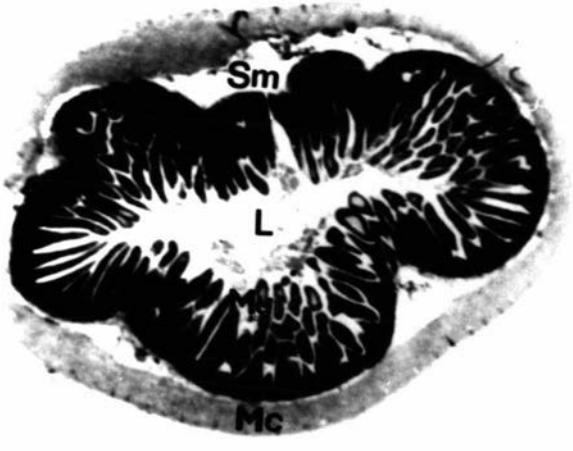
The intestinal villi are normal and the mucosa (Mu) is fairly closely applied to the muscle layers (Mc), so that the submucosa (Sm) is not particularly prominent.

C. Semi-macrophotograph of Stained Sections of the Toxin-containing Loop:

The villi are shrunken and there is severe oedema of the submucosa.

Stain (B & C)	H & E	
Magnification	A	1.6
	B & C	8


B V
B V
Broth
Toxin


Sm
L
Mc


L
Mc
Sm

When examined by light microscopy the mucosa of the intestinal loops, which had contained nutrient broth only, was histologically normal in all animals. There was a superficial mild inflammatory infiltration of the serosal surface of the intestine in the animals which had received the lower dose of toxin. In contrast to these findings, the loops which had contained the higher dose of toxin had almost completely lost the superficial epithelium of the mucosa. In addition there was a severe inflammatory oedema of the submucosa and a diffuse acute inflammatory infiltration in the mucosa and submucosa. These changes are illustrated by the series of photomicrographs in Figure 11.2. The epithelial changes produced by the lower dose of toxin were not as severe as those produced by the higher dose but there was a severe diffuse acute inflammatory infiltration of the mucosa and submucosa and inflammatory oedema of the submucosa. The lymphatics were also distended with fluid.

Electron microscopic examination confirmed the severity of the epithelial damage produced by high concentrations of toxin and only occasional degenerated epithelial cells remained in the sections of these loops. There was also severe endothelial damage in the capillaries of the loops which had contained high concentrations of toxin but there was no evidence of capillary damage in the broth containing loops from these animals (Figure 11.3).

The Effect of Epsilon Toxin on the Mucosa of Ligated Loops of Intestine in Lambs

The levels of toxin which were employed in the rabbit experiments described above were similar to those which may be encountered in the intestinal contents of field and experimental cases of enterotoxaemia in lambs. It appeared that damage of the type just described could be expected to occur in the mucosa of isolated loops of intestine in the latter species. The experiments described below were therefore carried out.

Figure 11.2: The Effect of Epsilon Toxin on the Intestine of the Rabbit.

A B

C D

A. Sections of Control Broth-containing Intestinal Loop:

Villi (Vi) are prominent and the mucosa , submucosa (Sm), muscle coats (Mc) and serosa (Se) appear to be normal.

L = lumen.

B. Section of Toxin-containing Loop:

There is a large amount of cellular debris in the intestinal lumen, the villi have become shortened and flattened and the submucosa is oedematous .

C. Mucosa and Submucosa of Broth-containing Loop:

The epithelium of the villi is intact and the mucosa and submucosa appear normal. The intestinal lumen is empty.

D. Mucosa and Submucosa of Toxin-containing Loop:

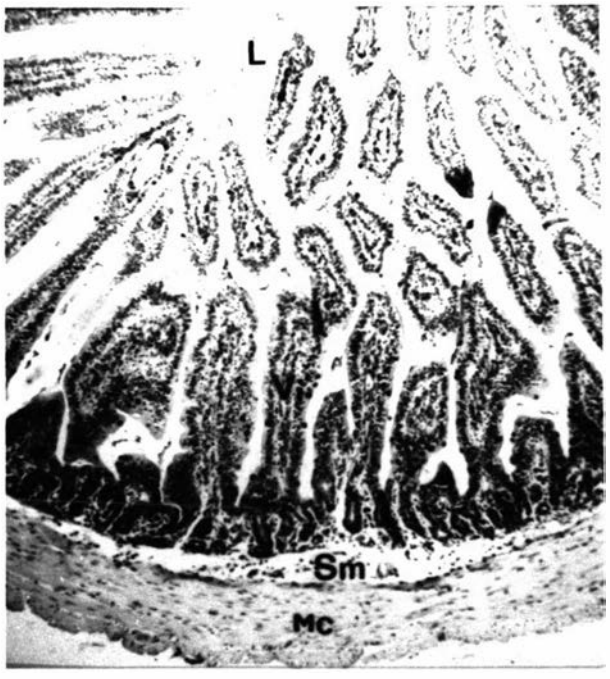
The lumen of the intestine contains large numbers of desquamated epithelial cells and acute inflammatory cells. The epithelium of the mucosa has been entirely lost apart from a few remaining cells in the glandular crypts. There is a severe diffuse acute inflammatory infiltration of the mucosa. The submucosa is oedematous and the lymphatics (Lc) are distended.

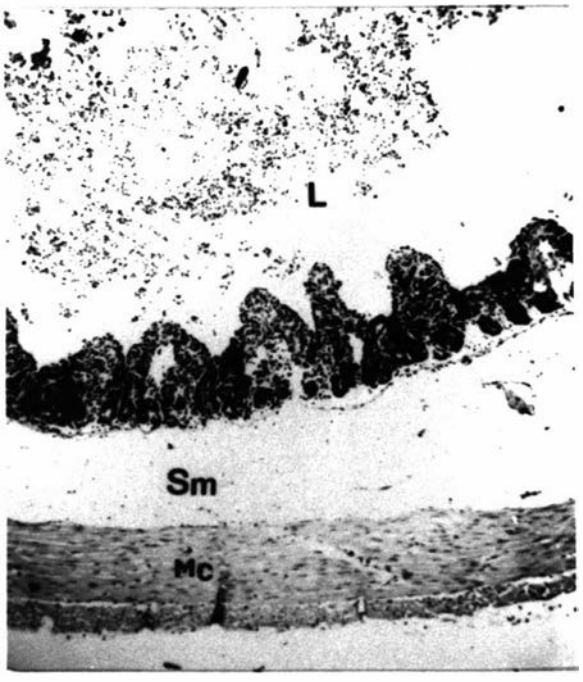
BV = Blood vessel

Stain H & E

Magnification A & B 50

C & D 120


L
Sm
Mc


L
Sm
Mc


L
Sm


L
Lc
Bv
Oe
Mc

Figure 11.3: Epithelial and Endothelial Damage Produced by Epsilon Toxin in Ligated Loops of Rabbit Intestine

A B

C D

A. Epithelium of Control, Broth-containing Loop:

The epithelium is composed of the normal tall columnar type of cell (Ep) with prominent microvilli (Mv) on the free border.

N = nucleii

B. Mucosa and Submucosa of Toxin-containing Loop:

The epithelial cells are severely damaged and are only recognisable by their position and the few remaining distorted microvilli. Portion of an area where epithelial cells have been completely lost can be seen at X. In addition to the epithelial changes there is oedema (Oe) of the interstitial tissue around the blood vessels (BV) and some extravascular erythrocytes (E).

C. Capillary from Broth-containing Loop:

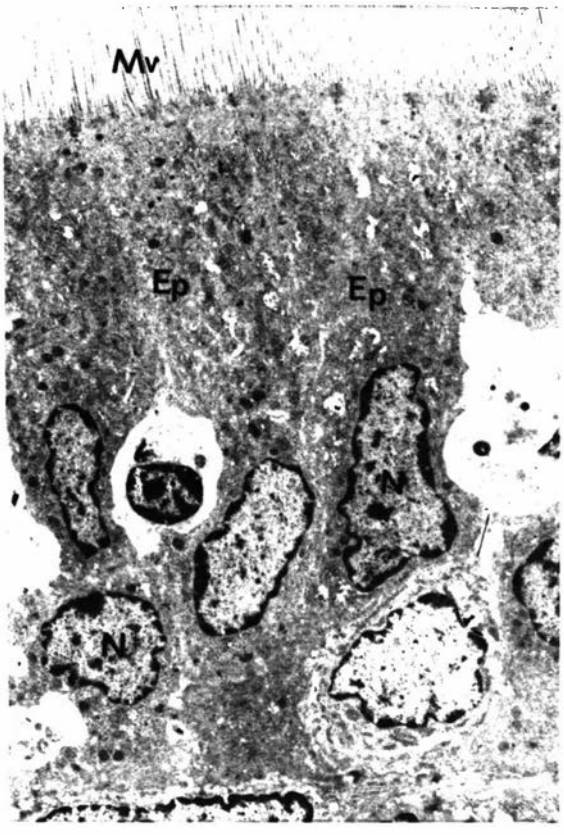
The cytoplasm of the endothelium (En) of this vessel appears to be normal.

D. Capillary from Toxin-containing Loop:

This loop was immediately adjacent to the loop from which C was taken. There is most severe endothelial damage with the cytoplasm being reduced to a thin, electron-dense band containing scattered unrecognisable organelles.

Magnification A & B 3,550

C & D 15,500


Mv
Ep
Ep
M
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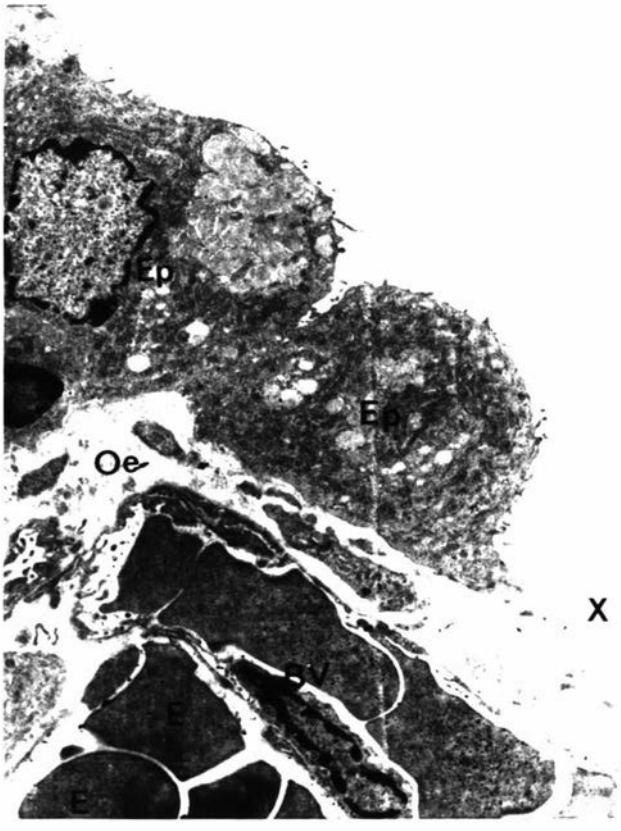
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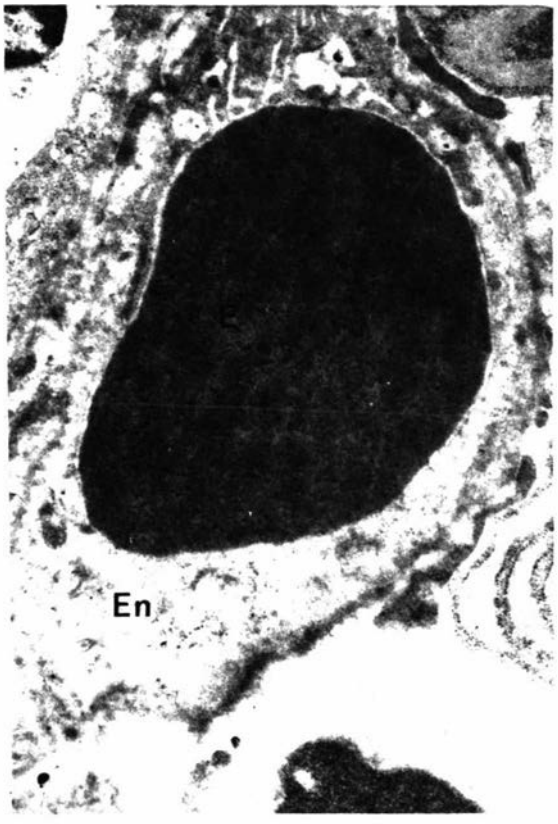
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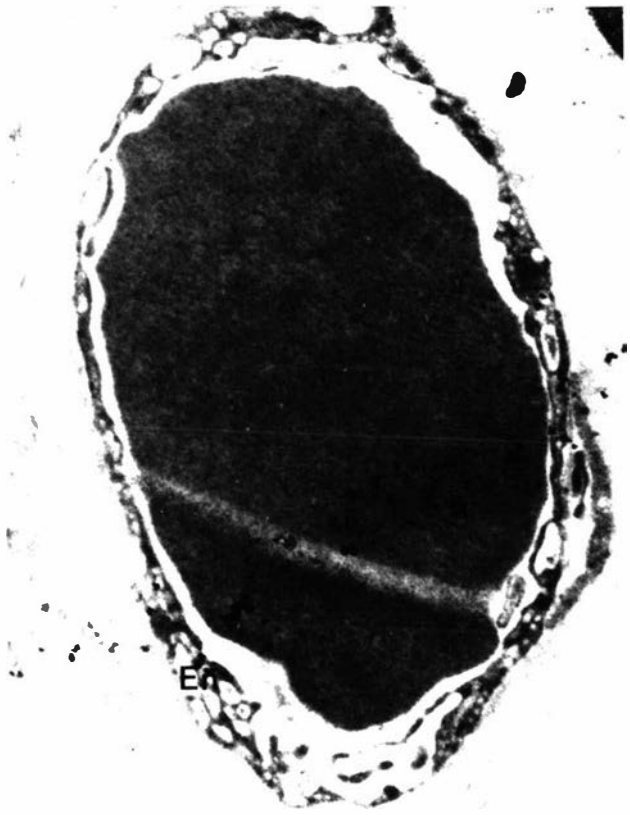

En

Materials & Methods

Two 2 week old female lambs, with epsilon antitoxin levels of less than 0.08 units/ml of serum, were anaesthetised and three intestinal loops with their blood supply intact, were isolated by ligation in each animal. The anaesthetic used and surgical procedures employed were similar to those used for the rabbits and described in Appendix I. Three mls of nutrient broth were introduced into the middle intestinal loop in each animal. Two solutions of batch CWD epsilon toxin in nutrient broth containing 4mg toxin/ml and 0.5mg toxin/ml respectively were prepared and 3ml of the higher concentration of toxin was introduced into one of the remaining intestinal loops and 3ml of the lower concentration of toxin into the other loop in each animal.

As soon as the animals were killed the section of intestine containing the loops was removed and photographed. The contents of the toxin containing loops was removed and stored. The volume of contents obtained from the loops which had contained the higher concentration of toxin was also measured. Pieces of intestine from each of the loops were fixed and sections prepared for light and electron microscopy and assays of epsilon toxin in the intestinal contents were carried out as described previously.

Results

Both animals recovered from the anaesthetic and thereafter remained normal for six hours from the time of the laparotomy. They were then destroyed by the intracardiac administration of 4ml of 'Euthatal' (May & Baker, Dagenham, England).

Necropsy of both animals revealed that all the loops containing either toxin or broth were bound together with fibrinous adhesions. The loops themselves were all distended with fluid and there was no difference in the appearance of the three loops in each animal. When sections of these loops were examined by light microscopy there were no detectable changes in the mucosa or submucosa which could be attributed to the action of the toxin

on the intestine. The only lesion was an early acute fibrinous peritonitis of the serosal surface of the intestine in all loops from both animals.

Electron microscopic examination did not reveal any evidence of damage to epithelial or endothelial cells in loops of intestine which had contained either high or low doses of toxin. In both instances the appearance of the mucosa was essentially the same as that of the broth containing loop (Figure 11.4)

All the intestinal loops had been empty prior to the introduction of the 5ml of broth or toxin solution. At the time of death, the volume of fluid in the loop which had contained the higher concentration of toxin was 25ml in one animal. Similar volumes of fluid were also present in the loops which had contained the lower concentration of toxin and in the loops which had contained broth only. The fluid from the loop which had been inoculated with 3ml of toxin solution containing 4mg toxin/ml (4,000 mouse doses/ml approx.) was found to contain 250 mouse lethal doses per ml i.e. a total of 6,250 mouse lethal doses of toxin when the lamb was killed 6 hours after inoculation of the loops.

The Morphological Changes Produced in the Intestine of Lambs by Experimental Cl. perfringens type D Enterotoxaemia

Materials & Methods

The animals employed and the method used to produce experimental enterotoxaemia have described in Chapter 6. Pieces of intestine were removed from these animals as soon as possible after death, although removal of this tissue was deferred until all other tissue samples had been taken.

Tissue sections were fixed and prepared for light and electron microscopy as described previously.

Results

There was no evidence of damage to the intestinal mucosa detectable by light microscopy in any of these animals. At the ultrastructural level there was some early autolytic change in the intestine but in animals where levels of toxin of over 1,000 mouse lethal doses of toxin were known to have been in contact with the mucosa for more than 3 hours (animal 81-see Chapter 6) there was no evidence of mucosal damage attributable to epsilon toxin detectable by electron microscopy. The cells themselves appeared to be unaffected and the junctions between them were intact. An electron photomicrograph of the intestinal mucosa of this animal is included in Figure 11.4.

Discussion

In view of the known necrotising action of epsilon toxin, its irritant effect upon the intestine and the increase in permeability to protein molecules which occurs when the mucosa is affected by this toxin, some morphological changes were to be expected. The severity of the lesions produced in the loops of rabbit intestine confirmed that such damage does occur in this species. The type of lesion, viz, an acute inflammatory necrosis is similar to that described by Todorov & Trifonov (1962) although they did not consider that this lesion developed when levels of toxin as low as 250 mouse lethal doses/ml were present in the intestine.

One finding in the experiments with rabbits is of particular interest. While severe capillary endothelial damage occurred in the mucosa of the toxin-containing loop, it was not present in the adjacent broth-containing control loop although the amount of toxin absorbed was lethal to the animal. Because the loops were ligated so that their blood supply and drainage were largely independent of one another there would be little or no direct transfer of toxin from the capillary network of one loop to that of the other. Under these circumstances, while the concentration of toxin entering the blood stream of the toxin containing loop could be quite high, the level in the adjacent loop would be

Figure 11.4: The Effect of Epsilon Toxin on the Mucosa of Lamb Intestine

A B

C D

A. Control:

Normal intestinal mucosa from a loop of lamb intestine which had been ligated for 6 hours and had been inoculated with 3 ml. of sterile nutrient broth.

Mv = microvilli

N = nucleii

BV = small blood vessel or lymphatic.

B. Mucosa from a Ligated Loop of Intestine which had been Inoculated with 3 ml. of a Solution Containing 500 Mouse Lethal Doses of Epsilon Toxin/ml:

The appearance of this tissue is essentially similar to the normal mucosa in A.

C. Mucosa from an Intestinal Loop which had been Inoculated with a Solution Containing 4,000 Mouse Lethal Doses of Epsilon Toxin/ml:

Again there is no evidence of pathological change in this section.

D. Mucosa from the Intestine of a Case of Experimental Enterotoxaemia:

One thousand or more mouse lethal doses of epsilon toxin had been present in the intestinal contents of this animal for at least 3½ hours before death.

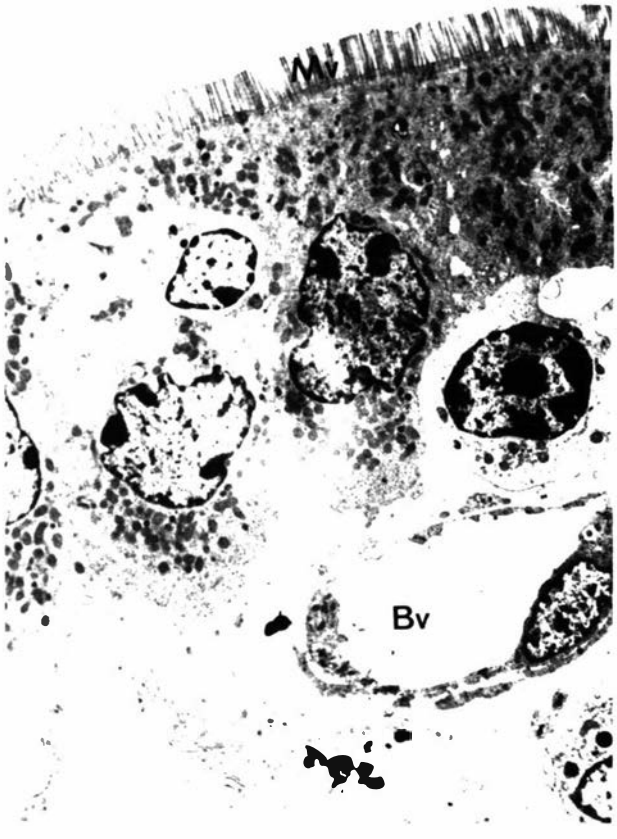
Although there appears to be some early autolysis in this section (see text), there are no changes which could be attributed to the action of epsilon toxin.

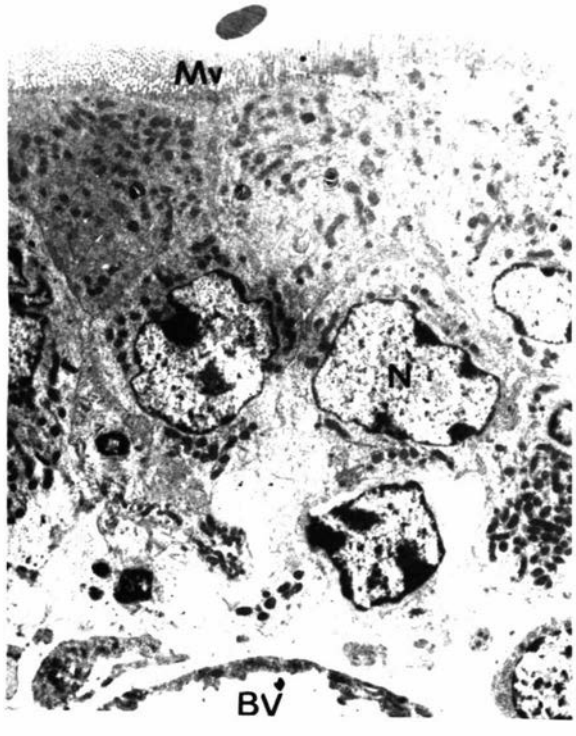
Eo = eosinophil

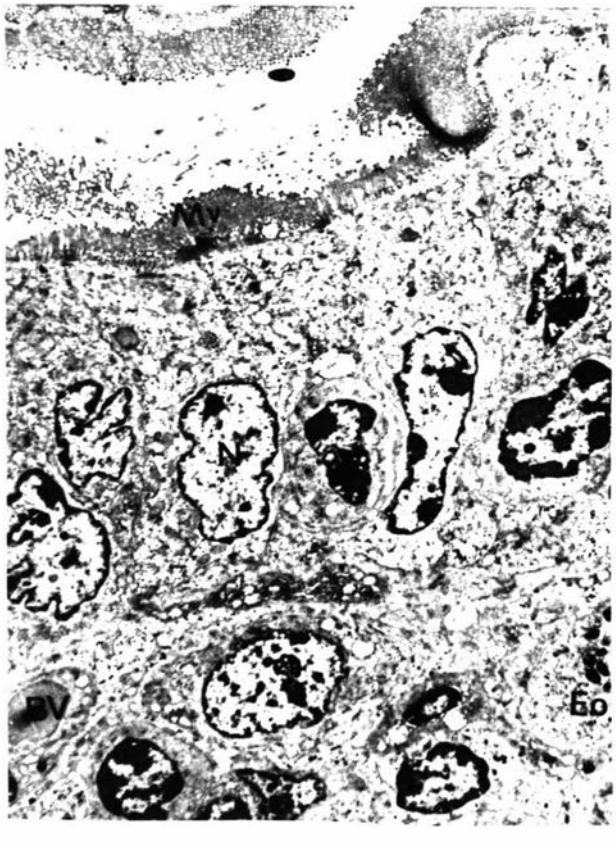
These figures should be compared with the sections of rabbit intestine in Figure 11.3.

Magnification 2,800


Mv
N
N
BV


Mv
Bv


Mv
N
BV


Mv
N
BV

the same as that in the rest of the systemic circulation. No tissues, apart from the intestine, were taken from these rabbits but it appears that the vascular damage which develops in this species during intoxication may not be as severe as that which occurs in lambs.

The fibrinous peritonitis which occurred around the intestinal loops of the rabbits which survived for 7 hours and also around the intestinal loops of the lambs was almost certainly the result of the intestinal ligation. It could be expected to occur in any situation where intestinal obstruction developed and was therefore not the result of the administration of epsilon toxin. It had been expected that this type of lesion would occur if animals were permitted to survive for many hours and was the reason why surviving animals were killed 6 - 7 hours after intestinal ligation in these experiments.

The lack of any detectable damage to the mucosa of loops of lamb intestine which could be attributed to the action of epsilon toxin was very surprising. It could be argued that, since neither of the animals died, the level of toxin in the intestinal loops was not maintained at a high enough concentration for sufficient time for either intoxication or intestinal damage to develop. This is borne out to some extent by the fact that, when examined 6 hours after inoculation, the amount of toxin in one of the loops which originally contained 4,000 mouse lethal doses/ml had fallen to the region of 250 mouse lethal doses/ml. Nevertheless this decrease had occurred over a period of 6 hours and it was probable that levels of 1,000 mouse doses/ml could have been present for at least an hour after inoculation. On the other hand it is difficult to explain how a susceptible lamb could absorb the amount of toxin which had disappeared from the intestinal loop without exhibiting signs of intoxication and it is possible that some inactivation of the toxin had occurred in the intestinal lumen. Nedyalkov and Stoyanov (1965) have suggested that this may occur. Todorov and Trifonov (1962) consider that both the level of toxin and the surface area available for absorption are important in epsilon toxin intoxication and in the present instance the length of the

loops, which was approximately 20cm/loop, may have been insufficient for adequate absorption of toxin to occur although this should not have affected its action on the intestinal mucosa.

Despite these possible shortcomings of the intestinal loop experiments in lambs the fact remains that evidence of intestinal damage was also absent from lambs in which enterotoxaemia had been induced by the infusion of Cl. perfringens type D culture plus carbohydrate. High levels of toxin were demonstrated in the intestinal contents of these animals and had been shown to have been present there for several hours prior to death in at least one of the animals examined. This suggests that the findings in the lamb intestinal loop experiments were in fact valid and reflect the fact that there may be a considerable difference in the action of epsilon toxin on the intestinal mucosa of rabbits and sheep.

From these experiments it does not appear that there is a detectable morphological basis for the altered intestinal permeability in the sheep intestine in epsilon toxin intoxication or enterotoxaemia.

Conclusions

1. Epsilon toxin produces a severe acute inflammatory necrosis of the mucosa and severe inflammatory oedema of the submucosa of ligated loops of rabbit intestine.

2. When introduced into ligated intestinal loops at a level of 2,000 mouse lethal doses/ml epsilon toxin is lethal to rabbits but it is non-lethal when 250 mouse lethal doses is given in the same manner.

3. Severe vascular endothelial damage occurs in the capillaries of toxin-containing loops of rabbit intestine but the endothelium of capillaries in adjacent control loops is not obviously affected even when lethal levels of toxin have been absorbed into the circulation. Rabbit endothelium may therefore, like that of mice, be less susceptible than that

of lambs to the action of epsilon toxin.

4. Levels of epsilon toxin of 12,000 mouse lethal doses were non lethal when a single inoculum, containing 4,000 mouse lethal doses/ml, is introduced into ligated intestinal loops of susceptible lambs. This may be due to inactivation of the toxin in the intestinal lumen, inadequate concentration of toxin for a sufficient period of time or insufficient surface area for absorption of lethal levels of toxin.

5. No morphological damage was detectable in the mucosa of ligated loops of lamb intestine when the animals were destroyed 6 hours after the loops had been inoculated with 12,000 mouse lethal doses of epsilon toxin in 5ml of nutrient broth.

6. There was no detectable morphological damage to the intestinal mucosa of lambs in which enterotoxaemia had been induced by the infusion of Cl.perfringens type D culture plus carbohydrate, even when toxin levels in the intestine exceeded 1,000 mouse lethal doses/ml for several hours.

Chapter 12: THE MORPHOLOGICAL CHANGES IN SKIN, PANCREAS, THYROID, ADRENAL, MUSCLE, PITUITARY AND LIVER PRODUCED BY THE ADMINISTRATION OF CL. PERFRINGENS TYPE D EPSILON TOXIN.

Most of the major morphological changes which occur during epsilon toxin intoxication have now been covered and in this chapter the changes which were seen in other tissues will be described and discussed.

No completely satisfactory explanation for the hyperglycaemia which occurs as a result of the administration or absorption of epsilon toxin has been put forward by previous workers and it is important to note that, with the exception of skin, all the tissues in this chapter are involved to a greater or lesser extent in the maintenance of a stable blood sugar level. For example the Islets of Langerhans in the pancreas are probably one of most important single organs in glucose homeostatis. Insulin, which is produced by the beta cells of these islets has an anabolic action, leading to the transfer of glucose from the bloodstream to the glycogen reserves in the skeletal muscle and liver. The effects of this hormone are balanced by those of glucagon from the alpha cells of the pancreatic islets, adrenalin from the adrenal medulla, growth hormone from the pituitary and steroid hormones from the adrenal cortex. The adrenal medullary and cortical hormonal secretions provide short and long term influences respectively on glucose mobilisation and Trifinov & Sotirov (1967) and Sotirov (1967) have suggested that epsilon toxin produces disturbances in both medullary and cortical adrenal function.

It has also been suggested by Jubb & Kennedy (1963) that there is some experimental evidence that the hyperglycaemia of enterotoxaemia results from a mobilisation of hepatic glycogen reserves. Further, Trifinov & Todorov (1965) have produced evidence that hepatic damage occurs when epsilon toxin passes through the liver.

To complete the present study it was necessary to examine the effects of the toxin on both thyroid and pituitary morphology although it was unlikely that damage to these organs would affect blood glucose levels in a short term intoxication.

The Morphological Features of the Cutaneous Damage Produced by the Intradermal Injection of Epsilon Toxin into Guinea Pig Skin.

Although the appearance of the skin lesions produced by the intradermal injection of epsilon toxin is important in the assay and identification of the toxin, those lesions are usually only examined visually and no description of the light or electron microscopic appearance of them appears to have been published. While it was beyond the scope of the present project to undertake a comprehensive study of pattern of histopathological changes produced by the various toxins of Cl. perfringens strains, it was of interest to determine whether the changes which occurred in skin, after the intradermal injection of epsilon toxin, differed in any important respect from those which occurred in other tissue following intravenous administration ot this substance.

Materials & Methods:

A series of cutaneous lesions were produced by the intradermal injection of 5ug of batch CWD epsilon toxin in 0.1ml saline into the clipped lateral abdominal wall of an adult male guinea pig. The injections were made at 3 hour intervals with a final injection one hour before the animal was destroyed by neck dislocation. Sections of skin for light microscopy were taken diametrically through each of the lesions including both the full thickness of the skin and a margin of normal tissue on either side of the lesion. The tissues were fixed in Zenker's fixative followed by appropriate washing in Iodine and Sodium thiosulphate solutions (Culling, 1963). Processing and staining of sections was carried out as described in Chapter 8.

Tissue for electron microscopy was taken from the area of increased capillary permeability in each lesion and sections were prepared as described in Chapter 8.

Results:

The gross appearance of lesions of different durations, produced in guinea pig skin by the intradermal injection of epsilon toxin were as

follows. At three hours post-inoculation there was intense erythema and increased capillary permeability around the site of the injection. After a further 3 hours had elapsed the central part of the lesion was becoming devitalised and this area was pale. It was surrounded by a zone of erythema, and increased capillary permeability was still prominent at this time. By 9 hours after injection the central devitalised zone was approximately 1cm in diameter and the surrounding zone of hyperaemia was less marked. Finally, by 12 hours post-inoculation, when the experiment was concluded, the central area was obviously necrotic and the epidermis had become firm and dry to the touch.

Light microscopic examination of these lesions showed that, one hour after the injection of the toxin, there was some inflammatory oedema in the subcutaneous connective tissue. Thereafter the most notable feature was an acute inflammatory infiltration around the blood vessels. Along with the inflammatory oedema, this became progressively more severe and diffuse as the duration of the lesion increased.

Unfortunately skin is very difficult to prepare for electron microscopy (Rhodin, 1963). Several attempts were made to prepare suitable sections from the skin lesions by using different hardnesses of epoxy resin and sectioning with a diamond knife on the ultramicrotome but the definition of the epidermis was poor in all sections. In the dermis there were no marked changes apart from the oedema and inflammatory cells seen by light microscopy. The capillary endothelium appeared to be normal in all the sections which were examined.

Morphological Changes in Liver, Pancreas, Thyroid, Adrenal, Pituitary and Skeletal Muscle of Intoxicated Mice and Lambs.

Materials & Methods:

The following tissues were taken for light and electron microscopic examination:

i. Muscle, liver, skeletal muscle and pancreas from the control and intoxicated mice described in Chapter 5.

ii. Liver, pancreas, adrenal, thyroid and pituitary from control lambs and those which received parenterally administered epsilon toxin (Chapter 6).

iii. Liver from the lambs which received enteric origin toxin (Chapter 6).

Sections were prepared for light and electron microscopy as described in Chapter 8. In addition paired liver sections from the control and intoxicated animals were treated with diastase (May & Baker) according to the method described in Culling (1963).

Results:

Liver:

Apart from the vascular endothelial damage, which was present to some extent in all tissues from intoxicated animals, there were no differences, in the appearance of the livers of intoxicated and control mice, detectable by either light or electron microscopy. There was lipid present in the hepatic cells and the amount varied considerably from animal to animal in both control and intoxicated groups. Unlike the situation in the kidney, the accumulation of lipid did not appear to be associated with starvation.

No consistent differences were noted in the P.A.S. staining affinity of the hepatocytes of control and intoxicated mice. This was in direct contrast to the situation in lambs. The hepatocytes of all control lambs contained abundant P.A.S. positive granules, which were confirmed as glycogen by diastase digestion. These granules were either greatly reduced in number or entirely absent from the hepatic cells of intoxicated animals. This reduction occurred irrespective of whether the toxin was parenterally administered or of enteric origin.

The light microscopic appearance of representative P.A.S. stained liver sections from intoxicated and control animals are shown in Figure 12.1.

Glycogen granules are very electron dense in sections stained with lead stains. They tend to have a fairly typical appearance in electron photomicrographs and are usually abundant in the hepatic cells of normal animals. Electron dense granules of this type were abundant in the hepatocytes of liver sections from control lambs in the present study but were almost entirely absent from the hepatic cells of intoxicated animals (Figure 12.2).

Figure 12.1: The Loss of Glycogen from the Livers of Intoxicated Lambs - Light Microscopy.

A B

C D

A. Liver - Control Lamb:

The hepatocytes, particularly around the centrilobular veins contain large amounts of PAS positive material resembling glycogen (Gn).

CV = centrilobular vein

PT = Portal triad

B. Liver - Control Lamb - after Treatment of the Section with Diastase:

The identity of the PAS positive material in A as glycogen is confirmed as it has been removed from this section, from the same block of liver, by diastase digestion.

H = Hepatocytes

C. Liver - Intoxicated Lamb:

There is no PAS positive material, resembling that seen in A, present in this section.

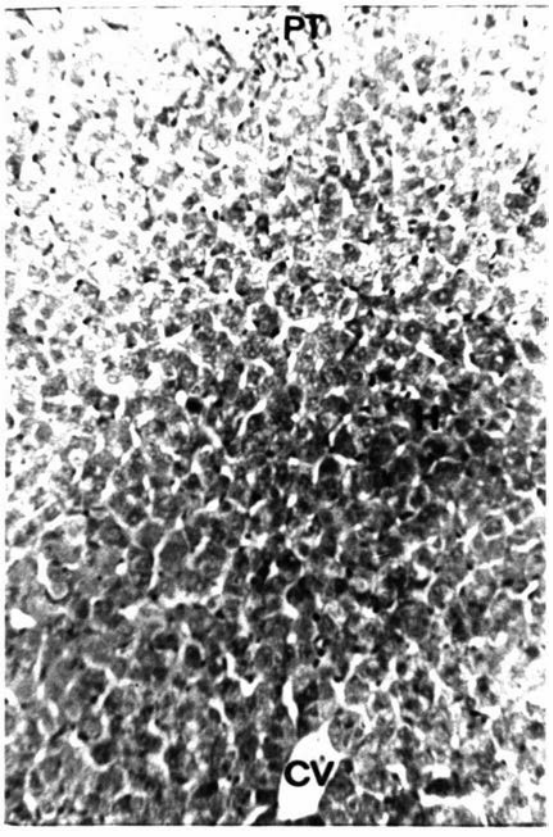
D. Liver - Intoxicated Lamb - after Treatment of the Section with Diastase:

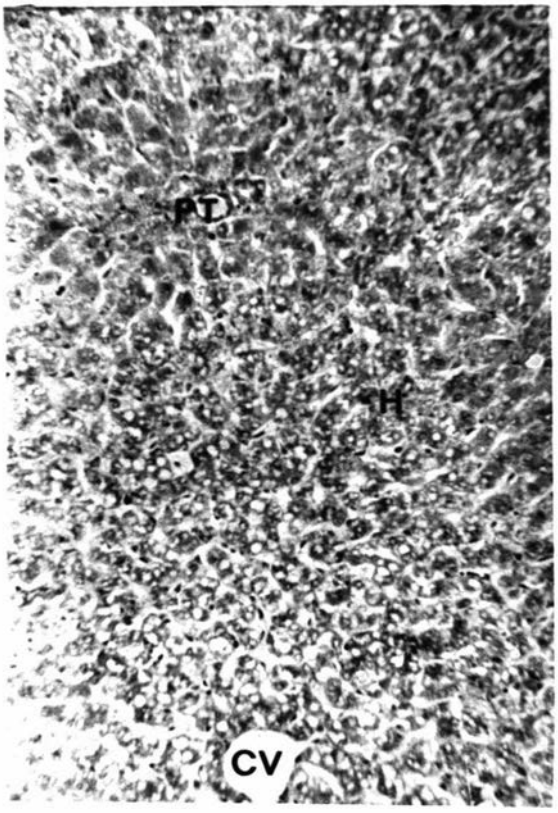
Diastase digestion has not noticeably affected the appearance of this section which is from the same block as C.

Stain PAS

Magnification 120


PT
CV


PT
H
CV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CV

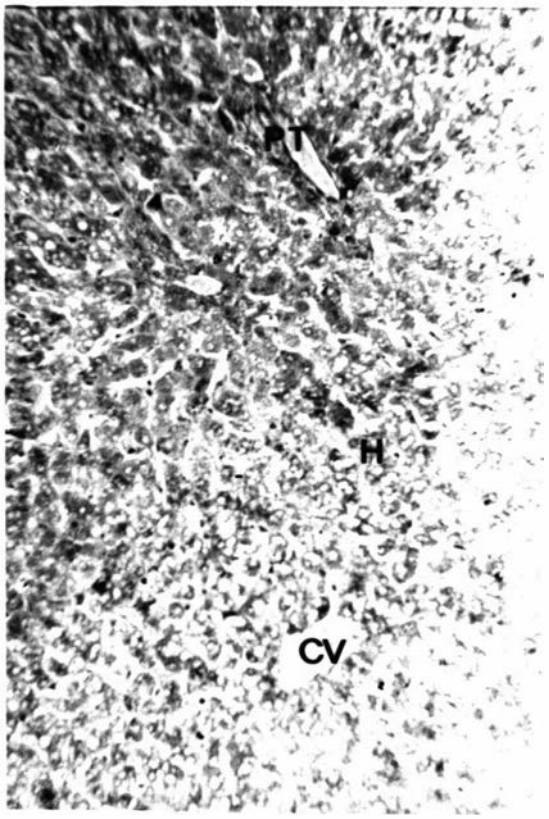

PT
H
CV

Figure 12.2: The Loss of Glycogen from the Livers of Intoxicated Lambs - Electron Microscopy.

A

B

A. Liver - Control Lamb:

Very large numbers of aggregations of electron-dense granules (Gn), resembling glycogen granules, are present in the cytoplasm of this hepatocyte.

N = nucleus

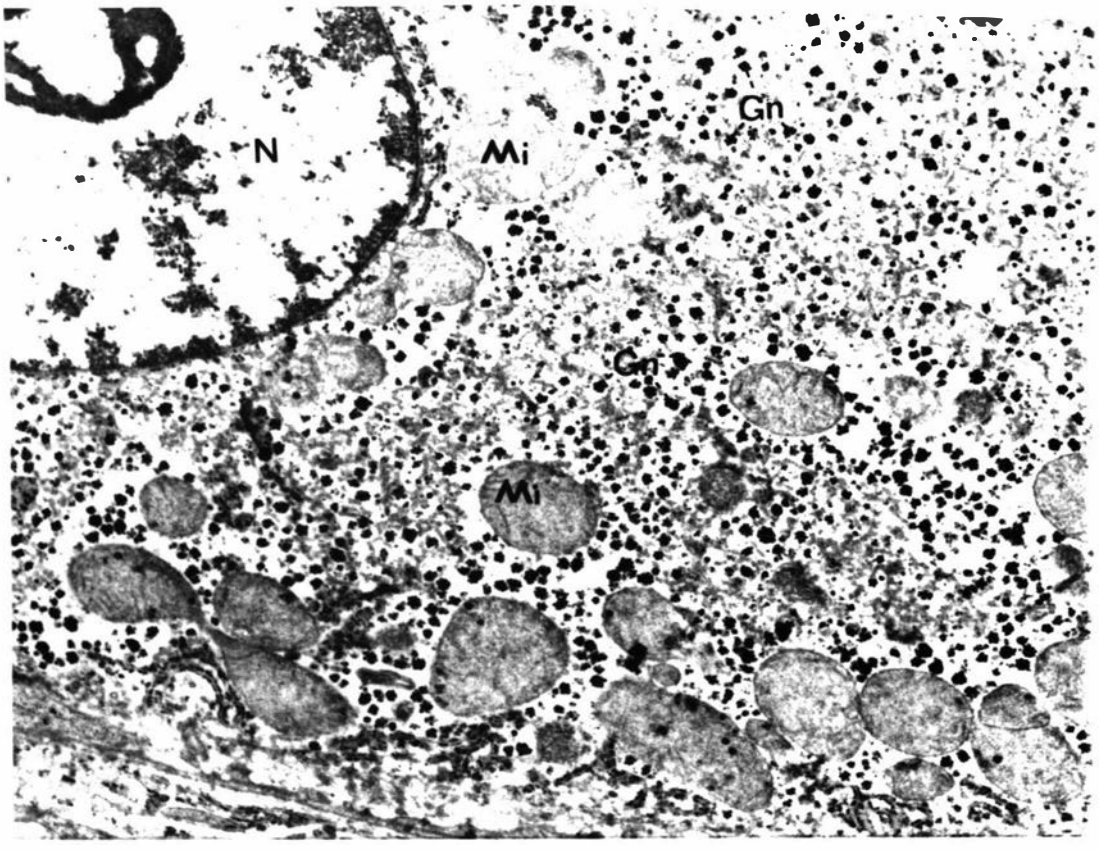
Mi = mitochondria

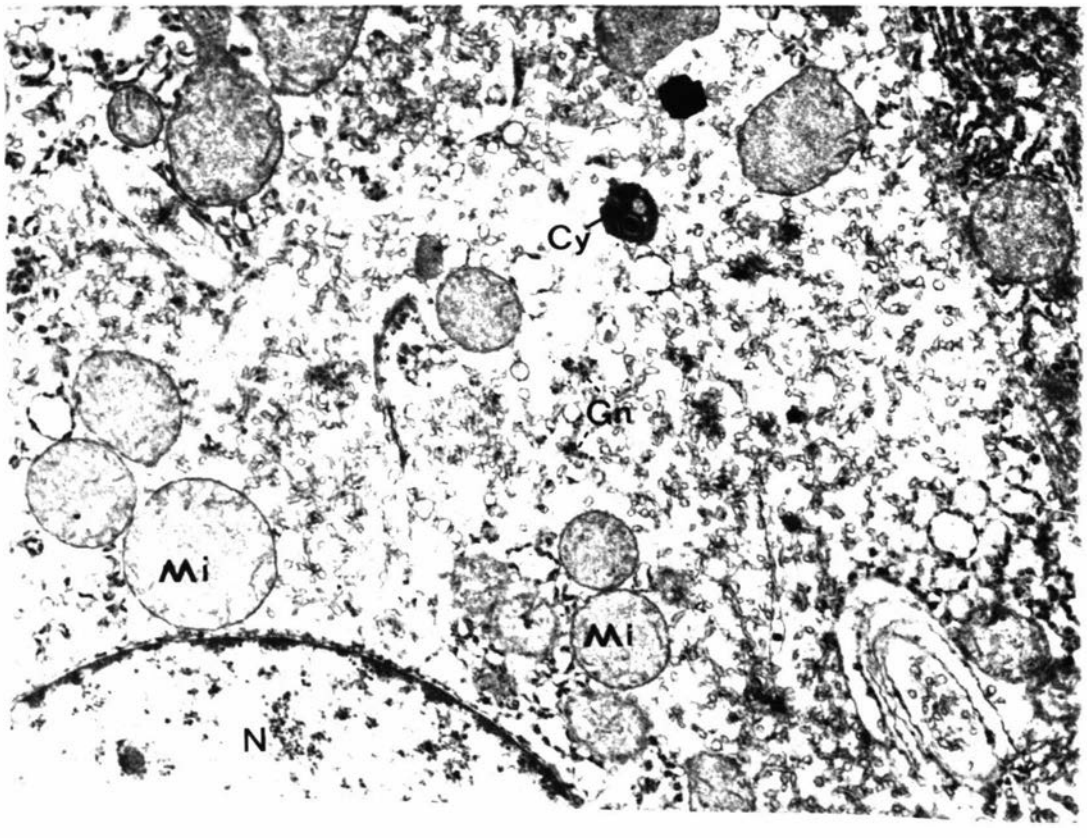
B. Liver - Intoxicated Lamb:

Only a few glycogen-type granules (Gn) are present in this hepatocyte.

Cy = cytosome

Magnification 12,000


N
Mi
Gn
Gn
Mi


Cy
Gn
Mi
Mi
N

Pancreas:

Vascular endothelial damage was also present in the pancreas of intoxicated animals. No other lesions were seen in either the glandular portion of the organ or in the Islets of Langerhans. Particular attention was paid to the appearance of the different cell types in the latter, endocrine portion of the gland. The appearance of both the alpha and beta cells, which are currently regarded as being the cell types which produce glucagon and insulin respectively, was similar in control and intoxicated animals.

Adrenal, Thyroid and Pituitary:

As in other tissues from intoxicated animals there was evidence of vascular endothelial damage in the thyroid, pituitary and adrenal sections taken from intoxicated animals. Apart from these lesions however, there were no differences between the appearance of these organs from intoxicated and control animals detectable by light and electron microscopy.

Skeletal muscle:

Skeletal muscle was not taken from intoxicated lambs. However, in intoxicated mice where there was vascular endothelial damage in the skeletal muscles there was no evidence of oedema or intracellular fluid accumulation in the muscle cells.

Discussion

Keratinised tissues are difficult to prepare for light microscopy, as well as electron microscopy, and fixatives such as formalin and Bouins fluid either penetrate poorly, giving inadequate fixation, or else make the tissues brittle and hard to section. In the present instance Zenker's fixative was employed and gave good fixation and preservation of cytological detail as well as reasonable sectioning characteristics.

In view of the apparent severity of the gross appearance of the skin lesions produced by the intradermal injection of epsilon toxin it was rather surprising to find that they were not more prominent when examined histologically. This finding emphasises the importance of careful visual

assessment of cutaneous lesions where, despite prominent clinical signs of irritation and gross lesions, the histological appearance may be practically normal. The absence of capillary endothelial damage in these sections suggests that the protein molecule of epsilon toxin may not be capable of passing into the vascular lumina when injected into the tissue substance. To some extent the lack of capillary endothelial damage may also reflect the site from which the tissues for electron microscopic examination were taken. As pointed out earlier, they were always taken from the erythemic margin of the lesion rather than from the same site relative to the point of injection which, in retrospect, would probably have been the better procedure.

Turning now to the changes in other tissues which have been described in this chapter. It is noteworthy that there was a general lack of detectable damage to cells, other than those of vascular endothelium. This was to be expected when the findings in previous chapters concerned with morphological changes are considered. In most instances the lesions associated with intoxication in mice and lambs have been referable to the vascular endothelial damage.

It appears therefore that the clinical signs and biochemical and physiological changes which occur during enterotoxaemia may be due to disturbances in tissue metabolism, brought about by inadequate transfer of nutrients and metabolites, as a result of the vascular damage, rather than any direct effect of the toxin on other tissue cells. Further consideration of these points however can be conveniently postponed until the functional status of the tissues has been assessed.

No quantitative estimations of the glycogen content of the livers of control or intoxicated animals have been carried out in the present study. However it appears, from the light and electron microscopic changes described in this chapter, that hepatic glycogen reserves are reduced very rapidly in intoxicated lambs. It is interesting to note that Berg & Levinson (1959) based their hypothesis, that the hyperglycaemia which occurs in experimental Cl. perfringens type A alpha toxin intoxication is due to mobilisation of hepatic glycogen reserves, on the fact that they demonstrated a reduction in the number of P.A.S. stained glycogen granules

in the livers of their animals. Although Jubb & Kennedy (1963) refer to the above findings in the relevant chapter on the pathological changes which occur in Cl perfringens type D enterotoxaemia, it is not clear from their bibliography whether this forms the basis for the statement, that the hyperglycaemia in the latter condition may be due to the mobilisation of hepatic glycogen, or whether other experimental evidence was also available.

In the present study further investigations were made into the possible role of hepatic glycogen and other factors in the pathogenesis of the hyperglycaemic response to epsilon toxin and these will be described in Chapters 19 and 20.

Conclusions

1. The skin lesion which is produced by the intradermal injection of epsilon toxin is an acute inflammatory oedema of the dermis followed by necrosis of the overlying epithelium. These changes do not appear to be associated with direct damage to the vascular endothelium.

2. Zenker's fixative is useful for the preparation of skin sections for light microscopy.

3. On the basis of light and electron microscopic findings, it appears that the glycogen content of the liver of lambs is rapidly reduced under the influence of Cl. perfringens type D epsilon toxin. The reduction occurs irrespective of whether the toxin is parenterally administered or of enteric origin.

4. Morphological changes in muscle, thyroid, adrenal, pituitary and pancreas in intoxicated animals were confined to vascular endothelial damage similar to that which has been described in the tissues discussed in earlier chapters.

CHAPTER 13: THE MORPHOLOGICAL FEATURES OF FIELD CASES OF CL.PERFRINGENS TYPE D ENTEROTOXAEMIA IN LAMBS

One of the stated aims of the present project was to relate the changes produced by the parenteral administration of epsilon toxin or experimentally induced enterotoxaemia to the changes which occur in field cases of enterotoxaemia. Unfortunately no field cases of acute enterotoxaemia were presented alive during the period over which these investigations were carried out and this precluded any extensive study of the 'clinical' pathology of the natural disease. However, the close similarity between the methods that are employed to induce enterotoxaemia experimentally and the conditions under which it occurs in the field made it most unlikely that biochemical changes in the natural disease would differ markedly from those which occur during experimental intoxication.

One field case of acute enterotoxaemia was presented a short time after death and it was therefore possible to study the morphological changes which occurred in this animal. A limited amount of biochemical information was also obtained from it and will be discussed along with the experimental data in the relevant chapters (Chapters 19 & 22). However, the morphological changes in tissues of this animal are more conveniently all studied together in the present chapter rather than in association with the individual tissues of experimental animals.

In addition to the acute case of enterotoxaemia, one case of focal symmetrical encephalomalacia was presented alive. The clinical signs and morphological changes which occurred in this animal will also be described here. Because this particular animal had been affected for some weeks before it was presented for examination, the lesions were of such a duration as to be hardly relevant to the present investigation and the discussion of this case will therefore be very brief.

Veterinarians and field officers of the Department of Agriculture in the Manawatu area which surrounds Massey University were asked to submit, to the university, any live cases of suspected

enterotoxaemia which they encountered. As a result, a number of lambs with clinical signs of neurological disturbance were submitted. These included cases of *Sphaerophorus sp.* spinal abscess and *Escherichiacoli* meningoencephalitis as well as the case of focal symmetrical encephalomalacia mentioned above.

Acute Enterotoxaemia Case:

History:

An unvaccinated 2 week-old male Romney/down cross lamb weighing 7.7kg was presented for examination approximately 2 hours after death was believed to have occurred.

Material & Methods:

Necropsy of this animal was carried out as soon as it arrived at the university and sections of cerebral cortex, thalamus, midbrain, cerebellum and medulla of the brain plus kidney, and liver, were fixed and prepared for light and electron microscopy as described for tissues of experimental animals. In addition, urine and heart blood were also collected for biochemical examination. The water content of sections of cerebral cortex, thalamus and cerebellum and lung was also determined as described in Chapter 8.

The intestinal contents were mixed with an equal volume of saline and examined for the presence of epsilon toxin by the mouse protection test described in Chapter 4.

Results:

Gross Pathology:

The carcase was that of a good condition lamb and the stomach contained a quantity of milk curd. The lungs were intensely congested and slightly oedematous. There were

approximately 50mls of clotted straw-coloured fluid in the pericardial sac. The kidneys were congested and soft although they still retained their normal shape. All other organs appeared to be grossly normal. The intestinal contents were sparse and of a thick creamy consistency and colour. They were toxic for mice and the toxicity was specifically neutralised by Cl.perfringens type D antiserum.

The brain of this animal weighed 70grams and the water content of the various tissues was as follows:

Cerebral cortex	4.53mgH_2O/mg.D.M.
Thalamus	4.43 " / "
Cerebellum	4.13 " / "
Lung	4.19 " / " (No total lung weight was recorded)

Light Microscopy:

The light microscopic appearance of H&E stained sections of formalin fixed kidney from this animal were similar to those previously mentioned as being considered to be typical of enterotoxaemia. There was 'necrosis' of the proximal tubular epithelium, and extensive interstitial 'haemorrhage' between these tubules. These changes have been illustrated in Figure 10.1 (Chapter 10), and formed the most prominent lesion which was seen in the tissues from this animal. There was also evidence of myocardial oedema, detectable by light microscopy in sections of heart.

No detectable lesions were seen in the brain of this lamb.

Electron Microscopy:

There was some evidence of autolytic change in all the tissues from this animal which were examined by electron microscopy. This was less severe in the brain than in tissues such as kidney.

In the brain sections there was evidence of severe vascular endothelial damage. This was similar in all respects to that which

occurred in experimentally intoxicated animals and was associated with swelling of astrocyte foot processes. The vascular lesions in the brain of this field case of acute enterotoxaemia are illustrated in Figure 13.1.

The degenerative changes in the kidney of this animal were essentially similar to those seen in the later stages of autolysis following experimental enterotoxaemia (see Chapter 10). There was extensive degeneration of the capillaries between the proximal tubules which had resulted in the presence of intact erythrocytes in the interstitial tissues (Figure 13.2).

Focal Symmetrical Encephalomalacia Case

History:

The affected animal was a 6½ week-old female crossbred (Romney X Southdown) lamb weighing 9.5Kg which had shown clinical signs of central nervous system damage for 3½ weeks prior to submission. Initially it had been found lying in a recumbent position exhibiting extensor spasms. Some improvement occurred over the next few days and from then on it moved in circles and pressed its head against objects in its path. Hand feeding had been necessary during this time as it was unable to suckle normally from its dam. The lamb was from a flock of 180 ewes. No vaccination against enterotoxaemia had been carried out, and no deaths, suspected as being associated with this disease, had been reported.

Materials & Methods:

Jugular blood samples were taken from this animal into heparinised, plain and perchlorate treated 'Vacutainers' in the same manner as for the lambs described in Chapter 15. The animal was then destroyed with intravenously administered sodium pentobarbitone ('Euthatal'-May & Baker). Necropsy was performed immediately and sections of a similar selection of tissues. to that taken from the

Figure 13.1: Vascular Endothelial Damage in the Cerebral Cortex of a Field Case of Acute Enterotoxaemia.

The endothelial cytoplasm (En) has been reduced to a thin electron-dense band. There is also severe swelling of astrocyte foot processes (AFP) around the capillary.

The changes in this electron photomicrograph should be compared with those in Figure 8.7 which were produced by experimental enterotoxaemia and the parenteral administration of epsilon toxin.

Magnification 29,500

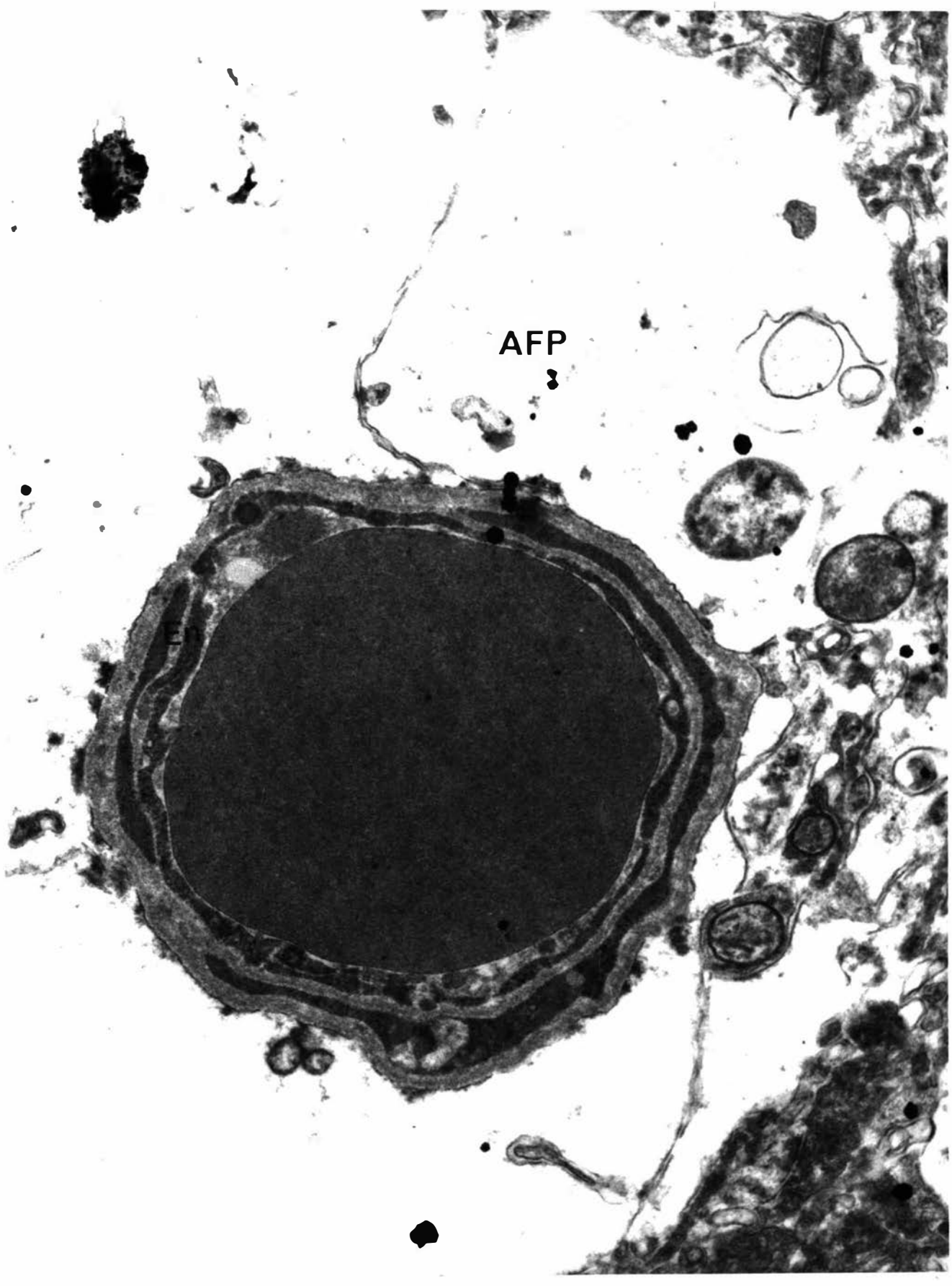

AFP
En

Figure 13.2: Autolytic Changes and Vascular Damage in Kidney from a Field Case of Acute Enterotoxaemia.

A B C

A. The proximal tubular epithelium (Ep) in this section still contains a recognisable nucleus (N) and microvilli, although there is a considerable amount of debris in the capillary lumen (L). The endothelium of the capillary (BV) appears to be damaged although the vessel wall does not appear to have been disrupted.

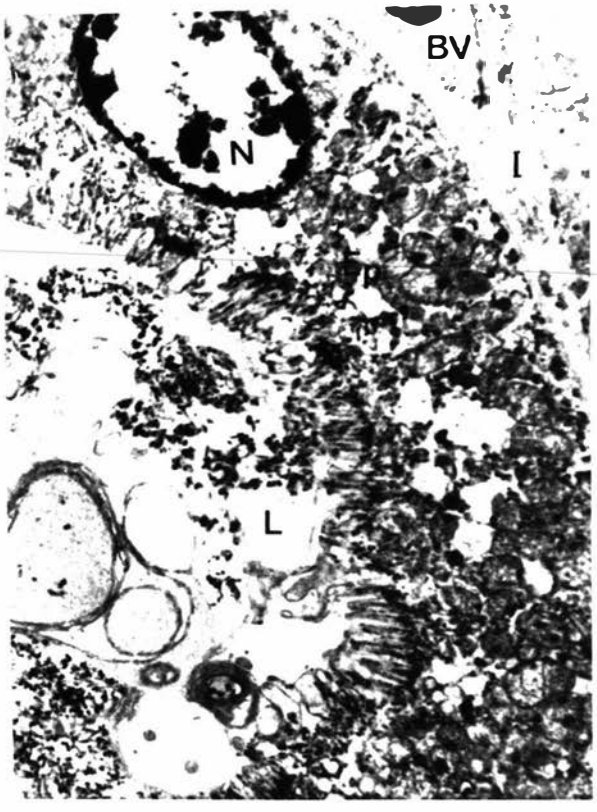
E = erythrocytes
I = Interstitium

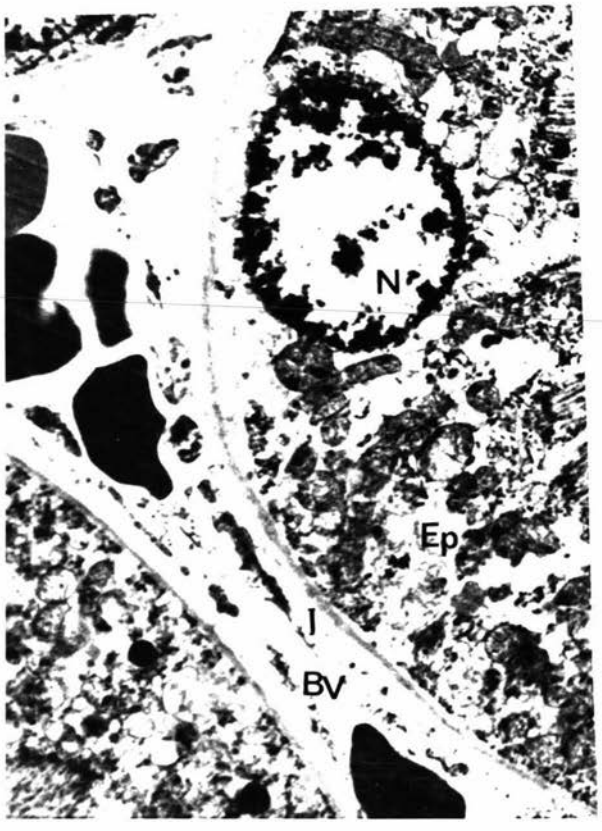
B. This area was adjacent to A above and in the same tubule. The epithelial changes are similar in both sections but, in the present instance, the capillary wall has disintegrated.

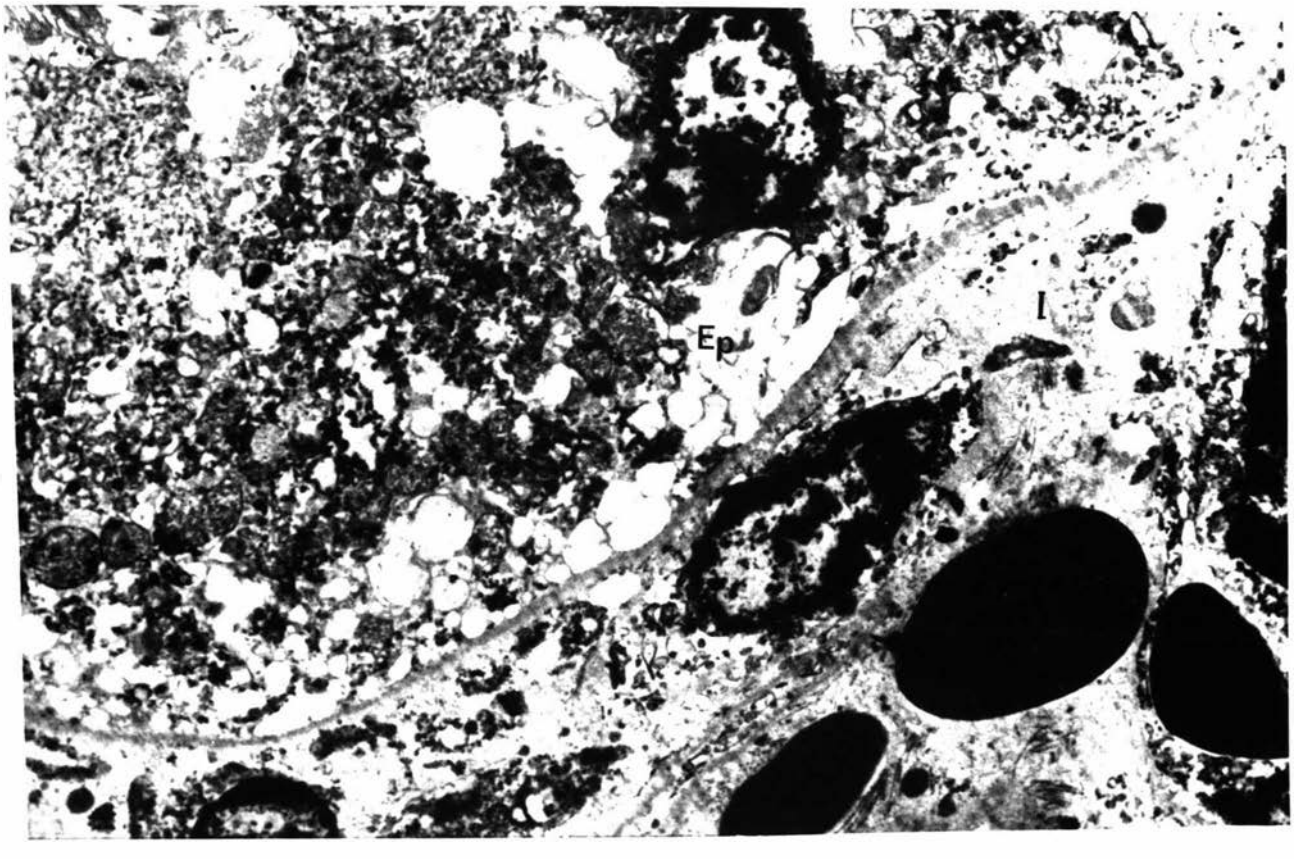
C. More advanced changes are present in this section which was from another area of the same kidney from which A and B were taken.

Degenerative changes in the tubular epithelium appear to be more advanced and erythrocytes are lying free in the interstitium

Magnification 5,300


BV
N
I
L


N
Ep
I
BV


Ep
I

acute case of enterotoxaemia described above, were taken for light microscopic examination. Urine was also collected by bladder puncture, and sections of brain were taken for water content determinations.

Results:

Gross Pathology:

The animal was in fair condition only. No gross lesions were detectable in any organ except the brain. In the brain there were lesions consistent with focal symmetrical encephalomalacia. These were bilaterally symmetrical pale fawn $\frac{1}{4}$-$\frac{1}{2}$cm diameter rather ill-defined areas in the internal capsule adjacent to the lentiform and caudate nucleii and continued into the mid-brain. Lesions were also present in the cerebellar peduncles. They were very hard to detect visually.

The water content of the various samples of brain were:-

Cerebellum	3.50mg	water/mg	D.M.	
Thalamus	3.49	"	/	"
Cerebral Cortex	3.71	"	/	"

Light Microscopy:

The only significant lesions in the tissues from this animal, which were examined by light microscopy, were in the regions of the brain where gross FSE had been seen. In these areas there was evidence of old standing malacia in the form of cavitation, focal proliferation of fibrous astrocytes and capillaries, plus scattered gitter cells.

These lesions are illustrated in Figure 13.3.

Figure 13.3: The Thalamic Lesions from a Case of Focal Symmetrical Encephalomalacia.

A

B

A. Low Magnification Photomicrograph:

Between the relatively normal tissue (NT) of the thalamus and the area of cavitation (Ca) which has resulted from earlier damage, there is a zone of marked glial proliferation (GP). This suggests that the lesion has been in existence for some time and is undergoing resolution.

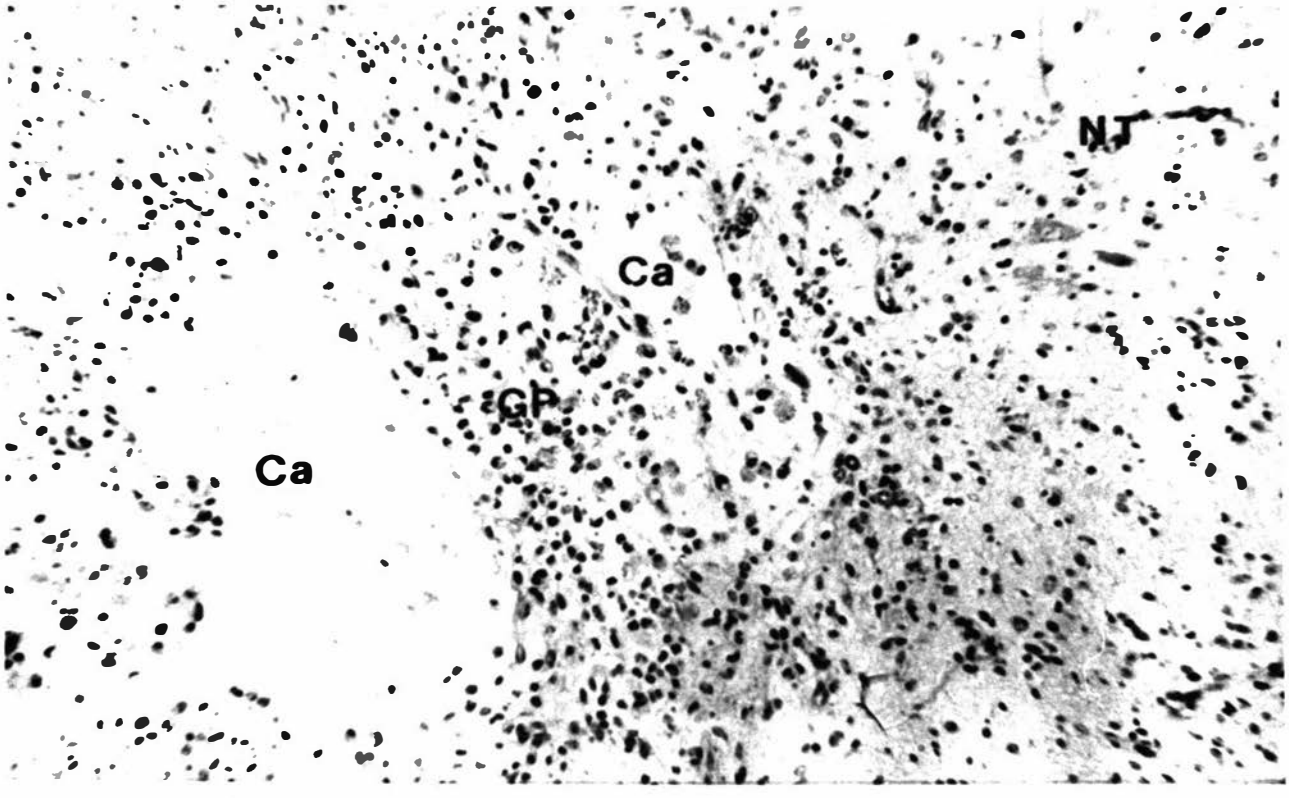
B. Higher Magnification of an Area of A:

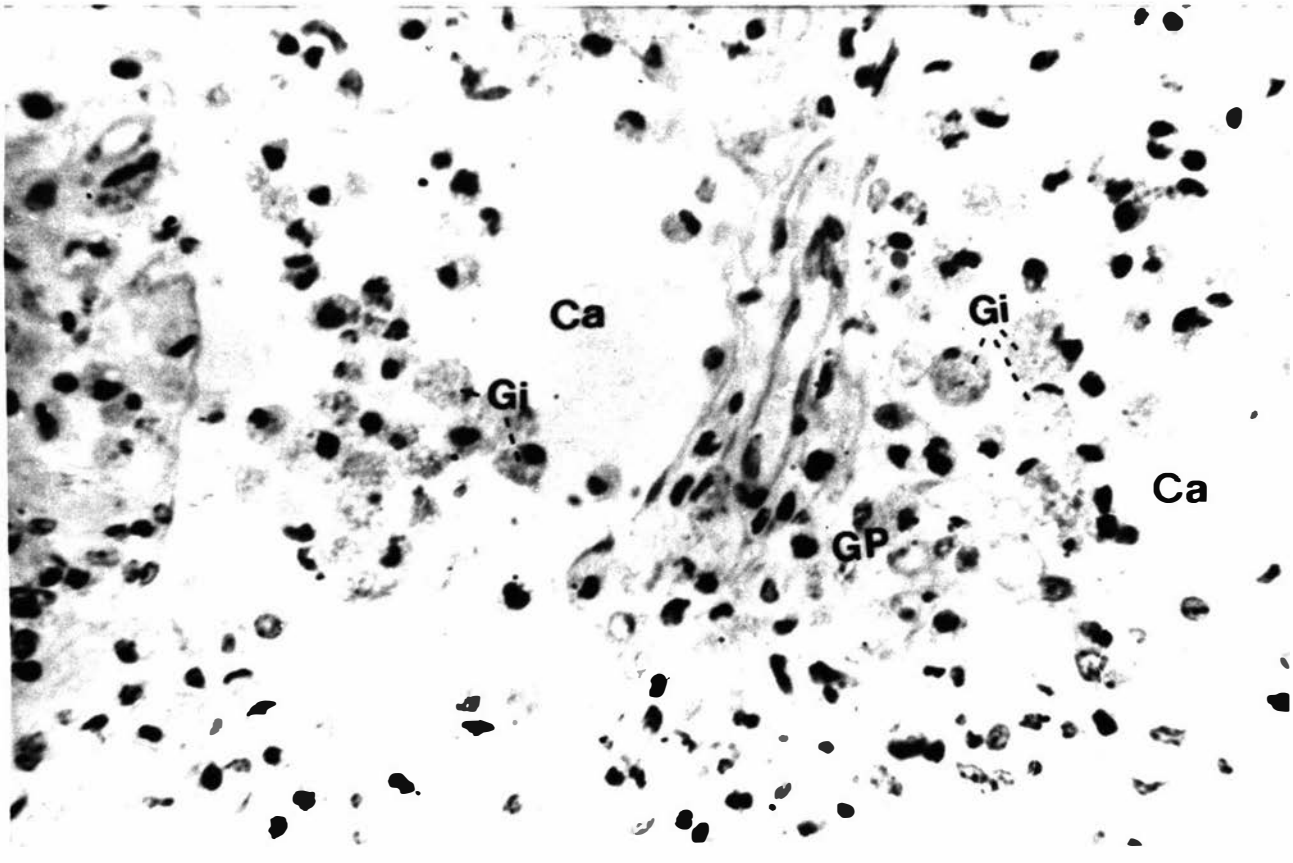
Numerous gitter cells (Gi) can be seen in the tissues adjacent to the areas of cavitation.

Stain PAS

Magnification A 180

B 450


NT
Ca
GP
Ca


Ca
Gi
Gi
Ca
GP

Discussion

It is an interesting fact that, over two consecutive lambing seasons, a number of veterinarians and Agriculture Department personnel apparently did not see any live lambs which they considered to be typical cases of acute enterotoxaemia. This emphasises the comparative rarity of the occasions on which live cases of this disease are seen. A number of different factors have led to this situation. Firstly, there is the acute nature of the condition. The short survival time of intoxicated animals makes it unlikely that live affected animals will be seen unless a considerable number of cases of the disease are occurring. The latter situation is much less common now than in the days of Gill (1933) and Hopkirk (1928), as widespread vaccination is carried out and large outbreaks of the disease are seldom seen. In addition, in the present instance, although the Manawatu region has a large sheep population, seasonal conditions during the period in question were not particularly favourable for the development of enterotoxaemia. There was a severe drought in the first season which made food supplies less abundant than usual. In the second year the food supply was better, but seldom plentiful, and the ewes themselves were still recovering from the effects of the drought and probably produced less milk than in a normal season.

The opportunity to examine the morphological changes in even one typical field case of acute enterotoxaemia was extremely valuable as it provided confirmation of the fact that the lesions which occur in the natural disease are indistinguishable from those which occur in experimental enterotoxaemia and epsilon toxin intoxication. In both instances the basic lesion appears to be severe vascular endothelial damage to which subsequent changes are referable. Ideally, particularly in the kidney, lesions in which autolytic changes were less advanced would have been preferable, but the pattern of change so closely resembles those in experimental enterotoxaemia and is so different from the autolytic changes in normal kidney that this limitation of the present study was less important than it might otherwise have been. The extent

of the autolysis in an animal which was supposed to have been dead for 2 hours was rather surprising since it was of a similar severity to changes which occurred after 6 hours or more of autolysis in experimentally intoxicated animals. It is possible that the carcase may have been lying in direct sunlight or that the animal had been in convulsions for some time prior to death. Since the animal was not recorded as having been seen alive it is also possible that it may have been dead for rather longer than was reported.

One feature of interest is the fact that the water content of the various regions of the brain of the acute case of enterotoxaemia was within the normal range established for lambs of this age (see Chapter 8). It may be recalled that this was also the case with animals which received parenterally administered epsilon toxin.

The water content of the various regions of the brain of the animal with focal symmetrical encephalomalacia was lower than for the above animals. This may have been due to the fact that the fluid intake of an animal, which was being hand fed and was also rather debilitated, may have been less than normal, but it is interesting to note that this animal was also older than the foregoing animals and that the brain water content of the normal older lambs has also been shown to be less than in the young lambs (see Chapter 8).

The extended duration of the clinical course and the resultant old standing nature of the lesions in the case of F.S.E. did not render it particularly suitable for the purposes of the present investigation. The lesions produced by the toxin had largely healed and were thus no longer relevant to a study directed primarily at the in vivo action of the toxin itself. It is appropriate to mention before leaving this case that Gill (1927) has also mentioned the apparent regression of the clinical signs of neurological damage in animals which survive the initial acute phase of the disease. As in most other cases of central nervous system damage in man and animals this is probably an adaptive phenomenon rather than a true recovery of nervous function in the

damaged areas.

Conclusions.

1. The lesions in the brain of field case of acute enterotoxaemia were similar to those in experimental enterotoxaemia and consisted basically of vascular endothelial damage.

2. The 'typical' renal lesions also appeared to be the result of the autolytic breakdown of the damaged vasculature in the field case of the disease.

3. An old-standing case of focal symmetrical encephalomalacia was also examined. The distribution of lesions was similar to those seen previously and described in the literature. Regression of clinical signs of nervous damage, also described in the literature, was seen in this animal.

CHAPTER 14: THE EFFECT OF EPSILON TOXIN ON TISSUE FUNCTION

The severity, and rapidity of onset, of the vascular endothelial damage described in Chapters 8 - 12 is remarkable and it is surprising that in lambs, apart from the swelling of astrocytes in the central nervous system, the damage is confined to the endothelium with no alterations in the subcellular architecture of the other tissues examined.

It has been shown in Chapter 8 that protein can escape from the vascular lumen in intoxicated mice and it could therefore be expected that epsilon toxin would also escape and come into contact with other cells, unless it is selectively bound to endothelial membranes. If this is the case it might explain both the severity of the endothelial damage and the lack of change elsewhere.

There are two commonly used ways in which the detection of the binding of substances to cell membranes can be approached. Firstly, membranes known as red cell stroma, which are frequently used in quantitative studies on the surface properties of plasma membranes, may be employed. However *in vitro* procedures, using these erythrocyte membranes, will not detect specific surface binding to other tissues and different methods must be employed when studying structures such as vascular endothelium *in vivo*.

Fluorescent antibody techniques provide a sensitive means of detecting a wide range of antigens in tissues, the main limitations of the technique being autofluorescence of tissue components and non-specific binding of the fluorescein-conjugated gamma globulin. It can be seen that both procedures have certain advantages, as well as limitations, and both were therefore used in the present investigation.

A large number of toxic agents produce severe disease or death, without being bound to plasma membranes, by acting directly or indirectly upon the pathways of energy metabolism. The complexity of the investigations necessary to determine a precise biochemical site of action of epsilon toxin place this objective well beyond the scope of the present project. Nevertheless techniques are available for studying many aspects of tissue function *in vitro* and some of these were employed in the present studies to determine whether epsilon toxin affects tissue energy metabolism.

Probably the most widely used techniques for studying the oxidative metabolism of tissues are those based on respirometry, employing instruments such as Warburg respirometers, Thunberg tubes or, more recently, oxygen electrodes for measuring oxygen uptake. Of these the Warburg respirometer is still the most widely used and also probably the most suitable for the study of tissue slices. Although homogenates and mitochondrial preparations have been used extensively in studies leading to the elucidation of metabolic pathways, simple tissue slices yield information on the overall efficiency of metabolism in organised tissue which is not obtainable when other preparations are used.

In vitro Studies of the Possible Binding of Epsilon Toxin to Cell Membranes Employing Red Cell Stroma

Preliminary Experiment

A simple preliminary experiment was carried out to determine whether or not there is demonstrable binding of epsilon toxin to cell membranes.

Materials & Methods:

A sample of red cell stroma was prepared by collecting 20ml of heparinised blood from a sheep with less than 0.08 units of epsilon antitoxin/ml of serum. The blood was centrifuged at 1800G for 20 minutes, the plasma discarded and the erythrocytes lysed by resuspending them in 50ml of distilled water. The resultant lysate was centrifuged at 2,500G for 30 minutes and the supernatant discarded. The deposit was resuspended and washed three times with Krebs-Ringer-Phosphate solution with similar centrifugal separation between each wash.

After the final wash solution had been removed, the deposit of red cell stroma was mixed with 1.0ml of a solution of epsilon toxin, containing 15ug toxin/ml, and held at room temperature for 30 minutes. The stroma was then removed by centrifugation as above and 0.2ml of the supernatant was injected intravenously into each of 3 mice. The same volume of the untreated epsilon toxin solution

was injected into each of 3 similar mice.

Results:

All three mice which received the stroma-treated toxin survived while the 3 mice which received the untreated toxin solution had died 12 hours after inoculation.

Main Experiment

The result in the preliminary experiment suggested that the toxin had been removed from the solution by the red cell stroma and a further experiment was necessary to determine whether this was a specific or a non-specific phenomenon. Inactivation of toxin without specific binding to cell membranes could occur in this type of experiment if there was any residual epsilon antitoxin associated with the red cell stroma or if the toxin itself was adsorbed non-specifically to the stroma.

The possibility of residual antitoxin being present can be eliminated by using blood from a species which does not normally have circulating epsilon antitoxin. Non-specific adsorption phenomena can also be overcome by pretreating the stromal preparation with a heterologous protein such as ovalbumin or a polysaccharide like dextran. The experimental method given below was therefore used in the main experiment.

Materials & Methods:

Three separate 40ml heparinised blood samples were obtained, viz. one sample from a healthy male rabbit and two from the same sheep that was used in the preliminary experiment. All blood samples were used on the day that they were collected and the red cell stroma was prepared in the same manner as in the preliminary experiment. Each stromal sample was then divided into three equal aliquots and these were pretreated with 1% ovalbumin in saline, 1% dextran in saline or saline alone. The stroma samples were incubated in these reagents for 30 minutes then washed 3 times as in

the preparative procedure. Thereafter the stromal samples were each mixed with 1ml of an epsilon toxin solution containing 20ug toxin/ml and incubated as before. After centrifugal separation of the stroma 0.2ml of the supernatant from each sample was injected intravenously into mice.

Results:

All mice died in this experiment.

The results of the preliminary and main experiments are tabulated in Table 14.1.

Table 14.1

The Results Obtained from Experiments Aimed at Detecting Binding of Epsilon Toxin to Red Cell Stroma in vitro

Exp't	Toxin mg/ml*	No.of Mice/ Group	Cell Source	Pretreatment of Cell Stroma			Toxin Control
				Nil	1% Albumin	1% Dextran	
				Number of Mice Dying per Group			
Prelim.	0.015	3	Sheep	0	-	-	3
Main i	0.020	3	Rabbit	3	3	3	3
" ii	0.020	4	Sheep	4	4	4	4
" iii	0.020	5	"	5	5	-	5

\- Not Done

* 0.2ml/mouse

In vivo Studies of the Possible Binding of Epsilon Toxin to Cell Membranes Employing an Indirect Fluorescent Antibody Technique

Although commercially prepared and fluorochrome conjugated gamma globulins prepared from hyperimmune horse sera are available for the detection and identification of clostridia in tissues by using direct fluorescent antibody techniques, this type of reagent is less satisfactory for the detection of Cl. perfringens toxins in tissues (Batty I. - pers.comm.) The indirect fluorescent antibody technique is considerably more sensitive however and was therefore

used in an attempt to determine whether there is any binding of epsilon toxin to tissues, such as capillary endothelium, *in vivo*.

Preliminary Experiments to Determine the Activity and Specificity of Fluorescein Conjugated Rabbit 'anti-horse serum' Globulin in Indirect Fluorescent Antibody Procedures

Fluorescein conjugated rabbit 'anti-horse serum' gamma globulins are available commercially, but one sample obtained from Sylvana Chemical Co. (Orange, N.J., U.S.A.) did not react efficiently in preliminary experiments. A fresh sample of rabbit 'anti-horse serum' gamma globulin was therefore prepared as described in Appendix 9 and the species specificity and activity of this reagent were confirmed before it was used in studies on the tissues of experimental animals.

The criteria employed were as follows:

i That the globulin was specific for horse tissue and would not cross react with other species eg sheep.

ii That the globulin would produce demonstrable specific fluorescence when used in an indirect fluorescent antibody test with a known antigen-antibody reaction.

Materials & Methods:

The following air-dried smears were prepared:

i Three smears of normal horse blood.

ii Three smears of normal sheep blood.

iii Nine smears of *Cl.septicum* - containing bovine tissue exudate in which the presence of *Cl.septicum* had been confirmed by cultural isolation and direct fluorescent antibody methods using commercial fluorescein-conjugated *Cl.septicum* antiserum.

All the smears were fixed in absolute methanol for 10 minutes.

The smears of bovine tissue exudate were then pretreated as follows:

i Three smears were covered with normal horse serum.

ii Three smears were covered with commercial unconjugated Cl.septicum antiserum.

iii Three smears were covered with commercial unconjugated Cl.chauvoei antiserum.

These serum-covered smears of exudate were incubated at room temperature for 30 minutes in a moist chamber, then washed in 0.05M phosphate buffered saline pH8.0 for 10 minutes.

All the blood smears from the horse and the sheep, plus the above exudate smears, were covered with the conjugated rabbit 'anti-horse serum' glogulin and incubated in the moist chamber for 30 minutes at room temperature followed by a buffered saline wash as described above. Coverslips were placed on the slides using 90% glycerine in the phosphate buffered saline as a mountant. The smears were examined for fluorescence with a Reichert microscope fitted with an HBO 200 mercury vapour lamp using bright field illumination and an E2 exciter filter.

Results:

There was no detectable fluorescence in the sheep blood smears.

The horse blood smears showed fluorescence of the interstitial material with the erythrocytes appearing as dark circles therein.

The Cl.septicum cells in the exudate smears which had been pretreated with the homologous antiserum showed intense green fluorescence. There was no detectable fluorescence in the exudate smear which was pretreated with normal horse serum. The exudate smears pretreated with the heterologous Cl.chauvoei antiserum showed organisms with low grade green fluorescence.

Comment:

Since no Cl.chauvoei had been detected in the original smears of exudate with a commercial rhodamine conjugated Cl.chauvoei antiserum it appears that, in the present instance, there may have

been some cross reaction with this heterologous antiserum which was detected by the more sensitive indirect fluorescent antibody method. This could be expected as some antigens are known to be common to both Cl.septicum and Cl.chauvoei. (Smith & Holdeman, 1968)

The Use of Indirect Fluorescent Antibody Techniques to Detect Epsilon Toxin in Tissues from Intoxicated Mice

Having ascertained that the rabbit anti-horse serum globulin was species specific and also reacted in an indirect fluorescent antibody procedure it was possible to utilise it in an attempt to detect possible binding of epsilon toxin to tissue structures in vivo.

Materials & Methods:

Pairs of mice were given intravenous epsilon toxin at doses of 10 and 250ug. Fresh frozen sections of brain, lung and kidney, cut at 8u on a Bright's cryostat, (Bright's Refrigeration Services, Huntingdon, England) were obtained from these animals as soon as they died. A normal control mouse was also sacrificed at the same time and the tissues were treated in the same way. Sections of each organ from the intoxicated animals were picked up on the same slide as a similar section from the control animal.

The sections were air-dried and incubated, without prior fixation, with either commercial Cl.perfringens type D antiserum, Cl.perfringens type C antiserum or normal horse serum for 30 minutes at room temperature, followed by a 10 minute wash in the buffered saline. Thereafter they were incubated with the conjugated rabbit 'anti-horse serum' globulin for 30 minutes, washed for 10 minutes and examined for fluorescence as described in the preliminary experiment.

Results:

There was marked green fluorescence of certain structures in all sections, notably nucleii and vascular structures, but there was no detectable difference between control and intoxicated tissues

pretreated with the different sera. No detectable fluorescence of endothelium occurred in any of the sections.

Oxygen Uptake of Tissue Slices, from Control and Intoxicated Animals in Warburg Respirometers.

This work was aimed at determining whether epsilon toxin affects the overall respiratory activity of tissues or interferes with the metabolism of glucose in either the Emden Meyerhoff or Tricarboxylic Acid Pathways.

In the present investigation mouse kidney slices were used for most of the work in preference to liver as kidney tissue is more orientated toward catabolic metabolism and thus has a higher oxygen uptake per unit weight than liver. A small series of estimations were also made using guinea pig brain, lung and kidney to study the effect of the toxin in other tissues and in a second species. Several substrates which occur at key points on the energy yielding pathways were chosen in an attempt to detect any point at which inhibition occurred.

Two separate runs were carried out using tissues from animals which had received *in vivo* toxin and one run each was made using mouse kidney and guinea pig tissues by adding the toxin *in vitro* to flasks containing tissue slices from normal animals. Since epsilon toxin is a protein and is therefore unable to cross cell membranes by diffusion it was felt that it would be unlikely that there would be marked differences in the action of the toxin *in vivo* and *in vitro* if it exerts its effect directly upon the cells of the tissues studied.

Materials & Methods:

Conventional Warburg respirometry techniques and reagents were used, as described by Umbreit *et al* (1957) for tissue slice procedures. Krebs-Ringer-Phosphate solution pH 7.4 was the buffer used in all instances.

The substrates employed were glucose, sodium pyruvate, sodium dl lactate, sodium succinate, and sodium citrate.

Control flasks containing no substrate were included, to measure endogenous respiration of the tissue. All substrates were made up in 0.5M concentrations in the buffer and the pH was checked and readjusted to pH7.4 if necessary. Pyruvate and ketoglutarate solutions were made up daily and the others weekly. All reagents were stored at 4°C.

Two flasks containing the same substrate were set up in every experiment for each of the tissues used. One of these was used for the control tissue slices and the other for the 'toxin' tissue slices. Where epsilon toxin was added *in vitro* 2 ug of Batch CWD in 0.1ml saline was placed in the 'toxin' flask and the same volume of saline added to the control flask. When the toxin was given to the animal *in vivo* the tissues slices from the intoxicated animals were placed in the 'toxin' flask without any additional toxin.

The reagent volumes placed in the respirometer flasks were as follows:

10N KOH	0.2ml	(Centre well with wick)
Krebs Ringer Phosphate	2.8ml	(2.9ml in '*in vivo*' expts)
Substrate	0.1ml	
Epsilon toxin	0.1ml	)
or		) where applicable
Saline 0.85%		)

Preparation of the tissue slices for the experiments in which the toxin was added *in vitro* was carried out as follows:

Six 6 week old male mice were starved overnight to reduce endogenous glycogen reserves then killed by neck dislocation. The kidneys were removed immediately and placed in ice cold Krebs Ringer Phosphate solution for a few minutes. This hardened the organs and assisted in the preparation of tissue slices of the optimal thickness of 0.5mm with a Stadie-Riggs hand operated tissue slicer. A pool of slices was prepared and held in the above solution until sufficient were available for the experiment. A similar procedure was employed for the preparation of slices of lung, brain and kidney from a pair

of 3 month old guinea pigs.

Where epsilon toxin was given *in vivo* 10ug was administered intravenously to each of a group of twelve 6 week old male mice which had been starved overnight. The high dose of toxin resulted in a number of animals dying in convulsions at the same time, approximately one hour after inoculation. The kidneys from six of these animals were removed and tissue slices prepared as above. At the same time kidney slices were prepared from a similar group of normal control mice.

Two of the appropriate tissue slices were added to each of the 'control' and 'toxin' flasks for the various substrates employed. All flasks were placed on their manometers and flushed with oxygen via the sidearm for three minutes at a flow of approximately 500ccs/flask/minute, using a glass manifold. The respirometers were then placed on the heating bath and allowed to equilibrate at 37°C for 10 minutes. Thereafter readings of oxygen uptake were made every 10 minutes for 60 minutes.

At the conclusion of the run the tissue slices were removed from the flasks and placed in preweighed 4cm diameter plastic petri dishes, freeze dried for 24 hours and weighed to obtain a dry-matter content to which oxygen uptake could be related.

Results:

The results of these experiments are shown in Appendix 10 and illustrated in Figure 14.1a-f. There was no significant difference between the oxygen uptake of control and intoxicated mouse kidney slices utilising any of the substrates. Although there are differences in the metabolic activity of the different types of tissues used in the *in vitro* toxin experiments these do not appear to be influenced by epsilon toxin when it is in contact with the tissues during incubation.

Figure 14.1 Effect of Cl. perfringens type A exotoxin on the respiration of Tissue Slices

Figure 14.1a:

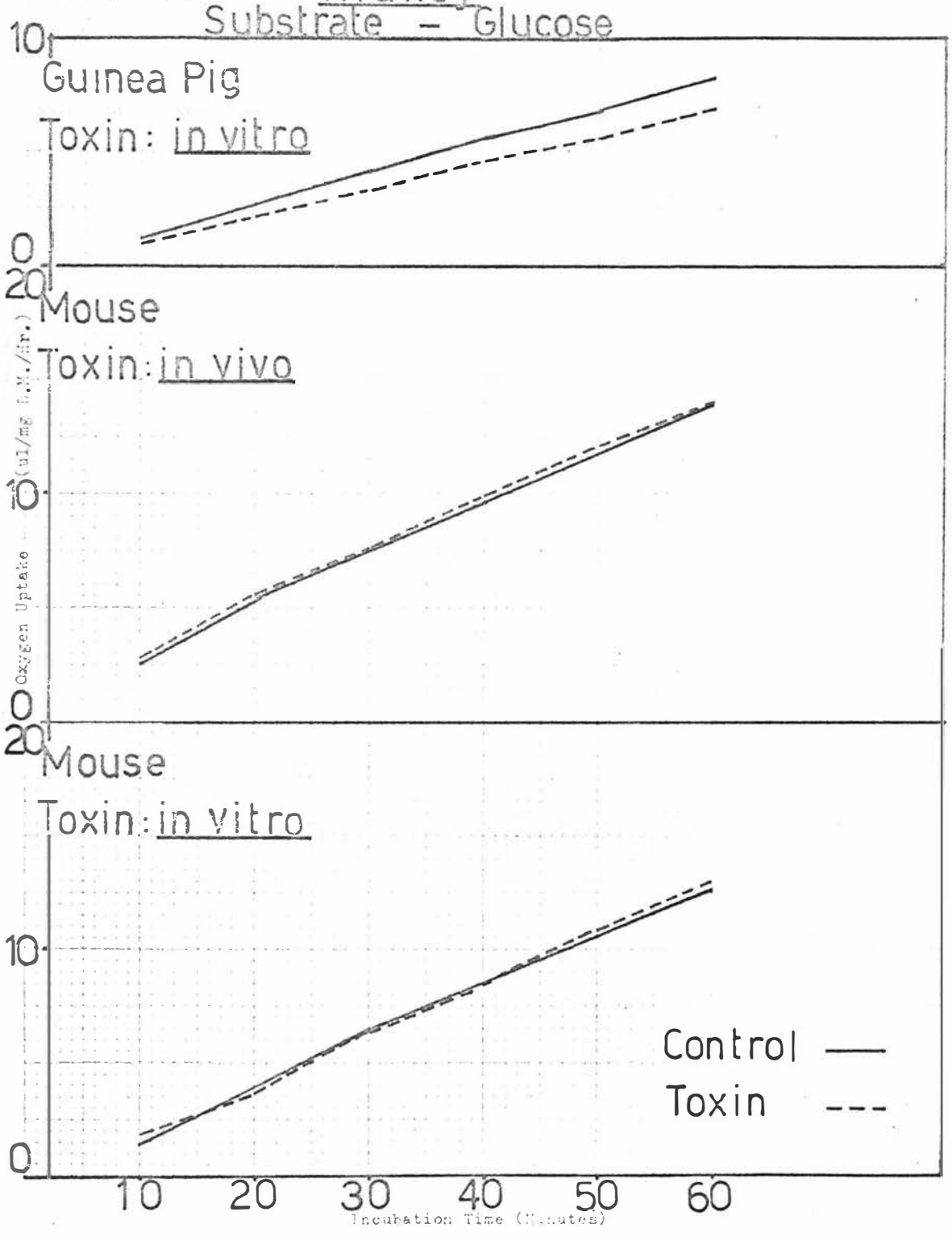


Figure 14.1(cont'd) Effect of Cl.perfringens type D Epsilon Toxin on the Respiration of Tissue Slices.

Figure 14.1b:

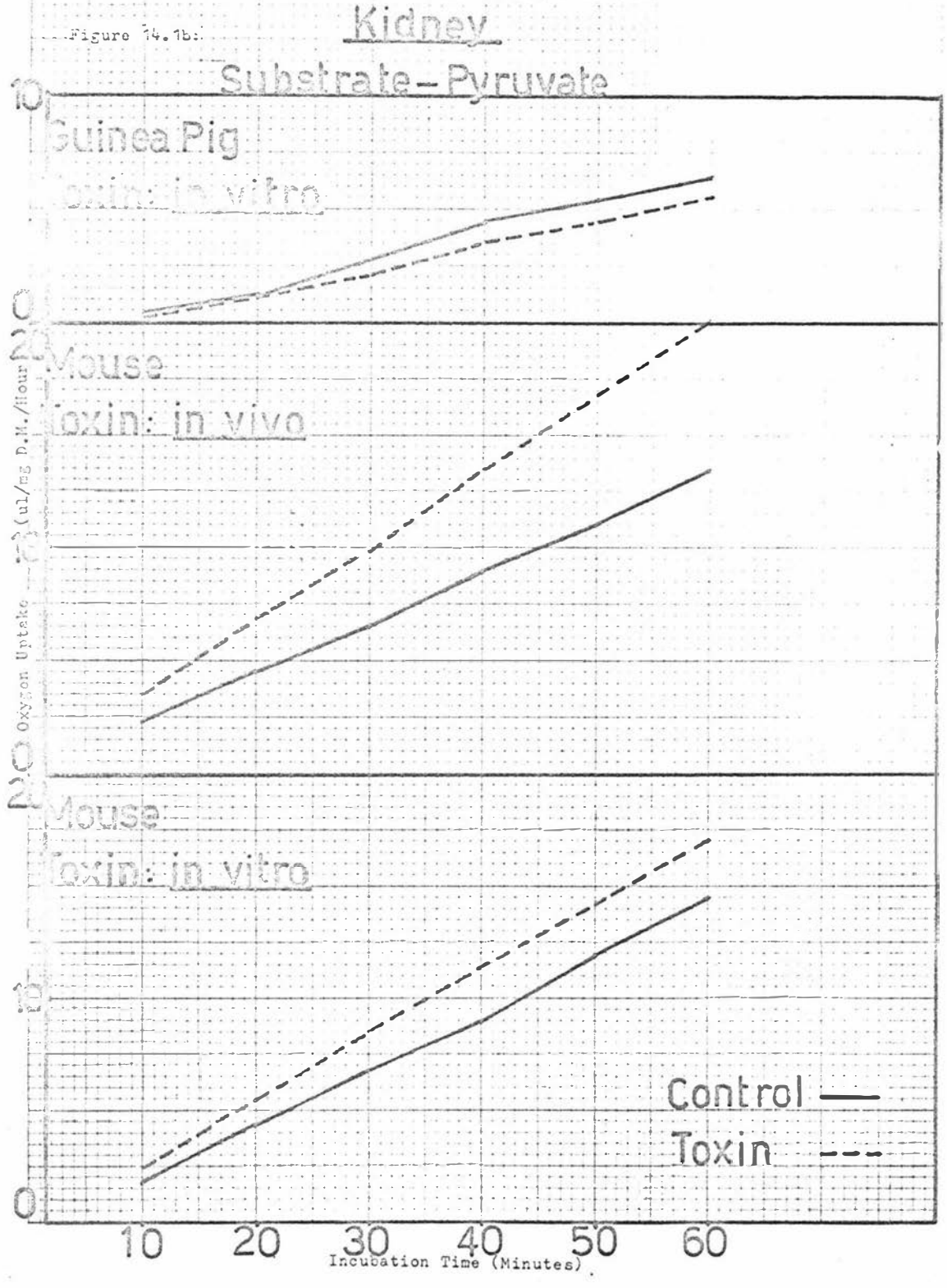


Figure 14.1(cont'd) Effect of Cl.perfringens type D Epsilon Toxin on the Respiration of Tissue Slices

Figure 14.1c:

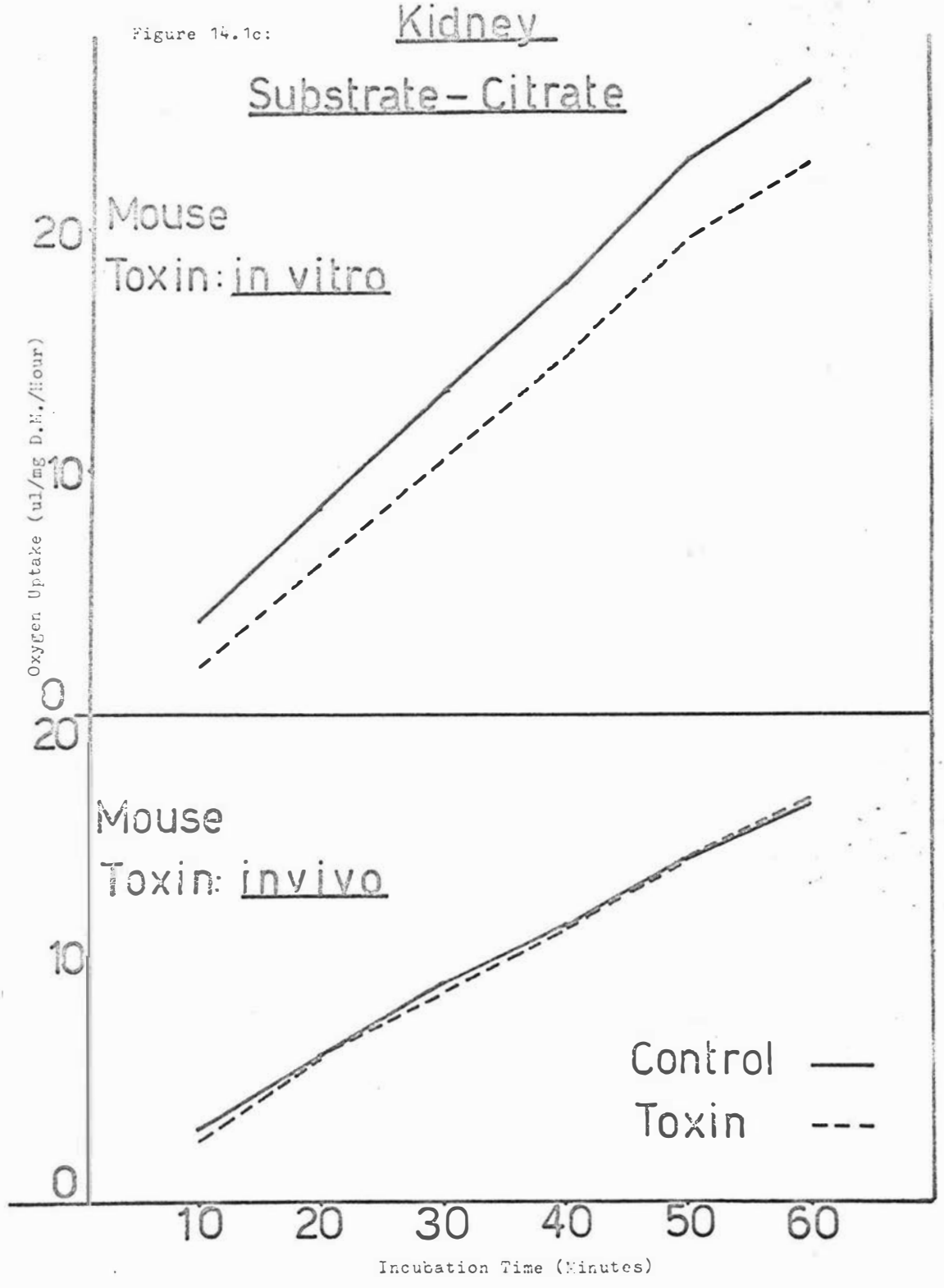
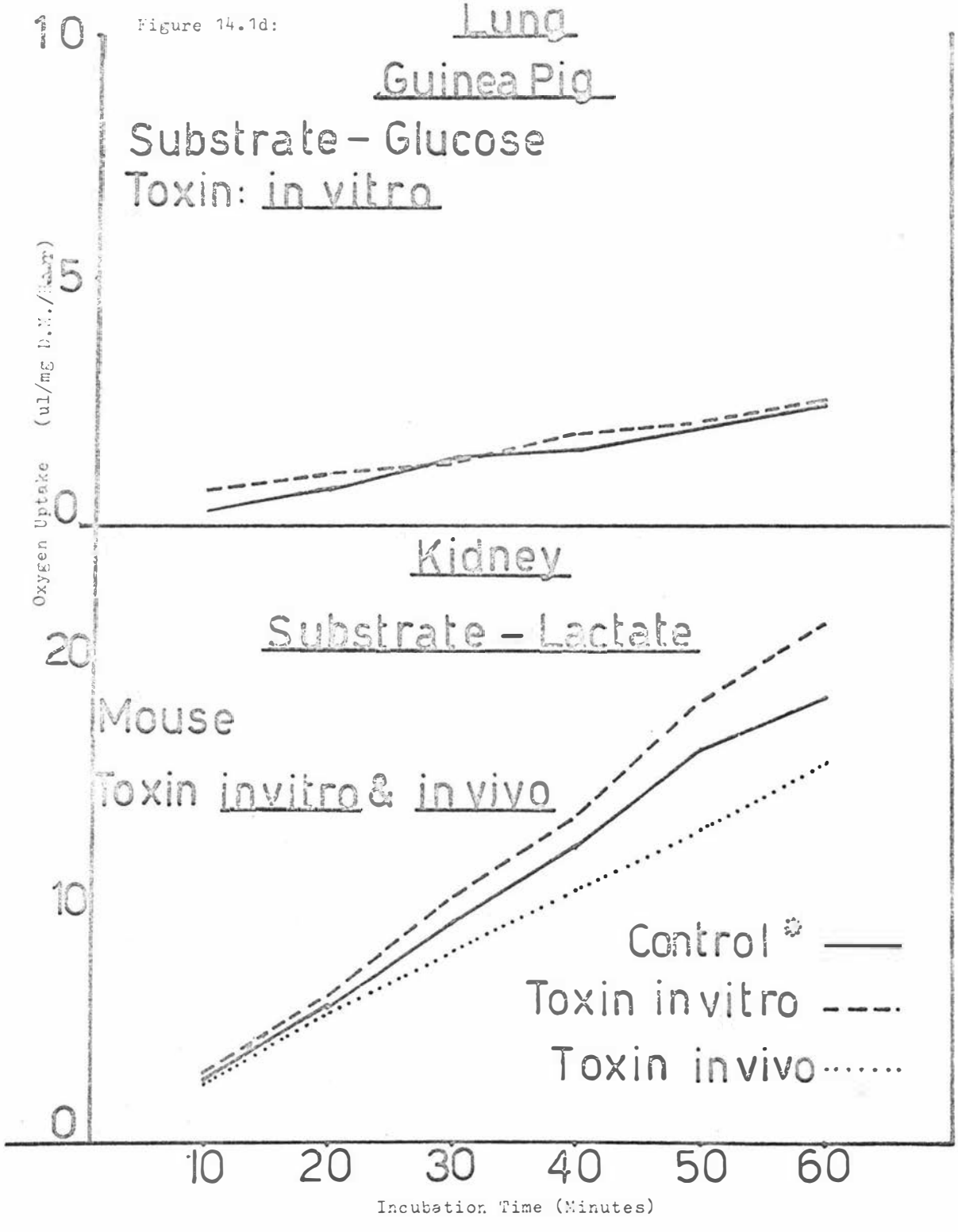


Figure 14.1(cont'd): Effect of Cl.perfringens type D Epsilon Toxin on the Respiration of Tissue Slices.



* ex in vitro exp't.

Figure 14.1(cont'd) Effect of Cl.perfringens type D Epsilon Toxin on the Respiration of Tissue Slices

Figure 14.1e: Brain

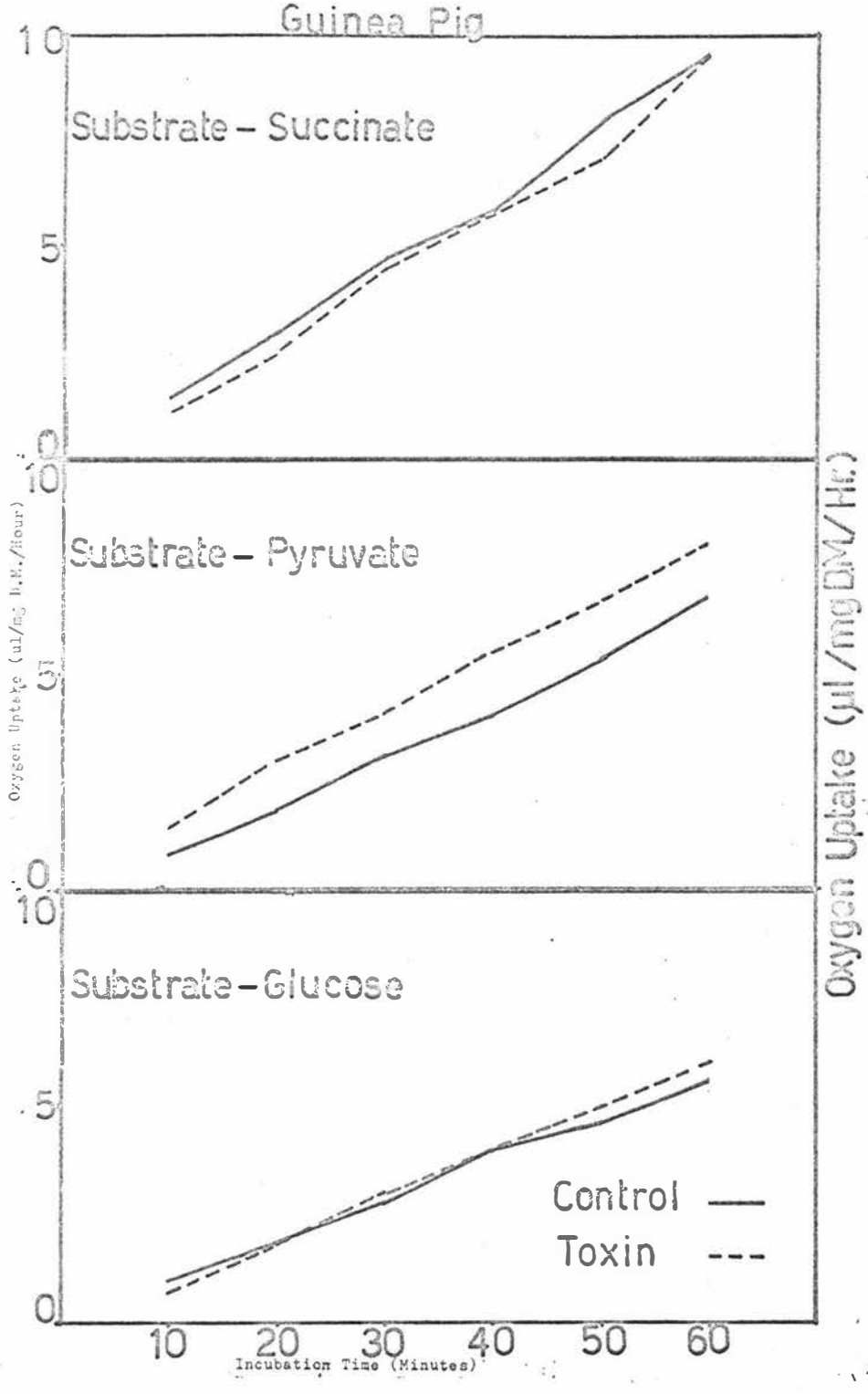
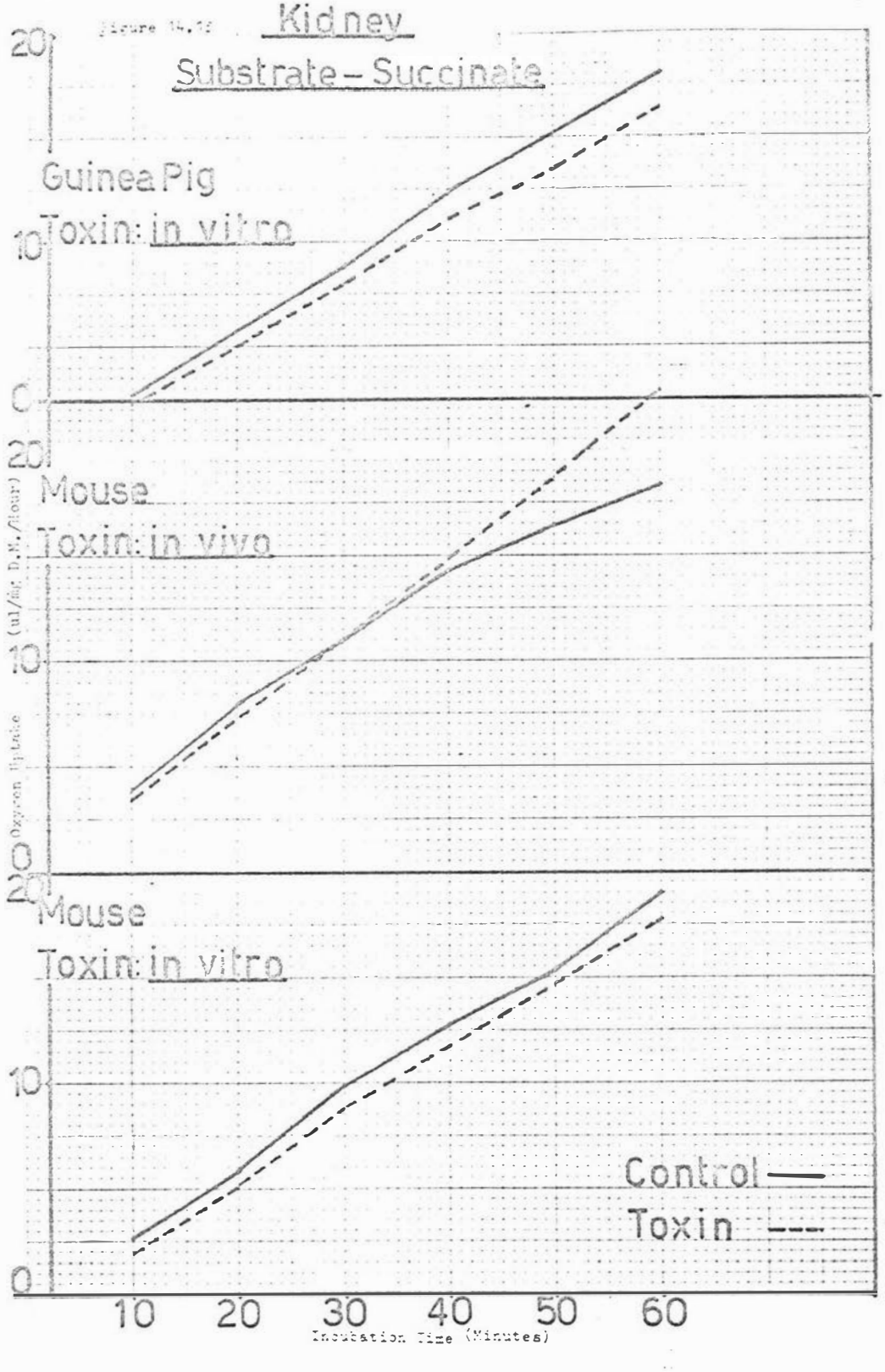


Figure 14.(cont'd.) Effect of Cl. perfringens type D Epsilon Toxin on the respiration of Tissue Slices



Discussion

It was not possible to demonstrate binding of epsilon toxin to cell membranes using the techniques employed in these experiments, nor does the toxin appear to affect the functional integrity of tissues to the extent that it is detectable by Warburg respirometry.

The ability of intoxicated tissues to utilise molecular oxygen at the same rate as control tissues implies that the oxidative electron transport chain is unaffected. Similarly, since all the substrates were dealt with equally effectively by intoxicated and control tissues, it is unlikely that the hyperglycaemia of enterotoxaemia is due to a metabolic block in either the Emden-Meyerhoff pathway or the Tricarboxylic Acid Cycle. It should be noted that the oxygen uptake figures in these experiments were similar to those given by Spector (1956) for mouse and guinea pig tissues.

Further information may have been gained from these experiments if post-incubation estimations of the actual substrates and some of the other intermediate compounds had been carried out since it is theoretically possible that the abnormal accumulation of an intermediate might have occurred. However, since a range of substrates from various parts of the normal metabolic pathways were employed and there was no evidence of an inability of intoxicated tissues to oxidise any of these substances, it is unlikely that useful information would have been obtained in this manner.

In the experiments using red cell stroma to detect toxin binding, the preliminary result was anomalous. The reason for this is not clear. It is unlikely that non-specific binding, residual antibody or dilution of the toxin by residual wash solution affected the results since they would have also affected the results in the main experiment. The most likely explanation is that a portion of the freshly made up toxin 'solution' was in fact still in an emulsified or finely divided particulate form and was removed with the stroma during centrifugal separation.

At the slightly higher level of toxin employed in the main experiments, the number of binding sites required per erythrocyte to

completely remove the toxin from the solution would be of the order of 5,000, in contrast to the 800,000 binding sites which are known to be available for the glucose molecule (Stein, 1968). On this basis, any significant binding of the toxin to red cell stroma should have been detected by the methods employed and it therefore appears that epsilon toxin is not bound to red cell stroma in vitro.

The increased sensitivity of the indirect fluorescent antibody technique did not provide any evidence that epsilon toxin is bound to vascular endothelium. With the massive dose of 250ug of epsilon toxin which was used in some animals there would be approximately 100 nanograms of toxin per cubic millimetre of blood. Since the indirect fluorescent antibody technique is capable of detecting quantities of antigen of the order of .014 - .028 nanograms/1mm3 tissue (Pressman, 1958; quoted by Kawamura, 1969) it appears that even if only a very small proportion of the total circulating toxin was bound to the endothelium it would be detectable by this technique.

The extent of the non-specific fluorochroming which occurred in the tissues of these animals confirms the impression that this procedure would not have been suitable for detecting any loss of protein from the bloodstream into the tissues of intoxicated animals.

Conclusions:

1. Detectable binding of epsilon toxin to red cell stroma does not occur in vitro.

2. Batches of commercial rabbit 'anti-horse serum' globulin with low activity can be encountered and all such materials should be tested for specificity and activity before use.

3. The indirect fluorescent antibody test using commercial unconjugated diagnostic clostridial antisera and a freshly prepared rabbit 'anti-horse serum' globulin is at least as sensitive, in detecting organisms in tissue exudates, as the direct fluorescent antibody test using commercial fluorescein-conjugated diagnostic clostridial antisera.

4. No binding of epsilon toxin to vascular endothelium or other structures could be detected using the indirect fluorescent antibody technique. In this respect the method is no more satisfactory than the less sensitive direct fluorescent antibody method used by other workers.

5. Kidney slices from intoxicated mice could utilise molecular oxygen, for the metabolism of glucose, pyruvate, succinate, citrate and lactate, as effectively as similar slices from normal control animals. Addition of epsilon toxin to the incubating medium did not affect the oxygen uptake of normal tissues utilising glucose, pyruvate, succinate or lactate.

6. In view of these findings it appears that the hyperglycaemia of enterotoxaemia is not due to an interference with the functioning of the Emden-Meyerhoff pathway or Tricarboxylic Acid Cycle by epsilon toxin.

Chapter 15: THE NORMAL VALUES OF A NUMBER OF BLOOD CONSTITUENTS OF LAMBS AND THE TECHNIQUES EMPLOYED FOR THEIR ESTIMATION

Some of the earlier biochemical and haematological investigations into the action of epsilon toxin, notably those of Gordon et al (1940) and Kellaway et al (1940), have shown that the major changes which occur during intoxication are a progressive haemoconcentation ('anhydraemia'), a considerable increase in the levels of glucose and phosphate in the blood and severe glycosuria. In the course of their investigations these workers examined a number of other blood constituents including certain ions, amino- and non-protein nitrogen; alkali reserve; oxygen content and haematological data.

In the present study it was not considered necessary to examine all of the parameters investigated by these workers as, in an acute intoxication such as enterotoxaemia, factors such as sedimentation rate, erythrocyte fragility and clotting time were unlikely to be affected. On the other hand there are some notable gaps in existing information on the action of the toxin that it was essential to fill. For example there is no published information on changes in the levels of plasma proteins during the course of intoxication and these could be expected to reflect the marked haemoconcentration. There is also a complete lack of information on whether blood levels of the metabolic products of glucose metabolism, e.g. pyruvate and lactate, are altered in association with the hyperglycaemia which occurs during the course of intoxication.

Modern techniques have extended both the range and accuracy of the information which can be obtained about electolyte balance, acid-base balance and respiratory exchange. In view of the pulmonary oedema described by other workers and the effusions which occur in the natural disease it was necessary to investigate the ionic status and acid-base balance of intoxicated animals more fully. Trifonov and Todorov(1965) have extended the biochemical studies on intoxicated animals to include estimations of the levels of some of the serum enzymes, primarily to ascertain whether the toxin produces hepatic damage. The range of enzymes examined by these workers was limited and it was extended, in the present study, to include a number of the enzymes which may be estimated in veterinary clinical

pathology.

Because the lambs used in the experiments in which epsilon toxin was administered parenterally were only 2-3 weeks of age, they were effectively simple stomached animals. Under these conditions the level of blood glucose and the pattern of excretion of some of the ionic constituents are different from those of the adult sheep. They are also in a state of flux as the change from a milk to a grass diet occurs. Thus it was essential to obtain an accurate picture of the normal range of the relevant constituents in a number of normal healthy lambs of this age. A great deal of this information could be obtained from the pre-inoculation samples from the animals which were to receive toxin and also from the paired uninoculated control animals. However, since it was envisaged that there would be a considerable number of pre- and post-inoculation samples to be examined for each constituent it had been decided to automate as many of the analytical procedures as possible. Under these circumstances it was possible to include a number of additional blood samples without the manipulative procedure becoming unmanageable. By including blood samples from a further group of lambs similar to those used in the experiments with epsilon toxin, it was possible to obtain additional information about the normal values for various biochemical and haematological parameters of young lambs in New Zealand.

At this stage it is convenient to describe and discuss the various methods which were employed to obtain this data from both the normal lambs and those which received parenterally administered epsilon toxin. The data obtained from normal lambs will also be recorded and discussed here while the changes which occur in the levels of the individual blood constituents during epsilon toxin intoxication will be investigated in subsequent chapters.

The Estimation of Biochemical and Haematological Parameters in Lambs.

Materials & Methods:

Animals:

Preinoculation blood samples were taken from the lambs which were

to receive parenterally administered toxin and also from the paired uninoculated control lambs described in Chapter 6. In addition, blood samples were taken from twenty eight 2-3 week old lambs from a crossbred flock on another farm, with a similar soil type and climate, situated about 5 miles from the Massey property.

Blood Samples:

'Vacutainers' were used for the collection of all blood samples as they are convenient to use and are particularly suitable for obtaining arterial blood samples from animals by percutaneous puncture of the femoral artery. They are available in a number of different sizes and with a number of different preservatives and anticoagulants added. Those containing heparin have been shown to be very suitable for the collection of blood samples intended for blood gas analyses. (Gambino, 1967). Because the procedures recommended for the enzymatic estimation of the levels of pyruvate or lactate require that the blood be deproteinised, with cold perchloric acid, as soon as the sample is collected, a number of plain 10ml 'Vacutainers' were opened and 3.5mls of a 6% solution of perchloric acid in distilled water were added to each tube. The stoppers were then reinserted and the tube re-evacuated with a 20ml syringe and a 20swg needle. These 'Vacutainers' were held in iced water for at least 30 minutes before use. 'Vacutainers' are manufactured by Becton Dickinson & Co, Rutherford, N.J., U.S.A.

Venous blood samples were taken from the jugular vein into each of the following 'Vacutainers'; 1 x 5ml heparinised - for blood gas analyses, haematological examinations and protein estimation; 1 x 7ml plain for serum ionic and enzymatic constituent estimations and epsilon antitoxin titration; 1 x 7ml perchlorate-containing 'Vacutainer' for glucose, pyruvate, lactate and phosphate estimation. A 3.5ml sample of blood was added to the latter container by adding blood to the level of a mark on the 'Vacutainer' holder with the tube and holder held in an upright position. Preliminary experiments had shown that the appropriate volume of venous blood from normal animals could be consistently added with an accuracy of better than 0.2 ml using this technique.

A similar series of arterial blood samples was taken from the lambs

which were to receive parenteral toxin and their paired controls only; i.e. no arterial samples were taken from the 28 lambs from the second property. The arterial samples were taken from the femoral artery using the same equipment as for the venous sampling, but blood was taken into heparinised and perchlorate treated 'Vacutainers' only. Percutaneous puncture of the femoral artery in the femoral triangle was carried out with the lamb held in a sitting position. Under these conditions some difficulty was encountered in accurately adding the required amount of blood to the perchlorate treated containers and this will be referred to again in the discussion section of this chapter. All blood samples were taken without occlusion of the blood vessels but pressure was subsequently applied to the femoral artery for several minutes after removal of the needle to limit haematoma formation. All blood samples were held in iced water in a vacuum flask until further preparative procedures or the actual estimations were undertaken. Blood gas analyses were carried out within 3 hours of sample collection and haematological examinations within 4 hours of collection. Separation of plasma and serum samples with subsequent storage at -20°C in a domestic deepfreezer was also completed within four hours of collection.

The perchlorate treated samples were shaken thoroughly at the time of collection and after centrifugal separation (1800G for 15 minutes) the supernatants were removed, neutralised in the cold with 5 N.KOH, with 0.05% methyl orange as internal indicator, and then stored at -20°C prior to analysis. These samples were in storage within 4 hours of collection.

Analytical Procedures:

Blood Gas Analyses:

Whole blood pH and pO_2 levels were determined on the arterial and venous blood samples using the appropriate electrodes of a 'Radiometer' Astrup-micro Blood Gas Analyser, Type AMEI (Radiometer - Copenhagen, Denmark). The pCO_2 and standard- and actual- bicarbonate levels of the blood samples were calculated from the pH of aliquots of the blood which had been equilibrated with O_2/CO_2 gas mixtures containing different partial pressures of CO_2 using the above instrument. The Sigaard-Andersen nomograms used for these calculations were those provided with

the instrument and calibration and operation were also according to the manufacturers instruction manual.

Estimation of Haematological Values:

The techniques employed for this purpose were standard procedures which are described in many laboratory manuals, e.g. Dacie & Lewis "Practical Haematology" and only the type of method employed will be described here.

Haemoglobin:

The cyanmethaemoglobin method was used and the results obtained with an EEL direct reading Haemoglobinometer (Evans Electro-selenium Ltd., Halstead, England) calibrated against cyanmethaemoglobin standard (Diagnostic Reagents Ltd., Thame, England).

Haematocrit (Packed Cell Volume):

Heparinised blood was placed in plain microhaematocrit capillary tubes and centrifuged for 5 minutes in an International Microcapillary centrifuge model MB. The results were read on an International Microcapillary reader (International Equipment Co., Needham Heights, Mass., U.S.A.)

Mean Cell Haemoglobin Concentration:

This was calculated from the haemoglobin and haematocrit values according to the usual formula viz.

$$\frac{\text{Haemoglobin (Gm\%)} \times 100}{\text{Haematocrit (\%)}} = \text{Mean Cell Haemoglobin Concentration}$$

Total White Cell Count:

Cell counts were carried out in Spencer bright line haemocytometers with an improved Neubauer ruling with 1% acetic acid solution as diluent. Duplicate counts were made on the two separate chambers of the haemocytometer.

Plasma Protein Fractionation:

Electrophoretic separation with Millipore 'Phoroslides' equipment (Millipore Corp., Bedford, Mass., U.S.A.) used according to the manufac-

turers instructions. Densitometric scanning of the uncleared, stained strips was carried out on a Photovolt densitometer model 520A with a 540 mu green filter in the scanning head. The densitometer was coupled to a 'Varicord' model 42B chart recorder and electronic integrator attachments (Photovolt Corporation, New York, U.S.A.).

Automated Biochemical Analyses:

Full details of the procedures used including flow charts, calibration data and a discussion of the technical aspects of the development and use of these methods are given in Appendix II but it is convenient to outline here the type of method that was used for each blood constituent. It is also important to note that all blood samples from the same animal were kept together in the analytical runs for each constituent.

Total Plasma Protein: Biuret method

Sodium and Potassium: Flame photometer

Chloride: Thiocyanate displacement from mercuric thiocyanate; measured as ferric thiocyanate

Phosphate: Production of phosphomolybdic acid with acid molybdate followed by stannous chloride/hydrazine sulphate reduction

Glucose: Glucose oxidase-Peroxidase-dianisidine method

Pyruvate: Lactic dehydrogenase/DPNH oxidation

Lactate: " " /DPN reduction with hydrazine sulphate trapping of the pyruvate formed.

Lactic dehydrogenase: DPNH oxidation with pyruvate as substrate

Isocitric dehydrogenase: TPN reduction with isocitrate as substrate

Glutamic oxaloacetic and Glutamic pyruvic Transaminases:

DPNH oxidation in coupled reactions with malic and lactic dehydrogenase respectively

Acid and Alkaline Phosphatase:

Detection of phenol liberated from phenyl phosphate with ferricyanide/amino-antipyrine at the appropriate pH.

Results:

The results obtained by the analysis of blood from normal 2-3 week old lambs when using the above methods were generally similar to those published in the literature for sheep, providing due allowance is made for the age of the animals. The mean values obtained for the various blood constituents in the present series of arterial and venous blood samples from normal lambs are tabulated in Table 15.1 where they are compared with published values. Details of the number of analyses, and standard deviations plus the sensitivity of the methodsused are also given in the table. The data are given in Appendix 12.

Discussion

It is appropriate to comment first upon the choice of some of the methods used to obtain the foregoing results, as some of the procedures still in current use have shortcomings which may influence the results obtained. For example, for many years it has been customary to take blood samples for blood gas analyses and store them under a layer of liquid paraffin to prevent gaseous exchange. It is only relatively recently that it has been found that carbondioxide will dissolve in the oil layer and thus lead to errors in the estimation of this component in the blood sample. In addition it was considered that only arterial blood samples were reliable for blood gas analysis, but more recently it has been found that capillary blood samples are equally satisfactory for the study of the blood gases in man and routine blood gas analyses are now frequently carried out on samples of capillary blood collected in heparinised capillary tubes without any contact with oil.

In domestic animals, adequate samples of true capillary blood are not always easy to obtain and, where samples have to be held for some time before analyses can be carried out, samples in capillary tubes are not particularly satisfactory as they are difficult to keep cool and the blood tends to clot. Gambino (1967) has shown that blood samples collected in vacuum tubes similar to 'Vacutainers' are very suitable for blood gas analyses and that samples collected in this way are stable for some hours if kept cool.

Table 15.1: The Haematological and Biochemical Parameters of Normal Two to Three Week Old Lambs

Constituent	Mean Values for Normal Sheep			Standard Deviation		No. of Analyses		Method Sensitivity
	Present Experiments		Published					
	VENOUS(V)	ARTERIAL	'NORMAL'+	(V)	(A)	(V)	(A)	
Haemoglobin Gm%	10.7	10.5	10.3,10.9 (10-11.8)	1.0	0.8	63	35	0.1
Haematocrit (%)	35.3	34.2	31.7 (29.9-33.6)	2.9	2.3	63	35	0.5
M.C.H.C.(%)	30.3	30.7	34.5	1.1	1.3	63	35	0.1
pH	7.36	7.40	7.44(arter,) (7.32-7.54)	0.03	0.059	56	28	0.01
pCO_2 (mmHg)	39.2	34.0	38.0	4.8	4.7	55	27	1
HCO_3-Actual	23.0	21.5	-	2.0	2.3	55	27	0.5
HCO_3-Standard	22.4	22.2	-	4.1	1.9	55	27	0.5
pO_2 (mmHg)	70	165	-	14	22	27	24	10
Total white cells (/mm³)	5,400	-	4,000-10,000	1700	-	52	-	50
Total Protein Gm%	6.7	-	5.7	0.64	-	63	-	0.1
Albumin %	36	-	54	5	-	21	-	
Globulin %	64		40	5		21	-	
Sodium(meq/l)	154.9	-	146-161	4.4	-	61	-	1
Potassium (meq/l)	5.2	-	4.8	0.4	-	61	-	0.1
Chloride (meq/l)	112	-	103,116 (98-109)	5	-	34	-	1
Phosphate (mg%)	4.3*	3.3*	6.9 (4-9)	1.7	1.5	59	35	0.1
Glucose(mg%)	80.2*	89.8*	70-120(lamb) 30-57(adult)	19.0	20.5	59	35	1
Lactate(mg%)	60*	71*	(5-20) (9-12)	3.0	2.9	55	34	1
Lactic Dehydrogenase(iu)	810	-	2,000	103	-	63	-	10
Isocitric Dehydrogenase(iu)	15.6	-	-	5.7	-	63	-	
Glutamic oxaloacetic Transaminase(iu)	43.5	-	31	8.7	-	63	-	1

continued.....

Table 15.1: continued.....

Constituent	Mean Values for Normal Sheep			Standard Deviation		No. of Analyses		Method Sensit-ivity
	Present Experiments		Published					
	VENOUS(V)	ARTERIAL	'NORMAL'+	(V)	(A)	(V)	(A)	
Glutamic pyruvic Transaminase (iu)	8.5	-	8-24	2.2	-	63	-	0.5
Alkaline Phosphatase (iu)	761	-	14-427	230	-	63	5	5
Acid Phosphatase (iu)	25	-		4.5	-	20	-	0.5

+ Adult sheep unless otherwise stated - Spector (1956)

* Estimated on perchlorate treated samples

Turning now to the methods which are currently employed for the estimation of the blood gas parameters themselves. The use of very sensitive microelectrodes, which are maintained at an accurately controlled temperature, for the estimation of pH and pO_2 measurements on blood are well documented in the literature and do not require further discussion here. On the other hand the measurement of pCO_2 and bicarbonate concentrations by equilibration of the blood sample with atmosphere containing two different partial pressures of carbon dioxide as in the Astrup technique does require comment. This is a well established procedure in human medicine and the rationale, methods of calibration and accuracy of the nomograms used for the calculation of the various parameters have been closely studied by workers in the fields of human physiology and human clinical pathology. Until recently, the procedure has been applied to domestic animals without any evidence that the nomograms developed for use in man are also valid for use in other animals, but it now appears from the work of Phillips (1970) that these techniques and nomograms are in fact suitable for use in sheep at least.

The techniques employed for the estimation of haemoglobin by the cyanmethaemoglobin method and packed cell volume by the use of a microhaematocrit centrifuge and reader have replaced most of the earlier methods and are now regarded as preferred procedures for these purposes. In the same way, the use of flame photometry for the estimation of sodium and potassium in blood has superseded the chemical methods previously employed for this purpose. The Technicon 'Autoanalyser' with flame photometer and similar instruments are widely used in medical laboratories for the automated estimation of these constituents. The simultaneous estimation of chloride with these ions, usually by the thiocyanate displacement method adds considerably to the usefulness of the automated procedure.

The estimation of total plasma protein by the Biuret reaction is widely used and the method has been adapted for automated analyses. More recently there has been a tendency to use refractometry to estimate plasma protein as it provides a quick result but it has not entirely superseded the Biuret method. Although the fractionation of plasma proteins by chemical means is still widely used to provide a measure of the albumin: globulin ratio, this method does not provide as much information as the

electrophoretic separation of protein constituents. Quantitation of the results obtained by the latter method is more difficult, but densitometric examination of the strip with associated integrated counting techniques provides results which are at least as reproducible as those obtained by chemical fractionation. Unfortunately the results obtained by chemical fractionation are not directly comparable with those obtained by electophoretic separation.

A wide variety of procedures have been employed for estimating the various enzymatic components of blood and in most instances more than one method is still currently employed for determining the activity of individual enzymes. In general the existing methods rely upon the enzyme converting its natural substrate or a similar artificial substrate through one or more steps to an end-product. The amount of product produced in a given time may be estimated by colorimetric or other means. Since accurate timing is necessary in order to obtain reproducible results, automated methods have distinct advantages for assessing enzymatic activity. It is known that a number of the enzymatic reactions require the presence of either the reduced or oxidised forms of one or the other of the phosphopyridine nucleotides (DPN or TPN) as hydrogen ion donors or acceptors. These nucleotides are quantitatively oxidised or reduced as the enzymatic reaction proceeds. The reduced forms of the nucleotides (DPNH and TPNH) have an absorption peak in the ultraviolet region at a wavelength of 340mμ and also fluoresce strongly when irradiated with light of this wavelength. This is not the case with the oxidised form of the nucleotide and thus the enzymatic reaction can be quantitated indirectly by spectrophotometric or fluorometric estimation of the amount of nucleotide which is oxidised or reduced in a given time. Where a number of different enzymes are being examined this method has certain advantages as the same compound is being assayed and, apart from the substrates, only comparatively minor alterations are needed to estimate a number of different enzymes by the same basic procedure. This makes the method very suitable for use in automated analyses and it was therefore used wherever possible in the present investigation. Automated methods of this type were developed for the estimation of isocitric dehydrogenase in serum. This new automated procedure and the necessary

alterations to published automated procedures to make them suitable for use in these experiments are described and discussed in Appendix 11

One complication which has arisen as a result of the multiplicity of methods available for the estimation of any given enzyme has been that a number of arbitrary systems of units have been developed for quantitating the enzymes. Many of these are still widely used but there is a gradual tendency to change to the international system of units which are based on the amount of substrate or nucleotide utilised in a given time. Nowadays, for convenience, the calibration curves employed in obtaining values for enzyme activity, regardless of the units employed, are obtained by using commercial natural or artificial 'serum' standards which contain normal or elevated levels of the various enzymatic constituents and this was the method employed in the present instance.

Before dealing with the techniques used for estimating the remaining blood constituents studied in the present investigation, viz. glucose, phosphate, lactate and pyruvate; some consideration must be given to the method of collection of the samples which required deproteinisation prior to analyses. Deproteinisation with perchloric acid is now widely used where enzymes are to be employed in the analytical procedure. The concentration of the perchloric acid and the ratio of the volume of sample to the volume of acid however varies considerably depending upon the published source of the method. For example, when discussing deproteinisation, Henry (1966) recommends 1 volume of blood to 9 volumes of 7% perchloric acid, but in his method for pyruvate he uses equal volumes of blood and 7% perchloric acid. It was therefore considered to be justifiable to use an equal volume of blood and 7% perchloric acid for all the procedures which required deproteinisation in the present investigation. It was realised that the sample:acid ratio was rather low by the standards of Bergmeyer (1963), but to enable constituents which were likely to be present in low concentrations to be detected it was desirable to take as much blood as possible consistent with adequate deproteinisation.

The use of a perchloric acid solution in 'Vacutainers' does not appear to have been recorded by other workers but as it had been proved to be a feasible method for obtaining blood samples from normal animals

it was felt that it would be very useful in the present instance as the different types of blood samples required at any given time viz. heparinised, plain and perchlorate-treated, could all be obtained with a single insertion of the needle into the appropriate blood vessel. In the case of arterial puncture this would greatly reduce the risk of haematoma formation.

Apart from the blood gas parameters the arteriovenous (A/V) differences in other constituents are generally small in normal animals but since both arterial and venous samples were being taken for gas analyses it was decided to compare arterial and venous values for some of the other constituents, as a measure of the accuracy of the manipulative procedures. While the agreement between arterial and venous values for the haematological parameters has already been seen to be good this did not prove to be the case with the components which were estimated on the deproteinised samples. With these more labile components, some of the differences can be physiological, and paired arterial and venous samples are not true duplicates in the analytical sense. The arteriovenous difference in blood glucose level in adult ruminants for example is usually of the order of 5mg%. However in simple stomached animals such as man it is generally of the order of 10mg% and may be considerably higher after a meal. As mentioned in Chapter 6 no attempt was made to deprive the present lambs of food prior to sampling. A similar situation to that occurring with glucose could also be expected to occur with the products of glucose metabolism e.g. lactate and pyruvate.

However, the main cause for concern was that the A/V differences were not always in the correct direction and in some instances were much greater than could be expected to be due to physiological differences. This suggested that there may have been manipulative errors in the deproteinisation procedure. Any 'single extraction' deproteinisation procedure can introduce errors into the final result, particularly if the compound to be estimated is at all difficult to extract from the sample (Bergmeyer, 1963.p 267). This may have accounted for some of the inconsistencies in the results obtained from arterial and venous samples taken from the same animal at the same time. Deproteinisation appeared to be complete although according to Henry (1966), perchloric acid does not remove certain

globulins and other substances which may have contributed to the background fluorescence. It was also found, after the work was completed, that Henry (p 171) states that perchlorate filtrates are unsuitable for glucose estimations. However, it appears likely that he was referring to chemical rather than enzymatic methods of analysis. There may also have been incomplete extraction of some of the plasma constituents with the large samples of blood relative to the amount of deproteinising agent. The difficulty which was encountered in accurately adding the required amount of arterial blood to the 'Vacutainers' may also have increased the error of the procedure. These factors would all be aggravated in the post-inoculation perchlorate treated samples described in Chapters 18 and 19 where struggling, circulatory failure and haemoconcentration all added to the difficulties in obtaining blood.

While the arteriovenous differences in these animals provide some measure of the errors present in the methods used to estimate the constituents in question, they have no direct bearing upon the changes induced by epsilon toxin administration since it will be seen, in Chapters 16-19, that the comparisons of pre- and post-inoculation levels of the various constituents, with the possible exception of the blood gases, were always made with either venous or arterial samples. No attempt was made to interpret arteriovenous differences. It will also be seen in Chapter 19 that the changes in glucose and lactate levels which occurred following the administration of epsilon toxin were so large that they completely overshadowed any inaccuracies introduced by the manipulative procedures.

One further point must be made regarding the handling of the deproteinised samples prior to actual analysis and that concerns the storage of the samples and the estimation of pyruvate. When using automated techniques it is preferable, if at all possible, to analyse all samples in a continuous run and in the present instance this meant that samples were stored at -20^{o}C for up to four weeks before the analyses were carried out. Although Henry (1966) states that pyruvate is stable for at least one month at 4 ^{o}C in perchloric acid filtrates of blood, the automated analysis of the stored samples in the present instance failed to yield reliable results for this metabolite. It appeared however, that storage did not affect the values obtained for glucose, phosphate or lactate,

To conclude the discussion on the biochemical methods used it is necessary to mention those employed to estimate the plasma constituents in the perchlorate treated samples. Glucose was estimated by the glucose oxidase-peroxidase-o dianisidine method. This is a procedure which estimates 'true' glucose rather than the assorted reducing substances detected by the older chemical methods. Techniques based on glucose oxidase have been replaced to some extent by o-toluidine methods but the former methods, being more specific, are still widely used and have been automated.

The Gomori modification of the Fiske and Subarrow method, (Gomori, 1942 quoted by Varley, 1962) is widely used for estimating blood inorganic phosphate and the available automated procedures employ a basically similar technique.

As stated earlier in this discussion, the estimation of pyruvate in the present series of samples was unsatisfactory. Considerable non-specific fluorescence occurred in some samples making fluorometric detection of the changes in the levels of DPNH impossible. The level of non-specific fluorescence was insufficient to affect the estimation of lactate (vide infra) but limited the usefulness of fluorometric detection where maximum sensitivity of the instrument was being used to detect the low levels of change in fluorescence produced in the estimation of pyruvate.

The estimation of lactate was also carried out by fluorometric detection of changes in the levels of DPNH when lactate is converted to pyruvate by lactic dehydrogenase and the pyruvate produced is trapped by hydrazine sulphate. The advantage of this method is that it is specific for the L_+ isomer of lactic acid which is almost entirely of metabolic origin from within the animal body.

Having discussed the methods employed for the estimation of the

biochemical and haematological parameters in the present series of animals, it is necessary to consider briefly the values obtained from normal young lambs before passing on to an examination of the effect of epsilon toxin on these values. Some comparisons are possible between the values obtained in the present study and those which are given in the literature for adult sheep, lambs and other species.

It appears that in lambs, in contrast to the young of some other species, the haemoglobin content of the erythrocytes may be somewhat lower than in the adult animal. As could be expected the haematological parameters did not differ significantly in paired arterial and venous blood samples.

All the blood gas parameters for arterial and venous samples fall within very narrow ranges and the differences between them are what could be expected to occur on the basis of the physiological transfers which occur as blood passes through the capillary beds in the tissues. There is a loss of oxygen from the blood and an increase in its CO_2 content. This results in a fall in p^H and bicarbonate. Although figures for the arteriovenous differences in these parameters are not readily available for normal unanaesthetised sheep, the differences in p^H and pCO_2 are of a similar order to those in man. The pO_2 on the other hand is higher in both arterial and venous samples than the values which are usually obtained in man.

The level of total protein in the present series of lambs was higher than the value given for adult sheep and this too is a feature of the young of other species. The albumin:globulin ratio was also different to the published figures but this may reflect the fact that the figures in the present study were obtained by integrated densitometry following electrophoretic separation instead of chemical separation. The overall pattern was generally consistent in all these animals and differed markedly from the plasma protein patterns of normal older lambs - see Chapter 17. It therefore appears that this may be a reflection of the immaturity of the present animals. Examples of the difference between the densitometric tracings of the electrophoretically separated plasma proteins of the present animals and those of the older lambs are shown in

Figure 15.1.

The concentrations of sodium, potassium and chloride were fairly constant and similar to those found in normal adult sheep. This point will be referred to again in Chapter 21 when the concentrations of these ions which are excreted in the urine are being discussed. Levels of phosphate on the other hand fluctuated rather widely but this probably reflects the limitations of the perchlorate deproteinisation and sampling procedure discussed earlier in this chapter as the A/V differences are also rather wide.

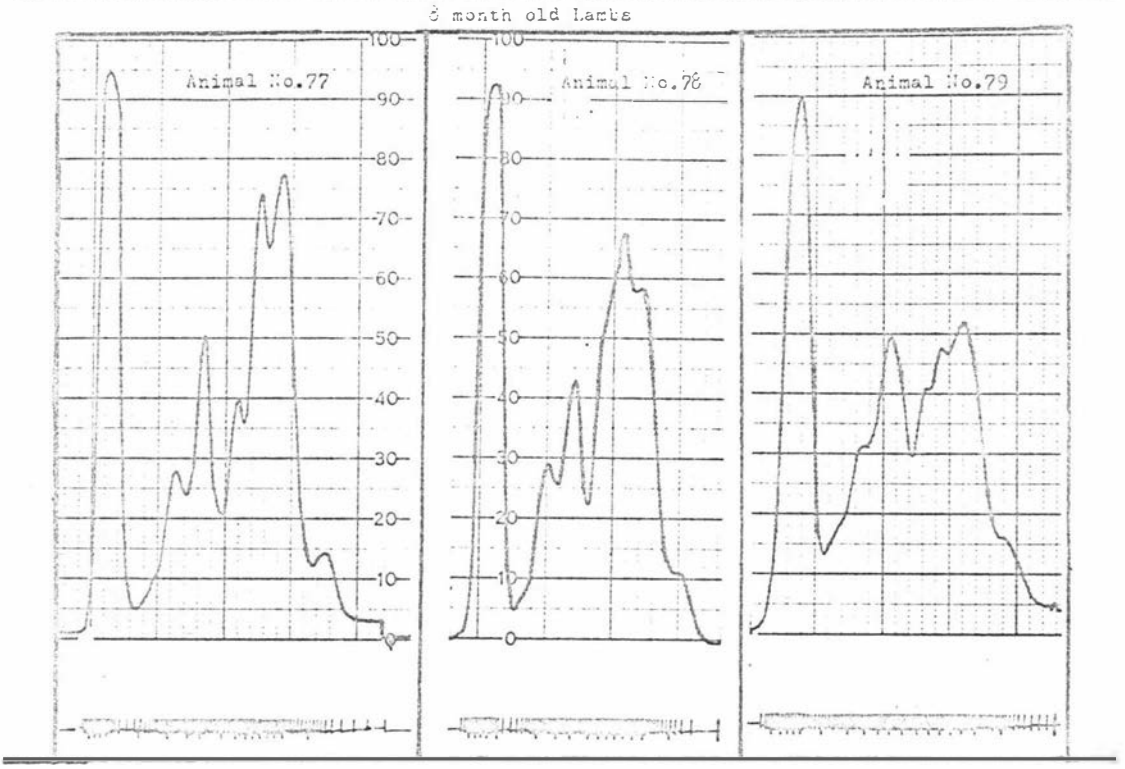
Although there were similar fluctuations in the levels of glucose and lactate in these samples which resulted in large standard deviations and arteriovenous differences the mean values obtained for levels of glucose were similar to those obtained by other workers.

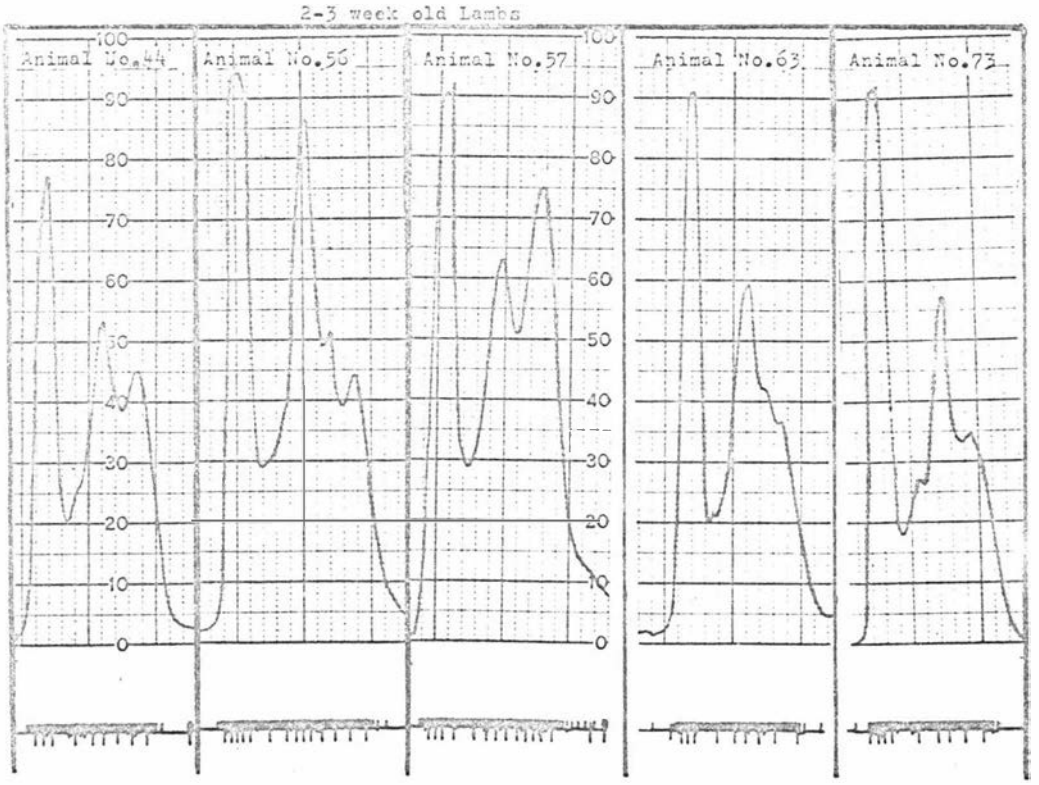
Finally the enzymatic constituents must be considered. Levels of some of these enzymes e.g. lactic dehydrogenase and alkaline phosphatase were extremely high relative to the values of these constituents in commercial standard 'serum' samples. In some instances the values were higher than the highest assayed levels of the enzyme in human serum samples with pathologically elevated levels of the particular enzyme. This meant that calibration of the methods in some instances had to be carried out by diluting one of the serum samples which showed a high activity, assessing its activity against a known standard, then using the undiluted sample to prepare the appropriate calibration curves and peaks. Nevertheless the values obtained were fairly consistent in all animals and it appears that the high levels could be partly due to species and partly to the fact that some enzymes, particularly the phosphatases, lactic dehydrogenase and isocitric dehydrogenase tend to be higher in young animals than in adults (Bergmeyer, 1963).

Conclusions

1. 'Vacutainers' are very convenient for the collection of both arterial and venous blood samples from lambs for blood gas analysis and haematological and biochemical examinations. The addition of deproteini-

Figure 15.1: Differences in the Electrophoretic Patterns of the Plasma Proteins of 2-3 week old and 8 month old Lambs.





sing solutions to these containers prior to sample collection however is not entirely satisfactory for the estimation of components by procedures which require deproteinisation.

2. Considerable care is necessary to ensure complete extraction of some blood constituents from the samples during deproteinisation and residual protein components in perchlorate filtrates can interfere with fluorometric analyses.

3. The haemoglobin content of the erythrocytes of young lambs is lower than that of the erythrocytes of older sheep. This is brought about by a higher haematocrit value and a similar haemoglobin content of the blood of the young lambs.

4. The blood pH and pCO_2 of the lambs in the present study was similar to the published values for adult sheep and the arteriovenous differences in these values were consistent and within physiological limits. The pO_2 of both arterial and venous blood was higher than in man.

5. Total plasma protein levels were higher in young lambs than in adult sheep and the pattern of the components obtained by electrophoretic separation of the proteins was different to published albumin:globulin ratios in the adult sheep. The electrophoretic pattern also contained fewer peaks in the young lambs.

6. Levels of sodium and potassium, obtained by automated flame photometric analysis of blood samples from these lambs, fell within a restricted range and corresponded closely with published values for these ions in the blood of adult sheep. A similar situation occurred with the chloride ion in these samples.

7. Levels of blood inorganic phosphate were variable and mean values tended to be lower than the published normal values for sheep. Some of the variability may have been due to errors introduced during deproteinisation.

8. Mean values for both lactate and glucose fell within normal limits.

Marked differences were encountered in the levels of these components in paired arterial and venous blood samples. These were partly physiological and partly inaccuracies introduced during sampling and deproteinisation. Pyruvate levels could not be estimated due to background fluorescence which interfered with fluorometric analyses.

9. Some enzymatic constituents e.g. lactic dehydrogenase and alkaline phosphatase were very high in young lambs.

10. Automated analyses with the Technicon 'Autoanalyser' provide a convenient means whereby a large number of samples can be subjected to a wide range of analytical procedures.

11. A wide range of serum enzymes can be estimated by following the oxidation or reduction of the phosphopyridine nucleotides during the course of the enzymatic reaction. The method is particularly suitable for automation where fluorometric or spectrophotometric detection can be employed. In the present instance a fluorometric method for the automated estimation of isocitric dehydrogenase based on the reduction of TPN was developed.

CHAPTER 16: THE EFFECT OF CL.PERFRINGENS TYPE D EPSILON TOXIN ON RESPIRATORY EXCHANGE AND ACID-BASE BALANCE IN LAMBS.

Kellaway et al (1940) have recorded a fall in 'Alkali Reserve' and oxygen saturation in the blood following the parenteral administration of epsilon toxin to lambs. Modern methods of blood gas analysis have made it possible to obtain more extensive and precise information on gaseous exchange and acid-base balance in animals and man than could have been obtained by these workers. During the present investigation it was intended to use the Astrup technique to follow the changes in blood pH, pCO_2, pO_2 and bicarbonate during the course of epsilon toxin intoxication in lambs. The data obtained in this way from lambs which had received parenterally administered toxin could then be compared with Kellaway's findings. It was hoped that the additional information obtainable in the present instance would assist in determining whether the acidosis (fall in 'Alkali Reserve') encountered by Kellaway et al was due entirely to deficiencies in respiratory exchange associated with the pulmonary oedema which occurred in their animals.

It was appreciated that there are a number of different causes of a lowered blood pH (acidosis) in animals. These fall into two broad types however. There is the so-called respiratory acidosis resulting from a failure of respiratory exchange and a fall in the efficiency of transfer of carbon dioxide from the blood to the atmosphere. On the other hand a metabolic acidosis can occur which may be associated with either a loss of basic components from the buffer system or an increase in acidic components in the blood. The latter compounds do not include carbonic acid resulting from increased concentrations of carbon dioxide, but refer to substances such as lactic acid produced by tissue metabolism or ingested in the diet. Thus it was of particular interest to determine whether there was an acidosis or alkalosis in intoxicated animals, in which respiratory exchange was not affected by the development of pulmonary oedema, as this would suggest that metabolic factors could affect the acid-base balance during the course of intoxication.

For several reasons the current work was intentionally confined to the changes in blood gas parameters which occurred in lambs which had received parenterally administered epsilon toxin. Firstly, in these animals it was possible to compare the influence of pulmonary factors such as oedema upon the blood gas status of the animal without the interference of other factors such as changes produced by high levels of carbohydrate in the diet, excitement etc. Secondly both arterial and venous samples could be obtained from these animals and A/V differences could therefore be examined to provide information on systemic and pulmonary aspects of gaseous exchange. Arterial blood sampling was difficult in the animals which received enteric-origin toxin. In addition, the period over which the blood samples were collected and the number of manipulative procedures to which the latter group of animals were subjected, made it technically difficult to obtain reliable blood gas data from them. Nevertheless it was considered desirable to follow the changes in blood gas parameters from at least one of these animals for comparison with the data from animals which had received parenterally administered toxin.

Materials & Methods:

Repetitive heparinised arterial and venous blood samples were taken with 'Vacutainers' from the lambs described in Chapter 6 both before and at intervals after the administration of toxin. The first post-inoculation samples were generally taken as soon as clinical signs of intoxication were evident ('Initial' samples) and a further sample was taken just before the death of the animal occurred ('Terminal' sample). The interval between these two samples varied between 15 minutes and 1 hour and additional samples were taken between the two above samples if the opportunity offered. Blood samples were also taken in the interval between inoculation and the onset of clinical signs of intoxication when this was prolonged. In some animals it was not possible to obtain the 'terminal' samples, due to circulatory collapse or death of the animal.

A series of heparinised blood samples was also taken from one of the animals (No. 77) which received enteric-origin toxin. These samples were taken prior to and during the course of the intraduodenal infusion and the development of clinical signs of intoxication.

The collection of blood samples and the analytical methods used for the blood gas analyses have been described in Chapter 15.

Results:

There were no detectable alterations in any of the blood gas parameters prior to the onset of clinical signs of intoxication. Following the development of depression etc., there was a marked progressive fall in the pH of both arterial and venous blood samples from all the animals which had received parenterally administered epsilon toxin. In many of these animals the fall in pH was associated with an increase in the pCO_2. Levels of 'standard' bicarbonate (bicarbonate calculated at a standard pCO_2 of 40mmHg.) and 'actual' bicarbonate (bicarbonate calculated at the pCO_2 of the sample) fell in the post-intoxication samples and this was associated with a fall in total buffer base concentration in the blood.

The changes which occurred in the mean values for these components are shown in Table 16.1 and illustrated by the histograms in Figure 16.1. The data is tabulated in Appendices 12 & 13.

Figure 16.1: The Effect of Intravenously Administered Epsilon Toxin on Blood pH and pCO_2 in Lambs

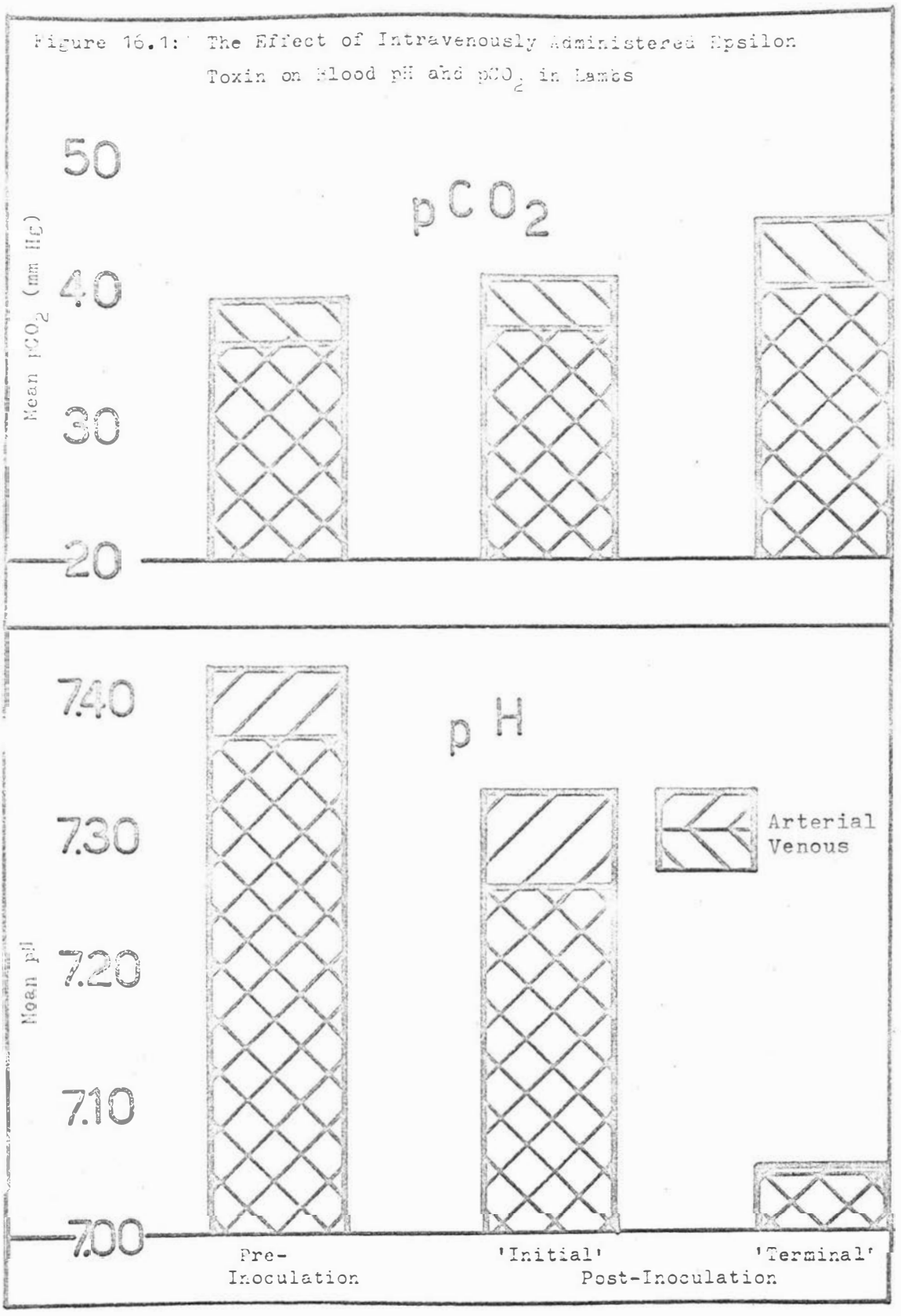


Table 16.1: Alterations in Blood Gas Parameters in Intoxicated Lambs - Time of Sampling

Means ± 1S.D.		Preinoculation	n	Onset of Intoxication	n	Time of Death	n
pH	Venous	7.35 ± 0.04	20	7.24 ± 0.11*	11	7.06 ± 0.18	17*
	Arterial	7.41 ± 0.04	19	7.31 ± 0.04*	8	7.11 ± 0.09	6*
pCO_2	Venous	41.2 ± 6.5	19	51.3 ± 7.1*	11	54.7 ± 21.7	15
	Arterial	33.0 ± 4.5	19	41.2 ± 5.7*	8	43.6 ± 17.0	6
Std HCO_3	Venous	21.7 ± 2.6	19	18.5 ± 3.5	10	17.9 ± 15.3	16
	Arterial	21.5 ± 4.2	19	19.0 ± 1.3	8	12.6 ± 5.1	7*
Actual HCO_3	Venous	22.3 ± 2.5	19	19.7 ± 3.3	10	16.7 ± 5.3	14+
	Arterial	20.7 ± 4.3	19	19.2 ± 2.0	8	14.7 ± 5.4	7
Buffer Base	Venous	42.3 ± 5.4	19	39.7 ± 5.2	10	31.2 ± 6.5	16*
	Arterial	42.5 ± 9.5	19	40.4 ± 4.9	8	30.8 ± 8.9	7

* t test between preinoculation figure and this value $p = < .01$
\+ t test between preinoculation figure and this value $p = < .05$

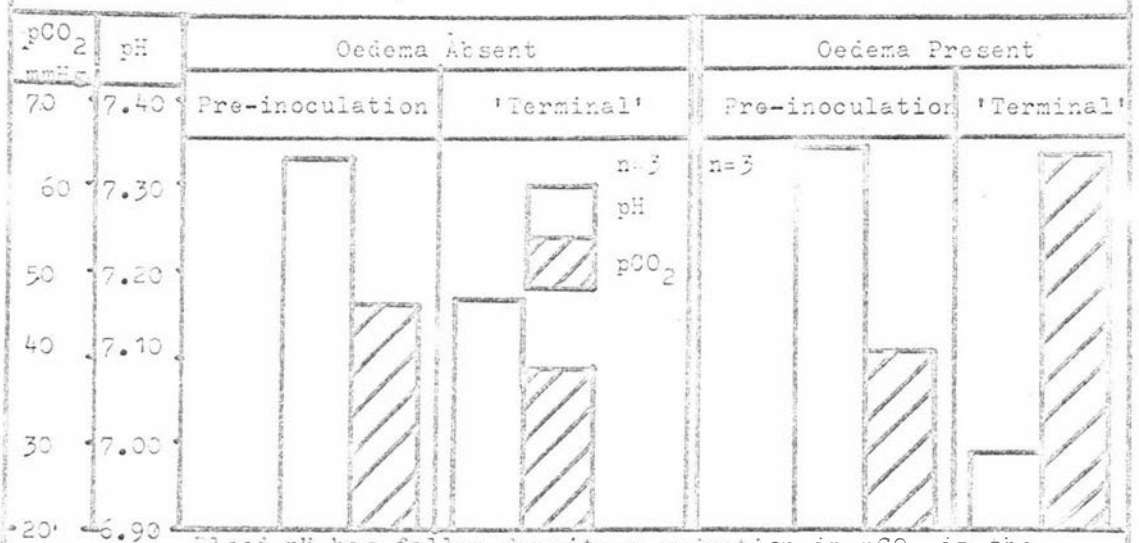
Although the fall in pH was very consistent, it was not always associated with rise in pCO_2 and this is reflected in the very large standard deviations which occur in the 'Terminal' pCO_2 column of the table. The rise in pCO_2 tended to be greatest in the animals with pulmonary oedema. In the animals which did not develop pulmonary oedema, and also in a few of the animals which did show this change, the rise in pCO_2 was either absent or very slight.

The different relationships between pH and pCO_2 in representative animals with and without pulmonary oedema are illustrated in Figure 16.2.

In these animals which had received parenterally administered toxin the levels of pO_2 fluctuated very widely in samples from the same animal during the course of intoxication. In some animals it was higher in the post-inoculation samples than in the pre-inoculation samples. On the other hand, in some animals the pO_2 fell to extremely low levels, particularly in the 'terminal' samples. The post-intoxication pO_2 results are tabulated in Appendix 13.

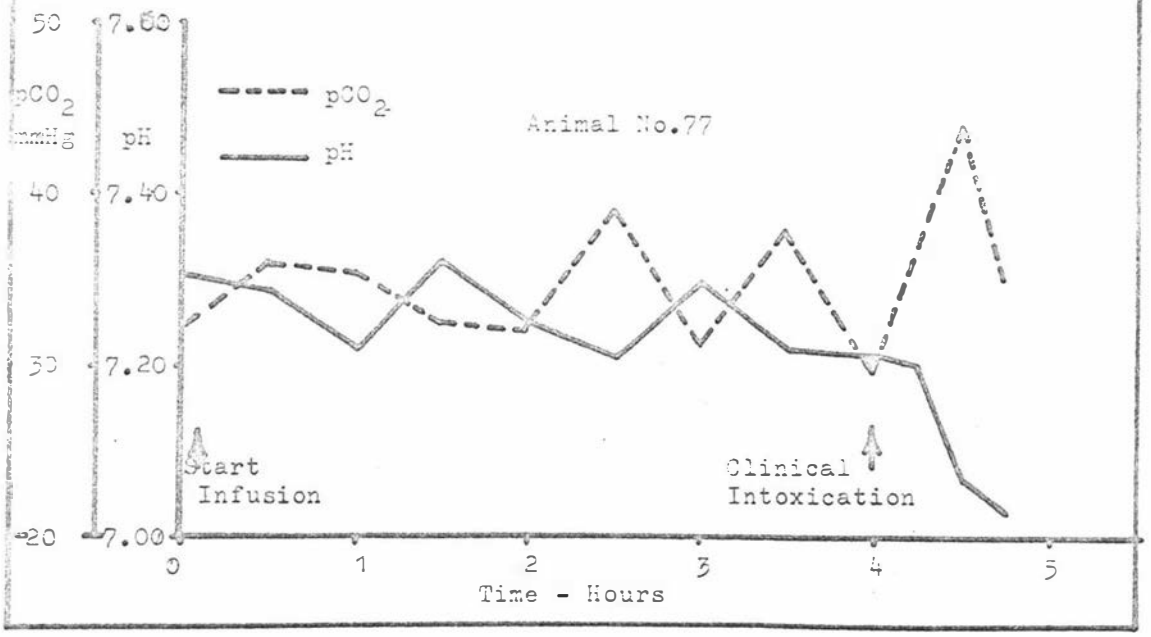
The pattern of changes in the blood gas parameters of the animal which received enteric-origin toxin were essentially similar to those described above. There were no consistent changes in any of the values prior to the onset of clinical signs of intoxication. At about the time that these signs developed there was a marked fall in blood pH from

Figure 16.2: The Influence of Pulmonary Oedema on the Changes which occur in Blood pH and pCO_2 in Intoxicated Lambs



Blood pH has fallen despite a reduction in pCO_2 in the animals where pulmonary oedema was absent. This suggests that there was a metabolic acidosis in these animals.

Figure 16.3: The Pattern of Change in Blood pH and pCO_2 in a Lamb with Experimental Cl.perfringens type D Enterotoxaemia



7.40 to 7.27. Subsequently there was a transient rise in pCO_2 from 30mmHg to 44mmHg but this had returned to almost the original level by the time the 'terminal' blood samples were taken. The pO_2 showed no marked alterations during the whole course of intoxication.

Results from this animal are given in Appendix 14 and illustrated by Figure 16.3.

Discussion

Severe acute pulmonary oedema, such as that which occurred in many of the animals in the present experiments, must of necessity interfere with respiratory exchange. Under these conditions a severe respiratory acidosis associated with high pCO_2 values and low blood pH could be expected to occur. In the present instance the levels of bicarbonate in the samples from the acidotic animals were also low indicating that the condition was uncompensated. This too could be expected in a rapidly progressive condition where renal compensatory mechanisms such as sodium retention would not have time to influence the plasma electrolyte system to any extent.

It is noteworthy that the pO_2 of the blood samples from many of these animals was not as markedly affected as the elevated pCO_2 values might suggest. The fact that in some instances they were actually higher than in the pre-inoculation samples suggests that a compensatory mechanism must have been in existence. The most likely explanation for this result appears to be that the increased haemoglobin content of the circulating blood resulting from the haemoconcentration has compensated in large measure for the decreased efficiency in respiratory exchange as far as oxygen is concerned. In addition, the increased respiratory stimulus resulting from the rising carbon dioxide levels in the blood would have increased the respiratory effort and assisted in the oxygenation of the available haemoglobin. The overall result would be an increase in pO_2 although, because of the inefficient respiratory exchange, the available haemoglobin was probably not fully saturated with oxygen. It is interesting to note in this respect that a similar situation appears to have occurred in the animal which Kellaway *et al* used to illustrate

their blood gas changes. In that animal too there was very little change in the oxygen content of the blood samples from the animal during the course of intoxication and the fall in oxygen saturation of the blood which they describe appears to have been associated with the increased haemoglobin present due to haemoconcentration.

While on the subject of oxygen content of the blood samples it should be mentioned that some of the very low pO_2 values encountered in the 'Terminal' samples from some of the animals in the present study were taken at a very advanced stage of intoxication when the animal was practically clinically dead and under these circumstances such low values could have been expected.

One of the most interesting features of the present series of estimations was the fact that in some of the intoxicated animals the fall in blood pH was not associated with a concomitant rise in pCO_2. This implies that, in these animals, the acidosis was not of respiratory origin. The basis of metabolic acidosis is often complex in natural disease states but in the present instance many of the possibilities could be discounted. In these animals there was a fall in bicarbonate and also in total buffer base levels and, since there was no severe diarrhoea or prolonged renal insufficiency, the most likely explanation appeared to be excessive acid formation in the body. This could only have been of metabolic origin and the acid under these circumstances could be expected to be lactic acid. This was even more probable in view of the hyperglycaemia which occurs in enterotoxaemia, but a discussion of the changes in the various products of glucose metabolism which occurred during the course of intoxication in the present animals can be conveniently deferred until Chapter 19.

Before leaving the blood gas analyses it is also important to note that in the animal which received enteric origin toxin there was a fall in blood pH which was not entirely referable to the levels of pCO_2 implying that in experimental enterotoxaemia too, there is a metabolic acidosis. Here again the details of the metabolic changes associated with altered glucose metabolism can be deferred.

Conclusions.

1. There were no consistent alterations in any of the blood gas parameters between the administration of epsilon toxin and the onset of clinical signs of intoxication.

2. A severe, progressive acidosis developed in both arterial and venous blood samples taken from animals which showed clinical signs of intoxication following the parenteral administration of epsilon toxin. This also occurred in an animal in which experimental enterotoxaemia had been induced.

3. There was an increase in the pCO_2 of the blood in many of the animals due to deficient respiratory exchange associated with the pulmonary oedema.

4. Plasma bicarbonate levels were low in all animals which developed acidosis indicating that the acidosis was uncompensated and that renal compensatory mechanisms had not come into effect.

5. Despite the elevated pCO_2 and deficient respiratory exchange the pO_2 did not fall consistently in all intoxicated animals. The compensatory mechanisms in this case were:i.Increased respiratory effort associated with stimulation of the respiratory centre by the rising pCO_2.

ii. Increased haemoglobin content of the blood due to the haemoconcentration. This resulted in a maintenance of normal or above normal partial pressures of oxygen in the blood despite a decrease in the saturation of the haemoglobin with oxygen.

6. In some intoxicated animals there was a severe acidosis with normal or reduced pCO_2 levels indicating that there was a metabolic acidosis present. In some animals, e.g. the animal in which experimental enterotoxaemia had been induced, there was evidence that both respiratory and metabolic acidoses were present.

CHAPTER 17: HAEMATOLOGICAL ALTERATIONS AND CHANGES IN THE LEVELS OF PLASMA PROTEINS IN LAMBS FOLLOWING THE ADMINISTRATION OR ABSORPTION OF CL.PERFRINGENS TYPE D EPSILON TOXIN

Gordon and his co-workers (1940) have shown that Cl. perfringens type D toxin produces a severe anhydraemia (haemoconcentration) in sheep. In the course of their studies, the alterations which occurred in a number of haematological parameters were examined. They failed to show any marked change in clotting time or erythrocyte fragility. This could be expected in an acute intoxication where little time was available for the production of erythrocytes or factors associated with the clotting mechanism to be affected. Similarly, alterations in erythrocyte numbers would generally be directly proportional to changes in haemoglobin level and haematocrit (packed cell volume - P.C.V.) in an acute non-haemolytic intoxication. Total erythrocyte counts were therefore considered to be unlikely to yield useful information.

It was decided to limit the haematological measurements in the present instance to values for haemoglobin, haematocrit and total white cell count. Calculation of the Mean Cell Haemoglobin Concentration (MCHC) from the first two values was considered to be desirable to confirm that the alterations in haemoglobin level were in fact associated with corresponding changes in packed cell volume. Although Gordon et al had demonstrated a slight leucopenia in their post-inoculation blood samples the main purpose of carrying out white cell counts in the present instance was as an indicator of possible intercurrent infection in the control and experimental animals.

As the name implies, the anhydraemia or haemoconcentration which was encountered by previous workers was believed to represent a loss of fluid from the circulation. For reasons which will be discussed in Chapter 18 it was unlikely that marked alterations in inorganic ions such as sodium, potassium or chloride would be encountered under these circumstances.

Whether or not changes in blood levels of colloidal substances, such as proteins, would occur would depend entirely upon the underlying basis for the fluid loss. Under these circumstances a study of the concentrations of the plasma protein components during the course of intoxication could provide useful information on the pathogenesis of the fluid loss and haemo-concentration. As these substances do not appear to have been studied by previous workers it was necessary to examine the changes in both total protein levels and the different protein fractions in the present investigation and to relate these to the morphological changes in capillaries.

Alterations In Haematological Parameters and Plasma Protein Levels in Lambs Following the Parenteral Administration of Epsilon Toxin

Material & Methods:

The animals used and the method of sample collection have been described in Chapter 15 along with the procedures used for determining the haematological parameters, total protein levels in plasma and the electrophoretic patterns of the plasma proteins. As in the previous chapter the 'Initial' post inoculation blood samples were those taken as soon as clinical signs of intoxication were evident and the 'Terminal' samples those taken just prior to the death of the animal.

Results:

There were no alterations in any of these parameters, referable to the action of the toxin, in post inoculation blood samples taken prior to the onset of clinical signs of intoxication. In some of the animals which survived for some time and were therefore sampled on several occasions there was, prior to the onset of signs of intoxication, a slight fall in the haematocrit and the levels of haemoglobin due to blood loss.

Following the onset of clinical signs of intoxication there

was evidence of a rapidly developing haemoconcentration in most animals. Grossly the haemoconcentration was detectable as a marked increase in the viscosity of the blood as it was being collected into the 'Vacutainers'and quantitatively it was reflected in an increase in the haemoglobin level of the blood and also in the haematocrit value. The changes in these two values were proportional, with the result that the mean cell haemoglobin concentration calculated from them remained almost the same as the preinoculation value.

The haemoconcentration was more severe in the animals which were subsequently shown to have developed pulmonary oedema and in some of these animals the packed cell volume and haemoglobin content of the 'Terminal' blood sample were more than double the pre-inoculation value. By comparison, the changes which occurred in these parameters in lambs which did not show lung oedema were fairly small. For example in two of the latter cases no significant change in either haemoglobin content or packed cell volume was detected. The overall changes which occurred in these parameters in venous blood during intoxication are shown in Table 17.1 and illustrated with the mean haematocrit values in Figure 17.1a. In addition the inter-relationship between the development of pulmonary oedema and the extent of the changes which occurred in the haematocrit are illustrated in Figure 17.1b. The post inoculation data on intoxicated lambs is presented in Appendix 13 .

Table 17.1:
Alterations in Haemoglobin, Haematocrit and Total Plasma Protein in Intoxicated Lambs

Mean ± 1 Std.Dev.	Time of Sampling					
	Pre-inoculation	n	Onset of Intoxication 'Initial'	n	Time of Death 'Terminal'	n
Haemoglobin Gm%	10.4 ± 0.9	26	11.3 ± 1.4	15	15.2 ± 0.8	24
Haematocrit %	34.6 ± 2.5	26	37.6 ± 4.6	15	51.4 ±14.0	24
Total Protein	6.5 ± 0.5	26	5.9 ± 0.5	15	6.3 ± 0.8	22

t test						
	Haemoglobin	Preinoc:Initial	p=< .05	Preinoc:Terminal	p = < .001	
	PCV	" "	p=< .05	" "	p = < .001	
	Protein	" "	p=< .001	" "	p = not significant	

There was an overall tendency for the total white cell count to be elevated in the post inoculation samples.

The level of total plasma protein tended to be slightly lower in most of the post-intoxication blood samples. The change was 0.5 Gm% or less in most instances, and the mean fall was not statistically significant. The changes which occurred in plasma protein values in intoxicated animals are also illustrated in Figures 17.1a where they are related to the changes in haematocrit.

Alterations in Haematological Parameters and Plasma Protein Levels in Lambs with Experimental Enterotoxaemia.

Materials & Methods:

The production of experimental enterotoxaemia in lambs is described in Chapter 6 i.e. the 'enteric-origin' toxin group of lambs. The method used for collection of blood samples, the estimation of haemoglobin etc. have already been described.

Total white cell counts were not carried out on these animals.

Total plasma protein levels in the blood samples were estimated by the manual 'Biuret' method described in Wootton (1964). The spectrophotometric measurements for this estimation were made at 540 nm with a Bausch & Lomb 'Spectronic 20' spectrophotometer (Bausch & Lomb Inc., Rochester Ill., U.S.A.). The standards employed were a sample of 'Versatol' serum standard (Warner Chilcott Morris, Plains N.J., U.S.A.) containing 7.2Gm% total protein

A preparation of 100mg fraction V bovine serum albumin in 1ml distilled water was also used.

Figure 17.1a: The Effect of the Parenteral Administration of Epsilon Toxin on the Haematocrit and Total Plasma Protein of Lambs.

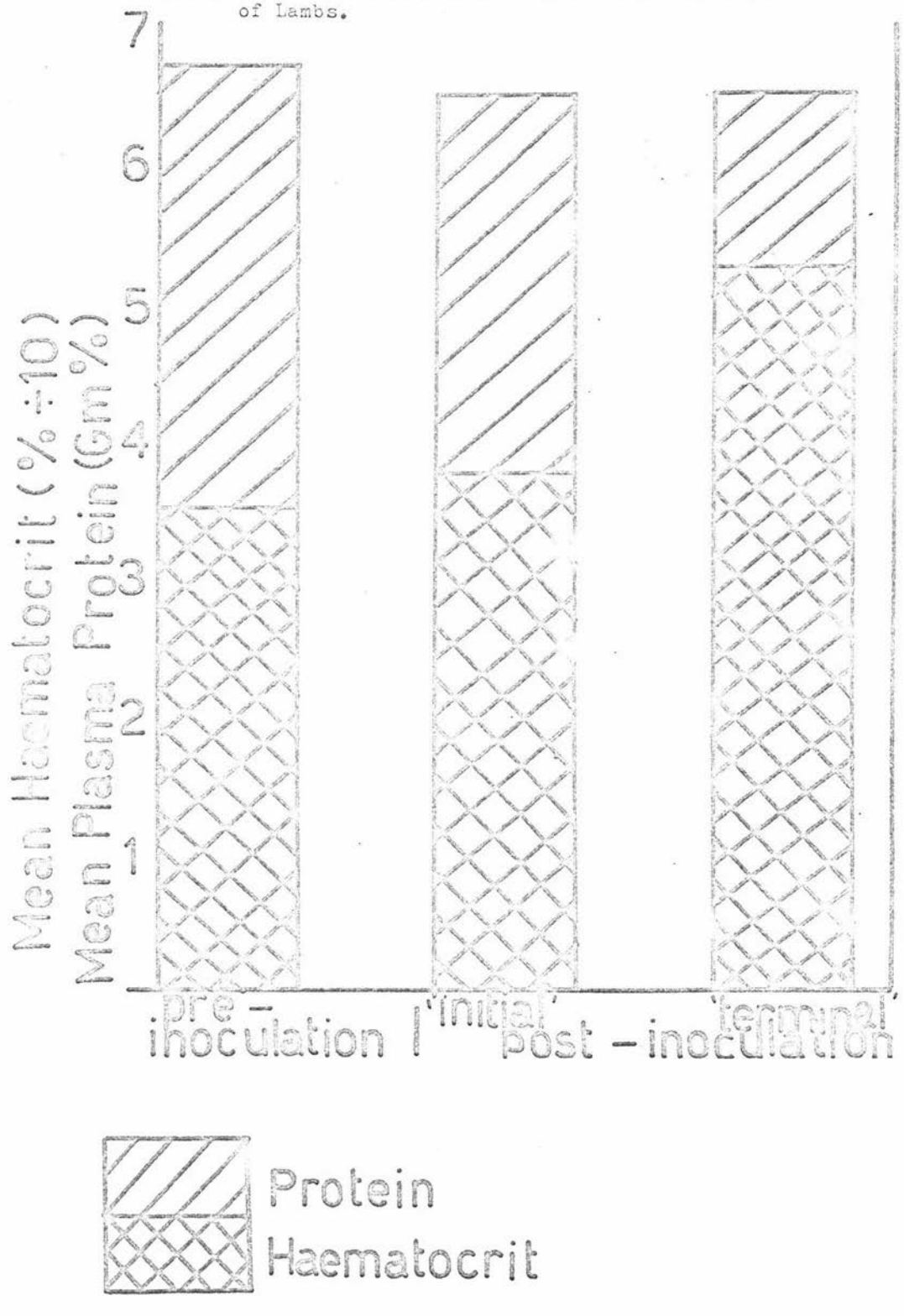
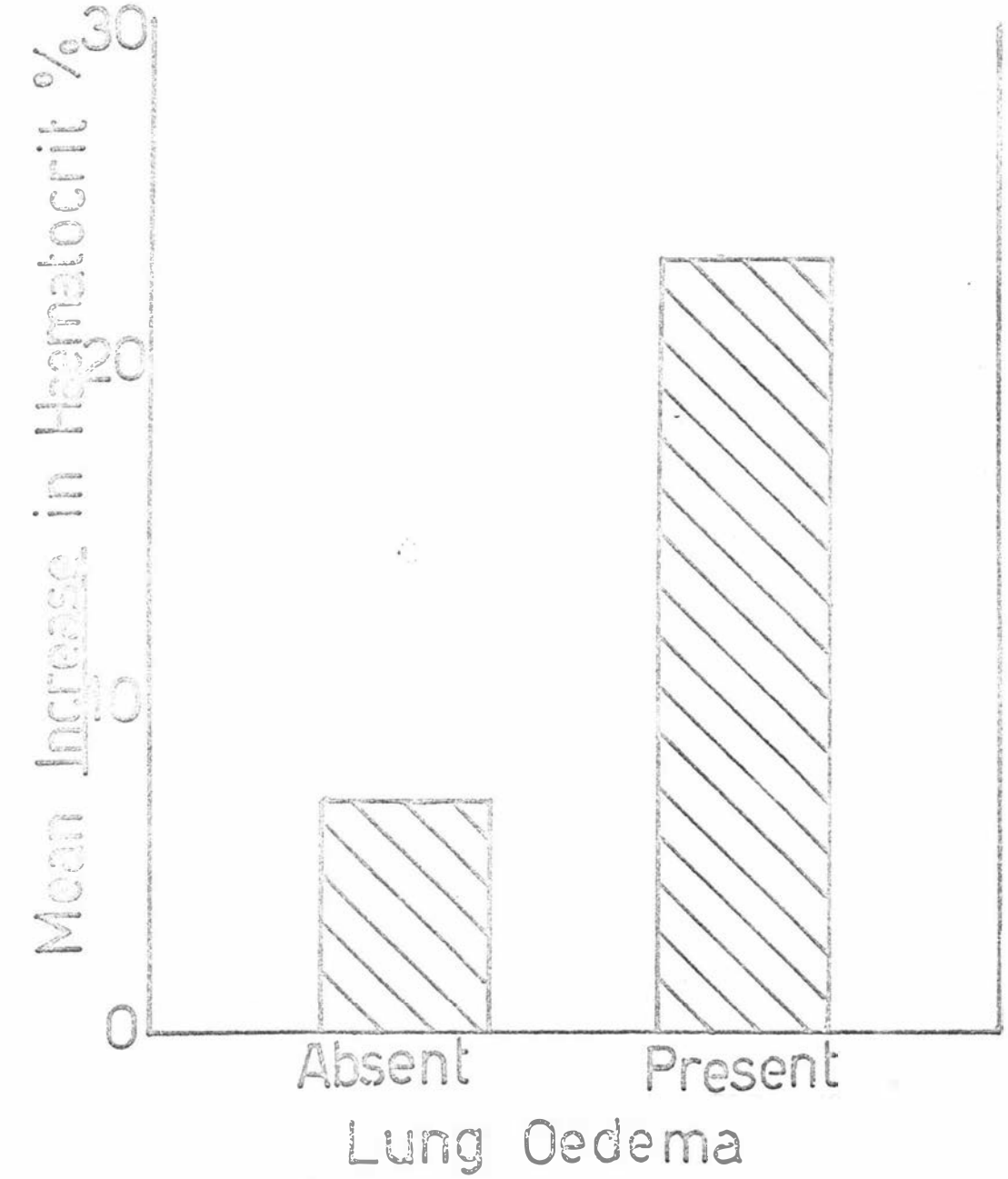


Figure 17.1c: The Effect of the Development of Pulmonary Oedema on the Changes in Haematocrit which Occur in Epsilon Toxin Intoxicated Lambs



Results:

No significant changes occurred in any of these parameters in any animal until the onset of clinical signs of intoxication even though several hours elapsed between the start of the intraduodenal infusion of Cl.perfringens type D culture plus carbohydrate, and the development of detectable intoxication.

Samples taken after clinical signs of intoxication were evident showed an increase in haemoglobin content and packed cell volume in three animals. However these parameters did not change significantly from the preinoculation level at any time in the fourth animal (No. 78) although it exhibited 'typical' signs of enterotoxaemia (see Chapter 7).

The changes in the levels of total protein were more variable in these animals than in lambs receiving parenterally administered toxin. Total protein rose slightly following the development of clinical signs of intoxication in all instances. In two animals this rise continued up to the time of death but in the others the level returned to normal or fell below the pre-inoculation level terminally.

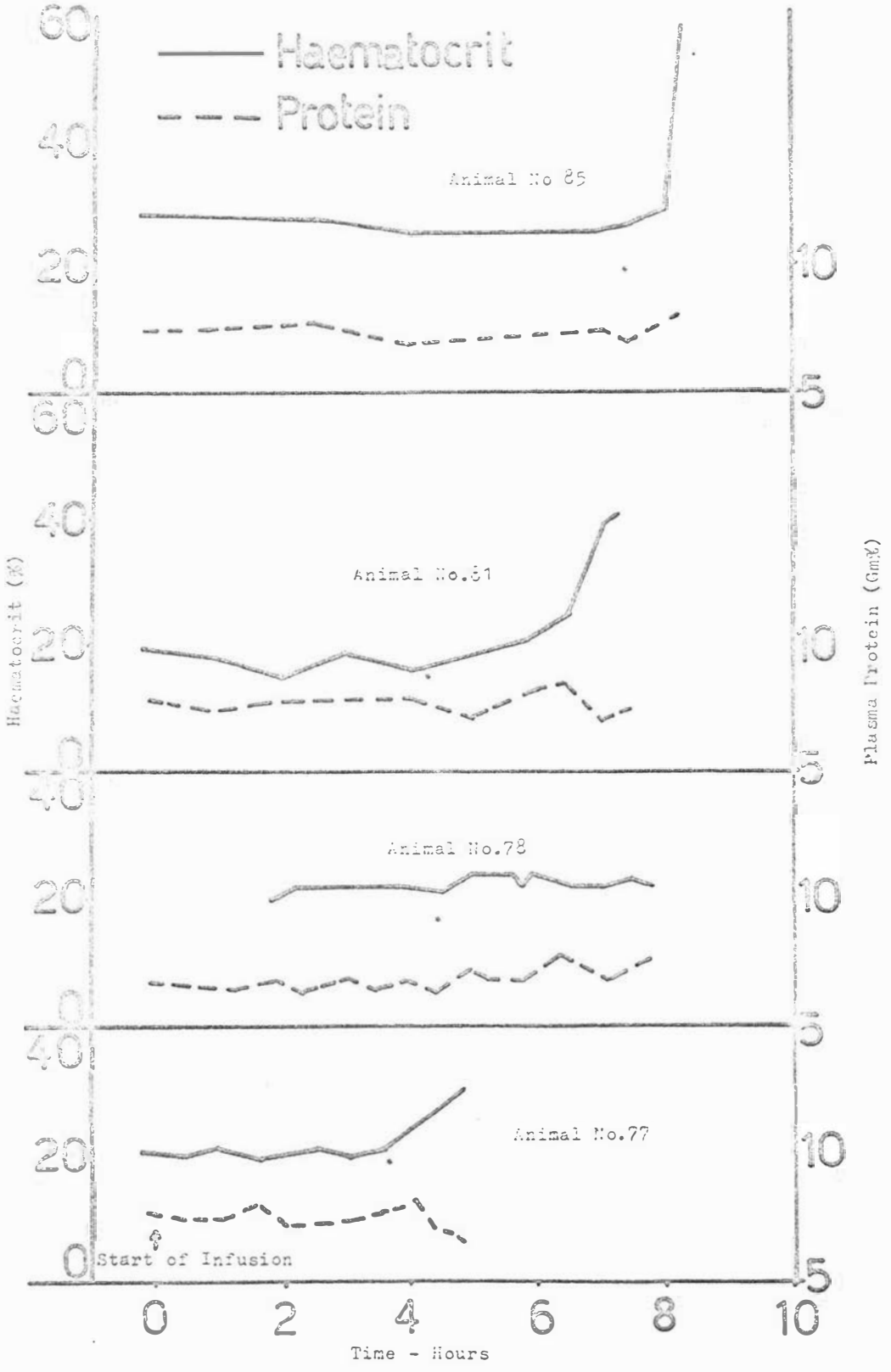
The progressive changes in these parameters from prior to the commencement of the introduodenal infusion until the time of death are illustrated in Figure 17.2 and the data is tabulated in Appendix 14. There were no consistent differences in the protein patterns obtained by electrophoretic separation of pre-or post-inoculation plasma samples.

As mentioned in Chapter 15, there were considerably more peaks in the patterns obtained from these animals than in the patterns from the young lambs used for experiments in which toxin was given by intravenous injection.

Discussion

From these findings it appears that the alterations which occur in the haematological parameters during intoxication are almost entirely due to changes in fluid balance between the blood stream and the extravascular fluid compartments. As could be

Figure 17.2: The Effect of Enterotoxaemia produced by the Intraduodenal Infusion of Cl.perfringens type D Culture plus Carbohydrate on the Haematocrit and Total Plasma Protein levels of Lambs.



* = Clinical signs of intoxication

expected in an acute intoxication in which intravascular haemolysis is not a prominent feature the mean cell haemoglobin concentration remained relatively constant and did not reveal any evidence of blood destruction in animals which received parenterally administered toxin thus confirming the earlier in vitro experiments which showed that alpha toxin was not present in appreciable quantities in batch CWD epsilon toxin (see Chapter 4).

The changes in the total white cell counts in intoxicated animals were small and could be explained on the basis of the haemoconcentration. This suggests that the toxin may not elicit an acute inflammatory response when administered intravenously. Two items of interest in regard to the white cell counts which do not emerge from the results given in this chapter are as follows: Two animals which were removed from the experiment on the basis of suspected intercurrent infections had very high white cell counts (38,000 and 63,000 white cells/mm^3) and one of these animals was subsequently shown to have a severe Sphaerophorus spp navel infection. On the other hand two of the field cases of neurological disease unassociated with enterotoxaemia which were examined viz. the case of E.coli meningoencephalitis and the cervical spinal abscess cases mentioned in Chapter 13 had white cell counts within the normal range (6,950 and 5,500 white cells/mm^3 respectively. The second interesting feature was the fact that the total white cell count rose for a pre-inoculation level of 5,800 cells/mm^3 to a figure of 13,800 cells/mm^3 in 5 hours in the lamb which received epsilon toxin by intraperitoneal administration. This was almost certainly due to the extremely severe peritonitis which was induced as a result of using this route of administration, (see Chapter 6).

The comparatively minor changes in haemoglobin and haematocrit values that occurred in the majority of the animals which did not develop pulmonary oedema after the parenteral administration of toxin suggests that haemoconcentration may not always occur in enterotoxaemia. This is also supported by the fact that no marked alteration to these parameters occurred in one of the animals which received enteric origin toxin (No. 78). It appears likely however that the extent of the haemoconcentration is almost entirely

dependent upon the severity of the vascular endothelial damage and, while this could be expected to vary from animal to animal, it is probable that in the majority of cases in which rapid death occurs the damage would be sufficiently severe to produce haemoconcentration. Animals No. 78 and 81 were the ones which survived the longest following the onset of clinical signs of intoxication and it is interesting to note that while the former animal did not show any haemoconcentration it was present in the latter animal.

Even in animals where pulmonary oedema did not occur there was usually some evidence of haemoconcentration and this suggests that fluid is lost into other tissues as well as the lungs. It has already been shown in Chapter 9 that severe myocardial oedema occurs and no doubt there are also other sites in which fluid is lost from the circulation. In this respect it was of considerable interest to find that there was a loss of protein from the circulation along with the fluid and that in fact the fluid which is extravasated must have a composition closely resembling whole plasma as not only did the level of total plasma protein tend to fall rather than rise but all the protein components appeared to have been affected to the same extent. Thus the condition is not, strictly speaking, an uncomplicated anhydraemia. The outpouring of proteins into the extravascular fluid must inevitably have a profound effect upon the colloid osmotic pressure of the extravascular fluid and this would tend to accelerate the extravasation of fluid and could contribute to the haemoconcentration and circulatory collapse.

These findings are not altogether surprising in view of the vascular endothelial damage which has been shown to occur in a wide variety of tissues in lambs and their occurrence could also have been anticipated from the loss of horse radish peroxidase from the circulation into the brain extracellular space in mice. It is possible that they may help to explain one of the rather anomalous results in Chapter 8. It was shown in that chapter that, while an increase in water content of brain tissue occurred in intoxicated mice, a similar change could not be demonstrated in intoxicated lambs. It appears possible that, since plasma contains

approximately 6% protein, if a fluid of this type were being extravasated into brain tissue which itself has a fairly high water content (84% in grey matter of adult sheep - Spector, 1956 - possibly higher in young animals - see Green 1966), then marked changes in fluid balance could occur before they were detectable, as a change in absolute water content of the tissue.

Some mention must also be made of the difference in the electrophoretic patterns of the plasma proteins in samples from the 2-3 week old lambs and in samples from 8 month old lambs. So far the preinoculation values for the haematological and biochemical parameters of the 8 month old lambs which were used for the experiments with enteric origin toxin have not been discussed extensively because in many instances they did not differ significantly from those of the 2-3 week old lambs which have already been described. There were certain notable differences however and these will be discussed as they arise. In particular there were very marked differences in the electrophoretic patterns of the plasma proteins.

The electrophoretic pattern of the young lamb's plasma with its few high globulin peaks probably reflects the considerable amount of protein which is absorbed from the dam's colostrum during the early life of the lamb. The pattern of permeability of the intestine to the different types of protein molecule is known to change rapidly during the first few days of life. In the rat, all types of protein are absorbed initially but there is a progressive selective reduction in protein absorption with that of globulins generally continuing for the longest time (Jordan & Morgan, 1968). In the lamb the absorption of intact globulins does not appear to continue for more than 48 hours after birth (McCarthy & McDougall, 1953; quoted by Cooper, 1967).

On the other hand, judging by the persistence of passive protection against certain diseases, which is conferred by colostrum derived antitoxins, the gamma globulins absorbed during the early life of the animal can persist at appreciable levels in the plasma until at least 12 weeks of age. From this time onwards the animals own

electrophoretic pattern of proteins will begin to emerge more clearly and will tend to increase in complexity with increasing maturity.

Finally, it appears that the loss of high molecular weight substances from the blood stream will be a major factor limiting the usefulness of many of the existing methods for studying the pattern of absorption of epsilon toxin from the intestine. It is obvious that under conditions where the toxin or tracer substances, such as the antibodies used by Bullen & Batty (1956, 1957) and the PVP used in the present study, are not being retained in the circulation following absorption from the intestine anomalous results are likely to be obtained when assays are carried out on blood samples. It appears probable therefore that the sudden fall in the levels of PVP in blood samples taken from animal no. 81 (see Chapter 6) following the onset of clinical signs of intoxication were due to loss of this tracer into the extravascular fluids via the damaged capillaries.

Conclusions

1. A severe haemoconcentration usually occurs following the onset of clinical signs of intoxication after parenteral epsilon toxin administration and also after the experimental induction of enterotoxaemia by the infusion of *Cl.perfringens* type D and carbohydrate into the duodenum of lambs. This change is not invariable and probably reflects the extent of the vascular damage which occurs.

2. The total plasma protein falls in association with the haemoconcentration indicating that protein is being lost from the circulation and this may help to explain the lack of detectable increase in water content of the brains of intoxicated lambs.

3. The electrophoretic pattern of the plasma proteins of normal 2-3 week old lambs is different from that in normal 8 month old lambs.

4. There were no detectable changes in the electrophoretic pattern of the plasma proteins after the onset of intoxication indicating that all protein fractions were being lost from the circulation.

5. The total white cell count is not influenced by intravenously administered epsilon toxin, but is elevated following the intraperitoneal administration of toxin due to the peritonitis which occurs.

6. The Mean Cell Haemoglobin Concentration is unaffected during epsilon toxin intoxication showing that the changes in the haematocrit and haemoglobin content of the blood are due to fluid loss and haemoconcentration only.

7. The loss of high molecular weight substances from the bloodstream of intoxicated animals explains the fall in plasma levels of PVP used in the present study to investigate the changes in intestinal permeability, which occur during enterotoxaemia.

CHAPTER 18: THE EFFECT OF CL.PERFRINGENS TYPE D EPSILON TOXIN ON THE CONCENTRATIONS OF SOME OF THE MAJOR IONIC CONSTITUENTS OF THE PLASMA OF LAMBS

Although a variety of cations occur in plasma the ones most commonly studied are sodium, potassium, calcium and magnesium. The latter two ions are important in a wide variety of physiological and biochemical processes and disturbances in their availability within the body can lead to serious disease and death. However, the clinical signs of enterotoxaemia do not suggest any major alterations of these ions. The findings of Kellaway et al (1940) support this impression, and these ions were not investigated further in the present instance.

Sodium and potassium on the other hand are the major cations involved in the acid-base balances which maintain the hydrogen ion concentration of the blood within physiological limits as well as being involved in a variety of other physico-chemical processes. They will be affected when there are major changes in acid-base balance or in disturbances of fluid equilibrium. Different concentrations of these ions occur in the intra-and extracellular fluid and the gradient is maintained by energy dependent processes. If any of the factors involved in maintaining these ionic gradients across cell membranes were affected by epsilon toxin this might be reflected as altered concentrations of the ions in plasma.

Along with the bicarbonate ion, chloride and phosphate form the major inorganic anions in blood and it was also necessary to examine the levels of these ions to enable a comprehensive picture to be obtained of any changes which occurred in the ionic status of lambs during the course of epsilon toxin intoxication. It was considered that this would provide information on both renal function and the cell membrane integrity of intoxicated animals.

Although phosphate ions do form part of the buffer base complex of blood, their role in this respect is minor compared to their function in bone and energy metabolism. For utilisation of glucose to occur in the animal body the formation of phophorylated intermediate compounds is essential and, in a situation where

hyperglycaemia occurred as a result of an interference with oxidative phosphorylation of glucose or its intermediates, there might also be an increase in circulating levels of inorganic phosphate. On the other hand, any changes in the level of phosphate ion in blood due to interference with the production of organic phosphate esters could be masked by changes in the equilibrium which exists with the phosphate in bone mineral.

The Effect of the Parenteral Administration of Epsilon Toxin on the Concentration of Sodium, Potassium, Chloride and Phosphate Ions in the Blood of Lambs.

Materials & Methods:

The blood samples used for this study were venous samples collected at the same time as the heparinised samples described in Chapters 16 & 19 and in the same way as the preinoculation and control blood samples described in Chapter 15.

Results:

There were no consistent changes in the levels of Na^+, K^+ or Cl^- which could be attributed to the action of epsilon toxin. Animals which had received prolonged intravenous infusions of toxin in saline and which had therefore received large quantities of sodium and chloride ions showed slight elevations of the concentration of these ions in serum but even these changes were not large in relation to the normal serum values, considering the amount of these ions which had been administered. The change in serum level was always less than 5% of the preinoculation level even in animals which had received approximately 400meq of Na^+ and 300 meq of Cl^-.

No consistent change in the level of inorganic phosphate was detectable in the blood of these lambs prior to or after the onset of clinical signs of intoxication.

The results obtained from the postintoxication ionic analyses

are tabulated in Appendix 13 and summarised in Table 18.1.

Table 18.1

The Effect of Epsilon Toxin on the Ionic Constituents in Blood of Lambs.

Mean ± 1std.dev.	Time of Sampling					
	n	Pre-inoculation	n	Onset of Intoxication	n	Time of Death
Sodium (meq/1)	25	152.6 ± 3.0	13	154.4 ± 4.7	9	151.3 ± 5.0
Potassium (meq/1)	25	5.16 ± 0.35	13	5.61 ± 1.29	9	5.81 ± 1.05
Chloride (meq/1)	25	111.6 ± 4.5	13	116.2 ± 4.9	9	113.2 ± 6.5
Phosphate (mg%) *	26	3.50 ± 1.82	15	3.17 ± 1.09	25	4.41 ± 1.61

* t test Phosphate Preinoc:Terminal p between .1 and .05

The Effect of Experimental Cl.Perfringens Type D Enterotoxaemia on the Blood Levels of Sodium, Potassium, Chloride and Phosphate Ions in Lambs

Materials & Methods:

The blood samples were obtained from the animals which received enteric origin toxin described in Chapter 6, and the automated methods described in Chapter 15 were used to determine the levels of sodium, potassium and chloride in these samples. Chloride was estimated on samples from two animals No's. 77 and 78, only.

Up to this point all the methods employed for sample processing have been the same for the animals which had received parenterally administered toxin and for those which received toxin of enteric origin. However, the latter group of animals were examined on an individual basis over an extended period of time. In view of the lability of some blood components and the previously encountered difficulties with deproteinisation in 'Vacutainers'

it was decided that samples which required deproteinisation or contained labile components from the present animals could preferably be dealt with by normal methods of deproteinisation and estimated by manual procedures as soon as possible after collection.

At the same time it was desirable to utilise basically similar methods in both the automated and the manual procedures and it will be seen in this and subsequent chapters that, where manual methods were employed on samples from animals receiving enteric origin toxin, they were similar to the automated method employed for the samples from animals which received parenterally administered toxin. Thus in the present instance inorganic phosphate was estimated by the Acid-Molybdate method (Gomori (1942) modification of the Fiske & Subarrow method described in Varley (1962).) Spectrophotometric measurements were made at 660nm in a Bausch & Lomb 'Spectronic 20' and the phosphate standards contained 4mg/100ml expressed as phosphorus.

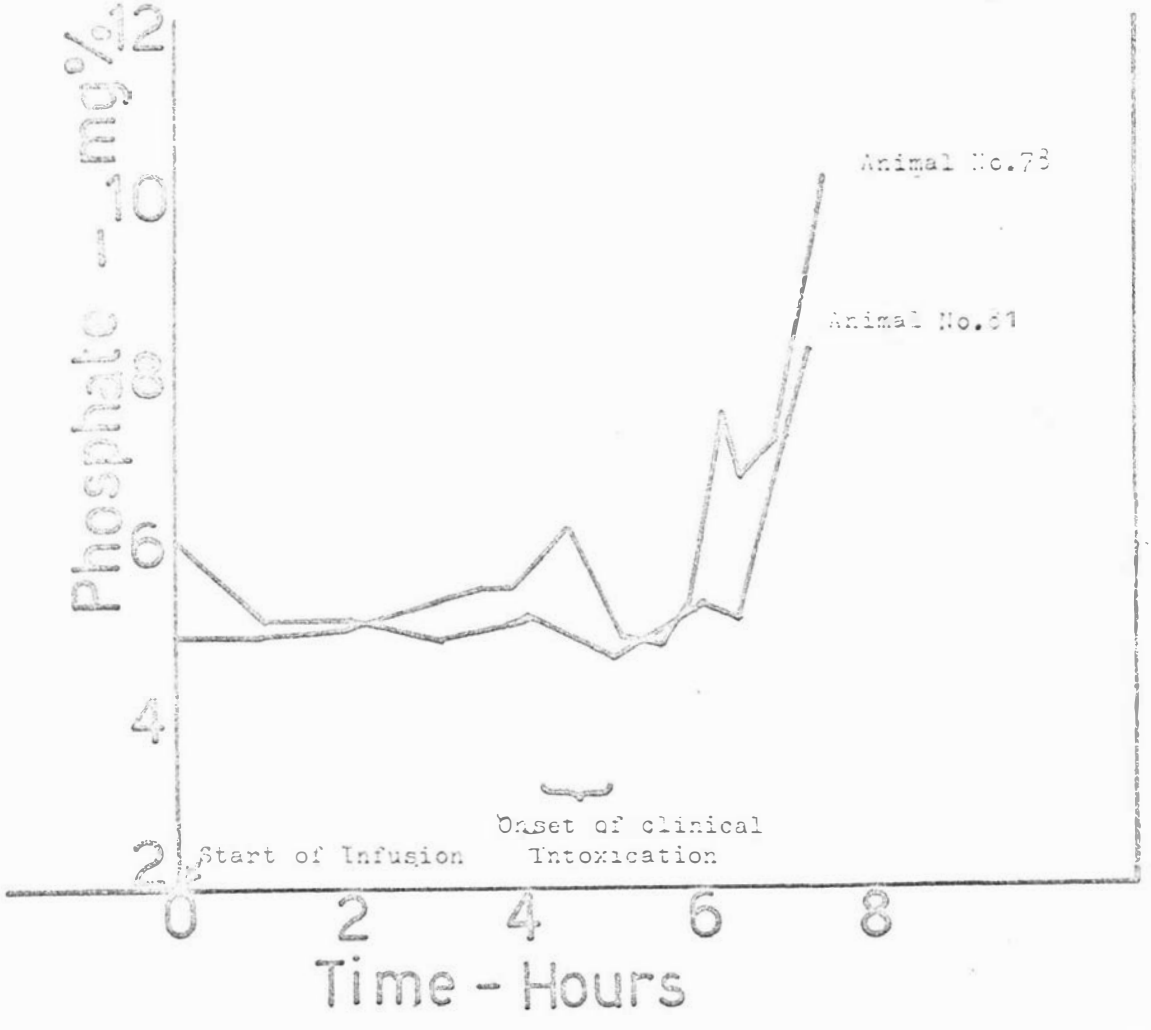
Results:

Again in these animals the levels of sodium, potassium and chloride did not show any consistent alterations during the course of intoxication. Inorganic phosphate was markedly elevated in blood samples taken from these animals late in the course of intoxication. The elevation only occurred in samples taken less than half an hour before death, even in animals where there were clinical signs of intoxication for an hour or more prior to death. The values for these blood constituents in the present animals are tabulated in Appendix 14 and the pattern of change in the blood inorganic phosphate levels of these animals during intoxication is illustrated in Figure 18.1.

Discussion

The largely negative results which were obtained in these investigations into the effect of epsilon toxin intoxication upon the concentrations of some of the important inorganic ions in plasma

Figure 13.1:
The Effect of Experimental Enterotoxaemia on Blood Levels of Inorganic Phosphate



were not altogether unexpected. These ions tend to diffuse freely to and from the blood stream so that an equilibrium exists between the concentration of these ions in the plasma and their concentration in extracellular tissue fluid. Thus in a disease where the primary lesion appears to be severe vascular endothelial damage, which does not really affect this equilibrium, there is unlikely to be any marked change in the levels of these ions in plasma.

Nevertheless the fact that such changes did not occur does provide some information which is of value in increasing our understanding of the action of epsilon toxin on tissue function. The maintenance of 'normal' concentrations of these ions in plasma is largely dependent upon the excretory efficiency of the kidney and the fact that the presence of relatively constant levels of these ions in the plasma of intoxicated animals, even when they had received considerable quantities of intravenously administered sodium and chloride ions, suggests that the kidneys ability to excrete these ions was not impaired by the action of epsilon toxin. Thus, although other workers have shown that this toxin causes a reduction in renal blood flow and have suggested that the glycosuria of enterotoxaemia may be associated with functional disturbances in the renal proximal tubules, it does not appear that other energy-dependent renal functions such as the 'Sodium pump' are markedly affected by epsilon toxin.

Since the maintenance of high concentrations of potassium within cells relative to the concentration of this ion in the extracellular fluid is also an energy dependent process and in addition requires intact plasma membranes it appears that in the absence of consistent alterations in plasma potassium concentrations in intoxicated animals, epsilon toxin has little direct effect upon the energy metabolism of cells or upon their plasma membranes. This provides some *in vivo* confirmation of the *in vitro* studies on tissue function described in Chapter 14.

Although there was a terminal rise in blood inorganic phosphate levels in the animals which received enteric origin toxin,

this did not appear to be associated in any way with the hyperglycaemia and probably reflects the mobilisation of phosphate to counter the metabolic acidosis. It is thus not the result of any uncoupling of oxidative phosphorylation. Due to the short clinical course of intoxication in the animals which received parenterally administered toxin any change in plasma inorganic phosphate levels would be small and would have been masked by the variability introduced by the deproteinisation procedures used prior to the automated analyses (see Chapter 15).

The fact that the other ions were unaffected in a situation where there was a moderate to severe metabolic acidosis indicates that the major ionic shifts which produced the fall in pH were between bicarbonate and lactate and that there was insufficient time for compensatory mechanisms to exert an effect. The metabolic acidosis is therefore largely uncompensated.

The changes which occur in the pattern of excretion of the inorganic ions in urine will be described and discussed in Chapter 21.

Conclusions

1. No consistent changes in the plasma levels of sodium, potassium or chloride occurred during the course of intoxication following the parenteral administration of epsilon toxin or the experimental induction of enterotoxaemia. The severe vascular damage therefore does not affect the equilibrium which exists between these ions in the bloodstream and the extracellular fluids.

2. The administration of considerable amounts of sodium and chloride ions to these animals did not markedly affect the plasma concentrations of these ions. This implies that renal control of the homeostasis of these ions is unaffected by epsilon toxin. However, there is no evidence of compensatory sodium retention to balance the acidosis produced by rising pCO_2 and lactate concentrations in the blood.

3. Levels of plasma inorganic phosphate rose late in the course of experimentally induced enterotoxaemia. The change in the concentration of this ion was unassociated with the development of hyperglycaemia and was probably a result of the acidosis, rather than an indication of interference with glycolysis by the action of the toxin.

4. The lack of change in plasma potassium concentrations during intoxication suggests that there is little or no loss of intracellular potassium. This indicates that there is no damage to plasma membranes or their energy-requiring ion transport mechanisms and strongly suggests that the overall cellular energy metabolism is normal.

Chapter 19: THE PATTERN OF CHANGES IN THE LEVELS OF BLOOD GLUCOSE, PYRUVATE, LACTATE, ALPHAKETOGLUTARATE AND KETONE BODIES FOLLOWING THE ADMINISTRATION OR ABSORPTION OF CL. PERFRINGENS TYPE D EPSILON TOXIN IN LAMBS.

Undoubtedly the most widely known of the biochemical changes associated with epsilon toxin intoxication and enterotoxaemia are the hyperglycaemia and glycosuria which occur. The pattern of change in glucose in blood and urine have been studied by a number of investigators but the hyperglycaemia does not appear to have been studied with the aim of elucidating its pathogenesis. It has been suggested by Jubb & Kennedy (1963) that it may be associated with a mobilisation of hepatic glycogen reserves.

In the absence of information on possible changes in the plasma levels of some of the compounds which result from the metabolism of glucose, e.g. lactate and pyruvate it is difficult to ascertain the possible basis of the hyperglycaemia which occurs in enterotoxaemia. Lactate values provide information upon whether glucose is being passed through the glycolytic steps of the Emden Meyerhoff pathway as far as pyruvate. When considered in association with pyruvate, lactate also provides information on the oxidative capacity of the tissues since the pyruvate:lactate ratio reflects the extramitochondrial redox potential (DPNH:DPN ratio) of the cell. This in turn is influenced by factors such as respiratory and circulatory function.

It has already been stated that for technical reasons it was not possible to obtain useful information on blood pyruvate levels in lambs which received parenterally administered epsilon toxin and in the present chapter this metabolite will be studied in animals where enteric origin toxin was used to produce intoxication. In these animals pyruvate can be studied in relation to both lactate and glucose.

Pyruvate occupies a key position in energy metabolism and there is an increasing awareness of the usefulness of estimating this compound in a number of clinical conditions where there is an interference with energy metabolism. Some of the conditions in which there is an increased production or decreased utilisation of pyruvate will be dealt with more fully

in the discussion section of this chapter.

Changes in the blood values for some of the other compounds involved in glucose metabolism have not been as widely studied as pyruvate and lactate in disease conditions, but it was considered that useful information might be obtained if one of the intermediate compounds which occurs late in the oxidative metabolic pathways could be estimated. This would provide information about the functional integrity of the Tricarboxylic cycle. One such substance, which can be conveniently estimated in blood is alpha-keto glutaric acid and it was decided to determine it in samples from some of the present animals.

Because some conditions in which hyperglycaemia and neurological disturbances occur e.g. diabetes mellitus, are also associated with marked ketonaemia it was desirable to examine blood samples from intoxicated animals for the presence of ketone bodies. This, in association with the temporal relationship between the hyperglycaemia and the onset of clinical signs of intoxication, would provide information on both the basis of the neurological signs of intoxication and also upon the possible relationship between hormones, such as insulin, and the hyperglycaemia of enterotoxaemia. There do not appear to have been any studies on the influence of either insulin or nutritional status on the changes in blood glucose levels induced by epsilon toxin but a consideration of these factors can be conveniently left until Chapter 20. Similarly the alterations in all urinary constituents including glucose can best be studied together and this has been deferred until Chapter 21.

Alterations in the Levels of Glucose and Lactate in the Blood of Lambs Following the Intravenous Administration of Epsilon Toxin

Materials & Methods:

The animals employed in these experiments have been described in Chapter 6. The methods used for the collection of blood samples and the automated estimation of glucose and lactate have been described and discussed in Chapter 15.

Results:

No significant alterations in the blood levels of either glucose or lactate occurred in the animals which survived the administration of epsilon toxin, nor were there any significant changes in these components in the interval between the administration of toxin and the onset of clinical signs of intoxication in the animals which succumbed.

Following the onset of clinical signs of intoxication there was a rapid rise in the concentration of glucose in blood samples taken from these animals. In some instances the level of glucose in the blood more than doubled in the interval between the administration of toxin and the death of the animal.

Similarly the levels of lactate in the blood rose sharply after the onset of clinical signs of intoxication and up to four fold increases in lactate content were found in some of the 'Terminal' blood samples .

The changes which occurred in mean values for glucose and lactate levels in arterial and venous blood samples from these animals during the course of intoxication are shown in Table 19.1 where they are compared with the preinoculation values. The pattern of the changes which occurred in these constituents are also illustrated by the histograms in Figure 19.1a. The basic data is presented in Appendices 12 & 13.

Table 19.1: The Effect of Intravenously Administered Epsilon Toxin on the Level of Blood Glucose and Lactate in Lambs.

Means ± 1std. dev.		Time of Sampling					
		Pre-inoculation	n=	Onset of Intoxication	n=	Time of Death	n=
Glucose	Venous	82.0 ± 22.3	26	127.3 ± 37.0	15	175.6 ± 66.9	24
	Arterial	90.5 ± 21.3	25	143.0 ± 68.0	8	181.3 ± 65.7	14
Lactate	Venous	64.4 ± 32.8	23	103.9 ± 61.0	15	173.1 ± 68.4	25
	Arterial	62.2 ± 27.7	25	78.4 ± 54.0	7	168.5 ± 53.3	14

t Tests: Venous glucose preinoc:Initial - $p = < .05$; preinoc:Terminal - $p = < .001$

Venous Lactate preinoc:Initial - $p = < .05$; preinoc:Terminal - $p = < .001$

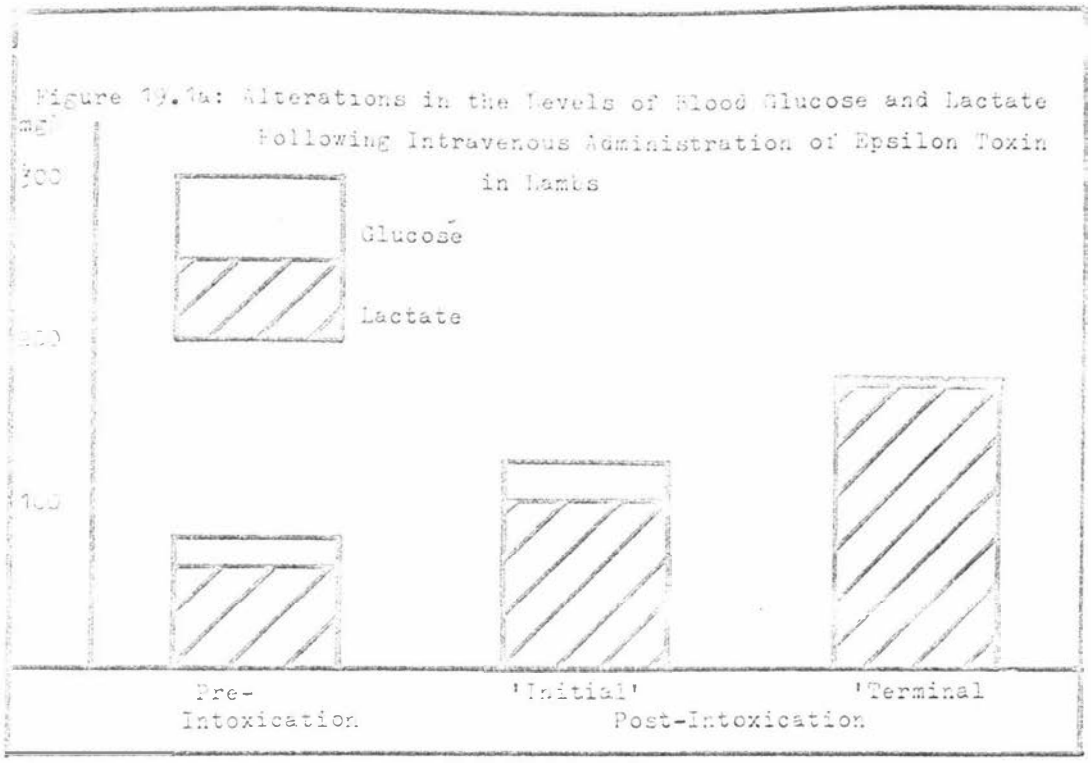


Figure 19.1a: Alterations in the Levels of Blood Glucose and Lactate Following Intravenous Administration of Epsilon Toxin in Lambs

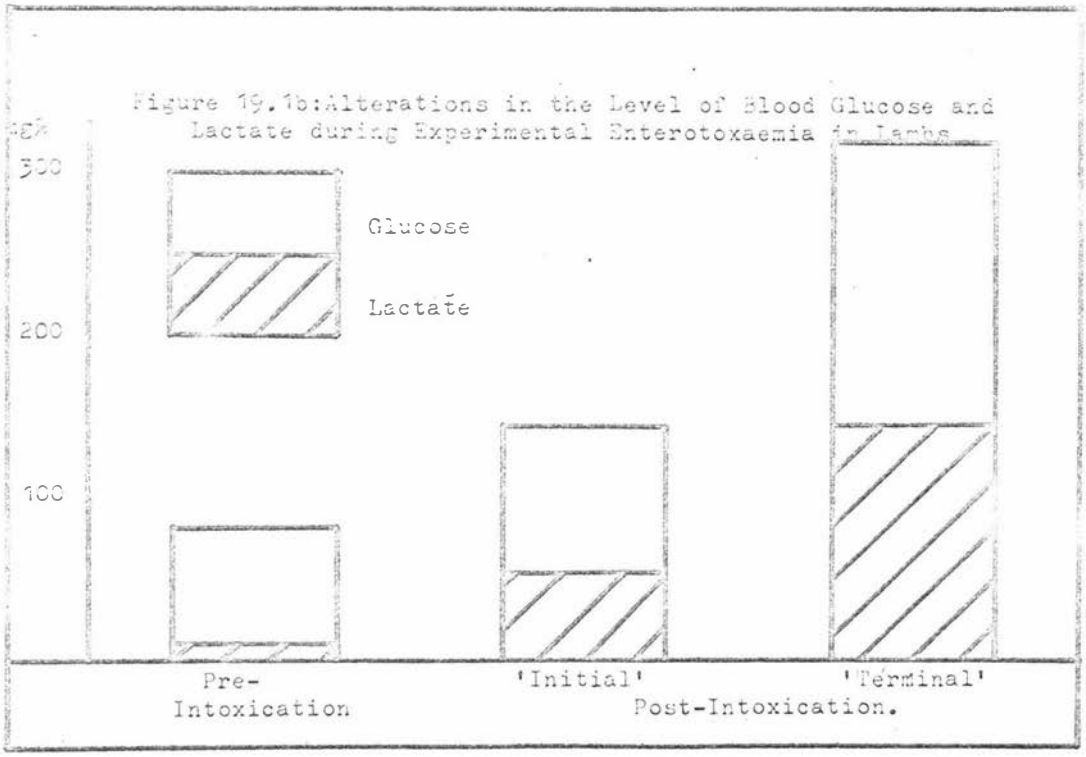


Figure 19.1b: Alterations in the Level of Blood Glucose and Lactate during Experimental Enterotoxaemia in Lambs

Figures 19a & b are based on mean values for venous blood samples.

Alterations in the Blood Levels of Glucose, Lactate, Pyruvate, Alphaketoglutarate and Ketone Bodies During Experimental Cl. Perfringens Type D Enterotoxaemia

Materials & Methods:

Blood samples were collected from the animals which received 'enteric-origin' toxin as described in Chapter 6. It should be noted that lamb No. 85 received 40 units Insulin B.P. I/V 15 minutes before death. This will be referred to again in Chapter 20. Manual methods were used for the evaluation of these constituents in the present series of samples and spectrophotometric measurements were made at the appropriate wavelength with a Bausch & Lomb 'Spectronic 20' spectrophotometer.

The following are descriptions of the methods used. Where appropriate they are manual versions of the automated procedures used for the samples from animals which received parenterally administered toxin.

Glucose:

Glucose was estimated by the glucose oxidase-peroxidase-dianisidine method using the reagents and procedure of the Sigma kitset no. 510A and the associated Technical bulletin 510 (Sigma Chemical Co. St. Louis, U.S.A.) Underproteinised plasma which had been separated from the blood sample by centrifugation, as soon as the samples were taken from the animal, was used. Plasma samples were stored in iced water prior to analysis.

Pyruvate:

Immediately after collection 5.0 ml of blood was added to 5.0 ml of ice cold 7% perchloric acid solution. After thorough mixing and standing for 10 minutes the supernatant was removed by centrifugation and neutralised with 5 N.KOH in the cold using 0.05% Methyl Orange as indicator. The supernatant was removed from the crystalline perchlorate deposit and pyruvate was determined using the reagents and method described in Henry (1966). This is a Lactic dehydrogenase/DPNH oxidation technique. Fresh standards containing 5 mg% of pyruvate were prepared from sodium pyruvate. These standards were treated in the same way as the samples prior to analysis. Spectrophotometric measurements were made at 340mu.

Lactate:

As soon as possible after separation, 0.1 ml of plasma was added to

0.7 ml of ice cold 7% perchloric acid solution. The supernatants were removed and neutralised as described for pyruvate. The estimation of L_+ lactate was carried out with the reagent and methods described in Bergmeyer (1963). This is a lactic dehydrogenase/DPN reduction procedure with hydrazine trapping of the formed pyruvate. Lactate standards (100mg%) were prepared from sodium dl lactate as described in Bergmeyer (p 271) and treated in the same way as the plasma samples prior to analysis. Spectrophotometric measurements were made at 340mu.

Alpha ketoglutarate:

Aliquots of the supernatants, which had been prepared for the pyruvate analyses from the blood samples of two animals, were used to measure the alpha ketoglutarate content of these samples. The method employed was that described in Bergmeyer (1963), with the exception that, after measuring the initial optical density and adding the enzyme solution, the reaction mixture was incubated at 37°C for 10 minutes, cooled in iced water and the change in optical density of the solutions at 340mu compared with that produced by a standard solution (containing 0.1mg%) of alpha-ketoglutaric acid which had been treated in the same way as the samples prior to the actual analysis. According to Henry (1966) this approach to enzymatic analyses is more reliable for routine use than methods which involve following changes in optical density with time

'Ketone Bodies'

Qualitative Rothera's tests were carried out by placing a drop of each plasma sample on powdered nitroprusside reagent (Sodium nitroprusside 1g; Ammonium sulphate 20g; anhydrous sodium carbonate 20g) on a spotting tile and observing the colour change after 30 seconds. Quantitative analyses were carried out using the alkaline vanillin method described by Henry (1966).

Results:

There were no consistent alterations in the levels of any of these blood components from the preinoculation values until clinical signs of intoxication were evident. At that time there was a marked elevation in the levels of glucose, pyruvate and lactate in all animals. The animal which failed to develop clinical signs of intoxication did not show any consistent change in blood levels of these components. Glucose rose from

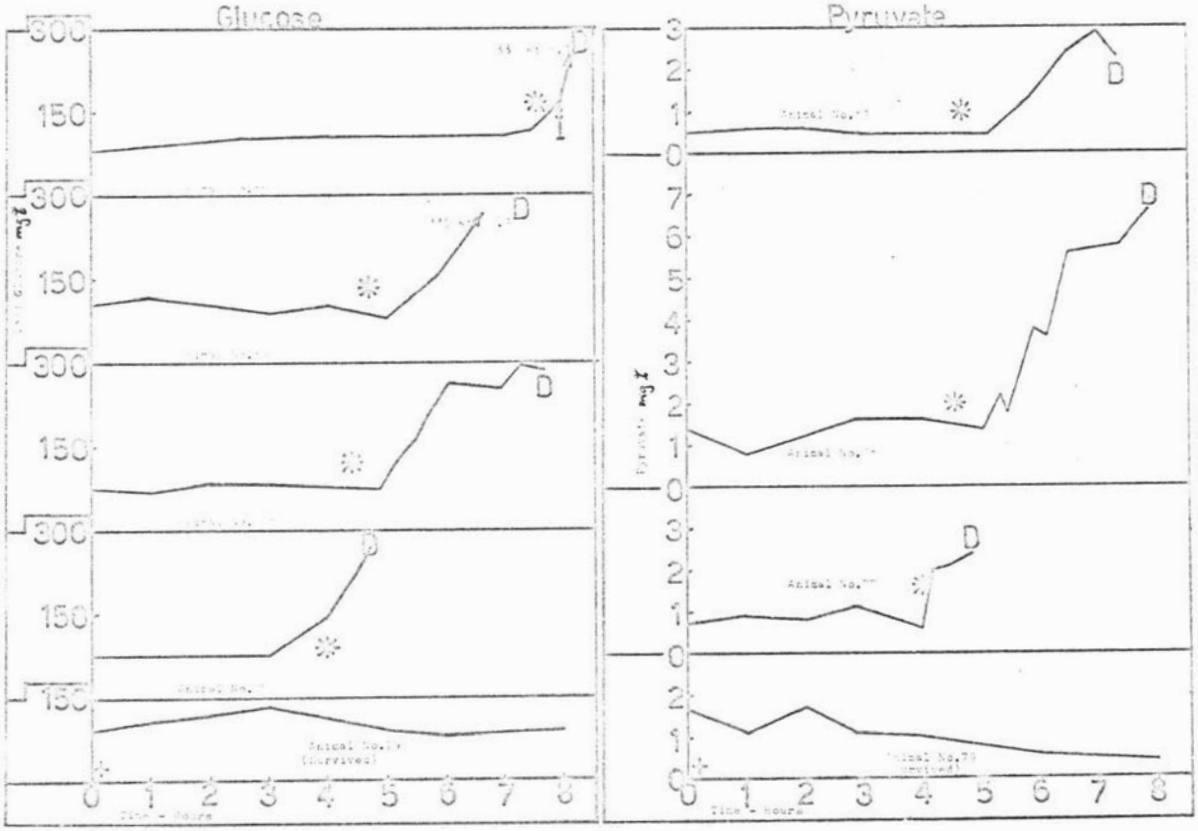
preinoculation levels of 70-100mg% to terminal values of 270-350mg%, while lactate rose from less than 20mg% to values over 100mg% in intoxicated animals. The increase in pyruvate followed a similar pattern, rising from approximately 1mg% in preinoculation samples to over 2mg% in the three animals in which it was estimated. There was no marked difference between the times at which the levels of each of these components began to rise in any individual animal although there was a slight tendency for the rise in glucose and lactate to precede that of pyruvate in two animals. No significant alterations in the levels of these components occurred in blood samples taken from the animal which survived (No. 79).

The results of the estimations of these substances are shown in Appendix 14 and the patterns obtained from these animals are illustrated in Figure 19.2. In addition a histogram, Figure 19.1b, has been prepared, of the changes in mean blood glucose and lactate levels which occurred, for comparison with the one for the animals which received parenterally administered toxin (Figure 19.1a).

Alphaketoglutarate normally occurs at very low concentrations in blood but in the two animals in which it was estimated there was a detectable rise in the level of this metabolite in blood samples taken late in the course of intoxication. In one animal (no. 78), the level in plasma, taken before the onset of clinical signs of intoxication developed, fluctuated between 0.05 and 0.09mg%. The first detectable rise in alphaketoglutarate was to a value of 0.14mg% and this occurred at about the same time as the first detectable rise in blood pyruvate. The concentrations of alphaketoglutarate in the terminal blood samples of this animal was 0.17mg%. In the other animal (no. 81), the pre-intoxication values for alphaketoglutarate fluctuated between 0.09 and 0.18mg% and rose terminally to 0.27mg%. The analytical results for alphaketoglutarate are also tabulated in Appendix 14 , along with those of glucose, pyruvate and lactate.

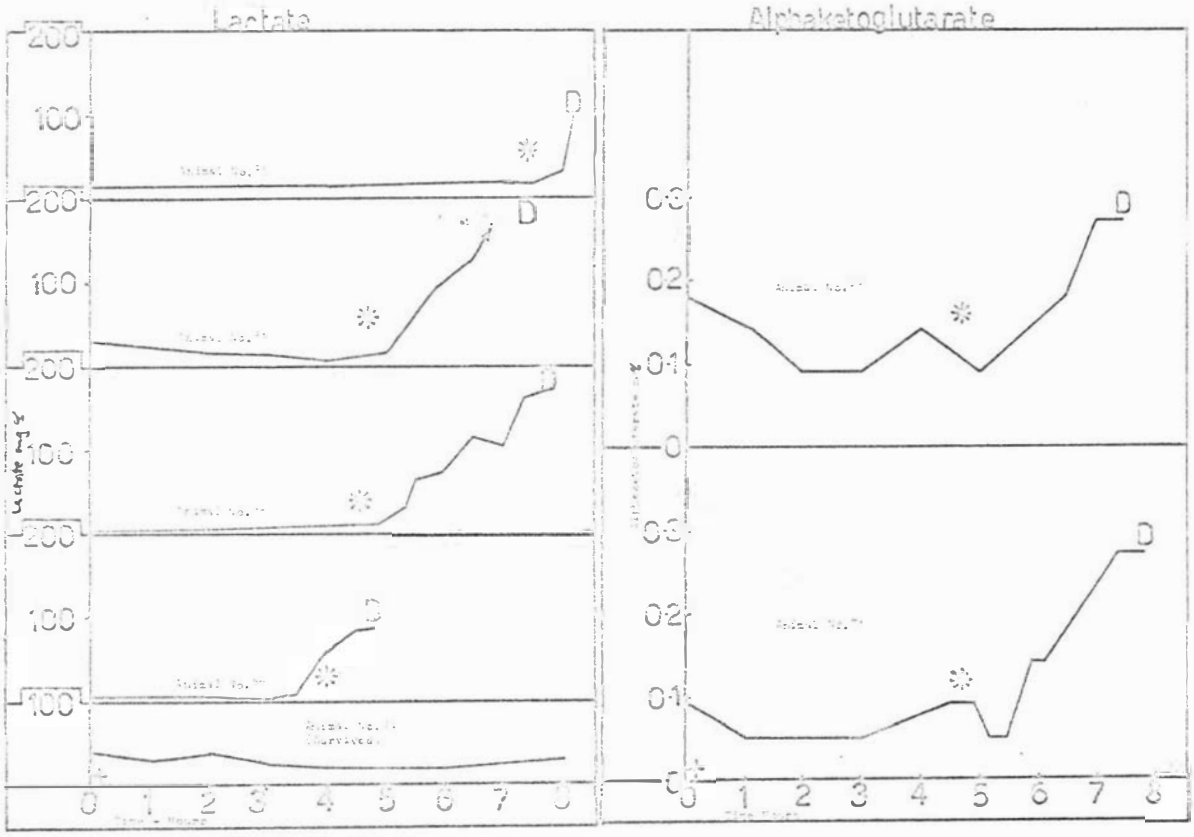
The results of the qualitative Rothera's tests for ketone bodies, carried out on plasma samples from these animals, were all negative with the exception of the tests carried out on the last three samples taken before death in animal no. 78 and the terminal sample from animal no. 81.

Figure 19 a: The Effect of Enterotoxaemia on Blood Levels of Glucose, Pyruvate, Lactate & Alphaketoglutarate



*= Start of intraduodenal Infusion
✻= Onset of Clinical Signs of Intoxication
D= Time of Death
I = Insulin Administered – see Chapter 20.

Figure 19.2b: The Effect of Enterotoxaemia on Blood Levels of Glucose, Pyruvate, Lactate & Alphaketoglutarate



+= Start of Intraduodenal Infusion
*= Onset of Clinical Signs of Intoxication
D = Time of Death
I = Insulin Administered—see Chapter 20.

In the latter samples there was a slight positive reaction which was readily distinguishable from the negative results of other samples on the same spotting tile. The lower limit of sensitivity of this test is generally taken to be 10mg% for acetone. However, when quantitative estimations of ketone bodies were carried out using the alkaline vanillin method the values for ketone bodies in all samples lay between 0 and 2.2mg%, expressed as acetone.

Glucose and Lactate Values in Post Morten Blood Samples from a Field Case of Enterotoxaemia

Materials & Methods:

The lamb from which samples were obtained was the acute case of enterotoxaemia described in Chapter 13. Blood was collected from the heart during necropsy, and estimations of glucose and lactate were made by the automated procedures employed for the samples from lambs which received parenterally administered toxin.

Results:

Blood glucose was 600mg% and lactate 820mg%.

Discussion

The fact that an elevation in blood glucose occurs during epsilon toxin intoxication and in experimental enterotoxaemia has been well documented by other workers and the time sequence of the change in blood sugar level in relation to the onset of clinical signs of intoxication have been described by Bullen & Scarisbrick (1957). In the present experiments with animals receiving parenterally administered or enteric origin toxin it was found that, in all instances, there was clinical evidence of intoxication before blood glucose levels were markedly elevated although Bullen & Scarisbrick appear to have found some elevation in blood sugar before obvious clinical intoxication occurred. The results of the present experiments suggest that the clinical signs are not the direct result of the elevated blood sugar levels or of concommitant changes in blood pyruvate, lactate or pH. However, in view of the lack of change in these metabolites in animals which survived experimental epsilon toxin intoxication and enterotoxaemia, it appears that the alterations are intimately

associated with the development of intoxication.

Although investigations of this type provide information on the time sequence and extent of the changes in blood glucose they do not, of themselves, provide any clue to the possible pathogenesis of the hyperglycaemia. It is only by studying related features, such as changes in intermediate products and the effect of other factors on the blood glucose response to toxin, that an insight can be gained into whether the hyperglycaemia is the result of increased mobilisation of glucose or some interference with its storage or metabolism.

Before leaving the discussion of blood glucose itself some mention must be made of the pre-inoculation levels of glucose in the animals which received enteric-origin toxin. Although these animals were 8 months old and had been weaned and at pasture for several months it can be seen that their blood sugar levels were similar to those of the 2-3 week old lambs and were thus considerably higher than the published values for adult sheep. The most likely explanation for this finding is that, because of severe drought conditions at the time, these animals had been in very poor condition and were therefore housed for several weeks prior to use. During this period they were fed on a ration high in concentrates and their body condition was improving markedly. Under these circumstances a higher than normal blood glucose level could have been expected.

In regard to the nutritional regime of these animals it is worthwhile emphasising the importance of using an analytical procedure which is specific for L_+ lactic acid when carrying out metabolic studies on ruminants. Under circumstances such as those mentioned above, where animals were on a high plane of nutrition with ample carbohydrate in the diet, blood levels of total lactate were liable to be markedly influenced by D-lactate which had been absorbed following microbial breakdown of the dietary carbohydrate in the rumen (Medway et al., 1969).

Interpretation of the results of biochemical estimations, carried out on post-mortem blood samples, is difficult as very rapid changes can occur in levels of labile components after death occurs. Thus, the extremely high levels of lactate in the blood from the field case of

enterotoxaemia are almost certainly largely due to post mortem glycolytic activity of erythrocytes. It is probable, however, that the elevated blood glucose level in this animal results from antemortem changes since true glucose tends to be lost, rather than gained, from the blood after death.

It was of considerable interest, although not unexpected, to find that levels of some of the intermediate products of glucose metabolism became elevated in association with the development of hyperglycaemia. The fact that they are elevated however rules out any possibility that the hyperglycaemia might be due to a disturbance of the Embden Meyerhoff pathway of glycolysis.

It is worthwhile considering some of the possible factors which can influence the levels of these metabolites in the blood of animals and man. In most clinical situations, particularly in ruminants, elevations in blood pyruvate have tended to be considered to result from conditions where there is an interference with the conversion of pyruvate to acetyl coenzyme A. This has been shown to occur in starvation, malabsorption syndromes and hepatic damage in a number of species (Medway et al., 1969). In addition there are conditions where there is believed to be an interference with thiamin availability or utilisation and thus of coenzyme function e.g. polioencephalomalacia of calves (Pill, 1967); or defective lipoic acid metabolism as in Subacute Necrotising Encephalomyelopathy of man (Clayton et al., 1967; Pena et al., 1970). Elevations of pyruvate occur in all of these conditions and it is interesting to note the similarity of the distribution of lesions in the latter disease to the distribution of the lesions in Focal Symmetrical Encephalomalacia of sheep.

However, it must be realised that elevation of blood pyruvate can also occur due to the increased formation of pyruvate since the oxidative portion of the pathways of glucose metabolism tend to be the limiting factor in normal animals. Thus there is an elevation in the level of blood pyruvate after feeding or exercise (Medway et. al., 1969) and under conditions of excess pyruvate formation it is rapidly converted to lactate prior to oxidative decarboxylation. The intravenous administration of glucose causes a rapid rise in blood pyruvate and it can therefore be seen

that, in a condition like enterotoxaemia where blood sugar is elevated, pyruvate would also be correspondingly elevated and that this does not necessarily indicate any reduction in the animal's ability to utilise this compound.

The same holds true for lactate since, as seen above, this compound will automatically increase in association with increased levels of glucose in the circulation. However, depending upon the redox state of the cell, the ratio between these two substances will change. In the resting state the pyruvate:lactate ratio is usually 1:4 or 1:5 and this may be increased to 1:20 after severe exercise, when DPNH oxidation is the limiting factor.

Apart from these largely physiological responses in the levels of lactate, blood levels of this substance will be greatly increased if there is any interference with normal oxidative processes as would occur in pneumonia or cardiac failure. Thus it can be seen that the high levels of lactate in the present animals may have been due to a number of different factors acting in combination. On the one hand there would have been an increase in lactate and pyruvate formation due to the high levels of blood glucose and on the other there was the severe pulmonary oedema in some animals affecting respiratory function, plus haemoconcentration, myocardial oedema and vascular damage, reducing circulatory efficiency. The end result of this combination of factors was a very high pyruvate: lactate ratio in intoxicated animals, which, in one of the animals which received enteric-origin toxin, rose to 1:80. In addition, the high levels of lactate in the blood led to the severe metabolic acidosis with the fall in plasma bicarbonate concentration and low blood pH values described in Chapter 16.

Although less is known about the clinical significance of elevated levels of alphaketoglutarate this is also almost certainly associated with an overall increase in the metabolism of glucose and reflects the limiting nature of some of the later steps in the oxidative pathways. However, the fact that some elevation of this metabolite occurred suggests that there was little interference with the Tricarboxylic Acid Cycle. Further, since the elevation in alphaketoglutarate level was not excessive, it appears to be unlikely that there was any interference with the terminal

electron transport chain of enzymes.

The different findings in the qualitative and quantitative tests for ketonebodies are of interest since it appears that the most likely explanation for this difference is related to factors which have already been discussed in this chapter. Since the Rothera's test is generally regarded as only being capable of detecting levels of ketone bodies of 10mg% or more in blood or other tissue fluids it might have been expected that the more sensitive alkaline vanillin test would react in all samples which yielded a positive result to the Rothera's test but this was not the case in the present instance. However, the alkaline vanillin test is a very specific test and will only detect acetone and acetoacetic acid, while the Rothera's test does show false positive reactions when high levels of keto acids such as pyruvate are present in the sample (Medway et al., 1969). The colour produced in the latter instance is stated to be royal blue rather than the purple which is produced with the ketone bodies. Because the reactions in the positive samples in the present instance were slight it was difficult to determine whether the colour produced was in fact blue or purple but the impression was that the colour tended to be more blue than usual. Thus it appears that the positive results obtained with the Rothera's test was probably due to the presence of higher than normal levels of pyruvate in the samples and the fact that this was a false positive reaction is borne out by the negative findings with the alkaline vanillin test.

Conclusions

1. A severe hyperglycaemia occurs following the intravenous administration of Cl. perfringens type D epsilon toxin and in experimental and field cases of enterotoxaemia.

2. The blood glucose level does not change until at or about the time that clinical signs of intoxication are evident, and the change does not occur following the administration of non-lethal amounts of toxin.

3. Associated with the hyperglycaemia, there is a concommitant rise in the concentrations of lactate, pyruvate and alphaketoglutarate in blood and a rise in the pyruvate:lactate ratio.

4. The changes in the latter group of components appear to be due to an increase in their formation in association with the increased level of circulating glucose. There is no evidence to suggest that they result from an interference with the catabolic metabolism of glucose.

5. The elevated levels of circulating lactate are responsible, in part, for the fall in blood pH and the displacement of plasma bicarbonate which occurs during intoxication. That is, a metabolic acidosis exists in affected animals.

6. The use of an analytical method which is specific for L_+ lactate is important when studying acidoses which may be of metabolic origin in ruminants, since large quantities of D- lactate may be absorbed from the rumen when animals are on a high carbohydrate diet.

7. There is no evidence of ketosis in experimental enterotoxaemia although false positive results may be obtained from the Rothera's (nitroprusside) qualitative test for ketone bodies, due to the high levels of keto-acids in the plasma of intoxicated animals.

Chapter 20: THE INFLUENCE OF INSULIN AND STARVATION UPON THE HYPERGLYCAEMIA INDUCED BY CL. PERFRINGENS TYPE D EPSILON TOXIN.

Jubb & Kennedy (1963) state that there is some experimental evidence to suggest that the hyperglycaemia which occurs in Cl. perfringens type D enterotoxaemia may be the result of increased glycogenolysis in the liver. If this is in fact the case then the blood sugar response during the course of this intoxication should be profoundly influenced by the extent of the hepatic glycogen reserves, and thus the short term nutritional status of the animal.

In the field, enterotoxaemia tends to occur most frequently in animals which are in good nutritional condition. Under these circumstances hyperglycaemia could be expected to occur regularly if the pathogenesis of this change is basically a mobilisation of hepatic glycogen. On the other hand, if the hyperglycaemia results, either wholly, or in part, from either a failure of the intoxicated animal to utilise glucose or an interference with one or more of the endocrine factors important in glucose homeostasis then the nutritional status of the animal would have little or no influence on the blood glucose changes following the administration of epsilon toxin. It was therefore important to determine whether depletion of the hepatic glycogen stores by starvation or other means, prior to the administration of toxin, would alter the animal's response to the toxin.

One of the important factors in maintaining relatively constant levels of glucose in the blood is the hormone insulin, produced by the beta cells of the pancreatic Islets of Langerhans. Insulin influences carbohydrate metabolism in a number of different ways. Perhaps the most important of these, in the short term, is the hormone's role in altering the permeability of the plasma membranes of skeletal muscle and adipose tissue cells to glucose thus allowing it to enter the cells where it is rapidly converted to glycogen or triglyceride. In conditions where there is a deficiency of insulin or an interference with its action, glucose tends to accumulate in the circulation and hyperglycaemia occurs. On the other hand, increased levels of insulin enhance the transfer of glucose into cells and this results in a severe hypoglycaemia which is

compensated for to some extent by a mobilisation of the hepatic glycogen reserves under the influence of glucagon. This latter hormone is produced by the alpha cells of the pancreatic Islets of Langerhans and released in response to low blood glucose levels. It is therefore possible to use insulin to enhance indirectly the effect of starvation in depleting hepatic glycogen reserves in an acute experiment. The rapid depletion of hepatic glycogen produced by a combination of starvation and insulin administration also tends to assist in overcoming side effects which could be produced by prolonged starvation alone. Since insulin is rapidly broken down in the animal body it does not persist for more than a few hours, unless 'adjuvants' are used, and its administration will therefore not interfere with subsequent experiments.

By studying the ability of insulin to influence the hyperglycaemia produced by the administration of epsilon toxin to unstarved animals it should also be possible to obtain some information on whether the toxin influences the action of this hormone on plasma membranes although there are certain limitations to this approach which will be dealt with in more detail in the discussion section of this chapter.

In the present experiments the response of lambs to insulin alone was compared with their response to similar doses of insulin plus toxin or toxin alone to obtain information on the possible interaction of these substances.

The Influence of Insulin and Starvation upon the Blood Glucose Response in Lambs Following the Parenteral Administration of Epsilon Toxin.

Materials & Methods:

A series of 2-3 week old female lambs similar to those described in Chapter 6 were screened for the presence of epsilon antitoxin and 8 lambs which had less than 0.08 units of epsilon antitoxin/ml. of serum were selected for use in the present experiment.

All of these animals were removed from their dams and held in small pens until used. One pair of lambs (No's. 92 & 96) was deprived

of food for 3 hours. A blood sample was then taken from these animals and 40 units of Insulin B.P. (Burroughs Wellcome & Co. (N.Z.) Ltd.) was administered intravenously to each lamb. These two lambs were then deprived of food until the second stage of the experiment was undertaken on the following day. They were therefore known as the 'starved' animals.

The other six lambs were given two feeds of an artificial milk preparation, 'Fostermilk' (Glaxo Laboratories, Palmerston North, N.Z.), at 8 hour intervals on the first day and a further feed on the morning of the following day, 2 hours before they were used.

In the second stage of the experiment blood samples were taken from all the animals. The 'starved' lambs (Nos. 92 & 96 above) were then each given 1.5 mg of batch CWD epsilon toxin in 10 ml saline intravenously. The same dose of toxin was also given to a further pair of lambs (Nos. 97 & 91) by the same route. The latter animals comprise the 'toxin' group. The third pair of lambs (Nos. 89 & 94) were also given the same dose of epsilon toxin intravenously but in addition they were given 40 units of Insulin B.P. I/V at the same time as the toxin. These were known as the 'Toxin/Insulin' group. Finally, the fourth pair of lambs (Nos. 90 & 93) were given 40 units of Insulin B.P. intravenously.

These treatments are summarised below:

Lambs	Group	First Day		Second Day		
		Fed	Insulin	Fed	Insulin	Epsilon
92 & 96	'starved'	No	40 units	No	Nil	1.5 mg
97 & 91	'Toxin'	Yes	Nil	Yes	Nil	1.5 mg
89 & 94	'Toxin/Insulin'	Yes	Nil	Yes	40 units	1.5 mg
90 & 93	'Insulin'	Yes	Nil	Yes	40 units	Nil

At intervals following the administration of toxin or hormone, blood samples were taken from the jugular veins of all animals. The samples were collected in heparinised 'Vacutainers' as described in

Chapter 15. The plasma was separated from the blood, by centrifugation as soon as possible after collection. Plasma samples taken from the 'starved' animals on the first day were held in a refrigerator at 4°C overnight. Estimation of blood glucose were carried out by the manual glucose oxidase method described in Chapter 19.

Results:

'Starved' lambs:

The administration of insulin alone did not produce marked clinical disturbance. Following the administration of the insulin the blood glucose levels of these animals fell rapidly. The decrease was detectable in blood samples taken 10 minutes after the hormone was given and fell to very low levels (less than 20 mg%) within an hour. It remained low for at least two hours but had risen to 74 mg% by the time 6½ hours had elapsed following administration of the hormone. It was still at this latter level on the following morning in one animal. The blood glucose level of the other animal which had received insulin was not followed for longer than 1 hour after administration but the blood glucose had risen to 100 mg% by the following morning.

Following the administration of epsilon toxin to these animals on the second day, one of the animals developed clinical signs of intoxication and died after 50 minutes. At the time that clincal signs of intoxication occurred there was a transient rise in blood glucose level from the preinoculation figure of 100 mg% to a value of 187 mg% but this rapidly fell again and remained at 91 mg% until death occurred. The blood glucose level of the other animal remained at 100 mg% or less throughout the course of intoxication and the animal died one hour after the administration of the toxin.

'Toxin' lambs:

The preinoculation blood glucose levels of these lambs were 94 120 mg% respectively. Following the administration of epsilon toxin, clinical signs of intoxication developed and a typical hyperglycaemic response was obtained. The blood glucose levels rose progressively throughout the course of intoxication to terminal values of 382 and 460mg%

and the animals died 45 and 85 minutes after the administration of the toxin.

'Toxin/Insulin' lambs:

The pre-inoculation blood glucose levels of these lambs were 100 and 216 mg%. One animal had a relatively short survival time of 50 minutes following the administration of the toxin and insulin. During intoxication the blood glucose level rose rapidly and reached a terminal value of 450mg%. In the other animal, there was a considerable lag (1 hour) before the onset of clinical signs of intoxication. During this period the blood glucose level progressively fell to a very low level (13mg%). Following the onset of clinical signs of intoxication however the blood glucose level rose rapidly to a terminal level of 280mg% 1½ hours after toxin administration.

The results of these experiments are tabulated in Table 20.1 and illustrated by the graphs in figure 20.1.

The Effect of Insulin upon the Hyperglycaemia which Occurs in Experimental Cl. perfringens Type D Enterotoxaemia.

Materials & Methods:

The 2-3 week old female lamb No. 85 which was cannulated and used in the experiments described in Chapter 6 was given 40 units of Insulin B.P. intravenously, 30 minutes after the onset of clinical signs of intoxication i.e. 15 minutes before the death of the animal.

The blood samples taken from this animal and the analytical procedures used for estimating the various blood constituents of this animal have already been described in Chapter 19.

Results:

The blood glucose values obtained from this animal during the course of intoxication have been given in Chapter 19. It is sufficient to state here that for several hours prior to the onset of clinical signs of intoxication the blood glucose level ranged from 100 to 108 mg%. When

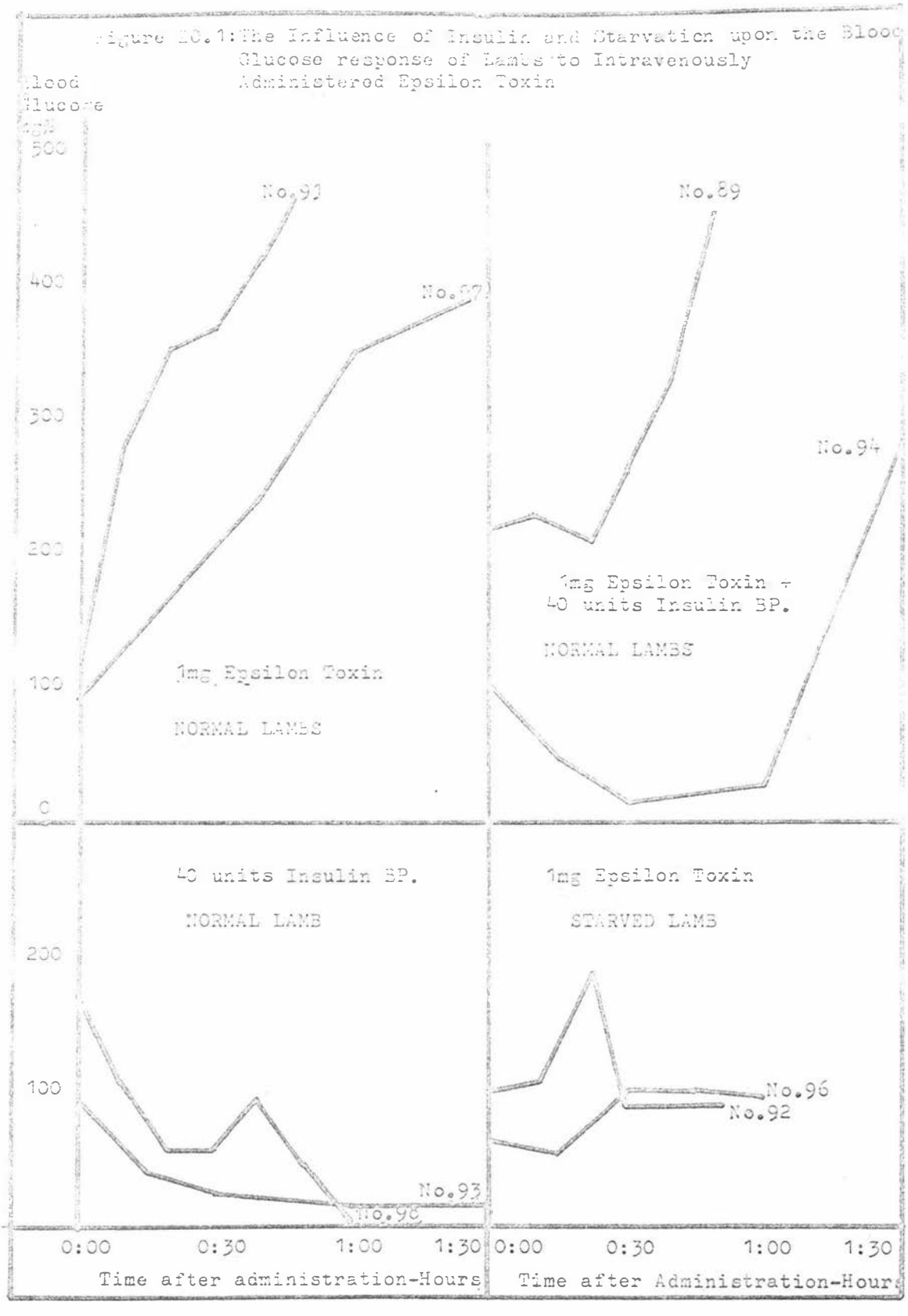


Figure 10.1: The Influence of Insulin and Starvation upon the Blood Glucose response of Lambs to Intravenously Administered Epsilon Toxin
Blood Glucose
mg%
500
400
300
200
100
0
No.91
No.97
1mg Epsilon Toxin
NORMAL LAMBS
No.89
No.94
1mg Epsilon Toxin + 40 units Insulin BP.
NORMAL LAMBS
40 units Insulin BP.
NORMAL LAMB
200
100
No.93
No.98
1mg Epsilon Toxin
STARVED LAMB
No.96
No.92
0:00
0:30
1:00
1:30
Time after administration-Hours
0:00
0:30
1:00
1:30
Time after Administration-Hours

clinical signs of intoxication became evident the blood glucose value was 125 mg% and 30 minutes later the hyperglycaemia was well established with a glucose level of 166 mg% in a blood sample taken just before the administration of the insulin. The animal only survived for a further 15 minutes after the insulin was given but at the time of death the blood glucose had risen to 355 mg%.

Discussion

The dramatic blood sugar response following administration of doses of insulin of the order of 4 units per Kg to normal 2-3 week old lambs suggests that they react to this hormone in a similar way to animals such as dogs. In the latter species the therapeutic dose of insulin recommended by the British Veterinary Codex (1965) is 5-50 units and this would be similar to the dose used in the present experiments if it were expressed on a bodyweight basis. In contrast to this it has been found that in order to produce a hypoglycaemic response in adult sheep it is necessary to use a dose level of 8 units of insulin/KgB.W. (Lapwood K. pers. comm.).

The pattern of the blood sugar response obtained when insulin and epsilon toxin were administered simultaneously was interesting. It appears that, while insulin was able to influence blood glucose levels during the prodromal stages of intoxication, it was not effective in blocking the hyperglycaemic response induced by epsilon toxin. A possible explanation for this finding would be that the toxin interferes with the action of insulin in allowing glucose to cross the plasma membrane of skeletal muscle or adipose tissue cells. It is rather unlikely that the toxin would have such a specific effect upon the hormone itself and, from the work described in previous chapters, it does not appear that epsilon toxin has a marked effect upon the morphological or functional efficiency of cell membranes. A more likely explanation would therefore appear to be that, due to vascular endothelial damage, the additional glucose in the bloodstream cannot pass to the muscle and adipose tissue cells sufficiently rapidly to enable insulin to reduce blood glucose levels. Thus it appears probable that the failure of insulin to control the hyperglycaemia induced by epsilon toxin is the result of physical factors

rather than any effect of the toxin on either the cell membrane or the action of insulin.

As far as the pathogenesis of the hyperglycaemia itself is concerned, the fact that it failed to develop in animals in which the hepatic glycogen stores had been depleted by a combination of starvation and the prior administration of insulin suggests that any elevation of blood glucose level during intoxication is dependent upon the presence of adequate hepatic glycogen reserves. On this basis and judging by the hepatic changes described in Chapter 12 it appears that, as Jubb & Kennedy (1963) have suggested, the hyperglycaemia of enterotoxaemia is due to a mobilisation of the hepatic glycogen reserves.

Conclusions

1. The dose of insulin required to produce a marked hyperglycaemic response in young lambs is similar on a bodyweight basis to that required to produce a similar effect in man and some other simple stomached animals. Lambs are therefore more sensitive to the hormone than adult sheep in which a higher dose rate is required.

2. Depletion of the hepatic glycogen reserves of young lambs by the administration of insulin, combined with starvation, results in a complete inhibition of the action of epsilon toxin on blood glucose levels. This implies that the hyperglycaemia of epsilon toxin intoxication and Cl. perfringens type D enterotoxaemia is dependent upon the mobilisation of hepatic glycogen reserves i.e. increased glycogenolysis.

3. Insulin was capable of depressing the blood sugar level of unstarved animals during the prodromal phase following the parenteral administration of epsilon toxin. It did not prevent the hyperglycaemia which subsequently occurs following the onset of clinical signs of intoxication, nor did its administration influence the course of an established hyperglycaemia in a case of experimental Cl. perfringens type D enterotoxaemia.

4. The inability of insulin to influence the blood sugar changes induced by epsilon toxin does not necessarily imply that the toxin affects

either the action of this hormone or the permeability of the plasma membranes of skeletal muscle cells. It appears more likely that the action of insulin, in lowering blood glucose levels, is rendered ineffective by interference with the transfer of glucose from the circulation to the muscle due to the vascular damage.

CHAPTER 21: THE EFFECT OF CL.PERFRINGENS TYPE D EPSILON TOXIN ON THE LEVELS OF SEVERAL SERUM ENZYMES IN SHEEP.

Alterations in the levels and proportions of a variety of enzymes have assumed considerable importance as diagnostic aids in the study of a number of human and animal diseases. In general changes in circulating enzyme levels and patterns in disease conditions are associated with their release from injured cells within the tissues. The relative proportion of the various enzymes in serum in any particular condition therefore tends to be a reflection of their concentration within the damaged tissues rather than a direct effect of the noxious agent upon the enzyme itself or its production.

A further consideration which limits the use of enzyme estimations in veterinary clinical pathology is that fact that, with a few notable exceptions, the changes which occur in the relative concentrations of the various enzymes in serum following injury to specific tissues have not been as extensively studied in domestic animals as they have in man. The use of organ specific enzyme patterns and specific isoenzymes therefore cannot be as widely used in veterinary diagnostic procedures as it is in human medicine.

In the present instance the situation was further complicated by the fact that vascular damage had resulted in loss of proteins from the bloodstream into the tissue fluids. For these reasons it was unlikely that a great deal of relevant information could be obtained by studying the changes in enzyme patterns which occurred during experimental epsilon toxin intoxication. Nevertheless it was considered desirable to estimate a number of enzymes to determine whether a consistent pattern of change emerged following the onset of intoxication. This information would be of value in extending the work of Trifonov & Todorov (1965) who have shown that epsilon toxin intoxication produces a rise in the level of glutamic-oxaloacetic transaminase and aldolase in serum.

The following enzymes were therefore estimated;

Glutamic oxaloacetic transaminase, Glutamic pyruvic transaminase,
Lactic dehydrogenase, Isocitric dehydrogenase,
Acid Phosphatase, Alkaline phosphatase.

Materials & Methods:

The methods employed for the collection of blood samples from the animals which received parenterally administered toxin have been described in Chapter 15 and the methods used for the automated determination of the various serum enzymes were also described in that chapter.

Results:

The changes which occurred following the onset of epsilon toxin intoxication were as follows;

Glutamic-oxaloacetic transaminase (SGOT)

There was fairly consistent increase in SGOT activity in serum samples taken after the onset of clinical signs of intoxication. The mean increase was of the order of 34%.

Glutamic-pyruvic transaminase (SGPT)

Again there was an elevation of the activity of this enzyme following the onset of clinical signs of intoxication. The mean rise in this instance was of the order of 44%.

Isocitric dehydrogenase (ICD)

The activity of this enzyme was also increased in serum samples from intoxicated animals. The mean increase on this occasion was of the order of 41%.

Lactic dehydrogenase (LDH)

The levels of activity of this enzyme in post-intoxication blood samples varied considerably from the preinoculation values. While the differences between serial samples from the same animal showed fairly consistent changes the results in different animals ranged from large increases to decreases to below the preinoculation level in others.

Acid and Alkaline phosphatases

Here again the values fluctuated widely, with elevations in activity in post-inoculations samples from some animals and decreases in others. These alterations did not reveal any statistically significant pattern of change for either enzyme.

The results of the post-intoxication enzyme analyses are given in Appendix 13 and these are summarised in Table 21.1.

Table 21.1

Alterations in the Activity of Some Serum Enzymes Following the Parenteral Administration of Epsilon Toxin to Lambs

Means ± 1std dev.	Time of Sampling					
	Pre-inoculation		n	Onset of Clinical Intoxication	n	Time of Death
Enzyme	No. of Animals	Enzyme Activity (International units.)				
Glutamic-oxaloacetic Transaminase	26	46.7 ± 10.0	18	56.5 ± 20.1	19	64.0 ± 23.3
Glutamic-pyruvic Transaminase	26	8.1 ± 2.1	14	10.6 ± 3.6	19	11.7 ± 5.3
Isocitric dehydrogenase	26	16.6 ± 4.5	18	25.9 ± 13.1	20	28.0 ± 11.9
Lactic dehydrogenase	26	863 ± 93	14	887 ± 97	24	881 ± 105
Alkaline Phosphatase	26	850 ± 213	14	840 ± 179	17	841 ± 179
Acid Phosphatase	15	25.8 ± 4.7	9	24.3 ± 3.5	9	23.5 ± 4.3

t test SGOT preinoc:terminal $p < .005$; SGPT preinoc:terminal $p < .01$
ICD " " $p < .001$

Discussion

With the profound changes in haemodynamics and loss of protein from the circulation, which occurred in intoxicated animals, a certain amount of variation could be expected to occur in serum enzyme levels. This would occur even in the absence of any specific release of enzymes from the tissues and it is probable that this explains the fluctuations in LDH and the phosphatases in the blood samples which were collected after the onset of clinical signs of intoxication. It also appears that some enzymes may have been less affected by the haemodynamic changes than others. In the case of the transaminases and isocitric dehydrogenase, the consistent increases which occurred suggest that these enzymes may have been released into the bloodstream by the tissues after intoxication occurred.

The factors which govern the time and pattern of release of the various enzymes from damaged tissues into the extracellular fluids are extremely complex. Thus the pattern of enzymatic change associated with any particular type of injury or damage to any particular organ may vary widely. It is interesting to note however that Schmidt *et al* in Bergmeyer (1963) have suggested that the retention of some enzymes intracellularly is dependent upon functional, as well as morphological, integrity of cells. They quote conditions in which interference with energy metabolism of the cells results in the release of enzymes before there is any evidence of morphological damage to the cells and also give examples of conditions in which morphological change is evident but enzymatic changes are minimal.

These factors could explain, in part, the differences in the changes in patterns of the individual enzymes in the present instance and also the fact that elevation of some of these enzymes occurred in the absence of detectable morphological damage to cells

in the major organs of the body.

This study has not provided any evidence to suggest that epsilon toxin interferes directly with the action of any of the enzymes which were examined.

Conclusions

1. There is rapid increase in the activity of SGOT, SGPT and ICD following the parenteral administration of epsilon toxin to lambs.

2. The activity of lactic dehydrogenase and both acid and alkaline phosphatases fluctuate widely but do not show a consistent rise or fall.

3. The fluctuation in enzyme levels in the post-inoculation samples may be due to the loss of protein from the bloodstream and the inefficient transfer of enzymes released from the tissues into the bloodstream.

4. Where elevations in enzyme levels occur they may indicate functional damage to the plasma membranes of cells.

5. There is no evidence that epsilon toxin interferes directly with the action of any of the enzymes examined in the present study.

Chapter 22: ALTERATIONS IN URINE CONSTITUENTS OF LAMBS FOLLOWING THE ADMINISTRATION OR ABSORPTION CL. PERFRINGENS TYPE D EPSILON TOXIN.

Although glycosuria is commonly encountered in field cases of enterotoxaemia in lambs it is not a constant finding (Smith, 1957). Sotirov (1965b) has carried out studies on the pathogenesis of this glycosuria and considers that it occurs as a result of failure of the proximal tubules to reabsorb glucose from the glomerular filtrate. On this basis the glycosuria is, to some extent at least, independent of the hyperglycaemia. However the blood glucose level frequently exceeds the renal threshhold for glucose in experimentally induced enterotoxaemia.

To investigate renal function effectively it is necessary to employ a variety of procedures including clearance tests with compounds which are excreted by different processes within the kidney but many of these tests are difficult to adapt for use in short term studies. In addition, the test compound itself may alter the pattern of excretion, and reabsorption of normal urinary constituents by saturating transport systems in the kidney. These factors rendered many of the available techniques unsuitable for use in the present study. Surgical cannulation of renal vessels and ureters of experimental animals was not considered to be desirable in this project.

The investigations into renal function in the present instance was therefore limited to a study of changes, in the levels of normal urinary constituents, which occur during the course of intoxication. It is interesting to note in this respect that, although other workers have investigated the glycosuria they do not appear to have examined the patterns of other urinary constituents such as inorganic ions during the course of intoxication. Since these latter substances are also excreted by energy dependent processes . . they should provide useful information on the functional status of the kidneys,

A number of factors can complicate the interpretation of the results of studies of this type. For instance the patterns of excretion of ionic constituents such as sodium, potassium and chloride differ

markedly in adult sheep and young milk fed lambs and vary considerably from animal to animal, depending upon diet and fluid intake. Physiological fluctuations also occur as a result of feeding and diurnal rhythms in adult sheep (Simpson, 1969) and it is possible that a similar situation could also occur in the immature lamb. Further, because the epsilon toxin that was used in the present study was diluted with physiological saline, the levels of sodium and chloride in the urine of experimental animals could reflect changes brought about by the intravenous administration of these ions.

The Urinary Constituents of 2-3 week old Lambs and the Influence of Parenterally Administered Epsilon Toxin upon Their Excretion.

Materials & Methods:

The lambs that were used in these experiments have been described in Chapter 6. Prior to the administration of toxin a sample of urine was collected from the bladder of these animals and the 10 paired control lambs. A metal bitch catheter and illuminated (Wappler model auriscope with bitch speculum attachment - Holborn Instrument Co., London, England) speculum designed for the catheterisation of bitches were used for this purpose and proved very satisfactory. The maximum volume of urine collected in the preinoculation sample was 30ml. No attempt was made to completely empty the bladder at this time although this occurred if less than 30ml urine was present.

Post-inoculation urine samples were collected in the same fashion or else by puncture of the bladder wall with a disposable hypodermic syringe and 20swg needle at necropsy if the animal died before a urine sample could be obtained. In animals which survived, or when the onset of clinical signs of intoxication were delayed, samples of urine were collected at intervals after the administration of toxin.

All urine samples were analysed for glucose by the automated glucose oxidase method described in Appendix II. In addition all terminal urine samples were checked for the presence of glucose at the time of collection with a semiquantitative dip-stick test ('Clinistix' -

Ames Co., Elkhart, Ind., U.S.A.). Ionic constituents were estimated with the automated methods described in Appendix II . The alterations to pump tube sizes necessary to deal with the levels of these constituents in urine instead of plasma are also given in that appendix. Specific gravity of the urine samples was measured with a 'Uricon' hand urine specific gravity refractometer (Atago Optical Works, Tokyo, Japan).

Results:

Pre-inoculation and Control Urine Values:

The values for the various constituents of urine from normal 2-3 week old lambs are shown in Table 22.1. In addition to the means and standard deviations, the range of values is also given as in some cases e.g. sodium, the data has a non-normal distribution. The urine data is given in Appendix 16.

Table 22.1: Values for Urinary Constituents of Normal 2-3 week old Lambs.

Constituent	No. of Animals	Mean $\pm$ 1 Standard deviation	Range
Sodium meq/l	35	3.27 $\pm$ 4.89	0.30 - 23.75
Potassium "	35	38.8 $\pm$ 23.6	7.5 - 98.0
Chloride "	35	20.9 $\pm$ 10.9	4.0 - 46.0
Phosphate mg%	33	40.0 $\pm$ 23.5	12.0 - 127.0
Bicarbonate meq/l	35	Not detectable in any sample	
Specific Gravity	35	1.005 $\pm$ 0.005	1.000 1.032
Glucose mg%	33	Not detectable in any sample	

Changes in Urine Constituents Following the Administration of Epsilon Toxin.

In animals which survived for less than an hour after the administration of toxin, and in which the bladder had been emptied at the pre-inoculation sampling, no urine was obtainable at the time of death by either catheterisation or bladder puncture.

In the urine samples obtained following the administration of epsilon toxin there was an increase in the concentrations of all the ionic constituents and also in the specific gravity in most instances. These increases occurred irrespective of whether clinical signs of intoxication were present or not.

The data obtained from urine samples of these animals is given in Appendix 16 . Because of the wide range of values in the data from the preinoculation samples, the alterations in the concentrations of the urine constituents following intoxication are more clearly seen when expressed as the difference between the pre- and post-inoculation values. This has been done in Table 22.2.

Table 22.2: Alterations in Urine Constituents Following the Parenteral Administration of Epsilon Toxin

Constituent	Time of Sampling			
	No. of Animals	Prior to onset of intoxication (Includes Animals which Survived)	No. of Animals	At time of Death
	Means Increase in concentration plus Range*			
Sodium meq/l	5	+ 21.7(-0.5 to +44.4)	19	+ 14.8(-0.3 to +70.4)
Potassium meq/l	5	+ 37.6(-8.5 to +84.5)	20	+ 27.6(-15.0 to +86.0)
Chloride meq/l	5	+ 15.5(-9.0 to +75.0)	20	+ 14.6(-5.0 to +69.0)
Phosphate mg%	5	+ 128(-41 to +316)	20	+ 86.8(-10 to +408)
Specific Gravity	5	+ 0.011(-0.003 to +0.032)	20	+ 0.010(-0.003 to +0.032)

* + = increase; - = decrease
Note - see table 22.1 for preinoculation values

Glucose was detectable in the terminal post-inoculation sample from one animal only. The level of glucose in this sample was 500mg% and gave a positive test with the 'Clinistix' dipstick. No glucose was detectable

in a urine sample taken from this animal 1 hour before death and 15 minutes after the onset of clinical signs of intoxication. The animal from which these samples were obtained was the one described in Chapter 6, which received divided doses of toxin but failed to develop pulmonary oedema.

The Urine Constituents of Lambs which Received Epsilon Toxin of Enteric Origin (Experimental Enterotoxaemia).

For technical reasons discussed shortly, the estimation of urinary constituents in these animals was confined to a study of changes in glucose levels.

Materials & Methods:

The lambs used for these experiments were the animals which received enteric origin toxin described in Chapter 6. As stated they were 8 month old male lambs. Catheterisation of male sheep is very difficult due to a tendency for the tip of the catheter to enter the orifice of the duct of the bulbourethral gland at the pelvic entrance (Weaver, 1969). After several unsuccessful attempts at penile catheterisation of the bladder under anaesthesia with fluoroscopic visualisation of the passage of the catheter, it was decided to implant catheters surgically in three of these animals by procedures which are described in Appendix 4.

Urine samples were collected from the catheters with a LKB 'Radirac' fraction collector (LKB Producter, AB - Stockholm, Sweden) and glucose estimations carried out by the manual glucose oxidase method described in Chapter 19. Samples were diluted where this proved to be necessary. 'Clinistix' were also employed to detect glucose in these samples.

Results:

Considerable difficulty was encountered in maintaining a continuous free flow of urine from the catheters, presumably because of occlusion of the internal orifices by the mucosa of the empty bladder. In addition, one animal developed a urinary tract infection after surgical implantation of the catheter. The infection was treated with antibiotics for several

days but treatment was withheld for a week before the main experiment and there was evidence of pyuria in the samples of urine taken at this time.

No glucose was detected in the terminal urine sample taken from uncatheterised animals (Nos. 81 & 85) at the time of necropsy. One of the catheterised animals (No. 79) was the animal which failed to develop clinical signs of intoxication. None of the urine samples from this animal contained detectable glucose. Glycosuria developed in Lamb No 77, fifteen minutes after the onset of clinical signs of intoxication. The actual concentration of glucose was 90mg% and this only rose to 95mg% in the terminal urine sample although the blood glucose level was over 200mg% at that time. The glucose in the urine of this animal was not detectable with 'Clinistix' dipsticks.

The pyuria in the fourth lamb (no. 78) made it necessary to centrifuge all urine samples from this lamb before attempting to estimate urinary glucose. There was no detectable glycosuria in this animal until approximately one hour after the onset of clinical signs of intoxication. From this time onward there was a severe continuous glycosuria with the urine containing between 5.5 and 8.5g glucose/100ml urine. Blood glucose levels at that time were of the order of 250mg%.

The sequence of changes in the blood and glucose levels in this animal are illustrated in Figure 22.1.

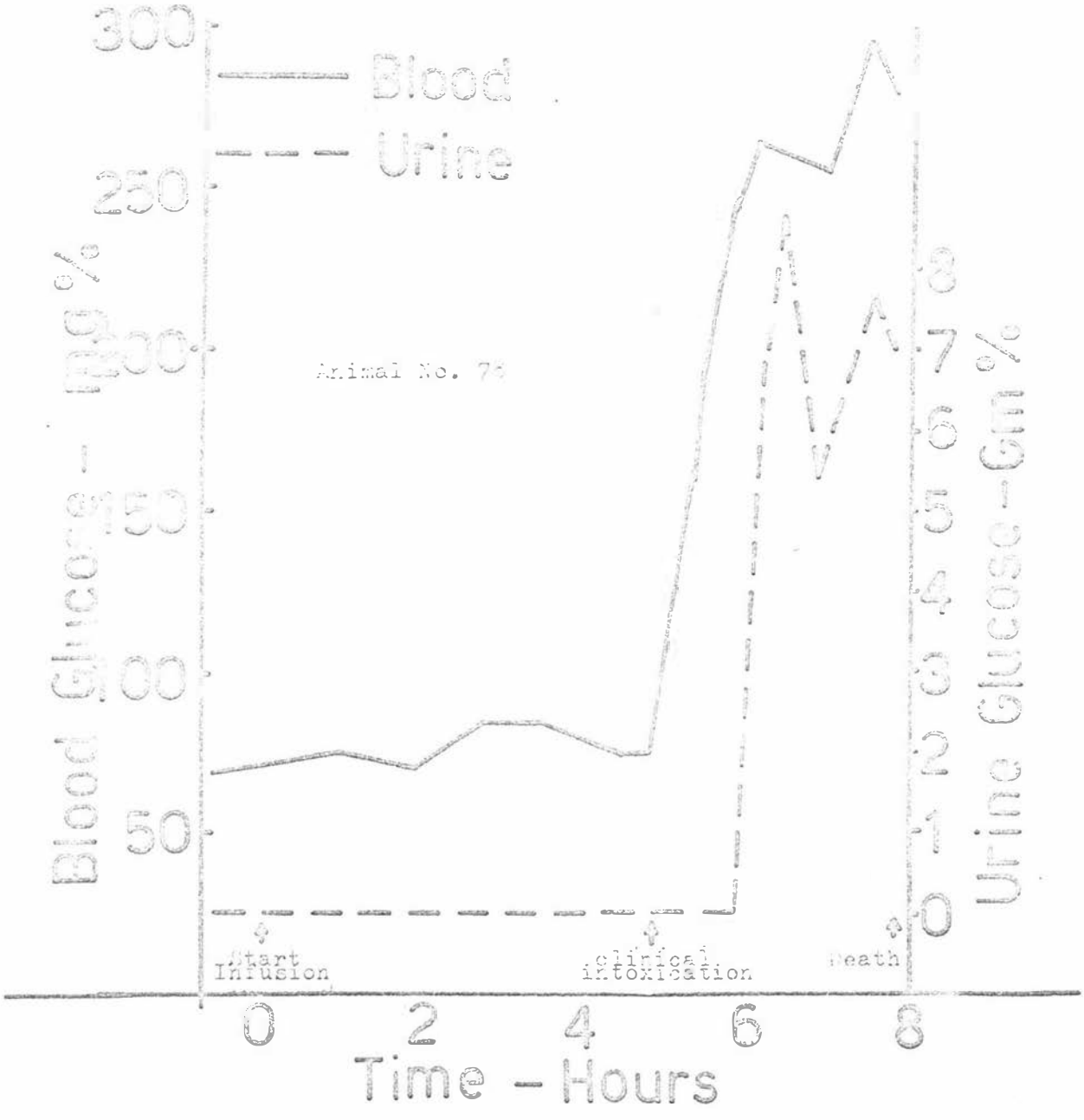
It should be noted that, despite the pyuria, there was no gross evidence of cystitis or pyelonephritis in this animal at necropsy. The bladder wall was not examined histologically, but the kidney did not show any inflammatory changes when examined by either light or electron microscopy.

The Urinary Constituents of a Field Case of Acute Enterotoxaemia.

Materials & Methods:

A urine sample was collected from the field case of acute enterotoxaemia described in Chapter 13. This sample was analysed at the same

Figure 22.1: Alterations in Blood and Urine Glucose during Cl.perfringens type D Enterotoxaemia



time as those from the lambs which received parenterally administered epsilon toxin. The same automated analytical procedures were used.

Results:

Sodium 3.8meq/l; Potassium 41.5meq/l; Chloride 20.5meq/l; Phosphate 43mg%. Glucose was present at a concentration of 800mg/100ml urine and a 'Clinistix' dipstick test for glucose was strongly positive.

Discussion

The values obtained for the urinary ionic constituents of these normal 2-3 week old lambs were very much lower than the values given in the literature for adult sheep. These are of the order of 60meq/l for Sodium and 130meq/l for Potassium and Chloride (Simpson, 1969). The fact that bicarbonate was not detected in the present samples was of interest since it is a major ionic constituent of the urine of adult sheep. The difference is almost certainly associated with dietary factors.

The marked increase in the concentration of ionic constituents in urine samples taken after toxin administration is probably not entirely the result of epsilon toxin intoxication since changes of a similar order of magnitude occurred in urine samples from animals which survived the administration of toxin and also in samples collected before there was any clinical evidence of intoxication. While some elevation in the levels of sodium and chloride ions could have been expected as a result of the saline solution used for the intravenous infusions this would not account for the concommittant rise in levels of potassium and phosphate in post-inoculation samples.

The concentration of ions in urine fluctuates widely under normal conditions since it is by excreting excess ions in the available water that the kidneys maintain ionic and fluid homeostasis within the body. Thus if fluid intake is reduced, or water loss by other routes is excessive, then the urine will tend to be more concentrated, although the total quantity of ions excreted per unit of time may be the same.

In the present instance, once the pre-inoculation sample of urine

had been taken from the bladder, the animals did not have access to fluid prior to the subsequent urine samples being taken. It was therefore likely that, under these conditions, more concentrated urine would be excreted. Epsilon toxin may have had some influence in increasing the ionic concentration of urine, since it has been shown to cause a loss of fluid from the blood stream and also reduces renal blood flow. However, in the absence of information on the volume of urine being excreted per unit time, in intoxicated and control animals while deprived of fluid, it was not possible to determine what effect if any this toxin had upon urinary ionic constituents. This data could not be obtained from the animals which had received parenterally administered toxin as it has already been seen that in small lambs the volume of urine excreted over the short duration of the intoxication was insufficient for ionic changes to be investigated in this way without employing special sampling and analytical methods. These were considered to be beyond the scope of the present investigation. It had been hoped that the use of indwelling catheters, in the lambs which received toxin of enteric origin, would overcome this problem but in this instance the irregular urine flow resulting from occlusion of the internal orifice of the catheter rendered volume data unreliable.

It is interesting to note however, that the values for ionic constituents in the urine from the field case of enterotoxaemia were close to the mean values for the normal animals. This implies that gross changes in ionic concentrations in the urine may not occur in the naturally-occurring disease. This information is of some importance because transfer of certain ions between blood and urine is, like the resorption of glucose by the proximal tubules, an energy dependent process. Under these circumstances, it could be expected that, if the glycosuria of enterotoxaemia is due to an interference with renal energy metabolism, some change in urine ion concentrations might also occur.

Turning now to the glycosuria of enterotoxaemia. It was surprising to find that detectable glycosuria only occurred in one of the 24 lambs which received parenterally administered epsilon toxin in which urine samples were obtained after the onset of clinical signs of intoxication. This may have been due to the short clinical course of the disease. In many instances too, due to the limited formation of urine during the period

of intoxication, the terminal urine samples probably consisted largely of urine which had been present in the bladder since before the toxin was adminstered. Nevertheless, even in animals in which the volume of urine in the bladder at the time of death was very small, no glucose was detectable by the glucose oxidase method which is 25-50 times more sensitive than the dip-stick procedures which are currently used for semiquantitative urinalysis.

A similar situation was found in the cases of experimentally induced enterotoxaemia where only one animal developed levels of glucose which could be detected by dip-stick tests and two animals did not develop detectable levels of urinary glucose although the blood glucose levels reached more than 300mg%. This suggests that there may be no glycosuria in some field cases of enterotoxaemia, particularly if the clinical course of intoxication is short. While this detracts from the value of urine glucose determinations as a diagnostic aids in suspected cases of enterotoxaemia, they may, as in the field case examined here, be of considerable value

Conclusions

1. Catheterisation of conscious young female lambs is readily accomplished by employing a bitch catheter and an illuminated bitch speculum. However, catheterisation of adult male rams is difficult or impossible, even in anaesthetised animals with fluoroscopic visualisation of the passage of the catheter.

2. The concentrations of sodium, potassium and chloride in the urine of 2-3 week old lambs are lower than in the urine of adult sheep.

3. Specific gravity and the concentration of sodium, potassium, chloride and phosphate were higher in urine samples taken after the parenteral administration of epsilon toxin, irrespective of whether or not clinical signs of intoxication occurred. This was probably associated with the excretion of more concentrated urine due to decreased fluid intake rather than an effect of the toxin alone.

4. Detectable glycosuria was not a constant feature of either parenterally administered epsilon toxin intoxication or experimental enterotoxaemia and when it did occur was a late feature of the intoxication, developing some time after the onset of hyperglycaemia.

5. The balance of evidence suggests that the glycosuria of enterotoxaemia is a direct reflection of the blood glucose level exceeding the renal threshold.

CHAPTER 23: GENERAL DISCUSSION.

Because the findings in each of the preceding chapters have already been discussed and conclusions drawn, it is unnecessary to consider individual results at this time. It therefore remains to examine the overall pattern which has emerged and to consider its relevance to the pathogenesis of enterotoxaemia. Special features of the investigation which have implications in fields of pathology beyond the immediate effects of epsilon toxin will also be mentioned.

Probably the most important single fact which has been found in this study is that epsilon toxin produces severe generalised vascular endothelial damage. This has been found to occur in cases of enterotoxaemia as well as when partially purified epsilon toxin is given intravenously. The apparently specific nature of these vascular lesions may allow the precise role of epsilon toxin in conditions such as ruminal bloat (tympanites) and overeating disease to be established in the future.

On the basis of the vascular endothelial damage it is possible to explain most of the morphological changes which occur following the administration or absorption of epsilon toxin.

The lesions which develop in the brains of intoxicated animals are of particular interest as these are relevant, not only to Focal Symmetrical Encephalomalacia (F.S.E.) which is directly attributable to the toxin, but also have wider potential applicability in the fields of neuropathology and neuroanatomy. The brain lesions will therefore be discussed before other aspects of the pathogenesis of enterotoxaemia are considered.

It is only comparatively recently that the different types of fluid accumulation which occur in the brain have been elucidated. For example, in 1965 Wechsler et al (in Klatzo & Seitelberger, 1967) proposed the first definition of cerebral oedema which included both intracellular and extracellular accumulations of fluid. Even at that time the presence or absence of a true extracellular space in the brain was the subject of debate. Horse radish peroxidase has proved to be particularly useful for demonstrating the distribution of protein when it leaves the blood-stream and it was the use of this substance which finally established

that a true extracellular space exists in the grey matter of the brain.

It is now known that the capillary endothelium, with its complete 'tight junctions' between cells, constitutes the major part of the so-called Blood-Brain Barrier (Brightman et al, 1970) and any interference with the functional integrity of this vascular lining will interfere with the normal transfer of metabolites to and from nervous tissue cells. However, the functional basis of the accumulation of fluid in the central nervous system in disease has not been fully clarified, although it is known that the osmotic pressure of brain tissue is higher than that of plasma. Energy is therefore required to maintain both ionic and fluid homeostatis (Quadbeck, in Klatzo & Seitelberger, 1967). Any interference with the transfer of nutrients, which are important in energy metabolism, from the bloodstream to the cells could therefore be expected to result in fluid accumulation.

The reason for the differential swelling of the protoplasmic astrocytes, in the grey matter type of brain oedema, and their precise role in the transfer of nutrients to and from the neurone also remains to be established. Epsilon toxin could therefore prove to be a useful substance for investigating the functional aspects of the grey matter type of brain oedema since it has been shown that it produces both endothelial damage and astrocytic swelling without apparently causing direct damage to neurones or other cell types. This could be of particular value as brain oedema can be a serious problem in human medicine (Klatzo & Seitelberger, 1967) and there are few experimental procedures which are suitable for the experimental investigation of generalised brain oedema, resulting from increased vascular permeability.

Some reference should also be made, at this time, to the occurence of brain oedema in domestic animals. It has not been widely recognised as a problem in veterinary medicine, possibly because traumatic head injuries are less common than in man and brain tumours and congenital status spongiosus are correspondingly rare. Nevertheless, it appears likely that brain oedema could account for some of the early clinical signs of neurological damage seen in conditions such as polioencephalomalacia of cattle and canine viral hepatitis. It will only be by careful studies of the ultrastructural morphological changes in brain tissue and the use

of tracers such as peroxidase, possibly associated with quantitiative studies on the fluid content of normal and abnormal nervous tissue, that the precise role of brain oedema in these disease syndromes will be established.

In modern neuroanatomy, considerable work is still required before the cell types that are seen by electron microscopy can be correlated with those seen by light microscopy. Studies by Mori & Leblond (1969a&b, 1970) have recently provided a useful basis from which to work but it appears that useful information could also be obtained on the distribution of cell types in regions such as the cerebellar granular layer, by the use of epsilon toxin to cause fluid accumulation in protoplasmic astrocytes.

It has been shown on a number of occasions in the present investigation that unexpected problems can arise in the interpretation of morphological changes in tissues from experimental animals. Perhaps the best examples were associated with the studies carried out on the changes which occur in the kidneys of intoxicated animals. The presence of free fat in the tubular epithelium of the kidneys from starved mice appears to have been known for some time but could prove to be a pitfall in experimental situations where food intake is inadvertantly reduced as the result of experimental procedures in this species. The rapid onset of post mortem autolysis in the kidneys of intoxicated lambs appears to have been misinterpreted as an antemortem necrosis of renal proximal tubular epitholium by some previous workers.

The sequence of changes in the intensity of histochemically demonstrable enzymes has proved useful for delineating areas of damaged tissue and also for estimating the duration of lesions in other pathological conditions. However it appears that it may be unwise to attempt to attribute specific biochemical actions to toxic agents on the basis of alterations in these enzymes at this stage of our knowledge. In the present investigation there were no observable differences in the staining intensity of a number of different enzymes in tissues such as liver, brain and kidney from intoxicated animals at the time of death and the alterations which occurred in levels of alkaline phosphatase in the kidney would therefore appear to be associated with the dissolution of the renal

tubular cells after death.

The development of the interstitial 'haemorrhage' in the kidney is of special interest. Traditionally, true haemorrhage is not regarded as occurring post mortem and, as far as is known, the present study is the first occasion on which a 'haemorrhagic' type of lesion has been shown to develop after death, albeit it is the result of autolytic degeneration of a pre-existing vascular lesion. It would be of considerable interest to determine whether a similar sequence of changes can occur following other forms of severe, acute generalised capillary damage, e.g. radiation damage or canine viral hepatitis.

In all these instances a sound explanation has been available for the morphological changes which occurred in intoxicated animals. There are however, still many gaps in our knowledge of the action of epsilon toxin upon tissues. So far no basis has been established for the endothelial damage which occurs. The toxin is not known to have any lecithinase activity, it does not appear to be bound to cell membranes and there is no evidence to suggest that it produces morphological or functional damage in specific cell structures such as plasma membranes or mitochondria. It is possible that more refined biochemical techniques, such as those used to elucidate the sites of actions of other toxins or enzymatic deficiency conditions, e.g. plague toxin, (Kadisetal, 1969) and subacute necrotising encephalomyelopathy (Cooper, 1969 and Pena et al 1970) may subsequently reveal a specific structural or biochemical site of action of epsilon toxin. In view of the severity of the endothelial damage caused by this toxin further investigations appear to be most desirable and it is possible that studies, possibly with tissue cultures of endothelium, could provide useful information on the precise effect of the toxin on the endothelial cell.

No entirely satisfactory explanation for the apparent species variation in the susceptibility of the intestinal mucosa to the action of epsilon toxin can be advanced, nor has the reason for the difference in the survival times of male and female mice been completely elucidated. In the latter instance it appears that the difference in survival time may have a hormonal basis, with testosterone being an important factor. As with sex differences in susceptibility to other toxic agents the

explanation of the difference in the effect of epsilon toxin on male and female mice must await further knowledge of the mode of action of sex hormones, such as testosterone on biochemical pathways.

Since epsilon toxin does not appear to have a marked effect on the metabolic efficiency of tissues, as judged by Warburg respirometry, some attempt must be made to explain the haematological and biochemical changes which occur in blood on the basis of the vascular endothelial damage.

The loss of protein from the blood stream is interesting since it is apparent that the haemoconcentration, which occurs during the course of intoxication, does not result from the loss of water and electrolytes alone. Unfortunately peroxidase cannot be used to demonstrate the abnormal loss of protein into the extracellular spaces of tissues, other than brain, since the presence of incomplete 'tight junctions' between the endothelial cells outside the brain allows peroxidase to escape under normal circumstances (Karnovsky, 1967). However, the presence of myocardial and pulmonary oedema, fibrin containing effusions and severe endothelial damage in all tissues of intoxicated animals suggest that loss of protein from the bloodstream occurs in many tissues.

It appears probable that the extent of the protein loss and also the haemoconcentration, in any given animal, will depend to a considerable extent on the severity of the vascular endothelial damage. It is therefore of interest to consider how these changes might be related to the various clinical syndromes of enterotoxaemia.

In very acute cases of enterotoxaemia, which are found dead with intense lung congestion and oedema, there has obviously been severe vascular endothelial damage. Under these circumstances extravasation of fluid would occur, leading to irreversible haemoconcentration and circulatory collapse. Death would therefore tend to occur rapidly, with repiratory distress and prostration as the most prominent clinical signs. Many field cases of enterotoxaemia probably fall into this category.

Where endothelial damage is less severe, protein loss and haemoconcentration would also be correspondingly less. The animals

might therefore survive for longer periods and, as a result of cerebral oedema etc., exhibit clinical signs of neurological disturbance such as convulsions and rigidity. It appears from personal observation of field cases of enterotoxaemia, which have been seen alive exhibiting this type of syndrome, that the lungs tend to be fairly normal when necropsies are performed on these animals. This, combined with the fact that the experimental animals which exhibited prominent signs of neurological damage did not as a rule show marked lung oedema or haemoconcentration, suggests that there is some factual basis for the hypothesis that this is a less severe manifestation of epsilon toxin intoxication.

Finally, in situations where the absorption of toxin was transient with small quantities being absorbed or where circulating epsilon antitoxin partially neutralised the absorbed toxin, vascular damage would be minimal and confined to those areas of the brain where the vasculature was particularly susceptible to toxic substances or anoxia due to selective vulnerability (Greenfield et al,1958 and Schade and McMenemy, 1963). Under these conditions F.S.E.-type lesions would develop.

In epsilon toxin intoxication, as in Cl.perfringens alpha toxin intoxication, there is a rapid mobilisation of hepatic glycogen reserves which probably accounts for all, or the greater part, of the hyperglycaemia. This is borne out by the lack of a hyperglycaemic response to administered epsilon toxin in animals in which hepatic glycogen reserves had been depleted by starvation and the administration of insulin. Further confirmation is provided by the histochemically demonstrable loss of glycogen from the liver of intoxicated animals.

Many investigations have been made into hepatic glycogenolysis. The biochemical pathways and enzymes that are involved have been elucidated and the influence of hormones such as insulin, glucagon and adrenalin in mediating this reaction in the liver have also been studied. There is however, practically no information on the cellular stimuli which lead to the mobilisation of hepatic glycogen reserves.

It is tempting to postulate that any interference with the respiratory activity of hepatic tissues would tend to favour catabolic pathways of glycogenclysis rather than the energy-requiring anabolic pathways of glycogenesis. Under these circumstances the vascular

endothelial damage produced by epsilon toxin could result in a decrease in the supply of oxygen and other nutrients to the hepatic parenchyma leading to glycogenolysis, gluconeogenesis and hyperglycaemia.

It must also be remembered that the results of enzymatic estimations on the sera of intoxicated animals, in this and other studies, suggest that epsilon toxin may have a direct effect upon hepatic cells and that a proportion of the toxin is retained by the liver. As mentioned earlier, it would be possible to explain the loss of intracellular enzymes on the basis of a reduced supply of available nutrients since the impermeability of plasma membranes to these enzymes is maintained by energy-dependent processes.

The background to the elevations in blood pyruvate and lactate levels which occur in intoxicated animals have already been discussed in some detail in Chapter 19. However, there are some general considerations which it is interesting to pursue further.

In a situation where there is an increased production of these substances due to increased amounts of available glucose, both pyruvate and lactate tend to be elevated as a reflection of the normal rate-limiting steps in the aerobic pathways of energy production and oxidative phosphorylation. Therefore an elevation of lactate and pyruvate under these circumstances does not imply that there is any direct interference with their utilisation. This is in direct contrast to the situation where normal amounts of these substances are being produced from glucose and the elevation in blood levels is due to an interference with steps in the oxidative Tricarboxylic acid (T.C.A.) cycle or cytochrome chain of enzymes. In the latter instance, either lactate alone or both lactate and pyruvate may accumulate depending upon the site of the metabolic block. Thus, if the block interferes with the complex steps between pyruvate and the passage of two carbon fragments into the T.C.A. cycle via acetyl coenzyme A, then pyruvate tends to accumulate with an associated increase in lactate. This occurs in conditions such as thiamine deficiency (West et al, 1966), subacute necrotising encephalomyelopathy (Clayton et al, 1967) and possibly also in polioencephalomalacia in cattle (Pill, 1967). If the block occurs at later stages as in various forms of anoxia then only lactate will increase to any extent, since the accumulation of the

reduced form of diphosphopyridine nucleotide alters the pyruvate:lactate equilibrium in favour of lactate.

Because lactate in the blood of domestic animals may be derived from either the alimentary tract or tissue metabolism the interpretation of lactate values in samples of blood from animals which are suspected of suffering from grain overload ('overeating' disease, excessive carbohydrate intake),may be difficult. However, the lactate produced by microbial action on carbohydrate in the intestine is mainly D-lactate and in these conditions estimation of lactate is usually based on the chemical assay of total DL lactate. From the present investigation it can be seen that the results of such an estimation could be misleading since high levels of metabolically produced L+ lactate, occurring in enterotoxaemia, could confuse the differential diagnosis of these clinically similar conditions. It would therefore appear to be desirable to either estimate both total DL lactate and L+ lactate or, preferably, to estimate D-lactate only in cases where excessive intake of carbohydrate is suspected (Medway et al, 1969 and Bergmeyer, 1963).

A rather similar situation to the above occurs when considering alterations in blood pH. It is obvious that estimation of blood pH alone is of little value as a diagnostic aid as it gives no indication of whether the acidosis or alkalosis is of respiratory or metabolic origin. It is equally difficult to interpret routine blood gas analyses in isolation. The present study clearly shows that, in many of the experimental animals blood gas analyses would have indicated a severe acidosis which was apparently explicable on the basis of inadequate repiratory gaseous exchange and it would have been quite possible to overlook the fact that there was also a severe intercurrent metabolic acidosis caused by high levels of lactate in the blood.

It can be seen from the foregoing that, while it is possible to explain most of the biochemical and haematological changes which occur in intoxicated animals on the basis of endothelial damage, formal proof of this is lacking in many instances. Further detailed biochemical studies will be necessary before this can be obtained but such investigations appear to be highly desirable as epsilon toxin intoxication appears to be a condition which could be usefully employed to increase our

understanding of a large number of biochemical and physiological phenomena.

The present investigation emphasises the importance of employing a rational, comprehensive range of haematological and biochemical parameters when investigating the clinical pathology of a disease syndrome. The tests which are selected in any given instance should be based on a reasoned approach to the problem and be sufficiently comprehensive to allow a meaningful picture to be built up. Any attempt to interpret the results of a random selection of tests must be fraught with difficulty and frequently lead to erroneous conclusions.

Before a sound approach can be made to the clinical pathology of the diseases of domestic animals a great deal more information is required on normal values for the different species and for different age groups within these species. Knowledge is also required on the effect of environmental conditions on many of these parameters. The range of substances in body fluids which can be estimated by automated methods has increased enormously in recent years and it should be possible to obtain reliable normal values for a wide range of blood constituents quickly and easily. In the present instance the use of automated procedures made it possible to obtain normal values and comparative data for control and intoxicated animals, from a considerably larger number of samples and for a wider range of constituents than would otherwise have been the case. It appears possible that, since economic considerations will always tend to influence veterinary clinical pathology, simultaneous, automated analyses of a number of relevant constituents of blood, urine etc., may prove to be economically sound for the large scale investigation of disease problems in livestock populations.

The overall pattern which has emerged from this work is that the disease syndromes that result from absorption of Cl.perfringens type D epsilon toxin from the intestine are largely due to damage to vascular endothelium caused by the toxin. The extent of the morphological damage depends upon the concentration of circulating epsilon toxin. Where endothelial damage is severe there is extravasation of protein-containing fluid into the extracellular spaces and this

may result in effusions into body cavities and oedema of the lungs, heart and brain. In addition the extravascular fluid accumulations and lack of vascular endothelial integrity may result in impaired transfer of substances between the bloodstream and the tissues, leading to depressed energy metabolism of the cells and intracellular fluid accumulation in the myocardium and brain. Selective vulnerability of the vasculature of areas of the brain stem to low levels of epsilon toxin could account for the occurrence of F.S.E. in animals which absorb sublethal amounts of toxin.

Although the renal changes appear to be post-mortem, autolytic phenomena, they are referable to pre-existing vascular endothelial damage.

The physiological, haematological and biochemical alterations which occur during the course of intoxication also appear to be associated directly or indirectly with the vascular damage. In the presence of fluid loss via the damaged capillaries, the haemoconcentration is self explanatory, but it is only when the extent of the associated protein loss is assessed that the severity of the damage and the self-perpetuating nature of the fluid loss can be appreciated.

The hyperglycaemia which occurs in intoxicated animals has been shown to be associated with the mobilisation of hepatic glycogen reserves and, while the exact basis for this is as yet uncertain, it appears possible that it may be a reflection of a stimulation of catabolic processes in the liver as a result of reduced transfer of substances between the bloodstream and the parenchyma in that organ. The resulting decrease in available energy within the hepatic cells may also result in an increase in cell membrane permeability leading to loss of intracellular enzymes. This is reflected in elevated levels of certain enzymes in serum.

The increased concentrations of intermediate compounds of glucose metabolism, such as pyruvate, lactate and alphaketoglutarate, in blood during intoxication are a reflection of the hyperglycaemia and do not indicate any direct action of epsilon toxin on particular metabolic processes.

From the foregoing it appears that the present investigation has

provided useful information on the pathology of enterotoxaemia and has gone some way towards elucidating the pathogenesis of certain of the haematological and biochemical changes which result from the action of Cl.perfringens type D epsilon toxin. The latter substance appears to have potential value as an agent for experimental investigation in the fields of neuroanatomy and neuropathology and also for studying the inter-relationships of many aspects of the cellular and clinical pathology of domestic animals.

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APPENDIX 1: Surgical and Manipulative Procedures - Laboratory Animals.

Mice

Castration of Males: The animals were warmed to ensure relaxation of the cremaster muscles, then anaesthetised with ether in a jar. The testes were removed by traction through bilateral vertical skin incisions over the scrotum.

Castration of Females: Anaesthesia was obtained with ether as above. The hair over the dorsal midline was then clipped and a 2cm longitudinal incision made in the skin over the lumbar region. The skin was then moved to one side and a paramedian incision made in the abdominal wall close to the lateral margin of the Longissimus dorsi muscles. The ovary on that side usually protruded through this incision or could be found immediately beneath it. The ovarian ligaments were clamped with small artery forceps and the ovary removed with a scalpel. After repeating the process on the other side of the midline, the skin incision was closed with a single monofilament nylon suture. Note: the muscle incisions did not require closure and no problems of sepsis were encountered with the procedure as described. Surgical instruments were held in 70% ethanol.

Rabbits

Intravenous Injections. The rabbit was held in a standard rabbit holding box and the injections made into the marginal ear vein with a 26 gauge needle.

Blood Sampling. Small volumes were obtained by pricking the marginal ear vein. Larger volumes were obtained by anaesthetising the animal with a 2.5% solution of sodium thiopentone intravenously to effect.

The animal was then held in dorsal recumbency and the blood removed by cardiac puncture using a 50ml. syringe and 14swg hypodermic needle.

Intestinal Ligation. Because these were very short term experiments in which the animals were not intended to survive for more than 12 hours, no aseptic precautions were taken. The animals were anaesthetised with

intravenous sodium thiopentone, the ventral abdominal wall was clipped and a 3cm midventral incision made posterior to the umbilicus. A portion of the intestinal mass as near to the middle of the ileum as possible was exposed and segments of the intestine with independent major blood supply and drainage were ligated with size 0 chromic catgut and the appropriate inocula introduced into the lumina of the loops with a hypodermic syringe and 26swg needle. The incision in the abdominal wall was closed with a series of interrupted full thickness sutures of size 0 chromic catgut. Recovery from the anaesthetic was generally almost complete within 2 hours.

Appendix 2: SEX:- SURVIVAL TIME DATA FOR MICE - Refer Chapter 5.

Survival Time - Hours

Males		Females	
2.45	4.00	2.75	7.33
2.45	4.25	2.92	7.33
2.75	4.25	3.25	7.66
2.75	4.25	3.75	8.25
2.75	4.33	3.92	8.66
2.75	5.33	3.92	9.00
2.92	5.33	3.92	9.00
3.00	5.33	4.33	9.17
3.00	5.91	4.41	9.50
3.00	6.17	4.75	10.00
3.17	6.17	5.00	10.00
3.33	6.17	5.00	10.33
3.33	6.66	5.41	10.33
3.50	8.58	5.41	10.42
3.58	8.58	5.41	11.00
3.58	9.00	5.75	11.00
3.58	10.33	6.08	12.42
3.58	12.75	6.41	12.42
4.00	12.75	6.92	
4.00	12.75*	7.08	

* Killed terminally.

Appendix 3: Hormonal Status: Survival Time Data for Mice - refer Chapter 5.

Oestrogen-Treated Males	Entire Males	Castrated Males	Entire Females	Testosterone Treated Females	Castrated Females
		Survival Time - Hours			
2.50	3.25	2.25	2.75	2.50	3.25
2.50	3.25	2.50	3.25	3.00	3.75
3.00	3.50	3.00	3.75	3.75	3.75
3.00	3.50	3.50	4.00	3.75	3.75
3.50	4.00	3.50	4.00	3.75	4.00
3.75	4.00	3.50	4.25	3.75	4.25
3.75	4.00	3.75	4.25	4.25	4.50
4.50	4.00	3.75	4.25	4.50	4.75
4.50	4.00	5.00	4.50	4.50	4.75
4.50	4.00	5.00	5.00	4.50	5.00
4.50	4.00	5.00	5.00	4.50	5.50
4.50	4.50	5.25	5.25	4.50	5.50
4.50	4.50	5.25	5.25	5.00	5.50
4.50	4.50	5.50	5.25	5.25	5.75
4.75	4.75	6.00	5.75	5.75	5.75
4.75	5.00	6.50	5.75	5.75	6.00
4.75	5.00	6.50	6.25	6.00	6.75
5.25	5.00	6.50	7.25	6.25	6.75
5.25	5.75	8.00	7.50	6.25	7.75
6.50	6.00	11.25	11.75	6.75	8.25
6.50	6.75	12.25	12.25	6.75	8.75
7.00	9.50	12.50*	12.50*	7.00	11.00
8.50	12.25	12.50*	12.50*	8.75	12.50
12.00	12.25		12.50*	9.25	
12.50*	12.50*			12.00	

* = Killed terminally

APPENDIX 4: Surgical and Manipulative Procedures - Sheep.

Intestinal Cannulae. Initially attempts were made to pass a cannula via the nasal passage, oesophagus, oesophageal groove and abomasum into the duodenum, using direct visualisation under an X-ray machine with image intensifier. Even when 2% Copper sulphate solution was passed down the tube or swabbed over the pharynx it was not possible to retain the tube in the oesophageal groove and the method was abandoned in favour of surgical implantation.

Animals were prepared for surgery by withholding food for 48 hours and water was also withheld for the last 12 hours of this period. The wool was clipped from the right side of the animal. It was then anaesthetised with intravenous sodium thiopentone (5% solution) and a cuffed McGill Tracheal tube immediately introduced. Anaesthesia was then maintained with fluothane/oxygen with a British Oxygen Co. (England) veterinary anaesthetic trolley with "Fluotec" vapouriser and closed circuit apparatus. The shorn right side of the animal was scrubbed and prepared for surgery. Full surgical aseptic techniques were used throughout the operation.

A 30cm skin incision was made over the ventro-lateral abdominal wall approximately 5 cm from the posterior margin of the rib cage and parallel to it. A muscle splitting approach was then used until the abdominal cavity was penetrated. The pyloric sphincter was located and the proximal part of the duodenum was then exteriorised and two bowel clamps applied to isolate a 5-10 cm segment of the latter as far as possible from the pylorus (usually 5-10 cm). A 1cm incision was made in the duodenum and a nylon duodenal cannula (with a temporary penetrating guide cap) was inserted and retained in place with a purse string suture through the serosa, muscle coats and submucosa of the intestine. A piece of omentum was tied over the base of the cannula and the penetrating cap of the cannula guided out through a stab incision in the abdominal wall anterior to the surgical incision and as close as possible to the normal site of the segment of duodenum. The cannula was retained at the skin surface by slipping a rubber, lamb castration ring over it. The penetrating cap of the cannula was replaced with the permanent cap, the bowel clamps removed and the layers of the abdominal wall closed individually with interrupted

size 1 chromic catgut sutures. Finally the skin incision was closed with Michelle clips and the animal given 500,000 units each of penicillin and streptomycin. Animals were allowed to recover from surgery for at least 14 days before use. Where an ileal cannula was inserted, exactly the same procedure was adopted with the cannula being inserted 10 cm from the ileocaecal valve and exteriorised at a point where no tension was placed on the cannulated area.

Catheterisation of Ram Lambs. Catheterisation of these animals is extremely difficult as the tip of the catheter generally enters the duct of the bulbourethral gland instead of following the curve of the urethra over the posterior margin of the pelvis. Several unsuccessful attempts were made to introduce catheters using x-ray visualisation as described previously, before recourse was made to surgical implantation.

Only two animals were catheterised. In one the catheter was tied into the bladder via an incision made 5cm anterior to the shaft of the ilium and parallel to the incision made for the duodenal cannulae. In the other animal a catheter was passed up the urethra to the pelvic brim and a midline incision made to expose the urethra 2cm below the anus. The urethra was opened and the reflected margins of the mucosa were sutured to the overlying skin. It was possible to pass a catheter into the bladder via this urethrotomy.

Restraint of Lambs During Intraduodenal Infusions. All animals were allowed to recover from the surgery for at least 14 days before being used in experimental procedures. They were then placed in crates made out of 1" galvanised water pipe. These crates were fitted with a wire mesh floor and adjustable head stall. The latter was used to retain the animal in the crate. In addition, a hessian sling was fitted which passed under the ventral areas of the animal. The legs passed through holes in the sling, thus allowing the animal to stand or rest in the sling, but not lie down. This was particularly important while animals were comatose and electrocardiograms were being recorded. The lambs were placed in the crates, without the sling, for several days before the experiments began to allow them to become familiar with handling and restraint.

Intestinal Loop Experiments:- The procedure used for the preparation of lambs with ligated intestinal loops was the same as that used for rabbits - see page 01.

Appendix 5: Total Body, Brain and Lung Weights, Epsilon Antitoxin Levels and Dosage of Epsilon Toxin for the 2-3 week old Experimental Lambs.

Control Lambs

Animal no.	Body-weight (Kg)	Brain weight (g)	Lung weight (g)	Epsilon antitoxin units/ml serum	Epsilon toxin Dose (mg) (approx.)
0	8.6	56	Not done	not done	
13	10.0	58	"	less than 0.2	
23	7.7	58	"	" " "	
26	8.6	60	110	" " "	
32	10.0	58	126	" " "	
36	9.5	60	120	" " "	
40	9.1	61	113	" " "	
43	915	59	107	" " "	
45	10.0	65	120	" " "	
49	10.4	68	176	0.4	

Lambs which received Divided Doses of Toxin

2	9.1	62	not done	less than 0.2	1*
6	10.4	65	"	" " "	1*
17	10.4	62	"	" " 0.4*	1*
33	8.2	61	258	" " 0.2	1*
38	8.2	60	220	" " "	0.50
42	10.4	64	225	" " "	0.25
44	8.6	60	220	" " "	0.25
46	10.4	60	275	" " 0.8*	0.25
48	9.5	61	290	" " " *	1.30
50	7.3	57	235	" " 0.2	0.25
51	10.0	68	104	" " "	0.25
53	8.6	60	245	" 0.2-0.4	1.50
55	9.1	64	280	" 0.4-0.8	8.25
56	9.5	69	340	0.8	4.75
57	8.6	65	330	less than 0.2	0.50
58	6.4	61	330	0.8-2.0	5.50
61	9.1	65	380	not done	0.25

Lambs which received Toxin by Continuous I/V Infusion

62	10.0	58	145	not done	0.25
63	9.1	60	156	less than 0.2	2.5
64	11.4	63	420	4 - 8	18.0
66	9.1	64	128	less than 0.2	1.0
70	10.4	60	220	4 - 8	9.0
71	9.1	60	180	0.4 - 0.8	3.0
72	10.0	65	410	0.4	4.0
73	13.2	74	332	0.8	3.0
75	10.0	59	147	less than 0.2	2.0

* lowest level tested.

Appendix 6: Levels of I_{125} Polyvinyl Pyrrolidone in Plasma Following Infusion of this Compound into the Duodenum of Normal Sheep and Sheep with Experimental Enterotoxaemia - see Chapter 6.

Animal no. 78

1.9ml of I_{125}PVP (5.5mg/ml) administered in 750 ml of inoculum (Saline)
1.5 ml sample of inoculum gave 115,700 counts/minute - equivalent to 5.5 counts/ngI_{125}PVP

Hours after start of Infusion	Counts/min. (1.5ml plasma)	ngI_{125}PVP/ml Plasma
0:00	4	0.49
0:45	6	0.73
1:00	24	2.90
1:30	28	3.40
2:00	21	2.55
2:30	38	4.60
3:00	76	9.20
3:30	70	8.50
4:00	85	10.30
4:30	110	13.30
5:00	103	12.45
5:30	130	15.80
6:00	119	14.40

Animal no. 77

1.75ml of I_{125}PVP (5.5mg/ml) administered in 700 ml of inoculum (Cl. perfringens type D culture + starch)
1.5 ml sample of inoculum gave 97,280 counts/minute - equivalent to 4.7 counts/ngI_{125}PVP

Hours after start of Infusion	Counts/min (1.5ml plasma)	ngI_{125}PVP/ml Plasma
0:00	0	0.00
0:30	8	1.13
1:00	39	5.51
1:30	53	7.50
2:00	75	10.60
2:30	78	11.05
3:00	114	16.20
3:30	120	17.00
4:00	133	18.90
4:15	136	19.30
4:30	148	21.00
4:45	194	27.50

cont'd.....

Appendix 6: (cont'd.)

Animal no. 81 Day 1

1.9ml of I_{125}PVP (2.5mg/ml) administered in 500ml of inoculum (Saline)

1.0ml sample of inoculum diluted 1:50 gave 17,670 counts/10mins. - equivalent to 93counts/ngI_{125}PVP

Hours after start of Infusion	Counts/10mins. (1.0ml plasma)	ngI_{125}PVP/ml Plasma
0:00 (9am)	0	0.00
1:00	58	0.62
2:00	366	3.92
3:00	374	4.04
4:00	683	7.35
5:00	806	8.65
6:00	990	10.60
7:00	875	9.40

Day 2

1.8ml of I_{125}PVP (2.5mg/ml) administered in 500ml of inoculum (Cl. perfringens type D culture + Dextrin)

1.0ml sample of inoculum diluted 1:50 gave 25,236 counts/10mins. equivalent to 141 counts/ngI_{125}PVP

Hours after start of Infusion	Counts/10mins. (1.0ml plasma)	ngI_{125}PVP/ml Plasma
0:00 (9am)	892	6.35
1:00	1222	8.70
2:00	1239	8.80
3:00	1104	7.80
4:00	1474	10.40
5:00	1351	9.60
5:50	1308	9.30
6:30	1241	8.80
7:00	115	0.82
7:15	119	0.79

Appendix 7a: SURVIVAL TIME: BRAIN WATER CONTENT DATA FOR MICE

Preliminary Experiment - see Chapter 8.

Control		Toxin
Water Content mg. H_2O/mg. D.M.	Survival Time (hours)	Water Content mg. H_2O/mg. D.M.
3.24	2.50	3.29
3.12	3.00	3.44
3.21	3.00	3.26
3.25	3.25	3.26
3.16	3.25	3.32
3.33	4.00	3.32
3.33	4.00	3.40
3.13	4.25	3.33
3.27	4.25	3.38
3.37	5.75	3.38
3.32	5.75	3.14
3.37	7.00	3.64
3.31	8.50	3.44
3.32	10.50	3.51
3.26	10.50	3.71
3.31	12.00	3.45
3.12	12.75*	3.55
3.24	12.75*	3.15
Mean 3.26+		3.39+
Std. dev. 0.11		0.16

+ t test $p = < .005$

* Killed terminally.

Appendix 7b: Survival Time: Brain Water Content Data for Mice

Main Experiment - see Chapter 8.

Males				Females			
Survival time-hours	Water content mg H_2O/mg D.M.			Survival time-hours	Water content mg H_2O/mg D.M.		
	Control	Toxin	Water I/P +		Control	Toxin	Water I/P +
2	3.32	3.34	3.50	2	3.62	3.40	3.63
2	3.58	3.51	3.67	4	3.90	3.63	3.93
2	4.09	3.31	3.60	4	3.52	3.75	3.59
2	3.57	3.52	3.44	4	3.38	3.62	3.21
2	3.34	3.44	3.55	4	3.54	3.65	3.89
2	3.45	3.46		4	3.40	3.42	
2	3.34	3.48		4	3.36	3.55	
3	3.58	3.46		5	3.48	3.40	
3	3.50	3.46		5	3.57	3.74	
3	3.58	3.42		5	3.44	3.46	
3	3.48	3.50		5	3.52	3.52	
3	3.36	3.44		5	3.52	3.52	
3	3.28	3.21		6	3.52	3.50	
3	3.38	3.30		6	3.48	3.56	
4	3.36	3.26		7	3.44	3.72	
4	3.36	3.38		8	3.65	3.82	
4	3.25	3.26		8	3.50	3.64	
5	3.36	3.60		10	3.53	3.75	
5	3.35	3.45		10	3.44	3.60	
5.5	3.60	3.50		11	3.44	3.88	
5.5	3.80	3.44		11	3.30	4.25	
5.5	3.43	3.30		12*	3.16	3.62	
5.5	3.24	3.20		12*	3.50	3.66	
6	3.44	3.52		12*	3.42	3.70	
6	3.54	3.44		12*	3.50	3.46	
6	3.36	3.46		12*	3.46	3.40	
8	3.40	3.34		12*	3.42	3.43	
9	3.52	3.67		12*	3.87	3.77	
12	3.32	3.49		12*	3.60	3.51	
12	3.25	3.53		12*	3.68	3.42	
				12*	3.39	3.47	
Mean	3.45	3.42	3.55		43.50	Δ3.61	3.65
Std.dev.	0.19	0.10	0.08		0.10	0.19	0.19

Δ t test $p = < .01$

* Killed terminally.

\+ All died within one hour of inoculation.

BRAIN WATER CONTENT DATA FOR 2-3 WEEK OLD LAMBS - see Chapter 8.

APPENDIX 8a:

Control				Toxin in Divided Doses				Toxin by Continucus I/V Infusion			
Animal No.	Cortex Water Content	Thalamus	Cerebellum	Animal	Cortex Water Content	Thalamus	Cerebellum	Animal	Cortex Water Content	Thalamus	Cerebellum
	$mgH_2C/mgD.M.$				$mgH_2O/mgD.M.$				$mgH_2O/mgD.M.$		
00	4.27	3.81	4.01	02	4.50	4.10	4.12	62	4.55	4.30	4.00
13	4.55	3.35	3.83	06	4.16	3.32	4.05	63	4.35	4.00	3.92
23	4.39	4.22	4.10	17	4.22	3.86	4.00	64	4.49	4.01	4.22
26	4.20	3.98	3.94	30	4.16	4.15	3.87	65	4.82	4.11	4.76
32	4.57	4.23	4.22	33	4.22	3.99	3.92	66	4.40	4.05	3.95
36	4.43	4.31	4.06	38	4.39	4.13	3.92	67	4.31	4.45	3.97
40	4.56	4.37	4.13	42	4.35	4.02	4.10	70	4.43	4.13	3.81
43	4.45	4.06	4.70	44	4.38	3.93	3.85	71	4.12	3.89	3.06
45	4.34	4.37	4.51	46	4.32	4.02	4.11	72	4.53	4.00	4.11
49	4.49	4.29	3.59	48	3.88	3.94	3.83	73	4.30	4.09	3.91
				50	4.33	4.17	3.90	74	3.94	3.96	3.83
				51	4.50	4.50	3.85	75	4.29	4.09	3.87
				53	4.51	4.02	4.07				
				55	4.12	3.96	3.61				
				56	4.50	4.04	3.87				
				57	4.45	4.26	4.18				
				58	4.09	4.10	3.80				
				60	4.16	3.97	3.65				
				61	4.00	4.06	4.31				

Appendix 8b: Tissue Water Content Data for 8 month old Lambs.

Control Lambs

	Cerebral Cortex	Thalamus+	Cerebellum	Heart
	3.62	2.18	3.74	3.67
	4.57	1.63	3.98	3.45
	3.23	2.50	3.79	3.61
	4.22	2.33	3.50	3.69
	3.50	1.78	3.61	3.98
Mean	3.83	2.08	3.72	3.68*
Std. Dev.	0.55	0.37	0.18	0.19

Lambs with Experimental Enterotoxaemia

Lamb No.				
81	3.61	3.20	3.74	4.76
77	3.05	3.38	3.31	4.97
78	3.49	3.52	3.88	3.90
Mean	3.39	3.53	3.64	4.46*
Std. Dev.	0.28	0.34	0.30	0.49

* t test; p = less than 0.05

\+ Note: The water contents of the samples of thalamus from the control animals are consistently lower than those of the other areas of the brain in these animals. They were also lower than those of thalamus from intoxicated animals. It appears probable that this may have been a sampling error with thalamic samples from the control animals consisting predominantly of white matter.

APPENDIX 9: FLUORESCENT ANTIBODY METHODS

Preparation of Conjugated Globulin:

i. Horse serum (0.3ml) emulsified in 0.4ml Freunds adjuvant was injected intramuscularly into each of two rabbits on 3 occasions at weekly intervals, ('Complete' Freunds adjuvant was used for the initial inoculum and thereafter 'Incomplete' Freunds adjuvant was used to overcome the severe local tissue reaction which can be encountered if the complete adjuvant is used throughout the entire course of injections.)

ii. One week after the final injection a 2ml sample of blood was collected from the ear veins and put up against horse serum as antigen in a precipitin test according to the schedule shown below, which, using horse serum diluted 1:1000, provides approximately 100ug equine globulin/ml of antigen suspension.

Tube	1	2	3	4	5	6
Antigen ml	0.2	0.4	0.6	0.8	1.0	1.2
Saline ml	1.0	0.8	0.6	0.4	0.2	0
Antiserum	0.1	0.1	0.1	0.1	0.1	0.1

Precipitation was allowed to occur overnight at room temperature.
(A serum which gives a precipitin reaction in tubes four, five and six is regarded as satisfactory for the preparation of conjugated globulin - Dr. K. Moriarty pers. comm.)
Both these sera gave a reaction in all six tubes.

iii. The rabbits were anaesthetised with sodium thiopentone intravenously and 50 ml of blood was removed by cardiac puncture with a sterile syringe.

The blood was allowed to clot and the serum removed and centrifuged at 3,000rpm for 10 minutes.

iv. The serum was cooled to 4°C and an equal volume of saturated ammonium sulphate added (with constant stirring). The mixture was stood overnight at 4°C to separate the globulin fraction.

v. The mixture was then centrifuged, the supernatant discarded and the deposit dissolved in a minimum volume of distilled water.

vi. An equal volume of saturated ammonium sulphate was added and the solution recentrifuged. This was repeated twice.

vii. The deposit was resuspended in a minimum volume of distilled water and dialysed in Cellulose casing dialysis tubing (Visking Co., Division of Union Carbide Co., Chicago) against 0.85% saline. Four changes of saline were used and the total dialysis time was 24 hours and the volume of saline used was 2 litres.

viii. Freedom of the dialysate from ammonium sulphate was confirmed by adding an equal volume of saturated $BaCl_2$ solution to a sample of the fluid.

ix. The protein content of the globulin sample was found to be 2% with a refractometer and an equal volume of saline was added to bring the protein concentration to the recommended 1%.

x. Ten percent of the volume of the globulin solution of carbonate/bicarbonate buffer pH9.0 ($NaHCO_3$ 4.2Gm/100ml adjusted to pH9.0 with Na_2CO_3 5.3Gm/100ml) was added.

xi. The mixture was chilled in an ice bath and 0.05mg Fluorescein isothiocyanate /mg protein (i.e. 10mg/20ml) added with constant stirring. The stirring continued overnight at $4^{\circ}C$.

xii. The solution was then passed down a 30 cm column of Grade G25 'Sephadex' (Pharmacia, Uppsala, Sweden) (6Gm of sephadex washed twice with saline and allowed to swell for at least 2 hours before packing into a 1cm diameter glass chromatography column). The globulin solution was washed through the column with saline. An alternative procedure is to dialyse the globulin first against saline then against 0.01M phosphate buffered saline pH7.5 for 2-3 days.

xiii The solution was mixed with liver powder 100mg/ml (Sylvana) and held at $4^{\circ}C$ for 4 hours.

Fluorescent Antibody Method (continued)

xiv. It was then clarified through asbestos pads (grade EKS Carlson Ford, Ashton-under-Lyne, England) with a Hemmings (Gallenkamp) filter.

Reference:

'Fluorescent Antibody Techniques in the Diagnosis of Communicable Diseases' Public Health Service Publication 729 by Cherry W.B., Goldman M., Carski T.R. 1960. United States Department of Health, Education and Welfare, Public Health Service, Bureau of State Services, Communicable Disease Centre, Atlanta, Georgia.

Appendix 10: The Oxygen Uptake of Tissue Slices - see Chapter 14.

Tissue	Mouse Kidney-Toxin in vitro								Mouse Kidney Toxin in vivo		G'pig kidney Toxin in vitro	
	Substrate: GLUCOSE											
Time	Oxygen Uptake (ul/mg. D.M.)											
Minutes	Control				Toxin				Control	Toxin	Control	Toxin
	a	b	c	d	a	b	c	d				
10	2.1	1.7	1.6	1.1	2.1	1.1	1.3	2.1	2.6	2.8	1.2	0.9
20	4.6	4.2	3.9	3.3	4.4	3.4	3.4	4.3	5.3	4.4	2.6	2.0
30	6.9	7.2	6.4	5.0	7.1	6.1	5.8	6.6	7.5	7.5	4.0	3.2
40	8.9	9.5	8.4	7.7	9.2	8.3	7.8	8.9	9.5	9.6	5.5	4.5
50	11.5	11.9	10.6	9.2	11.4	10.8	10.2	11.5	11.7	12.0	6.7	5.5
60	13.5	14.4	12.2	10.3	13.7	13.1	12.3	14.0	14.0	14.0	8.2	6.8
	Substrate PYRUVATE											
10	1.0	0.7	1.8	1.7	3.0	2.8	1.7	2.4	2.2	3.5	0.3	0.1
20	4.7	1.8	4.4	4.2	5.8	6.2	3.9	5.9	4.4	6.8	1.2	1.2
30	8.7	2.8	6.6	6.9	9.5	9.8	6.6	9.2	6.5	9.9	0.5+	2.1
40	12.0	3.3	9.0	9.1	12.6	12.8	8.4	11.8	-+	13.5	4.4	3.6
50	17.7	4.6	12.2	11.9	16.1	15.5	11.2	14.6	11.7	16.8	5.3	4.4
60	21.5	5.8	14.6	14.5	18.6	19.8	13.7	16.5	13.5	20.1	6.5	5.5
	Substrate SUCCINATE											
10	2.6	2.5	2.2	3.1	3.0	1.3	1.4	2.7	3.8	3.4	2.6	2.2
20	6.5	6.1	5.7	6.5	7.2	3.7	4.3	5.8	8.0	7.3	5.7	5.0
30	10.6	9.8	9.8	9.1	11.6	6.7	7.9	9.4	11.0	11.0	8.7	7.9
40	14.3	13.0	13.2	10.3	15.3	9.2	10.6	13.1	13.7	14.7	12.4	11.1
50	18.2	15.4	16.6	11.1	19.1	11.8	13.5	16.9	16.4	18.6	15.1	13.4
60	21.7	20.7	20.1	13.6	22.5	14.3	16.7	19.9	18.3	22.9	18.0	16.3
	Substrate CITRATE											
10	3.8				8.9	1.8				2.7	2.4	
20	8.4				14.1	6.1				5.7	5.9	
30	13.2				13.0	10.5				8.7	8.4	
40	17.7				18.4	14.6				11.1	11.2	
50	22.9				19.4	19.6				14.0	14.1	
60	26.3				18.7	22.7				16.3	16.5	

continued.....

Appendix 10: continued.....

Tissue	Mouse Kidney-Toxin in vitro		Mouse Kidney Toxin in vivo		G'pig kidney Toxin in vitro	
Time Minutes	Oxygen Uptake (ul/mg. D.M.) Control	Toxin	Control	Toxin	Control	Toxin
	Substrate LACTATE					
10	2.5	2.7	-*	2.4		
20	5.4	5.9	-	5.1		
30	8.7	9.9	-	7.6		
40	11.9	13.2	-	10.1		
50	15.9	17.9	-	12.6		
60	18.0	21.0	-	15.2		
	Substrate ABSENT					
10	2.2	1.8	2.3	3.7		
20	4.0	3.6	4.1	10.3		
30	6.4	5.4	6.8	10.5		
40	8.4	6.5	8.8	10.4		
50	10.5	8.4	11.1	10.5		
60	12.0	9.6	13.0	10.1		

Tissue	Guinea Pig Brain				Guinea Pig Lung			
Time	Substr.Glucose		Substr.Pyruvate		Subs.Succinate			Substrate Glucose		
Minutes	Control	Toxin	Control	Toxin	Control	Toxin		Control	Toxin	
10	0.9	0.7	0.8	1.4	1.4	1.1	0.9	0.2	0.0	0.7
20	1.8	1.7	1.8	3.0	3.0	2.8	2.8	0.7	0.2	1.0
30	2.7	2.9	3.1	4.1	4.7	3.9	3.9	1.3	0.6	1.2
40	3.8	3.9	4.2	5.5	5.8	5.7	5.7	1.5	1.2	1.8
50	4.6	5.0	5.3	6.7	8.0	7.0	7.1	2.0	1.6	2.1
60	5.6	6.0	6.8	8.1	9.6	8.4	8.5	2.4	2.0	2.5

\+ = Reading Error

* = Faulty Manometer

APPENDIX 11: AUTOMATED BIOCHEMICAL ESTIMATIONS

A wide variety of clinical biochemical methods have been developed to make use of the Technicon 'Autoanalyser', (Technicon Equipment Pty. Ltd. Ireland) and similar instruments. The main advantages of these machines are rapid sample processing and high reproducibility of results. When adequate standardisation is carried out the methods are at least as accurate as comparable manual ones.

Because a large number of pre and post inoculation samples had to be examined and comparisons between pre and post inoculation values made, the high reproducibility of the Autoanalyser had considerable advantages.

One disadvantage of many of the published methods is that they are not particularly economical of either samples or reagent. As it was hoped that a number of different estimations would be carried out, the sample size had to be limited to avoid introducing alterations in the blood picture of the animal due to blood loss. Some of the published methods were therefore modified to economise on the sample. In addition, since many of the published methods are basically similar, comparatively small alterations to reagent concentrations and pump tube sizes enabled the same manifold to be used for several different estimations.

The following pages are set out to show:

1. A reference to the method which was used.
2. A list of the reagents.
3. A diagram of the flow pattern, using standard 'Autoanalyser' symbols. In cases where the same manifold was used for more than one method a reference is given to the appropriate page.
4. A note of modifications applied to the published method.
5. A copy of the 'calibration peaks' and a representative portion of the sample peaks obtained using the method.
6. Any relevant comments on the procedure which have not been discussed previously.

The 'Autoanalyser' modules used were:

1 x 'Sampler-II' automatic sample changer.
2 x Proportioning pumps.
1 x Dialyser bath.
1 x 40' heating coil.
2 x Colorimeters.
1 x 'Turner'* Fluorimeter modified with flow through sample cuvette.
2 x 2-pen chart recorders.
1 x Flame III Flame photometer.

* Turner Fluorimeter - Turner & Associates, Palo Alto, Calif., U.S.A.

Blood Glucose

Automated Estimation:

Reference: Saifer A. & Robin M. in 'Automation in Analytical Chemistry' - Technicon Symposia 1965. Mediad, New York. p. 589-592.

"Determination of Glucose in Biologic Fluids with an Automated Enzymatic Procedure".

Reagents: Glucose Standard. (Standard Solution Sigma Cat no. for Blood Glucose estimation)

Buffer (Stock): 23.3Gm Maleic Acid dissolved in 800ml hot distilled water. Add 24.2Gm tris hydroxymethyl aminomethane, cool and make up to 1 litre.

Buffer (working): 250ml stock buffer + 48ml 1N.NaOH made up to 1 litre with distilled water and adjusted to pH7.4.

Enzyme/Dye: 400ml Glycerol, 10ML Glucose Oxidase-peroxidase (Sigma cat. no 510-6) 600mg o-dianisidine dihydrochloride made up to 1 litre with distilled water.

Sulphuric acid: 30% v/v solution.

Wash solution: 1% sulphuric acid.

Flow Chart:

see page 025, 026

Calibration and Sample Peaks:

Modifications to published method: Since preliminary deproteinisation of samples had been carried out a dialyser was unnessary and was not included. Similarly, in the absence of fluoride in the samples the Magnesium sulphate solution was not required to overcome inhibition of the glucose oxidase. The sensitivity of the method using a heating bath at 37°C (as required for the simultaneously run Lactate estimations) was sufficient to obviate the necessity of using a temperature of 50°C as recommended in the published method. With prior dilution of the sample during deproteinisation in association with the increase in sensitivity due to elimination of the dialysis step, the pump tube sizes were adjusted to compensate for these factors and thus retain the approximate glucose: reagent ratios and concentrations of the original method.

Blood Pyruvate

Automated Estimation:

Reference: Minaire Y., Forichon J. & Studievic C. in 'Automation in Analytical Chemistry' - Technicon Symposia 1966, Mediad New York, Vol. II p 145-149.

"Dosage Fluorometrique Enzymatique de L'Acide Pyruvique par l'Auto-analyzer".

Reagents:

Buffer: 6.05Gm tris hydroxymethyl aminomethane in 900ml distilled water adjusted to pH7.6 with concentrated hydrochloric acid and made up to 1 litre.

Lactic dehydrogenase: as for lactate.

Reduced diphosphopyridine nucleotide 1mg/25ml of the above buffer.

Pyruvate standards Stock 24.6mg Sodium Pyruvate in 100ml distilledwater. (Equivalent to 20mg pyruvic acid per 100ml. Working standards of 0.5,1.0,1.5,2.0,2.5,3.0,4.0mg/ml were prepared from this standard).

Flow Chart:

see page 025, 027

Calibration and Sample Peaks:

Modification to the Published Method: Dialysis was not required and a 40foot heating coil at 37°C was inserted in its place. This increased the sensitivity of the method and pump tube sizes were adjusted accordingly. The fluorometer was set at 340nm.

Notes: This method gave satisfactory results with the standards, but due to a high level of non-specific fluorescence in the samples, possibly derived from incomplete deproteinisation in the 'Vacutainers' and the instability of pyruvate in stored samples, the results of the estimations of pyruvate made on them are not regarded as being sufficiently reliable to be of value.

Blood Lactate

Automated Estimation:

Reference: Minaire Y., Studievic C., & Foucherand F. (1965) Pathologie et Biologie 13 : 1170 "Dosage Automatique de L'Acide Lactique -".

Reagents:

Lactate standards: containing 10, 20, 50, 100 and 200mg% L + lactate - prepared from sodium dl lactate - see Bergmeyer (1963) p 271.

Glycine/Hydrazine Buffer: 0.5M Glycine in distilled water. Add 0.1M Hydrazine Sulphate and adjust to pH 9.0 with 10N NaOH.

Lactic dehydrogenase: 2.0 ml lactic dehydrogenase solution (Sigma Type II LDH ex Rabbit muscle) to 100ml of the above buffer.

Diphosphopyridine Nucleotide: 0.9Gm DPN in 100ml buffer.

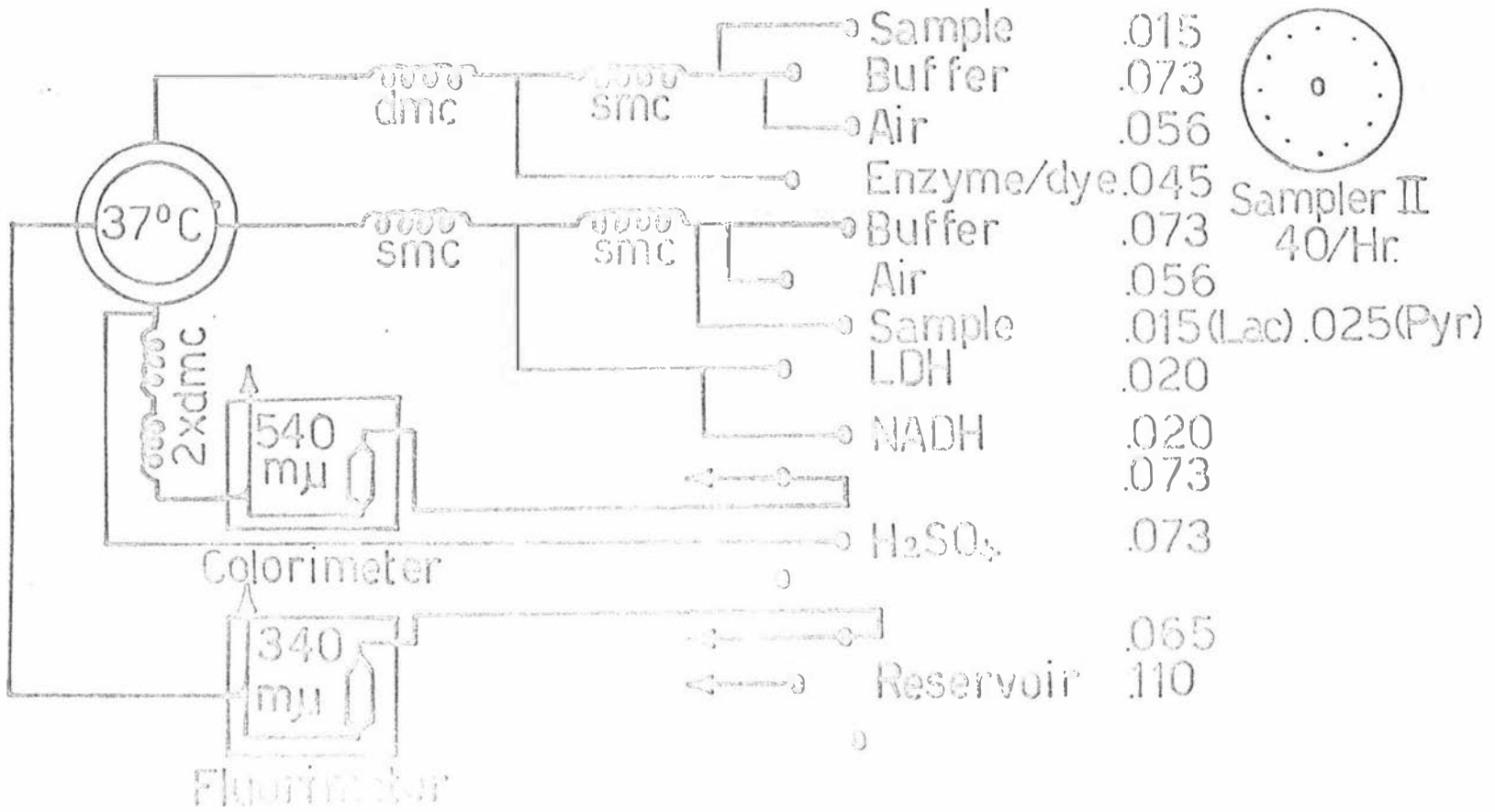
Flow Chart:

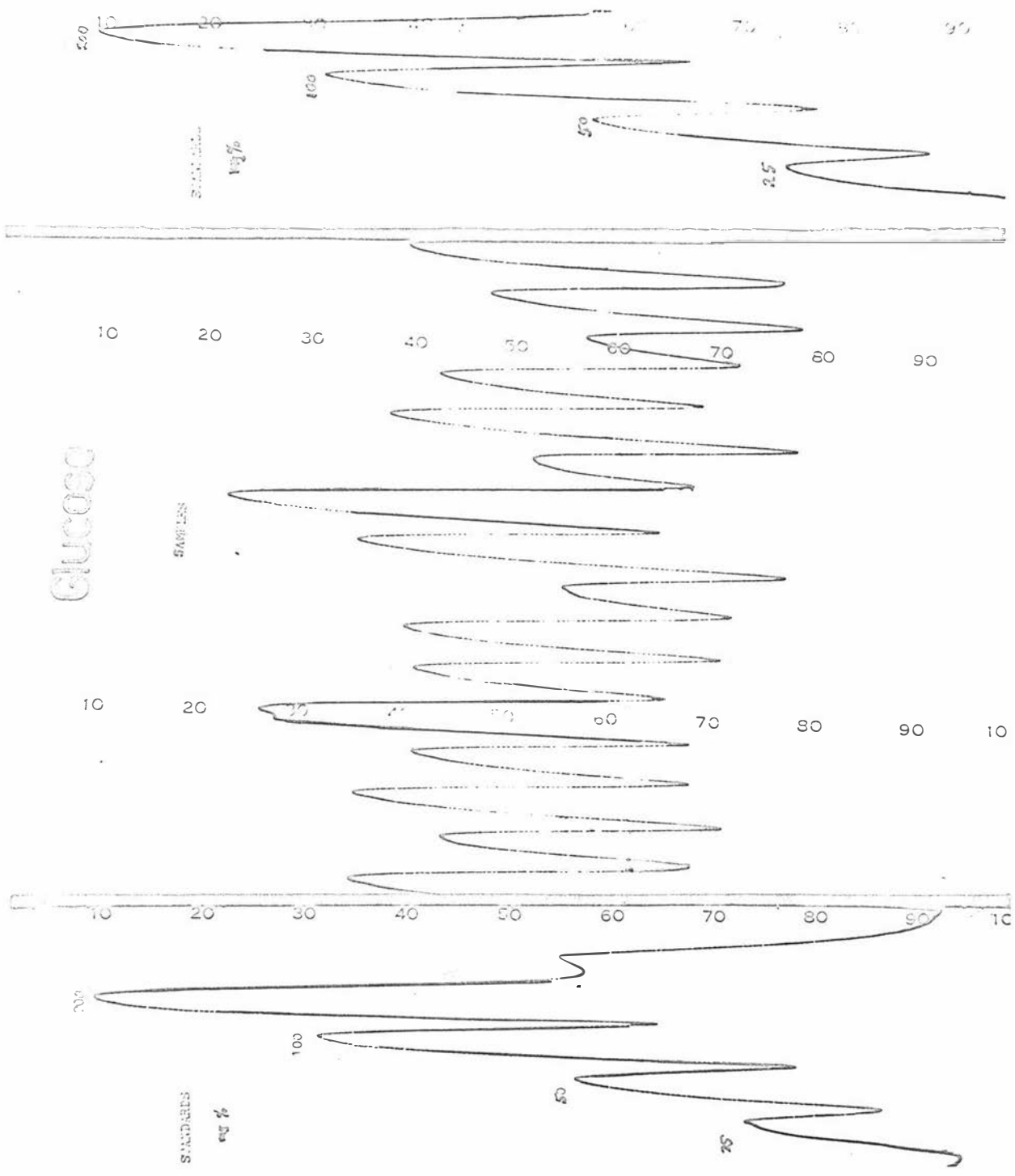
see page 025, 028

Calibration and Sample Peaks:

Modifications to published Method: No dialysis was necessary as the samples were protein free (see glucose method). Mixing coils were inserted to ensure sample dilution and reagent mixing prior to incubation. Pump tube sizes were adjusted to give similar reagent:sample concentrations to the published method in the absence of dialysis.

Glucose, Lactate & Pyruvate




glucosa
10
20
30
40
50
60
70
80
90
100

Pyruvate
Samples
Standards
mg%
10
5
2.5
1.25
Standards
mg%
10
5
2.5
1.25
60
70
80

STANDARDS
SAMPLES
Lactate

Serum Glutamic Oxaloacetic and Glutamic Pyruvic Transaminases

Automated Estimation:

Reference: Levine J.B. & Hill J.B., in 'Automation in Analytical Chemistry'.- Technicon Symposia (1965), Mediad New York p 569-574.

"Automated Fluorometric Determinations of Serum Glutamic Oxalacetic Transaminase and Serum Glutamic Pyruvic Transaminase."

Reagents: As described in the original method with the following exceptions - Albumin solution was not used to dilute the nucleotide and enzymes as the fluorescence of many albumin samples can seriously increase the background fluorescence. The detergent used was Brij 35, and the stock lactic and malic dehydrogenases were supplied by Sigma Chemicals Ltd.

Standards were prepared by diluting 'Enzatrol' (Dade Reagents, Miami, U.S.A.) to give standards of 10, 20, 31, 41 int. units of SGOT and 10,20,30,40.5 int. units of SGPT respectively.

Flow Chart:

see page 031, 032, 033

Calibration and Sample Peaks:

Modifications to Published Method: To economise on the amount of sample and reagents required, the pump tube sizes were all reduced proportionately. It was found that, while retaining a sampling rate of 40/hour, good separation and washout characteristics were obtained. A portion of the enzyme substrate mixtures were removed after mixing to be used for total serum protein estimations.

Total Protein

Automated Estimation:

Reference: 'Autoanalyser' Methodology File N-14b.

Reagents: Biuret reagent - as described.

Protein standards. Serial dilutions of a commercial serum standard - ('Versatol', Warner Chilcott, Morris Plains, N.J. U.S.A.) containing 6.9% protein/100ml serum.

Flow Chart:

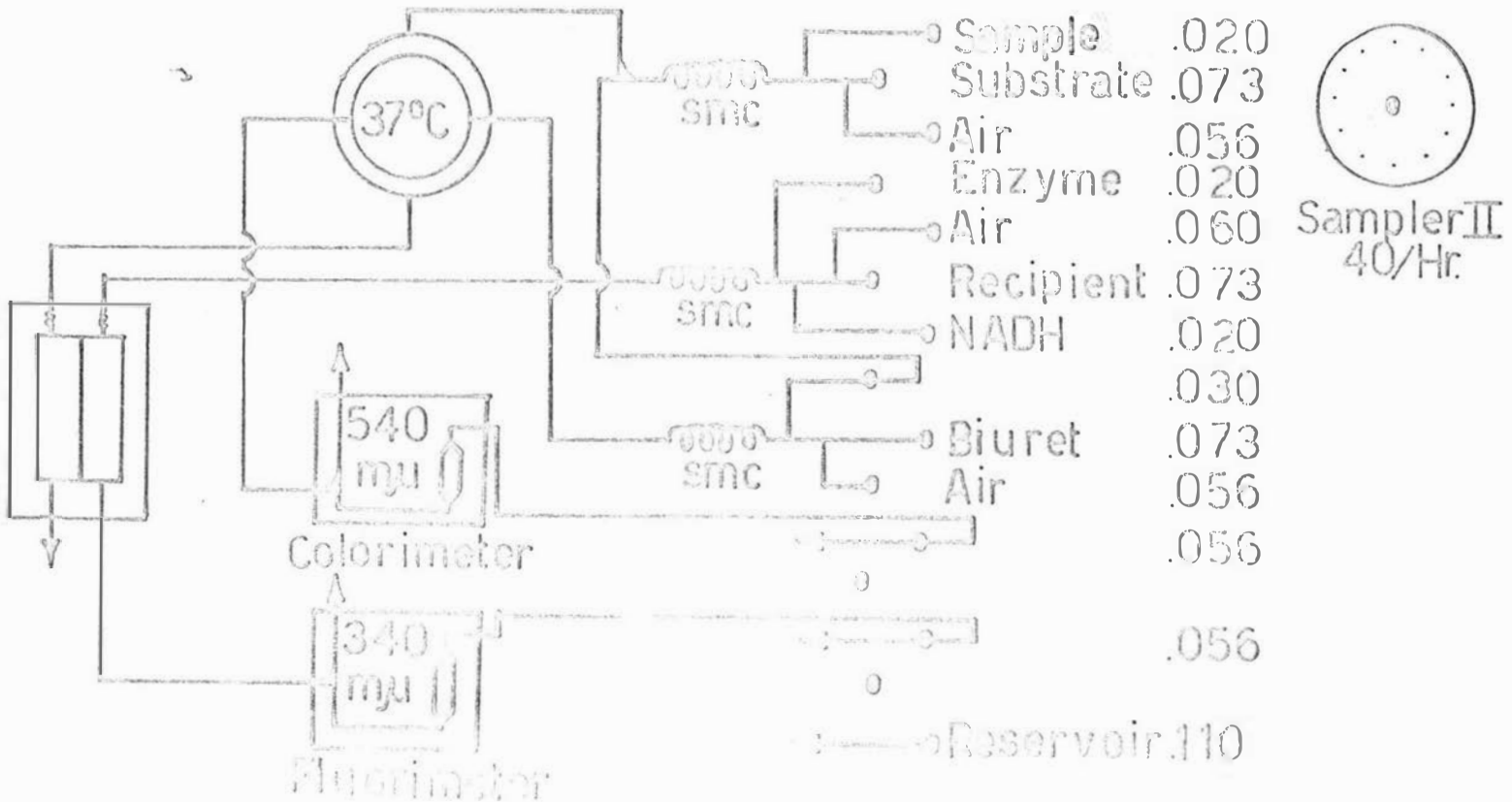
see page 031, 032

Calibration and Sample Peaks:

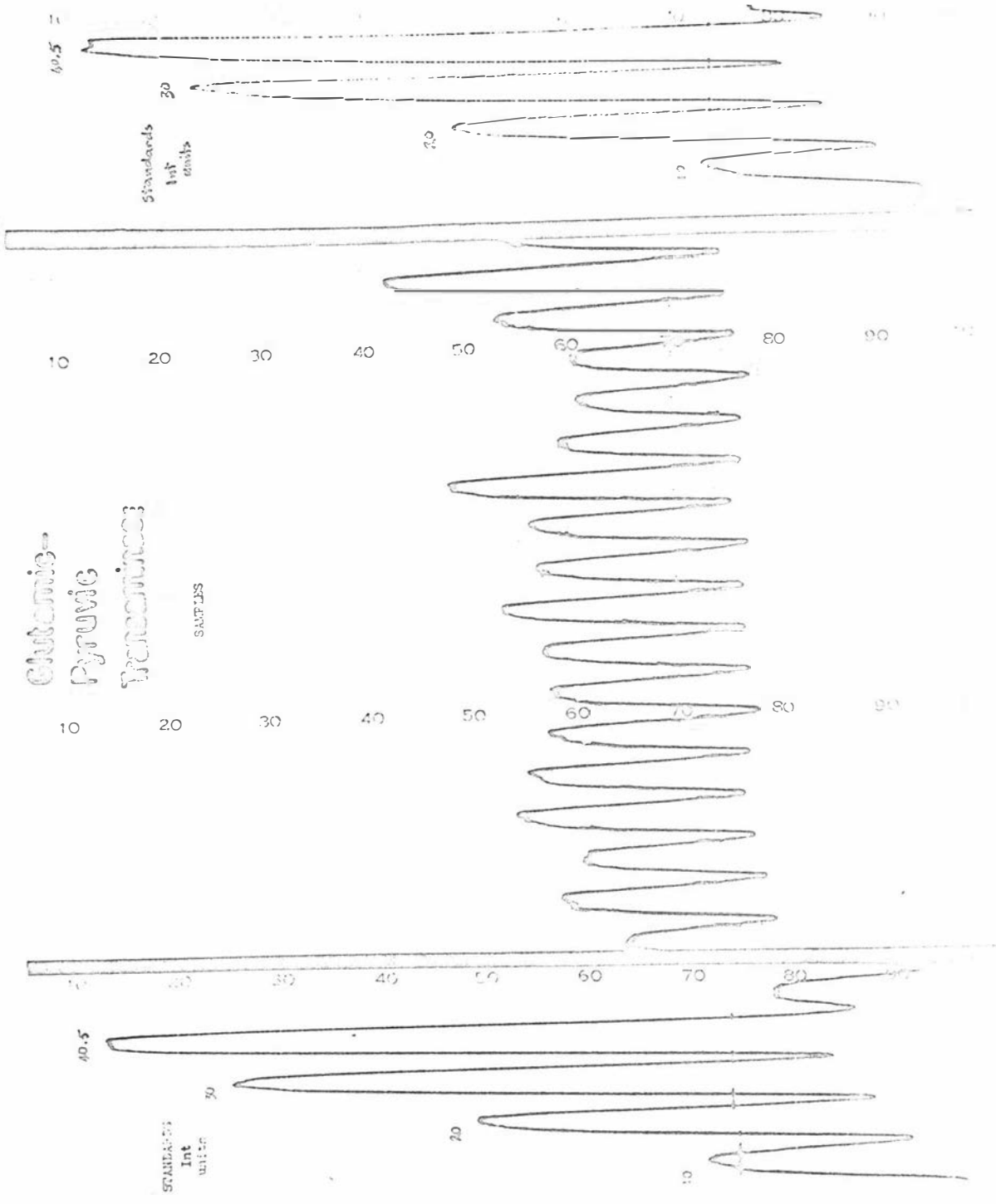
Modifications to Published Method: Dilution of the sample was with the substrate used for the estimation of glutamic-oxaloacetic transaminase. The diluted sample for protein estimation was removed (after mixing) via the bottom arm of a debubbler and a pump tube of the appropriate diameter. It was then mixed with the biuret reagent and passed through a 40 foot heating coil at 37°C.

Notes: The reaction was not influenced by either the substrate used for dilution nor by the incubation at 37°C.

Glutamic-oxaloacetic & Glutamic-pyruvic Transaminases + Serum Protein



Protein
10
20
30
40
50
SAMPLES
STANDARDS
6.6
6.3
5.2
3.5
1.7
Glutamic-
Oxalacetic
transaminase
SAMPLES
41
40
50
60
70
80
STANDARDS
Int.
units
41
31
20
10


Standards
Int units
SAMPLES
10
20
30
40
50
60
70
80
90

Serum Isocitric Dehydrogenase

Automated Method:

Reference: Wolfson S.K. (Jr.) & Williams-Ashman H.G. (1957) Proc. Soc. Exp. Biol. Med. 96: 231-234.

Reagents: The above reference is to a manual method as no published methods for isocitric dehydrogenase could be found in the literature. Reagent solutions were therefore made up so that, using the manifold described for lactic dehydrogenase, the final concentrations of reagents would be similar to those used in the manual method.

Tris Buffer: 0.1M, pH7.6 This was the same buffer as described in the Pyruvate method.

Substrate: 2.61Gm Sodium isocitrate (0.1M), 300mg Manganese chloride (0.01M) in 600 ml of Tris Buffer.

TPN (NADP) 100mg triphosphopyridine nucleotide (nicotinamide adenine dinucleotide) in 75ml tris buffer.

Recipient Tris Buffer.

Standards: Serial dilutions of Enzatrol - Dade were used. The lamb sera showed higher activity than these standards. A lamb serum was therefore diluted with buffer, measured against the commercial standard, then used to prepare a calibration curve for the remaining samples.

Flow Chart:

see page 036, 037

Calibration and Sample Peaks:

Modifications to the Published Method: The present method was developed from the above manual method and a published automated method for lactic dehydrogenase which employs dialysis and subsequent fluorimetric estimation of the reduced nucleotide formed by the enzyme during incubation with the appropriate substrate - see Lactic dehydrogenase method.

Notes: It can be seen from the standard and sample peaks that, while the automated estimation of the isocitric dehydrogenase does not appear to have been described previously, adaptation of existing manual methods appears to have provided a useful automated procedure with good linearity and washout characteristics.

Serum Lactic Dehydrogenase

Automated Estimation:

Reference: Passen S. & Gennarc W. (1966) Am. J. Clin. Path. 46: 69-85 "An Automated System for the Fluorometric Determination of Serum Lactate Dehydrogenase".

Reagents: As described in the original method.

Standards: "Enzatrol" - Dade was reconstituted and serial dilutions were used as standards. In addition a series of human bloods with high values were obtained from the Palmerston North Hospital and used as standards. As in other methods where samples exceeded the standards, the sample was diluted and re run to obtain its true value.

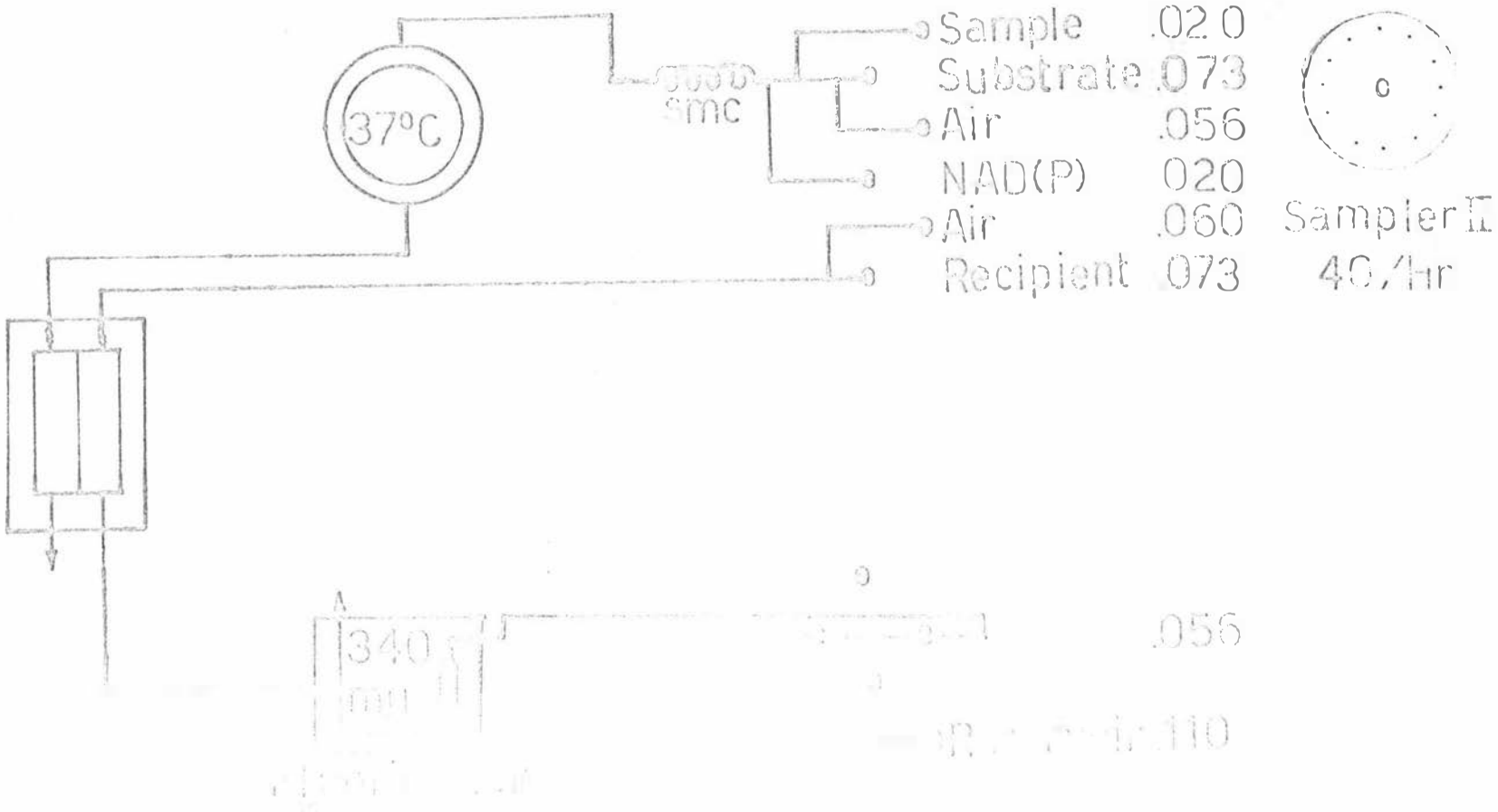
Flow Chart:

see page 036, 038

Calibration and Sample Peaks:

Modifications to the Original Method: Slight proportional alterations were made to pump tube sizes to economise on reagents and sample. These did not affect the washout characteristics or peak patterns to a noticeable extent since a slower flow rate of 40 instead of 60 samples per hour was being used.

Lactic & Isocitric Dehydrogenases,



STANDARDS
Int.
11.2 units
8.4
5.6
2.8

Serum Acid and Alkaline Phosphatase

Automated Estimation:

Reference: Green S., Thomas J.G., & Fishman W.H. in 'Automation in Analytical Chemistry' - Technicon Symposia (1966) Mediad, New York vol. 1 p 480 - 488.

"Automated Differential Analysis of Several Serum Phosphatase Isoenzymes".

Reagents i Acid Phosphatase: As described under 'Manifold 2' in the original method (note that only total acid phosphatase was being estimated therefore the reagents for the estimation of 'tartrate sensitive acid phosphatase viz, citrate tartrate buffer and solutions of naphthyl- and phenyl-phosphate in this buffer, were not required.)

Alkaline Phosphatase: As described under 'Manifold 3' in the original method (note that only total alkaline phosphatase was being estimated and both d- and l-phenylalanine were omitted) with the exception that due to the high levels of alkaline phosphatase activity encountered in the samples it was found necessary to use the concentrations of aminoantipyrene and potassium ferricyanide given for the acid phosphatase to overcome exhaustion effects encountered when the concentrations recommended in the alkaline phosphatase method were used.

Standards of phenol and also dilutions of the commercial serum standard 'Enzatrol' (Dade) were used.

Flow Chart:

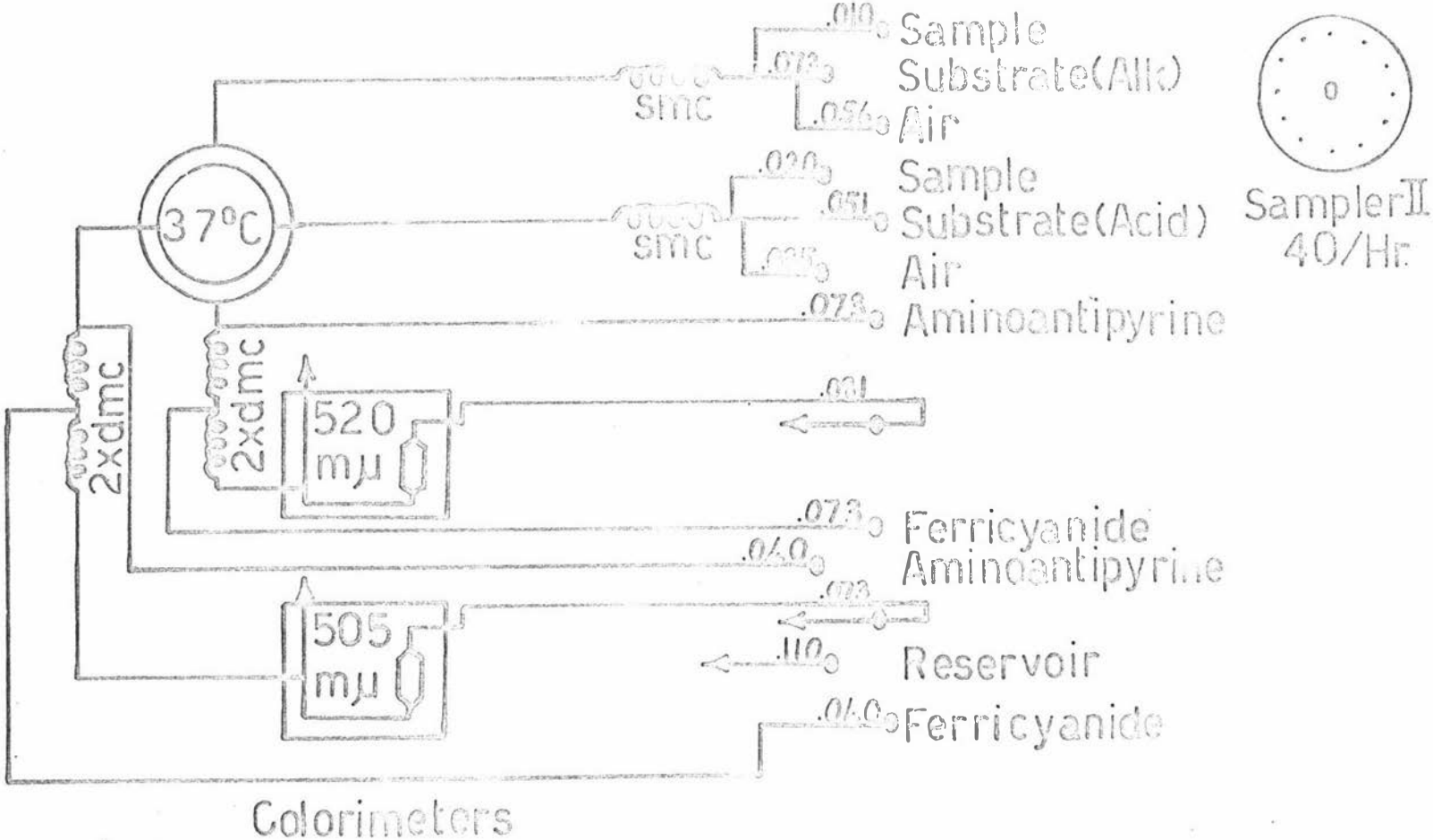
see page 041, 042, 043

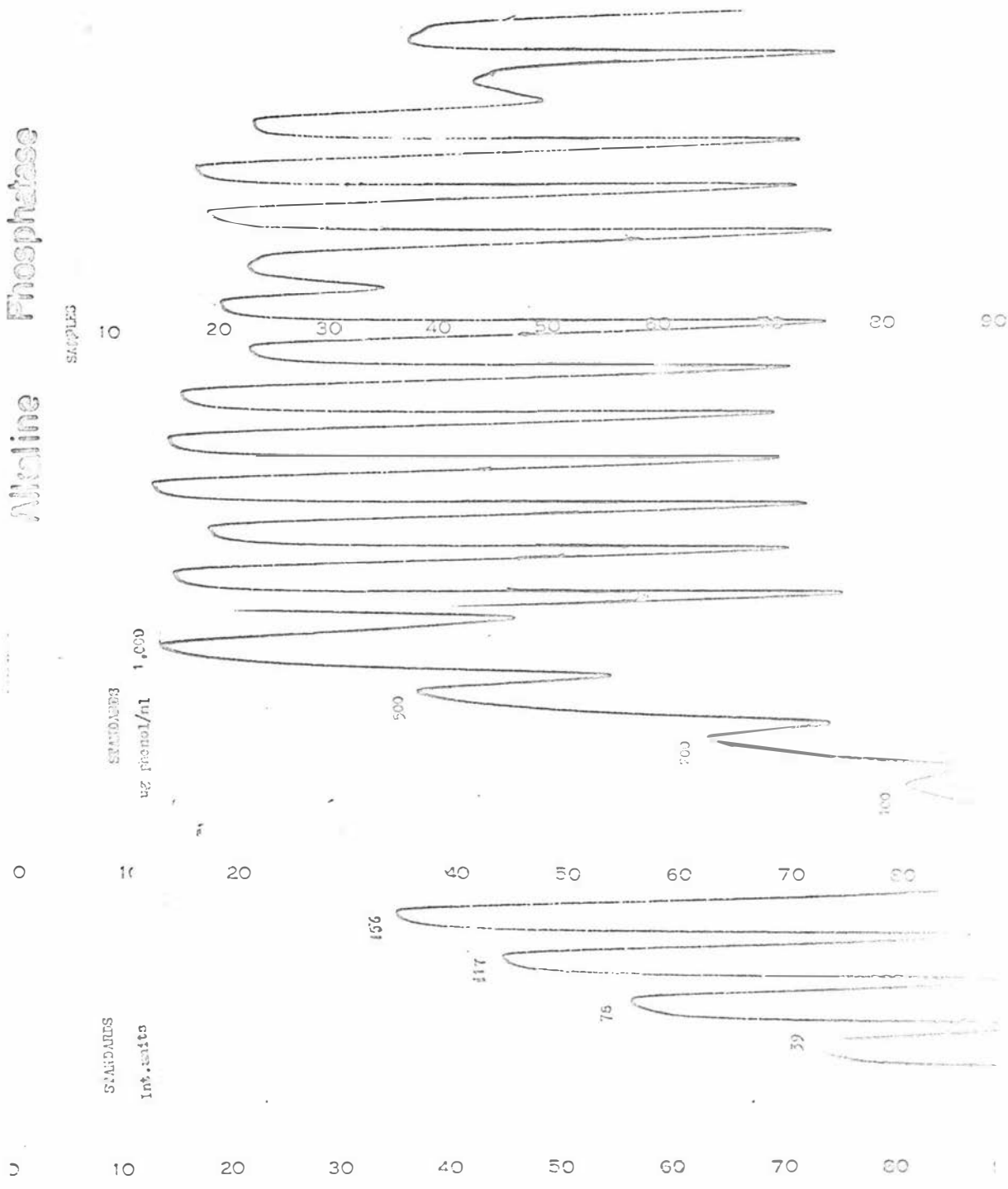
Calibration and Sample Peaks:

Modifications to the Original Method: Apart from the reagent alterations described above, the manifold consists of the portions of the original manifolds which deal with the estimation of the 'total' acid and alkaline phosphatase, with slight modifications to the pump tube sizes to give suitable sensitivity with the high levels of activity encountered. The manifold shown closely resembles that given in N28-29 methodology in the 'Autoanalyser' manual. In the absence of a pair of 505mu filters it was necessary to use a 520mu filter in the alkaline phosphatase colorimeter, but since the colour produced has a broad peak this did not seriously affect the sensitivity of the colorimeter response.

Notes:- A technical fault developed in the acid phosphatase manifold while samples were being run. Results from a number of samples were lost and there was insufficient serum left to repeat the estimations.

Acid & Alkaline Phosphatases




Alkaline Phosphatase
SAMPLES
10
20
30
40
50
60
70
80
90
STANDARDS
ug phenol/ml
1,000
500
100
0
10
20
40
50
60
70
80
156
117
78
39
STANDARDS
Int.units
0
10
20
30
40
50
60
70
80

Acid Phosphatase
SAMPLES
STANDARDS
µg Phenol/ml
100
50
20
10
5
Blanks
90°

Serum & Urine, Sodium, Potassium, Chloride & Bicarbonate

Automated Estimation:

Reference: Autoanalyser Methodology File N21b.
'Electrolyte Determination - Flame III'.

Reagents: as given in reference.

Flow Chart:

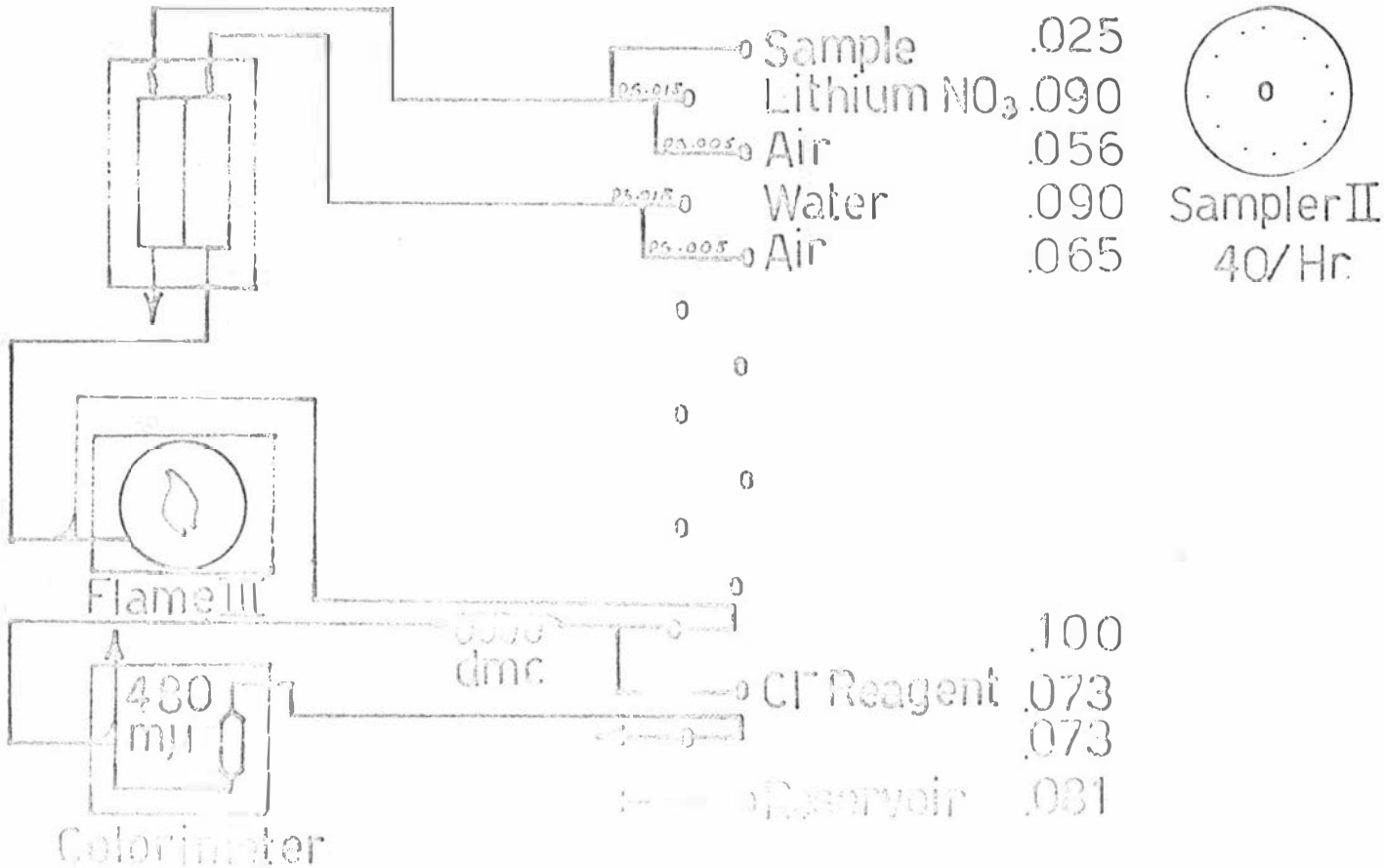
see page 045 - 048

Calibration Peaks:

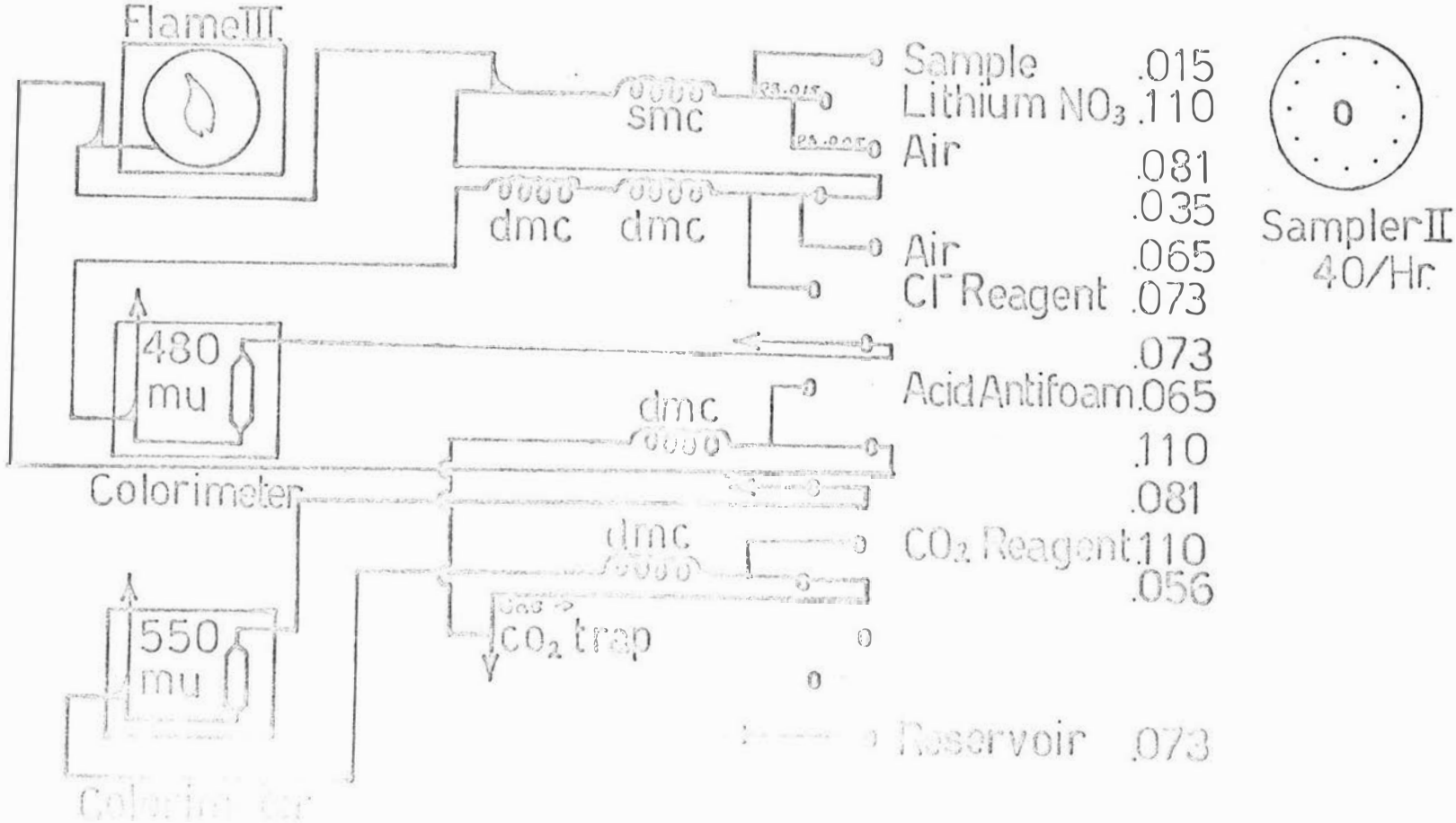
Modifications to Published Methods: Pump tube sizes and ionic concentrations of standards were adjusted to suit the range of values encountered in the samples of sheep urine. Bicarbonate estimations were omitted from the serum samples.

Note: No bicarbonate was detectable in any urine sample from these lambs.

Serum Sodium, Potassium & Chloride



Urine Sodium, Potassium, Chloride & Carbon dioxide



Chloride
Sodium

Potassium
SAMPLES
STANDARDS
STANDARDS

Serum Inorganic Phosphate

Automated Estimation:

Reference: 'Autoanalyser' Methodology file N-4c
Technicon Corporation, New York, 1968.

Reagents: Acid Molybdate - Stannous chloride - Hydrazine method as described. Note however that, in the 'General Description' of the method, it is stated that "Serum is mixed with, and also dialysed into 1% H_2SO_4 " and this was therefore done.

Flow Chart:

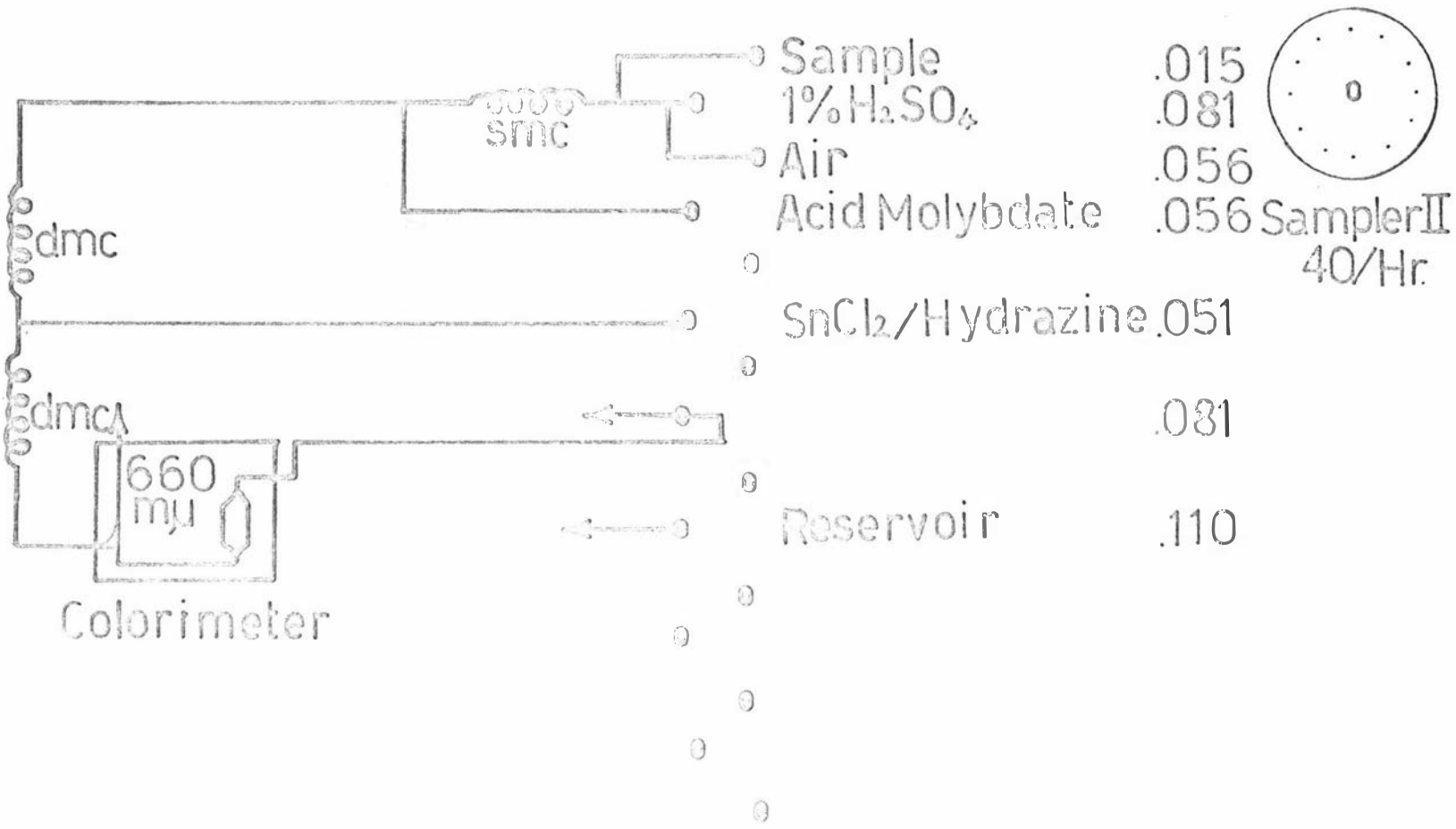
see page 050, 051

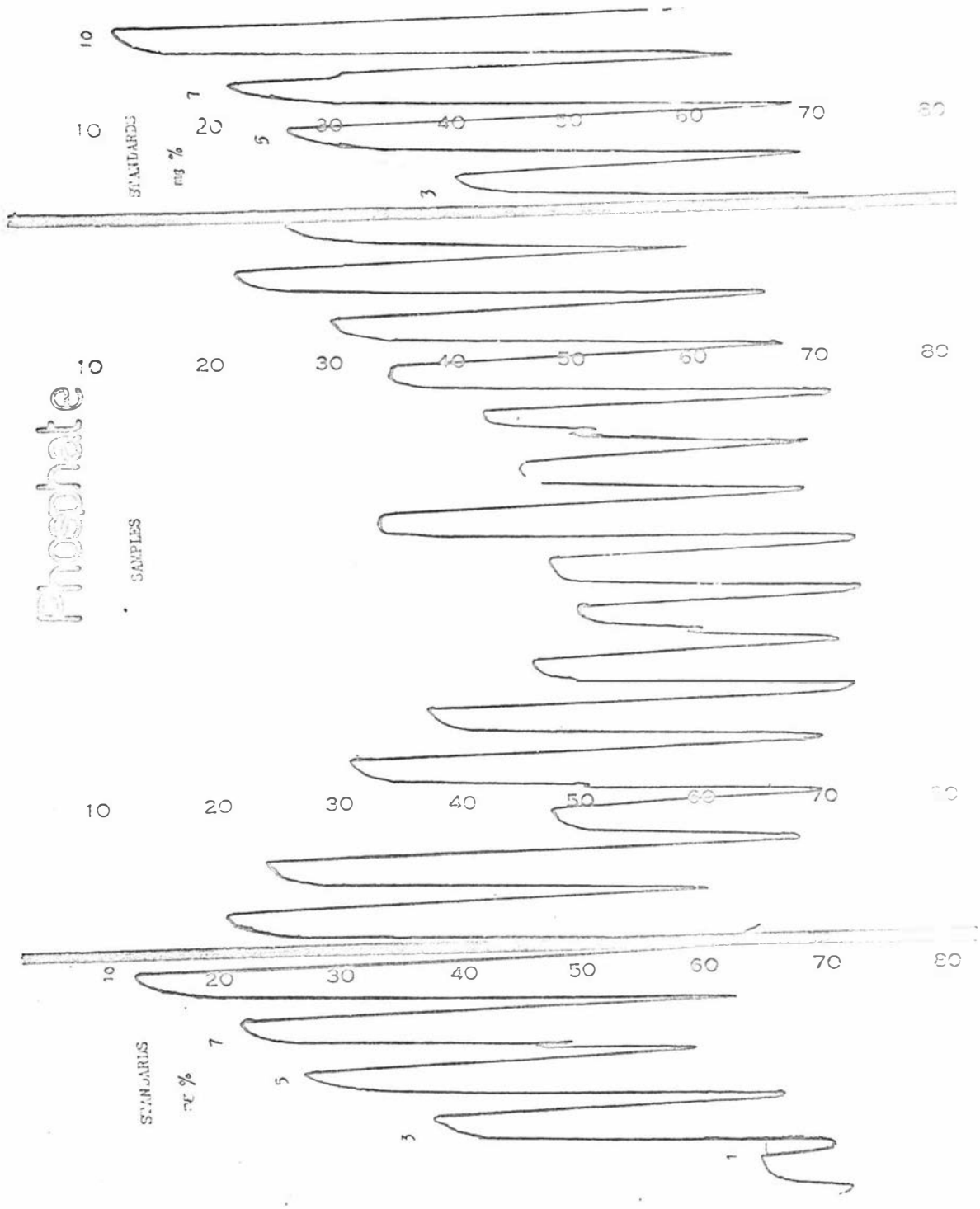
Calibration and Sample Peaks:

Modification to published methods: Since this estimation was carried out on the deproteinised samples dialysis was unnecessary and this was omitted. Pump tube sizes were adjusted to allow for this.

Notes: The use of H_2SO_4 is not shown in the list of reagents, nor in the published flow diagram. The method will not function satisfactorily if saline is used instead of the acid as the phosphates precipitate in the mixing coils and flow cuvette.

Phosphate


Sample .015
1% H_2SO_4 .081
Air .056
Acid Molybdate .056
SamplerII
40/Hr
smc
dmc
$SnCl_2$/Hydrazine .051
dmcA
.081
660
mμ
Reservoir .110
Colorimeter


Phosphate
SAMPLES
STANDARDS
mg %
10
20
30
40
50
60
70
80
1
3
5
7

Appendix 12:

CONTROL HAEMATOLOGICAL AND BIOCHEMICAL DATA -- VENOUS BLOOD

GROUP-- SECOND PROPERTY

ANIMAL	HB GM 0/0	PCV 0/0	PH	PCO2 MM HG	NA+ MEQ/L	K+ MEQ/L	PHOSPH MG 0/0	BUFFER MEQ/L	ST.HCO3 MEQ/L	AC.HCO3 MEQ/L	WCC /MM3
1	12.2	39.	7.43	32.	160.	53.	3.4	46.0	23.0	22.0	0.
3	9.9	35.	7.42	34.	160.	50.	5.0	45.0	23.5	23.0	0.
4	9.9	32.	7.41	36.	157.	54.	5.6	46.0	24.5	24.5	0.
7	11.6	37.	7.45	31.	154.	51.	0.0	46.5	24.0	22.5	0.
8	12.5	39.	7.38	31.	154.	51.	3.0	47.0	23.0	23.0	4800.
9	10.2	38.	7.41	32.	154.	56.	3.1	46.0	22.5	21.5	9250.
10	9.6	32.	7.37	36.	159.	50.	7.2	45.0	22.5	22.5	0.
11	10.0	33.	7.38	40.	155.	51.	3.0	45.5	24.0	24.5	0.
12	13.1	41.	7.40	35.	164.	54.	2.6	47.0	23.0	22.5	8200.
14	11.2	38.	7.39	34.	155.	52.	0.0	46.0	22.5	22.0	0.
15	9.3	29.	7.37	29.	152.	57.	6.4	45.0	20.5	19.0	5350.
16	13.0	39.	7.40	40.	157.	52.	9.0	46.5	24.5	24.5	0.
18	10.9	35.	7.39	36.	154.	49.	5.6	46.0	23.5	23.5	0.
19	12.9	40.	7.42	39.	160.	55.	4.6	47.0	25.0	25.0	6850.
20	9.8	33.	7.41	36.	146.	45.	7.8	46.0	24.5	24.5	0.
21	12.2	37.	7.45	30.	152.	52.	3.5	46.5	23.5	21.5	5450.
22	8.6	29.	7.37	38.	157.	50.	5.8	45.0	23.0	23.0	6250.
24	9.9	34.	7.38	44.	156.	60.	8.2	45.0	24.0	24.5	0.
25	12.0	38.	7.40	38.	161.	55.	5.5	46.0	24.0	24.0	0.
27	10.5	34.	7.36	40.	164.	56.	8.5	46.0	23.5	24.5	4950.
28	10.2	36.	7.40	43.	155.	51.	5.5	46.0	25.5	26.0	6150.
29	10.6	34.	7.36	40.	164.	57.	0.0	46.0	22.5	21.5	3450.
34	11.7	36.	7.36	39.	155.	54.	6.4	46.5	23.5	24.5	0.
35	11.4	36.	7.45	35.	152.	49.	4.1	46.0	25.5	25.0	5550.
37	11.4	36.	7.45	35.	167.	55.	7.3	46.0	23.5	23.5	7850.
39	11.9	39.	7.43	32.	155.	55.	6.0	46.5	23.5	22.0	6500.
41	10.9	36.	7.42	43.	158.	56.	2.8	46.0	26.0	26.0	0.
MEAN	11.01	35.7	7.402	36.2	156.9	52.9	5.37	46.00	23.64	23.35	6200.
STD DEV	1.21	3.1	.029	4.1	4.5	3.2	1.87	.60	1.14	1.59	1563.
NUMBER	27	27	27	27	27	27	24	27	27	27	13

CONTROL HAEMATOLOGICAL AND BIOCHEMICAL DATA -- VENOUS BLOOD

GROUP-- SECOND PROPERTY

ANIMAL	LDH IU	ICD IU	SGOT IU	SGPT IU	GLUCOSE MG 0/0	LACTATE MG 0/0	ALKPHOS IU	PROTEIN MG 0/0
1	850.	13.	54.	7.	73.	6.0	1025.	7.0
3	1040.	34.	51.	8.	90.	2.3	1120.	6.8
4	810.	14.	35.	7.	76.	2.2	585.	6.9
7	790.	15.	34.	8.	0.	0.0	420.	6.5
8	650.	10.	43.	8.	73.	0.0	790.	6.7
9	680.	9.	39.	8.	43.	0.0	555.	6.0
10	820.	10.	41.	9.	83.	0.0	790.	6.5
11	790.	10.	37.	8.	57.	3.8	625.	7.7
12	750.	13.	35.	7.	56.	4.3	750.	7.5
14	700.	15.	33.	10.	0.	0.0	655.	7.8
15	650.	16.	46.	18.	0.	12.7	615.	9.2
16	850.	9.	40.	7.	97.	4.4	710.	6.5
18	940.	17.	37.	11.	98.	4.7	1120.	7.1
19	660.	12.	50.	8.	68.	3.2	380.	7.0
20	710.	16.	42.	8.	93.	3.2	535.	7.7
21	680.	9.	49.	7.	65.	2.9	340.	6.2
22	730.	15.	38.	9.	72.	3.1	1120.	7.1
24	740.	23.	33.	9.	99.	5.4	1000.	6.3
25	820.	18.	37.	10.	58.	2.1	495.	5.8
27	720.	13.	37.	9.	86.	3.2	895.	6.9
28	660.	8.	28.	8.	53.	2.6	350.	7.1
29	810.	14.	42.	8.	0.	0.0	430.	6.3
34	760.	10.	43.	10.	80.	5.8	865.	6.1
35	810.	11.	55.	8.	74.	3.4	585.	6.6
37	870.	9.	54.	10.	92.	3.8	790.	6.8
39	610.	12.	56.	8.	68.	6.6	310.	6.1
41	730.	21.	44.	5.	48.	3.6	895.	6.3
MEAN	764.0	13.9	41.9	8.6	74.0	4.25	694.	6.83
STD DEV	97.0	5.4	7.6	2.2	16.5	2.31	251.	.71
NUMBER	27	27	27	27	23	21	27	27

Note: Zero values indicate no sample.

Appendix 12: (cont'd)

CONTROL HAEMATOLOGICAL AND BIOCHEMICAL DATA — VENOUS BLOOD

GROUP— PAIRED CONTROL

ANIMAL	HB GM 0/0	PCV 0/0	PH	PCO2 MM HG	NA+ MEQ/L	K+ MEQ/L	PHOSPH MG 0/0	BUFFER MEQ/L	ST.HCO3 MEQ/L	AC.HCO3 MEQ/L	MCC /MM3	P02 MM HG	CL- MEQ/L
0	10.4	34.	7.34	37.	0.	0.	5.8	46.0	21.5	20.5	3150.	90.	0.
13	11.6	38.	7.30	42.	147.	51.	5.3	46.5	21.5	24.0	4600.	60.	104.
23	9.4	31.	7.31	46.	146.	46.	3.7	43.5	21.5	22.5	5100.	80.	108.
26	9.3	31.	7.29	46.	169.	59.	2.1	49.0	20.5	23.0	9250.	72.	124.
32	11.5	39.	7.32	47.	158.	53.	5.5	42.5	22.5	25.0	0.	76.	116.
36	11.8	40.	7.37	43.	162.	62.	3.2	48.0	24.0	25.0	4000.	70.	117.
40	10.7	35.	7.36	44.	160.	52.	2.5	47.5	23.5	25.0	3600.	80.	117.
43	10.6	36.	7.35	47.	150.	48.	2.0	45.0	23.5	25.0	6150.	50.	112.
45	11.0	37.	7.33	41.	155.	50.	2.7	44.5	20.5	20.5	6700.	40.	114.
49	11.1	37.	0.00	0.	153.	55.	5.0	0.0	0.0	0.0	4100.	0.	114.
MEAN	10.74	35.8	7.330	43.6	155.5	52.8	3.78	45.83	22.11	23.39	5183.	68.6	114.0
STD DEV	.85	3.0	.027	3.3	7.5	5.1	1.48	2.15	1.31	1.88	1914.	15.9	5.7
NUMBER	10	10	9	9	9	9	10	9	9	9	9	9	9

CONTROL HAEMATOLOGICAL AND BIOCHEMICAL DATA — VENOUS BLOOD

GROUP— PAIRED CONTROL

ANIMAL	LDH IU	ICD IU	SGOT IU	SGPT IU	GLUCOSE MG 0/0	LACTATE MG 0/0	ALKPHOS IU	PROTEIN MG 0/0
0	1050.	42.	41.	8.	90.	4.1	575.	6.7
13	640.	11.	30.	8.	102.	5.7	565.	8.0
23	830.	17.	41.	10.	89.	3.1	945.	6.9
26	560.	10.	29.	11.	83.	13.9	260.	7.0
32	890.	22.	60.	8.	116.	3.8	905.	7.3
36	940.	20.	38.	7.	84.	14.5	915.	7.1
40	830.	16.	44.	12.	78.	12.4	700.	6.6
43	750.	12.	39.	7.	100.	8.0	875.	6.2
45	710.	11.	34.	13.	101.	16.0	585.	7.3
49	820.	12.	38.	6.	52.	0.0	800.	7.2
MEAN	802.0	17.3	39.4	9.0	89.5	9.05	712.	7.03
STD DEV	143.8	9.6	8.6	2.3	17.3	5.15	218.	.48
NUMBER	10	10	10	10	10	9	10	10

Note: Zero values indicate no sample.

CONTROL HAEMATOLOGICAL AND BIOCHEMICAL DATA -- VENOUS BLOOD

GROUP-- DIVIDED DOSE OF TOXIN... PREINOCULATION VALUES

ANIMAL	HB GM 0/0	PCV 0/0	PROTEIN MG 0/0	NA+ MEQ/L	K+ MEQ/L	CL- MEQ/L	PH	PCO2 MM HG	PO2 MM HG	LACTATE MG 0/0	GLUCOSE MG 0/0	PHOSPH MG 0/0
2	9.6	32.	7.4	148.	49.	103.	7.34	32.	91.	155.	84.	3.0
6	11.3	37.	6.2	149.	48.	107.	7.37	34.	96.	74.	73.	2.9
17	11.2	37.	6.8	148.	50.	105.	7.32	49.	60.	0.	61.	3.7
33	11.7	38.	6.7	152.	47.	112.	7.24	51.	82.	0.	112.	4.0
38	10.0	36.	6.6	154.	50.	119.	7.30	40.	70.	63.	119.	4.8
42	10.1	33.	6.7	152.	49.	113.	7.34	47.	80.	0.	97.	3.4
44	10.5	35.	6.9	153.	43.	110.	7.39	39.	50.	124.	108.	2.1
46	8.6	29.	6.7	152.	50.	110.	7.36	39.	60.	85.	63.	4.0
48	11.0	36.	7.0	159.	50.	122.	0.00	0.	0.	120.	79.	2.6
50	9.7	34.	5.8	150.	54.	116.	7.22	54.	0.	120.	123.	2.3
51	10.6	36.	6.6	158.	51.	117.	7.30	48.	0.	83.	90.	5.1
53	10.4	35.	6.6	150.	52.	108.	0.00	0.	0.	46.	82.	3.6
55	10.6	35.	6.9	156.	61.	112.	0.00	0.	0.	22.	75.	5.9
56	8.9	30.	6.1	151.	49.	111.	0.00	0.	0.	22.	50.	1.6
57	10.3	34.	6.8	152.	58.	108.	0.00	0.	0.	20.	79.	4.1
58	9.4	29.	6.1	153.	54.	109.	0.00	0.	0.	44.	63.	1.4
61	10.4	35.	6.4	150.	55.	107.	7.37	30.	70.	43.	64.	1.4
MEAN	10.25	34.1	6.60	152.1	51.1	111.1	7.326	42.0	73.1	72.9	83.7	3.66
STD DEV	.83	2.7	.38	3.1	4.2	5.0	.049	8.1	15.0	43.4	21.2	1.10
NUMBER	17	17	17	17	17	17	11	11	9	14	17	17

GROUP-- DIVIDED DOSE OF TOXIN... PREINOCULATION VALUES

ANIMAL	LDH IU	ICD IU	SGPT IU	SGOT IU	ALKPHOS IU	BUFFER MEQ/L	ST.HCO3 MEQ/L	AC.HCO3 MEQ/L	ACPHOS IU	WCC /MM3
2	880.	27.	12.	37.	555.	460.0	205.0	205.0	0.	3650.
6	860.	15.	10.	78.	670.	465.0	215.0	210.0	0.	4200.
17	760.	16.	7.	26.	525.	455.0	255.0	245.0	0.	4750.
33	830.	17.	4.	36.	975.	450.0	215.0	250.0	0.	3650.
38	820.	16.	8.	40.	1070.	430.0	190.0	190.0	0.	4150.
42	930.	13.	8.	54.	820.	445.0	235.0	250.0	0.	4000.
44	860.	13.	11.	51.	730.	440.0	230.0	230.0	0.	7850.
46	820.	21.	13.	39.	810.	435.0	215.0	215.0	0.	5500.
48	610.	12.	9.	35.	380.	0.0	0.0	0.0	0.	6150.
50	760.	12.	7.	33.	875.	425.0	180.0	210.0	205.	6250.
51	830.	17.	9.	54.	780.	445.0	215.0	230.0	220.	8100.
53	850.	19.	7.	54.	1060.	0.0	0.0	0.0	299.	5100.
55	860.	20.	4.	44.	1080.	0.0	0.0	0.0	310.	5050.
56	960.	21.	5.	45.	1110.	0.0	0.0	0.0	310.	6350.
57	900.	14.	8.	61.	1090.	0.0	0.0	0.0	345.	5250.
58	790.	17.	7.	46.	670.	0.0	0.0	0.0	170.	10000.
61	1050.	33.	10.	56.	1040.	255.0	145.0	170.0	310.	6250.
MEAN	845.2	17.8	8.1	46.4	837.6	427.72	209.09	218.63	271.1	5702.
STD DEV	94.1	5.5	2.5	12.5	226.4	58.58	29.48	25.40	63.2	1701.
NUMBER	17	17	17	17	17	11	11	11	8	17

CONTROL HAEMATOLOGICAL AND BIOCHEMICAL DATA -- VENOUS BLOOD

GROUP-- CONTINUOUS INFUSION OF TOXIN... PREINOCULATION VALUES

ANIMAL	HB GM 0/0	PCV 0/0	PROTEIN MG 0/0	NA+ MEQ/L	K+ MEQ/L	CL- MEQ/L	PH	PCO2 MM HG	PO2 MM HG	LACTATE MG 0/0	GLUCOSE MG 0/0	PHOSPH MG 0/0
62	11.0	36.	6.9	0.	0.	0.	7.39	30.	70.	40.	73.	4.6
63	11.8	38.	5.5	155.	54.	112.	7.38	40.	80.	84.	94.	1.4
64	10.4	34.	6.3	154.	53.	111.	7.35	45.	60.	48.	101.	2.2
66	10.9	34.	6.6	150.	52.	111.	7.35	0.	50.	76.	69.	1.6
70	10.2	34.	6.4	152.	53.	112.	7.38	42.	80.	42.	103.	4.3
71	12.6	39.	7.0	151.	53.	111.	7.41	39.	85.	40.	96.	7.1
72	10.7	37.	5.2	155.	56.	111.	7.37	42.	70.	52.	73.	3.3
73	10.2	34.	5.6	152.	51.	112.	7.40	40.	55.	44.	33.	2.1
75	9.4	33.	6.5	158.	49.	120.	7.38	42.	60.	58.	68.	2.3
MEAN	10.80	35.4	6.22	153.3	52.6	112.5	7.378	40.0	67.7	53.7	78.5	3.21
STD DEV	.94	2.1	.63	2.6	2.0	3.0	.020	4.4	12.2	16.1	23.0	1.34
NUMBER	9	9	9	8	8	8	9	8	9	9	9	9

GROUP-- CONTINUOUS INFUSION OF TOXIN... PREINOCULATION VALUES

ANIMAL	LDH IU	ICD IU	SGPT IU	SGOT IU	ALKPHOS IU	BUFFER MEQ/L	ST.HCO3 MEQ/L	AC.HCO3 MEQ/L	ACPHOS IU	WCC /MM3
62	800.	12.	10.	41.	760.	315.0	175.0	170.0	0.	6100.
63	960.	18.	8.	57.	1110.	455.0	235.0	235.0	250.	6200.
64	900.	14.	6.	44.	1040.	470.0	230.0	245.0	290.	6800.
66	910.	17.	8.	46.	1060.	0.0	0.0	0.0	300.	2100.
70	870.	14.	7.	55.	675.	430.0	240.0	240.0	190.	5150.
71	810.	17.	7.	46.	760.	430.0	240.0	240.0	225.	5600.
72	1100.	15.	8.	45.	1020.	420.0	230.0	230.0	295.	7950.
73	850.	10.	9.	46.	585.	390.0	240.0	240.0	245.	9000.
75	860.	13.	9.	45.	845.	415.0	240.0	240.0	260.	4750.
MEAN	895.5	14.4	8.0	47.2	872.7	415.62	228.75	230.00	256.8	5961.
STD DEV	91.2	2.6	1.2	5.2	190.1	47.39	23.16	24.64	37.9	1972.
NUMBER	9	9	9	9	9	8	8	8	8	9

Note: Zero values indicate no sample.

Note 2: Coding error in standard and actual bicarbonate values. All values shown are x10 actual value e.g. 205.0 = 20.5

Appendix 12: (cont'd)

CONTROL HAEMATOLOGICAL AND BIOCHEMICAL DATA -- ARTERIAL BLOOD

GROUP-- PAIRED CONTROL

ANIMAL	HB GM 0/0	PCV 0/0	PH	PCO2 MM HG	PO2 MM HG	GLUCOSE MG 0/0	PHOSPH MG 0/0	LACTATE MG 0/0	BUFFER MEQ/L	ST.HCO3 MEQ/L	AC.HCO3 MEQ/L
0	10.4	34.	7.43	30.	186.	73.	2.2	5.0	46.0	22.5	20.5
13	11.4	36.	7.41	31.	170.	107.	2.9	6.7	46.5	22.5	20.5
23	9.4	30.	7.40	38.	160.	81.	3.0	7.8	44.0	22.0	21.0
26	9.7	30.	7.41	31.	160.	69.	2.7	10.2	44.0	21.5	20.0
32	11.2	37.	7.47	30.	190.	76.	3.2	9.4	43.0	23.5	22.0
36	12.0	39.	7.45	35.	180.	82.	2.8	10.6	48.0	25.5	24.5
40	10.7	34.	7.42	37.	180.	95.	1.6	14.2	46.0	24.0	23.5
43	10.7	35.	7.41	35.	170.	95.	2.2	7.9	47.5	23.0	23.0
45	11.3	37.	7.45	24.	160.	128.	6.2	16.1	40.0	19.5	17.0
49	10.8	36.	0.00	0.	0.	73.	2.6	0.0	0.0	0.0	0.0
MEAN	10.76	34.8	7.427	32.3	172.8	87.9	2.9	9.7	45.0	22.6	21.3
STD DEV	.78	2.9	.023	4.3	11.6	18.5	1.2	3.5	2.5	1.6	2.2
NUMBER	10	10	9	9	9	10	10	9	9	9	9

CONTROL HAEMATOLOGICAL AND BIOCHEMICAL DATA -- ARTERIAL BLOOD

GROUP-- DIVIDED DOSE OF TOXIN... PREINOCULATION VALUES

ANIMAL	HB GM 0/0	PCV 0/0	PH	PCO2 MM HG	PO2 MM HG	GLUCOSE MG 0/0	PHOSPH MG 0/0	LACTATE MG 0/0	BUFFER MEQ/L	ST.HCO3 MEQ/L	AC.HCO3 MEQ/L	ACPHOS IU	WCC /MM3
2	9.4	34.	7.38	34.	180.	126.	7.6	5.6	46.0	22.5	21.5	0.	0.
6	11.3	36.	7.48	26.	200.	86.	3.5	5.7	46.0	22.5	20.0	0.	0.
17	10.6	34.	7.40	43.	160.	97.	2.9	4.9	49.0	25.5	26.0	0.	0.
33	11.3	36.	7.44	32.	240.	82.	5.4	4.0	46.0	23.5	22.0	0.	0.
38	10.6	36.	7.34	38.	220.	119.	2.5	13.4	43.0	20.5	20.5	0.	0.
42	10.5	33.	7.39	41.	140.	112.	3.2	8.3	46.0	24.0	24.0	0.	0.
44	11.0	30.	7.39	38.	130.	103.	2.1	11.0	42.0	22.5	22.5	0.	0.
46	9.2	30.	7.39	35.	170.	0.	0.0	0.0	44.0	21.5	20.5	0.	0.
48	10.5	35.	0.00	0.	0.	88.	2.6	11.0	0.0	0.0	0.0	0.	0.
50	10.0	33.	7.31	37.	0.	90.	4.2	10.0	39.0	18.5	18.0	0.	0.
51	10.9	36.	7.38	36.	0.	116.	3.0	5.9	40.5	21.5	21.5	0.	0.
53	10.6	34.	0.00	0.	0.	127.	2.4	8.6	0.0	0.0	0.0	0.	0.
55	10.6	34.	0.00	0.	0.	57.	3.6	3.2	0.0	0.0	0.0	0.	0.
56	8.9	31.	0.00	0.	0.	96.	2.3	4.9	0.0	0.0	0.0	0.	0.
57	10.4	34.	0.00	0.	0.	74.	3.8	3.0	0.0	0.0	0.0	0.	0.
58	0.0	0.	0.00	0.	0.	92.	3.0	0.0	8.3	2.4	5.2	0.	0.
61	10.4	35.	7.44	37.	160.	100.	2.0	5.6	55.0	25.5	24.5	0.	0.
MEAN	10.38	33.8	7.394	36.0	177.7	97.8	3.38	7.00	42.06	20.86	20.51	0.0	0.
STD DEV	.69	2.0	.046	4.5	36.3	19.1	1.42	3.17	11.41	6.14	5.28	0.0	0.
NUMBER	16	16	11	11	9	16	16	15	12	12	12	0	0

CONTROL HAEMATOLOGICAL AND BIOCHEMICAL DATA -- ARTERIAL BLOOD

GROUP-- CONTINUOUS INFUSION OF TOXIN... PREINOCULATION VALUES

ANIMAL	HB GM 0/0	PCV 0/0	PH	PCO2 MM HG	PO2 MM HG	GLUCOSE MG 0/0	PHOSPH MG 0/0	LACTATE MG 0/0	BUFFER MEQ/L	ST.HCO3 MEQ/L	AC.HCO3 MEQ/L	ACPHOS IU	WCC /MM3
62	10.6	36.	7.38	35.	0.	88.	1.6	10.3	43.0	21.0	20.0	0.	0.
63	0.0	0.	0.00	0.	0.	70.	3.8	3.5	0.0	0.0	0.0	0.	0.
64	9.6	33.	7.45	23.	130.	70.	3.0	4.7	57.0	22.0	15.5	0.	0.
66	10.8	33.	7.42	0.	110.	85.	2.3	4.6	0.0	0.0	0.0	0.	0.
70	10.5	35.	7.48	31.	120.	122.	6.5	4.2	42.5	23.5	22.0	0.	0.
71	11.8	37.	7.40	40.	0.	95.	7.4	4.8	39.0	22.5	22.5	0.	0.
72	10.7	37.	7.43	35.	130.	67.	3.1	5.7	40.0	23.5	23.5	0.	0.
73	10.7	34.	7.47	32.	140.	30.	1.5	2.4	40.0	23.0	22.5	0.	0.
75	9.2	33.	7.48	35.	160.	80.	5.8	5.2	42.0	23.0	22.0	0.	0.
MEAN	10.48	34.7	7.438	33.0	131.6	78.5	3.88	5.04	43.35	22.64	21.14	0.0	0.
STD DEV	.79	1.7	.037	5.2	17.2	24.8	2.17	2.19	6.19	.89	2.70	0.0	0.
NUMBER	8	8	8	7	6	9	9	9	7	7	7	0	0

Note: Zero values indicate no sample.

Appendix 13:

POST-INOCULATION HAEMATOLOGICAL AND BIOCHEMICAL DATA -- VENOUS BLOOD

GROUP-- DIVIDED DOSE OF TOXIN... INITIAL VALUES

ANIMAL	HB GM 0/0	PCV 0/0	PROTEIN MG 0/0	NA+ MEQ/L	K+ MEQ/L	CL- MEQ/L	PH	PCO2 MM HG	PO2 MM HG	LACTATE MG 0/0	GLUCOSE MG 0/0	PHOSPH MG 0/0
2	0.0	0.	0.0	0.	0.	0.	0.00	0.	0.	0.	0.	0.0
6	0.0	0.	0.0	0.	0.	0.	0.00	0.	0.	0.	0.	0.0
17	12.4	38.	6.9	150.	48.	109.	7.35	34.	200.	102.	158.	3.1
33	0.0	0.	0.0	0.	0.	0.	0.00	0.	0.	0.	0.	0.0
38	10.8	36.	0.0	0.	0.	0.	7.28	41.	80.	113.	158.	2.3
42	12.2	40.	6.9	154.	55.	118.	7.16	59.	75.	148.	200.	3.9
44	12.4	40.	6.6	146.	46.	111.	7.21	53.	50.	61.	152.	3.7
46	10.8	37.	6.6	150.	70.	110.	7.33	45.	20.	128.	138.	2.5
48	9.7	32.	6.6	157.	49.	124.	0.00	0.	0.	64.	80.	2.4
50	11.2	38.	5.5	154.	55.	121.	7.21	51.	0.	64.	108.	2.5
51	9.6	35.	6.6	165.	87.	126.	6.94	64.	0.	280.	158.	2.7
53	11.5	39.	6.4	162.	58.	118.	0.00	0.	0.	32.	72.	2.3
55	13.1	47.	6.5	0.	0.	0.	0.00	0.	0.	114.	88.	3.2
56	9.1	30.	6.0	153.	48.	114.	0.00	0.	0.	100.	64.	1.9
57	0.0	0.	0.0	0.	0.	0.	0.00	0.	0.	0.	0.	0.0
58	0.0	0.	0.0	0.	0.	0.	0.00	0.	0.	0.	0.	0.0
61	0.0	0.	0.0	0.	0.	0.	0.00	0.	0.	0.	0.	0.0
MEAN	11.16	37.4	6.46	154.5	57.3	116.7	7.211	49.5	85.0	109.6	125.0	2.81
STD DEV	1.30	4.4	.42	6.0	13.3	6.1	.138	10.3	68.5	66.0	44.7	.60
NUMBER	11	11	10	9	9	9	7	7	5	11	11	11

GROUP-- DIVIDED DOSE OF TOXIN... INITIAL VALUES

ANIMAL	LDH IU	ICD IU	SGPT IU	SGOT IU	ALKPHOS IU	BUFFER MEQ/L	ST.HCO3 MEQ/L	AC.HCO3 MEQ/L
2	0.	0.	0.	0.	0.	0.0	0.0	0.0
6	0.	0.	0.	0.	0.	0.0	0.0	0.0
17	730.	19.	9.	26.	800.	47.0	22.5	24.5
33	0.	0.	0.	0.	0.	0.0	0.0	0.0
38	0.	27.	0.	0.	0.	41.0	18.5	19.0
42	860.	16.	11.	54.	945.	39.0	16.5	20.0
44	850.	15.	10.	51.	790.	0.0	0.0	0.0
46	830.	24.	8.	40.	840.	46.0	22.0	23.5
48	760.	17.	11.	41.	370.	0.0	0.0	0.0
50	750.	16.	7.	36.	905.	39.0	17.5	19.5
51	990.	20.	21.	85.	865.	26.0	9.8	11.8
53	920.	36.	8.	75.	965.	0.0	0.0	0.0
55	1050.	47.	17.	60.	915.	0.0	0.0	0.0
56	1050.	53.	8.	68.	1010.	0.0	0.0	0.0
57	0.	0.	0.	66.	0.	0.0	0.0	0.0
58	0.	25.	0.	57.	0.	0.0	0.0	0.0
61	0.	21.	0.	0.	0.	0.0	0.0	0.0
MEAN	879.0	25.8	11.0	54.9	840.5	39.66	17.8	19.71
STD DEV	119.7	12.2	4.5	17.2	179.8	7.52	4.58	4.48
NUMBER	10	13	10	12	10	6	6	6

GROUP-- CONTINUOUS INFUSION OF TOXIN... INTITIAL VALUES

ANIMAL	HB GM 0/0	PCV 0/0	PROTEIN MG 0/0	NA+ MEQ/L	K+ MEQ/L	CL- MEQ/L	PH	PCO2 MM HG	PO2 MM HG	LACTATE MG 0/0	GLUCOSE MG 0/0	PHOSPH MG 0/0
62	0.0	0.	0.0	0.	0.	0.	0.00	0.	0.	0.	0.	0.0
63	13.2	43.	5.2	152.	50.	112.	7.25	43.	65.	156.	149.	1.6
64	0.0	0.	0.0	0.	0.	0.	0.00	0.	0.	0.	0.	0.0
66	0.0	0.	0.0	0.	0.	0.	0.00	0.	0.	0.	0.	0.0
70	10.8	36.	5.8	153.	44.	115.	7.33	40.	80.	65.	122.	3.1
71	12.8	41.	6.9	155.	48.	115.	7.28	43.	85.	68.	145.	7.3
72	0.0	0.	0.0	0.	0.	0.	0.00	0.	0.	0.	0.	0.0
73	0.0	0.	0.0	0.	0.	0.	0.00	0.	0.	0.	0.	0.0
75	9.2	32.	6.3	156.	71.	117.	7.35	41.	60.	64.	118.	4.6
MEAN	11.50	38.0	6.05	154.0	53.2	114.7	7.302	41.7	72.5	88.2	133.5	4.15
STD OEV	1.85	4.9	.72	1.8	12.0	2.0	.045	1.5	11.9	45.1	15.7	2.43
NUMBER	4	4	4	4	4	4	4	4	4	4	4	4

GROUP-- CONTINUOUS INFUSION OF TOXIN... INTITIAL VALUES

ANIMAL	LDH JU	ICD IU	SGPT IU	SGOT IU	ALKPHOS IU	BUFFER MEQ/L	ST.HCO3 MEQ/L	AC.HCO3 MEQ/L
62	0.	16.	0.	0.	0.	0.0	0.0	0.0
63	950.	23.	10.	65.	1000.	40.5	17.5	18.5
64	0.	0.	0.	50.	0.	0.0	0.0	0.0
66	0.	0.	0.	45.	0.	0.0	0.0	0.0
70	930.	53.	8.	110.	700.	42.0	20.5	20.5
71	850.	21.	11.	41.	680.	38.5	18.5	18.5
72	0.	0.	0.	0.	0.	0.0	0.0	0.0
73	0.	0.	0.	0.	0.	0.0	0.0	0.0
75	900.	17.	9.	47.	985.	38.0	21.5	21.5
MEAN	907.5	26.0	9.5	59.6	841.2	39.75	19.50	19.75
STD DEV	43.4	15.3	1.2	25.9	174.9	1.84	1.82	1.50
NUMBER	4	5	4	6	4	4	4	4

Note: Zero values indicate no sample.

Appendix 13: (cont'd)

POST-INOCULATION HAEMATOLOGICAL AND BIOCHEMICAL DATA -- VENOUS BLOOD

GROUP-- DIVIDED DOSE OF TOXIN... TERMINAL VALUES

ANIMAL	HB GM 0/0	PCV 0/0	PROTEIN MG 0/0	NA+ MEQ/L	K+ MEQ/L	CL- MEQ/L	PH	PCO2 MM HG	PO2 MM HG	LACTATE MG 0/0	GLUCOSE MG 0/0	PHOSPH MG 0/0
2	9.0	29.	7.5	140.	56.	102.	7.27	35.	100.	120.	0.	3.9
6	15.8	51.	5.8	0.	0.	0	0.00	0.	0.	260.	210.	3.0
17	13.0	43.	6.8	150.	77.	109.	7.15	44.	40.	320.	240.	6.6
33	8.6	63.	0.0	0.	0.	0.	6.84	47.	50.	330.	201.	6.8
38	15.4	56.	6.2	0.	0.	0.	6.95	60.	50.	180.	310.	4.2
42	15.5	54.	6.9	0.	0.	0.	6.87	81.	50.	230.	330.	4.0
44	14.0	48.	6.1	0.	0.	0.	7.11	46.	60.	210.	265.	4.2
46	0.0	0.	8.9	0.	0.	0.	0.00	0.	0.	210.	100.	3.4
48	15.3	52.	6.3	153.	44.	120.	0.00	0.	0.	148.	131.	4.6
50	20.0	71.	5.4	0.	0.	0.	0.00	0.	0.	148.	123.	3.4
51	9.5	33.	6.5	158.	43.	121.	7.19	30.	0.	280.	265.	2.1
53	0.0	0.	0.0	0.	0.	0.	0.00	0.	0.	280.	128.	6.4
55	16.6	57.	0.0	0.	0.	0.	0.00	0.	0.	200.	130.	3.2
56	22.8	76.	5.7	0.	0.	0.	0.00	0.	0.	0.	0.	0.0
57	16.6	56.	6.6	0.	0.	0.	0.00	0.	0.	103.	129.	2.7
58	10.3	33.	5.7	156.	54.	116.	0.00	0.	0.	62.	67.	1.1
61	18.9	62.	5.6	0.	0.	0.	7.10	65.	35.	113.	134.	3.3
MEAN	15.42	52.2	6.42	151.4	54.8	113.6	7.060	51.0	55.0	199.6	184.1	4.24
STD DEV	3.88	13.5	.92	7.0	13.7	8.0	.155	16.7	21.4	80.4	81.6	1.35
NUMBER	15	15	14	5	5	5	8	8	7	16	15	16

GROUP-- DIVIDED DOSE OF TOXIN... TERMINAL VALUES

ANIMAL	LDH IU	ICD IU	SGPT IU	SGOT IU	ALKPHOS IU	BUFFER MEQ/L	ST.HCO3 MEQ/L	AC.HCO3 MEQ/L	ACPHOS IU	WCC /MM3
2	1050.	54.	0.	64.	820.	46.0	20.5	23.0	0.	3500.
6	900.	32.	11.	88.	1070.	0.0	0.0	0.0	0.	0.
17	860.	38.	9.	74.	635.	34.0	22.5	24.5	0.	5050.
33	760.	24.	0.	39.	0.	25.0	8.8	0.0	0.	11050.
38	790.	0.	8.	42.	0.	24.5	8.7	9.7	0.	6750.
42	910.	25.	10.	71.	905.	24.5	9.0	14.0	0.	7150.
44	880.	26.	11.	80.	730.	31.0	12.8	13.5	0.	7150.
46	780.	44.	0.	80.	0.	0.0	0.0	0.0	0.	5800.
48	770.	21.	14.	47.	420.	0.0	0.0	0.0	0.	6850.
50	730.	17.	8.	35.	905.	0.0	0.0	0.0	0.	7750.
51	940.	23.	34.	90.	740.	30.0	12.4	13.0	205.	8900.
53	0.	0.	0.	0.	0.	0.0	0.0	0.0	0.	5600.
55	0.	0.	0.	0.	0.	0.0	0.0	0.0	0.	10550.
56	1040.	42.	9.	63.	0.	0.0	0.0	0.0	0.	17900.
57	800.	15.	11.	0.	1160.	0.0	0.0	0.0	315.	6050.
58	920.	0.	8.	0.	645.	0.0	0.0	0.0	170.	10650.
61	1050.	0.	0.	49.	0.	37.0	14.0	20.0	0.	9800.
MEAN	878.6	30.0	12.0	63.2	803.0	31.50	13.58	16.81	230.0	8156.
STD DEV	108.1	12.0	7.4	19.0	218.3	7.45	5.30	5.65	75.6	3388.
NUMBER	15	12	11	13	10	8	8	7	3	16

GROUP-- CONTINUOUS INFUSION OF TOXIN... TERMINAL VALUES

ANIMAL	HB GM 0/0	PCV 0/0	PROTEIN MG 0/0	NA+ MEQ/L	K+ MEQ/L	CL- MEQ/L	PH	PCO2 MM HG	PO2 MM HG	LACTATE MG 0/0	GLUCOSE MG 0/0	PHOSPH MG 0/0
62	11.4	37.	6.8	0.	0.	0.	7.22	40.	70.	141.	129.	3.
63	18.6	64.	5.0	0.	0.	0.	6.88	70.	50.	148.	205.	7.
64	18.0	60.	6.0	0.	0.	0.	6.77	0.	70.	68.	170.	5.
66	10.8	34.	6.4	150.	68.	111.	7.11	0.	50.	130.	180.	4.
70	13.6	44.	5.9	155.	68.	119.	7.06	62.	60.	140.	165.	4.9
71	17.1	57.	6.8	0.	0.	0.	7.05	50.	45.	200.	220.	5.
72	21.0	76.	0.0	0.	0.	0.	6.85	115.	40.	108.	109.	3.4
73	10.9	37.	5.6	150.	57.	112.	7.41	34.	55.	45.	95.	5.0
75	11.6	40.	6.1	150.	56.	109.	7.16	44.	90.	156.	180.	3.8
MEAN	14.77	49.8	6.07	151.2	62.2	112.7	7.056	59.2	58.8	126.2	161.4	4.72
STD DEV	3.91	14.7	.60	2.5	6.6	4.3	.200	27.5	15.5	46.8	42.3	1.18
NUMBER	9	9	8	4	4	4	9	7	9	9	9	9

GROUP-- CONTINUOUS INFUSION OF TOXIN... TERMINAL VALUES

ANIMAL	LDH IU	ICD IU	SGPT IU	SGOT IU	ALKPHOS IU	BUFFER MEQ/L	ST.HCO3 MEQ/L	AC.HCO3 MEQ/L	ACPHOS IU	WCC /MM3
62	770.	0.	11.	43.	0.	36.5	15.5	15.5	0.	4400.
63	930.	28.	12.	65.	935.	24.5	8.8	14.0	0.	12000.
64	840.	25.	11.	0.	935.	24.0	84.0	0.0	0.	10450.
66	860.	18.	10.	0.	1060.	0.0	0.0	0.0	280.	4800.
70	920.	52.	12.	130.	790.	33.5	13.0	17.0	235.	2100.
71	770.	24.	15.	46.	675.	30.0	11.4	13.0	190.	6300.
72	1100.	18.	0.	0.	0.	26.0	9.2	20.5	0.	6950.
73	870.	15.	7.	55.	925.	38.0	21.5	21.0	265.	5300.
75	890.	19.	12.	55.	945.	34.5	14.2	15.0	240.	5000.
MEAN	883.3	24.8	11.2	65.6	895.0	30.87	22.20	16.57	242.0	6366.
STD DEV	99.2	11.7	2.2	32.4	124.6	5.54	25.29	3.11	34.3	3087.
NUMBER	9	8	8	6	7	8	8	7	5	9

Note: Zero values indicate no sample.

Appendix 13: (cont'd)

POST-INOCULATION HAEMATOLOGICAL AND BIOCHEMICAL DATA -- ARTERIAL BLOOD

GROUP-- CONTINUOUS INFUSION OF TOXIN... INITIAL VALUES

ANIMAL	HB GM 0/0	PCV 0/0	PH	PCO2 MM HG	PO2 MM HG	GLUCOSE MG 0/0	PHOSPH MG 0/0	LACTATE MG 0/0	BUFFER MEQ/L	ST.HCO3 MEQ/L	AC.HCO3 MEQ/L	ACPHOS IU	WCC /MM3
62	0.0	0.	0.00	0.	0.	0.	0.0	0.0	0.0	0.0	0.0	0.	0.
63	0.0	0.	0.00	0.	0.	0.	0.0	0.0	0.0	0.0	0.0	0.	0.
64	0.0	0.	0.00	0.	0.	0.	0.0	0.0	0.0	0.0	0.0	0.	0.
66	0.0	0.	0.00	0.	0.	0.	0.0	0.0	0.0	0.0	0.0	0.	0.
70	10.8	36.	7.34	39.	140.	147.	3.2	0.0	38.0	20.0	20.0	0.	0.
71	0.0	0.	0.00	0.	0.	129.	4.2	11.5	0.0	0.0	0.0	0.	0.
72	0.0	0.	0.00	0.	0.	0.	0.0	0.0	0.0	0.0	0.0	0.	0.
73	0.0	0.	0.00	0.	0.	0.	0.0	0.0	0.0	0.0	0.0	0.	0.
75	9.8	33.	7.38	40.	125.	68.	3.5	2.8	49.5	23.0	23.0	0.	0.
MEAN	10.30	34.5	7.360	39.5	132.5	114.6	3.63	7.15	43.75	21.50	21.50	0.0	0.
STD DEV	.70	2.1	.028	.7	10.6	41.4	.51	6.15	8.13	2.12	2.12	0.0	0.
NUMBER	2	2	2	2	2	3	3	2	2	2	2	0	0

POST-INOCULATION HAEMATOLOGICAL AND BIOCHEMICAL DATA -- ARTERIAL BLOOD

GROUP-- DIVIDED DOSE OF TOXIN... INITIAL VALUES

ANIMAL	HB GM 0/0	PCV 0/0	PH	PCO2 MM HG	PO2 MM HG	GLUCOSE MG 0/0	PHOSPH MG 0/0	LACTATE MG 0/0	BUFFER MEQ/L	ST.HCO3 MEQ/L	AC.HCO3 MEQ/L	ACPHOS IU	WCC /MM3
2	0.0	0.	0.00	0.	0.	0.	0.0	0.0	0.0	0.0	0.0	0.	0.
6	0.0	0.	0.00	0.	0.	0.	0.0	0.0	0.0	0.0	0.0	0.	0.
17	12.0	35.	7.33	48.	240.	235.	5.9	11.2	41.0	19.0	17.5	0.	0.
33	0.0	0.	0.00	0.	0.	0.	0.0	0.0	0.0	0.0	0.0	0.	0.
38	10.0	35.	7.34	30.	350.	0.	0.0	0.0	32.5	16.5	15.5	0.	0.
42	12.4	39.	7.35	43.	120.	230.	6.5	7.1	39.0	17.5	18.0	0.	0.
44	12.3	39.	7.28	46.	110.	200.	2.1	15.2	43.5	19.5	21.0	0.	0.
46	11.8	39.	7.25	48.	30.	0.	0.0	0.0	42.0	18.5	20.5	0.	0.
48	10.6	34.	0.00	0.	0.	70.	2.4	4.4	0.0	0.0	0.0	0.	0.
50	10.8	38.	7.32	36.	0.	69.	6.7	2.7	38.0	18.0	18.0	0.	0.
51	0.0	0.	0.00	0.	0.	0.	0.0	0.0	0.0	0.0	0.0	0.	0.
53	0.0	0.	0.00	0.	0.	0.	0.0	0.0	0.0	0.0	0.0	0.	0.
55	0.0	0.	0.00	0.	0.	0.	0.0	0.0	0.0	0.0	0.0	0.	0.
56	0.0	0.	0.00	0.	0.	0.	0.0	0.0	0.0	0.0	0.0	0.	0.
57	0.0	0.	0.00	0.	0.	0.	0.0	0.0	0.0	0.0	0.0	0.	0.
58	0.0	0.	0.00	0.	0.	0.	0.0	0.0	0.0	0.0	0.0	0.	0.
61	0.0	0.	0.00	0.	0.	0.	0.0	0.0	0.0	0.0	0.0	0.	0.
MEAN	11.41	37.0	7.295	41.8	170.0	160.8	4.72	8.12	39.33	18.16	18.41	0.0	0.
STD DEV	.93	2.2	.040	7.3	125.4	84.4	2.27	5.09	3.89	1.08	2.03	0.0	0.
NUMBER	7	7	6	6	5	5	5	5	6	6	6	0	0

Note: Zero values indicate no sample.

Appendix 13: (cont'd)

POST-INOCULATION HAEMATOLOGICAL AND BIOCHEMICAL DATA -- ARTERIAL BLOOD

GROUP-- DIVIDED DOSE OF TOXIN... TERMINAL VALUES

ANIMAL	HB GM 0/0	PCV 0/0	PH	PCO2 MM HG	PO2 MM HG	GLUCOSE MG 0/0	PHOSPH MG 0/0	LACTATE MG 0/0	BUFFER MEQ/L	ST.HCO3 MEQ/L	AC.HCO3 MEQ/L	ACPHOS IU	WCC /MM3
2	8.9	28.	7.23	45.	174.	174.	0.0	12.5	46.0	20.5	23.0	0.	0.
6	8.5	28.	0.00	0.	0.	270.	4.1	28.0	0.0	0.0	0.0	0.	0.
17	0.0	0.	0.00	0.	0.	325.	0.0	32.0	0.0	0.0	0.0	0.	0.
33	18.8	66.	6.93	80.	70.	166.	5.0	14.1	25.0	14.0	11.4	0.	0.
38	14.8	52.	7.08	31.	70.	230.	4.1	14.4	28.0	10.3	8.3	0.	0.
42	0.0	0.	0.00	0.	0.	0.	0.0	0.0	0.0	0.0	0.0	0.	0.
44	14.4	48.	7.10	37.	125.	0.	0.0	0.0	31.0	11.4	11.0	0.	0.
46	0.0	0.	0.00	0.	0.	0.	0.0	0.0	0.0	0.0	0.0	0.	0.
48	0.0	0.	0.00	0.	0.	102.	4.1	18.2	0.0	0.0	0.0	0.	0.
50	0.0	0.	0.00	0.	0.	0.	0.0	0.0	0.0	0.0	0.0	0.	0.
51	0.0	0.	0.00	0.	0.	0.	0.0	0.0	0.0	0.0	0.0	0.	0.
53	0.0	0.	0.00	0.	0.	0.	0.0	0.0	0.0	0.0	0.0	0.	0.
55	0.0	0.	0.00	0.	0.	0.	0.0	0.0	0.0	0.0	0.0	0.	0.
56	0.0	0.	0.00	0.	0.	0.	0.0	0.0	0.0	0.0	0.0	0.	0.
57	0.0	0.	0.00	0.	0.	133.	2.1	12.4	0.0	0.0	0.0	0.	0.
58	0.0	0.	0.00	0.	0.	0.	0.0	0.0	14.7	1.9	9.2	0.	0.
61	23.0	76.	7.02	65.	30.	118.	3.2	11.2	34.5	11.6	21.0	0.	0.
MEAN	14.73	49.6	7.072	51.6	93.8	189.7	4.81	18.10	29.86	11.61	14.06	0.0	0.
STD DEV	5.61	19.5	.110	20.4	56.1	78.5	2.28	7.74	10.39	6.01	6.25	0.0	0.
NUMBER	6	6	5	5	5	8	7	8	6	6	6	0	0

POST-INOCULATION HAEMATOLOGICAL AND BIOCHEMICAL DATA -- ARTERIAL BLOOD

GROUP-- CONTINUOUS INFUSION OF TOXIN... TERMINAL VALUES

ANIMAL	HB GM 0/0	PCV 0/0	PH	PCO2 MM HG	PO2 MM HG	GLUCOSE MG 0/0	PHOSPH MG 0/0	LACTATE MG 0/0	BUFFER MEQ/L	ST.HCO3 MEQ/L	AC.HCO3 MEQ/L	ACPHOS IU	WCC /MM3
62	0.0	0.	0.00	0.	0.	0.	0.0	0.0	0.0	0.0	0.0	0.	0.
63	0.0	0.	0.00	0.	0.	220.	2.2	14.2	0.0	0.0	0.0	0.	0.
64	0.0	0.	0.00	0.	0.	190.	4.7	20.0	0.0	0.0	0.0	0.	0.
66	0.0	0.	0.00	0.	0.	215.	6.6	30.0	0.0	0.0	0.0	0.	0.
70	0.0	0.	0.00	0.	0.	0.	0.0	9.0	0.0	0.0	0.0	0.	0.
71	0.0	0.	0.00	0.	0.	0.	0.0	0.0	0.0	0.0	0.0	0.	0.
72	0.0	0.	0.00	0.	0.	142.	4.1	15.7	0.0	0.0	0.0	0.	0.
73	12.6	39.	7.33	36.	0.	106.	3.4	9.4	36.0	18.5	18.0	0.	0.
75	0.0	0.	0.00	0.	0.	0.	0.0	0.0	0.0	0.0	0.0	0.	0.
MEAN	12.60	39.0	7.330	36.0	0.0	174.6	4.20	16.38	36.00	18.50	18.00	0.0	0.
STD DEV	0.00	0.0	0.000	0.0	0.0	49.2	1.63	7.83	0.00	0.00	0.00	0.0	0.
NUMBER	1	1	1	1	0	5	5	6	1	1	1	0	0

Note: Zero values indicate no sample.

Appendix 14: Haematological and Biochemical Data of Lambs which Received Intraduodenal Infusions of Cl. Perfringens Type D plus Carbohydrate

Hours after start of Infusion	Haemoglobin Gm%	P.C.V. %	Total Protein Gm%	Glucose mg%	Pyruvate mg%	Lactate mg%	Phosphate mg%	Sodium meq/l	Potassium meq/l	Chloride meq/l
Animal 77 - 8 month male										
0:00	6.2	20.5	7.5	76	0.7	4	3.7	146	4.0	105
0:30	6.5	20.0	7.0	76	1.1	7	4.0	143	4.0	108
1:00	7.0	21.5	7.0	75	0.9	4	3.3	140	4.5	109
1:30	6.2	19.0	7.5	75	1.1	4	3.5	141	4.0	106
2:00	6.0	-	6.8	76	0.8	4	3.1	140	4.2	108
2:30	6.4	20.5	6.8	75	0.7	4	2.8	145	4.4	107
3:00	6.0	19.0	7.0	74	1.1	1	3.2	145	4.4	110
3:30	6.3	20.5	7.2	73	1.0	4	3.3	147	4.3	110
4:00	7.6	-	7.7	145	0.7	60	3.6	141	4.5	103
4:15	7.6	26.0	7.2	180	2.0	84	4.3	-	-	-
4:30	8.8	29.0	7.0	225	2.1	88	4.0	143	4.5	101
4:45	8.7	30.0	6.5	270	2.4	88	5.3	146	6.5	108
Animal 78 - 8 month male										
0:00	-	-	6.7	70	1.4	1	4.9	154	4.2	116
1:00	-	-	6.4	77	0.8	-	4.9	152	4.0	111
1:55	8.0	20.0	6.7	65	1.2	6	5.0	153	4.4	112
2:15	8.4	22.0	6.4	72	1.2	4	4.8	144	4.1	112
2:50	8.6	22.0	6.7	85	1.6	-	5.2	143	4.2	112
3:20	8.6	22.0	6.4	85	1.4	1	5.5	149	3.9	110
3:50	8.5	22.0	6.7	82	1.6	1	5.6	145	3.7	113
4:35	8.7	21.0	6.4	75	2.0	1	6.1	150	3.5	110
4:50	9.2	24.0	7.1	75	1.8	10	5.3	146	3.5	113
5:10	9.4	23.5	6.7	115	1.4	22	4.9	150	3.5	107
5:25	9.6	24.0	6.7	160	2.2	32	4.8	150	3.3	107
5:35	9.2	22.0	6.7	195	1.8	56	5.1	149	3.2	109
5:55	9.4	24.0	6.7	245	2.8	74	5.2	148	3.2	106
6:10	9.4	23.0	7.5	265	3.6	80	7.5	144	3.3	110
6:30	8.5	22.0	7.2	260	5.6	116	6.7	147	6.3	110
6:55	8.8	22.0	6.7	255	-	106	7.2	151	2.9	111
7:20	9.6	23.0	7.0	295	5.8	167	10.2	150	7.8	106
7:45	9.4	22.0	7.5	280	6.6	172	12.4	138	4.8	102

cont'd.....

Appendix 14: (cont'd.)

Hours after start of Infusion	Haemo-globin Gm%	P.C.V. %	Total Protein Gm%	Glu-cose mg%	Pyru-vate mg%	Lac-tate mg%	Phos-phate mg%	Sodium meq/l	Potas-sium meq/l	Chlo-ride meq/l
Animal 81 - 8 month male										
0:00	10.8	29.0	7.7	100	0.6	31	6.0	153	5.0	
1:00	10.0	28.0	7.4	118	0.6	25	5.1	152	4.9	
2:00	9.8	25.0	7.7	109	0.6	16	5.1	151	4.4	
3:00	10.6	29.0	7.7	91	0.5	14	4.9	152	4.4	
4:00	10.0	26.0	7.7	109	0.5	8	5.1	152	4.5	
5:00	10.3	29.0	7.4	82	0.5	16	4.7	151	4.5	
5:50	12.3	31.5	8.2	154	1.3	95	5.3	152	5.1	
6:30	12.8	35.0	8.5	245	2.5	125	5.1	151	4.1	
7:00	18.0	49.0	6.9	320	2.9	230	7.2	-	-	
7:15	18.0	51.0	7.7	355	2.3	220	8.2	-	-	
Animal 85 - 3 week female										
0:00	-	39.0	7.6	83	-	14	6.8	149	4.9	
2:30	-	38.0	7.8	100	-	14	6.3	142	4.3	
4:00	-	36.0	7.1	104	-	17	6.6	136	4.2	
7:00	-	36.0	7.6	108	-	23	6.6	143	4.1	
7:30	-	37.0	7.3	125	-	19	5.6	151	3.9	
8:00	-	39.0	7.6	166	-	33	5.4	140	3.9	
8:15	-	68.0	8.2	335	-	100	12.1	-	-	

Appendix 15: Blood Glucose: Survival Time Data for Starved and Unstarved Lambs which Received Insulin and/or Epsilon Toxin - see Chapter 20.

The Effect of 1mg Epsilon Toxin Administered Intravenously

Animal no. 97		Animal no. 91	
Hours post-Inoculation	Blood Glucose mg%	Hours Post-Inoculation	Blood Glucose mg%
0:00	91	0:00	120
0:40	236	0:10	275
1:00	246	0:20	350
1:25	382	0:30	365
		0:40	415
		0:45	460

The Effect of 40 units Insulin B.P. Intravenously

Animal no. 90		Animal no. 93	
Hours Post-Inoculation	Blood Glucose mg%	Hours Post-Inoculation	Blood Glucose mg%
0:00	175	0:00	94
0:10	108	0:15	40
0:20	58	0:30	27
0:30	58	1:00	17
0:40	92	2:00	13
0:50	46	6:30	74
1:00	undetectable		

The Effect of 40 units Insulin B.P. + 1mg Epsilon Toxin Intravenously

Animal no. 89		Animal no. 94	
Hours Post-Inoculation	Blood Glucose mg%	Hours Post-Inoculation	Blood Glucose mg%
0:00	216	0:00	100
0:10	225	0:15	47
0:20	208	0:30	13
0:30	258	1:00*	27
0:40	325	1:30	280
0:50	450		

* No clinical intoxication at this time

cont'd.....

Appendix 15: (cont'd).

The Effect of 1mg Epsilon Toxin Intravenously 24 Hours after 40 units Insulin and Starvation.

Animal no. 92

Hours Post-Inoculation	Blood Glucose mg%	Hours Post-Inoculation	Blood Glucose mg%
0:00	100	0:00	67
0:10	108	0:15	54
0:20	187	0:30	100
0:30	91	0:45	100
0:40	91	1:00	94
0:50	91		

Appendix 16.

BIOCHEMICAL DATA -- URINE

GROUP-- DIVIDED DOSE OF TOXIN... PREINOCULATION VALUES

ANIMAL	SP.GR.	PHOSPH MG O/O	NA+ MEQ/L	K+ MEQ/L	CL- MEQ/L
2	1.010	9.0	8.5	74.0	27.0
6	1.007	4.3	3.5	47.5	19.0
17	1.008	2.4	5.5	42.0	21.5
33	1.000	1.9	5.0	14.5	9.5
38	1.000	2.0	3.0	13.5	11.5
42	1.000	1.2	3.5	16.0	10.5
44	1.020	0.0	7.5	81.0	4.0
46	1.000	1.2	35.5	15.0	10.0
48	1.005	3.1	20.0	21.0	13.0
50	1.010	8.1	125.0	82.5	46.0
51	1.003	3.4	26.0	55.0	21.5
53	1.016	6.2	23.0	66.0	31.5
55	1.005	7.2	46.0	31.0	21.0
56	1.006	5.4	25.0	38.0	24.0
57	1.000	4.2	7.5	16.5	12.5
58	1.000	4.1	20.0	23.5	22.5
61	1.000	2.8	5.0	22.5	23.0
MEAN	1.005	4.15	21.73	38.79	19.29
STD DEV	.006	2.42	29.47	24.63	10.06
NUMBER	17	16	17	17	17

GROUP-- DIVIDED DOSE OF TOXIN... INITIAL VALUES

ANIMAL	SP.GR.	PHOSPH MG O/O	NA+ MEQ/L	K+ MEQ/L	CL- MEQ/L
2	0.000	0.0	0.0	0.0	0.0
6	0.000	0.0	0.0	0.0	0.0
17	1.013	3.3	17.5	57.0	25.5
33	0.000	0.0	0.0	0.0	0.0
38	0.000	0.0	0.0	0.0	0.0
42	0.000	0.0	0.0	0.0	0.0
44	0.000	0.0	0.0	0.0	0.0
46	0.000	0.0	0.0	0.0	0.0
48	1.025	12.0	22.0	61.0	31.0
50	0.000	0.0	0.0	0.0	0.0
51	1.035	35.0	490.0	120.0	18.0
53	1.031	19.2	25.0	145.0	30.0
55	1.008	7.8	20.0	43.0	48.0
56	0.000	0.0	0.0	0.0	0.0
57	0.000	0.0	0.0	0.0	0.0
58	0.000	0.0	0.0	0.0	0.0
61	0.000	0.0	0.0	0.0	0.0
MEAN	1.022	15.46	114.90	85.20	30.50
STD DEV	.011	12.39	209.70	44.57	11.04
NUMBER	5	5	5	5	5

GROUP-- DIVIDED DOSE OF TOXIN... TERMINAL VALUES

ANIMAL	SP.GR.	PHOSPH MG O/O	NA+ MEQ/L	K+ MEQ/L	CL- MEQ/L
2	1.030	27.5	29.5	124.0	22.0
6	0.000	0.0	0.0	0.0	0.0
17	1.010	2.9	25.0	54.0	25.5
33	0.000	5.8	0.0	0.0	0.0
38	1.012	10.4	26.5	63.0	43.0
42	1.011	5.4	15.0	69.0	33.0
44	1.027	37.3	92.5	123.0	8.5
46	1.000	1.7	22.0	16.5	9.0
48	1.025	16.8	24.5	64.0	33.0
50	1.025	8.3	48.0	115.0	115.0
51	1.032	29.0	730.0	115.0	19.5
53	0.000	0.0	0.0	0.0	0.0
55	1.026	48.0	60.0	127.0	32.0
56	1.027	29.0	440.0	124.0	74.0
57	1.001	4.1	12.0	32.0	20.0
58	1.010	7.4	280.0	44.0	40.0
61	1.000	3.7	11.0	32.0	32.0
MEAN	1.016	15.82	129.71	78.75	36.17
STD DEV	.011	14.63	212.59	40.79	27.89
NUMBER	14	15	14	14	14

GROUP-- PAIRED CONTROL

ANIMAL	NA+ MEQ/L	K+ MEQ/L	CL- MEQ/L	SP.GR.	PHOSPH MG O/O
0	4.0	27.5	8.0	1.001	3.2
13	13.5	50.5	21.0	1.010	5.6
23	4.0	19.5	14.0	1.000	2.0
26	120.0	18.0	27.5	1.004	3.1
32	107.5	24.5	22.5	1.005	5.0
36	15.5	38.5	25.5	1.005	0.0
40	45.0	43.5	25.0	1.004	2.6
43	2.5	24.0	10.0	1.005	1.8
45	237.5	33.5	35.0	1.005	3.5
49	87.5	22.5	15.0	1.004	2.6
MEAN	63.7	30.20	20.35	1.0043	3.26
STD DEV	75.9	10.90	8.48	.0026	1.28
NUMBER	10	10	10	10	9

GROUP-- CONTINUOUS INFUSION OF TOXIN... PREINOCULATION VALUES

ANIMAL	SP.GR.	PHOSPH MG O/O	NA+ MEQ/L	K+ MEQ/L	CL- MEQ/L
62	1.001	1.8	4.0	18.0	10.0
63	1.000	1.2	4.0	7.0	5.5
64	1.013	6.8	38.0	65.0	41.0
66	1.024	10.7	37.0	81.0	43.0
70	0.000	0.0	0.0	0.0	0.0
71	1.000	3.6	23.0	38.0	29.0
72	1.005	5.0	6.0	40.0	13.5
73	1.011	5.7	7.5	98.0	37.0
75	1.004	2.7	21.0	48.0	23.0
MEAN	1.007	4.68	17.56	49.37	25.25
STD DEV	.008	3.10	14.34	30.72	14.54
NUMBER	8	8	8	8	8

GROUP-- CONTINUOUS INFUSION OF TOXIN... INITIAL VALUES

ANIMAL	SP.GR.	PHOSPH MG O/O	NA+ MEQ/L	K+ MEQ/L	CL- MEQ/L
62	0.000	0.0	0.0	0.0	0.0
63	0.000	0.0	0.0	0.0	0.0
64	0.000	0.0	0.0	0.0	0.0
66	0.000	0.0	0.0	0.0	0.0
70	0.000	0.0	0.0	0.0	0.0
71	0.000	0.0	0.0	0.0	0.0
72	0.000	0.0	0.0	0.0	0.0
73	0.000	0.0	0.0	0.0	0.0
75	0.000	0.0	0.0	0.0	0.0
MEAN	0.000	0.00	0.00	0.00	0.00
STD DEV	0.000	0.00	0.00	0.00	0.00
NUMBER	0	0	0	0	0

GROUP-- CONTINUOUS INFUSION OF TOXIN... TERMINAL VALUES

ANIMAL	SP.GR.	PHOSPH MG O/O	NA+ MEQ/L	K+ MEQ/L	CL- MEQ/L
62	1.005	4.2	9.5	25.0	21.5
63	0.000	0.0	0.0	66.0	0.0
64	0.000	0.0	0.0	0.0	0.0
66	1.017	10.0	730.0	0.0	130.0
70	1.006	3.2	120.0	45.5	33.0
71	1.007	5.9	120.0	50.5	30.0
72	1.005	4.0	7.5	38.0	8.5
73	0.000	0.0	0.0	0.0	0.0
75	1.006	3.6	18.0	60.0	30.0
MEAN	1.007	5.15	167.50	47.50	42.16
STD DEV	.004	2.55	280.65	14.88	43.94
NUMBER	6	6	6	6	6

Note: Zero values indicate no sample.