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"COMPARATIVE PATHOLOGY OF INFLAMMATION IN THE SHEEP"

A thesis
presented in partial fulfilment of the requirements
for
the degree of Doctor of Philosophy
at Massey University, Palmerston North
New Zealand

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Summary

(1) The inflammatory response in the sheep has been investigated using experimental methods developed in similar work on laboratory animals.

(2) A biphasic increase in vascular permeability occurs following turpentine and thermal injury; an early phase seems to be mediated by histamine. In contrast, mediation of the delayed phase, which appears to be the major part of the response, remains uncertain. Histamine, a "Globulin Permeability Factor" and possibly kinins, probably participate. 5-Hydroxytryptamine does not appear to be a natural mediator of inflammatory reaction in the sheep.

(3) Histamine is readily released in sheep skin, and in response to a wide variety of stimuli.

(4) The occurrence of the delayed phase does not depend on the operation of the histamine - mediated early phase.

(5) Strophanthin-G, which interferes with ionic transport across cell membranes, suppresses the early histamine phase of inflammation in the sheep.

(6) Cutaneous antigen-antibody reaction of the Arthus-type has been found to be associated with histamine release in the sheep.

(7) The effects of sodium salicylate, cortisone and indomethacin on the permeability response in the sheep are recorded. In all cases, a significant suppression of exudate formation was obtained in the delayed phase of the permeability response following intrapleural injection of turpentine. These effects differ from those reported in the rat.

(8) A factor which increases vascular permeability both by the release of histamine and apparently through another mechanism, has been isolated from lymph node cells of normal sheep. This is termed "lymph-node-permeability factor".

(9) Most of the increase in vascular permeability in the inflammatory reaction in the sheep is associated with venules and comparatively little with capillaries.

(10) Increased vascular permeability and emigration of leucocytes from blood vessels are dissociated phenomena.

(11) Permeability substances activated or released by injury do not appear to exert much effect on the emigration of leucocytes.

(12) Mast cells appeared locally more numerous in the sheep skin following turpentine injury and the injection of various permeability-increasing substances.

(13) Histamine in doses of 100 µg attracted eosinophiles to the site of injection in the sheep skin.

(14) Total denervation did not affect the permeability response in inflammatory reaction to turpentine injury in sheep skin.

(15) Toxic doses of compound 48/80 and of histamine produced sudden death in sheep. The post-mortem picture has been compared to that seen in clostridial infections in sheep, and the suggestion is made in these infections release of histamine in large or even toxic amounts may contribute to the cause of death.

(16) The results obtained in the sheep suggest a closer similarity between that animal and the guinea-pig than between sheep and rat. Certain features are common to all.

(17) The present work has revealed that there are clear differences between species in the inflammatory mechanism. While information obtained in one species may provide some information for another, inter-species differences may be wide and basic, and it is not possible to extrapolate results between species. Extensive work is required to categorise the mechanisms in domestic animals. This would provide a better understanding of the pathogenesis and pathology of diseases in them. It is felt that sheep may prove a useful animal in exploring certain basic problems of the inflammatory reaction.

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INTRODUCTION

INTRODUCTION

The inflammatory reaction is fundamental to the survival of the organism. It cannot be over-emphasised that without it there could be no protection against the effects of noxious external stimuli nor repair of damaged tissue (see Ebert, 1965). The inflammatory response, however, has attracted renewed interest in recent years with the realisation that inflammation may become aberrant and considerably more harmful to the body than the noxious stimulus which initiated the reaction. Thus, in some diseases a large portion of tissue damage results from the inflammatory response itself. Allergic and rheumatic diseases fall into this category (see Spector and Willoughby, 1963a). Ebert (1965) suggests that " ..... within certain limits the inflammatory reaction is stereotyped and it cannot distinguish between those instances in which the process protects the host and those in which the host is harmed".

Inflammation is difficult to define. Spector and Willoughby (1963a) have suggested that, "In vertebrates it could perhaps be described as the local reaction to injury of the living microcirculation and its associated tissues, in which would be included blood leucocytes and such features of perivascular tissue as mast cells and histiocytes". More recently, Ebert (1965) in an attempt to define inflammation pointed out that "It is difficult to give a definition which is neither so all-inclusive as to be meaningless nor so specific that it is too restrictive.", and suggested that "Inflammation is a process which begins following a sublethal injury to tissue and ends with complete healing".

The inflammatory reaction can be provoked by any noxious stimulus. The reaction is a complex response and involves a series of events rather

than a single event. Thus, the vascular permeability response e.g. in thermal injury, which includes both early and late responses, each of which probably evoked by a different substance, provides an indication of the complexity of the inflammatory reaction (see Wilhelm, 1965). However, the reaction follows a course which is broadly uniform although different types of injury do lead to variation in the relative intensity and duration of particular aspects of the reaction (see Spector and Willoughby, 1963a; see Wilhelm, 1965).

The events in inflammation were described by Spector and Willoughby (1963a) as possibly divisible into two main categories, involving the fluid and cellular phases of the circulation, respectively: they wrote, "The fluid-phase reaction consists of a transient vasoconstriction followed by sustained dilation of arterioles, capillaries, and venules, during which blood flow is increased and subsequently decreased, and permeability to plasma protein is raised. The cellular response consists of swelling of histiocytes and tissue macrophages and emigration of leucocytes from the vessels, preceded by swelling of vascular endothelial cells and the adhesion to them of leucocytes".

Vascular response in inflammation

Increased vascular permeability:

The endothelial wall of capillaries and venules forms a semipermeable barrier that allows free movement of small molecules but normally restricts the passage of plasma protein. A cardinal feature of inflammation is the striking increase in the permeability of these vessels to plasma protein. This is referred to as increased vascular permeability. It has been

suggested by Spector and Willoughby (1963a), " ..... that in structures such as the skin the addition of increased permeability to hyperaemia and vascular dilation represents the transition from a 'physiological' to a 'pathological' response". The importance of increased vascular permeability has made it a focus for research on vascular changes in inflammation. The study of increased vascular permeability poses two separate problems; the structural and functional changes occurring in the vessel wall, and the intermediary mechanism by which injury brings these changes about (see Spector and Willoughby, 1963a).

Chemical mediators of increased vascular permeability:

The study of the chemical mediation of inflammation as a whole has been largely directed at the increase of vascular permeability. This is because increased permeability is a key response in the inflammatory reaction and is readily demonstrated by experiments, which has made it a popular indicator reaction in the search for mediators of the inflammatory process.

There are certain features of the permeability response which strongly suggest that chemical mediators rather than physical effects (see Cohnheim, 1882, and Krogh, 1929, as cited by Wilhelm, 1965; Landis, 1946) are responsible for the vascular changes. And, it is now clear that between injury and vascular reaction there occur a series of chemical reactions, in particular the release or activation of vasoactive substances whose effects are responsible for the increased vascular permeability characteristic of acute inflammation (see Wilhelm, 1962). The evidence for the participation of chemical mediators has been summarized by Spector

and Willoughby (1963a) as follows: (1) the reversible nature of the inflammatory process which occurs only in living tissues; (2) the constancy of the nature and pattern of response in various types of injury; (3) the suppression by drugs of, at least, part of the response; and in particular, (4) the existence of a latent period between the early and late phases of increased permeability. Further support is provided by the isolation of chemical mediators from both normal and inflamed tissue. These substances are able to reproduce the vascular events of inflammation, and their vascular effects can be suppressed by specific antagonists (see Wilhelm, 1962).

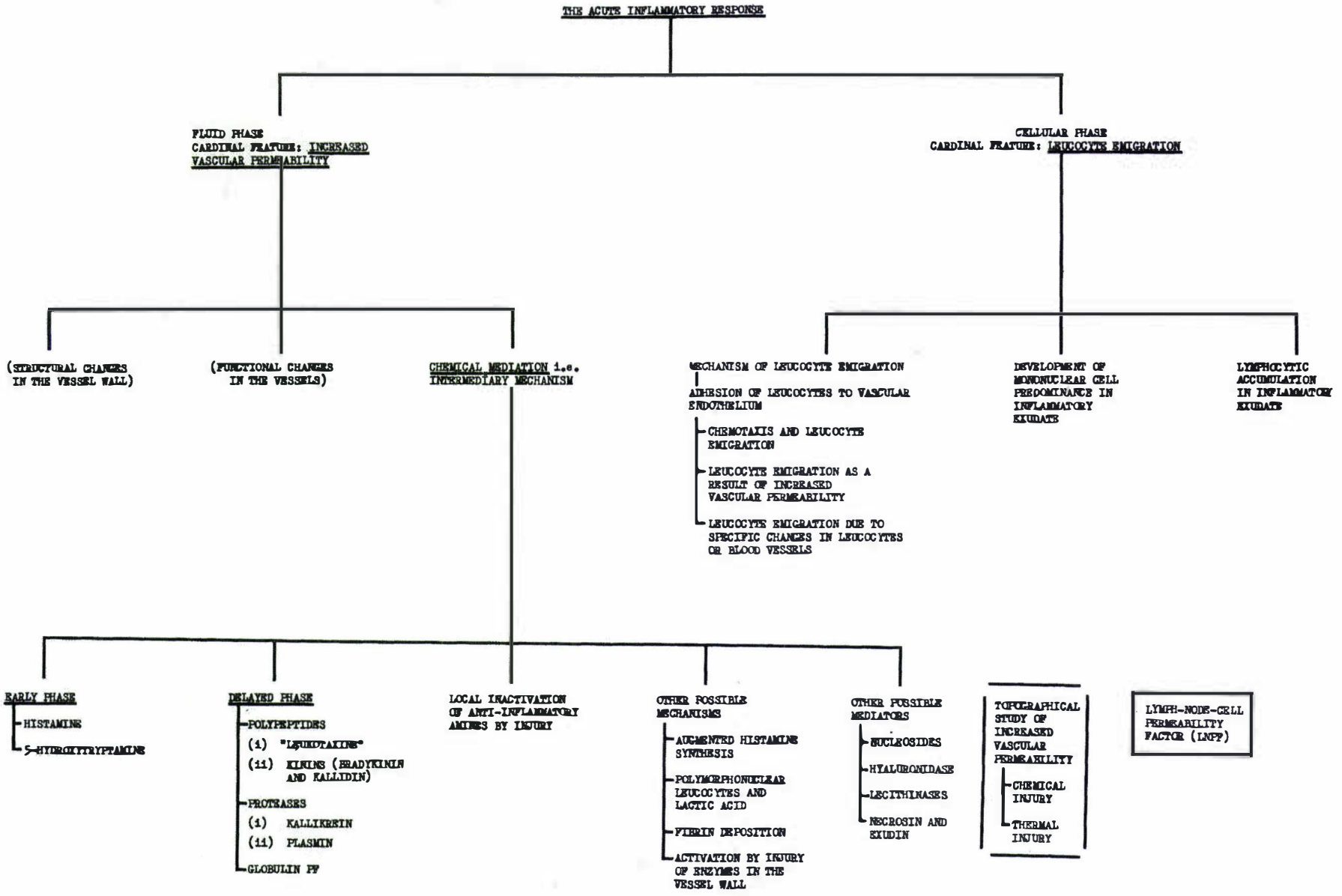
Much attention has, therefore, been devoted to the chemical mediation of the vascular changes and this has resulted in laying down of certain criteria which must be fulfilled before accepting that a substance is acting as a mediator of inflammation. Criteria defined have been: " (1) The substance should possess properties which qualify it as a mediator of inflammation, (2) The substance should be demonstrably present during the inflammatory reaction and absent when the reaction subsides, (3) Inhibition of the substance by specific antagonists should lead to a diminution of that aspect of the inflammatory reaction for which the substance is assumed to be responsible, (4) Depletion of the tissues of the suspected mediator prior to the injurious stimulus likewise should suppress that part of the inflammatory reaction for which the substance is believed to be responsible" (Spector and Willoughby, 1965). The authors suggested that the criteria may be considered idealistic and it is only in rare instances that they can be successfully applied. They believed that the third of the above criteria is normally the most convincing (Spector and Willoughby, 1959a) and on that basis demonstrated a method of identifying vascular

permeability factors by the use of antagonists (Spector and Willoughby, 1963b).

Sevitt (1958) has thrown light on the complex nature of the permeability changes, by demonstrating that reaction to injury may be a double phenomenon, and that in thermal injury the permeability response is biphasic. The reaction of the skin of guinea-pigs to heating at 54°C for 20 seconds shows this point well. There is an immediate increase in vascular permeability lasting for less than ten minutes and a delayed increase beginning in about one hour. Sevitt's work on thermal injury was confirmed in the guinea-pig, rat and rabbit (Spector and Willoughby, 1958b, 1959b; Wilhelm and Mason, 1958, 1960; Wilhelm, 1959; Allison and Lancaster, 1959), and later work has provided further details of this biphasic increase of vascular permeability.

Sevitt's (1958) report of a biphasic permeability response in thermal injury stimulated a renewed investigation of the vascular changes. Shortly, there appeared the independent report on biphasic permeability response to bacterial infection by Burke and Miles (1958). Similar biphasic permeability responses have since been demonstrated in chemical (turpentine) (Spector and Willoughby, 1959a), ultrasonic (J.F. Burke, unpublished - see Wilhelm, 1962), X-ray (Willoughby, 1959a), and ultraviolet injuries (Logan and Wilhelm, 1963, 1966a). Earlier, Elder and Miles (1957) had noted a biphasic increase in permeability following injection of certain clostridial toxins in the guinea-pig skin, which has been confirmed recently by Logan and Wilhelm (1966c). More recently, Steele and Wilhelm (1966) have shown that xylol, benzene, chloroform, carbon tetrachloride, dilute phenol and dilute acetic acid all induce a biphasic increase in vascular permeability

TABLE 1


THE ACUTE INFLAMMATORY RESPONSE
FLUID PHASE
CARDINAL FEATURE: INCREASED VASCULAR PERMEABILITY
CELLULAR PHASE
CARDINAL FEATURE: LEUCOCYTE EMIGRATION
(STRUCTURAL CHANGES IN THE VESSEL WALL)
(FUNCTIONAL CHANGES IN THE VESSELS)
CHEMICAL MEDIATION i.e. INTERMEDIARY MECHANISM
MECHANISM OF LEUCOCYTE EMIGRATION
ADHESION OF LEUCOCYTES TO VASCULAR ENDOTHELIUM
CHEMOTAXIS AND LEUCOCYTE EMIGRATION
LEUCOCYTE EMIGRATION AS A RESULT OF INCREASED VASCULAR PERMEABILITY
LEUCOCYTE EMIGRATION DUE TO SPECIFIC CHANGES IN LEUCOCYTES OR BLOOD VESSELS
DEVELOPMENT OF MONONUCLEAR CELL PREDOMINANCE IN INFLAMMATORY EXUDATE
LYMPHOCYTIC ACCUMULATION IN INFLAMMATORY EXUDATE
EARLY PHASE
HISTAMINE
5-HYDROXYTRYPTAMINE
DELAYED PHASE
POLYPEPTIDES
(i) "LEUKOTAXINE"
(ii) KININS (BRADYKININ AND KALLIDIN)
PROTEASES
(i) KALLIKREIN
(ii) PLASMIN
GLOBULIN PF
LOCAL INACTIVATION OF ANTI-INFLAMMATORY AMINES BY INJURY
OTHER POSSIBLE MECHANISMS
AUGMENTED HISTAMINE SYNTHESIS
POLYMORPHONUCLEAR LEUCOCYTES AND LACTIC ACID
FIBRIN DEPOSITION
ACTIVATION BY INJURY OF ENZYMES IN THE VESSEL WALL
OTHER POSSIBLE MEDIATORS
NUCLEOSIDES
HYALURONIDASE
LECITHINASES
NECROSIN AND EXUDIN
TOPOGRAPHICAL STUDY OF INCREASED VASCULAR PERMEABILITY
CHEMICAL INJURY
THERMAL INJURY
LYMPH-NODE-CELL PERMEABILITY FACTOR (LNPF)

early phase
- histamines
- serotonin

delayed phase.

Polypeptides
(i) leucotoxin
(ii) toxin – bradykinin
– ~~kallikrein~~
– kallidin

Proteases
(i) Kallikrein
(ii) Plasmin

Globulin PF

mechanism of
emigration

adhesion of abc
endothelium
- chemotaxis
- a result of
- due to

Cellular Phase

cardinal feature – leukocyte emigration

ukocyte

in vascular

predominance in inflammatory exudate

lymphocytic accumulation

↑

ejection of ova or endothelium

following their application to the guinea-pig skin.

Some of the evidence for the natural role of different permeability factors in the vascular changes and other related phenomena will now be reviewed. An outline of a number of views on what factors are involved is presented in table 1.

Endogenous substances in the early vascular changes of injury:

Histamine: In 1910, Barger and Dale demonstrated histamine to be a constituent of intestinal mucosa. Its pharmacologic properties were first described by Dale and Laidlaw (1910, 1911, 1918).

Histamine is widely distributed throughout the body in most tissues and also in many physiologic fluids (see Feldberg, 1956). Under normal circumstances histamine is found in the tissues closely associated with mast cells (Riley and West, 1953; West, 1955; Riley, 1959), and the presence of histamine in mast cells is now well established (Riley, 1953, 1959; West, 1956; Benditt et al, 1956; Keller, 1957, cited by Bloom, (1965); Sjoesdsna et al, 1957). Furthermore, this amine appears to be located in the mast cell granule (Mota et al, 1954; Hagen et al, 1959; Green and Day, 1960).

In 1927, Sir Thomas Lewis described the occurrence, following mild injurious stimulation, of three distinct events (the triple response), in the skin:- (i) a primary and local dilatation of the minute vessels; (ii) a wide-spread dilatation of the neighbouring arterioles brought about entirely through a local nervous mechanism (axon reflex); and (iii) local increased permeability of the vessel walls. Lewis noted that a variety of stimuli including pressure, heat, cold and ultraviolet light could

produce this reaction and postulated that these changes were mediated by a histamine-like material which he termed 'H-substance'. He studied the response in the skin to intradermal histamine and found that it was not only identical in appearance to the triple response, but the time relationships were the same. He, thus, assumed that histamine was the chemical mediator of inflammation. Subsequently, the demonstration of the ability of histamine to reproduce the acute vascular changes of injury, its widespread distribution, and the ease with which it is released from its bound form in the body, added to the belief that the inflammatory reaction is brought about by the action of histamine. It is now clear, however, that histamine is not the only mediator. Many endogenous substances have been found which affect vascular permeability. The problem is to find out which of these, if any, play a part in the natural inflammatory response, and also as suggested by Wilhelm (1965), to discover other potential mediators.

Evidence of histamine release in inflammatory response:

Histamine is released after a wide variety of noxious stimuli, that is, thermal, chemical, X-ray and anaphylactic injury and bacterial invasion (see Spector, 1958; Wilhelm, 1962, 1965; Spector and Willoughby, 1963a, 1965). Many of these observations have been found difficult to interpret based as they are on either a rise or a fall in the histamine content of the involved tissues, which is difficult to assay accurately (see Spector and Willoughby, 1965). The authors found histamine to be present in pleural exudates from rats following the intrapleural injection of turpentine. The histamine concentration became maximal by 1 hour after the injection of turpentine, after which the histamine content of exudate fell rapidly (Spector and Willoughby, 1957b). Wilhelm and Mason (1960) demonstrated

release of histamine by heat *in vitro* in skin samples obtained from guinea-pig, rat and rabbit.

In irradiation injury, the part played by histamine has long remained controversial. Thus, Kawaguchi (1930), cited by Spector and Willoughby (1965), demonstrated an increase in histamine content of skin after irradiation, a finding which differed from that of Ellinger (1928, 1930 cited by Spector and Willoughby (1965)) and of Ungar and Damgaard (1954). Willoughby (1959a, 1960) reported a loss of histamine from the intestine of rats following irradiation during the first 24 hours after abdominal X-irradiation. More consistent results have come from the assay of blood levels of this amine after irradiation. Irradiation is accompanied by a rise in blood histamine level (see Spector and Willoughby, 1965). A review by Ellinger (1951), cited by Spector and Willoughby (1965), supported the theory that histamine is the main contributory factor to the vascular changes after irradiation injury. However, Willoughby (1960) has suggested that histamine is merely the first detectable pharmacologically active amine released and that its role is as transitory in irradiation injury as it is in other types of inflammation. He, thus, concluded that its release is merely the first in a sequence of events. More recently, Logan and Wilhelm (1966b) have demonstrated release of histamine in guinea-pig skin after ultraviolet injury.

Evidence of the role of histamine by inhibition studies:

(i) Antihistamines:

Spector and Willoughby (1959a) showed that in turpentine-induced pleurisy in the rat, the volume of exudate obtained 30 minutes after the intrapleural injection of turpentine was reduced from a mean volume

of 1.0 ml to a mean of 0.1 ml in those animals pretreated with the antihistamine, mepyramine maleate. However, after 1 hour, i.e., the period during which histamine was demonstrable, the volume of exudate rose rapidly, and after 2 - 3 hours was not significantly different from that in control animals, despite repeated administration of the antihistamine drug. Together with analysis of inflammatory exudates showing the presence of histamine in the first 30 minutes after such chemical insult (Spector and Willoughby, 1957b) but not at later times, these results indicate that endogenous histamine is responsible for the initiation of the vascular changes in this form of injury, other mechanisms being then required to maintain these changes. After injury of guinea-pig skin by xylol, chloroform, benzene, or barium sulphide, the antihistamines triprolidine and mepyramine both moderately antagonised the early part of the permeability response (Miles and Wilhelm, 1960a; Wilhelm, 1965).

Following thermal injury in rats, Spector and Willoughby (1958b, 1959b) showed that the earliest changes in vascular permeability were also suppressed by small intravenous doses of antihistamine drugs. However, after this initial suppression of increased vascular permeability, repeated administration of the antihistamine failed to affect the increase as assessed by oedema formation, suggesting that histamine was released only during the earliest phase of the inflammatory reaction. Similar results were obtained in guinea-pigs and rabbits by Wilhelm and Mason (1960).

Following X-irradiation of rats, a marked loss of histamine from the intestine occurs during the first 24 hours after irradiation and is accompanied by a local increase in vascular permeability. Pre-treatment of rats with small doses of antihistamine drugs causes a suppression of

this increase in vascular permeability. However, the effect of antihistamine is short-lived, and only the earliest permeability changes are abolished by this treatment (Willoughby, 1959b).

There is, thus, close agreement between the results of administering antihistamine drugs in thermal, chemical and irradiation injury in the rat; in each case, only the initial phase of the vascular reaction is suppressed, although the duration of this phase varies greatly, being 20 minutes, 1 hour, and 24 hours, respectively (see Spector and Willoughby, 1963a).

The role of histamine in injury induced by bacterial infection has been difficult to elucidate. Smith and Miles (1960) failed to modify the inflammatory response to experimental bacterial peritonitis in rats by using antihistamines, and they found no significant release of histamine into the peritoneal fluid. Spector and Willoughby (1963a) concluded in their review that, viewing the inflammatory process as a whole histamine plays a minor role, and its relative importance may vary with different types of injury. These authors have suggested that in certain experimental systems, the failure of antihistamines to modify the inflammatory response may be misleading. Thus, in the Arthus reaction, antihistamine drugs have failed to influence events (see Cochrane, 1965), but here Spector and Willoughby (1964c) suggested that the reaction may be of too explosive a type for histamine to exert a separate effect. In passive cutaneous anaphylaxis, the vascular reaction is peracute and has run its course in 20 minutes. Spector and Willoughby (1963a) suggested that because events are compressed into a short space of time any release of histamine would almost certainly coincide with activation of other vasoactive substances, e.g. plasma kinins. It is not surprising, therefore, that Brocklehurst et al

(1960) found that antihistamine drugs failed to diminish increased vascular permeability in passive cutaneous anaphylaxis.

Recently, Logan and Wilhelm (1966b) showed that after ultraviolet injury, the early permeability response in the guinea-pig was suppressed by antihistamine, but not the late phase. The early phase was believed to be due to histamine.

(ii) Alloxan

Experimental evidence now favours the view that the release of histamine, at least from mast cells, is an enzymic process (see Mongar and Schild 1962; Uvnas, 1964) and that both NH_2 (Uvnas and Thou, 1961) and SH groups (Ednam and Mongar, 1961) are essentially involved in the mechanism. Alloxan, which inactivates sulphydryl groups, (Barron, 1951) has been found to prevent histamine release, and it has been suggested that it does so by preventing the degranulation of mast cells (see Spector and Willoughby, 1965). Spector, Willoughby and Frears (1963) investigated the role of SH groups in the release of histamine in the rat, using turpentine-induced pleurisy as a test-system (see also Spector and Willoughby, 1964a). Inactivation of the SH groups was achieved by administering alloxan monohydrate. This treatment in the rat led to an effect similar to that seen following administration of antihistamine drugs, i.e., an initial suppression of increased vascular permeability which was apparent only up to 1 - 2 hours after the injection of turpentine. To implicate the SH groups further, Spector et al (1963) gave cysteinamine 30 minutes before the dose of alloxan. Cysteinamine is a source of SH groups and such treatment led to a complete reversal of the effect of alloxan, i.e., animals receiving cysteinamine plus alloxan developed a normal volume of exudate during the first hour

after the injection of turpentine. These results supported the view of Ednam and Mongar (1961) that SH groups are vital for the process of histamine release.

The above results further support evidence obtained in the rat that endogenous histamine is responsible for the initiation of the vascular changes following turpentine pleurisy, other mechanisms being then required to maintain these changes.

Evidence of the role of histamine by depletion studies:

Compound 48/80: Compound 48/80 causes the degranulation of mast cells and releases histamine (Feldberg and Talesnik, 1953; Parratt and West, 1957a; Riley, 1959). In addition to the demonstration of histamine in inflammatory exudates and the action of antihistamine drugs leading to the suppression of the inflammatory reaction, it has also been possible to apply the injurious stimulus to animals depleted of histamine by Compound 48/80.

Chemical and thermal injury, when applied to histamine-depleted rats, resulted in a delayed onset of increased vascular permeability comparable with that seen when the animals were treated with antihistamine drugs (Spector and Willoughby, 1958a; 1959a, b). It has been found that certain "classic" histamine liberators release agents other than histamine; Compound 48/80 leads to a depletion of tissue histamine as well as tissue 5-HT in the rat (Bhattacharya and Lewis, 1956a, b). In turpentine-induced pleurisy in the rat, the effect of histamine and 5-HT depletion can be observed after 48/80 treatment. In this case the suppression of increased vascular permeability persists slightly beyond the period of the inflammatory response believed to be due to histamine, and probably has an effect on the part of the inflammatory reaction suspected to be mediated by 5-HT (Spector and

Willoughby, 1959a).

Similarly, Sheldon and Bauer (1960) found that rats, depleted of histamine by treatment with 48/80, failed to develop the local increase in vascular permeability which occurred in untreated rats, following the cutaneous inoculation of mucormycetes.

5-hydroxytryptamine (5-HT, Serotonin): 5-HT is a vasoconstrictor in the rat in which species low concentrations induce increased vascular permeability (Sparrow and Wilhelm, 1957; see also Spector, 1958). It is considered a vasodilator in man, but it does not produce a significant change in vascular permeability (see Ebert, 1965). This amine is widely distributed in mammalian tissues, particularly in all cells belonging to the enterochromaffin system, in the spleen and the central and peripheral nervous systems (Collier, 1958) and in thrombocytes (Humphrey and Jaques, 1954; Stacey, 1957).

The presence of 5-HT in mast cells was first shown in the rat by Benditt and co-workers (1955). Parratt and West (1957a) studied mast cells from different species and noticed that although 5-HT appeared to be a common component of rat and mouse mast cells, it was not present in significant amounts in mast cells of guinea-pig, dog, man, rabbit, cow, hamster, or cat. Hagen et al (1959) by fractionation-separation procedures showed in the mouse that 5-HT resided in the mast cell granules along with heparin and histamine.

Rowley and Benditt (1956) observed that, on injection in microgram amounts, 5-HT rapidly caused oedema in rat paws. Spector and Willoughby (1957b, see also c) found that 5-HT caused vasoconstriction in rat skin in concentrations above 50 microgram/ml, but that it increased vascular permeability to plasma protein in lower concentrations. The development of

turpentine-induced pleurisy in the rat was associated during the first hour after injection of turpentine with a high concentration of 5-HT in the exudate. It was not possible, however, to reduce the formation of pleural exudate by the administration of 5-HT antagonists. On the other hand, the oedema provoked in the rat by injection of egg white and dextran was considerably lessened as a result of administration of these compounds (Parratt and West, 1957b; Halpern et al, 1959). The 5-HT antagonist BOL 148 (bromolysergic acid diethylamide tartrate) has been reported by Brocklehurst et al (1960) also to have some effect on passive cutaneous anaphylaxis in the rat, but the significance of this result remains doubtful. Nevertheless, it is believed that release of 5-HT is likely to be important in certain specialized vascular reactions provoked in the rat by injection of egg white or dextran (see Spector and Willoughby, 1963a).

The authors suggested that the failure of 5-HT antagonists to diminish the vascular reaction to chemical and thermal injury in the rat (see Spector, 1958), need not necessarily exclude its participation, since similar inhibitors fail to influence the syndrome associated with argentaffinoma and thought to be due to excessive production of 5-HT. Depletion of histamine and 5-HT by repeated injections of Compound 48/80 leads to a longer delay in the onset of vascular changes in the inflamed rat pleura than does dosage with antihistamine drugs. This observation was regarded by Spector and Willoughby (1963a) as an indication of a complementary role for 5-HT in the early phase of the inflammatory reaction and suggested that in the rat 5-HT exerts a short-lived effect which probably follows that of released histamine. This hypothesis gains support from the observations by Willoughby (1959b, 1960) on the effects of irradiation on the rat intestine. It was found that the gut was depleted of histamine one day after irradiation

and of 5-HT by the second day. As in the case of turpentine-induced pleurisy, these permeability changes could not be modified by pretreatment with 5-HT antagonists.

Although 5-HT appears to play a contributory role in the rat, a comparable action in other species is at present doubtful. In guinea-pig and rabbit it does not significantly increase vascular permeability (Sparrow and Wilhelm, 1957). Recently, Logan and Wilhelm (1966b) found that in ultraviolet injury, whereas the early permeability response was mediated by 5-HT in the rat, histamine was the mediator in the guinea-pig.

Endogenous mechanisms possibly responsible for the delayed vascular changes of injury:

It is apparent from the above results that release of histamine or 5-HT can account for only the early phase of the vascular changes of inflammation. Some other explanation must be sought for the subsequent delayed onset of vasodilatation and increased vascular permeability. The situation was envisaged by Krogh (1929) who, as cited by Spector and Willoughby (1963a), postulated that tissue damage leads to the release both of a diffusible, readily destroyed substance akin to histamine and of a less diffusible, less readily inactivated principle which he called H-colloid. The evidence for the participation of certain endogenous substances in the delayed phase of increased vascular permeability, will now be considered.

(A) Polypeptides: The presence in inflammatory exudates and especially in pus of protein breakdown products has been known for a long time; and so also has been the effect of peptides in causing vasodilatation particularly in peptone shock (Pfeiffer, 1913; cited by Spector and Willoughby, 1963a).

(1) Leucotaxine: Modern investigation into the possible role of polypeptides in the local response to injury began with Menkin's observation that tryptic digests of albumin cause increased vascular permeability (Menkin, 1936; 1956). He demonstrated that 24 to 48 hour old pleural exudates, induced by intrapleural injection of turpentine in dogs, contained a factor that increased vascular permeability to circulating dye in the rabbit skin and also induced emigration of leucocytes. Menkin (1938, 1940) fractionated such exudates by a pyridine - acetone technique to obtain a polypeptide preparation which he termed 'leukotaxine' and proposed that this substance was a primary mediator in inflammation. Later work confirmed the presence of a comparable substance in similar exudates in goats (Cullumbine and Rydon, 1946).

However, Menkin's work subsequently came under criticism. Spector (1958) suggested that Menkin's fractionation technique was essentially a precipitation of protein with acetone, the pyridine acting as a buffer to prevent adsorption of peptides on the precipitated protein. Harris (1954) pointed out that Menkin failed to remove turpentine by-products during fractionation. Thus, Menkin's belief that his extraction procedures had produced a pure crystalline end product is now known to be incorrect (see Spector, 1958) and the status of leucotaxine as an entity, remains in doubt (see Wilhelm, 1965). Moreover, neither Menkin (1936, 1956) nor Cullumbine and Rydon (1946) realised that only a small proportion of the vascular activity in the exudate was due to the presence of polypeptides. By using turpentine-induced pleural exudates in rats, Spector (1956) showed that the great majority of vascular activity was nondialyzable and resided in the mixed globulin protein fraction. The exudate did contain some peptide nitrogen but this remained constant from 1 to 24 hours, whereas

the permeability-increasing activity of the globulins rose and fell in parallel with the inflammatory changes in the pleural vessels. This experiment, therefore, provided little support for the idea that freely dialyzable peptides of the 'leukotaxine' type proposed by Menkin (1936, 1956) play a part in inflammation (see Spector and Willoughby, 1963a).

Earlier, Miles and Miles (1962) had shown that blueing induced by 'leukotaxine', as with histamine, was antagonised by mepyramine. The authors concluded that 'leukotaxine' increased vascular permeability by liberating histamine (see also Miles and Wilhelm, 1960a).

(2) Kinins (Bradykinin and Kallidin):

Investigation of the role of polypeptides in inflammation was greatly advanced by the discovery in 1949 by Rocha e Silva and co-workers that a substance released from the pseudoglobulin fraction of blood after incubation with snake venom or trypsin was capable of stimulating contraction of smooth muscle, lowering blood pressure, and causing increased vascular permeability. They called this active agent 'bradykinin' because it caused a relatively slow contraction of muscle. Later, various other kinins closely resembling bradykinin were shown to be released from plasma (see Lewis, 1963). The group of such polypeptides is designated 'kinins' (Holdstock et al, 1957), with a prefix to denote their origin, e.g., 'plasma kinins' (see Lewis, 1960).

Plasma of many species, including man, contains a substrate in the serum globulins, often in the alpha-2 fraction from which the active peptides are formed (see Lewis, 1960). Plasma kinin can be formed from plasma not only by the action of trypsin and snake venon, but also by the actions of at least two enzymes present in the blood (Bhoala et al, 1960), kallikrein and plasmin (Lewis, 1958), in which are discussed later. In addition, similar

peptides are formed when plasma from a number of species is diluted with saline (Schachter, 1956) or brought into contact with glass (Armstrong et al, 1957). Plasma so treated causes a slow contraction of smooth muscle (Schachter, 1956), gives rise to pain on application to a blister base (Armstrong et al, 1957) and increases vascular permeability. Once formed, plasma kinins are rapidly destroyed by further contact with plasma, due presumably to the action of peptidases. They are destroyed also by chymotrypsin and are dialyzable (see Lewis, 1960; see Spector and Willoughby, 1963a).

The dispute as to whether bradykinin and plasma kinin kallidin were the same polypeptide and whether one or both were important chemical mediators of inflammation was solved by determining the chemical structures of both compounds. Bradykinin was found to be a nonapeptide (Boissonnos et al, 1963) and kallidin to be a decapeptide, i.e., a homologue of bradykinin containing an extra lysine residue (Webster and Pierce, 1963). It is now supposed that kallidin-10 is converted to kallidin-9 (or bradykinin) by an aminopeptidase present in plasma (see Ebert, 1965). The pharmacological activity of kallidin-10 almost parallels that of bradykinin but its in vivo activity does not appear dependent on its conversion to the nonapeptide (bradykinin) (see Wilhelm, 1965).

Role of Plasma kinins in inflammation: Beraldo (1950a) found an increased plasma kinin activity in the blood of dogs after peptone or anaphylactic shock, but not constantly. Brocklehurst and Lahiri (1962) confirmed this finding. Rocha e Silva and Rosenthal (1961) demonstrated plasma kinin activity in rat tissue after thermal injury. This experiment was performed by injecting saline into an air pouch whose wall was formed by the injured skin. Spector and Willoughby (1963a) suggested that this was open to the

objection that the kinin activity may have been a consequence of dilution of exuded plasma by the injected saline. Miles (1961) has reported a failure to detect kinin activity during the inflammatory response of rabbits to infection. However, Spector and Willoughby (1962) have demonstrated kinin activity in turpentine-induced pleural exudates in the rat. The rise and fall of slow-contracting activity of the exudates was accompanied by parallel fluctuations in the exudate's ability to increase vascular permeability and also paralleled the increased permeability of the pleural microcirculation and its subsequent reversion to normal. The slow-contracting activity was due to two components which could be separated by fractionation using Sephadex. One of these components had little or no action on vascular permeability and appeared to be indistinguishable from SRS 'A' (slow-reacting substance of anaphylaxis). The other was associated with the ability to increase vascular permeability and had some but not all of the features of plasma kinins. More recently, Lewis (1963) has proposed that bradykinin "appears to be a suitable candidate as mediator" of the vascular responses in the inflammatory reaction.

(B) Proteases: The protease systems most widely investigated include plasmin-plasmin inhibitor and kallikrein - kallikrein inhibitor.

(1) Kallikrein: In 1925 Frey (cited by Frey, 1963) discovered a vaso-depressor substance in urine, and subsequently also in the pancreas, (Frey and Kraut (1928), cited by Wilhelm (1962)), which they named kallikrein. Kallikrein has been shown to be an enzyme - an endogenous protease. These workers discovered that kallikrein existed in an inactive form in the blood which they called kallikreinogen. This inactive precursor could be activated by acidification or by acetone in a test-tube. Following the work of Rocha e Silva et al, (1949), Werle and his colleagues (Werle and Berek,

(1950); Werle et al, (1950); both cited by Wilhelm (1965)) discovered that kallikrein liberated a kinin which they called kallidin, from kallidinogen, an alpha-2 globulin in plasma, that was capable of producing increased vascular permeability.

Pancreatic, serum and urinary kallikreins were originally considered to be the same substance. However, the enzymes from different organs or species vary in their susceptibility to trypsin inhibitors or antibodies (Webster and Pierce, 1963), and it is now believed that the kallikreins are not a single substance but a family of closely related enzymes (see Wilhelm, 1965). Besides occurring in pancreas, blood and urine, kallikreins have also been identified in saliva (Werle and von Roden, 1936, cited by Wilhelm, 1965), sweat (Fox and Hilton, 1958), lachrymal secretions (Lewis, 1959) and cerebrospinal fluid (Chapman and Wolff, 1958). The dilution of serum or plasma produces proteolytic activity ascribed to kallikrein and results in the release of substances pharmacologically resembling kallidin and bradykinin (see Wilhelm, 1965). There is evidence both for and against Schachter's (1956) proposal that dilution of serum activates kallikrein (Lewis, 1960), but recent work suggests that kallikrein may be the factor in plasma responsible for the rapid release of kallidin (Schachter, 1963). In addition to dilution, activation of kallikrein can also be induced by contact with glass or other foreign substances (Margolis, 1957, 1958; Armstrong et al., 1957).

The evidence that kinin-forming enzymes or kallikrein are activated after injury is scanty. Kallikrein, unlike plasmin (discussed later), causes increased vascular permeability on injection into skin, due presumably to formation of kinins in vivo (Bhoola et al, 1960). The evidence yet available of the importance of kallikreins in the body concerns a

physiological rather than a pathological role (see Spector and Willoughby, 1963a). Attempts to demonstrate a role for kinin-forming enzymes in hyperaemia and inflammation have been unsuccessful. Thus, Hilton and Lewis (1958) were unable to demonstrate activity of the system during vasodilatation in the striated muscle of the tongue; and Miles (1961) has reported failure to demonstrate such enzymes in inflammation induced by bacteria in the rabbit. Spector and Willoughby (1962) did not find kinin-forming activity in inflammatory exudates collected from the rat pleural cavity from 30 minutes to 24 hours after injection of turpentine.

(2) Plasmin: Plasmin is another kinin-forming enzyme in plasma. It is a beta-globulin and is the blood fibrinolysin (Lewis, 1958, 1960). It occurs in mammalian blood as an inactive precursor - plasminogen (Kaplan, 1944; Christensen and MacLeod, 1945; Loomis et al, 1947; all cited by Wilhelm, 1965), which can be activated by chloroform or trypsin (see Astrup, 1956), by tissue extracts containing 'tissue activator' (Astrup and Permin, 1947; Permin, 1947) and by a plasminogen activator present in blood and tissue fluids (see Mullertz, 1955). Blood and tissue fluids also contain natural antagonists of plasmin. The plasmin and plasmin-inhibitor system and its possible role in inflammation has been reviewed by Macfarlane and Biggs (1948) and by Astrup (1956). They concluded that the details of plasmin activation remain unsettled, but the system in man appears to differ from that in various other animals.

Plasmin is activated in vitro by dilution of plasma in glass containers (Macfarlane and Pilling, 1946), the effect probably being due to contact of the plasma with glass (see Margolis, 1957, 1958). Although kallikrein and plasmin are both activated in plasma by dilution (Schachter, 1956; Macfarlane and Pilling, 1946), they appear to be distinct enzymes. A third factor, called globulin permeability factor, (discussed later), is also activated by

dilution, but is distinct from both kallikrein and plasmin (see Wilhelm, 1965). The contact of cell free plasma with glass also activates various other substances which accelerate blood coagulation (Margolis, 1957), increase vascular permeability (Margolis, 1957), and induce pain on injection (Armstrong et al, 1957). It is now clear that the above and other effects are induced by polypeptides (Beraldo, 1950b; Rocha e Silva, 1955a; Lewis, 1958, 1959; Hilton, 1960) but whether or not plasmin is responsible for the proteolysis releasing them is still disputed. Thus, Schachter (1963) cites work by Bhoola et al, (1960) suggesting that kallikrein, and not plasmin, is the factor responsible for the release of the corresponding polypeptides.

In conclusion, it is believed that the action of plasmin on protein substrates, other than fibrin, may have important implications in the inflammatory reaction (see Wilhelm, 1965). However, this lacks experimental support at the moment. Moreover, plasmin forms kinin relatively slowly, compared both with its action on fibrin and with other kinin-releasing enzymes. Unlike kallikrein, plasmin is a weak permeability factor and does not appear to be important as such in the blood of man (Mill et al, 1958), guinea-pig, rat or rabbit (Wilhelm et al, 1955, 1958; Bhoola et al, 1960; see also Miles and Wilhelm, 1960a).

(C) Globulin Permeability Factor (Globulin PF):

The discovery that plasma diluted with saline would increase vascular permeability was made by Miles and Wilhelm (1955) in the guinea-pig. They called the factor PF/dil. The permeability factor resided in the serum globulins. The PF has been demonstrated besides in the guinea-pig (Wilhelm, et al, 1955, 1957; Baggi and Cantelli, 1956 cited by Wilhelm, (1965)) in the plasma or serum of the rat (Spector, 1956, 1957; Wilhelm et al, 1958), the

rabbit (Paskhina, 1956 cited by Wilhelm, 1965; Wilhelm et al, 1958) and man (Stewart and Bliss, 1957; Elder and Wilhelm, 1958; Mill et al, 1958). The particular globulin fraction with which the PF is associated varies with the species (see Miles, 1961). There is a natural inhibitor of the globulin PF (IPF) and the two together constitute a system present in plasma, tissue fluid and lymph (Miles and Wilhelm, 1958) in all mammalian species studied (see Miles, 1958-1959; Miles and Wilhelm, 1960a, b). Globulin PF in mammalian plasma or serum exists as an inactive precursor (Mackay et al, 1953). This system has been investigated in most detail in the guinea-pig (see Wilhelm, 1965). Globulin PF is considered to be a protease (Miles and Wilhelm, 1960a and b). However, this does not appear to have been finally established as it has with plasmin and kallikrein (see Wilhelm, 1965).

Although globulin PF is a general feature of mammalian plasma, its mode of activation is not uniform. It may be activated by dilution of the plasma of the guinea-pig, rat, mouse, baboon and man, but not that of the rabbit, cat, dog, horse and ox (see Miles, 1961). A similar globulin PF has also been stated to be present in the serum of sheep, but no mention could be found in the literature regarding its activation by dilution, (see Miles and Wilhelm, 1960a; Miles, 1961). The precise mechanism of activation remains obscure, but it is believed that both alteration of the globulin molecule and removal of specific inhibitors are needed (Spector, 1957; see Miles and Wilhelm, 1960a; Miles, 1961). In guinea-pig serum preparations held at room temperature for 60-90 minutes, dilutions of 1:1 to 1:15 in 0.85% saline, Ringer's or Locke's solution has little permeability effect due to the presence of IPF. At dilutions of 1:100 or 1:400, the effect of the IPF was so reduced that maximum PF activity was observed (Miles and

Wilhelm, 1955). The essential basis of the dilution effect appears to be due to contact of the plasma with the glass container, since dilution in polyethylene containers is comparatively ineffective, and the mixing of serum with Ballotini glass beads considerably accelerates the effect (see Wilhelm, 1965).

Guinea-pig PF has been isolated: it is an alpha-2 globulin, relatively thermo-stable and resistant to changes of pH. On a weight basis, the PF has about the same permeability-activity as histamine in the guinea-pig, but on molar basis it is very much more active (Wilhelm et al, 1955; see Wilhelm, 1965).

The IPF has been investigated in less detail than the PF. It has been isolated in fractions containing alpha-globulins in the guinea-pig, albumins in the rabbit, and both proteins in the rat. The IPF present in human serum has not been characterized. Guinea-pig and rat IPF each has a similar order of activity when tested in the homologous species. Guinea-pig IPF is effective in both animals, but the rat factor only in the rat (Wilhelm et al, 1955; see Wilhelm, 1965).

Inhibitors of Proteases:

Natural inhibitors of plasmin, kallikrein and globulin PF have been demonstrated in serum, but their interrelationship is obscure. The natural inhibitor of globulin PF in serum has been mentioned above. A similar inhibitor of plasma kallikrein also exists, which is thermolabile, partially dialyzable, and destroyed by peptidases; the substance is thought to be a basic polypeptide (Frey et al, 1950 cited by Spector and Willoughby (1963a). Serum also contains antiplasmin, an inhibitor of plasmin (Jacobson, 1955). Antiplasmin also inhibits plasma kallikrein (Webster and Pierce,

1961) but not kallikrein prepared from exocrine glands, glandular secretions, and urine (Lewis, 1959).

Soya bean trypsin inhibitor (SBTI), an antiprotease and antiesterase, inhibits plasmin and plasma kallikrein (Webster and Pierce, 1961) but not kallikrein from other sources (Lewis, 1959). SBTI also inhibits the activation by dilution of kinin-forming enzymes in plasma (Schachter, 1956; Lewis, 1958), prevents the activation by dilution of globulin PF in guinea-pig and rat plasma and diminishes the effect on vascular permeability of globulin PF isolated from guinea-pig and rabbit (Wilhelm, et al, 1955, 1957). In addition to the inhibition due to SBTI, globulin PF and kallikrein are also susceptible to the trypsin inhibitors from lima bean and potato but insusceptible to that from ovomucoid (Miles and Wilhelm, 1960b).

Diisopropyl fluorophosphate (DFP), like SBTI, is an antiprotease and antiesterase. DFP too, inhibits the activation by dilution of globulin PF in guinea pig plasma and also the activity of isolated globulin PF from many other species (Becker et al, 1959; see Miles and Wilhelm, 1960b; Miles, 1961). In the rat activation of globulin PF follows plasma dilution and also incubation of plasma with minced tissues (Spector, 1957); DFP inhibits both types of activation (Spector and Willoughby, 1960a). DFP also prevents the action of plasma kallikrein (Webster and Pierce, 1961).

Sodium salicylate has also been shown to antagonize the kinin-forming properties of diluted serum and of salivary kallikrein in vitro (Northover and Subramanian, 1961). The activation of globulin PF by dilution of rat serum is lessened by the presence of salicylate. Similarly, the globulin PF activation following incubation of resolving inflammatory exudate with minced tissues is lessened by the addition of salicylate before incubation

commences (Spector and Willoughby, 1959a).

In summary, plasmin, kallikrein and globulin PF are all present in the plasma and all are activated by dilution. The evidence that plasmin and kallikrein are different, has already been reviewed. It has been clearly established that globulin PF and plasmin are separate entities. Globulin PF is an alpha-globulin, plasmin a beta-globulin; unlike globulin PF, plasmin attacks other globulins to form kinins (see Lewis, 1960). In the guinea-pig, rat, and rabbit, globulin PF is distinguished from plasmin by its greater heat stability (Wilhelm et al, 1955, 1958). The effects of dilution on human globulin PF and plasmin seem different (Elder and Wilhelm, 1958) and the globulin PF in man is distinguished from plasmin by its instability at pH 2 (Mill et al, 1958). It is thought that globulin PF and kallikrein are also distinct, although their properties do overlap and the distinction has not been absolutely defined. Globulin PF and kallikrein are both susceptible to DFP and the trypsin inhibitors mentioned above, but insusceptible to that from ovomucoid (see Miles and Wilhelm, 1960b), and both exhibit hypotensive and permeability effects (see Miles, 1961). However, it has been found in the guinea-pig that vascular activity attributable to kallikrein is associated with gamma-globulin fraction, whereas globulin PF is associated with alpha 2-globulins (Davies and Lowe, 1962 cited by Wilhelm (1965)). In man, there are permeability factors associated with both the beta- and gamma-globulins. The globulin PF resides in the beta-globulin fraction and the activity associated with the gamma-globulin is thought to be due to kallikrein (Kagen et al, 1963).

Role of Globulin PF in inflammation:

Wilhelm et al (1957) did not find changes in globulin PF in the serum of guinea-pigs subjected to various pathological conditions. Changes in

activity of globulin PF in inflammation were demonstrated by Spector (1956) in turpentine-induced pleurisy in the rat. Active globulin PF was found in pleural exudates collected at times when pleural vascular permeability to protein was at its height, i.e., 1 to 8 hours after induction. As the inflammation subsided and the permeability of the pleural capillaries reverted to normal (after 12 to 24 hours), the globulin PF in the exudate returned to its normal inactive state. These results have been criticized (Miles, 1961) on the grounds that the presence of globulin PF in the exudate could have been the result rather than a cause of increased vascular permeability. Spector and Willoughby (1963a) in turn have suggested that such a view does not account for the transformation of inactive precursor to active globulin PF followed by reversion to a completely inactive state, all in the space of 12 hours, and in parallel with the rise and fall of vascular permeability in the tissue from which the exudate arises (Spector, 1956). Active globulin PF at heated sites in guinea-pig skin has been demonstrated (Wilhelm and Mason, 1960). Small amounts of active globulin PF have also been found in bacterial inflammation in the rabbit (Miles, 1961).

Further work on turpentine-induced pleurisy in the rat by Spector and Willoughby (1962) revealed that the exudates contained two substances that caused a slow-contraction of isolated plain muscle. One of these substances did not affect blood vessels, but the other appeared closely related, in its time of appearance and in its properties, to the globulin PF of the exudates. It is possible that some of the permeability-increasing effect of these exudates, in addition to the globulin PF (Spector, 1956), might be due to this slow-contraction stimulating substance. Spector and Willoughby (1963a) speculated that if globulin PF is regarded as a type of kallikrein or kinin-forming enzyme, the slow-contracting substance could be considered as some

form of kinin formed by the action of globulin PF.

It has been found that guinea-pig PF does not liberate histamine *in vivo* or *in vitro* from rat tissues; rat and rabbit GPF's are similarly inactive in this respect (Wilhelm *et al*, 1958). The permeability response to guinea-pig PF persists after administration of antihistamines (Wilhelm *et al*, 1955, 1957). Moreover, human PF is insensitive to mepyramine in man (Stewart and Bliss, 1957) and in the guinea-pig (Mill *et al*, 1958). From this it has been concluded that globulin permeability factors do not owe their permeability increasing activity to the liberation of histamine (see Wilhelm, 1965). However, there is evidence which indicates that guinea-pig PF itself is a histamine antagonist (MacKay *et al*, 1953; Laborde *et al*, 1953; cited by Wilhelm, 1965), and Wilhelm *et al* (1958) found that the effects on vascular permeability induced by GPF's from rat and rabbit were moderately suppressed by antihistamines.

In general, it is believed that serum globulin PF has little direct relation to other factors proposed as mediators, being neither plasmin, producer of polypeptides, nor a liberator of histamine or 5-HT (see Miles and Wilhelm, 1960a).

Spector and Willoughby (1959a) found that salicylate, which inhibits the activation of globulin PF *in vitro*, when administered to the rat prior to intrapleural injection of turpentine, suppressed the increase in vascular permeability in the pleura after the first hour, and for the remaining five hours of experimental observation. The period of vascular permeability susceptible to salicylate followed that earlier phase of vascular change which was suppressed by antihistamine drugs. The later phase developed whether or not the 'histamine' phase had been suppressed by antihistamine. The vascular changes inhibited by salicylate constituted the major portion

of the inflammatory response, and comprised the delayed vascular response. In thermal injury, too, in the rat, a similar inhibitory effect of salicylate has been observed (Spector and Willoughby, 1960a). Administration of DFP, which also prevents the activation by dilution of globulin PF (Miles, 1961) suppressed the delayed increase in vascular permeability in rats after thermal injury to the skin and also following intrapleural injection of turpentine, (Spector and Willoughby, 1959b; 1960a). Willoughby (1960) found that DFP also suppressed the delayed increased vascular permeability in the rat intestine after irradiation. Further, Willoughby (1961) states that apart from its diminution of vascular permeability, DFP led to a striking reduction of the mortality consequent on intestinal damage and also of the associated blood-stained diarrhoea, which follows irradiation. Wilhelm and Mason (1960) failed to affect the delayed increase in vascular permeability following thermal injury in the guinea-pig, rat and rabbit with either DFP or trypsin inhibitors derived from soya bean, lima bean and potato. They found that DFP produced such general depression and other toxic signs in experimental animals that it was doubtful if it had any specific anti-inflammatory affect.

Spector and Willoughby (1963a) suggested that a real objection to interpreting the actions of salicylate and DFP as evidence in favour of the participation of globulin PF or kinin-forming enzymes in inflammation lies in their possession of other effects. Apart from inhibiting a wide range of enzymes and causing a number of metabolic disturbances, these compounds lead to a general suppression of vascular reactivity, so that after their administration the increased vascular permeability induced by intradermal injections of histamine, 5-HT, globulin PF, or polypeptides is much diminished, (Spector and Willoughby, 1960a; see also Northover and Subramanian, 1961; Wilhelm, 1962). Moreover, soya bean trypsin inhibitor

which *in vitro* inhibits both globulin PF and kinin-forming enzymes, has no affect on the vascular changes of inflammation (Wilhelm and Mason, 1960; see Miles, 1961).

In summary, in spite of the above limited evidence for the role of globulin PF, the lack of demonstration of the presence of kinin-forming enzyme in the inflammatory reaction, and the insufficient evidence for the participation of kinins, the common explanation for *the delayed phase* remains that discussed by Spector and Willoughby (1964c) which postulates activation of the globulin PF - kinin system that leads to the formation of plasma kinins. They suggested that the kinins would be formed from globulin substrates by the action of kinin forming-enzymes such as plasma kallikrein that exists normally in an inactive form and may be activated by injury. Globulin PF which, they suggested appears related to the kinin-forming system may be a precursor to the kinin-forming enzyme. However, these authors have pointed out that since salicylate possesses multiple and complex actions, the evidence for the activation of globulin PF-kinin system in the delayed phase based on the action of salicylate, cannot be accepted with complete confidence.

Histamine is not believed to participate in the delayed phase. However, in view of Schayer's (1963) findings of the increased histidine decarboxylase activity in the delayed response to injury (see page 33), Wilhelm (1965) has suggested that although the consensus of opinion favours the involvement of proteases and kinins, histamine cannot be entirely dismissed as a mediator.

Local inactivation of anti-inflammatory amines by injury:

So far the release and inactivation of endogenous substances that increase vascular permeability have been discussed. It is clear, however,

that the evidence implicating kinin, kinin-forming enzymes and globulin PF is not entirely conclusive. A search for alternative mechanisms continues.

It was considered possible that besides release of substances i.e., histamine or 5-HT, the vascular changes of inflammation could be brought about also by the local inactivation by injury of anti-inflammatory amines, such as adrenaline (epinephrine) whose presence would otherwise lead to vaso-constriction and reduced vascular permeability. Experiments to support the idea that such a mechanism contributes to the development of the inflammatory response have been reported by Spector and Willoughby (1960b, c).

Evidence for the existence of this mechanism is based on the results of administering inhibitors of monoamine oxidase (MAO) before injury. The vasoconstrictor amines, adrenaline, noradrenaline and 5-HT are destroyed in the body partly by catechol-o-methyl transferase and partly by MAO (see Axelrod, 1959). The inactivation of any suddenly increased amount of these amines has been attributed to MAO, whereas the normal regulation of levels is probably maintained by catechol-o-methyl transferase.

Spector and Willoughby (1960b) showed that MAO inhibitors were partially effective in suppressing increased vascular permeability following thermal injury in the rat, whereas pyrogallol, an inhibitor of catechol-o-methyl transferase had little or no effect. These workers further showed that if Dibenamine, an adrenolytic drug, was given simultaneously with the MAO inhibitor, no suppression of inflammatory oedema occurred. On the other hand, if bretylium tosylate which potentiates circulating adrenaline and noradrenaline, but prevents its release from nerve endings, was given simultaneously with the inhibitor of MAO, the anti-inflammatory effect was

potentiated. It was also found that systemic administration of adrenaline suppressed the inflammatory response after thermal injury. A similar effect was obtained with high doses of noradrenaline. However, the above authors stated that "These results suggest that adrenaline or noradrenaline was released at the site of injury and, when destruction of the amines was prevented, the inflammatory reaction was suppressed", (Spector and Willoughby, 1965). Northover (1963) found that inhibitors of MAO suppressed the permeability of the peritoneal vessels in the mouse. And, Rackallio (1963) has demonstrated by histochemical methods increased MAO activity in injured rat skin.

In addition to thermal injury the vascular changes following chemical injury (Willoughby and Spector, 1962, cited by Spector and Willoughby, 1965), and X-ray injury to the intestine in the rat (Willoughby, 1962) have also been suppressed by MAO inhibitors, the effect in every case being reversed by Dibenamine.

Experiments were then carried out to determine the source of adrenaline. Since adrenalectomy failed to modify the inhibitory action of the MAO inhibitors, medullary adrenaline was not considered to be involved. From their findings that bretylium tosylate did not reverse but potentiated the activity of MAO inhibitors, Spector and Willoughby, (1965) concluded that, "This would seem to eliminate adrenaline from the nervous system as a source of our anti-permeability hormone".

More recently, these workers have carried out investigations on the metabolic pathway for the formation of adrenaline (Spector and Willoughby, 1964a; Willoughby and Spector, 1964a). Inhibition of the enzymes which convert dopa to dopamine (dopa decarboxylase) and dopamine to noradrenaline and adrenaline (dopamine beta-oxidase) caused a reduction in inflammatory

exudate formation. It was concluded from this that the inflammatory reaction might be influenced not only by the destruction of adrenaline and noradrenaline but also of their precursors.

The enzyme inhibitors of MAO, also of dopa decarboxylase, and dopamine beta-oxidase did not diminish the increased vascular permeability induced by histamine, 5-HT, globulin PF, bradykinin and kallikrein. To explain this, Spector and Willoughby (1965) have suggested that the activity of the above enzymes is increased following injury and only under these circumstances are the inhibitors of these enzymes effective in suppressing permeability changes.

From the results obtained the following hypothesis has been put forward: Following injury there is increased MAO activity inactivating adrenaline and noradrenaline. At the same time there is increased activity of dopa decarboxylase and dopamine beta-oxidase which results in increased formation of the anti-inflammatory amines. During inflammation MAO activity predominates and allows the inflammatory reaction to proceed (see Spector and Willoughby, 1965). This interpretation of the results depends on the assumption that the substances employed as antagonists were specific in their actions.

Other possible endogenous mechanisms responsible for the vascular changes of injury:

(1) Augmented histamine synthesis: Yet another possible explanation of the delayed, sustained local vascular response to injury has been provided by Schayer (1960; 1961 a,b; 1963). In these investigations it was found that a variety of insults, including chemical irritation, allergic reactions and injection of endotoxin, led to a local increase in the histamine-synthesizing enzyme histidine decarboxylase. This increase reached its peak six hours after injury, which corresponds roughly with the development of the delayed

increase in vascular permeability seen in inflammation (Schayer, 1961a). It has been suggested that such an additional synthesis of histamine (as opposed to release of histamine from the existing stores), could account for the delayed vascular changes.

Since, however, these delayed changes are unaffected by antihistamine drugs, the acceptance of this hypothesis depends upon the assumption that this newly formed histamine is not susceptible to antihistamines. More recently, Schayer (1963) has postulated that the newly formed histamine, or what he termed the 'induced' histamine is the candidate most likely responsible for the delayed changes, as it is 'intrinsic' (Dale, 1948). He also postulated that this histamine would be rapidly destroyed or inactivated locally and, therefore, would not be detectable in exudates or in the blood circulation. At present there is no positive evidence to support the view that newly formed histamine is responsible for the later stages of acute inflammation.

(2) Polymorphonuclear leucocytes and lactic acid:

It has been noted that the delayed increase in vascular permeability, seen in many types of tissue injury, coincides with the onset of leucocyte emigration from the affected vessels (see Miles, 1961). This phenomenon has been observed in the Arthus reaction (Stetson and Good, 1951; Humphrey, 1955a), in bacterial infections (Burke and Miles, 1958) and recently in ultraviolet injury (Logan and Wilhelm, 1966a). Moreover, if the numbers of circulating polymorphonuclear leucocytes are reduced by injection of antileucocytic serum (Humphrey, 1955b) or administration of nitrogen mustard (Stetson and Good, 1951; Humphrey, 1955a; Page and Good, 1958), the oedema and leakage of circulating dye resulting from local injury by antigen-antibody union are considerably reduced. These results

indicated that polymorphonuclear neutrophils at the site of injury might yield a substance that contributes to the maintenance of altered vascular permeability. In the guinea-pig, however, extracts of granulocytes have no permeability-increasing effect, and may even be inhibitory (Miles, 1961). In view of this observation it has been suggested by Miles (1961) that granulocytes might increase vascular permeability indirectly by virtue of the lactic acid these cells produce in glycolysis. This is supported by the observation that 90 µg of lactic acid cause maximal vasodilatation in 1 g. of guinea-pig skin (Miles, 1961). Further, increased local lactic acid production has been shown in the Schwartzman reaction (Thomas and Stetson, 1949).

On the other hand, other types of tissue insult may cause intense leucocytic infiltration, without a demonstrable increase in glycolysis or lactic acid accumulation (Thomas and Stetson, 1949). Moreover, in the rat agranulocytosis induced with nitrogen mustard fails to lessen the inflammatory oedema consequent on thermal injury (Hurley and Spector, 1961a). Also, in the rat, delayed leucocyte emigration, caused by injection of saline or histamine is not accompanied by any increase in vascular permeability (Hurley and Spector, 1961b). Recently, Logan and Wilhelm (1966a) have reported that the late permeability response in ultra-violet injury in the guinea-pig, rat and rabbit does not depend on the associated tissue leucocytosis. These findings indicate that the accumulations of leucocytes in injured tissues have little influence on vascular permeability.

(3) <u>Fibrin deposition and the vascular response to injury</u>:

Jancso (1961) has suggested that increased vascular permeability after injury is in some way a consequence of fibrin formation on the inside of the small vessels. This hypothesis was based on the observation that, after

injury, colloidal silver adheres to the walls of small vessels, especially venules and is incorporated into the cytoplasm of the vascular endothelium. Jancso considered that this might be due to trapping of the colloidal particles by fibrin and showed that administration of certain anticoagulant drugs diminished this effect and also lessened oedema after many types of injury. He found also that rats rendered afibrinogenaemic showed similar suppression.

Spector and Willoughby (1963a) in their review of the subject presented a number of difficulties which make Jancso's interpretation of his results unacceptable. For example, they suggest that if Jancso's view were correct, it might be expected that electron micrographs of injured venules would reveal a fibrin layer such as he has postulated. This has not been detected (Alksne, 1959; Moore, 1959; Marchesi, and Florey, 1960; Florey and Grant, 1961; Palade, 1961; Williamson and Grisham, 1961). Other types of investigation based on the induction of hypofibrinogenaemia, too, have failed to find evidence involving fibrin or fibrinogen in the properties acquired by inflammed blood vessels (Allison and Lancaster, 1960, 1961).

(4) Activation by injury of enzymes in the vessel wall:

Spector and Willoughby (1963a) suggested that if this occurred histochemical investigations might reveal activation of enzymes in the vessel wall after injury.

Investigations of other possible mediators in inflammation:

1. Nucleosides: The ability of nucleosides to increase vascular permeability has been investigated in the rat and in man (Spector and Willoughby, 1957a). In the rat the nucleosides inosine, adenosine, and guanosine were active. Inosine was inactive in man but xanthosine, which was inactive in rats, caused a response in some human subjects although

not in others. Nucleosides increase vascular permeability by releasing histamine (Moulton et al, 1957; Spector and Willoughby, 1957a).

2. Hyaluronidase: Hyaluronidase has been stated both to increase vascular permeability and to have no effect on it and is probably of no importance in increasing vascular permeability in inflammation (see Florey, 1962). In the rat skin Spector (1958) found that purified hyaluronidase caused leakage of trypan blue at a concentration of 1 mg/ml but not below this level. He did not mention the amount injected. The author stated that most of the evidence is against a significant action of hyaluronidase on vascular permeability.

3. Lecithinases: Although investigations so far have failed to incriminate lecithinases in the late phase of the inflammatory response to thermal and ultraviolet injury (Wilhelm and Mason, 1960; Logan and Wilhelm, 1963, 1966c), several observations reviewed recently by Wilhelm (1965) suggest that they require further study.

4. Necrosin and exudin: Menkin's (1956) necrosin and exudin have properties similar to those of a tryptic tissue enzyme (Gorkin, 1957 cited by Wilhelm, 1965), kallikrein, and globulin PF (see Wilhelm, 1965).

Leucocyte Emigration

A prominent feature of the inflammatory response is the emigration of leucocytes from small blood vessels into the injured tissues and their accumulation at this site. The emigration is preceded by the movement of leucocytes in the blood vessel from the centre to the periphery of the blood stream (i.e. margination) and by the adhesion of the cells to the luminal surface of the vascular endothelium (see Florey, 1962).

There has been much speculation about the mechanism of leucocyte emigration. It is now believed that there are at least four mechanisms

whereby emigration might be brought about. These are (i) chemotaxis (i.e., a positive directional response to a chemical stimulus), (ii) the changes in the vessel wall which lead to increased permeability to protein, (iii) changes in the vessel wall other than those associated with increased permeability to protein, and (iv) changes in the leucocytes themselves leading to their migration (see Spector and Willoughby, (1963a).

Adhesion of leucocytes to vascular endothelium:

Leucocyte emigration depends upon adhesion of leucocyte and endothelium. Observations on living vessels in rabbit ear chambers and the delay which ensues between injury and onset of adhesiveness may indicate that the essential change, resulting in leucocyte adhesion, occurs in the endothelial cell (Grant et al, 1962). These authors concluded this from a consideration of the delay which occurs before leucocyte adhesion begins. They considered that this points to some effect on the static endothelial cell rather than on the leucocyte being carried along in the blood stream. No direct evidence was provided to support this. Spector and Willoughby (1963a) suggested that once such adhesion has occurred, the natural mobility of the leucocyte could lead to some degree of emigration.

The adhesion of leucocyte to endothelial cell has been most commonly explained on the basis of an adhesive coagulum deposited on the endothelial surface which traps the cells. However, electron micrographs of inflamed venules have failed to reveal the presence of such material (Marchesi and Florey, 1960: Florey and Grant, 1961; Williamson and Grisham, 1961). Moreover, this hypothesis fails to explain the relative immunity from adhesion exhibited by some types of blood cells, notably small lymphocytes and erythocytes. More recently, there have been several attempts to explain the phenomenon of leucocyte adhesion to endothelium in terms of electrochemical forces that may influence cell surfaces in such a way as to alter their

adhesive properties. In spite of these investigations, mechanisms responsible for the phenomenon, remain obscure (see Grant, 1965).

Chemotaxis and leucocyte emigration:

Chemotaxis of leucocytes, especially granulocytes, has received much attention. Harris (1954) reviewed the literature and concluded that much earlier work was of doubtful value because of inadequate techniques. In his experiments, Harris (1953) demonstrated a lack of correlation between the chemotactic powers of microorganisms in vitro and their ability to induce granulocytic accumulations in vivo. Thus, living Salmonella typhi and M.tuberculosis which were chemotactic to granulocytes in vitro were distinguished by their lack of effect on the emigration of granulocytes in the body. In addition, Strep. pyogenes, Bacillus anthracis and Klebsiella pneumoniae, which were not chemotactic in vitro, induced granulocytic accumulations in the body.

Findings of Meier and Schar (1957) cited by Spector and Willoughby (1963a), indicated that the chemotactic properties of micro-organisms might be due to their content of polysaccharide or lipopolysaccharide; they found such chemical compounds to be chemotactic to polymorphs. These observations are at variance with those of Harris (1953) that clumps of killed Staph. albus are not chemotactic to granulocytes. Finally according to Spector and Willoughby (1963a), many workers have attempted to show that various tissue fluids or tissue extracts are chemotactic to leucocytes, but by Harris's criteria they have not succeeded. Nevertheless, these experiments have demonstrated the ability of these fluids or extracts to accelerate the random movement of leucocytes. Spector and Willoughby (1963a) suggested that in the inflammatory response, an effect of this nature would be as important as true chemotaxis since an increase in random movement would increase

the chance of contact between the leucocyte and vascular endothelium. The importance of chemotaxis in leucocyte emigration is still uncertain.

Leucocyte emigration as a result of increased vascular permeability to protein:

Menkin (1938) was first to concentrate intensively on histological methods to demonstrate leucocyte emigration-inducing properties of his 'leukotaxine' preparation. Menkin's interpretation of his results led to some confusion which was only resolved when Hurley and Spector (1961b) emphasized the importance of time factors. They demonstrated that single injections of sterile pyrogen free saline and of other solutions will lead to significant leucocytic emigration at the injection site, provided that some hours are allowed to elapse between injection and histological examination. This, delayed leucocytic emigration and the absence of such a response in the initial two hours after injection, explained the discrepancies of the effect in vivo of various substances on the movement of leucocytes.

Single injections of various endogenous permeability factors in amounts sufficient to cause an increase in vascular permeability induce leucocyte emigration more than 45 minutes after injection. The increase in vascular permeability lasts 15-20 minutes (rabbit globulin PF is an exception to this which produces a prolonged effect in rabbit skin (Wilhelm et al, 1958; see Miles and Wilhelm, 1960a; Wilhelm, 1965). If leucocyte emigration coindiced with the permeability change it might be thought that all compounds that increase vascular permeability also induce leucocyte emigration. Menkin's leucotaxine is said to cause leucocyte emigration within 45 minutes of injection (see Spector and Willoughby, 1963a). Injections of large amounts of permeability factors are said to induce leucocyte emigration in less than 45 minutes but the doses necessary are so high as to throw doubt on the meaning of the result. In the guinea-

pig the amount of histamine needed for an effect on leucocytes is 50 to 100 times that which produces a strong increase in vascular permeability (Wilhelm et al, 1955) and a similar situation exists with regard to bradykinin (Lewis, 1961), globulin PF (Wilhelm et al, 1955) and peptide mixtures derived from fibrin (Spector, 1951). From these results Spector and Willoughby (1963a) concluded that it seems unlikely that release or activation of vascular permeability substances could be responsible for the considerable accumulation of leucocytes seen in most types of injury. More recently, Spector and Willoughby (1964b) provided further evidence which supports the view that increased vascular permeability to protein and emigration of leucocytes are separate phenomena. Gowland (1964) also supported the view from studies on leucocyte emigration in man and concluded that the mechanism for leucocyte emigration does not appear to be chemotactic or to depend on permeability-promoting substances.

Leucocyte emigration due to specific changes in leucocytes or blood vessels:

The idea that leucocytic emigration was related to neither chemotaxis nor to increased vascular permeability received little attention. However, it has become evident that there is no correlation between the ability of various extracts or chemical substances to increase vascular permeability and to affect leucocyte emigration. Spector and Storey (1958) found that extracts of oestrogenized mouse uterus had as much effect on permeability as comparable extracts of other tissues but possessed a relatively much greater activity in promoting migration of leucocytes. Hurley and Spector (1961b) demonstrated that following intradermal injections of histamine and plasma proteins, large-scale leucocytic emigration developed after the increased vascular permeability induced by these substances had returned to

normal. They also demonstrated a separation of the vascular and leucocytic events in thermal injury.

Because leucocyte emigration could not be explained in terms of chemotaxis or increased vascular permeability, the existence of a specific endogenous mechanism for leucocyte emigration has been postulated. Speculations on this mechanism have been reviewed by Spector and Willoughby (1963a). It has been suggested that injury may lead to an increase in the pseudopodial activity of the leucocytes, facilitating adhesion to the endothelium and accelerating their movement, so that the cells migrate through the vessel wall in large numbers. Alternatively, there may be an effect on the vessel wall, increasing the activity of the endothelium, attacking the integrity of basement membrane, opening up gaps at the interendothelial junctions, or merely rendering the endothelial surface 'adhesive' (or non-repulsive) towards leucocytes. At the moment, there is insufficient evidence to support these various possibilities.

In an attempt to define a specific endogenous mechanism for leucocyte emigration, Hurley and Spector (1961a) found that of saline extracts prepared from many different tissues only that obtained from granulocytes induced leucocytic emigration within 40 minutes of intradermal injection. A similar property was acquired by serum after its incubation with certain tissues, notably liver. From these results, Hurley and Spector (1961a) postulated that, plasma extravasated after injury (owing to increased vascular permeability) acquired the property of inducing leucocyte emigration through contact with damaged tissue cells or with granulocytes damaged directly or damaged as a consequence of adhesion to inflamed vessels. Spector and Willoughby (1964b) have supported this view. They suggested that emigration of leucocytes after injury may be due to the operation of specific chemical mediators. Serum extracts of rat liver (liver extract factor - LEF) and

lymph node permeability factor (LNPF), discussed later, were proposed as models for such substances. Recently, Ryan and Hurley (1966) from studies in an *in vitro* test system, suggested that damaged tissue may interact with serum to produce a factor which is chemotactic to polymorphs.

In summary, considering the above three possible mechanisms of leucocyte emigration after injury, i.e., chemotaxis, increased vascular permeability to protein, and the postulated specific endogenous mechanism, Spector and Willoughby (1963a) concluded that it seems likely that all three may play a role. The slight migration of leucocytes seen immediately after injury could be a result of increased vascular permeability. The much more extensive migration that develops later, could, in the case of bacterial infection, be due to chemotaxis, and, in non bacterial injury, could follow the operation of a specific endogenous mechanism such as that outlined above.

Development of mononuclear cell predominance in inflammatory exudates:

It is now well known that in the early stages of an inflammatory response the cellular exudate is dominated by polymorphonuclear neutrophils. As the reaction subsides mononuclear leucocytes replace polymorphs as the preponderant cell type. The duration of the phase of polymorph dominance depends on the cause of the inflammatory reaction. In bacterial infections associated with pus formation, polymorphs remain in the majority until resolution or organisation supervenes. In other bacterial infections, e.g., tuberculosis or typhoid fever, polymorphs are virtually absent from the cellular response seen in established lesions, the cell population being predominently mononuclear cells. It has, however, been established, at least in the case of tuberculosis (Bhoola *et al*, 1960) and brucellosis (Braude, 1951) that the very earliest stages of infection are accompanied

by an inflammatory exudate in which polymorphs are transiently predominant. Spector and Willoughby (1963a) concluded "It seems, then, that the replacement of polymorphs in the exudate by mononuclear cells is a constant feature of the inflammatory response".

The mononuclear cells of inflammatory exudate could be derived from either the blood or tissue cells. Studies to reveal transformation of connective tissue or endothelial cells into any form of mononuclear leucocyte have failed (Clark et al, 1936; Ebert and Florey, 1939). No mitotic activity has been noticed in tissue macrophages that might indicate that proliferation of these cells is the source for mononuclear cell preponderance (Paz and Spector, 1962). There is, on the other hand, a great deal of evidence to indicate that mononuclear cells of inflamed tissues are haematogenous (Ebert and Florey, 1939; Kolouch, 1939; Rebuck and Crowley, 1955; Kosunen et al, 1963; Volkman and Gowans, 1965a and b). Recently, Spector, Walters and Willoughby (1965a) have further supported the view of the haematogenous origin of mononuclear cells in inflammatory exudate by autoradiographic studies. Spector and Coote (1965) by using similar technique of labelling differentially various types of leucocytes found that in the reaction to paraffin oil almost all the macrophages, epithelioid cells and occasional giant cells were derived from what they described, as 'typical' blood monocytes. More recently, Spector and Lykke (1966) have shown that in granulomatous inflammation too, the mononuclear infiltration at the reaction site is due mainly to blood monocytes (see also Spector, 1967).

Considering that mononuclear cells of the inflammatory exudate are derived from blood leucocytes, there can be various possible explanations of their accumulation (see Spector and Willoughby, 1963a). The common view is that there is a selective emigration of monocytes and lymphocytes from injured vessels which takes place after similar emigration of polymorphs

has ceased. A number of reasons for this selective migration have been suggested, e.g., pH changes, but lack of evidence has prevented their acceptance. The observation that monocytes respond chemotactically to the same stimuli as do polymorphs (Harris, 1954) makes an explanation based on chemotaxis difficult to accept, considering the differential accumulation of cell types. The third possibility is based on the findings of Clark et al (1936), who concluded that the mononuclear predominance of late inflammatory exudates was the result of emigrated polymorphs changing to degenerate forms. This hypothesis is not accepted for several reasons discussed by Spector and Willoughby (1963a).

Evidence supporting the view that polymorphs and monocytes emigrate concurrently, the former then disappearing and the latter remaining, has been provided by Paz and Spector (1962). They found that polymorphs and mononuclear cells left the vessels concurrently, However, polymorphs left faster than did the mononuclear cells, so that a dominance of polymorphs was soon established in the tissues. Once emigration ceased, the polymorphs disappeared due to their disintegration and their movement further away from the blood vessels. The mononuclear cells, on the other hand, remained in the vicinity of the vessels and underwent transformation to macrophages and thence to special types whose nature depended on the stimulus. Boughton and Spector (1963) reported similar findings from studies on the tuberculin reaction in guinea-pigs. Recently, however, Hurley et al (1966) have reported results contrary to those of Paz and Spector. They found that instead of polymorphs and monocytes leaving the vessels concurrently but at different rates (Paz and Spector, 1962), there was a biphasic emigration of leucocytes into the inflammatory exudate - an early, relatively brief - escape of polymorphs being followed by a late,

more prolonged - emigration of mononuclear cells. From these results they concluded that the polymorph and mononuclear leucocytes migrate independently and successively into the exudates, and that a separate mechanism controls the escape of each cell type.

Lymphocytic accumulation in inflammatory exudate:

Lymphocytes are seen in long-standing inflammatory exudates and are present at the periphery of tubercles and other granulomatous lesions. They are also found in the mononuclear cell exudates of inflammatory responses which, although of several days or weeks duration, are not as chronic as those of infective granulomata. Collections of lymphocytes, often in a perivascular situation, are also observed in healing tissues where all other cellular reaction has ceased. They are also the only type of cells involved in certain mild inflammatory reactions, e.g., due to viral invasion or an immunological reaction.

Several possible explanations have been suggested for the accumulation of lymphocytes in inflammatory exudates. Clark et al (1936) considered that degenerate polymorphs may account for foci of lymphatic accumulations. Harris (1954) suggested that the accumulation might be due to a specific change in vessels leading to selective emigration, or to immobilization of lymphocytes in the process of circulating through the tissues, or to migration from lymphatics. Spector and Willoughby (1963a) have proposed that although these mechanisms may operate, once lymphocytes can be induced to adhere to the endothelium, emigration and local accumulation might occur without the necessity for further changes. They considered that the differences between the surface properties of lymphocytes compared with those of monocytes would make it more likely that lymphocytes will stick to endothelium. They suggested that specific antigen-antibody binding, could serve as the basis of a system causing adhesion of the lymphocytes.

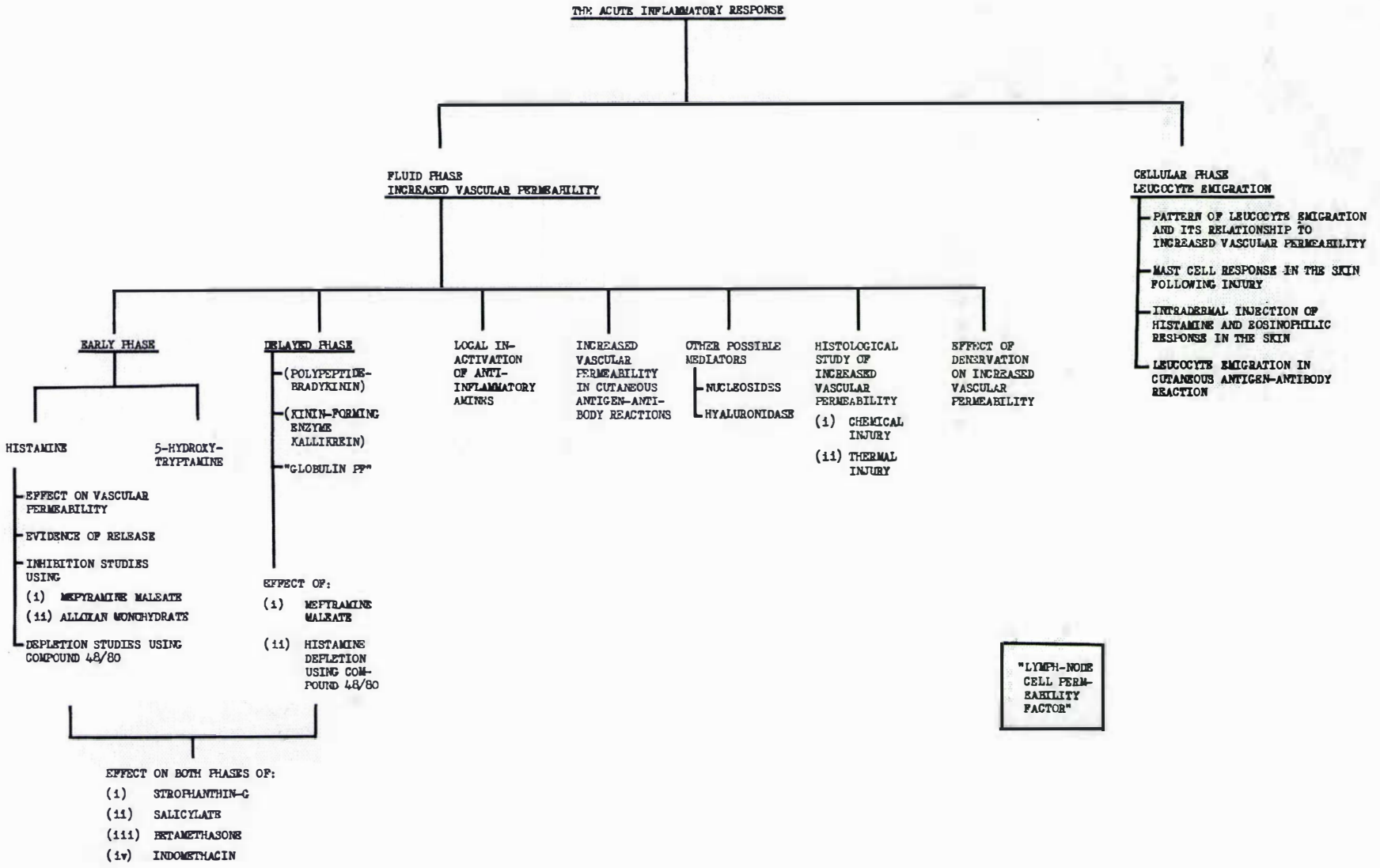
It was proposed that lymphocytes bearing antibody on their surface might adhere to and migrate through endothelium on whose surface the appropriate antigen is present. This hypothesis gains support from the observation that lymphocytes actually carry the antibodies causing many delayed hypersensitivity, homograft and heterograft reactions; all of these reactions are characterized by lymphocytic accumulations. Further, Najarian and Feldman (1961) showed that labelled sensitized lymphocytes accumulate at the site of injection of antigen, whereas non-sensitized labelled lymphocytes fail to do so. Spector and Willoughby (1963a) further suggested that lymphocytic accumulations in infective granulomata could also be attributed to antigen-antibody reactions, and those found in scars could be associated with protein synthesized locally in healing tissues, which might have a special affinity for the lymphocyte surface.

Summary and Conclusions:

It is clear from the above review that despite intensive experimental investigation in recent years into the inflammatory process, particularly in the rat and guinea pig, "our knowledge of inflammation remains largely descriptive, and we still have little precise information concerning the chemical mediation of the characteristic vascular reactions" (Wilhelm, 1965).

It is now clearly established that the permeability response is often biphasic - with early and late phases. The former is transient, whereas the latter is prolonged and probably the major part of the reaction. Histamine and/or 5-HT mediate the early phase. Although mast cell 5-HT has been found to play a certain role in the acute inflammatory response in the rat, this remains to be shown in other animals. In fact, the absence of 5-HT from mast cells of animals other than the rat and mouse suggests that it is unlikely to be a mediator of the permeability response in these

TABLE 2


THE ACUTE INFLAMMATORY RESPONSE
FLUID PHASE
INCREASED VASCULAR PERMEABILITY
CELLULAR PHASE
LEUCOCYTE EMIGRATION
PATTERN OF LEUCOCYTE EMIGRATION AND ITS RELATIONSHIP TO INCREASED VASCULAR PERMEABILITY
MAST CELL RESPONSE IN THE SKIN FOLLOWING INJURY
INTRADERMAL INJECTION OF HISTAMINE AND EOSINOPHILIC RESPONSE IN THE SKIN
LEUCOCYTE EMIGRATION IN CUTANEOUS ANTIGEN-ANTIBODY REACTION
EARLY PHASE
DELAYED PHASE
(POLYPEPTIDE-BRADYKININ)
(KININ-FORMING ENZYME KALLIKREIN)
"GLOBULIN PP"
LOCAL IN-ACTIVATION OF ANTI-INFLAMMATORY AMINES
INCREASED VASCULAR PERMEABILITY IN CUTANEOUS ANTIGEN-ANTI-BODY REACTIONS
OTHER POSSIBLE MEDIATORS
NUCLEOSIDES
HYALURONIDASE
HISTOLOGICAL STUDY OF INCREASED VASCULAR PERMEABILITY
(i) CHEMICAL INJURY
(ii) THERMAL INJURY
EFFECT OF DENERVATION ON INCREASED VASCULAR PERMEABILITY
HISTAMINE
5-HYDROXY-TRYPTAMINE
EFFECT ON VASCULAR PERMEABILITY
EVIDENCE OF RELEASE
INHIBITION STUDIES USING
(i) MEPYRAMINE MALEATE
(ii) ALLOXAN MONOHYDRATE
DEPLETION STUDIES USING COMPOUND 48/80
EFFECT OF:
(i) MEPYRAMINE MALEATE
(ii) HISTAMINE DEPLETION USING COMPOUND 48/80
"LYMPH-NODE CELL PERMEABILITY FACTOR"
EFFECT ON BOTH PHASES OF:
(i) STROPHANTHIN-G
(ii) SALICYLATE
(iii) BETAMETHASONE
(iv) INDOMETHACIN

other species.

The mechanism of the delayed phase in the increase of vascular permeability remains uncertain. In spite of the powerful effects on vascular permeability of plasma kinins and the enzymes which form them, their role in inflammation in still to be established. Further, the inter-relationships of globulin permeability factors, kinins and kinin-forming enzymes are far from clear. The significance of histamine in the delayed phase is not settled. The mediators of the delayed phase have still to be certainly identified.

On leucocyte emigration, "whereas there is agreement on a few basic facts, there is disagreement on most of the critical issues" (Grant, 1965). The mechanisms responsible for the phenomenon remain obscure.

The investigations reviewed indicate that the mediation of the inflammatory reaction is probably not the same in different animal species. Virtually no information is available concerning this in domesticated animals.

The present work on the comparative pathology of inflammation in the sheep was undertaken to provide information on the nature of the inflammatory process in this species. Further, it was hoped that the results would help to explain certain species differences in the inflammatory reaction and so contribute to an understanding of the process as a whole.

Table 2 outlines the experimental work.

MATERIALS AND METHODS

MATERIALS AND METHODS

Only those materials and methods are mentioned here that relate to all the experiments. These will be referred to many times. The special materials and methods that pertain to particular experiments are described with those experiments.

Animals: Romney x Cheviot lambs, of either sex, weighing about 20 kg. and 6 - 10 months of age were used throughout. In these animals skin was thin, soft and pliable and especially suited for permeability experiments. Older sheep with thick and tough skin were not used.

Induction of inflammation: Injections of turpentine were used to cause chemical injury and so induce a sterile inflammatory response. Turpentine was either injected intradermally in volumes of 0.1 ml., or intrapleurally in volumes of 0.5 ml. The intrapleural injection resulted in pleurisy.

Intrapleural injections were made in the following way:- The lamb was held lying down on the left side. A wide bore 15 gauge needle was pushed slowly through the clipped skin over the lower half of the 4th to 8th intercostal space. The entrance of the needle into the pleural cavity was accompanied by a hissing sound produced by the inward rush of atmospheric air. The sound was easily detected by an ear kept close to the needle. A smaller needle (21 gauge), attached to a tuberculin syringe that contained turpentine, was then carefully introduced into the 15 gauge needle, and 0.5 ml. turpentine injected into the pleural cavity. The needle was then slowly withdrawn. The 15 gauge needle was also withdrawn carefully. Its open end was kept closed with a finger to prevent efflux of turpentine. The whole procedure took about 5 minutes.

Animals whose lung was punctured accidently by the needle while in-

jecting, were discarded. Such a mishap was characterized by efflux of frothy blood from the needle.

Pleural exudate was collected from animals killed after the appropriate interval. The right pleural space was opened and the exudate withdrawn with a sterile 10 ml. syringe and transferred to a sterile receptacle. At no stage was blood allowed to contaminate the exudate when the thorax was opened. After collection the exudate was either used at once or stored at -10°C until required. The colour, the presence of cells in the exudate, its coagulation after collection and the fibrinous nature of the pleurisy were noted and are discussed later.

(2) Thermal injury. The standard injury was produced by applying the end of a copper rod (16 mm. diameter) at 70°C for one minute to the clipped skin of the back. The rod was heated to this temperature in a water bath and maintained at 70°C until applied to the sheep. The response to thermal injury was studied only in limited cases, because of the difficulty of producing a consistent reaction each time. This was due to movement of the animal while the copper rod was being applied to the skin. Since it has been shown that the vascular permeability response is reduced by general anaesthesia (Miles and Miles, 1952) and could as well be affected by other pharmacologically depressant agents, experiments were not undertaken in animals under the influence of general anaesthesia or other drugs.

Had these been used in restraint it would still have been necessary to carry out experiments in normal animals to establish the effects of the pharmacological agents used on the inflammatory response.

Skin biopsy: These were taken for histological study by a circular dermatome of internal diameter 1 cm.

Estimation of increased vascular permeability:

(1) Macroscopically: The method most commonly used depends on the fact that dyes such as pontamine blue, trypan blue and Evan's blue do not pass through normal capillary walls after intravenous injection because they become attached to plasma proteins - particularly albumin (Rawson, 1943). The movement of protein molecules coupled with dye out of the blood vessels is associated with colouring of the tissue surrounding the vessels, and indicates an increase in permeability of the smaller vessels. The dye test seems to have been introduced by Ramsdell (1928).

To test the effect of various substances on vascular permeability this method was used as follows:- First a solution of the dye was injected intravenously into the animal. Then the substance to be tested, dissolved in sodium chloride solution of suitable tonicity, was injected intradermally into the previously shaved skin. An increase in vascular permeability was produced by the intradermal injection of a number of substances. These caused leakage of dye into the tissues, forming a coloured patch around the point where they were injected (fig. 12). Both the diameter of the patch, and the intensity of colour, gave an estimate of the increased vascular permeability produced by the solution injected. An advantage of this method is that a number of injections can be made at different sites at the one time in the same animal. In this way the activity of different substances and different amounts of the same substance can be examined under similar conditions and compared (Florey, 1962). The size of the sheep permitted the same animal to be used both to test the permeability-increasing effect of a substance, and the possible suppression of its effect by antagonists. This reduced variations in results due to differences between individual animals.

All the present observations on sheep were made on the skin of the back;

experiments were started within an hour of shearing and clipping the area. Sheep were injected intravenously with pontamine sky blue 6BX (Edward Gurr Ltd., London) 25 mg/kg body weight in a 5 per cent solution in 0.85 per cent saline. The solution was freshly prepared and filtered for each experiment. Animals injected with the dye are referred to as 'blued' and were used within 10 minutes after injection. In those cases where the skin had been injured during shearing the skin became bright blue within a few minutes of 'blueing'. Such areas were not used for injections of test substances or their antagonists.

All solutions injected were made up in 0.85 per cent saline, which by itself was found not to induce blueing. Test solutions were injected intradermally in 0.1 ml. volume, which initially raised a small bleb. Injections were made with a one ml. tuberculin syringe using a 26 gauge needle. A separate syringe was used for each test substance. The sites of injections were marked with a red grease pencil. The insertion of the needle produced a traumatic blueing 0.5 - 2 mm in diameter but never greater than this. When the diameter of the area of blueing exceeded 4 mm. the increase in vascular permeability was considered significant and to be due to the injection of the test substance. The diameter of the blue areas was measured with a Vernier Calliper. In individual animals differences in the intensity of the colour were assessed in grades of ascending intensity; +, ++, +++ and ++++ (Miles and Wilhelm, 1955 used a similar scale).

The method described worked well in sheep. The injection of substances which increased vascular permeability produced a well-defined area of blueing and with standard doses of test substances there was a satisfactory degree of reproducibility of results both within and between individual sheep.

(2) Microscopically: To evaluate increased permeability of blood vessels

histologically, the method of Majno, Palade and Schoefl (1961) was used. These workers used colloidal carbon as an indicator of increased vascular permeability in the rat; they found that carbon was trapped in the wall of the particular vessels through which plasma protein was escaping and so marked them.

Preliminary observations showed that the same technique was applicable to the sheep. To demonstrate from which vessels the protein leaked, in the sheep, a colloidal suspension of carbon (supplied by Gunther Wagner Pelikan Werke, Hanover, Germany: batch C 11/1431 a) was injected intravenously (in a dose of 1 ml per kg) at various intervals after chemical or thermal induction of inflammation. Twenty minutes after the injection of carbon the sheep were killed. This is the time interval Hurley and Spector (1965) found gave optimal results in the rat. Samples of inflamed skin and diaphragm were removed, fixed in formalin and processed for histology as described below.

Histological Methods: Skin and other tissues were collected either fresh in the post-mortem room, or as biopsy samples (skin only) and were cut into slices, not more than 3.5 mm. thick and put at once into 10 per cent neutral formol saline for fixation. Tissues were processed with the aid of a histokinette. They were dehydrated through ascending grades of alcohol, cleared in chloroform and embedded in paraffin. Where skin was found hard to cut after routine paraffin embedding, tissues were double embedded-first in one per cent celloidin in methyl benzoate and then in paraffin, following Gurr (1962). The use of "Mollifex", a B.D.H. Ltd. product for softening tissues embedded in paraffin, facilitated the section cutting. All sections were cut at a thickness of 6 μ and stained with haematoxylin (Ehrlich's) and eosin. In addition the following special stains were used:

(A) For mast cells (in the skin):

1. Toluidine blue-eosin (Gurr, 1962): Tissues were fixed in the fixative recommended for mast cells (Gurr, 1962), the composition being formaldehyde 40 per cent (10 ml.), alcohol 95 per cent (90 ml.) and calcium acetate (1 g.). It did not yield satisfactory results in sheep.

2. Polychrome methylene blue for mast cells (Gurr, 1962): For this, tissues were fixed in absolute alcohol. The method was not satisfactory in the sheep.

3. Aldehyde fuchsin for mast cells (Gurr, 1962): The use of any fixative except those containing dichromate was recommended. The tissues were embedded in paraffin only. Celloidin was avoided, as it is impervious to the stain (Gurr, 1962). This method too, proved unsatisfactory for sheep tissues.

4. Iron haematoxylin - Fast green - Safranin O: (Lillie, 1954): Weigert's iron haematoxylin was used. Tissues were formalin fixed. This method gave good results.

5. Leishman's stain (Culling, 1963): Tissues were formalin fixed. Staining was done at pH 6.8. This method gave the most satisfactory results.

(B) For eosinophiles (in the skin):

Tissues were fixed in saturated aqueous mercuric chloride (6 - 7 per cent) for not more than 48 hours. Prior to staining, sections were treated by the standard technique to remove the mercuric precipitate (Culling, 1963).

Staining methods used:

1. Leishman stain (Culling, 1963): Sections were stained at pH 6.8 and also tried within a range of 6.0 to 7.0. It did not yield satisfactory results with sheep tissues.

2. McNeal's Method (Charleston, 1965): This also proved unsatisfactory for the skin of sheep.

3. Methyl blue-eosin (Mann's stain) (Gurr, 1962): This did not yield satisfactory results with sheep tissues.

4. Lendrum's phloxine-tartrazine stain (Culling, 1963): The staining was slightly modified for sheep. In the place of alum haematoxylin, Weigert's iron haematoxylin was used to provide better nuclear contrast and the sections were stained with phloxine for 10-15 minutes only, instead of 30 minutes. This method gave good results.

5. Haematoxylin and eosin: Ehrlich's haematoxylin and water soluble eosin (one per cent) were used. Sections were stained in haematoxylin for 10 minutes and in eosin for 10 - 15 seconds only. This method gave the best results.

Guinea-pig ileum preparation: Assays of histamine were performed on a piece (1 - 2 cm. segment) of guinea-pig terminal ileum suspended in a 10 ml. bath of oxygenated Tyrode solution (Boughton and Schild, 1962) at 38°C. Contractions were recorded with the conventional kymographic method.

Dialysis: Cellulose dialysis tubing (Arthur H. Thomas Co., Catalogue No. 4465-A2) was used. This has an average pore size of 48°A and while restricts the passage of protein molecules allows free permeability of smaller molecular weight solutes. Dialysis was carried out at 4°C against three changes of saline.

The following substances were used:

Histamine (Koch-Light Laboratories) as the acid phosphate in an aqueous solution.

5-Hydroxytryptamine (Koch-Light Laboratories) as the serotonin creatine sulphate in an aqueous solution.

Compound 48/80 (Burroughs Wellcome and Co. Ltd.) in an aqueous solution.

Histamine, 5-HT and 48/80 solutions were freshly prepared each time.

Mepyramine maleate (Anthisan Veterinary; May and Baker Ltd.) as the manufacturer's 5 per cent solution.

Promethazine hydrochloride (Phenergan Veterinary; May and Baker Ltd.) as the manufacturer's 5 per cent solution.

Bradykinin (Sandoz Ltd.) as the manufacturer's synthetic preparation supplied in 1 ml. ampoule containing 0.1 mg.

Nucleosides (Edward Gurr Ltd.) Adenosine, guanosine, inosine and xanthosine in an aqueous solution.

Kallikrein (Glumorin; Bayer Ltd.) supplied in ampoules of 10 biological units in a powder form to be dissolved immediately before use in the sterile physiological saline solution supplied. It contained pancreatic kallikrein.

Hyaluronidase (Koch-Light Laboratories) supplied as purified hyaluronidase prepared from ovine testes (batch No. 15225); 1 mg. contained 300 I.U. It was used in an aqueous solution.

Sodium Salicylate as a 40 per cent aqueous solution.

Strophanthin - G (Ouabaine Arnaud; Laboratoire Nativelle, Paris): as supplied in 1 ml. ampoules containing 0.25 mg. of strophanthin - G.

Indomethacin (Indocid (1-p-chlorobenzoyl) -5-methoxy -2-methyl-indole -3-acetic acid)

(Merck, Sharp and Dohme) supplied as powder which contained 10.4 per cent of indomethacin in lactose. A 20 per cent aqueous suspension (i.e., 2.08 per cent of actual indomethacin) was used. The powder was mixed to a paste prior to adding the final quantity of water as advised by the manufacturer.

Betamethasone (Betsolan, Veterinary; Glaxo Ltd): as the manufacturer's 'Betsolan' injections in 50 ml. bottles (2 mg/ml).

Ribonucleic acid Different types of RNA were used. Highly polymerised RNA from yeast and RNA sodium salt were as obtained from B.D.H. Ltd; RNA prepared from wheat-germ and pea-seed were obtained from the Plant Chemistry Division of the D.S.I.R., Palmerston North. Each RNA was dissolved in 0.85 per cent saline. Since sodium RNA was found only sparingly soluble in saline, the amounts of this RNA injected in 0.1 ml.

volume are, therefore, only approximately known.

Alloxan monohydrate (B.D.H.Ltd.) as a 10 per cent solution in 0.85 per cent saline.

Isocarboxazid (Marplan: 3-benzylhydrazino Carbonyl-5-methyl-iso xazole) (Roche Ltd.) supplied in packs of 250 tablets, each of which contained 10 mg. isocarboxazid. Tablets were ground; a 2.5 per cent aqueous suspension was used.

Lactic acid (CH_3CH (OH) COOH) (May and Baker Ltd.).

RESULTS

RESULTS

Section 1

Studies on inflammation - using skin in the sheep as a test system

Since histamine and/or 5-HT are clearly involved in the mediation of the 'early' permeability changes in the inflammatory response in the rat and guinea-pig, and both these amines are present in mast cells, it was decided to begin with a study of mast cells in the normal sheep. As observations on the inflammatory changes in the skin were restricted to the skin of the back, studies on the mast cells were similarly restricted.

Mast cells in the skin:

Mast cells in the sheep skin were found difficult to stain. The well-established method of toluidine blue, so effective in staining mast cells of rat and mouse, did not yield satisfactory results in the sheep skin. Of the methods tried, Leishman's stain and iron-haematoxylin fast green-safranin stain proved satisfactory.

Mast cells in the sheep skin were much less numerous than in the skin of the rat and the mouse (figs. 1, 2 & 3). Most of them appeared in the middle-third of the dermis. A few were present scattered diffusely throughout. They were particularly numerous along the blood vessels and showed a characteristic perivascular distribution (fig. 1). An occasional mast cell could be seen in groups of other mononuclear cells, or surrounding the sebaceous glands or hair follicle. In size they were smaller than the mast cells of the rat and the mouse. In shape they varied from round to oval and in some cases were fairly elongated. (In the rat and mouse the majority of mast cells are elongated, and may even be filiform, (figs. 2 & 3)). Although numerous granules were present in the cytoplasm in the sheep's mast cells,



Fig.3 Section of normal mouse skin showing mast cells, (arrows). Compare with figures 1 and 2.

Toluidine blue x 250

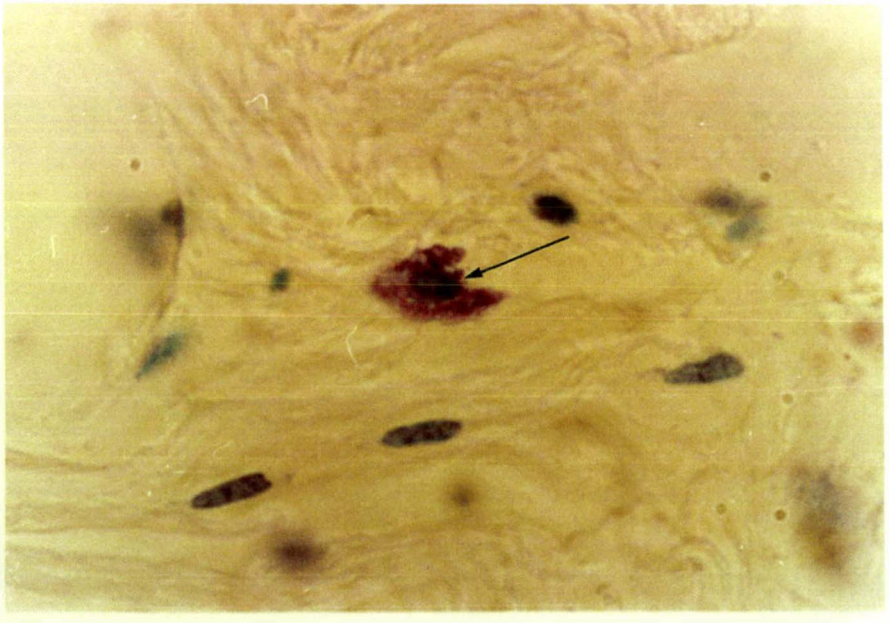


Fig.4 Section of normal sheep skin. A mast cell under oil immersion showing round-to-oval nucleus (arrow). Note difficulty in distinguishing granules in cytoplasm.

Leishman stain x 1000 (oil immersion)



Fig.1 Section of normal sheep skin showing mast cells (arrows) in perivascular areas.

Iron-haematoxylin - fast green - safranin x 250

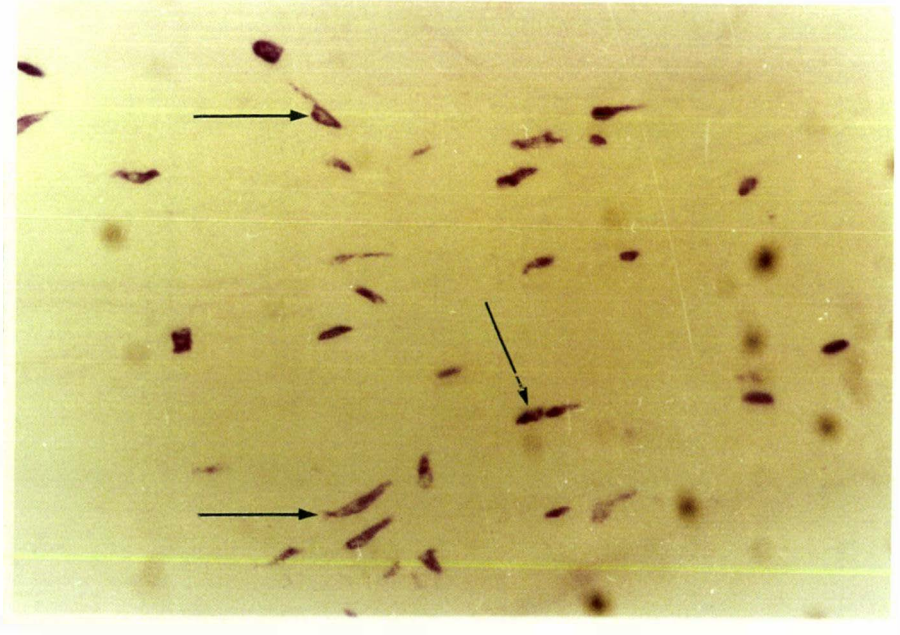


Fig.2 Section of normal rat skin showing elongated mast cells, (arrows). Compare their shape with those in the sheep skin in fig. 1.

Toluidine blue x 250

the nucleus was not usually obscured; it was centrally-placed in the cell and round-to-oval in shape (fig. 4). The granules were finer than those of the rat and mouse mast cells and it was more difficult to distinguish individual granules.

Experiments:

(i) Histopathological changes following intradermal injection of turpentine:

Turpentine has been used extensively in the rat to produce experimental acute inflammation. The inflammatory changes following intradermal injection of turpentine were studied in the sheep; in particular the effects were observed on the mast cells in skin sections after these injection.

0.1 ml. volumes of turpentine were injected intradermally into the dorsal skin of a lamb. Skin biopsies were taken at 15 min., 30 min., 1 hr., 2 hr., 4 hr., 6 hr., and 24 hr. intervals following the injections. Skin samples were fixed, sectioned and stained with haematoxylin and eosin, and for mast cells as described previously.

15 min. after the intradermal injection of turpentine there was congestion of the small vessels in the dermis. The dilated hyperaemic vessels showed margination of polymorphs (i.e., appearance in the marginal stream and adhesion to the vessel wall). There was oedema (characterised by separation of tissue elements by a pale, pink-staining, granular material) but very slight emigration of leucocytes.

Mast cells were increased in number (fig. 10a). Some were swollen and showed well-defined granules (fig. 5). There was slight degranulation. This was indicated by the development of vacuoles (a region of cytoplasm free of granules) within some mast cells (fig. 6) and/or the presence of granules in the surrounding tissues.

After 30 min. margination of the polymorphs in the venules was more marked, but still the cells were largely confined to blood vessels (fig. 7).

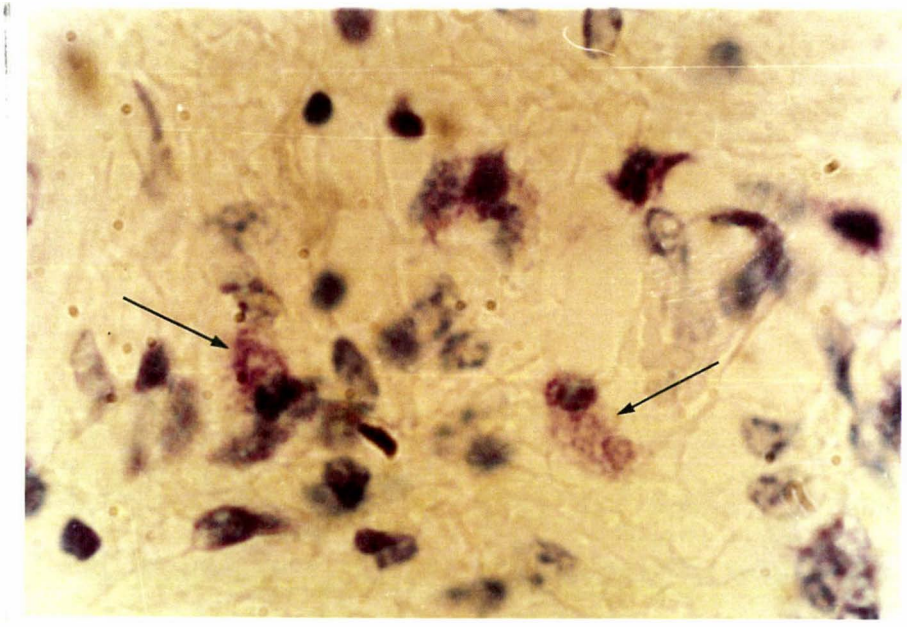


Fig.5 Section of sheep skin 15 min. after intradermal injection of 0.1 ml. turpentine. Mast cells showing varying degrees of swelling and well-defined granules (arrows). Compare with fig. 4.

Leishman stain x 1000 (oil immersion)

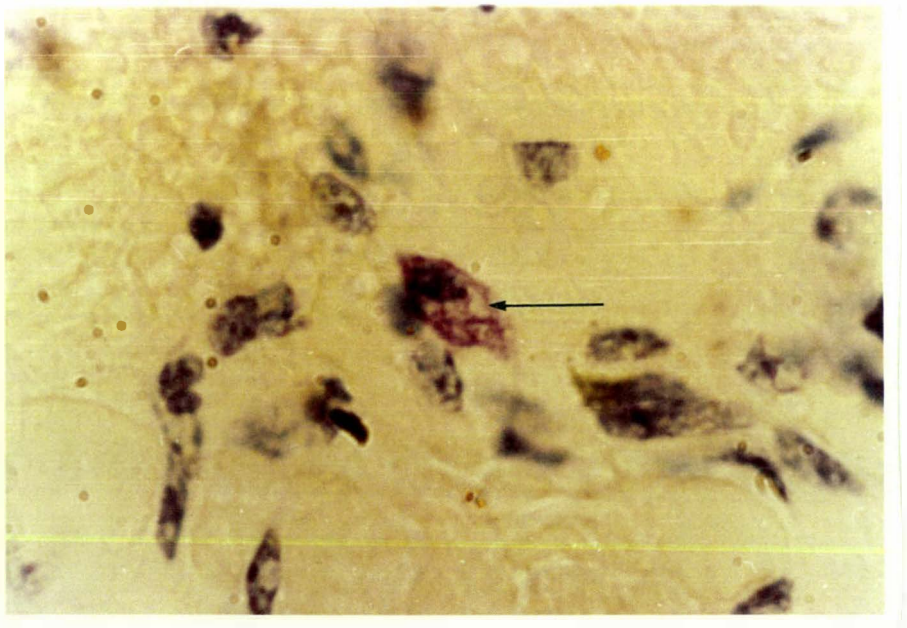


Fig.6 Section of sheep skin 15 min. after intradermal injection of 0.1 ml. turpentine showing "vacuolation" (i.e., loss of granules from an area of cytoplasm) in a mast cell, (arrow).

Leishman stain x 1000 (oil immersion)

Congestion of the blood vessels and oedema had increased.

No increase in mast cell numbers was seen, compared with sections taken at 15 min. Degranulation was more marked (fig. 7). Occasionally a basophil leucocyte was seen inside a blood vessel in the skin section (fig. 8).

After 1 hr., emigration into and infiltration of the tissues with polymorphs had commenced. Congestion of the blood vessels had further increased and oedema of the subdermal connective tissue was more extensive.

Mast cells appeared to have decreased in number i.e., compared with sections taken at both 15 and 30 min. Some of the few remaining mast cells showed vacuolation. The presence of numerous granules characteristic of mast cells, in association with nuclear fragments, suggested that the disruption of mast cells was well advanced at this stage (fig. 9).

After 2 hr. changes were essentially similar to those seen at 1 hr. but the leucocyte emigration was more advanced.

Mast cells appeared more numerous compared with the number seen during the preceding stage. Degranulation and disruptive changes in them were, however, less marked. All showed varying degrees of swelling.

After 4 hr., there was further leucocytic infiltration of the tissue spaces which appeared distended with fluid. Dense collections of leucocytes, which were largely perivascular in distribution, were seen. These were mainly polymorphs. Many blood vessels were still dilated and hyperaemic.

Mast cells appeared to have decreased in number at this stage and to be present in about those numbers seen 1 hr. after injection. They showed varying stages of swelling, degranulation and disintegration but were not detected in the areas of dense leucocytic infiltration.

After 6 hr., further increases in the leucocytic emigration were noticed



Fig.7 Section of sheep skin 30 min. after intradermal injection of 0.1 ml. turpentine showing degranulation in a mast cell (d). Note dense accumulation of polymorphs inside the venule (v); at this early stage in the reaction they are still confined to the vessel.

Leishman stain x 1000 (oil immersion)

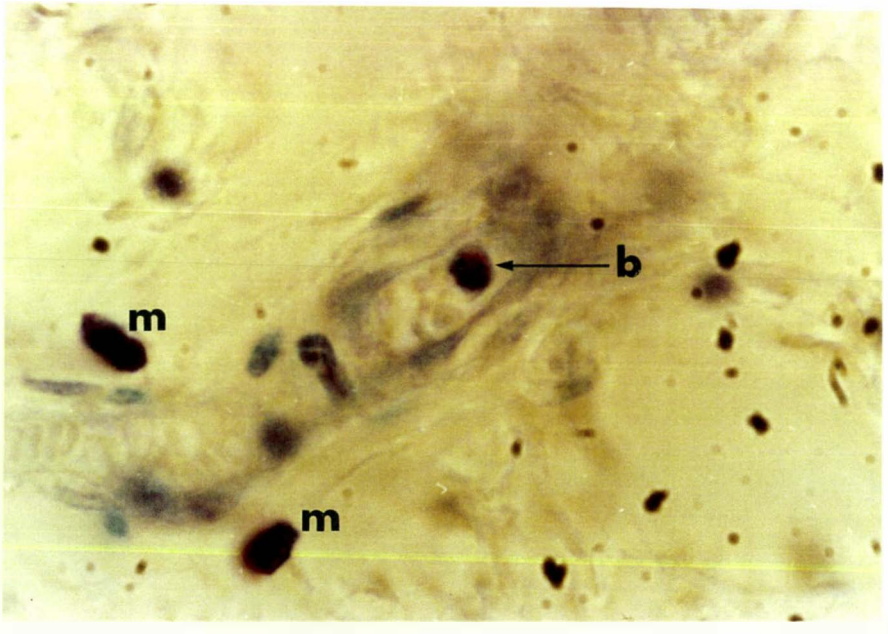


Fig.8 Section of sheep skin 30 min after intradermal injection of 0.1 ml. turpentine showing a polychromatic cell (b), presumably a basophil, in a blood vessel. Two mast cells (m) are present outside the blood vessel.

Leishman stain x 1000 (oil immersion)

(fig. 10b). Polymorphs showed early degenerative changes. Haemorrhages had occurred into the tissue spaces. Inflammatory oedema, especially of the sub-dermal connective tissue, was considerable.

Mast cells were further decreased in number. Those remaining revealed changes similar to those seen 4 hr. after the injection. After 24 hr., leucocytic emigration and infiltration of the tissues was at its height. The neutrophiles showed marked degenerative changes with fragmentation of nuclei (karyorrhexis). Haemorrhage had occurred into the tissue spaces, specially at the periphery of the area occupied by dead and degenerating cells. Oedema of the tissues was marked.

Mast cells once again appeared to show some increase in number at this stage compared with sections taken at 6 hr. However, they were not seen in areas of dead and degenerating neutrophiles. Some of them still showed varying degrees of swelling and degranulation.

(ii) The effect of compound 48/80 on the mast cells:

Of the wide variety of agents which release histamine from the mast cells, compound 48/80 - a condensation product of p-methoxyphenethylamine and formaldehyde, is the most specific and least toxic (Paton, 1951; Fawcett, 1954). It also produces degranulation of the mast cell (Riley, 1959; Horsfield, 1965).

The effect of 48/80 on mast cells in the sheep skin was studied. Two dose levels were used: 1 mg. in 0.1 ml. saline (1%) and 20 mg. in 0.2 ml. saline (10%) were injected intradermally. The lower dose was injected at four sites in one sheep. 30 min. after the injections, skin biopsies were taken from each site. Skin samples from the rat and mouse, after similar treatment, were also collected for comparison. Skin samples were fixed, sectioned and stained with H & E and for mast cells as described previously.

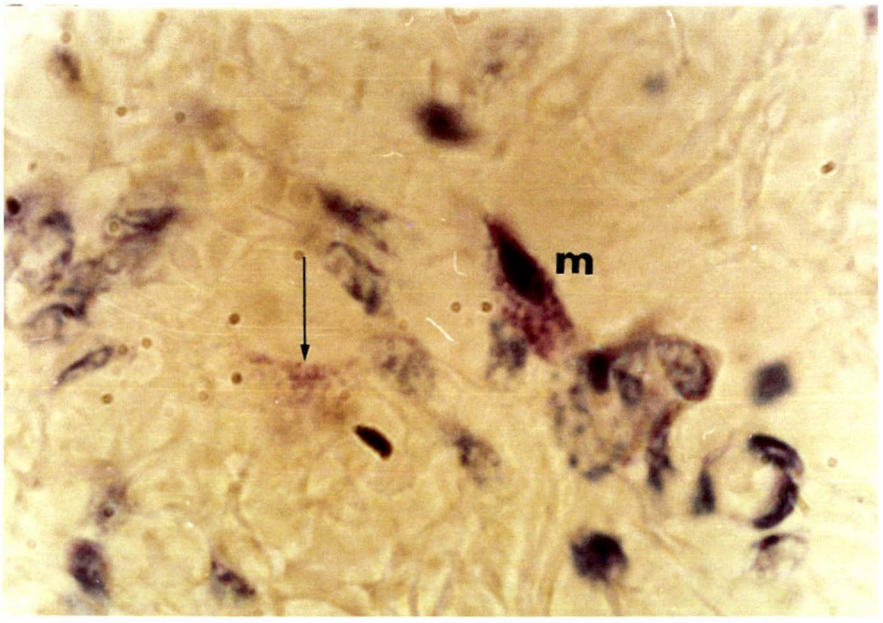


Fig.9 Section of sheep skin 1 hr. after intradermal injection of 0.1 ml. turpentine showing two mast cells. One of them is swollen but still intact (m); the other has disrupted and the cytoplasmic granules are scattered (arrow).

Leishman stain x 1000 (oil immersion)

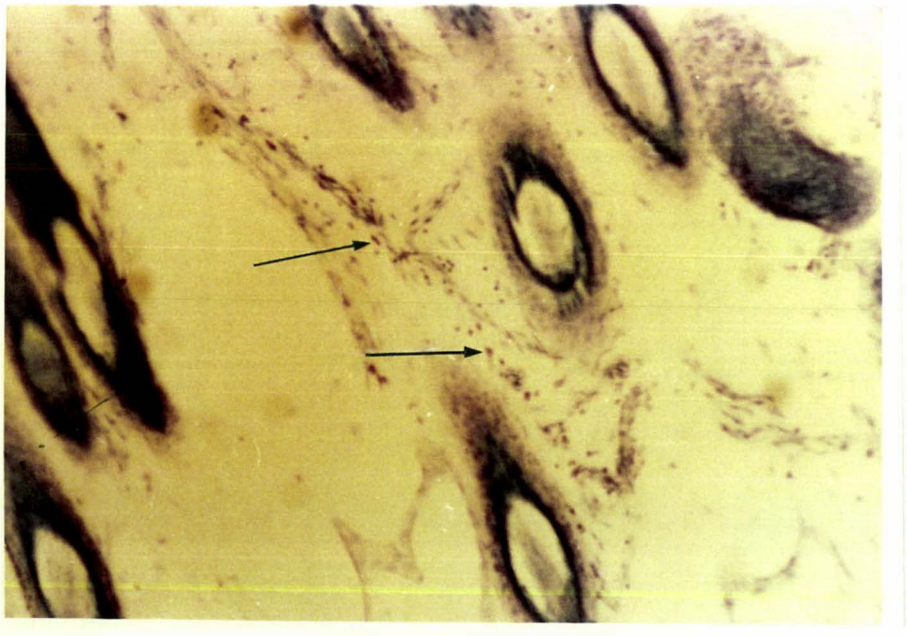


Fig.10a Section of sheep skin 15 min. after intradermal injection of 0.1 ml. turpentine showing more numerous mast cells (arrows) than in normal skin. Interstitial oedema is present, but slight leucocyte emigration.

Leishman stain x 100

20 mg. 48/80 was injected intradermally at each of four sites in a second sheep. Skin biopsies were taken sixty minutes after the injection. This animal died one hour after injecting the total of 80 mg. 48/80. This is discussed later in connection with attempts to induce local depletion of histamine in the skin.

In sections of skin taken from sites of intradermal injection of 1 mg. of 48/80, there was vascular dilatation and hyperaemia. Hyperaemic vessels showed margination of polymorphs. Oedema was also present.

Mast cells had increased in number compared with those seen in sections of untreated skin; they were swollen and had well defined cytoplasmic granules; degranulation had occurred as indicated by the presence of vacuoles in the mast cells and/or granules in the surrounding tissues. Some cells were completely disrupted and granules scattered in the interstitial tissue (fig. 11). The above changes were seen in all the three species but were most marked in the rat.

The effect of the local injection of 20 mg. 48/80 on the skin was seen in skin sections in which there were dilated and hyperaemic blood vessels showing margination of polymorphs. There was some leucocytic emigration evidenced by collections of leucocytes at a few places. The cellular collections tended to be sub-epidermal. Oedema, especially of the subdermal connective tissue, was pronounced and contributed to an increased thickness of the skin.

Mast cells had significantly increased in number compared with those seen in sections of untreated skin and showed varying degrees of swelling and degranulation. However, unlike sections from the skin receiving 1 mg. of 48/80, most of the mast cells in these sections appeared slightly swollen and showed very little degranulation. Granules were so numerous in the cytoplasm as to obscure the nuclei. Mast cells could not be detected in areas of leucocytic infiltrations.

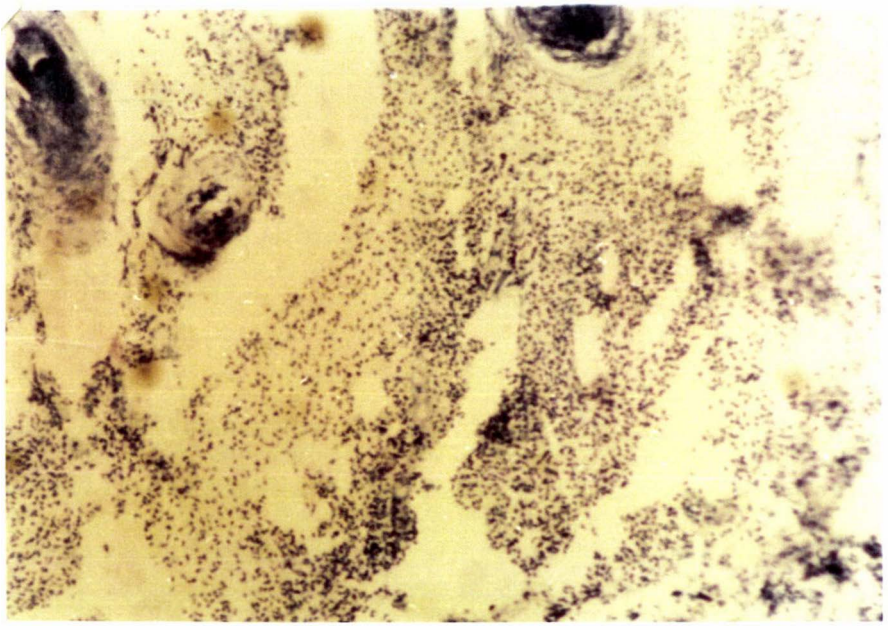


Fig.10b Section of sheep skin 6 hr. after intradermal injection of 0.1 ml. turpentine showing marked neutrophilic infiltration.

Leishman stain x 100

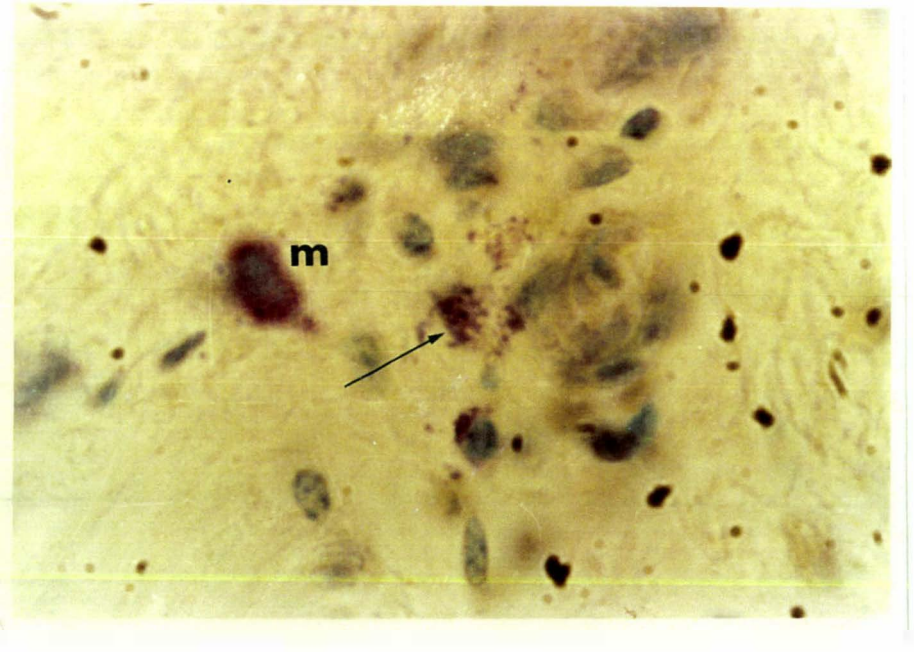


Fig.11 Section of sheep skin 30 min. after intradermal injection of Compound 48/80 (1 mg.) showing two mast cells. One of them (m) is swollen but still intact; the other has disrupted and its granules have scattered.

Leishman stain x 1000 (oil immersion)

(iii) Permeability-increasing effects of turpentine and Compound 48/80:

Turpentine was injected intradermally at four sites in 0.02, 0.05, 0.1 and 0.25 ml. volumes. A 1.0 per cent solution of compound 48/80 in 0.85 per cent saline was injected at two further sites in volumes of 0.05 and 0.1 ml, i.e., in 0.5 mg. and 1 mg. amounts respectively. The size of the blued area and intensity of the colour were recorded (table 3). Saline and distilled water were injected at another four sites in a volume of 0.1 ml. as a control. This pattern of injections was carried out on the right and left sides of each of two animals.

Results:

The results were closely comparable in the two animals and the results from one animal are summarised in table 3. Saline and distilled water by themselves induced no blueing. As can be seen from table 3 and fig. 12, intradermal injection of either turpentine or compound 48/80, caused a well-marked leakage of dye. Blueing appeared after a latent period of 1-3 min. The size of the blue area and the intensity of colour increased for about 60 min. Thereafter very small increases occurred at some sites up to 3 hr. and no detectable increase occurred after this time.

Table 3 - The effect on skin blueing of turpentine and compound 48/80 in the sheep.

Time after intradermal injection	Size of the blue area (mm.) (length x breadth)					
	Turpentine (ml.)				48/80 (mg.)	
	0.02	0.05	0.1	0.25	0.5	1.0
15 min.	6x4	7x5	10x5	20x10	4x2	5x3
30 min.	30x20	35x25	40x30	45x35	15x10	25x15
1 hr.	30x20	35x35	50x35	45x40	20x10	40x20
3 hr.	30x20	50x40	50x40	50x45	25x15	40x20



Fig.12 Lamb with pontamine blue in the circulation showing blue staining of the skin induced by intradermal injections of turpentine and Compound 48/80, and the application of a heated copper rod. Photograph taken 30 min. after the following treatment:

Top row: Three intradermal injections of turpentine (0.1 ml.)

Middle row: Three applications of a heated copper rod (70°C for one min.)

Bottom row: Three intradermal injections of Compound 48/80 in 1 mg. amounts.

There was a relation between the dose of a substance and the size of the blue lesion; a larger dose caused a greater area of blueing. No such relation could be established with the intensity of the colour; a smaller dose produced a lesion of about the same colour intensity as the larger, this depended on a subjective assessment.

(iv) Effect of mepyramine maleate (Anthisan) on the increased vascular permeability induced by turpentine and compound 48/80:

Mepyramine maleate, an antagonist of histamine, was used to try to suppress the increased vascular permeability caused by intradermal injections of 48/80 and of turpentine. The suppression of the increased vascular permeability caused by both substances would indicate mediation of these changes by histamine.

Mepyramine was injected intradermally in doses of 10 µg., 50µg., 100 µg., 1 mg., 2 mg., and 5 mg. in 0.1 ml. volumes. Two sheep were treated in this way. Each sheep received two such series of injections on each side. Mepyramine treatment was carried out 15 min. before the proposed injection of test substances.

It was found that mepyramine itself induced blueing and could not therefore be used in this way. Mepyramine induced the leakage of dye in doses of 1 mg. or more. Systemic administration was subsequently tested.

The effect of systemic mepyramine was tested by injecting it intramuscularly in a single dose of 25 mg./kg. body weight 15 min. before injection of the test substances. Two sheep were treated in this way.

The following series of intradermal injections was made:- turpentine, 0.1 ml., compound 48/80, 10 µg., 50 µg., 100 µg., 500 µg, 1 mg., (all in 0.1 ml. volumes). Each series was repeated three times at different sites on both right and left sides of each sheep.



Fig.13 Same lamb as in fig. 12 showing the effect of mepyramine maleate-pretreatment on the blueing induced by the intradermal injections of turpentine and of Compound 48/80, and the application of a heated copper rod.

Summary of procedures:

(a) 0 min: Mepyramine maleate injected intramuscularly (25 mg./kg.)

(b) 15 min: following treatment carried out:

Top row: Three intradermal injections of turpentine (0.1 ml.)

Middle row: Three applications of a heated copper rod (70°C for one min.)

Bottom row: Three intradermal injections of Compound 48/80 in 1 mg. amounts.

(c) 30 min: Photograph taken. Note suppression of blueing at the sites of turpentine injection; return of blueing at the application of heated copper rod, and incomplete suppression of blueing at sites of Compound 48/80 injections.

The systemic treatment with mepyramine abolished the increase in vascular permeability previously shown to occur after the intradermal injection of doses of 10 - 500 µg. of compound 48/80. The suppression of increased permeability due to 48/80 was incomplete when 1.0 mg. was injected (fig. 12 & 13). The leakage of dye after turpentine injection was delayed for about one hour after its injection following the administration of mepyramine. Where suppression of the effect of 48/80 occurred no blueing developed within an observation period of eight hours. In those areas where blueing was suppressed an area of reddening was seen.

An attempt was made to exclude the possibility that the development of blueing one hour after injection of turpentine was due to a fall in the concentration of mepyramine in the blood and tissues. This was made by giving two further intramuscular injections of 5 mg/kg of mepyramine. One such injection was given one hour and another two hours after the initial injection of mepyramine. These further doses of mepyramine failed to suppress the blueing after turpentine injection, which was (as described above) delayed for about one hour after mepyramine injection.

(v) Effects of histamine, 5-hydroxytryptamine and bradykinin on vascular permeability:

Suppression of dye-leakage induced by turpentine and compound 48/80 by mepyramine maleate indicated that these substances might have increased vascular permeability in the sheep through histamine release. Histamine and/or 5-HT act as mediators of 'early' permeability changes in inflammation in the rat and guinea-pig and bradykinin may be involved in the 'delayed' changes (see Spector and Willoughby, 1963a; Wilhelm, 1965). The effects of histamine, 5-HT and bradykinin were tested on sheep skin.

Histamine, 5-HT and bradykinin were each injected intradermally in 0.05, 0.1, 0.5, 1.0, 2.5, 5.0 and 10 µg. amounts. Two sheep were used in



Fig.14 Lamb with pontamine blue in the circulation showing blueing induced by 10 µg amounts of histamine, 5-Hydroxytryptamine and bradykinin and the effect of prior administration of mepyramine maleate on it.

Summary of procedures:

From right:

(a) 0 min: following injections were made:

- Top row: First three sites: Histamine injected intradermally in 10 µg amounts.
- Middle row: First three sites: 5-HT injected intradermally in 10 µg amounts.
- Bottom row: First three sites: Bradykinin injected intradermally in 10 µg amounts.

(b) 15 min: Mepyramine maleate injected intramuscularly (25 mg./kg.)

(c) 30 min: following injections were made:

- Top row: Last three sites: Histamine injected intradermally in 10 µg amounts.
- Middle row: Last three sites: 5-HT injected intradermally in 10 µg amounts.
- Bottom row: Last three sites: Bradykinin injected intradermally in 10 µg amounts.

(d) 45 min: Photograph taken. Note blueing at sites of injection of histamine, 5-HT and bradykinin before mepyramine maleate treatment. After mepyramine treatment, blueing is suppressed at the sites of injection of histamine and of 5-HT but not of bradykinin.

this experiment. Each sheep received on both right and left sides the complete series of injections of each test substance.

With all three of the substances there was a noticeable increase in permeability within 1-3 min. of their injection; the least effective doses used were 0.1 µg. histamine, 2.5 µg. of 5-HT and 0.5 µg. of bradykinin. Judged by the intensity of colouration of the skin the maximal responses were obtained with 10 µg. of each of these substances.

(vi) The duration of the permeability change was measured by injecting into one clipped lamb 10 µg. amounts of histamine, 5-HT and bradykinin 2 hr., 1½ hr., 1 hr., 45 min., 30 min., 15 min., 10 min., and 5 min. before the intravenous injection of pontamine sky blue, after the technique of Miles and Miles (1952). Thus, when the dye was injected after 2 hr., the skin bore lesions varying in age from 0 to 2 hr. and those sites of injection at which the vessels were no longer permeable did not blue. One series of injections at the above timings for each permeability factor was made on each side of the lamb.

It was found that the increased permeability due to histamine and 5-HT decreased after 30 min. and the normally low permeability was restored after one hour. In contrast bradykinin caused an increased permeability which lasted for only 15 min. The subsequent decrease in enhanced permeability was abrupt; normal permeability was restored after 30 min.

(vii) Effect of mepyramine maleate ('Anthisan') on the increased vascular permeability caused by histamine, 5-hydroxytryptamine and bradykinin:

In this experiment mepyramine maleate was used in an attempt to suppress the increased vascular permeability caused by histamine, 5-HT and bradykinin injections. If the mepyramine suppressed the effect of histamine, and also, suppressed the effects of 5-HT and bradykinin it was argued this would indicate that the actions of these substances could be ascribed to the relase of histamine.



Fig.15 Lamb with pontamine blue in the circulation showing effect of mepyramine maleate-pretreatment on the blueing induced by 10 µg of bradykinin injected intradermally.

Summary of procedures:

(a) 0 min: Mepyramine maleate injected intramuscularly (25 mg./kg.)

(b) 15 min: Bottom row: Three intradermal injections of bradykinin were made (10 µg)

(c) 20 min: Middle row: Three intradermal injections of bradykinin were made (10 µg)

(d) 25 min: Top row: Three intradermal injections of bradykinin were made (10 µg)

(e) 30 min: Photograph taken. Thus,

Top row: sites 5 min. after mepyramine treatment-blueing suppressed.
Middle row: sites 10 min. after mepyramine treatment-appearance of blueing
Bottom row: sites 15 min. after mepyramine treatment-maximum blueing

In each case mepyramine maleate was injected intramuscularly in a single dose of 25 mg/kg body weight 15 min. before the test substances were injected. Histamine, 5-hydroxytryptamine and bradykinin were injected intradermally in 10 µg. amounts. Two sheep were used. Each sheep was given three injections of each test substance on each side.

Identical results were obtained in all injection series. Mepyramine completely suppressed the leakage of dye caused by the previous injections of the intradermal injection of 10 µg. of histamine and 10 µg. 5-HT (fig. 14). The onset of increased vascular permeability due to bradykinin was delayed for approximately 10 min. after this time marked leakage of dye took place (fig. 15): reaching a maximum 15 min. after bradykinin injection.

(viii) In a study of the effect on vascular permeability of thermal injury two sheep were used. Thermal injury was inflicted as described earlier at six sites on the left side of each blued sheep.

To determine the effect of antihistamine both the lambs were injected intramuscularly with mepyramine maleate (25 mg/kg) one hour after the end of the above observations. 15 min. later thermal injury was inflicted at six sites on the right side of each sheep. The results are shown in table 4.

Table 4 - Summary of results of 12 exposures to thermal injury in each of two sheep - 6 before and 6 after mepyramine administration.

Time after thermal injury	Leakage of dye (intensity of colour)	
	Normal sheep	After mepyramine-treatment
5 min.	+	none
10 min.	+++	+
15 min.	++++	+++
30 min.	++++	++++

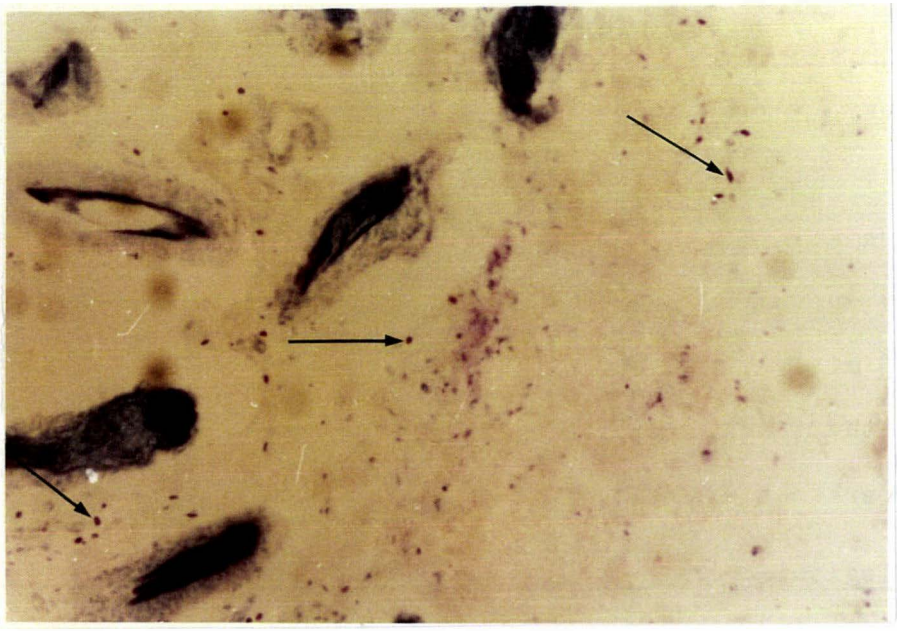


Fig.16 Section of sheep skin 15 min. after intradermal injection of 10 µg histamine showing more numerous mast cells (arrows) than in normal skin.

Leishman stain x 100

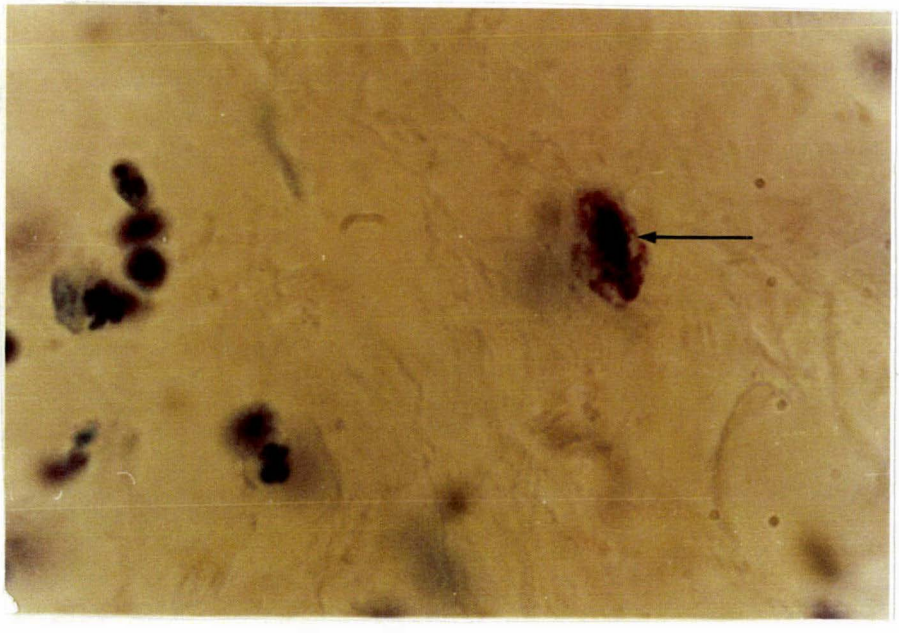


Fig.17 Section of sheep skin 30 min. after intradermal injection of 10 µg histamine showing vacuolation in a mast cell, (arrow).

Leishman stain x 1000 (oil immersion)

Thermal injury caused marked leakage of dye after a latent period of 1-3 min. There was an early blanching at the heated site. The blueing was first seen as a ring of colour at the periphery of the heated site, and then it extended inwards. The entire lesion had become evenly coloured after 15 min. (fig. 12). The previous administration of mepyramine delayed the leakage of dye by 8-15 min. only (fig. 13; table 4): after this time marked blueing took place.

(ix) Histopathological changes following intradermal injection of histamine, 5-hydroxytryptamine and bradykinin:

It has been demonstrated in the rat that there is little relation between the ability of substances to induce leucocyte emigration and their ability to increase the vascular permeability. Further the leucocyte emigration occurs when vascular permeability has returned to normal and the two phenomena therefore are separated in time (Hurley and Spector, 1961a, b).

The histopathological changes following the intradermal injection of histamine, 5-HT and bradykinin were studied in sheep to determine their effects on leucocyte emigration and to see if the emigration was delayed and dissociated from the increased vascular permeability. Mast cell response was also studied.

Histamine, 5-HT and bradykinin were injected intradermally in 10 µg amounts into one sheep. Each compound was injected at seven sites. 0.1 ml. of 0.85% saline was injected intradermally as a control procedure. Skin biopsies were taken 15 min., 30 min., 1 hr., 4 hr., 6 hr., and 24 hr. after the injections. Sections were stained with H & E and for mast cells.

At sites where saline was injected no significant changes were seen 30 min. and 1 hr. after injection except a few hyperaemic vessels showing some margination of polymorphs. Significant leucocytic emigration was seen 3 hr. and 6 hr. after injection of saline.

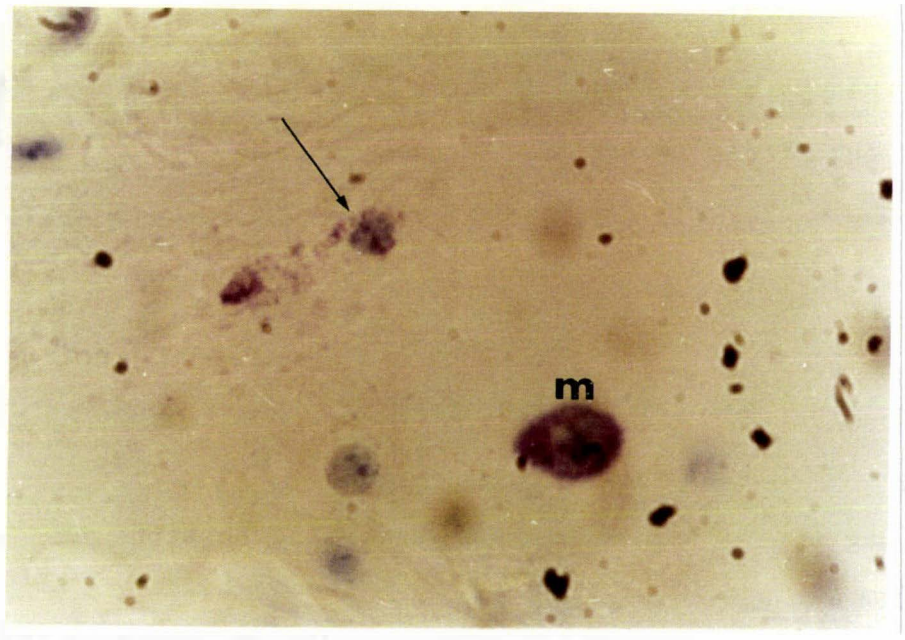


Fig.18 Section of sheep skin 30 min. after intradermal injection of 10 µg histamine showing two mast cells. One of them (m) is swollen and shows some vacuolation, but still intact; the other has disrupted (arrow).

Leishman stain x 1000 (oil immersion)

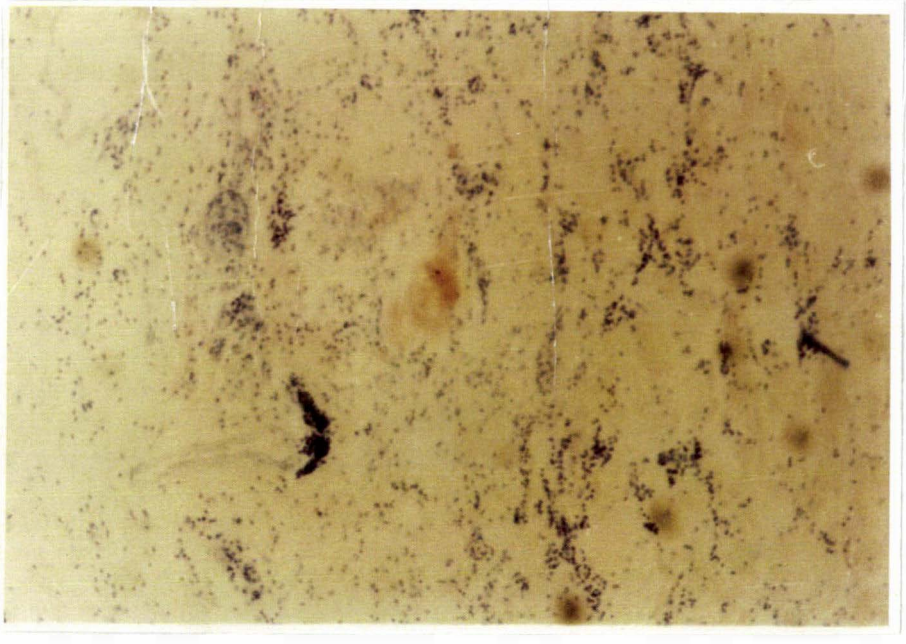


Fig.19 Section of sheep skin 6 hr. after intradermal injection of 10 µg histamine showing diffuse polymorphonuclear neutrophilic infiltration of tissues.

Leishman stain x 100

Sections taken 15 min. after histamine injection revealed vascular dilatation and congestion. Hyperaemic vessels showed margination of polymorphs. There was oedema but only slight emigration of leucocytes into the tissues. Mast cells were increased in number (fig, 16) compared with those seen in sections of untreated skin. Most of them showed varying degrees of swelling with well-defined cytoplasmic granules. Only a few were seen degranulated.

Similar changes were seen in the corresponding sections from areas treated with 5-HT and bradykinin.

30 min. after the injection of histamine, 5-HT and bradykin, congestion of blood vessels and oedema had slightly increased. No significant change was noticed in leucocyte emigration. Mast cells appeared to have further increased in number compared with the preceding stage and many showed vacuolation (fig. 17). Degranulation in them was marked. A few mast cells had disrupted (fig. 18).

1 hr. after the injection of histamine, 5-HT and bradykinin margination of the polymorphs was more marked but the cells were still largely confined to the blood vessels. Mast cells showed a further increase in number, and revealed changes similar to those seen in sections taken 30 min. after injection.

2 hr. after injection of all three substances, emigration and infiltration of the tissues with polymorphs had commenced. The leucocytes at this stage were mostly perivascular in distribution. No further increase in mast cells was noticed: both vacuolation and degranulation of mast cells was a prominent feature.

In sections taken 4 hr. after the injections were made, leucocytic emigration and infiltration of the tissues with polymorphs had markedly increased. Leucocyte emigration was more marked with 5-HT than with histamine and was even greater with bradykinin. The cells were no longer confined to the perivascular areas, and were seen diffusely scattered in the

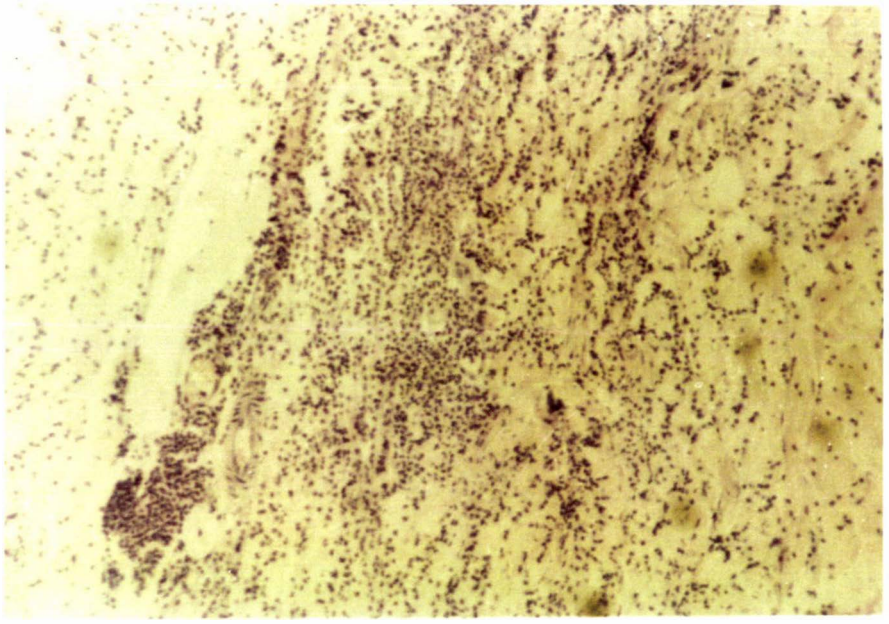


Fig.20 Section of sheep skin 6 hr. after intradermal injection of 10 µg bradykinin showing polymorphonuclear neutrophilic infiltration of tissues. The infiltration was more marked compared to that induced by histamine (10 µg) and 5 - Hydroxytryptamine. (10 µg)

Leishman stain x 100

dermal tissues. Oedema had increased as shown by separation of the skin structures. Mast cells appeared to have decreased in number and were particularly lacking in areas of leucocytic infiltration. Degranulation was more marked than in all the earlier stages.

6 hr. after injections of histamine, 5-HT and bradykinin further increases in the leucocytic infiltration of the tissues were noticed. The same relative differences in the degree of leucocyte emigration due to the different compounds prevailed (figs. 19, 20).

Mast cells again appeared to be increasing in number compared with sections taken at 4 hr. They were still lacking in areas of leucocytic infiltrations, and revealed changes similar to those seen at 4 hr. - degranulation.

24 hr. after the injections were made the amount of leucocyte emigration showed no increase over that seen at 6 hr. The polymorphs revealed mild degenerative changes. Mast cells revealed varying degree of swelling with distinct granules. Degranulation was less marked than at 6 hr.

(x) The migration of eosinophil leucocytes to sites of intradermal injection of histamine:

As noted above histamine injected intradermally in 10 µg amounts caused a delayed leucocytic emigration in the sheep, the cells being polymorphonuclear neutrophiles. In these experiments, the effect of histamine on eosinophiles was further studied by injecting histamine intradermally in 10 µg and in 100 µg amounts and giving single and repeated injections. Later, when it was found that eosinophiles migrated into the skin at those sites where histamine was injected in 100 µg amounts, histamine-inactivation in vivo by eosinophiles was attempted by producing local eosinophilia.

In one sheep histamine was injected intradermally in 10 µg amounts at four sites as set out in table 5. A series of biopsies were taken six hours

after the first histamine injection from sites that had received 1, 2, 3 or 6 histamine injections at the intervals shown. Each of the four injection series was repeated four times, two on each side of the sheep providing sixteen biopsy samples.

In a second sheep the same procedure was carried out using injections of 100 µg histamine as shown in table 5. Skin samples were fixed, sectioned and stained H & E and for eosinophiles. Both formalin and mercuric chloride fixatives were used.

Table 5 - The dose, time interval and the number of intradermal histamine injections given to investigate the effect on eosinophiles.

S. No. of intradermal injection	Time after intradermal injection (hr.)	Injections in 10 µg amounts				Injections in 100 µg amounts			
		Site 1	Site 2	Site 3	Site 4	Site 1	Site 2	Site 3	Site 4
1	zero	10	10	10	10	100	100	100	100
2	one	-	-	-	10	-	-	-	100
3	two	-	-	10	10	-	-	100	100
4	three	-	10	-	10	-	100	-	100
5	four	-	-	10	10	-	-	100	100
6	five	-	-	-	10	-	-	-	100
At	six	biopsy	biopsy	biopsy	biopsy	biopsy	biopsy	biopsy	biopsy

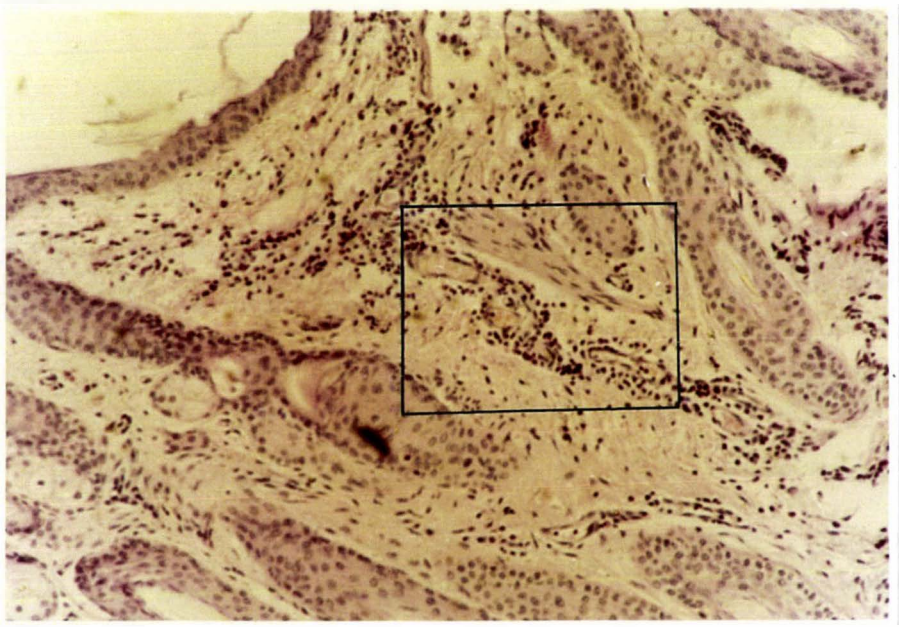


Fig.21 Section of sheep skin 6 hr. after intradermal injection of histamine (100 µg) showing diffuse infiltration of tissues with eosinophiles. Part of this section (square) is shown at a higher magnification in fig. 22.

H.E. x 100

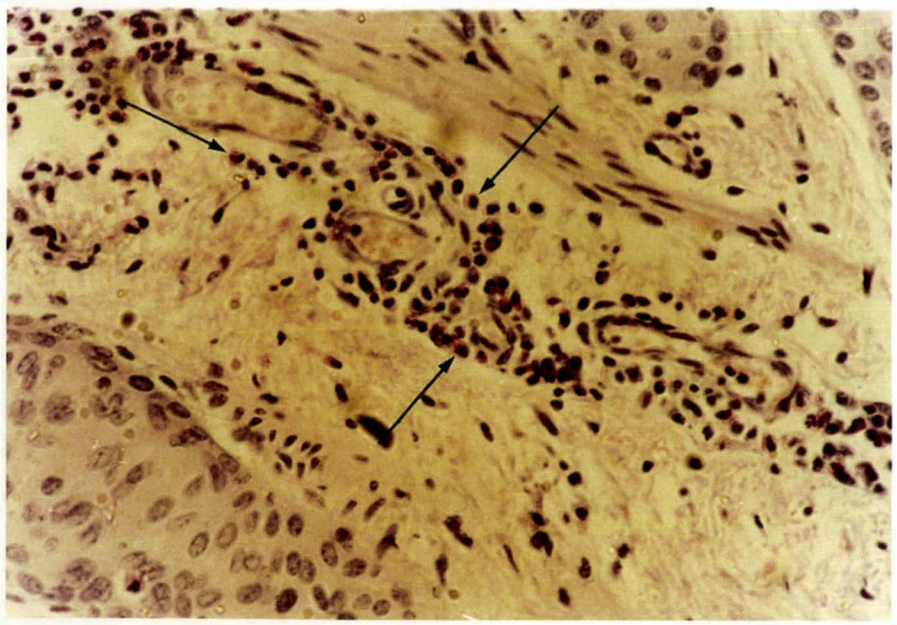


Fig.22 Same as fig. 21, showing eosinophiles (arrows) under a higher magnification.

H.E. x 250

The staining of eosinophiles in sections was found to be most satisfactory with the following procedure. The skin samples were fixed in the saturated aqueous mercuric chloride. The sections were stained for 10 min. in Ehrlich's haematoxylin and for 10-15 secs. in water soluble eosin. Lendrum's phloxine-tartrazine stain was also satisfactory.

In the skin samples that received 10 µg of histamine, either as single or repeated injections, very little tissue eosinophilia was produced. A large number of polymorphonuclear neutrophiles were seen in the tissues, particularly in perivascular locations. Only occasional eosinophiles were seen scattered in the tissues.

In all the skin samples receiving histamine in 100 µg amounts, either as single or repeated injections, marked local infiltration of eosinophiles was seen (figs. 21,22). A few neutrophiles were also present. The eosinophilic infiltration was greatest in the skin samples that received six repeated injections of histamine at hourly intervals (figs. 23, 24). At several places in and around blood vessels dense collection of eosinophiles was seen with scarcely any other cell types visible. The cells did not show degranulation. The eosinophilic infiltration was diffuse in the deeper tissues and the neutrophiles tended to be more numerous at this level but were fewer than eosinophiles.

Since histamine injected intradermally in 100 µg amounts was found to attract eosinophiles, its effect on eosinophiles was examined in another sheep at earlier stages. Skin biopsies were taken 15 min., 30 min., and 1 hr. after intradermal histamine injections, but very little eosinophilia had occurred at these times.

Histamine inactivation <u>in vivo</u> by eosinophiles was attempted by producing local eosinophilia. Six repeated injections of histamine in 100 µg

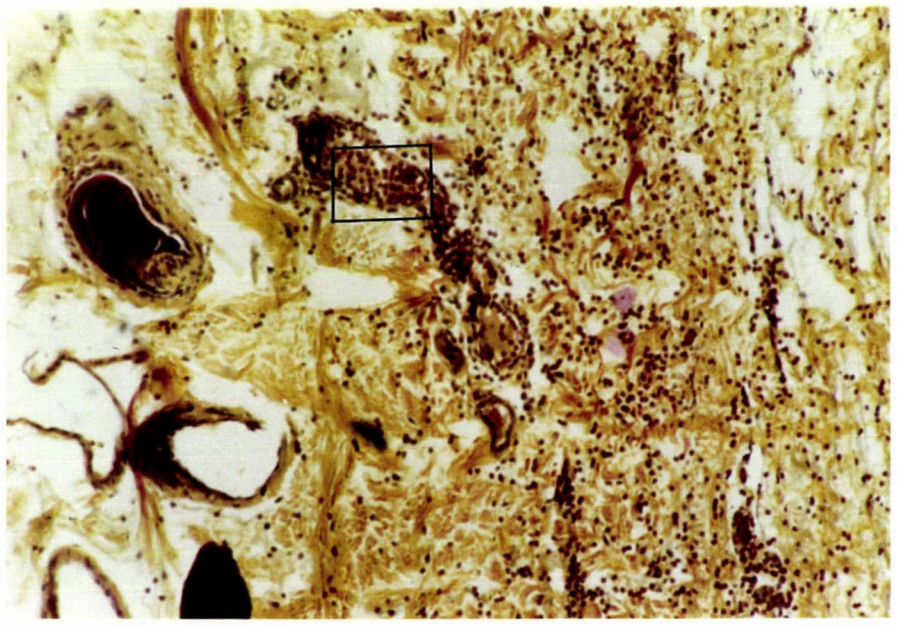


Fig.23 Section of sheep skin 6 hr. after six intradermal injections of histamine in 100 µg amounts of hourly intervals, showing marked infiltration of the tissues with eosinophiles. Part of this section (square) is shown at a higher magnification in fig. 24.

Lendrums' phloxine tartrazine x 100

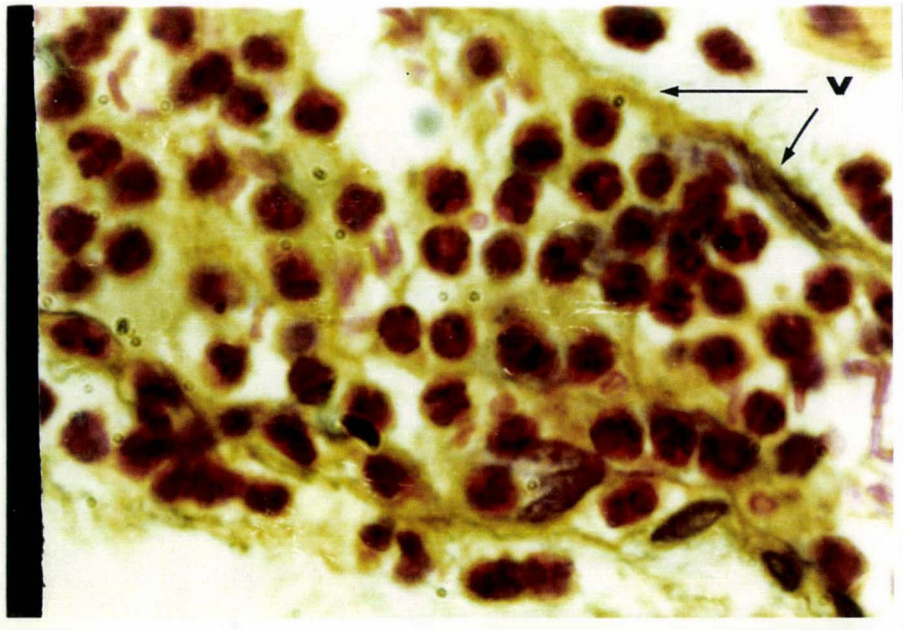


Fig.24 Same as fig. 23, under oil immersion showing a small vein (v) filled with eosinophiles.

Lendrums' phloxine tartrazine x 1000 (oil immersion)

amounts were given intradermally at hourly intervals as described in the above experiment to produce local eosinophilia. Eight such injections were made on either side of one sheep. Next morning, after taking skin biopsies from two places, the lamb was blued. The skin samples were fixed, sectioned and stained H & E, and for eosinophiles.

No blueing occurred at the injected sites indicating that the increased vascular permeability that follows the intradermal injection of histamine had returned to normal. At these and at other untreated sites, which served as controls, histamine in 10 µg amounts was injected intradermally. The extent of blueing and its colour intensity were recorded. Blueing in the treated sites was not significantly different to that obtained at the untreated sites. Skin samples from the treated sites revealed marked eosinophilia on histological examination. The results indicated that the injected histamine at these places was fully active and did not suffer any inactivation.

Next it was decided to inject histamine intradermally daily in 200 µg amounts to see if the local swelling, which normally followed single injections of histamine, decreased and then to repeat the above experiment. Another lamb was used. Histamine was injected intradermally, morning and evening, in 100 µg amounts at seven sites on either side for twelve days beginning with an evening dose. By the 12th day, the size of the local swelling, as judged subjectively, resulting from each successive injection of histamine, had decreased considerably. Therefore on the 13th morning, two skin biopsies were taken from treated sites - one from each side. The lamb was then blued.

No blueing occurred at the injected sites indicating that the increased vascular permeability had returned to normal. At these and at other untreated

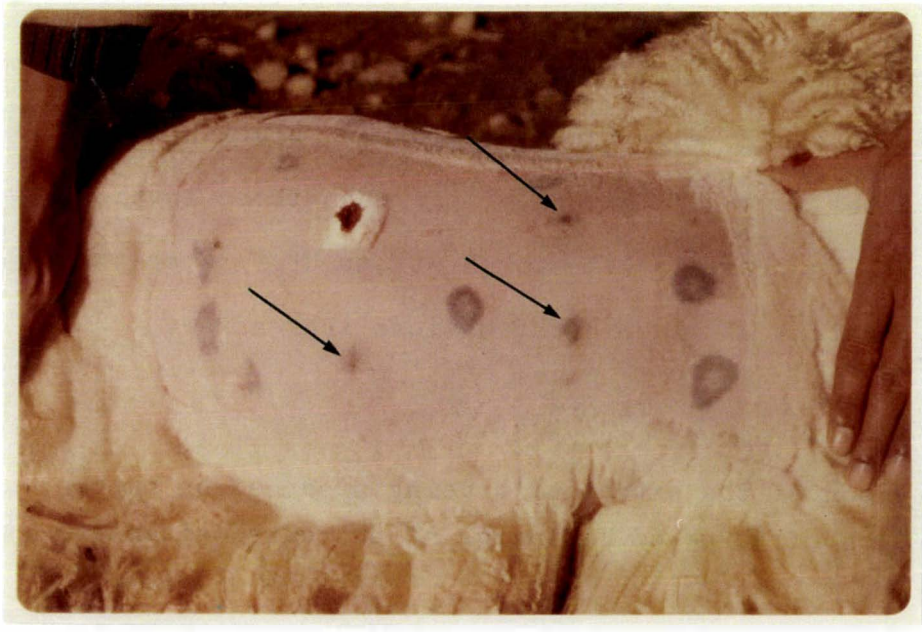


Fig.25 Blueing produced by intradermal injection of 10 µg of histamine. Lamb showing less blueing at sites previously treated with histamine over a period of 12 days (arrows) than at the untreated control sites. The wound is the site of a previous skin biopsy.

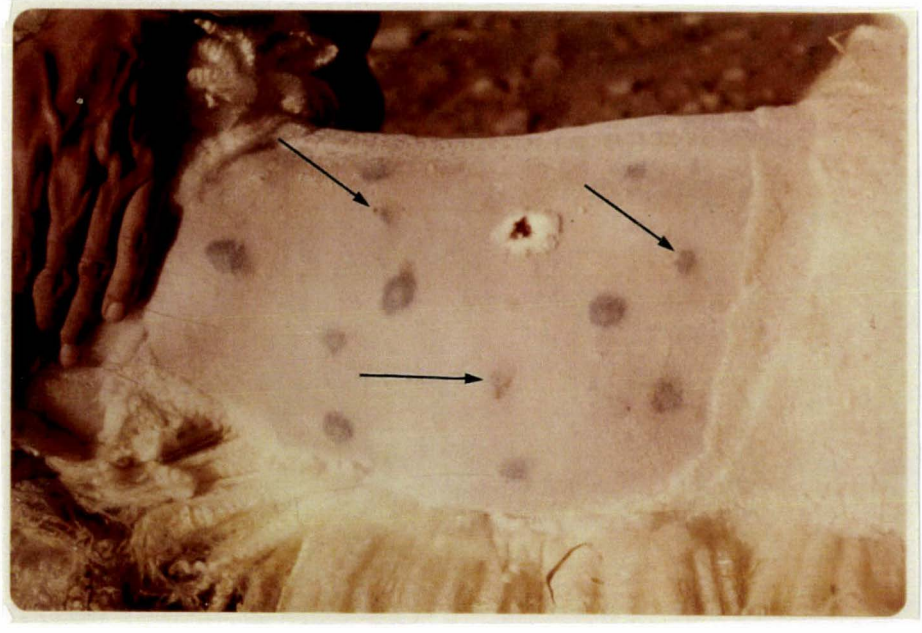


Fig.26 Same lamb as in fig. 25, showing similar blueing reactions on its other side.

sites, which served as controls, histamine was injected in 10 µg amounts. Table 6 and figs 25 and 26 show that the areas of blueing in all the twelve treated sites were significantly less than those in the corresponding untreated sites. Measurements were taken at one hour after the injection; no change in the extent of blueing was noticed thereafter.

Table 6 - Comparison of the extent of blueing produced by intradermal injection of 10 µg of histamine at untreated sites and at sites previously given repeated injections of histamine over a period of 12 days.

S. No. of intradermal injection	Maximum diameter of blue area (mm.)	
	Untreated site	Treated site
1	25	7
2	24	10
3	20	12
4	23	10
5	20	15
6	20	17
7	22	8
8	18	10
9	19	10
10	20	5
11	21	8
12	22	11

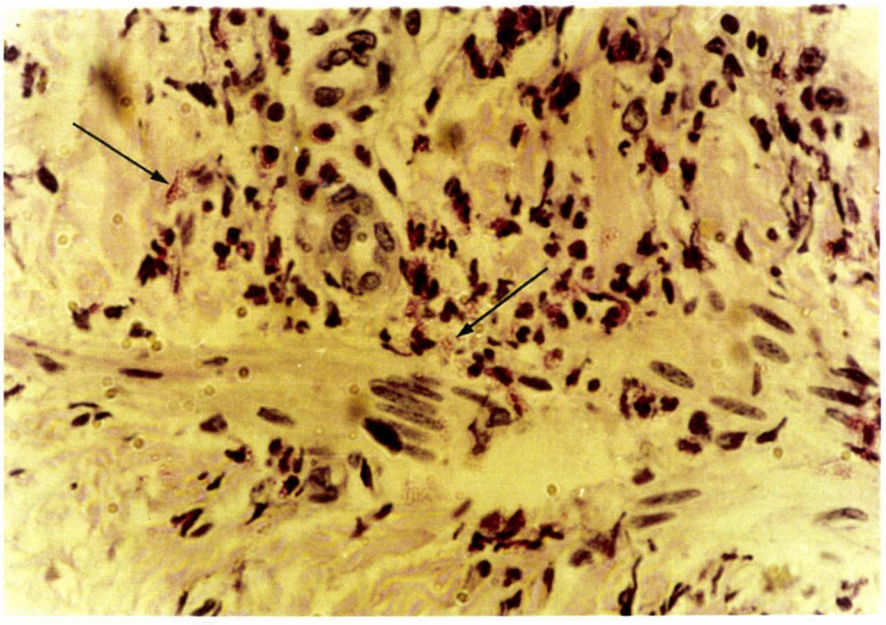


Fig.27 Section of sheep skin after 12 intradermal injections of histamine (200 µg daily for 12 days), showing extensive degranulation of eosinophiles (arrows). Part of the section is shown at a higher magnification in fig. 28.

H.E. x 400

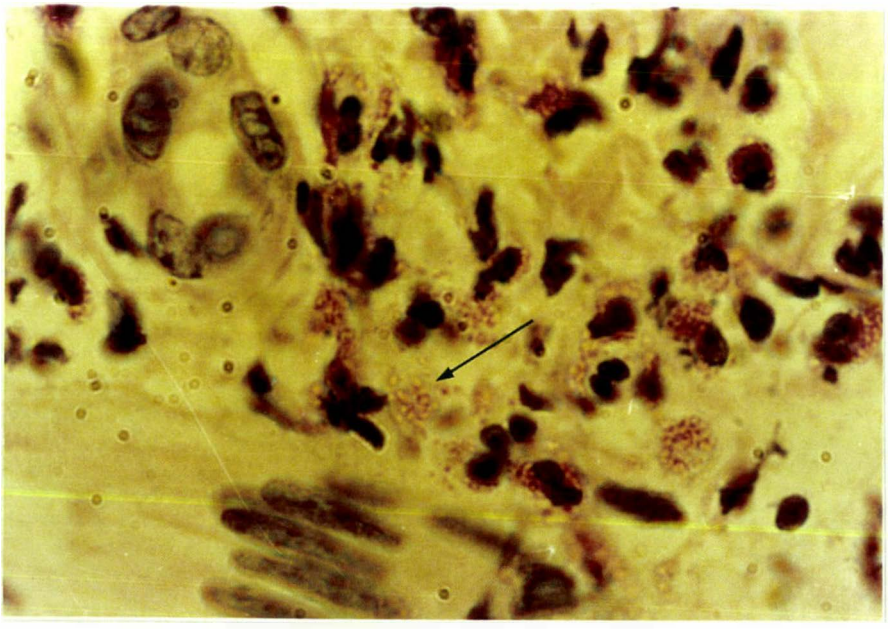


Fig.28 Same as fig. 27, showing degranulation of eosinophiles (arrow), under a higher magnification.

H.E. x 1000 (oil immersion)

The two skin samples taken from the treated sites, before blueing, revealed marked eosinophilic infiltration. A high proportion of the eosinophiles seen were extensively degranulated (figs. 27,28) unlike those seen six hours after histamine injections. The eosinophiles were most numerous in the superficial regions of the dermis. Only slight proliferation of fibrous tissue was seen in the dermis.

(xi) Effect on vascular permeability of nucleosides and its suppression by mepyramine:

Some nucleosides increase vascular permeability in some species but not in others. In the rat this is attributed to histamine release (Moulton, et al, 1957; Spector and Willoughby, 1957a).

Nucleosides adenosine, guanosine, inosine and xanthosine were injected intradermally in 10µg amounts. Two sheep were used. Each received three series of such injections on each side.

Although much less marked compared with the effect of intradermal histamine all the nucleosides increased vascular permeability. Blueing produced by inosine and xanthosine was only half the intensity of that produced by adenosine and guanosine.

Two other sheep were injected intramuscularly with mepyramine maleate in a single dose of 25 mg/kg body wt. 15 min. before the intradermal injections of nucleosides. Each received three series of injections on each side, as in the previous experiment. It was found that pretreatment with mepyramine abolished the increase in vascular permeability caused by the intradermal injection of adenosine, guanosine, inosine and xanthosine. Blueing did not develop at the sites of injection during eight hours observation.

(xii) Effect of pH on vascular permeability:

The leakage of dye caused by the intradermal injection of mepyramine

maleate solution "Anthisan" noted earlier suggested that the solution had a permeability-increasing effect. To ascertain if this was due to the action of the mepyramine itself or the vehicle in which it was dissolved, more information was sought from Messrs May and Baker Ltd. It was stated that the "Anthisan" solutions of all strengths were slightly acidic. It was considered possible that "Anthisan" may have increased vascular permeability through the acidic nature of the solution. To investigate this possibility in the sheep, the effect of varying pH on vascular permeability was tested. It has been suggested that some antihistamines themselves release histamine (Arunlakshana, 1953); and this possibility has been borne in mind.

Saline (0.85 per cent), which by itself induced no blueing, was adjusted to a range of pH values of 3,4,5,6,6.4,7,8,9 and 10. The pH of saline itself was 6.4. The adjustment of pH was made using N/10 HCl and N/10 NaOH and tested on a Beckman pH meter. Phosphate buffers were not employed, since it was thought possible that the presence of buffer salts might affect the results.

The pH of the "Anthisan" solution, that induced leakage of dye when injected intradermally, was found to be between 5-5.1. All injections were made in 0.1 ml. volumes. One sheep was used. On each side it received duplicate injections of each pH interval.

No increase in vascular permeability resulted from the injection of the saline at any pH.

To obtain an idea whether mepyramine induced increased vascular permeability by itself or by releasing histamine, the effect of systemic mepyramine was tested on the leakage of dye caused by mepyramine injected intradermally. Two sheep were injected intramuscularly with mepyramine (25 mg/kg body wt.). Each sheep received in addition six separate

injections of 1 mg., of 2 mg. and of 5 mg. of mepyramine intradermally, 15 min. after the intramuscular dose of mepyramine.

The blueing caused by mepyramine maleate solution given intradermally was completely suppressed by the systemic administration of the same agent.

(xiii) Effect of kallikrein on vascular permeability:

Some of the properties of kallikrein have been discussed in the Introduction. Intradermal injection of kallikrein in the rat has been found to cause increased vascular permeability; this is presumed to be due to the formation of kinins in the skin (Bhoola et al, 1960).

Kallikrein was injected intradermally in amounts of 1 and 2 biological units. Two sheep were used. Each received six injections at each dose rate on each side.

Kallikrein caused marked leakage of dye. The blueing appeared after a latent period of about 4 minutes. There was a distinctly greater response to the larger dose in that a more intense blueing developed.

Mepyramine maleate was used to try to suppress the increased vascular permeability caused by kallikrein. It was injected intramuscularly in a single dose of 25 mg/kg body wt. 15 min. before intradermal injections of kallikrein. Two sheep were used. Each received three injections of one and two biological units on each side of the body.

Mepyramine pretreatment delayed the leakage of dye for about 15 min. Blueing then gradually increased from 15 min. on until at 30 min. the intensity of colour was similar to that produced in untreated animals.

(xiv) Effect of ribonucleic acid on vascular permeability:

The ability of the ribonucleic acids to increase vascular permeability has been reported recently in the rat (Willoughby and Walters, 1965): of

the four different types of RNA tested, the highly polymerised substance (RNA,HP) was found to be most active. In the present experiments different types of RNA were tested to see if they increased vascular permeability in the sheep.

RNA of five different types was injected intradermally in amounts of 100 µg and 500 µg. Two sheep were used. Each received one injection of 100 µg and 500 µg of each RNA type on each side.

In contrast to results obtained in the rat (Willoughby and Walters, 1965), and also compared with the effect of intradermal histamine, 5-HT and bradykinin in the sheep skin, the RNA of different types produced only a slight increase in vascular permeability in the skin, and then only with 500 µg doses. Blueing appeared after a latent period of 15-120 min. according to the RNA type. No significant change continued to occur after four hours.

Sodium RNA was completely without effect. With yeast, wheat-germ and pea-seed RNA blueing was first seen about 15 min. after the injection. Wheat-germ and pea-seed RNA appeared to be of the same order of effectiveness. Blueing induced by yeast RNA and the highly polymerised RNA, although still feeble, was slightly more intense than those produced by wheat-germ and pea-seed RNA.

(xv) Effect of hyaluronidase on vascular permeability:

There is some uncertainity about the significance of the action of hyaluronidase on vascular permeability. An effect has only been found at high dose rates.

Hyaluronidase was injected intradermally in 10 µg, 50 µg, 100 µg, 500 µg, 1 mg. and 2 mg. amounts. Two sheep were used. Each received two complete series of injections on each side. The observations of colour intensity were made 30 min. after injection of the hyaluronidase.

Hyaluronidase produced slight blueing at 500 µg and this increased with larger doses. No blueing occurred at doses less than 500 µg. The blueing appeared after a latent period of 5-7 min. in all cases.

Mepyramine maleate was used to try to suppress the permeability effect of hyaluronidase. It was injected intramuscularly in a single dose of 25 mg/kg. body wt. Hyaluronidase was injected intradermally in 500 µg, 1 mg. and 2 mg amounts. Two sheep were used. Each received three series of injections on each side. The recordings were made after 30 min. observation.

Mepyramine completely suppressed the increase in vascular permeability caused by the hyaluronidase. No blueing had developed 8 hr. after the hyaluronidase injection, when it was preceded by mepyramine administered systemically.

To investigate the spreading effect of hyaluronidase, non-blueing amounts, i.e., 10 µg, 50 µg and 100 µg were each injected with 1 µg and 10 µg of histamine, and the size of the blued area was measured after 30 min. This was compared with the blueing produced by 1 µg and 10 µg of histamine alone and the difference recorded. Two sheep were used. Each received two series of injections on each side as indicated in table 7 which summarises the results.

Table 7 - The spreading effect of hyaluronidase.

Size of the blue area in mm. (length x breadth)			
1 µg Histamine	1 µg Histamine + 10 µg Hyaluronidase	1 µg Histamine + 50 µg Hyaluronidase	1 µg Histamine + 100 µg Hyaluronidase
5 x 3	11 x 9	11 x 10	12 x 10
10 µg Histamine	10 µg Histamine + 10 µg Hyaluronidase	10 µg Histamine + 50 µg Hyaluronidase	10 µg Histamine + 100 µg Hyaluronidase
17 x 15	25 x 20	25 x 20	25 x 22

The same dose of histamine caused a greater area of blueing when injected with hyaluronidase than when injected alone. It was further noticed that an increase in the dose of hyaluronidase did not cause a corresponding increase in the extent of blueing.

(xvi) Effect of lactic acid on vascular permeability:

Miles (1961) suggested that the accumulation of granulocytes in tissues might increase vascular permeability indirectly by producing lactic acid. Since lactic acid has been shown to cause vasodilatation in guinea pig skin (Miles, 1961), its effect on vascular permeability in the sheep skin was tested.

A 4%, 2% and 1% solution of lactic acid (v/v) was prepared in saline and injected intradermally in volumes of 0.1 ml; the pH for each strength being 1.88, 1.90 and 1.97 respectively, as checked on a Beckman pH meter. One sheep was used. It received six injections of 4% solution on the left side and another six injections of 2% and 1% solution on the right side.

In all strengths used 0.1 ml. of lactic acid injected intradermally induced leakage of dye that appeared within 1-3 min. after the injections. There also appeared a small swelling at sites of all the injections. Maximal effects were observed with the 4% solution: 2% and 1% solution appeared to be of the same order of effectiveness.

Mepyramine maleate was used to try to suppress the increased vascular permeability caused by lactic acid. Mepyramine was injected intramuscularly in a single dose of 25 mg/kg 15 min. before the intradermal injections of lactic acid, in the percentages referred to above. One sheep was used. It received three injections of each solution of lactic acid.

It was found that in all sites mepyramine failed to suppress the leakage of dye.

(xvii) Effect of Strophanthin-G on the increased vascular permeability induced by turpentine, histamine, 5-HT and bradykinin:

Following the report by Judah et al, (1965) of the similarity of action between the antihistamine promethazine and the cardiac glycoside strophanthin-G, the latter was used as a possible suppressor of the early inflammatory changes in the sheep induced by turpentine.

Strophanthin-G (Ouabaine) was injected intravenously into three sheep in a single dose of 100 µg/kg body weight, 10 min. before the intradermal injection of the following substances: turpentine, 0.1 ml; histamine, 5-HT and bradykinin in 10 µg amounts. Each sheep received two complete series of intradermal injections on each side.

Systemic strophanthin-G delayed the increase in vascular permeability normally caused by turpentine, histamine, 5-HT and bradykinin. The suppression of increased vascular permeability caused by turpentine, histamine and 5-HT lasted for nearly an hour, after which time leakage of dye took place at the site of turpentine injection and to a lesser extent at the sites of histamine and 5-HT injections. Permeability response to bradykinin was delayed for only about 10 min., after which time blueing occurred. In all places where blueing was suppressed, an area of reddening was seen.

(xviii) Effect of Sodium Salicylate on the increased vascular permeability induced by turpentine, histamine, 5-HT and bradykinin:

A 40% solution of sodium salicylate was injected intraperitoneally into two sheep in a dose of 0.5 g/kg and into two others at 0.6 g/kg body weight. 30 min. later each sheep was injected intradermally with 0.1 ml. turpentine and 10 µg of histamine, 5-HT and bradykinin. Each sheep received two series of these injections. The increase in vascular permeability indicated by the leakage of dye was recorded for up to one hour after

the intradermal injections were given. The same four sheep were also injected intradermally with 0.1 ml. of turpentine-induced pleural exudate, the response to which was suppressed by salicylate. This is described later in section 3.

Salicylate failed to suppress completely the leakage of dye induced by turpentine, histamine, 5-HT and bradykinin. Blueing with turpentine and 5-HT and to some extent with histamine and bradykinin, though not suppressed, was delayed for 7-10 min. Thereafter blueing appeared gradually, reaching a peak by one hour which was only about half or one-third as intense as that obtained in untreated sheep.

(xix) Effect of betamethasone ("Betsolan", Glaxo Ltd.) on the increased vascular permeability induced by turpentine, histamine, 5-HT and bradykinin:

Betamethasone is a recently synthesised cortisone derivative and is approximately five to eight times more potent than prednisolone as an anti-inflammatory agent (see "Betsolan", Glaxo literature).

Betamethasone was injected intramuscularly in a single dose of 2 mg/kg 15 min. before the intradermal injection of 0.1 ml. turpentine, 10 µg of histamine, 5-HT or bradykinin and 0.1 ml. of turpentine-induced pleural exudate. Two sheep were used. Each received two complete series of intradermal injections on each side. The leakage of dye was recorded after 30 min. observation.

Betamethasone failed to suppress the leakage of dye induced by turpentine, histamine, 5-HT and bradykinin; the response to turpentine pleural exudate was suppressed; this is described later in section 3.

(xx) Effect of alloxan on the increased vascular permeability induced by turpentine:

In the rat alloxan prevents the release of histamine from mast cells,

and suppresses the early increase in vascular permeability which follows the intrapleural injection of turpentine (Spector et al, 1963).

Four sheep were used. A 10 per cent solution of alloxan monohydrate in 0.85 per cent saline was employed. Four different doses were tried. First, it was given intraperitoneally, one hour before intradermal injection of 0.1 ml. of turpentine at a dose of 200 mg/kg body wt., following its use in the rat (Spector et al, 1963). Next, it was given intraperitoneally at 600 mg/kg body wt. It was also injected intravenously at 200 mg/kg and 600 mg/kg body wt. 15 min. before the intradermal turpentine. The same four sheep were also injected intrapleurally with 0.5 ml. of turpentine, before alloxan administration.

Alloxan (200 or 600 mg/kg) failed to suppress the reaction to the intradermal injection of 0.1 ml. turpentine and the pleural exudation induced by turpentine. This is described later in Section 4.

(xxi) Effect of indomethacin ("Indocid", Merck Sharp and Dohme) on the increased vascular permeability induced by turpentine, histamine, 5-HT, bradykinin and thermal injury.

Indomethacin is an anti-inflammatory agent and has been found particularly effective in man.

A 20% suspension made up in distilled water was used. It was given intraperitoneally in a dose of 60 mg/kg body weight 30 min. before the intradermal injection of test substances, and the production of thermal injury. Two sheep were used. Turpentine was injected intradermally in 0.1 volumes. Histamine, 5-HT and bradykinin were injected in 10 µg amounts. Each sheep received two complete series of intradermal injections on each side. Thermal injury was induced at two sites on each side of the sheep.

Indomethacin failed to suppress the leakage of dye normally caused by

turpentine, histamine, 5-HT, bradykinin and thermal injury.

(xxii) Effect of promethazine ("Phenergan", May & Baker Ltd.) on the increased vascular permeability induced by turpentine, histamine 5-HT, bradykinin and thermal injury:

Promethazine hydrochloride is an antihistaminic.

Two sheep were injected intramuscularly with 25 mg/kg of promethazine hydrochloride 15 min. before the intradermal injection of test substances and the infliction of thermal injury. Turpentine was injected in 0.1 ml. volumes. 5-HT and bradykinin were injected in 10 µg amounts. Histamine was injected in 10 µg and 100 µg amounts. Each sheep received two complete series of intradermal injections on each side. Thermal injury was induced at two sites on each side of the sheep.

Promethazine hydrochloride suppressed the leakage of dye for about one hour after intradermal turpentine; for about 10 min, after intradermal bradykinin, and for about 8-15 min. after thermal injury. No blueing developed at places where histamine and 5-HT had been injected in 10 µg amounts. However, in contrast to results obtained with mepyramine maleate blueing was not completely suppressed after the intradermal injection of histamine in larger doses, i.e., in 100 µg amounts. It was only delayed for about 10 min.

(xxiii) Effect of isocarboxazid ("Marplan", Roche Products Ltd.) on the increased vascular permeability caused by turpentine, histamine, 5-HT, bradykinin and thermal injury:

The effect of isocarboxazid, an inhibitor of monoamine oxidase (MAO) was tested in the sheep. In the rat, MAO inhibitor was found to suppress increased vascular permeability after chemical and thermal injury (Spector and Willoughby, 1960b,c).

Two sheep were used. The first was injected intraperitoneally with 150 mg/kg and the second with 200 mg/kg of a 2.5% suspension of isocarboxazid 30 min. before the intradermal injection of test substances and the infliction of thermal injury. Turpentine was injected intradermally in 0.1 ml volumes. Histamine, 5-HT and bradykinin were injected in 10 µg amounts. Each sheep received two complete series of intradermal injections and thermal injury on each side. The results were recorded up to one hour.

Neither dose of isocarboxazid suppressed the increased vascular permeability caused by turpentine, histamine, 5-HT or bradykinin. However, both doses reduced the leakage of dye following thermal injury. The intensity of leakage of dye after turpentine injection was slightly diminished.

Section 2

Histamine release in cutaneous antigen-antibody reactions in the sheep:

The earlier results indicate that histamine is a mediator of the early vascular changes in inflammation in the sheep. Histamine appears to be released by many agents (viz., turpentine, thermal injury, 5-HT, mepyramine maleate; also by turpentine-induced pleural exudate and lymph node cell extract (described later) which suggests that histamine release in the sheep skin is easily initiated. The following experiments were carried out to see if histamine is released in the antigen-antibody reactions in the sheep skin. It was proposed to produce the Arthus reaction and passive cutaneous anaphylaxis (PCA).

1. Active sensitisation (Active Arthus Reaction):

Four lambs were used in the experiment. The antigens used were: horse serum (HS) and bovine gamma globulin BGG; Hyland Laboratories). Two lambs were given six sensitising injections of 0.1 ml. of horse serum intradermally on each side. The other two lambs were similarly injected with BGG in 5 mg. amounts, contained in 0.2 ml. saline. The above injections were repeated daily. From the fourth day onward in the case of HS and from the sixth day onward with BGG, the injection sites started showing cutaneous reaction indicated by swelling within a few minutes of the injection of antigen. Blood samples were taken on the 9th day from all the lambs and the sera were later tested for precipitating antibody by double diffusion in agar gel.

After the collection of blood samples the lambs were blued. No blueing occurred at the injection sites indicating that the vascular permeability was normal. In each animal at all sites on the right side the homologous antigen was injected. Blueing appeared within 1-4 min. at all sites and

became most marked by 30 min. It was found that a similar marked leakage of dye occurred when antigen was injected at untreated sites. Leakage of dye was more marked in the case of HS than with BGG. The blueing obtained at sites not previously injected with the antigens suggested that the reaction might be due to precipitating circulating antibodies.

After two hours' observation, the lambs were injected with mepyramine maleate intramuscularly (25 mg/kg) and 15 min. later again injected the homologous antigen at the six sites of previous injections on the left side and also at untreated sites. Blueing was temporarily suppressed; leakage of dye did not occur until after one hour and was most marked at 2 hr.

Agar diffusion tests using sera from all four sheep against the homologous injected antigen failed to give precipitation lines, despite the indication that circulating precipitating antibodies might be involved in the cutaneous antigen reaction.

2. Reversed Passive Arthus Reaction:

The Arthus reaction depends on the circulating precipitating type of antibody and the ability to respond to the antigen is lost when this is removed (Benaceraff and Kabat, 1950; see Cochrane, 1965). The negative results of the gel diffusion test did not confirm the precipitating nature of the antibodies, and therefore whether or not the cutaneous antigen-antibody reaction described above was of the Arthus type, remained unknown.

The possibility of producing a Reversed Passive Arthus Reaction was then considered to test the efficacy of mepyramine maleate in its role of suppressing the increased vascular permeability that follows the antigen-antibody reaction in the skin. Arthus reaction was produced by the reversed passive method described by Ovary and Bier (1952) and used by Brocklehurst

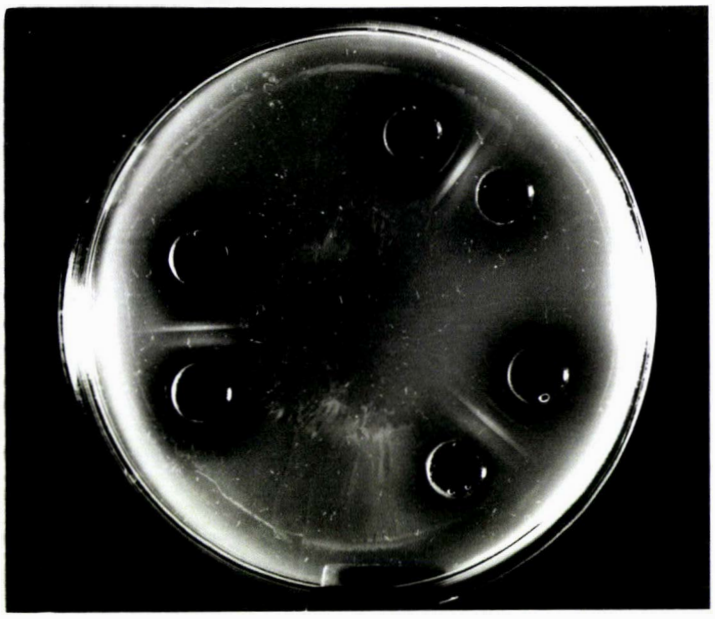


Fig.29 A double diffusion test in agar gel showing precipitation lines between adjacent wells, one containing horse serum and the other anti-horse serum obtained from rabbit.

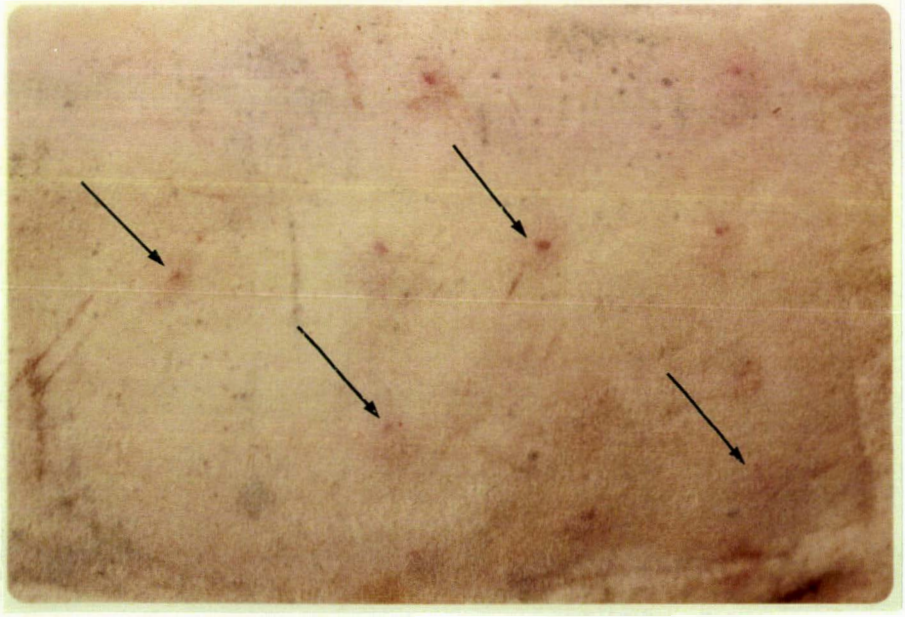


Fig.30 Reversed Passive Arthus Reaction in the skin of a sensitised sheep. Photograph taken one hour after mepyramine maleate-pretreatment (25 mg./kg. given intramuscularly). Note complete suppression of dye-leakage at sites of intradermal injection of the antibody (i.e., anti-horse serum obtained from rabbit), but presence of a local "flare" (arrows).

et al (1955). It was thought possible from the negative gel diffusion results that, as in guinea-pig (Humphrey, 1955b), sheep sera may not contain sufficient precipitating antibody to give readily measurable macroscopic lesions in RPA-reaction, and consequently rabbit antisera were used. The antigen used was horse serum (HS). Rabbit anti-horse serum was produced in two different ways.

(i) Two rabbits were used. Each was injected with 0.4 ml. of HS intravenously. On the 8th day each rabbit received 0.4 ml. of HS intraperitoneally and on the 9th day 0.2 ml. intravenously. Blood samples were taken on day sixteen and the antisera were tested for precipitating antibodies by double diffusion in agar gel. This procedure led to the production of precipitating antibody, the gel diffusion test giving clear precipitation lines (fig. 29).

(ii) Two rabbits were used. On day one each was injected intramuscularly with 0.4 ml. of HS emulsified in an equal volume of complete Freund's adjuvant (Difco). Three weeks later the animals were injected intravenously with 0.2 ml. of HS emulsified in an equal volume of a 2% solution of aluminium hydroxide gel diluted 1 in 10 with saline. Eight days later blood samples were taken and the antisera were tested for precipitating antibodies by double diffusion in agar gel. The test gave distinct precipitation lines.

Two lambs (20 kg. body weight) were used to produce RPA-reaction. Rabbit anti-HS serum was injected intradermally in volumes of 0.1 ml. at twelve sites in each lamb, all on left side. Immediately thereafter excess of antigen (40 ml. of HS) was given intravenously and the lambs were blued. Results were recorded.

To test the effect of antihistamine both the lambs were injected two hours later with mepyramine maleate intramuscularly (25 mg/kg) and 15 min.

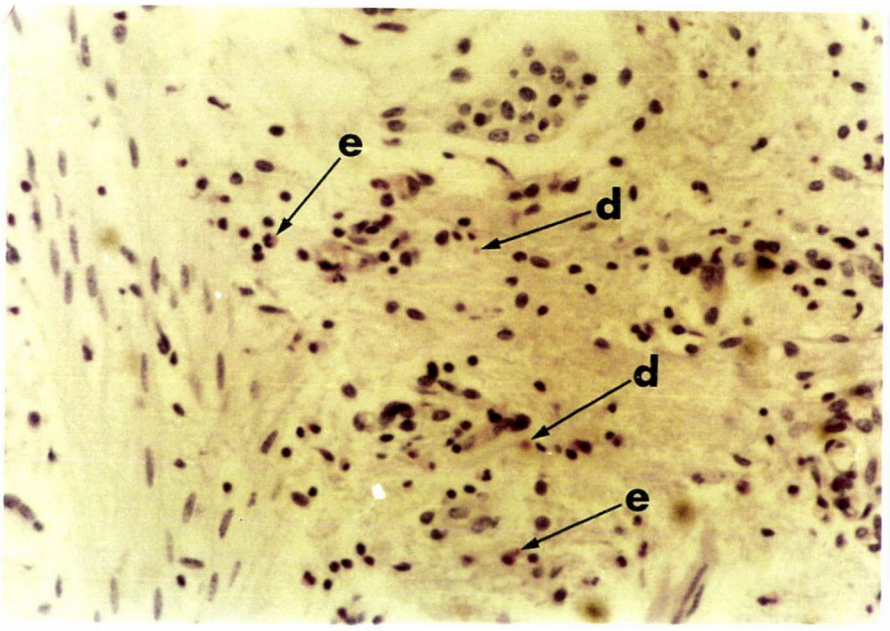


Fig.31 Section of sheep skin 30 min. after the production of Reversed Passive Arthus Reaction (RPA) showing diffuse infiltration of tissues with mononuclear cells and eosinophiles (e). Degranulation of eosinophiles is also seen (d).

H.E. x 250

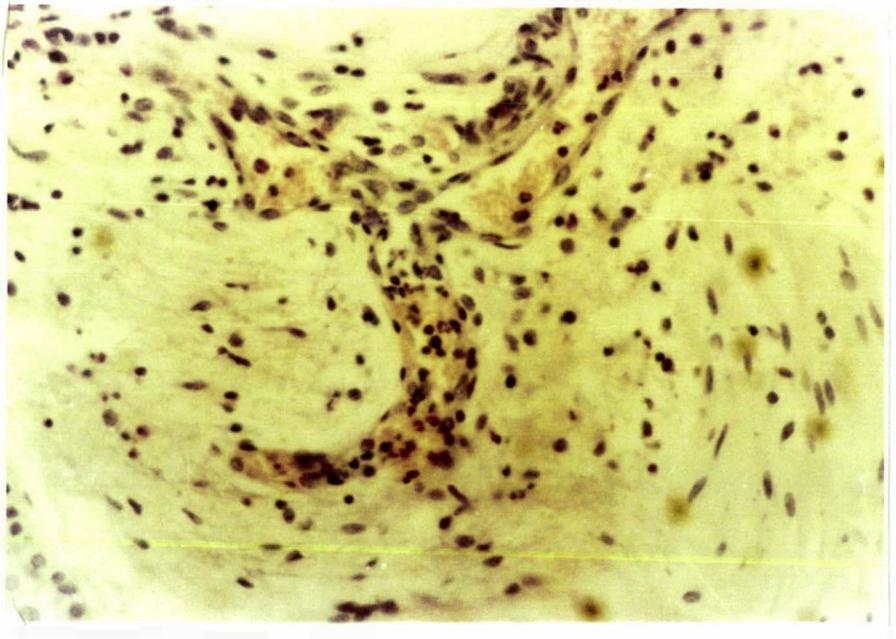


Fig.32 Section of sheep skin 3 hr. after the production of RPA-reaction showing margination of polymorphs in hyperaemic blood vessels. Many eosinophiles are also seen in the perivascular tissues.

H.E. x 250

after this treatment, antibody was injected intradermally at twelve sites on the right side.

Normal horse and rabbit serum did not cause leakage of dye by themselves as was previously shown by injecting them in another blued sheep in 0.1 ml. volumes. The intradermal injection of antibody in the sensitized sheep before mepyramine treatment was followed by leakage of dye within 1-3 min. The size of the blue areas varied from 10 to 15 mm. in diameter at the end of one hour. After mepyramine-pretreatment, the leakage of dye was completely suppressed following the intradermal injections of antibody. No blueing developed even after twelve hrs, and the sites of injections showed reddening (flare) instead(Fig.30).

3. Histopathological changes in the RPA-Reaction:

RPA reaction was produced by injecting antibody intradermally in 0.2 ml. volumes in a lamb previously sensitized with horse serum. Sixteen injections were made, eight on either side. Skin biopsies were taken, one from each side at 15 min., 30 min., 1 hr., 3 hr., 6 hr., 8 hr., 24 hr., and 72 hr. after the injections. Skin samples were fixed, sectioned and stained H & E and for eosinophiles.

The following results were obtained at the given time intervals.

15 min: Sections revealed vascular dilatation and hyperaemia. There was slight infiltration of mononuclear cells, mostly lymphocytes. A few eosinophiles were seen.

30 min: Congestion of blood vessels and emigration of mononuclear cells had slightly increased. Eosinophiles had significantly increased; many showed degranulation (fig. 31).

1 hr: Further increases in the above changes were seen, mainly in the tissues close to the base of the epidermis.

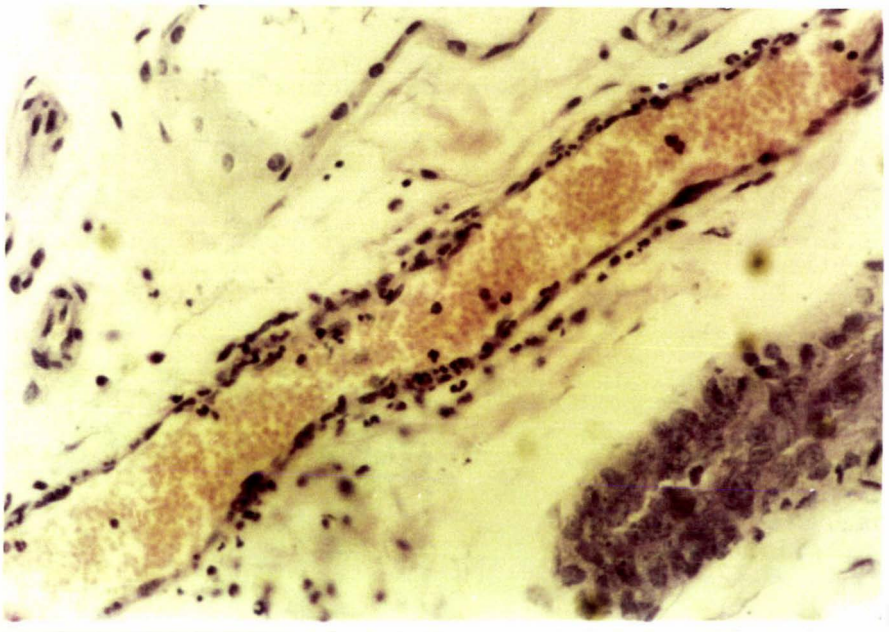


Fig.33 Section of sheep skin 6 hr. after RPA-reaction showing an early stage in the accumulation of neutrophiles in the wall of a small vein.

H.E. x 250

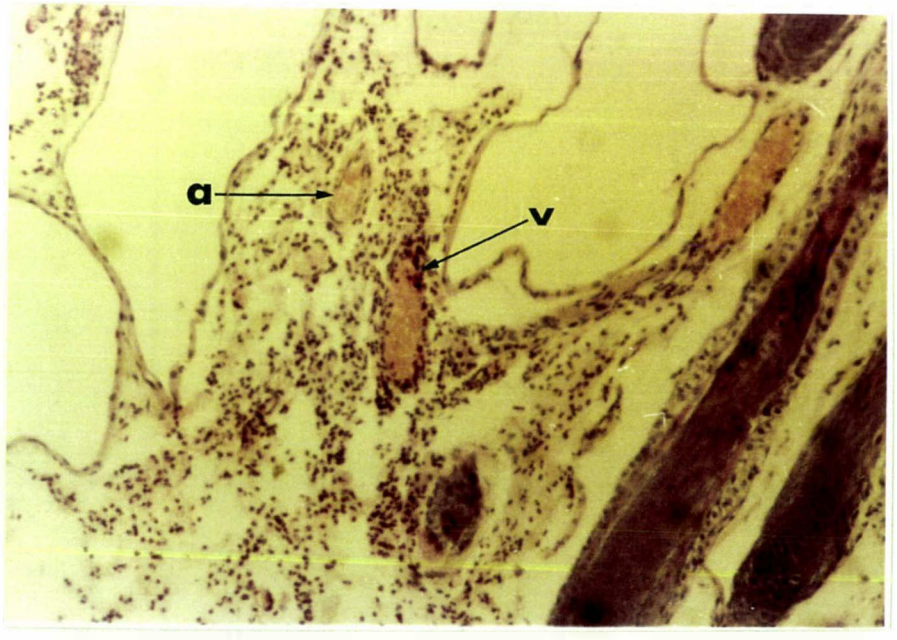


Fig.34 Section of sheep skin 8 hr. after RPA-reaction showing infiltration of tissues with neutrophiles. A small vein (v) in the centre has an accumulation of neutrophiles in its wall, but a small artery (a) above it is free from such accumulation.

H.E. x 100

3 hr: This stage was characterized by a change in the type of emigrating cells. The hyperaemic vessels showed margination of polymorphs (fig. 32). Only a few mononuclear cells were seen and were replaced by the polymorphonuclear neutrophiles in the tissues. Eosinophiles were seen in significant numbers as noted above, and showed degranulation. The changes now also involved the deeper tissues.

6 hr: The polymorphonuclear neutrophilic emigration had slightly increased. The stage was characterized by the beginning of accumulation of neutrophiles in the walls of small veins and venules (fig. 33).

8 hr: At this stage the first large-scale emigration of neutrophiles had occurred, considerably more than at the earlier stages (fig. 34). Accumulation of neutrophiles in the vessel walls was most marked at this stage (fig. 35) and was seen in small veins and venules only; arteries and arterioles were not involved (fig. 34).

24 hr: Neutrophilic emigration and infiltration of the tissues was maximal at this stage. The accumulation of neutrophiles in the vascular walls, though marked, was less than at the preceding stage and more cells were in the tissues surrounding the vessels than in the vessel walls. Large numbers of neutrophiles were in the lumina of the vessels. Eosinophiles were still present in the tissues but showed no increase in number.

48 hr: There was marked reduction in the cellular infiltration of the tissues. Most of the neutrophiles had disappeared and the cytological picture was once again predominently mononuclear, mainly lymphocytic. Blood vessels still revealed small numbers of neutrophiles in the walls. Eosinophiles were seen in significant numbers in the tissues.

72 hr: The above changes had largely subsided; skin had returned almost to normal at this stage. Blood vessels did not show any significant changes. In places there were slight infiltrations of lymphocytes. Eosinophiles

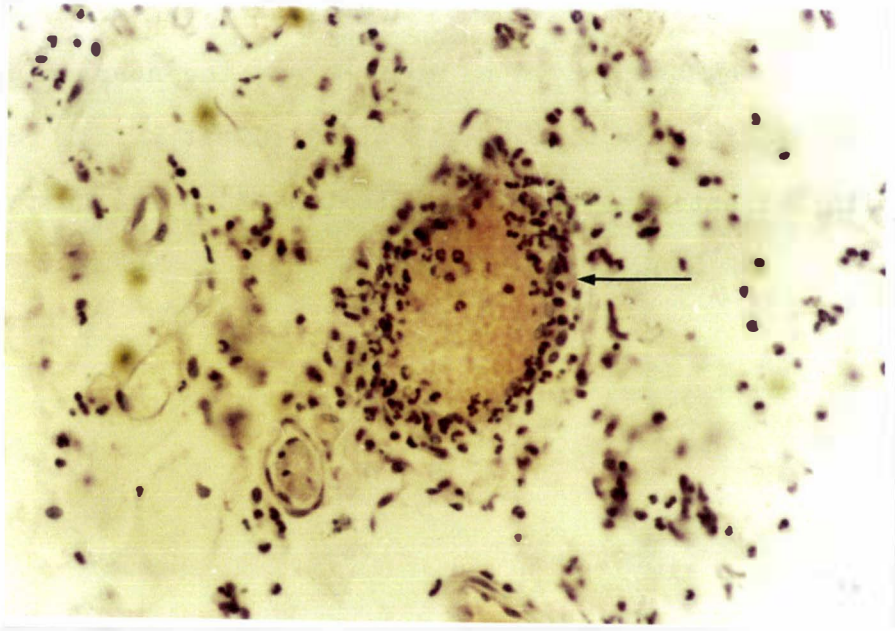


Fig.35 Same as fig. 34, another area showing marked accumulation of neutrophiles in the wall of a small vein (arrow).

H.E. x 250

were seen scattered among the lymphocytes.

4. Passive Cutaneous Anaphylaxis (PCA):

The Arthus reaction is dependent upon the continued presence of circulating precipitating antibody (Benacerraf and Kabat, 1950; see Cochrane, 1965). In the case of cutaneous anaphylaxis, however, the precipitating antibody may be removed by antigen and the nonprecipitating circulating or tissue 'fixed' antibody will still elicit a full reaction upon challenge with the antigen (Ovary and Bier, 1953). The test is based on the fact that this nonprecipitating antibody shows a marked tendency to become bound to cells. In order to elicit the PCA reaction a suitable interval, termed 'latent period' is required between the injection of antibody into the skin and the administration of antigen intravenously (Ovary, 1958). During the latent period the antibody attaches to the tissue cells in such a way that its subsequent interaction with antigen will damage the tissue. Tissue in this state is termed 'sensitised' (see Cochrane, 1965). The following experiment was carried out to study if histamine was released in the PCA-reaction in the sheep.

The PCA-reaction was produced by the 'reversed' technique described by Ovary and Bier (1952) and used by Brocklehurst et al, (1955). The same antigen-antibody system as described for the RPA-Reaction was used. Antibody was injected intradermally in 0.1 ml. volumes. Ten injections were made on each side in one lamb (20 kg. body weight). 4 hr. later excess antigen (40 ml. of HS) was given intravenously, and immediately thereafter the lamb was blued. Results were recorded.

No leakage of dye occurred at the sites of previous intradermal injections of antibody.

5. Histopathological changes following intradermal injection of immune complexes:

This experiment was carried out to see if immune complexes exerted any eosinophilotactic effect in the sheep. Two approaches were attempted. Firstly, preformed insoluble antigen-antibody complexes were injected intradermally. Secondly, superimposed injections of both antibody and antigen were given intradermally in an endeavour to induce the formation of antigen-antibody complexes in vivo, i.e., Local Passive Arthus Reaction (LPAR) (Fischel and Kabat, 1947). BGG and anti-BGG, and also horse serum and anti-horse serum were used as the antigen-antibody systems. Both antisera were prepared in rabbits. The preparation of anti-BGG antiserum followed the same schedule as that described for preparing anti-horse serum. Both antisera were shown to contain precipitating antibodies by double diffusion in agar gel.

The insoluble antigen-antibody complexes were prepared by tube precipitation in liquid medium. The precipitates were washed twice with cold ($4^{o}C$) 0.85% saline solution. For each system 0.1 ml. volumes of complex were injected intradermally at four sites in a lamb. At four other sites 0.2 ml. of anti-horse serum followed by 0.2 ml. of horse serum was injected intradermally to induce LPAR. Two skin biopsies were obtained from the sites of injection of each antigen-antibody complex and from the LPAR site 15 min. and 6 hr. following intradermal injections. Skin samples were fixed, sectioned and stained with H & E and for eosinophiles.

Section taken 15 min. after injection of preformed immune complex and induction of LPAR revealed vascular dilatation and hyperaemia. There was slight infiltration of mononuclear cells, mostly lymphocytes. In the case of LPAR, hyperaemic vessels showed margination of polymorphs. Eosinophiles were not seen in significant numbers.

After 6 hr. there was large-scale emigration of neutrophiles which was most marked in the case of LPAR and least with the BGG immune complexes. In all cases only an occasional blood vessel showed slight accumulation of neutrophiles actually in their walls. Eosinophiles in significant numbers were not seen.

Section 3

Studies on inflammation - using turpentine-induced pleurisy in the sheep as a test system.

Studies on the pleural exudates:

A criterion to be met before accepting that a substance is a mediator in inflammation is that the substance should be demonstrably present during the inflammatory reaction.

Spector (1956) suggested that if blood vessels whose permeability had been altered were to be in close contact with their exudate, then active compounds (i.e., chemical mediators of inflammation) should diffuse into this exudate and be recoverable from it, except where they were rapidly destroyed or inactivated. He gave turpentine-induced pleurisy as a suitable example of inflamed blood vessels lying in close contact with their exudate and demonstrated that in the rat this test system gave reproducible results in providing adequate exudate for examination at various times (in hours) after turpentine injection. In the present experiments, pleurisy has been produced in the sheep by the intrapleural injection of turpentine as described previously. Samples of pleural exudate were obtained at different times and their permeability-increasing properties were studied. Pleural exudate was collected 30 min., 2 hr., 3 hr., 6 hr., and 24 hr. after turpentine injection from a total of five sheep, each killed at the appropriate interval after the intrapleural injection of turpentine. The effect of undiluted and diluted pleural exudate on vascular permeability was examined. Results were compared with the reaction produced by intradermal injection of undiluted and diluted normal serum to investigate the globulin permeability factor.

Two 'blued' sheep received two intradermal injections of samples of undiluted exudate taken at each of the intervals after injection mentioned

above. They also received paired injections of undiluted serum from a normal sheep. Five 'blued' sheep were used to test the effect of saline dilutions of the exudates. Each sheep was given, on each side, a double series of injections starting with undiluted exudate taken at a given interval after turpentine injection, and with dilutions of this exudate as indicated in table 8. One 'blued' sheep was injected in the same way with undiluted and serially diluted serum from a normal sheep.

The inflammatory pleural exudate induced by turpentine was pale yellow in colour; in a few cases it was slightly red presumably due to haemolysed erythrocytes. The presence or absence of such haemolysis did not influence the properties of the exudate. Fibrin deposition was marked and this led to a fibrinous coating of the pleura and some adhesion between the visceral and parietal pleura seen at 24 hr. There were few leucocytes in exudates obtained in the early stages but large numbers were present in the 24 hr. old exudate. They were mostly neutrophiles.

The exudate obtained at all the intervals after intrapleural injection of turpentine, coagulated soon after its collection. Evidence of intrapleural clotting, was present in some cases in that flakes of fibrin were present in the exudate as it was being collected. The clotted fibrin was removed and the remaining fluid was used to test its effects on intradermal injection. This fluid had approximately the same activity in increasing vascular permeability as the uncoagulated exudate when tested in a 'blued' sheep. The term 'exudate' used hereafter, therefore, refers to an exudate from which the fibrin has been removed. Attempts to prevent coagulation of the exudate with the anticoagulants, ammonium and potassium oxalate, EDTA, trisodium citrate and heparin were not successful.

The 2 hr., 3 hr., and 6 hr. exudates were alkaline (pH 8.2 - 8.5) and the alkalinity was increased at 24 hr.; (pH 8.5 to 8.8).

Permeability-increasing activity of the inflammatory exudates:

Effect on vascular permeability of undiluted pleural exudates:

Exudates were injected intradermally in 0.1 ml. amounts. Blueing appeared after a latent period of 1-4 minutes. Table 8 records the effect on vascular permeability of undiluted exudates collected from 30 min. to 24 hr. after intrapleural injection of turpentine.

Table 8 - The effect of diluted and undiluted pleural exudates and sheep serum on vascular permeability.

Material	Leakage of dye (colour intensity)						
	Undiluted	Diluted (with 0.85 per cent saline) to					
		1 in 10	1 in 25	1 in 50	1 in 100	1 in 200	1 in 400
30 min. Exudate	++	+	+	0	0	0	0
2 hr. Exudate	++	++	++	+	+	0	0
3 hr. Exudate	+++	+++	++	++	++	+	0
6 hr. Exudate	++++	+++	++	++	++	+	+
24 hr. Exudate	0	0	0	0	+++	++	++
Serum	0	+	+	++	+++	++	++

30 min., 2 hr., and 3 hr., exudates caused marked leakage of dye on intradermal injection and with 6 hr. exudate this activity was maximal. Exudates collected at 24 hr. were inactive. The subsequent experiments were carried out on 6 hr. and 24 hr. exudate since these showed maximal and minimal activity respectively. The activity of the exudate was examined to see whether histamine or globulin permeability factor were involved as mediators. Histamine was sought by testing the effect of the inflammatory exudates on isolated guinea-pig ileum. Both 6 hr. and 24 hr. exudates were found inactive in that they failed to induce contractions of the guinea-pig ileum. It was found later with studies on the lymph-node-permeability-factor that as little as 0.002 µg of histamine in a 5 ml. organ bath induced a significant contraction of the guinea-pig ileum in sensitive preparations.

Table 8 shows the effect on vascular permeability of diluted exudates collected from 30 min. to 24 hr. after intrapleural injection of turpentine. Undiluted 24 hr. exudate had no effect on vascular permeability. When 0.1 ml. volumes of 1:10, 1:25 and 1:50 dilutions were injected intradermally into 'blued' sheep, they failed to induce leakage of dye. However, intradermal injections of 1:100, 1:200 and 1:400 dilutions caused well-marked leakage of dye. Blueing appeared gradually at the end of an hour and became most marked by $1\frac{1}{2}$ hr. As can be seen from the table, maximum activity resided in the 1:100 dilution. A similar study with 30 min., 2 hr., 3 hr., and 6 hr. exudates showed that their activity decreased with dilution (table 8).

To see if the permeability factor in the 24 hr. exudate that was activated by dilution was of a large molecular size, the active 1:100, 1:200 and 1:400 dilutions of 24 hr. exudate were dialysed as described previously. Undiluted 24 hr. and undiluted 6 hr. exudate were also dialysed. Dialysis would remove small molecular substances like histamine and 5-HT

and prevent the escape of larger molecular substances like proteins. The results showed that dialysis did not affect the activity of dilutions of the 24 hr. exudates in increasing vascular permeability. Similar results were obtained when the undiluted 24 hr. exudate was first dialysed and then diluted. Dialysis did not alter the activity of the undiluted 6 hr. exudate.

Storage at room temperature of 1:100 dilution of 24 hr. pleural exudate for an hour did not affect its activity: nor did the degree of heating which inactivates complement, i.e., 30 min. at 56°C.

Undiluted sheep serum gave a feeble blueing response. On dilution with 0.85 per cent saline, the activity increased and reached a maximum at a dilution of 1:100 (table 8). As with diluted 24 hr. exudate, the blueing appeared gradually at the end of an hour and became most marked by 1½ hr. Unlike the 24 hr. exudate, serum dilutions of 1:10, 1:25 and 1:50 also showed some activity. Dialysis of the 1:100 serum dilution and also keeping it at room temperature for an hour did not affect its activity in increasing vascular permeability. The same results were obtained in sheep tested with its own diluted serum.

The lack of activity of undiluted 6 hr. pleural exudate on guinea-pig ileum indicated that it did not contain histamine. To see whether its effect on vascular permeability was due to release of histamine <u>in vivo</u>, mepyramine maleate was used as a means of suppressing possible histamine effects. The effect was tested on the increased vascular permeability induced by undiluted 6 hr. pleural exudate, and both diluted 24 hr. exudate and serum (each reduced to a concentration of 1:100 in saline). Three sheep were used. One sheep was used for each test substance and each received nine injections on each side. Mepyramine maleate was injected intramuscularly in a single dose of 25 mg/kg body wt. 15 min. before injections of test substances.

Undiluted 6 hr. exudate was first dialysed and tested on guinea-pig ileum to confirm that it was free from histamine - this was found to be the case. When 0.1 ml. volumes of undiluted dialysed 6 hr. exudate were injected intradermally into 'blued' sheep pre-treated with mepyramine, the leakage of dye was completely suppressed. Mepyramine maleate-pre-treatment suppressed the blueing for about 2 to 3 hr. in the case of 6 hr. exudate, and delayed it by about 1½ hr. in the case of activated, i.e., diluted 24 hr. exudate and serum. In all cases blueing again appeared after 2 - 3 hr.

Earlier, in connection with skin tests described under section one, reference was made to the effect of sodium salicylate and betamethasone ('Betsolan', Glaxo Ltd.) on the increased vascular permeability caused by intradermal injection of pleural exudate. It was found that the leakage of dye normally induced by the undiluted 6 hr. pleural exudate and both diluted 24 hr. exudate and sheep serum (each reduced to a concentration of 1:100 in saline), remained suppressed after the prior administration of sodium salicylate (0.5 g/kg.) given intraperitoneally 30 min. before, and betamethasone (2 mg/kg.) given intramuscularly 15 min. before, the intradermal injections of test substances. No leakage of dye was seen in 8 hr. of observation.

The suppression by mepyramine maleate of blueing induced by 6 hr. pleural exudate for up to 2 - 3 hr., suggested that this early phase might be mediated by the release of histamine <u>in vivo</u>. To test this possibility further the histamine-releasing effect of undiluted 6 hr. exudate was examined <u>in vitro</u>.

<u>In vitro study of the histamine-releasing activity of 6 hr. pleural exudate using chopped guinea-pig and sheep lung</u>:

Histamine was assayed on guinea-pig ileum according to procedures described previously. The study on the pleural exudate was carried out

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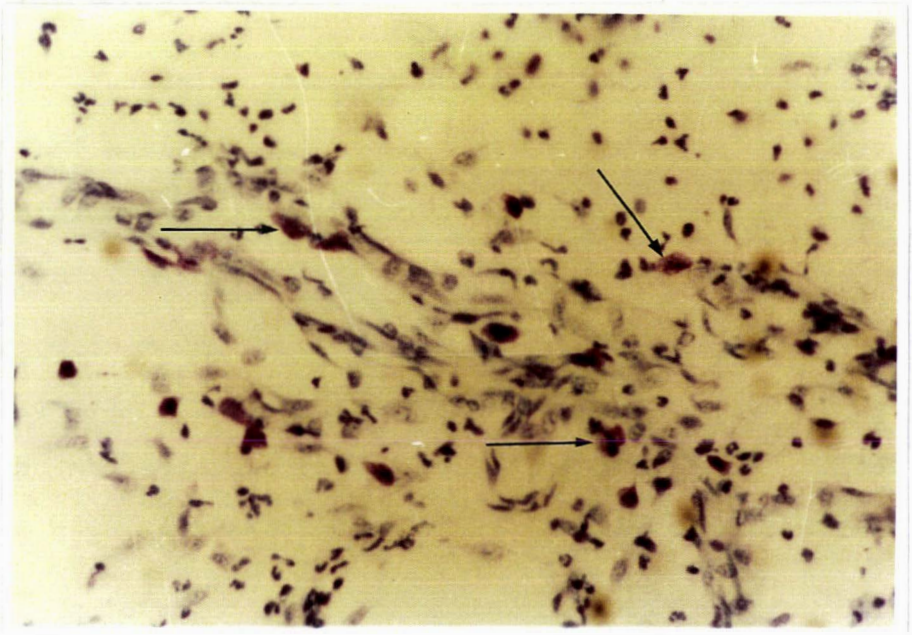


Fig.36 Section of sheep skin 15 min. after intradermal injection of 6 hr. turpentine-induced pleural exudate (0.1 ml.) showing more numerous mast cells (arrows) than in normal skin. Varying degrees of swelling of the mast cells is also seen.

Leishman stain x 250

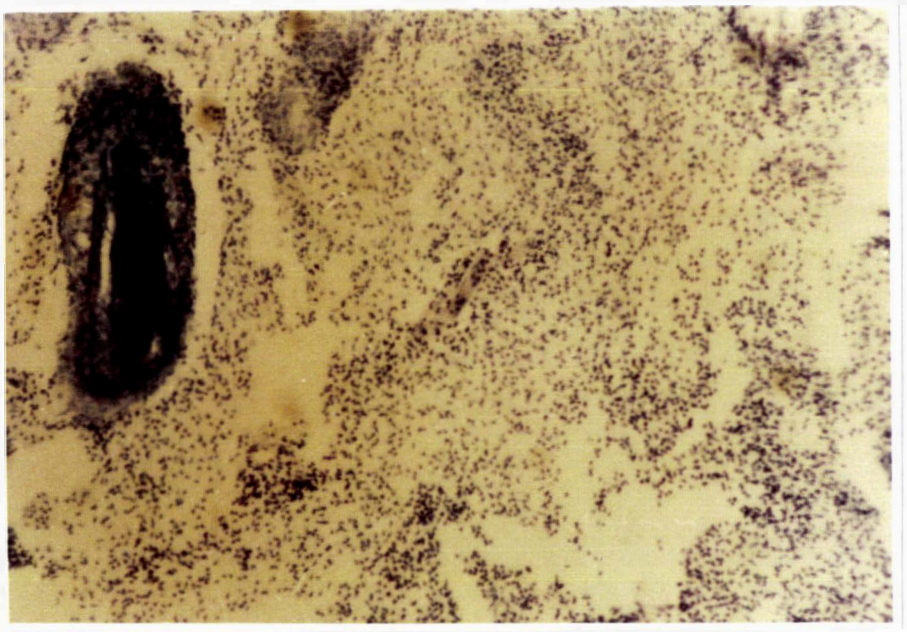


Fig.37 Section of sheep skin 6 hr. after intradermal injection of 6 hr. turpentine-induced pleural exudate (0.1 ml.) showing extensive neutrophilic infiltration of the tissues.

Leishman stain x 100

together with a similar study on the lymph node cell extracts. In all, four guinea-pig and four sheep lungs were used. It was found that undiluted dialysed 6 hr. pleural exudate induced some histamine release from chopped guinea pig-lungs, but the effect was not marked. In only one case in experiments with sheep lungs was there any release of histamine produced by the exudate. There was a marked difference in the height of the ileum contractions between that induced by the exudate-treated lung and the corresponding control samples treated with Krebs' Ringer solution.

Histopathological changes following intradermal injections of pleural exudate:

0.1 ml. volumes of undiluted 6 hr. pleural exudate were injected intradermally into the dorsal skin of a lamb. Skin biopsies were taken 15 min., 30 min., 1 hr., 2 hr., 4 hr., 6 hr., and 24 hr. following the injections. Skin samples were fixed, sectioned and stained H & E and for mast cells. The results are summarised as follows:-

15 min: Sections revealed vascular dilatation and engorgement. Hyperaemic vessels showed margination of polymorphs. No leucocytic emigration was seen. Mast cells appeared more numerous than in normal skin (fig. 36).

30 min: Margination of the polymorphs was more marked, but still the cells were confined to blood vessels. Mast cells appeared to be even more numerous. Occasionally, a basophil leucocyte could be seen inside a blood vessel.

1 hr: Emigration and infiltration on the tissues with polymorphs had commenced. Mast cells showed degranulation.

2 hr: Changes were essentially similar to those seen at 1 hr.; leucocytic emigration was more marked.

4 hr: There was further neutrophilic infiltration of the tissue spaces which appeared distended with fluid. Blood vessels were dilated with

lining endothelial cells swollen. Degranulation in mast cells was marked and the cells appeared less numerous than at 30 min.

6 hr: The further progression of the above changes especially neutrophilic infiltration was seen (fig. 37).

24 hr: Leucocytic emigration and infiltration of the tissues was at its height. The infiltration of polymorphonuclear neutrophilic leucocytes, many of which were degenerate, appeared nearly as intense as that produced by turpentine.

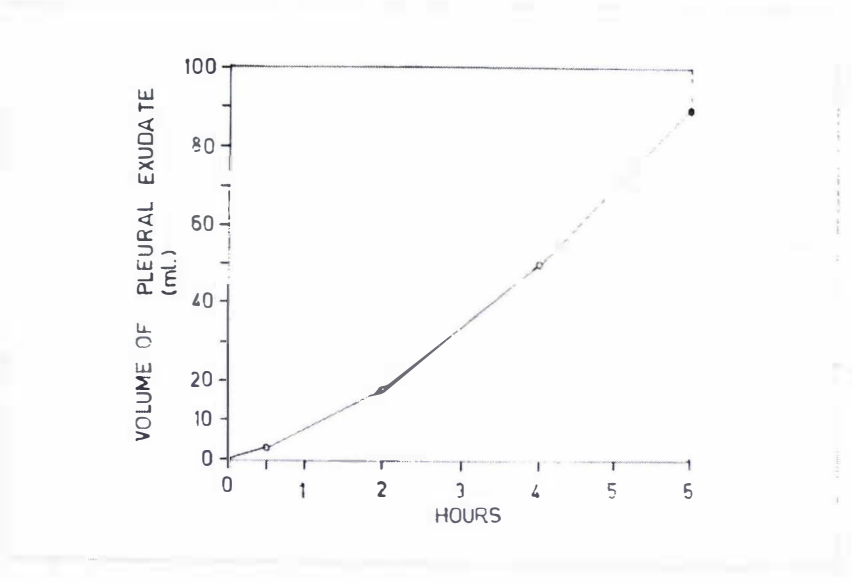


Fig.38 The volume of pleural exudate produced at different times after intrapleural injection of turpentine (0.5 ml.). The volumes represented at 2 hr. and at 6 hr. are the means of volumes produced in three sheep in each case. The volumes shown at 30 min. and at 4 hr. are those obtained in one sheep in each case.

Section 4

Studies on inflammation - using turpentine-induced pleurisy in the sheep as a test system

Suppression of the increased vascular permeability by specific antagonists of chemical mediators, anti-inflammatory agents and other substances:

The criterion that inhibition of the substance by specific antagonists should lead to a diminution of that aspect of the inflammatory reaction for which the substance is assumed to be responsible, is normally the most convincing evidence that a substance is acting as a mediator in inflammation.

In the following experiments the effects of mepyramine maleate and other substances were tested using turpentine - induced pleurisy as a test-system to confirm the apparent mediation by histamine of the early vascular changes. The effects of several other anti-inflammatory agents were also tried on the turpentine-induced pleural exudate to try to provide further information on the delayed vascular changes.

Turpentine pleurisy was produced and the exudates collected at varying intervals by procedures described previously. The effects of mepyramine maleate, sodium salicylate, strophanthin-G, indomethacin, betamethasone, alloxan monohydrate and isocarboxazid were tested on the production of pleural exudates. Lambs weighing about 20 kg. were used. Large differences in the weights of the sheep were avoided to minimize variations in the volume of pleural exudate due to the weight. The results of all the experiments are summarised in table 9.

Table 9 and fig. 38 show the volumes of pleural exudates collected from 30 min. to 6 hr. after intrapleural injection of turpentine into sheep. Since it was found that the antihistamine mepyramine maleate gave almost complete suppression of pleural exudation for up to two hours only, the pleural exudates mostly examined were those obtained 2 hr. and 6 hr.

after turpentine injection.

Table 9 - The volumes of pleural exudates developed in turpentine-induced pleurisy in relation to various treatments.

Time after intrapleural turpentine (0.5 ml.)	Group	Number of animals	Volume of exudate (ml.)
30 min.	Control	1	3.0
2 hr.	Control	3	17.0) (mean 17.0) 18.0 20.0) ml.)
2 hr.	Mepyramine maleate ("Anthisan") 25 mg./kg. intramuscularly 15 min. before intrapleural turp.	3	2.0) (mean 1.0) 2.0 3.0) ml.)
2 hr.	Sodium salicylate 0.5 g./kg. intraperitoneally 30 min. before intrapleural turp.	1	17.0
2 hr.	Sodium salicylate + Mepyramine maleate ("Anthisan") (given separately)	1	1.0
2 hr.	Strophanthin-G (Ouabaine) 100 µg./kg. intravenously 10 min. before intrapleural turp.	3	1.0) (mean 3.0) 2.0 2.0) ml.)
2 hr.	Indomethacin ("Indocid") 60 mg./kg. intraperitoneally 30 min. before intrapleural turp.	1	18.0
2 hr.	Indomethacin + Mepyramine maleate ("Anthisan") (given separately)	1	3.0
2 hr.	Indomethacin + Strophanthin-G (Ouabaine) (given separately)	1	2.0
2 hr.	Betamethasone ("Betsolan") 2 mg./kg. intramuscularly 15 min. before intrapleural turp.	1	20.0
2 hr.	Alloxan monohydrate 200 mg./kg. intraperitoneally 1 hr. before intrapleural turp. 600 mg./kg. " " " 200 mg./kg. intravenously 15 min. " 600 mg./kg. " " "	4	17.0) 16.0 ((mean 17.0 ml.) 19.0) 16.0)
2 hr.	Isocarboxazid ("Marplan") 150 mg./kg. intraperitoneally 30 min. before intrapleural turp. 200 mg./kg. " " "	2	17.0) (mean 15.0) 16.0 ml.)
4 hr.	Control	1	50.0
4 hr.	Mepyramine maleate ("Anthisan")	1	20.0
4 hr.	Sodium salicylate	1	25.0
4 hr.	Betamethasone ("Betsolan")	1	30.0
6 hr.	Control	3	80.0) (mean 90.0) 90.0 100.0) ml.)
6 hr.	Mepyramine maleate ("Anthisan")	3	53.0) (mean 50.0) 50.0 *47.0) ml.)
6 hr.	Sodium salicylate	2	42.0) (mean *38.0) 40.0 ml.)

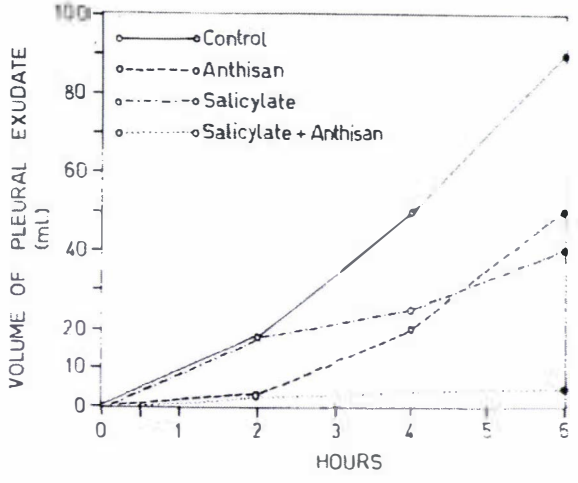


Fig.39 The effect of "Anthisan" (mepyramine maleate), sodium salicylate, and "Anthisan" plus sodium salicylate on the volume of pleural exudate produced at different times after intrapleural injection of turpentine (0.5 ml.).
The volumes represented at 2 hr. and at 6 hr. after "Anthisan" are the means of volumes obtained in three sheep in each case; one of the sheep at the 6 hr. interval was given multiple injections of "Anthisan". The volume represented at 4 hr. after "Anthisan" is that obtained in one sheep.
The volumes represented at 2 hr. and at 4 hr. after sodium salicylate are those obtained in one sheep in each case; and that at 6 hr. is the mean of volumes obtained in two sheep, one of which was given multiple injections of the salicylate.
The volume represented at 2 hr. after sodium salicylate plus "Anthisan" (each given separately in a single injection), is that obtained in one sheep; and that at 6 hr. is the mean of volumes obtained in three sheep.

6 hr.	Sodium salicylate + Mepyramine maleate ("Anthisan") (given separately)	3	4.0) (mean 3.0) 4.0 5.0) ml.)
6 hr.	Strophanthin-G (Ouabaine)	2	43.0) (mean 45.0) 20.0 ml.)
6 hr.	Indomethacin ("Indocid")	2	21.0) (mean *19.0) 20.0 ml.)
6 hr.	Indomethacin + Mepyramine maleate ("Anthisan") (given separately)	1	19.0
6 hr.	Indomethacin + Strophanthin-G (Ouabaine) (given separately)	1	18.0
6 hr.	Betamethasone ("Betsolan")	1	40.0

Note: The volumes marked thus (*) were obtained from sheep which had received multiple injections of the respective compounds indicated in the table.

(i) Effect of the antihistamine mepyramine maleate ('Anthisan') on pleurisy:

Mepyramine maleate (25 mg/kg) was given intramuscularly 15 min. before induction of pleurisy. In the sheep pretreated with mepyramine there was a substantial suppression of exudation for up to 2 hr. after the intrapleural injection of turpentine (table 9 and fig. 39). 4 hr. and 6 hr. after turpentine injection the exudation was not completely suppressed but the volumes of exudates remained significantly lower than in the corresponding control animals. To exclude the possibility that the development of exudate 2 hr. after injection of turpentine was due to a fall in the concentration of mepyramine in the blood and tissues, two further intramuscular injections of mepyramine at a dose rate of 5 mg/kg body weight were given. Injections were given at intervals of 2 hr. between the time of injection of turpentine and the collection of exudate at 6 hr. No difference between the two mepyramine treatments was evident.

(ii) Effect of Sodium salicylate on pleurisy:

Sodium salicylate (0.5 g./kg was given intraperitoneally 30 min. before induction of pleurisy. Table 9 and fig. 39 show that salicylate failed to

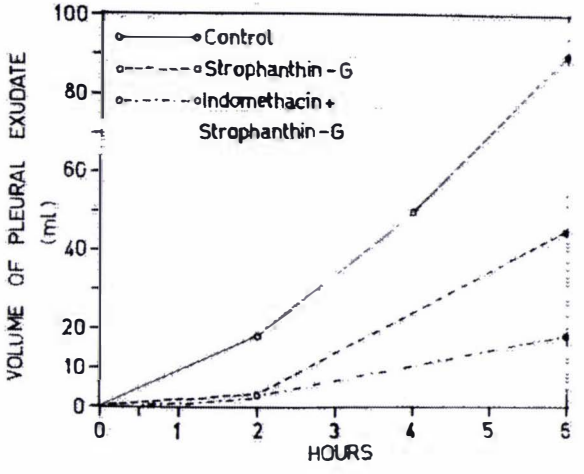


Fig.40 The effect of strophanthin-G, and indomethacin plus strophanthin-G on the volume of pleural exudate produced at different times after intrapleural injection of turpentine (0.5 ml.). Strophanthin-G and indomethacin were given separately in single injections. The volumes represented at 2 hr. and at 6 hr. after strophanthin-G are the means of volumes obtained in three sheep and in two sheep respectively. The volumes represented at 2 hr. and at 6 hr. after indomethacin plus strophanthin-G are those obtained in one sheep in each case.

suppress exudate formation at 2 hr. after administration of turpentine. 6 hr. after turpentine injection the exudation was markedly suppressed. To exclude the possibility that complete suppression of exudate formation at 6 hr. was not obtained due to a fall in the concentration of salicylate, two further intraperitoneal injections of salicylate at a dose rate of 0.2 g/kg body weight were given after turpentine injection at the same intervals as mepyramine was given. Despite this treatment, salicylate failed to give complete suppression of exudate formation at 6 hr. On the other hand, as can be seen from fig. 39 that sheep receiving salicylate and treated with mepyramine beforehand showed the most marked suppression of exudate formation both at 2 hr. and 6 hr.

(iii) Effect of Strophanthin-G (Ouabaine) on pleurisy:

Strophanthin-G (100 µg/kg) was injected intravenously 10 min. before induction of pleurisy. The results summarised in table 9 and fig. 40 show that in the sheep pretreated with strophanthin-G there was almost complete suppression of exudate formation up to 2 hr. after intrapleural injection of turpentine. 6 hr. after turpentine injection the exudation was not completely suppressed though the volumes of exudates were lower than in the corresponding control animals. On the other hand it was found that sheep receiving Strophanthin-G and pretreated with indomethacin showed almost complete suppression of exudate formation 6 hr. after intrapleural injection of turpentine.

(iv) Effect of indomethacin on pleurisy:

Indomethacin (60 mg/kg) was given intraperitoneally 30 min. before induction of pleurisy. As shown in the summarised results given in table 9 and fig. 41 indomethacin failed to suppress exudate formation at 2 hr. after intrapleural injection of turpentine. 6 hr. after turpentine injection the exudation was markedly suppressed, in the lamb used. To exclude the

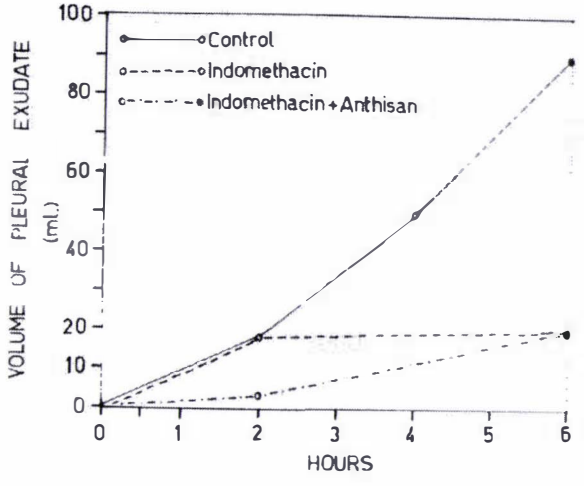


Fig.41 The effect of indomethacin, and indomethacin plus "Anthisan" (mepyramine maleate) on the volume of pleural exudate produced at different times after intrapleural injection of turpentine (0.5 ml.).
The volume represented at 2 hr. after indomethacin is that obtained in one sheep given a single injection of indomethacin and that at 6 hr. is the mean of volumes obtained in two sheep, one of which received multiple injections of indomethacin.
The volumes represented at 2 hr. and at 6 hr. after indomethacin plus "Anthisan" (each given separately in single injections) are those obtained in one sheep in each case.

possibility that complete suppression of exudate formation at 6 hr. was not obtained due to a fall in the concentration of indomethacin in the blood and tissues, two further intraperitoneal injections of indomethacin 20 mg/kg body weight were given to a second lamb after turpentine injection at the same intervals as mepyramine was given. Despite this treatment, indomethacin failed to give complete suppression of exudate formation at 6 hr. When sheep receiving indomethacin were treated beforehand with mepyramine almost complete suppression of exudation was obtained up to 2 hr. No significant difference was noticed in the volume of pleural exudate produced at 6 hr. from that obtained from sheep treated with indomethacin alone, and indomethacin plus mepyramine maleate.

(v) Effect of betamethasone ('Betsolan') on pleurisy:

Betamethasone (2 mg/kg) was given intramuscularly 15 min. before induction of pleurisy. The results recorded in table 9 and fig. 42 indicated that betamethasone did not suppress exudate formation at 2 hr. after intrapleural injection of turpentine. In one sheep at 4 hr. and another 6 hr. after turpentine injection the exudation was found to be much reduced compared to the exudation in the control animals.

(vi) Effect of alloxan on pleurisy:

Alloxan prevents the release of histamine from mast cells. The effect of alloxan on turpentine pleurisy in the sheep was tested in the early histamine-mediated phase only, i.e., up to 2 hr. by both intraperitoneal and intravenous routes, using two sheep for each route. Alloxan monohydrate was given intraperitoneally in a dose of 200 mg/kg and 600 mg/kg one hour before the induction of pleurisy. It was also injected intravenously in the same doses 15 min. before the induction of pleurisy. It was found that in all doses alloxan failed to reduce the amount of exudate formed by 2 hr. after intrapleural injection of turpentine (table 9).

(vii) Effect of isocarboxazid ('Marplan') on pleurisy:

Isocarboxazid (150 mg and 200 mg/kg) was given intraperitoneally 30 min. before the induction of pleurisy.

Table 9 shows that isocarboxazid failed to reduce the volume of exudate formed by 2 hr. after intrapleural injection of turpentine.

Section 5

Studies on inflammation - depletion experiments with Compound 48/80:

The suppression by the antihistamine mepyramine maleate of the early phase of increased vascular permeability in the inflammatory reaction in the sheep indicated that this phase of the reaction was probably mediated by histamine. Depletion of the tissues of a suspected mediator prior to the injurious stimulus should suppress that part of the inflammatory reaction for which the substance is believed to be responsible. To further investigate such a mediator role for histamine, turpentine injury was applied in the following experiments to sheep depleted of histamine so that reactions dependent on the release of histamine would be prevented. Compound 48/80 was used to deplete the sheep of their stores of histamine. Previous use of 48/80 in the skin experiments indicated that 48/80 was an effective liberator of histamine in the sheep.

In an attempt to deplete the skin locally of its histamine content, a 10 per cent solution of 48/80 in saline was injected intradermally in 0.2 ml. volumes, i.e., 20 mg. Four such injections were made in a 20 kg. lamb. 48/80 in the above dose proved toxic and the lamb died one hour after the intradermal injections with symptoms of acute respiratory distress. Post-mortem examination was conducted; the findings are described in the next Section in connection with toxicity of 48/80. Systemic histamine depletion was then attempted.

Sheep were depleted of their stores of histamine by repeated injections of Compound 48/80 using the method of Spector and Willoughby (1959). A 0.1 per cent w/v solution of Compound 48/80 in saline was given intraperitoneally, morning and evening, in ten doses beginning with an evening dose. The dose used was 0.6 mg/kg body weight for the first eight injections and 1.2 mg/kg for the last two injections. Two hours after the

last injection of Compound 48/80, sheep were blued and skin tests were carried out (see below). Turpentine pleurisy was also produced at the same time and the exudate collected at varying intervals after the intrapleural injection of turpentine. Suppression of blueing following intradermal injections of Compound 48/80 and turpentine was taken to indicate that the sheep had been depleted of histamine. A total of four sheep was used. After intradermal injections of the test substances, colour intensity of the blue area was recorded. The permeability-increasing effect of turpentine, 'Anthisan' solution, histamine, 5-HT, bradykinin, nucleosides and 6 hr. pleural exudate were tested in the depleted sheep. The lymph node cell extracts were also tested. The production of these extracts and their effects in untreated sheep are described later in Section 10.

When 0.1 ml. of turpentine, 10 µg of 5-HT, 10 µg of bradykinin, 10 µg of nucleosides, 0.1 ml. of 6 hr. pleural exudate, 0.1 ml. of dialysed lymph node cell extract and 0.1 ml. of 5% 'Anthisan' were injected intradermally in blued sheep depleted of histamine, blueing was delayed. However, intradermal injection of 10 µg of histamine produced less leakage of dye than in untreated (i.e., non-depleted) animals. In all places where blueing was suppressed, an area of local reddening (flare) was seen. It was found that the leakage of dye at the sites of injection was suppressed for a limited period which varied with the vascular permeability factor concerned, after which blueing took place. With turpentine the blueing was delayed for one hour; with bradykinin, blueing occurred in about 10 - 15 min., whereas in the case of 6 hr. pleural exudate and dialysed lymph node cell extract blueing remained suppressed for from 1 to 2 hr. No blueing developed at the sites of 5-HT, nucleoside and 'Anthisan' injections.

The results of histamine depletion on the increased vascular permeability induced by turpentine and other test substances closely resembled

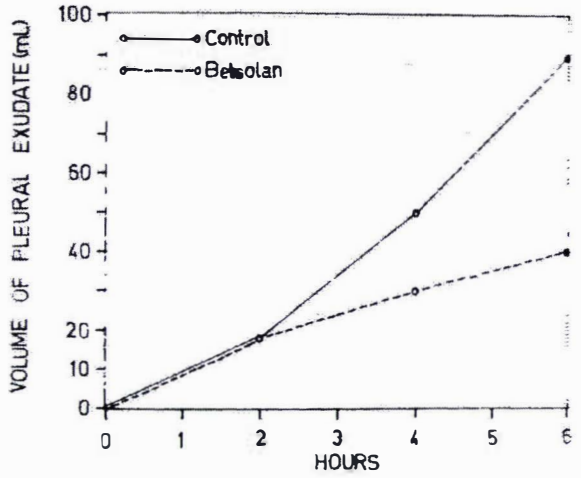


Fig.42 The effect of "Betsolan" (betamethasone) on the volume of pleural exudate produced at different times after intrapleural injection of turpentine(0.5 ml.). The volumes represented at 2, 4 and 6 hr. are those obtained in one sheep in each case, after a single injection of "Betsolan".

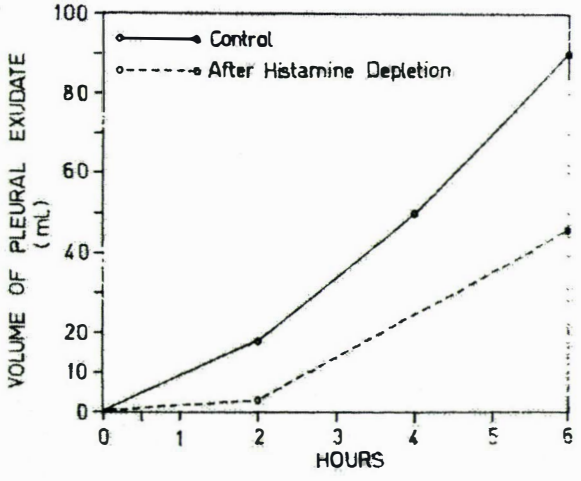


Fig.43 The effect of histamine depletion on the volume of pleural exudate produced at 2 hr. and at 6 hr. after intrapleural injection of turpentine (o.5 ml.). The volumes represented from the depleted sheep are the means of volumes produced in two sheep in each case.

the results of mepyramine maleate treatment already described.

The results summarised in table 10 and fig. 43 show that in the sheep depleted of histamine there was almost complete suppression of exudate formation up to 2 hr. after the intrapleural injection of turpentine. By 6 hr. after turpentine injection the exudation was not completely suppressed although the volumes of exudates were significantly lower than in the corresponding control animals. The results of histamine depletion on the pleural exudation also closely resembled those obtained with mepyramine maleate treatment.

Table 10 - Effect of histamine depletion on the volume of pleural exudate produced after intrapleural injection of turpentine.

S. No. of Sheep	Time after intrapleural turpentine	Volume of Exudate (ml.)	
1	2 hr.	2.0	
			(Mean 2.5 ml.)
2	2 hr.	3.0	
3	6 hr.	43.0	
			(Mean 46.0 ml.)
4	6 hr.	49.0	

Section 6

Toxicity of Compound 48/80 and histamine in sheep

As described previously the intradermal injection of a total of 80 mg. of compound 48/80 into a 20 kg. lamb caused acute respiratory distress and proved fatal. The death of the lamb may have been due to the release of histamine by this compound in amounts lethal to the animal. To investigate the sensitivity of the sheep to histamine, the toxicity of compound 48/80 and histamine were tested.

Toxicity of Compound 48/80: A 5 per cent w/v solution of Compound 48/80 in saline was given intravenously. A total of 12 lambs each weighing about 20 kg., was injected. Doses ranging from 3 mg. down to 1 mg/kg body weight were given. Differential leucocyte counts were made before the injection of 48/80 and after death of the animal. Table 11 shows the dose of 48/80 given to each animal, and the time of death following its injection.

Table 11 - Dose of 48/80 administered and the time of death of the animal following its injection.

S. No.	Weight (kg.)	Total dose of 48/80 (mg.)	Dose per kg. body wt.(mg.)	Time of death after i/v injection of 48/80
1	20	60	3	5 min.
2	19	57	3	6 min.
3	18	45	2.5	7 min.
4	20	50	2.5	6 min.
5	20	40	2	7 min.
6	21	42	2	6 min.
7	18	27	1.5	9 min.
8	20	30	1.5	10 min.
9	19	19	1	Survived
10	20	20	1	
11	21	21	1	
12	20	20	1	

The results showed that doses from 1.5 mg. to 3 mg/kg produced death. Death was sudden and occurred only within a few minutes following the injections. Salivation, acute respiratory distress, passage of faeces and general collapse preceded the death in all cases. There was marked distension of the abdomen. No significant change could be noticed in the differential leucocyte counts made before the injection of 48/80 and after the animal died. At post-mortem of all sheep that died subcutaneous vessels were intensely congested. Lungs appeared slightly inflated and did not collapse when the thorax was opened; they were pale in colour, but showed a few petechial haemorrhages on the surface. A little frothy fluid was present when the lungs were cut open and squeezed. The interlobular septa appeared slightly oedematous. Trachea and main bronchi also contained a little frothy fluid which was not blood stained. A small amount of serous fluid was present in the pericardial sac, but not in the chest or abdominal cavities. The right side of the heart was filled with soft blood clots, the left side contained only a little frothy blood. The endocardium of both sides of the heart showed mild congestion and at places small sub-endocardial haemorrhages. The portal veins of the mesentery and vessels in the serosa of the intestinal tract were markedly hyperaemic. The serosal surface of the small intestine was dark red in colour. On opening the gut, the mucosal surface especially of the small intestine showed intense hyperaemia and was dark red in colour. The intestinal contents were not blood stained. The liver appeared normal. On cutting them,open, kidneys showed slight congestion. No significant changes were seen in other organs.

Histological examination revealed vesicular emphysema in the lungs characteristed by distension and at several places rupture of the alveoli. Capillaries in the interalveolar septa at several places were engorged and their rupture had caused haemorrhage into the alveoli. Interlobular septa

were oedematous. Myocardial blood vessels were congested, congestion becoming more marked towards endocardium. At places small subendocardial haemorrhages were seen. The mucosa in the small intestine was severely congested. Congestion of the blood vessels was also seen in the kidney, mainly in the cortex. In the liver, sinusoids were dilated and engorged.

In an attempt to determine the site of action of 48/80 a lamb was first blued with pontamine blue and then injected intravenously with a lethal dose of 48/80. It was hoped that the sites of histamine release would differentiate by the selective accumulation of dye at these places due to increased vascular permeability induced by histamine. This could then be seen at post-mortem. At post-mortem the lungs showed accumulation of dye throughout, which was more marked at some places than at others. A heavy concentration of dye was seen in the intestinal tract, mainly in the mucosa of the small intestine. Here again it tended to be more marked at some places than at others. A few scattered blue spots were seen on the serosal surface of the rumen. The abomasum appeared normal. The heart revealed a few spots of dye beneath the endocardium. Dye accumulation in the liver was difficult to assess, since similar accumulation in the liver of control animals in connection with other experiments was seen which may be due to its high blood content. No accumulation of dye was seen in other organs including brain.

Toxicity of histamine: A 5 per cent w/v solution of histamine acid phosphate in saline was given intravenously. A total of 12 lambs were, weighing about 20 kg., injected. Doses ranging from 15 mg. down to 1 mg/kg body weight were given. Table 12 shows the dose of histamine given to each animal, and the time of death following its injection.

Table 12 - Dose of histamine administered and the time of death of the animal following its injection:

S. No.	Weight (kg.)	Total dose of histamine (mg.)	Dose per kg. body wt. (mg.)	Time of death after i/v injection of histamine
1	19	285	15	9 min.
2	21	315	15	13 min.
3	20	200	10	10 min.
4	19	190	10	14 min.
5	19	95	5	15 min.
6	18	90	5	14 min.
7	20	60	3	20 min.
8	20	60	3	23 min.
9	18	36	2	Survived
10	20	40	2	Survived
11	18	18	2	Survived
12	20	20	1	Survived

The results showed that doses from 3 mg. to 15 mg/kg produced death. Death occurred within 30 min. of the injections. The symptoms, prior to death, were similar to those seen in the sheep given 48/80. However, in all cases the animal struggled violently on the ground, kicked with its hind legs, and exhibited muscular spasms before it died. No significant change could be noticed in the differential leucocyte counts made before the injection of histamine and after the animal died. The post-mortem findings

were similar to those in sheep given 48/80. However, in all these cases more petechial haemorrhages were seen on the surface of the lungs. When cut, lungs contained more frothy fluid. There was about 5 ml. of serous fluid in the pericardial sac, 10 ml. in the chest cavity and 25 ml. in the abdominal cavity. The most important finding, in contrast to 48/80 cases, was the presence of extensive sub-endocardial haemorrhages in all cases which appeared apparently confined to the left ventricle. Another difference in these animals was the much smaller involvement of the intestinal tract. The mucosa of the small intestine was only slightly congested. The serosa was normal except for a few petechial haemorrhages which were also seen on caecum and colon. Histological changes were similar to those for the sheep given 48/80. In all cases, however, the heart revealed extensive sub-endocardial haemorrhages.

The location of the site of action was attempted as with 48/80. The lungs revealed less accumulation of dye than in the case of 48/80. Again, in contrast to 48/80, the heart showed marked blueing at the sites of sub-endocardial haemorrhages in the left ventricle. Slight leakage of dye was seen throughout the mucosa of the small intestine. A few scattered blue patches were seen on the serosal surface of small intestine, caecum and colon and also on the rumen. Other organs, including brain did not reveal any significant leakage of dye.

Section 7

Histological study of increased vascular permeability in turpentine-induced inflammation of the skin and pleura:

All substances at present known to increase vascular permeability appear to act on the venules. This has been shown recently by the colloidal carbon technique (Majno *et al*, 1961; Hurley, 1963; Cotran and Majno, 1964; Hurley and Spector, 1965). Majno, Palade, and Schoefl (1961) demonstrated that colloidal carbon injected intravenously was a good indicator of increased vascular permeability in the rat and clearly labelled the particular vessels through which plasma protein was escaping. The carbon accumulated between endothelial cells and their basement membrane. They further showed that the action of histamine and 5-HT was confined to venules and small veins ranging from 10 μ to 80 μ in diameter; arterioles and capillaries were not affected. Hurley (1963) confirmed these findings in the rat skin. Cotran and Majno (1964) showed that the permeability factors histamine, bradykinin, 5-HT and globulin PF acted almost exclusively on the venules. More recently, Hurley and Spector (1965) from a topographical study of increased vascular permeability in acute turpentine-induced pleurisy in rat further supported the view of Majno *et al* (1961) that all endogenous substances at present known to increase vascular permeability induce carbon leakage only in venules. They found that the leakage of carbon from venules was biphasic; the first phase, starting within a few minutes of turpentine injury, was substantially completed by 1 hr.; the second phase commenced 2-3 hr. after injury and reached a maximum at about 5 hr. After injury capillary leakage also occurred; it was seen first about 45 min. after injury and was at its maximum between 2 and 5 hr. The permeability antagonists suppressed leakage of carbon only in venules; leakage from capillaries was unaffected. In discussing this the authors, (Hurley and Spector, 1965), suggested that leakage of carbon from capillaries may be due to direct damage by the noxious stimulus or to an indirect mechanism as yet obscure.

The following experiments describe the use of the colloidal carbon technique in a histological study in sheep of increased vascular permeability in acute turpentine-induced inflammation of the skin and pleura.

The colloidal carbon technique has been referred to previously. A colloidal suspension of carbon was injected intravenously into four sheep at intervals of either 15 min., 1 hr., 3 hr., or 6 hr. after intrapleural and intradermal injections of turpentine. One sheep was used for each interval. Each sheep was killed 20 min. after the injection of carbon. Portions of skin and diaphragm from the experimental sites were removed, fixed, sectioned and stained H & E and with 0.1 per cent safranin to study the carbon labelled blood vessels. To test the effects of inhibitors of increased vascular permeability on carbon leakage five sheep were used. In three sheep antihistamine mepyramine maleate was injected intramuscularly (25 mg/kg) 15 min. before turpentine was injected intradermally and intrapleurally. The effect was tested 15 min., 1 hr., and 3 hr., after turpentine injection, when the sheep was killed and tissues collected. One sheep was used for each interval. In two sheep sodium salicylate (0.5 g/kg) was given intraperitoneally 30 min. before turpentine and mepyramine maleate 15 min. after salicylate. The combined effect of salicylate and mepyramine was tested at 3 hr. and 6 hr. after turpentine injection. When testing was carried out at the 6 hr. interval, sheep received two further injections of both mepyramine (5 mg/kg) and salicylate (0.2 g/kg) given at intervals of 2 hr. between the time of injection of turpentine and the injection of colloidal carbon at 6 hr. The above intervals and combination of drugs to see the effects on carbon leakage was chosen on the basis of the earlier results, which showed that mepyramine maleate gave almost complete suppression of pleural exudation for up to 2 hr. only, whereas combined treatment of salicylate and mepyramine suppressed exudate formation for up to 6 hr. The carbon labelling was assessed separately in venules and capillaries,

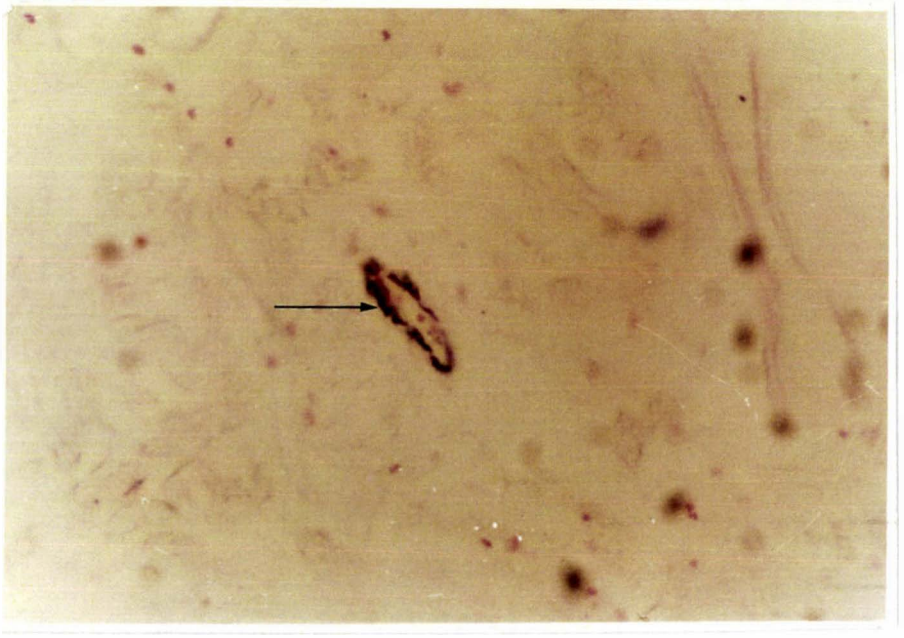


Fig.44 Section of sheep skin showing labelling of a venule wall with carbon (arrow) 15 min. after intradermal injection of turpentine (0.1 ml.). Note in this early stage carbon is sharply confined within the vessel wall. Interstitial oedema is seen but no leucocytic emigration.

Safranin x 250

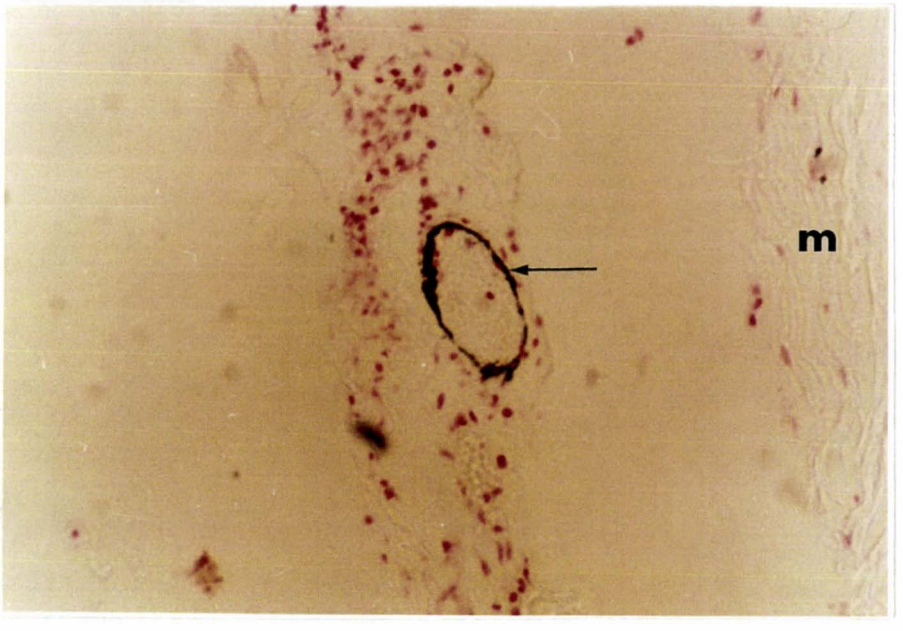


Fig.45 Section of sheep diaphragm showing labelling of a venule (in diaphragmatic pleura) with carbon (arrow), 1 hr. after intrapleural injection of turpentine (0.5 ml.). Accumulation of fluid beneath the pleural surface separates the latter from the underlying muscles (m) seen on the right. Carbon is seen sharply confined within the vessel wall. Slight leucocytic emigration seen.

Safranin x 250

The term capillary in tissue sections refers to blood vessels of 4-6 μ in diameter. Blood vessels larger than 7 μ and less than 100 μ in diameter of a histologically venous character were classified as venules. The classification is the same as used by Hurley and Spector (1965), and by Willoughby, Walters and Spector (1966).

Vascular labelling after acute turpentine-induced inflammation of the skin and pleura was recorded at the following intervals: In the sheep given carbon 15 min. after intradermal and intrapleural injections of turpentine and killed 20 min. after the carbon injection, vessels in both the skin and diaphragm were labelled with carbon. Veins and venules labelled with carbon could be clearly distinguished, particularly in sections stained with safranin.

In the skin labelling was confined to small veins and venules only ranging from 11 to 90 μ in diameter (fig. 44). True capillaries of 4-6 μ in diameter showed no carbon labelling of their walls. Veins over 100 μ in diameter and arterioles were not labelled. The deposits were sharply confined to the walls of the affected vessels and showed no tendency to extend into the adjacent tissues. Blood vessels were hyperaemic. Oedema was present but very little emigration of leucocytes. Similar changes were seen in the diaphragm. The carbon deposition involved more vessels than in the skin. The labelling was confined to the vessels of the diaphragmatic pleura. Only small veins and venules were labelled; capillaries showed no carbon deposits. Congestion of blood vessels and oedema were less marked than in the skin. No significant emigration of leucocytes was seen.

In skin taken from sheep injected with colloidal carbon 1 hr. after the turpentine injections carbon labelling of veins and venules was more

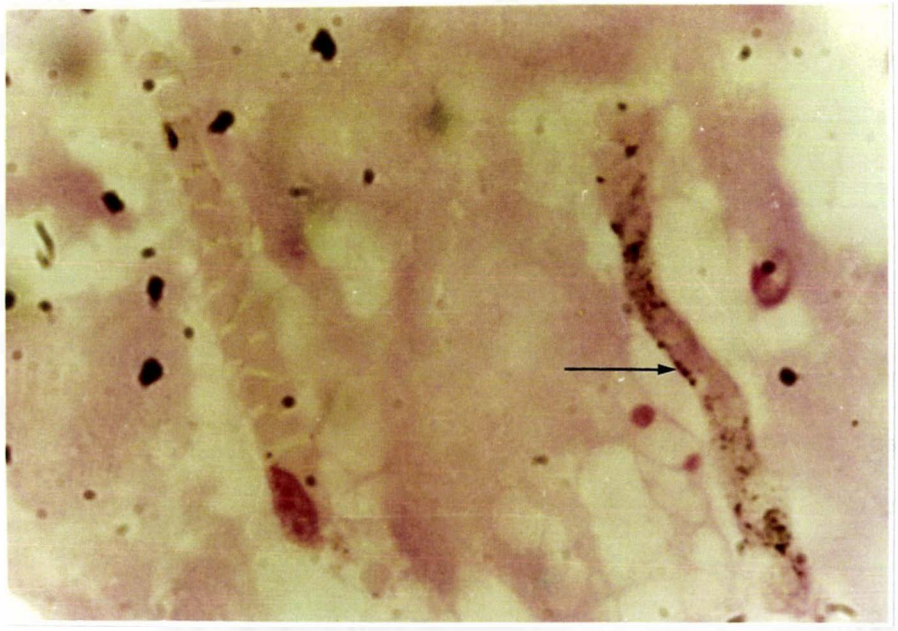


Fig.46 Section of sheep diaphragm 1 hr. after intrapleural injection of turpentine (0.5 ml.) showing labelling of a capillary wall with carbon (arrow).

Safranin x 1000 (oil immersion)

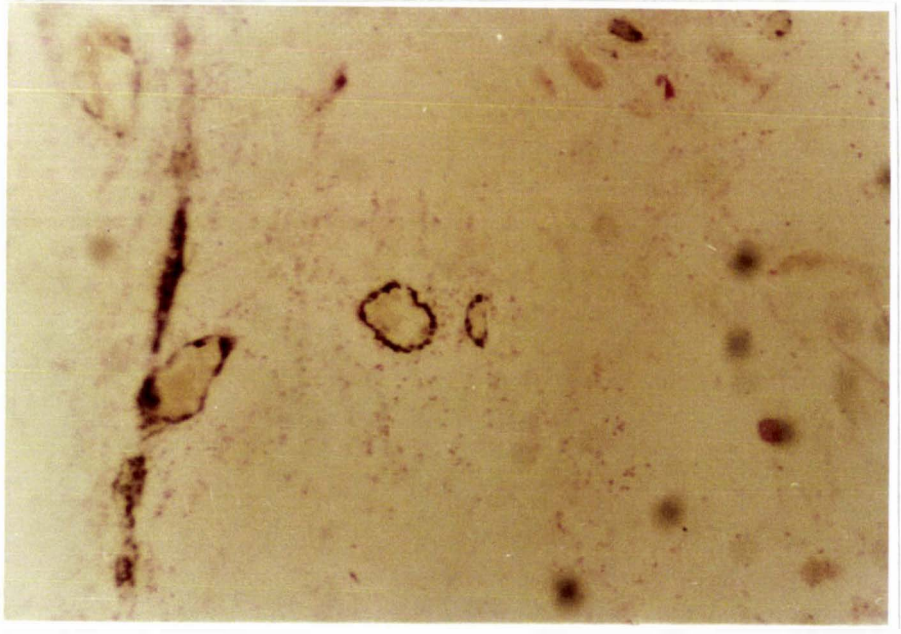


Fig.47 Section of sheep skin 3 hr. after intradermal injection of turpentine (0.1 ml.) showing widespread labelling of venules. Leucocytic emigration in interstitial tissues is also seen.

Safranin x 100

marked. In addition a few blood vessels of 7 to 9 μ diameter showed carbon deposits at this stage in the sections examined, but true capillaries of 4-6 μ in diameter showed no carbon deposition in their walls. The carbon deposition in the venules were confined to the vessel wall as at 15 min. Congestion of blood vessels had increased. Oedema of the subcutaneous tissues was more extensive but only a slight increase in leucocytic emigration was seen. At this stage too, carbon labelling of veins and venules was more extensive in the diaphragm than in the skin and was still mostly confined to the vessels of the diaphragmatic pleura (fig. 45). In the diaphragm, however, an occasional true capillary of 5 - 6 μ in diameter, lying in contact with the individual muscle fibres, also showed some carbon labelling (fig. 46). Oedema had very much increased and accumulation of fluid beneath the pleural surface separated the latter from the underlying muscles. Leucocytic emigration had only slightly increased.

Carbon injected 3 hr. after injection of turpentine revealed extensive labelling of vessels in both skin and diaphragm. In the skin labelling of small veins and venules was maximal at this stage (fig. 47). A few capillaries of 5 - 6 μ in diameter also showed carbon deposits. The most obvious feature at this stage was significant emigration of leucocytes. Dense collections of leucocytes, mainly polymorphs, were seen and were largely perivascular in distribution. In many venules active leucocytic emigration was in progress, and in a few some carbon particles had passed into the perivascular tissues and were no longer entirely confined to the walls of the vessels as they were 15 min. and 1 hr. after injury (fig. 48). The deposits appeared as an irregular extension of the carbon in the venule wall or as discrete speckled deposits some distance from the outer border of the vessel and lying in the dense collections of leucocytes (see fig. 50).

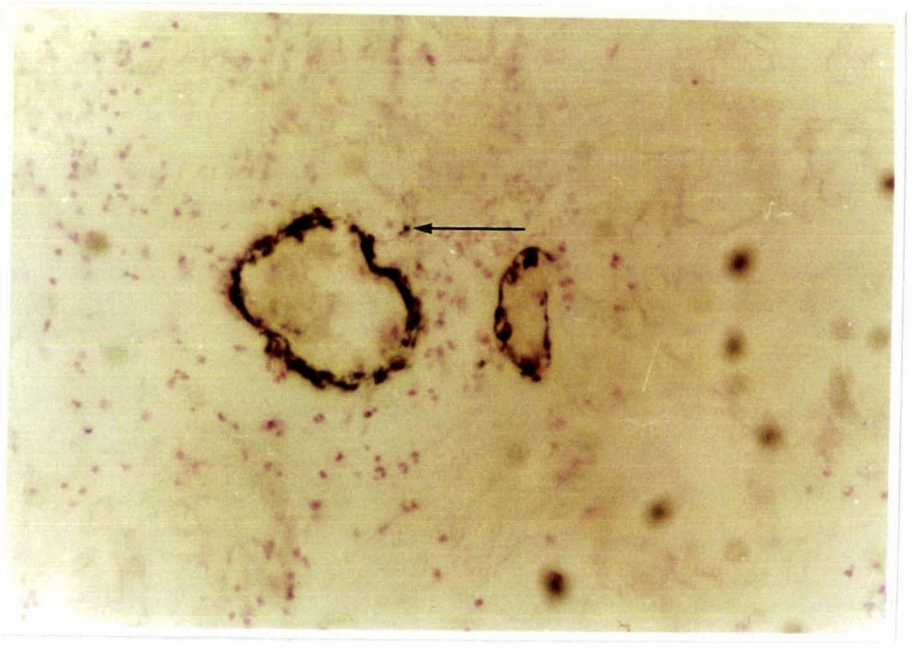


Fig.48 Same as fig. 47 showing two labelled venules under higher magnification. The venule on the left is showing the beginning of the spread of carbon particles (arrow) from its wall into the perivascular tissues.

Safranin x 250

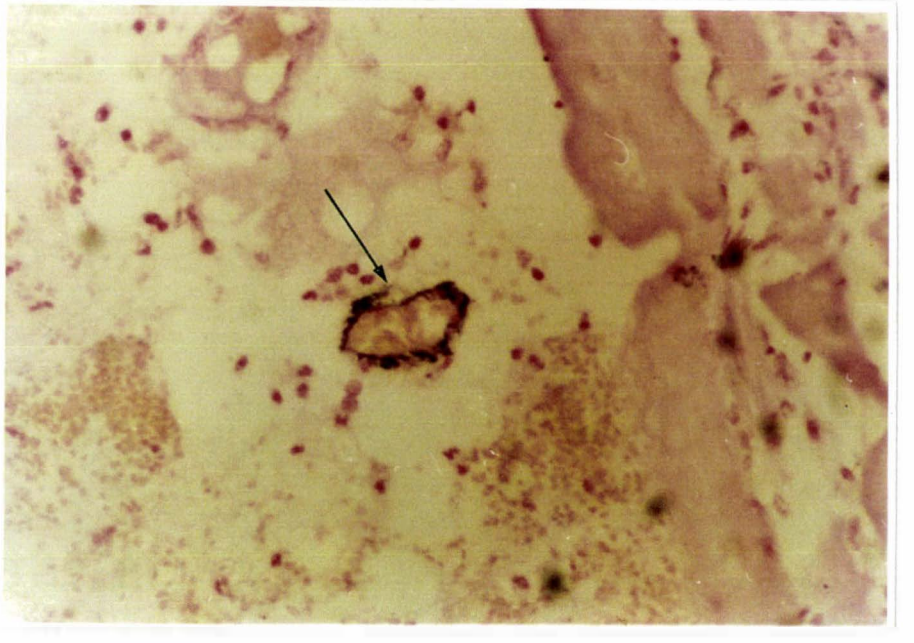


Fig.49 Section of sheep diaphragm 3 hr. after intrapleural injection of turpentine (0.5 ml.) showing labelling of a venule with carbon (arrow). The venule also shows the beginning of the spread of carbon into perivascular areas. Interstitial oedema is seen but only a few leucocytes are seen in the perivascular tissues.

Safranin x 250

Spector (1965). Carbon was seen lying free and not in leucocytes or macrophages. 'Smudging' was observed only from venules 11 - 90 μ in diameter; it could not be detected in capillaries. Congestion of blood vessels and oedema of the subcutaneous tissue had markedly increased. The diaphragm revealed changes essentially similar to those of skin. Carbon labelling of small veins and venules although significantly increased at this stage, was slightly less extensive than in the skin. Labelling also involved small veins and venules of the diaphragmatic muscles (fig. 49) and was no longer confined to vessels of the diaphragmatic pleura only. No significant increase was seen in the labelling of capillaries. 'Smudging' occurred as in the skin (fig. 50). Leucocyte emigration though significantly greater than earlier times was less marked than in the skin. Congestion of blood vessels and oedema were more pronounced than in skin.

Carbon injected 6 hr. after the turpentine injection produced slightly less extensive labelling of small veins and venules in the skin than that seen at 3 hr. No significant change could be observed in the labelling of the capillaries. Acute inflammatory changes were most marked at this stage, mainly in the subcutaneous tissue. Venules in the underlying muscles were labelled. Oedema was very marked. Emigration of leucocytes was at it s height at this stage. The dense collections of leucocytes were still largely perivascular and many small veins surrounded by them showed carbon labelling. 'Smudging' was more marked than at the preceding stage (see fig. 51). Leucocytic emigration was also seen to be taking place from venules that were not labelled with carbon and were presumably therefore not abnormally permeable to protein. In contrast to the skin, labelling of small veins and venules in the diaphragm showed a further increase. The vessels of diaphragmatic pleura, the diaphragmatic muscles and the tendinous portion of the diaphragm were extensively labelled. Capillary labelling showed no

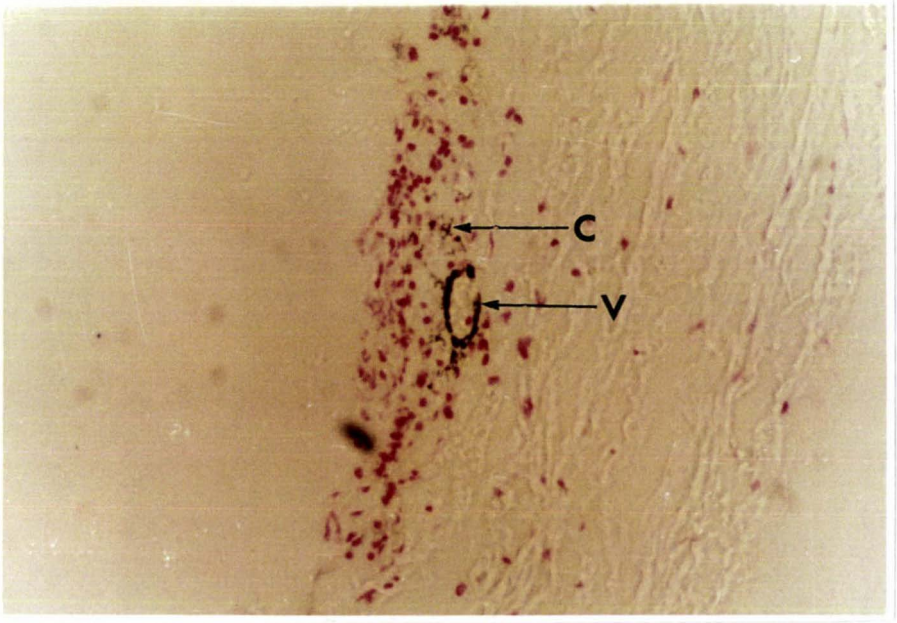


Fig.50 Section of sheep diaphragm 3 hr. after intrapleural injection of turpentine (0.5 ml.) showing another labelled venule (v) in which carbon (c) has further extended into the adjacent tissues. Leucocytic emigration is also seen in this area.

Safranin x 250

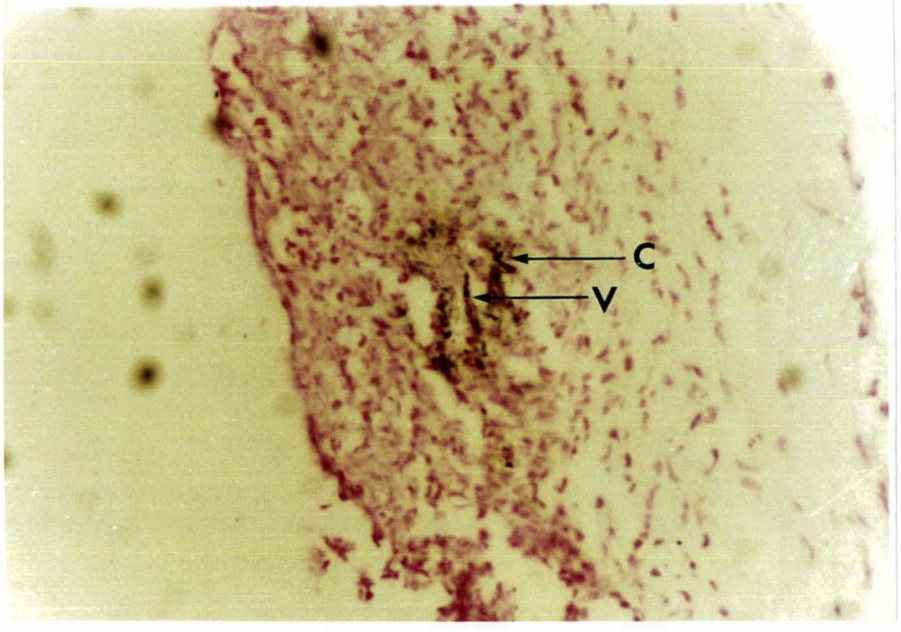


Fig.51 Section of sheep diaphragm 6 hr. after intrapleural injection of turpentine (0.5 ml.) showing marked spread of carbon particles (c) from the venule wall (v) into adjacent tissues. Massive leucocytic emigration in progress.

Safranin x 250

detectable change. Inflammatory changes were most marked at this stage as in the skin and affected the muscles more than the pleura. Leucocytic infiltration was at its height (fig. 51). There was marked oedema. Bundles of muscle fibres were dissected out by oedema and the affected areas showed some necrotic changes characterized by loss of staining in muscle cells. Polymorphs showed mild degenerative changes. 'Smudging' from labelled venules ~~(fig. 53)~~ and leucocyte emigration from venules that were not labelled with carbon was seen as in the skin.

The effects of inhibitors on carbon leakage:

Mepyramine maleate: Carbon labelling of venules was largely suppressed in the early stages of the inflammation, i.e., up to 1 hr. in the skin and 3 hr. in the diaphragm. Leakage of carbon from capillaries was unaffected. Only a few labelled capillaries were seen as in untreated cases. In the sections of both skin and diaphragm, though the carbon labelling in the small veins and venules was largely suppressed, the congestion of blood vessels was affected only slightly and the leucocyte emigration remained unaffected, by the prior administration of mepyramine. At both 15 min. and 1 hr. many hyperaemic but unlabelled blood vessels were seen and at 3 hr. leucocytic infiltration was as prominent as in the untreated sheep.

Sodium salicylate: The combination of sodium salicylate and mepyramine maleate markedly suppressed the venular labelling at both 3 and 6 hr., i.e., in the delayed phase. Carbon labelling of capillaries was unaffected. Labelled capillaries were very few and scattered as in untreated cases. As with antihistamine, pretreatment with sodium salicylate and mepyramine maleate failed to suppress either the vascular hyperaemia or the leucocyte emigration. In sections examined at this stage, both in the skin and diaphragm, active migration of leucocytes was seen taking place from both labelled and unlabelled venules.

Section 8

Histological study of increased vascular permeability in thermal injury:

It has been mentioned in the previous Section that blood vessels in a state of increased permeability are blackened when colloidal carbon is injected into the circulation. Normal vessels are not labelled (Majno et al, 1961). Wells and Miles (1963) were the first to apply the colloidal carbon technique to thermal injury. They found that the carbon leakage in the immediate phase of increased vascular permeability occurred almost entirely from venules, whereas the delayed phase of vascular change was marked mainly by leakage from true capillaries. Cotran and Majno (1964) confirmed these findings, particularly the association of capillary leakage with the delayed phase of increased vascular permeability. From these results it was suggested that most of the inflammatory oedema of thermal injury was due to increased capillary (as opposed to venular) permeability. Since all known potential mediators of inflammatory changes affect the venules almost exclusively and have little effect on capillaries, which has been described in the previous section, the above results raised the possibility that endogenous mediators were of little importance in thermal injury. The situation has been clarified recently by Spector, Walters and Willoughby (1965b). These authors also found that after thermal injury in the rat leakage of carbon from venules occurred mainly in the early stages; capillary leakage was prominent in the delayed phase of increased vascular permeability. However, the anti-inflammatory drugs salicylate and iproniazid (an inhibitor of monoamine oxidase), suppressed oedema formation throughout the experiment but inhibited the carbon leakage from venules only in the first hour after injury. The drugs had no effect on residual venular leakage from 1 to 3 hr., or on capillary leakage. On the basis of these results the authors suggested that the inflammatory oedema of thermal injury is due largely to the increased permeability in venules that occurs

in the first hour or so. The continual leakage from true capillaries and the low-grade leak from venules after the first hour were thought to make little contribution to the fluid exudate. They supported the view that endogenous chemical mediators are important in causing inflammation after thermal injury. The following experiments describe the use of the colloidal carbon technique in a histological study of the increased vascular permeability in thermal injury in the sheep.

Thermal injuries were produced as described previously in a single sheep 6 hr., 4 hr., 2 hr., 1 hr., 30 min., 15 min., 10 min., and 5 min. before the intravenous injection of a colloidal suspension of carbon. 20 min. after carbon was injected, skin biopsies from the sites of thermal injury were taken. Skin samples from two areas of thermal injury were obtained for each time interval. Skin biopsies from untreated sites were also obtained at the start of the experiment as controls. The skin samples were weighed immediately after their removal and the inflammatory oedema assessed tentatively by the increase in weight. The skin samples were then fixed, sectioned and stained with H & E and with 0.1 per cent Safranin to study the carbon labelled blood vessels. To test the effects of inhibitors on carbon leakage two more sheep were used. In one sheep the antihistamine mepyramine maleate (25 mg/kg) was injected intramuscularly 15 min. before thermal injury. In the other sheep sodium salicylate (0.5 g/kg) was given intraperitoneally 30 min. before thermal injury. Skin samples were obtained at all intervals given above. Two further injections of both mepyramine (5 mg/kg) and salicylate (0.2 g/kg) were given at intervals of 2 hr. between the time of infliction of the thermal injury and the collection of skin samples at 6 hr. The carbon labelling was assessed separately in venules and capillaries. The criteria for distinguishing capillaries and venules have been described in the previous section. Table 13 summarises the results of all the experiments.

Table 13 - Weight of skin samples (gm. of one cm. diameter skin biopsy specimens) collected at different times after thermal injury (70^{o} for 1 minute) and the effect on them of prior administration of mepyramine maleate and sodium salicylate.

Time after thermal injury	Mean Weight of two skin samples (gm. of 1 cm. diameter skin biopsy specimens)		
	Thermal injury	Mepyramine-pretreatment then thermal injury	Sodium salicylate-pretreatment then thermal injury
Control-sample (i.e., without injury)	0.1	0.1	0.1
5 min.	0.12	0.12	0.12
10 min.	0.16	0.12	0.13
15 min.	0.2	0.16	0.15
30 min.	0.21	0.17	0.16
1 hr.	0.25	0.21	0.2
2 hr.	0.29	0.23	0.22
4 hr.	0.3	0.26	0.22
6 hr.	0.30	0.30	0.23

The degree of vascular labelling after thermal injury was recorded at different time intervals. The results are given below:

5 min.: Skin collected from the site of thermal injury showed an increase in weight (table 13) and oedema was histologically evident. Carbon labelling was confined to small veins and venules only, ranging from 11 to 90 μ in diameter. True capillaries (4 - 6 μ diameter) showed no deposition of carbon in their walls. Small blood vessels were dilated and hyperaemic but no margination or emigration of leucocytes was seen in them.

10 min.: There was a further increase in the weight of the skin samples

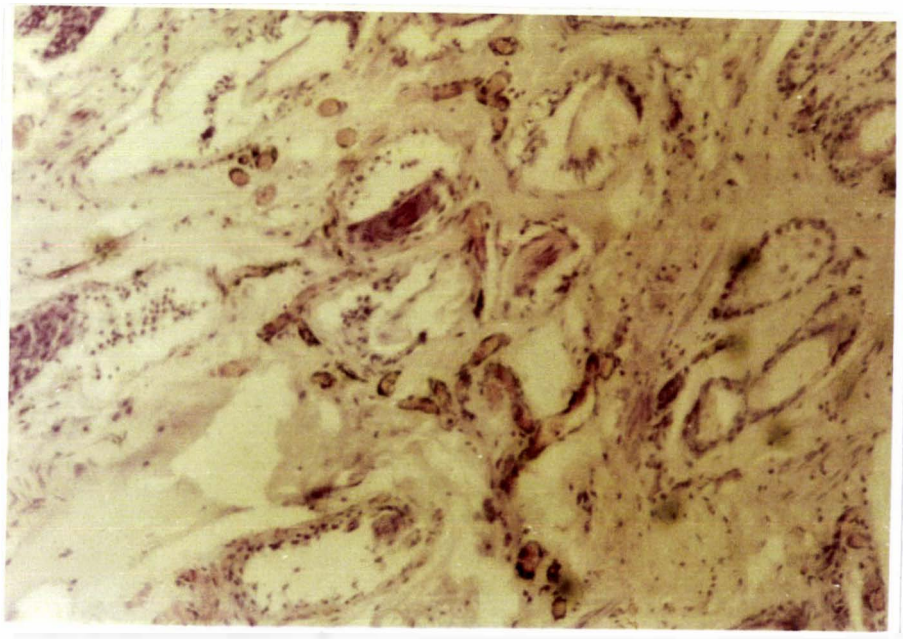


Fig.52 Section of sheep skin 15 min. after thermal injury showing extensive carbon labelling of venules. Interstitial oedema is present, but no leucocytic emigration.

H.E. x 100

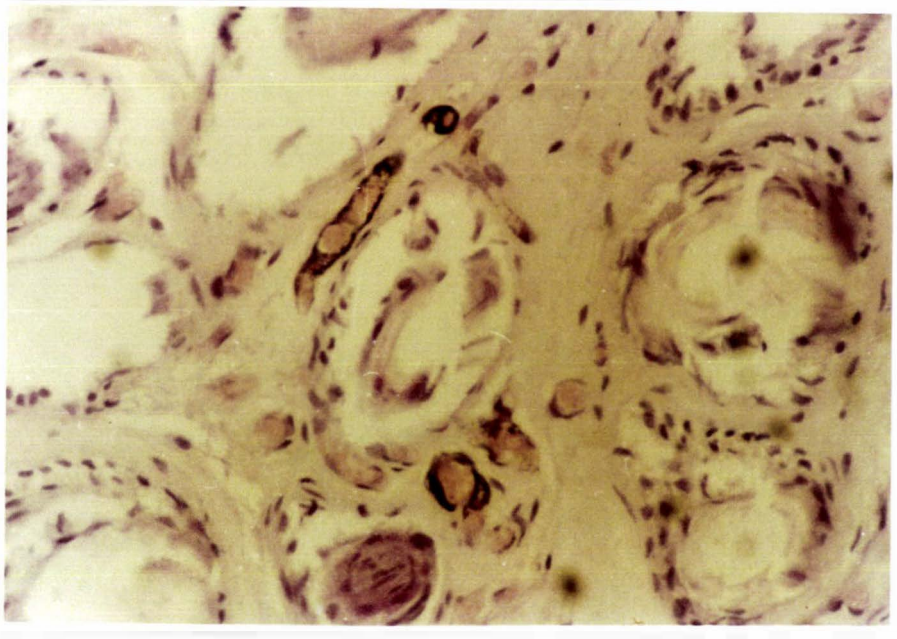


Fig.53 Same as fig. 52, showing carbon labelling of venules under higher magnification. Blood vessels are hyperaemic but no margination or emigration of leucocytes is seen. Interstitial oedema is present.

H.E. x 250

obtained at this stage (table 13). Carbon labelling of small veins and venules was more extensive than at 5 min. In addition an occasional capillary revealed carbon deposits. Other changes were similar to those seen at 5 min.

15 min.: The mean biopsy weight was now twice that of the control samples (table 13). Labelling of small veins and venules was maximal at this stage (fig. 52). A few capillaries of 5 - 6 μ in diameter also showed carbon deposits. Massive oedema was present throughout the whole thickness of the dermis; the small blood vessels were dilated and hyperaemic but neither margination nor emigration of leucocytes was seen (fig. 53).

30 min.: There was slight further increase in the weight of the skin samples (table 13). Carbon injected at this time resulted in slightly less extensive labelling of small veins and venules than that seen at 15 min. No significant change could be detected in the labelling of the capillaries. Other changes were similar to those seen at 15 min. No leucocyte emigration was seen.

1 hr.: Weight of the skin samples had further increased (table 13). Carbon labelling of small veins and venules did not reveal any noticeable change at this stage as compared to the preceding sample (fig. 54). It became increasingly difficult with increasing time after injury to find capillaries labelled with carbon. Oedema, vascular dilatation and hyperaemia were prominent. Some adhesion of leucocytes to the vessel walls was now present but significant emigration of leucocytes was still not seen. Only a few leucocytes had escaped into the perivascular tissues (fig. 54).

2 hr.: Weight of the skin samples showed a further increase (table 13). There was a further fall in the extent of carbon labelling of small veins and venules. Labelling of capillaries remained difficult to detect. A few more leucocytes appeared to have escaped into the perivascular tissues. The emigration, however, was not marked. Other changes were as seen at 1 hr.

4 hr.: No increase in the weight of the skin samples was noticed (table 13).

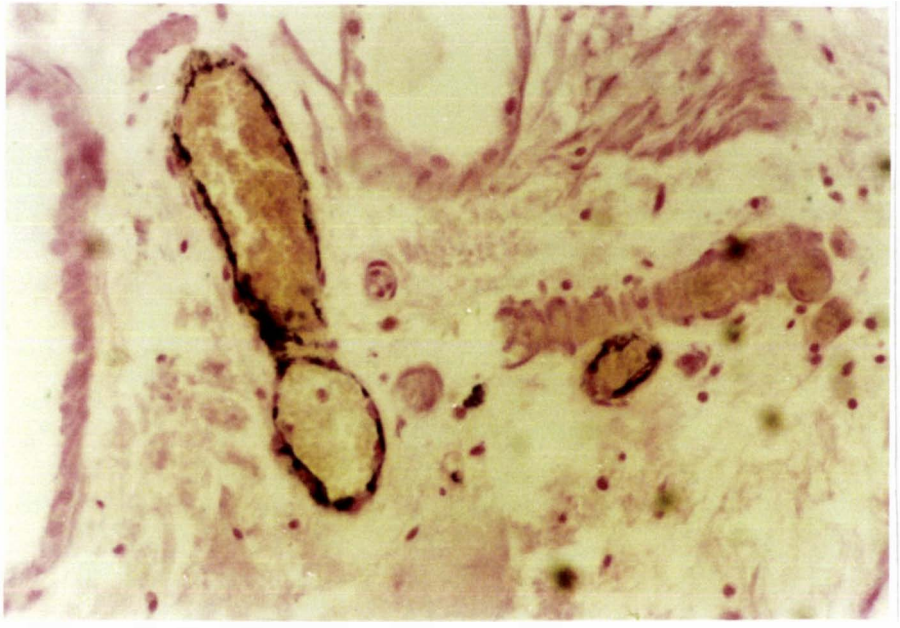


Fig.54 Section of sheep skin 1 hr. after thermal injury showing carbon labelling of venules. Interstitial oedema is present but only a few leucocytes are seen in the perivascular tissues.

Safranin x 250

No further decrease in the carbon labelling of venules was seen. Labelled capillaries were scarce and leucocyte emigration was as noted at 2 hr.

6 hr.: There was a further increase in the weight of the skin samples (table 13). Histologically these samples were not different to the 4 hr. samples.

Effect of prior administration of mepyramine maleate:

In table 13 is recorded the effect of prior administration of mepyramine maleate on the skin biopsy weight. Increase in weight of the skin samples was suppressed significantly in the early stages. The effect started to wane 2 hr. after the injury and at 6 hr. no difference could be seen from the corresponding untreated (i.e. without mepyramine pretreatment) samples. Mepyramine had, moreover, a striking but temporary effect on venular labelling. The main peak of labelling between 15 min. and 1 hr. after injury was greatly reduced: the persistent but relatively weaker labelling from 1 hr. to 6 hr. remained unaffected.

Effect of prior administration of sodium salicylate:

The effects of prior administration of sodium salicylate on skin biopsy weights after thermal injury are shown in table 13. There was a reduction in the weight of the skin samples at all stages with no return to the levels found in the untreated animals. An inhibiting effect on the venular labelling was first noticeable 30 min. after injury and persisted thereafter.

Section 9

Effect of denervation on the increased vascular permeability:

It has been noted earlier that whereas mepyramine maleate suppressed the early phase of the increased vascular permeability following intradermal injections of turpentine in a 'blued' sheep, an area of reddening (flare) was still seen at the sites of injections. Histology from these sites indicated that the reddening was due to local hyperaemia and vascular dilatation. Thus, in these experiments, although the response to the locally released histamine was abolished by the antihistamine-pretreatment, the nerve supply of the area remained intact to influence the local inflammatory process. It was considered possible that the local hyperaemia and vascular dilation, which persisted after antihistamine treatment following intradermal injection of turpentine, may depend on nervous control. Following the suggestion by Humphrey (1955a) that capillary tone and to some extent permeability are under nervous control, the effect of denervation on the vascular permeability in the sheep was tested.

It was thought that the inflammatory response in an area of skin immediately after cutting its nerve supply might be affected by the surgery involved. It was therefore decided to study the inflammatory response both immediately after denervation and after the animal had recovered for some days from the surgery. A six days interval was chosen for its convenience. On the first day the sheep was anaesthetised with "Nembutal" (Abbott Laboratories Ltd.) and the successive spinal nerves T8 to L5 were exposed immediately lateral to the longissimus dorsi muscle. A loop of stainless steel wire was passed around each of the first five nerves and section 1 cm. long was removed from each of the remaining nerves. On the sixth day the looped nerves were located and cut. In this procedure, 5 to 10 ml. of local anaesthetic (xylocaine) was sprayed into the wound; general anaesthesia was not used because of possible effects on the inflammatory



Fig.55 Effect of denervation on increased vascular permeability.
IC = Control zone; IT = Immediately denervated; 6T = 6 day old denervation.
Photograph taken 30 min. after intradermal injection of turpentine (0.1 ml.) into a blued sheep showing marked leakage of pontamine blue in all the three zones.



Fig.56 Effect of mepyramine maleate-pretreatment plus denervation on increased vascular permeability. Same sheep as in fig. 55 showing the same side after mepyramine administration
IC = mepyramine-pretreated zone
IT = mepyramine-pretreated zone plus immediate denervation
6T = mepyramine-pretreated zone plus 6 day old denervation
Photograph taken 30 min. after intradermal injections of turpentine (0.1 ml.). Note appearance of blueing in the denervated zones. In all zones, at sites where blueing is suppressed, a local flare is still seen (arrows).

responses (Miles and Miles, 1952). These procedures produced an anterior band of immediate denervation and a posterior band that had been denervated for six days. The control zones were taken both cranial and caudal to these denervated areas. Severance of each nerve well away from the spinal column meant that the denervation was total, i.e., both motor (including autonomic) and sensory fibres were cut. The zones of denervation were mapped on the sheep by pricking the skin with a pin; only the central area of each zone well away from the wound was used to study the inflammatory response. The nerve section was checked at necropsy.

Two lambs were denervated as described above and blued on the 7th day. Turpentine was injected intradermally in 0.1 ml. volumes in each zone, i.e., from anterior - control, immediately denervated and six day denervated zones (fig. 55). The colour intensity and the extent of blueing, in all the three zones were recorded. After one hour observation, each lamb was injected with mepyramine maleate intramuscularly at the dose rate of 25 mg/kg. Turpentine was again injected in 0.1 ml. volumes in each zone. Blueing at all injected sites was recorded.

It was found that increased vascular permeability was not suppressed in the denervated areas. Marked leakage of dye took place in all the three zones, and covered a larger area in the denervated zones, (fig. 55).

Mepyramine maleate-pretreatment when combined with denervation suppressed the leakage of dye for about an hour. It, however, still failed to abolish the reddening (flare) at the sites of turpentine injection that was noticed when leakage of dye was suppressed by mepyramine-pretreatment alone (fig. 56). Moreover, the blueing seemed to return slightly more quickly after treatment with mepyramine in denervated areas than in those whose

nerve supply was intact.

The colloidal carbon technique, described earlier, was used in two more sheep to examine histologically the effect of denervation on the increased vascular permeability that followed intradermal injection of turpentine. The lambs were denervated as before. On the 7th day after operation one of them was injected intradermally with turpentine in 0.1 ml. volumes in all the three zones described above 30 min., 15 min., 10 min., and 5 min. before the intravenous injection of a colloidal suspension of carbon. 20 min. after carbon was injected, the lamb was destroyed and skin samples were collected from the sites of turpentine injection. Two samples were obtained for each time interval.

The other lamb was treated similarly and the skin samples obtained at the same intervals. But it was injected with mepyramine maleate intramuscularly (25 mg/kg) 15 min. prior to the intradermal injections of turpentine. Skin samples were fixed, sectioned and stained H & E and with 0.1 per cent safranin to study the carbon-labelled blood vessels.

Denervation, both immediate and 6-day old, failed to suppress the carbon labelling of the blood vessels that occurred when turpentine was injected intradermally. This has been described in Section 7. At sites with an intact nerve supply but subject to mepyramine-pretreatment, carbon labelling was almost completely suppressed; only an occasional vessel was labelled. In sites that were both denervated (immediate and 6 day old) and pretreated with mepyramine, the picture was similar to that noted with mepyramine-treatment alone, i.e., no further suppression of labelling in the vessels was achieved.

Section 10

Lymph-Node-Cell Permeability Factor:

Extracts of lymph node cells from various species are capable of producing considerable increases of vascular permeability (Willoughby, Boughton, Spector and Schild, 1962). These authors described the factor present in the lymph node extract as the lymph-node-cell permeability factor (LNPF). The factor has since been demonstrated in other tissues in the rat (Walters and Willoughby, 1965a). The pharmacological properties of LNPF differ from those of other known permeability-increasing substances (Willoughby, Boughton, and Schild, 1963). In addition to its effect on vascular permeability, LNPF has been shown to possess the more unusual property of inducing an immediate large-scale emigration of leucocytes in the rat (Spector and Willoughby, 1964b; see Willoughby and Spector, 1964c). Another sequel to the intradermal injection of LNPF in the rat is the formation of an eosinophilic deposit with a resemblance to connective tissue fibrinoid (Willoughby and Spector, 1964b), which gives a positive stain for RNA during the first 20 min. after injection of the extract (Willoughby, Walters and Spector, 1964). LNPF has been shown to be present in the developing tuberculin reactions and to disappear as the reactions subside (Willoughby, Spector and Boughton, 1964). More recent work on the properties of LNPF has revealed many similarities and some differences between LNPF and RNA and the findings suggest that RNA, or more likely a derived oligonucleoside may account for the effects of lymph node extracts (Willoughby and Walters, 1965). Results of further investigations on the role of LNPF, have led to the belief that it may be associated with delayed hypersensitivity and auto-immune reactions (see Willoughby and Spector, 1965; Willoughby, Walters and Spector, 1965; Willoughby, 1966; Willoughby and Coote, 1966; Schild and Willoughby, 1967).

The following experiments demonstrate the presence of a "LNPF" in the

lymph node cell extracts of normal sheep and attempt to describe some of its properties.

(i) Effect of lymph node cell extracts on vascular permeability:

To prepare lymph node cell extract grossly normal lymph nodes (prescapular, prefemoral, mediastinal and mesenteric) were collected from five sheep. A separate extract was prepared from each individual animal using the method of Willoughby, Boughton and Schild (1963). Lymph nodes were freed of fat and the cells teased out with forceps into Tyrode solution. The percentage composition of the Tyrode solution was the same as used by Boughton and Schild (1962), i.e., NaCl 0.8 per cent, KCl 0.02 per cent, CaCl2 0.02 per cent, $MgCl_2$ 6.H20 0.01 per cent, NaH2P042.H20 0.005 per cent and glucose 0.1 per cent. The suspension was freed of coarse debris by passage through fine nylon and the cells collected by centrifugation at 4000 r.p.m. for 10 min. The pad of cells was washed twice by resuspension in Tyrode solution and the number of cells estimated using a haemocytometer counting chamber. The final suspension contained approximately 500 million cells per ml. and was treated in one of the following ways: (a) Cell suspensions were frozen and thawed ten times until microscopic smears showed no whole cells but only debris which was removed by centrifugation at 4000 rev./min. for 15 min. Most of the experiments were carried out with the supernatant obtained in this way. (b) After treating cells with an ultrasonic disintegrator of 20,000 cycles/sec. for two minutes, microscopic examination revealed no intact cells. The supernatant obtained in this way had the same permeability-increasing activity as that obtained by freeze thawing. The lymph node cell extract was either used at once or stored at -10°C until required.

Dialysis of the cell extract is necessary to remove any histamine present. This was carried out by exposing the extract to three changes of

saline in dialysis tubing at 4°C.

Two blued sheep were made to test the effect on vascular permeability of the extract. Both undialysed and dialysed extract was injected intradermally in 0.1 ml. volumes, representing an extract of 5×10^7 cells. Nine injections of each extract were made and distributed on both sides of the sheep.

Leakage of dye occurred after a latent period of 1 - 3 min. The intensity of blueing reached its maximum in about 15 min. When the undialysed lymph node extract was tested on guinea-pig ileum it showed some histamine-like activity; no such activity was seen after the dialysis. The dialysed lymph node extract showed similar permeability-increasing activity as the undialysed extract: in both cases it was evident within 5 min. and reached a maximum 15 min. after intradermal injection. The permeability activity of the extract was apparently unchanged when the extract was left standing at room temperature for one day or when stored at 4°C for two weeks, or stored at -10°C for 4 months.

(ii) Comparison of the area of dye leakage induced by lymph node extract and other permeability factors;

Lymph node extract was injected intradermally in a blued sheep in volumes of 0.1 ml. Histamine, 5-HT, bradykinin and Compound 48/80 were injected in 10 µg amounts in volumes of 0.1 ml. 6 hr. pleural exudate was injected in 0.1 ml. volumes. Each of the vascular permeability factors was injected once in a series on each side of one sheep, using a different syringe for each test substance. The diameter of the blue areas that developed following the intradermal injections of the permeability factors was recorded after 15 min., 1 hr., and 5 hr. The results are summarised in table 14.



Fig.57 Skin of a lamb showing blueing induced by lymph node cell extract and other permeability factors. Photographed 15 min. after the intradermal injections of the following substances. From left: 6 hr turpentine-induced pleural exudate (0.1 ml.), histamine (10 µg), Compound 48/80 (10 µg), lymph node cell extract (0.1 ml. representing an extract of 5×10^7 cells), 5-HT (10 µg) and bradykinin (10 µg).

Table 14 - Comparison of the area of dye leakage induced by lymph node extract and other permeability factors.

Time after intradermal injection	Blue area (maximum diameter mm.)					
	Pleural exudate 6 hr. (0.1 ml.)	Histamine 10 µg	48/80 10 µg	Lymph node $5x10^7$ cells	5-HT 10 µg	Bradykinin 10 µg
15 min.	11	16	11	15	15	18
1 hr.	14	19	15	18	17	19
5 hr.	43	50	35	45	18	20

In the early stages (i.e., up to one hr.) the diameter of the blue areas produced by lymph node extract and other permeability factors were similar (fig. 57), The intensity of the blueing differed slightly with each test substance; 48/80 produced blueing of the lowest intensity. 5 hr. after injection the areas differed markedly.

(iii) Histopathological changes following intradermal injection of lymph node extract:

The ability of the lymph node cell extracts to induce leucocyte emigration was studied by intradermal injections of the dialysed extract into a sheep in 0.1 ml. volumes. Skin biopsies were taken 15 min., 30 min., 1 hr., 2 hr., 4 hr., 6 hr., and 24 hr. after the intradermal injections. Skin samples were fixed, sectioned and stained with H & E and for mast cells. The results are described under the heading of each time interval.

15 min: Sections revealed vascular dilatation and congestion. Hyperaemic vessels showed margination of polymorphs. There was oedema but very little emigration of leucocytes. Mast cells appeared more numerous than in the normal skin. They were smaller and contained well-defined cytoplasmic

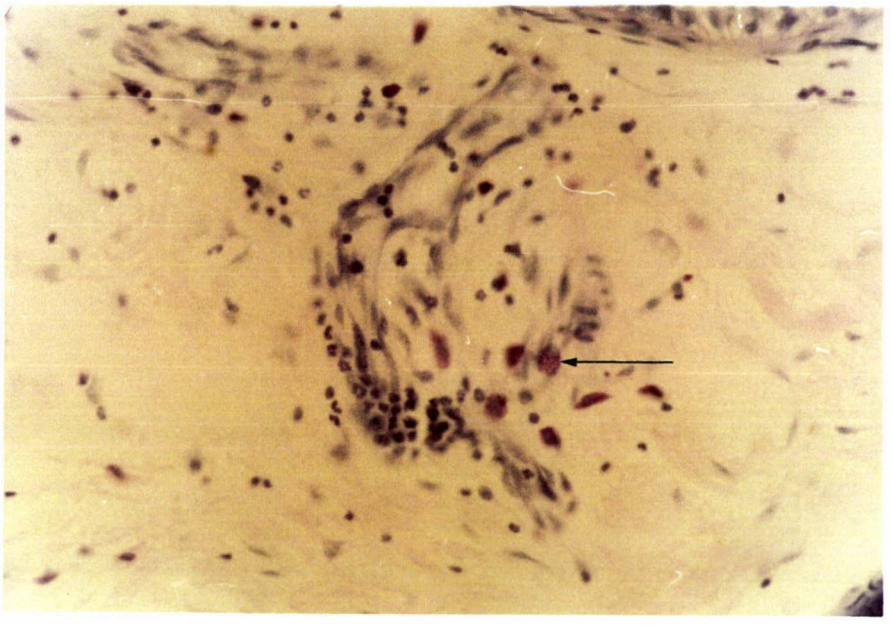


Fig.58 Section of sheep skin 30 min. after intradermal injection of lymph node cell extract (0.1 ml., representing an extract of $5x10^7$ cells) showing margination of polymorphs but only slight emigration. More numerous mast cells (arrow) are seen than in the normal skin; they show varying degrees of swelling.

Leishman stain x 250

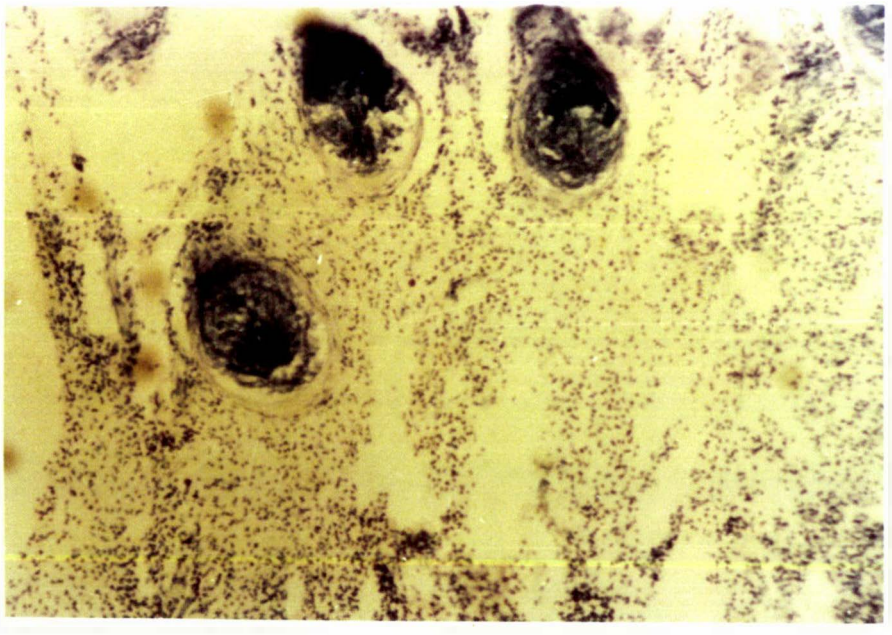


Fig.59 Section of sheep skin 6 hr. after intradermal injection of lymph node cell extract (0.1 ml., representing an extract of $5x10^7$ cells) showing extensive neutrophilic infiltration of tissues.

Leishman stain x 100

granules. Some of the cells showed vacuolation and mild degranulation.

30 min: Margination of the polymorphs was more marked but still the cells were mostly confined in the blood vessels (fig. 58). Congestion of blood vessels and oedema had slightly increased. Mast cells were increased in number; vacuolation and degranulation was more marked. Occasionally, a basophil leucocyte could be seen lying inside the blood vessels with polymorphs.

1 hr: Emigration and infiltration of the tissues with polymorphs had commenced. These cells were at this stage mostly in the perivascular areas. Mast cell changes ranged from swelling and degranulation to disruption of the cells with scattering of the granules.

2 hr: The changes were essentially similar to those seen at 1 hr. Leucocyte emigration had markedly increased; the cells had now moved away from their perivascular sites and were diffusely scattered throughout the dermal tissues. Mast cells revealed changes similar to those seen at 1 hr.

4 hr: There was further leucocytic infiltration of the tissue spaces which appeared distended with fluid. Degranulation of mast cells was marked and the cells appeared less numerous. They were not seen in areas of leucocytic infiltrations.

6 hr: Further increases in the above changes were noticed particularly in leucocyte emigration (fig. 59). Fluid distension, especially of the subdermal connective tissue was considerable. Similar changes in mast cells were seen as were noted at 4 hr.

24 hr: Leucocyte emigration and infiltration of the tissues was at its height at this stage, many of the polymorphnuclear neutrophils being degenerate. Oedema of the tissues was marked. Mast cells appeared more numerous than in the proceding stage and still showed varying stages of swelling and degranulation. As noted earlier the cells could not be detected in areas

of leucocytic infiltration and oedema.

Lymph node extract appeared to induce more extensive emigration of leucocytes than either histamine, 5-HT or bradykinin, but the emigration was less extensive than that induced by 6 hr. pleural exudate.

(iv) Effect of mepyramine maleate on the increased vascular permeability induced by lymph node extract:

Two blued sheep were used. Mepyramine maleate was injected intramuscularly in a single dose of 25 mg/kg body wt. 15 min. before intradermal injections of dialysed lymph node extract in volumes of 0.1 ml. Six intradermal injections were made on each side of each sheep. The dialysed lymph node extract had been shown to have no histamine-like action in guinea-pig ileum.

Pretreatment with mepyramine delayed the increase in vascular permeability which normally followed the intradermal injection of 0.1 ml. volumes of lymph node extract. The leakage of dye was suppressed for 2 - 3 hr. only , after which marked blueing took place, reaching a maximum in about 2 hr.

(v) In vitro study of the histamine-releasing activity of lymph node extract using chopped guinea-pig and sheep lung:

The ability of the lymph node extracts and other test solutions to release histamine from chopped guinea-pig and sheep lungs was tested using a modification of the method of Monger and Schild (1953). Histamine was assayed on the guinea-pig ileum.

Lung preparation: The histamine-releasing activity of the lymph node extract was tested on the guinea-pig and sheep lungs separately, and was compared with the activity of 6 hr. pleural exudate and compound 48/80 tested in the same way. A total of four guinea-pig and four sheep lungs was used. The fresh lung was first chopped into small pieces. Four samples of equal weight

(500 mg.) were incubated separately at 37°C (in a water bath) with 2 ml. of Krebs' Ringer solution in a 10 ml. beaker for 30 min. The contents of the beakers were kept stirred and at the end of 30 min. the Ringer solution was discarded to remove any spontaneously released histamine. The discarded Krebs' Ringer was replaced in three of the four lung samples with either 1 ml. dialysed lymph node extract or dialysed 6 hr. pleural exudate or Krebs' Ringer containing 1 mg/ml. of compound 48/80. All sclutions were kept at 37°C. To the fourth lung sample 1 ml. Krebs' Ringer was added to check for spontaneous release of histamine. Before the experiment commenced the dialysed lymph node extract, dialysed pleural exudate and the Compound 48/80 solution were shown to exert no histamine-like action on guinea-pig ileum.

The lung samples were incubated for a further period of 30 min. at 37°C. At the end of this time fluid was removed from each sample with a pipette and examined for the presence of any released histamine by testing against guinea-pig ileum.

Guinea-pig ileum preparation: A segment (1-2 cm.) of terminal ileum was suspended in oxygenated Krebs' Ringer solution in a 5 ml. bath at 37°C. Histamine was assayed using an automatic apparatus.

It was found that when 0.1 ml. volumes of fluid samples were added to 5 ml. organ bath, all the treated solutions including the control (i.e., containing Krebs' Ringer only) induced contraction of the ileum. This suggested that the control Krebs' Ringer solution contained some spontaneously released histamine. To overcome this difficulty all the treated solutions, as well as the control Krebs' Ringer were diluted five times with Krebs' Ringer Solution. When tested on the ileum, whereas the other solutions still induced the contractions of ileum, the dilution nearly abolished the activity of the "control" Krebs' Ringer Solution. All the solutions were

then assayed for histamine.

The contractions of the ileum induced by the test solutions were compared with those produced by 0.002 µg, 0.01 µg, and 0.2 µg histamine. This was done by adding histamine to the 5 ml. organ bath in 0.1 ml. volumes in a concentration of 0.02 µg/ml., 0.1 µg/ml. and 2 µg/ml. respectively in Krebs' Ringer Solution. After the histamine contractions, the effects of lung-treated samples, i.e., control Krebs' Ringer, Krebs' Ringer containing 48/80, 6 hr. pleural exudate and lymph node extract were recorded, in that order. Each solution was added in 0.1 ml. volumes to the organ bath and maximum height of the contraction recorded. After testing effect of each sample, the organ bath was washed with Krebs' Ringer for 10 seconds.

To show that the activity of the solutions was due to the presence of histamine and not 5-HT, the effect of mepyramine was tested. Mepyramine in concentration of 0.4 µg/ml. abolished contractions of the ileum induced by all the test solutions. Further, when the test solutions were tested on the rat uterus for 5-HT, they were found inactive.

Dialysed lymph node extract induced some histamine release from chopped guinea-pig lungs, but the effect was not marked. On the other hand, when tested on the sheep lungs, lymph node extract showed marked histamine-releasing activity. Similar results were obtained with all four lung samples. As judged from the guinea-pig ileum assay, compound 48/80 appeared to induce more histamine release from sheep lung than from guinea-pig lung: the lungs from both species having been treated in the same manner.

D I S C U S S I O N

Discussion

The present results have shown that a biphasic increase in vascular permeability develops in the inflammatory reaction in the sheep following turpentine and thermal injury; an early phase seems to be mediated by histamine. In contrast, mediation of the delayed phase, which appears to be the major part of the response, remains uncertain. Histamine, a "Globulin PF" and possibly kinins, probably participate. The results further suggest that permeability substances activated or released by injury, do not appear to exert much effect on the emigration of leucocytes, and, thus, support the idea of a separate endogenous mechanism for this - separate, that is, from that leading to increased permeability to protein.

One of the most striking results from the present experiments, is the apparent ease with which histamine can be released in sheep skin and with a wide variety of stimuli. The situation is quite different to that obtaining in the rat and, perhaps, more akin to that in the guinea-pig, though there is less information in the latter species.

The contribution histamine made in the inflammatory reaction was revealed by the application of a number of different but complementary techniques. The major techniques which have been used to investigate the role of histamine include:

(i) The use of antihistamines

(ii) Depletion of, or attempts to deplete histamine

(iii) Blocking of histamine release

The first of these has been employed in relation to studies on the reaction to exogenously derived, i.e., injected histamine, to injections of the histamine liberator, compound 48/80, and on the reaction to turpentine injury, thermal injury, or cutaneous antigen-antibody reactions. Studies

made by using antihistamines required their careful selection, since many antihistamines are not specific in their action in blocking histamine. Mepyramine maleate is well known for its high specificity as an antihistamine and has not been shown to antagonise any other compound which increases vascular permeability (Halpern et al, 1950; Spector and Willoughby, 1957a, 1959a, 1963b; Parratt and West, 1958a and b; see also Miles and Wilhelm, 1960a; Wilhelm,1962). In the sheep mepyramine maleate suppressed the increased vascular permeability induced by high doses of histamine (i.e., in 100 µg amounts). In contrast, promethazine hydrochloride failed to give a complete suppression. The results confirm in the sheep specific antagonism of histamine by mepyramine maleate.

Of the wide variety of agents which release histamine from mast cells, compound 48/80 is considered the most specific (Paton, 1951; Fawcett, 1954). Marked species differences have been noted with the histamine-releasing activity of 48/80 (Sparrow and Wilhelm, 1957, Riley, 1959). The increased vascular permeability induced by 48/80 injected in small amounts in the sheep, and its suppression by mepyramine maleate indicates that 48/80 releases histamine in the sheep. It was later used to deplete tissues of histamine and then study the inflammatory response. The failure of mepyramine maleate to block the response induced by high doses of 48/80 (i.e., in 1 mg. amounts) could be explained on the basis of the large amount of histamine released by this high dose quantitatively over-riding the effect of the antihistamine available. Alternatively, it could be suggested that at this dose 48/80 released some substance in addition to histamine which affects vascular permeability and is not antagonised by mepyramine maleate. Similar observations have been made by other workers in other species with large doses of 48/80 (Sparrow and Wilhelm, 1957; Paton, 1957; Miles and Wilhelm, 1960a)

In an attempt to suppress locally the permeability response that followed intradermal injection of turpentine by mepyramine maleate, it was found that mepyramine maleate solution ("Anthisan"), by itself, increased vascular permeability. Solutions with a pH ranging between 3 and 10 did not increase vascular permeability. It seems the mepyramine maleate solution did not increase permeability due to its acid pH. No increase in permeability was induced by intradermal injection of mepyramine maleate in those sheep previously given mepyramine maleate, systemically. This may indicate that the antihistamine solution increased permeability by causing a release of histamine.

Release of histamine by antihistamines has been reported. Arunlakhna (1953) showed that the antihistamine diphenhydramine and antazoline liberated histamine from guinea-pig tissue, but she did not test mepyramine. Paton and Schachter (1951) reported that the acid gastric secretion induced by 48/80 in dogs was greater after mepyramine treatment; this was probably because mepyramine liberated histamine. Sparrow and Wilhelm (1957) found that in guinea-pigs doses of mepyramine just below the dose which would cause blueing has a peculiar enhancing effect on compound 48/80 when injected intradermally at the same site. The authors suggested that this effect of mepyramine may have been due to the fact that it is a histamine liberator. These findings are supported by the results obtained in the sheep.

The previous administration of mepyramine maleate gave almost complete suppression of exudate formation in the 2 hr. following the intrapleural injection of turpentine. However, the continued reduction in the volumes of exudate obtained 4 and 6 hr. after intrapleural injections of turpentine suggests a continuing contribution of histamine in the later phases of the inflammatory reaction in the sheep. This contrasts with the situation in

the rat in which mepyramine gives complete suppression of exudate formation for not longer than 30 min: no significant difference was seen in the volumes of exudates formed in mepyramine-treated rats and control animals 4 hr after the intrapleural injection of turpentine (Spector and Willoughby, 1959a).

In contrast to turpentine injury, the permeability response to thermal injury in the skin of the sheep was suppressed by mepyramine maleate for only 8 - 15 min. This indicated that in thermal injury the release of histamine was of a much shorter duration. This appears similar to the situation in the rat (Spector and Willoughby, 1959b), and also guinea-pig and rabbit (Wilhelm and Mason, 1960). Direct comparison of the sheep with other species is difficult, since the amount of heating used in sheep is more than that used in the laboratory animals. It has been shown in the laboratory animals that the onset of delayed permeability response, which is not dependent on histamine, is hastened by the use of higher temperatures. The suggestion has been made that in high temperature injury the pharmacological picture may be complicated by non-specific breakdown products (Wilhelm and Mason, 1960), or that there will have been direct physical damage to the blood vessels in the skin (Spector and Willoughby, 1959b). However, these observations do not seem to confound the present results in sheep, for two reasons. Firstly, the leakage of dye followed a latent period as in the rat and guinea-pig. And secondly, after this initial latent period, the permeability response was suppressed by mepyramine maleate. This is consistent with results obtained in the laboratory animals, and indicates the specific mediation of the reaction by histamine. It is suggested that the degree of thermal injury inflicted in the sheep was comparable with that used in the rat and the guinea-pig to produce burns of moderate severity. A <u>relative</u>

insusceptibility of the sheep to the heat applied may be due to the greater thickness of the skin or to the fact that, although the skin was closely clipped, it was not subjected to depilatives, as it was in the experiments on the laboratory animals.

Apart from the above, the variation in results obtained by different workers in response to similar degrees of heating in the same species, introduces yet another difficulty in comparing the effects of thermal injury. Spector and Willoughby (1959b), and Wilhelm and Mason (1960) obtained different results with almost similar degrees of heating in the rat. The present results suggest greater involvement of histamine in the sheep than in the rat, and in this way the sheep resembles the guinea-pig (Wilhelm and Mason, 1960). Some suppression of oedema formation in response to thermal injury in the sheep for up to 3 hr. after mepyramine maleate-pretreatment, further suggests that histamine continues to have some effect over a long period in the sheep. This contrasts with the situation reported both in the rat (Spector and Willoughby, 1959b), and the guinea-pig (Wilhelm and Mason, 1960).

In addition to turpentine and thermal injury, mepyramine maleate was used to try to suppress the permeability response resulting from antigen-antibody reactions. A complete absence of increased vascular permeability in the Reversed Passive Arthus Reaction (RPA) after mepyramine maleate-pretreatment, indicated that in this reaction too the permeability response was mediated by histamine. No development of increased permeability over 12 hr suggested that the permeability response, which normally occurs within this period, was mediated by histamine only.

Release of histamine in Arthus reaction in other animals has not been clearly demonstrated. Antihistamine drugs failed to modify the inflammatory

response in the Arthus reaction in rat and guinea-pig (Brocklehurst et al, 1955; see also Cochrane, 1965). Histamine mediation of the RPA-reaction in the present study may indicate a species difference; or may have been because very small quantities of antibody were used, and that the reaction produced was very mild. It has been shown in passive cutaneous anaphylaxis (PCA) that when small doses of antibody are used, mepyramine maleate suppresses the reaction (Ovary and Bier, 1953; Halpern et al, 1959); but neither mepyramine nor triprolidine suppress the more intense reactions (Ovary and Bier, 1953; Alberty and Takkunen, 1957; Brocklehurst et al, 1960; Craig and Wilhelm, 1963). Cochrane (1965) pointed out that although evidence suggests that the release of histamine plays little part in the development of Arthus reaction, the relatively great quantities of antigen and antibody employed necessitates caution in this interpretation. Spector and Willoughby (1964c) suggested that in Arthus reaction, the reactions produced may be too explosive a type for histamine to exert a separate effect. It is likely that in a severe RPA-reaction in the sheep, and in the more explosive active type, the permeability response might not entirely depend on histamine. Any release of histamine might also be associated with activation of other vasoactive substances like plasma kinins. Results obtained in attempts to produce the Arthus reaction in the sheep, also suggest the possibility that in a severe Arthus reaction the increase in vascular permeability may be biphasic. The early phase may be an anaphylactic-like response mediated by histamine; and the Arthus reaction may be superimposed on it. This suggestion has been made for laboratory animals (Humphrey, 1955a, b; Hayashi et al, 1964; see Cochrane, 1965).

The same antigen-antibody system for the RPA-reaction was used to produce passive cutaneous anaphylaxis, but the reaction was not obtained as judged by the leakage of dye. This may have been because the antiserum

which contained precipitating antibody, as shown by the double diffusion in agar gel, perhaps, lacked the tissue "fixed" antibody on which the cutaneous anaphylactic reaction depends (Ovary and Bier, 1953). It is possible that the rabbit antibody employed to produce both RPA- and PCA-reaction might have failed to sensitize the sheep for PCA. Antisera have been found capable of provoking Arthus but not cutaneous anaphylactic responses and vice versa (see Cochrane, 1965). It cannot be suggested and, seems unlikely, that it is difficult to elicit PCA in the sheep, unless antisera containing known tissue "fixed" antibody are tested.

To recapitulate, with a single antigen-antibody system an RPA-reaction was produced in the sheep but PCA could not be produced. The reasons for this failure have been discussed. The Arthus reaction was very mild, possibly since very small amounts of antibody were used. It was mediated by histamine, although in a more severe reaction other vasoactive substances might be released together with histamine.

Interest in the mode of action of antihistamines led to the use in the present experiments of the cardiac glycoside strophanthin-G. This followed the report by Judah, Ahmed and McLean (1965) of the similarity of action between the antihistamine promethazine and strophanthin-G. Suppression of the early inflammatory changes in the skin and pleura by strophanthin-G suggested that strophanthin-G may block the effects of local histamine release.

The protective effects of antihistamines in preventing liver cell necrosis have been reported (Gallagher et al, 1961) and interpreted to be due, in part, to their interaction with the system in the cell membrane that regulates active transport of sodium and potassium ions (Judah et al, 1964). Both, the antihistamine promethazine and strophanthin-G have been reported to protect the rat liver against thioacetamide induced liver necrosis (Gallagher et al, 1956; Judah et al, 1965). More recently, it has

been reported that promethazine and strophanthin-G show approximately equal activity in inhibiting potassium ion transport (Judah et al, 1965). In the sheep the suppression by strophanthin-G of the early inflammatory changes induced by histamine could be due to inhibition of the sodium/potassium ion transport mechanism of the vascular endothelium and this could, in return, suggest that part of the action of the antihistamine mepyramine maleate against histamine may be due to its action on the ion transport mechanism of the endothelial cell membrane. It is suggested that strophanthin-G may prove useful in the study of inflammatory reaction, particularly histamine liberation and the mechanism of exudate formation in the sheep.

Histamine depletion studies:

Different animal species do not show the same sensitivity to histamine. The present findings that histamine produced death in the sheep in doses from 3 mg. to 15 mg/kg are consistent with those reported by Alexander et al, (1967). Compared with cattle (Deslieus, 1958, cited by Nilsson, 1963) and pigs (Smith and Alpert, 1955), the sheep appears somewhat less sensitive to histamine, but more sensitive than the dog (Halpern, cited by Nilsson, 1963). No information concerning the toxicity of 48/80, or the release of histamine by various stimuli used in the present study, could be found in the literature relating to either sheep or other species of domesticated animals. In view of the present findings of the apparent ease with which histamine is released in the sheep and the rapidity of death following toxic doses of 48/80 and histamine, it is suggested that these findings may help to explain sudden deaths in sheep, which are common in certain acute toxaemic diseases like clostridial infections. It is of interest that the post-mortem picture seen following lethal doses of 48/80, resembled very closely those in clostridial

infections (see Jubb and Kennedy, 1963). It is possible that clostridial infections in sheep may involve sudden widespread release of histamine, mainly from the intestinal tract, in amounts lethal to the animal, and, thus, contribute to the sudden deaths.

When turpentine-induced inflammation was produced in sheep depleted of histamine by 48/80 given systemically, similar results to those found by using mepyramine maleate were obtained. The results supported previous ones indicating a continuing, though after the early stages, a decreasing role for histamine in the inflammatory reaction and a greater significance of histamine in inflammation in the sheep than in the rat.

In histamine depleted sheep, histamine produced less reaction in the skin than in control animals. This may have been due to reduced sensitivity of the skin to histamine following prolonged 48/80 treatment. This has been reported to be so in the rat skin, where Brocklehurst et al, (1955) found that depletion of skin histamine by subcutaneous injections of 48/80 reduced the effect of intradermally injected histamine by about 50 per cent (see also Wilhelm, 1962). In the present experiments sheep were treated over a slightly longer period with 48/80 than in similar experiments in rats (Spector and Willoughby, 1959a).

The conclusions which can be drawn concerning histamine from the present experiments depend in part on the specificity of the drugs used. It has been suggested that even mepyramine maleate can be shown to have some non-specific action in high doses (see Spector and Willoughby, 1965). Although mepyramine has been used at 25 mg/kg in the present experiments, it appeared a specific antagonist to histamine in the sheep. Even, in much higher and fatal doses (50 mg/kg), mepyramine still did not suppress the increased vascular permeability in the delayed phase of inflammation. Moreover,

results obtained from histamine depletion using compound 48/80 were consistent with those using mepyramine. It was also found that whereas, mepyramine antagonised the action of histamine in the skin, it consistently failed to suppress the action of bradykinin and kallikrein. Failure of mepyramine maleate to suppress the action of these permeability factors, leads us to a consideration of endogenous substances responsible for the delayed phase of the increased vascular permeability: this is discussed later.

It is interesting to speculate on the possible explanations for the species differences observed. The apparent ease with which histamine is released in the sheep skin when compared with rat may be due to differences in sensitivity of the mechanism of histamine release. The greater sensitivity of the sheep skin to histamine and the larger participation of histamine in the inflammatory reaction in the sheep may, theoretically, be due to some or all of the following possible differences in species:

(i) in the enzyme-system that inactivates histamine; (ii) in the amount of histamine released in response to a standard injury, its rate of release and rate of degradation; (iii) in local increase of the synthesis of histamine following injury; or (iv) in the responsiveness of the blood vessels to the same amount of histamine.

Blocking of histamine release:

The release of histamine from mast cells is now believed to be an enzymic process. The presence of both SH and NH_2 groups is considered necessary in the enzyme system that releases this histamine (see Uvnas, 1964). The failure of alloxan, an SH-blocking agent (Barron, 1951) to suppress histamine release in the sheep, suggests that the mechanism of histamine release may differ in the sheep and the rat. However, since

SH-blocking agents other than alloxan have not been tried in the present experiments, this limits such an interpretation. Alternatively, the failure of alloxan to suppress histamine release in the sheep may be due to differences in penetration into the cells of the various SH-blocking compounds or to variations in susceptibility of the SH-groups to different types of inactivation. In the rat, Spector et al, (1963) found that use of an SH-blocking agent other than alloxan failed to diminish the exudate formation and offered the above explanation for its failure. It is not clear whether there are species differences in the mechanism of histamine release or not. Studies on various inhibitors of SH and NH2 groups may prove useful. Results with strophanthin-G (ouabain) suggest yet another possibility. Strothanthin-G suppresses the early histamine-mediated response in the sheep. On the other hand, it has been reported not to affect the inflammatory response in other (unspecified) animals (see McLean, Ahmed, and Judah, 1964). Investigations into the ion transport mechanism of the cell membrane in relation to histamine release should prove a useful adjunct to studies on inflammation.

The points enumerated as (i) - (iv) on page 147 will now be considered in the same sequence as listed earlier.

(i) The most important means of inactivation of histamine is an oxidizing deamination by the enzyme diamine oxidase (histaminase), which normally destroys free, unbound histamine and prevents its toxic effect (see Tabor, 1954). The concentration of histaminase in the tissues varies in different animals (see Rocha E Silva, 1955b). It can be argued that since sheep skin is poor in mast cells, it may contain less histamine, and possibly therefore need a lower concentration of histaminase than the rat. The same argument can be applied to the guinea pig and the rabbit which also contain fewer mast cells in the skin and also less histamine than the rat (see Riley, 1959).

This would suggest that the same amount of histamine injected intradermally would be subjected to less inactivation in the sheep, guinea-pig, and the rabbit than in the rat, and would thus produce more reaction. This appears to be the case, and may partly explain the greater sensitivity of the sheep skin to histamine. The use of inhibitors of histaminase in sheep may throw further light on this.

(ii) In relation to the apparent greater importance of histamine in the inflammatory reaction in the sheep, several possibilities may be considered. Whereas the released histamine may be subjected to less inactivation, as considered above, it is possible that histamine in comparatively larger amounts may be released in response to a standard injury. Mast cells in the sheep skin appeared more numerous following an injury than in normal skin. This may be associated with a corresponding increase in the histamine content of the inflamed skin, which could contribute to a greater role for histamine.

Mast cells contain histamine, but not all the histamine in the body is held in the mast cells (West, 1956). Paton (1956) also suggested that all of the histamine in the body is not contained in the mast cells, and gave examples of epidermis in the cat and in man (Harris, 1927; cited by Paton, 1956). In the pig, histamine in the stomach is bound directly to the gastric mucosa and not contained in the mast cells (Riley and West, 1956). The apparent release of histamine by hyaluronidase in the sheep skin may suggest some source of histamine outside the mast cells. Histamine may be released in the depolymerization of the connective tissue mucopolysaccharides, which hyaluronidase is known to cause. Hyaluronidase is said to accumulate in inflamed tissues (see Florey, 1962).

(iii) Another factor of importance could be an increase in local synthesis

of histamine following injury. Schayer (1960, 1961a and b, 1963) has shown that following injury there is increased activity of the histamine-synthesizing enzyme histidine decarboxylase, which he assumes leads to an increased histamine synthesis. This is considered possible in the sheep, but remains to be investigated: use of inhibitors of histidine decarboxylase may prove useful.

(iv) Differences in the responsiveness of the blood vessels should also be considered. Spector and Willoughby (1963a) have stated that hyaluronic acid is probably a major constituent of the basement membrane, and perivascular ground substance. In the rat, Spector (1958) found that hyaluronidase increased vascular permeability at a concentration of 1 mg/ml but not below this level. As the amount injected was not stated, it is possible that the lowest dose of 500 µg of hyaluronidase needed to produce increased vascular permeability in the sheep may not be the same as in the rat. Since hyaluronic acid can reduce the rate of filtration of colloids like albumin (Ogston and Sherman, 1961), it can be argued that a low concentration in the vessels may facilitate an increase in permeability to plasma proteins, i.e., render them more responsive to the action of permeability-increasing substances. It is also possible that instead of a quantitative difference, the hyaluronic acid molecule may differ in its location in the vessel wall. Investigation of factors that control the responsiveness of blood vessels, may help to explain different response produced by the same amount of histamine in different animals.

It may also be that inter-species differences in blood vessel structure, perhaps, at a molecular level, account for variation in the type of vascular response in inflammatory reaction as indicated by the predominantly venular response in sheep and minimal capillary involvement.

In contrast, in the rat although there is greater involvement of venules, capillaries also show significant increase in vascular permeability (Hurley and Spector, 1965; Spector, Walters and Willoughby, 1965b).

Until more information is available on the way in which histamine is released, on how it actually produces an increase in vascular permeability and the way in which antihistamine drugs work, it will not be possible to explain the species differences discussed. Studies in these different species may contribute to obtaining the explanations.

Factors other than histamine concerned in the permeability changes:

The possibility that factors other than histamine contribute to the mediation of the early permeability response: and, studies on the delayed phase, will not be considered. Antihistamines again proved of value in these studies - in attempting to determine whether histamine or some other factor was involved.

Suppression by mepyramine maleate of the increased vascular permeability induced by 5-Hydroxytryptamine in the sheep, in contrast to its failure to do so in the rat (Spector and Willoughby, 1963b), indicated that 5-HT in the sheep produced its activity by releasing histamine. Histamine release by 5-HT has been observed in certain experimental conditions. Sparrow and Wilhelm (1957) noted histamine release by 5-HT in the excised skin and perfused hind quarters of rat and concluded that in the rat 5-HT increases permeability, at least in part, by the release of histamine. A point worth re-investigation in the rat may be the release or not under *in vivo* conditions of histamine by 5-HT. Other *in vitro* studies by Feldberg and Smith (1953) showed that histamine was liberated in perfused isolated skin flaps from the cat and dog on treatment with 5-HT. These *in vitro* observations support the results in the sheep.

Species differences in the amount of 5-HT in the mast cells have been reported. It is found in only rat and mouse mast cells, and is not present in significant amounts in mast cells of other animals: no mention was made of the position in the sheep (Parratt and West, 1957a). It is possible that mast cells in the sheep may similarly lack 5-HT. The present results suggest that 5-HT is not a natural mediator of the inflammatory reaction in the sheep, unlike the rat (see Spector and Willoughby, 1963a).

Results obtained with various nucleosides and hyaluronidase, used in conjunction with mepyramine maleate, also indicate that in sheep these substances produce their activity by releasing histamine. Release of histamine by nucleosides in the sheep is similar to findings in the rat (Moulton et al, 1957; Spector and Willoughby, 1957a).

The relationship between hyaluronidase and histamine is not clear. Though there are conflicting reports regarding the mode of action of hyaluronidase, its action through histamine has been suspected. Elster et al (1949) found that tripelennamine, an antihistamine, inhibited the effects of hyaluronidase in the rat. These workers suggested that histamine or histamine-like substances may be released by the rat in response to hyaluronidase and tripelennamine may be blocking the action of histamine. The results obtained in the sheep support their observations. However, hyaluronidase has been reported to have no significant action on the vascular permeability (see Spector, 1958). It has been further suggested by Florey (1962) that hyaluronidase is of no importance in inflammation.

A spreading effect of hyaluronidase on the increased vascular permeability produced by histamine, was observed in the sheep. However, with increased doses of hyaluronidase, still within the non-blueing range, the blue area did not increase in size. This suggested that although

hyaluronidase exerted a spreading effect, it did not potentiate the permeability activity of histamine.

The failure of mepyramine maleate to suppress the increased vascular permeability induced by bradykinin and kallikrein, indicates that the permeability response induced by these substances is not dependent on the release of histamine. However, the brief delay in the leakage of dye noticed in the early stages, both in the case of bradykinin and kallikrein, does point to some histamine release by these substances. This contrasts with the results obtained in the rat (Spector and Willoughby, 1963b). No mention of histamine release by bradykinin could be found in the literature. However, polypeptides have been reported to possess weak histamine-releasing properties and certain basic peptides are histamine liberators (see Spector, 1958). Further, "leucotaxine" which is considered to be a polypeptide has been found to increase vascular permeability by releasing histamine (Miles and Miles, 1952; Miles and Wilhelm, 1960).

The slightly longer latent period of 3 - 4 min. after which an increase in vascular permeability was noticeable in the case of kallikrein, and a gradual increase thereafter, contrasts with the results of histamine, 5-HT and bradykinin. Kallikrein is a kinin-forming enzyme present in plasma. Bhoola et al (1960) suggested that increased permeability caused by the injection of kallikrein into the skin is, presumably, due to the formation of kinins in vivo. This may explain the results obtained in sheep and could account for the slightly longer latent period noticed with kallikrein, because of the time needed for the formation of kinins in the skin. Some release of histamine by kallikrein may be explained on the basis of the previous result showing that bradykinin has a weak histamine-releasing action at the site of intradermal injection in the sheep.

Further investigation into the mediation of the delayed permeability response included a study of the inflammatory exudates, induced by intrapleural injection of turpentine. Failure of dialysis to eliminate the permeability-increasing activity of the undiluted and diluted pleural exudates and the diluted sheep serum, indicated that they contained a permeability factor of a large molecular size. The factor, however, was not identified by fractionation in the present work. The results in the sheep closely resemble those of Miles and Wilhelm (1955) using diluted guinea-pig serum, and of Spector (1956) using diluted rat serum and turpentine-induced pleural exudate. These workers found the factor concerned to be a globulin. This suggests a similarity, or possible identity, between the Globulin PF isolated by them and the vasoactive factor found in diluted sheep serum and pleural exudate.

Suppression of the increased vascular permeability due to "Globulin PF" by mepyramine maleate further indicates that "GPF" releases histamine in the sheep skin. Some histamine release by pleural exudate *in vitro* from the guinea-pig and sheep lungs provides further support for this proposition. A failure of mepyramine to suppress the increased permeability for longer than 2 - 3 hr, after the intradermal injection of "GPF", indicates an increase in vascular permeability apparently through another mechanism in the later stages. The relationship between GPF and histamine is not entirely clear. "The overall evidence indicates that globulin PF does not owe its permeability-increasing activity to the liberation of histamine" (Wilhelm, 1965). This conclusion refers particularly to guinea-pig GPF, and there is evidence that it may actually antagonise histamine (Mackay *et al*, 1953). Moreover, it has been found that reactions to rat and rabbit GPF are moderately susceptible to antihistamine (Wilhelm *et al*, 1958; see Miles and Wilhelm, 1960a). Results obtained in the sheep are somewhat similar. An explanation

for the release of histamine by "GPF" in the sheep, remains to be provided. Miles and Wilhelm (1960a) suggested that it was theoretically possible that GPF released leucotaxine-like polypeptides. They discounted this possibility since guinea-pig GPF activity was not affected by antihistamine treatment; much of the effect of "leucotaxine" on vascular permeability is through the release of histamine. It may be argued that if in the sheep "GPF" released "leucotaxine" or leucotaxine-like polypeptides, this could account for the apparent release of histamine by "GPF".

The activation of GPF *in vitro* is inhibited by salicylate, which also has a relatively specific effect *in vivo* in suppressing the action of activated GPF on vascular permeability in the rat (Spector and Willoughby, 1959a). The suppression of pleural exudate formation for up to 6 hr. by salicylate in sheep pretreated with mepyramine, compared with only up to 2 hr. by mepyramine alone, suggests a delayed role for "GPF" found in the pleural exudate. Development of pleural exudates after 2 hr. in both mepyramine-pretreated sheep and in sheep depleted of histamine by 48/80, further indicates that the salicylate-sensitive mechanism that succeeds the histamine phase is not dependent for its appearance on the successful operation of the early histamine phase. This is consistent with results obtained after turpentine injury in the rat (Spector and Willoughby, 1959a), after thermal injury in the guinea-pig, rat and rabbit (Wilhelm and Mason, 1960) and after ultraviolet injury (Logan and Wilhelm, 1966b).

In sheep pretreated with salicylate there was a significant suppression of oedema formation after thermal injury of the skin. This indicates that in thermal injury, too, a salicylate-sensitive mechanism, possibly activated "GPF", comes into operation, after the brief early histamine phase and, perhaps, mediates the major portion of the inflammatory response.

Although it has been shown that salicylate depresses the activity of the GPF in the rat, both the present results of skin tests in the sheep and those obtained in the rat (Spector and Willoughby, 1959b), further indicate that salicylate may have some general depressant effect on the vascular permeability. However, the failure of salicylate to suppress pleural exudate formation at two hours after its induction, indicates that this was not of sufficient magnitude to interfere with the early phase of the reaction. From work in the rat, Spector and Willoughby (1959b, 1963a) concluded that the interference with GPF activity was more significant than a general depressant effect on the vascular permeability.

Taken together, these results suggest that in the sheep a "GPF" may be involved in the maintenance of increased vascular permeability, initiated by histamine and when the effect of which is declining, i.e., in the delayed phase of inflammation.

Significant suppression of pleural exudate formation in the delayed phase was produced in the sheep by either indomethacin, cortisone, or salicylate, all given without antihistamine or any other treatment. This contrasts with findings in the rat (Spector and Willoughby, 1959a; Walters and Willoughby, 1965). The above compounds are not specific antagonists of any particular permeability-increasing substance and the reason for the species difference is obscure. All these substances are potent anti-inflammatory agents in man, but their mode of action is not known.

From the observed interference in the delayed phase of inflammation in the sheep by mepyramine maleate, it has already been suggested that, in the sheep, histamine is involved in more than the early inflammatory response. It is likely that some of the histamine release in the delayed phase in the sheep is caused by the "GPF". Histamine is not generally

believed to be involved in the delayed phase in the rat, guinea-pig, and rabbit. However, from a consideration of Schayer's (1963) findings of increased histidine decarboxylase activity in the delayed response to injury, Wilhelm (1965) has suggested that histamine cannot be entirely dismissed as a mediator of the delayed phase. The results obtained in the sheep can be taken to support Wilhelm's contention. It is suggested that a study of histidine decarboxylase activity in the sheep may further help to explain the present results.

It does not appear, however, that histamine plays a major role in the delayed phase in the sheep, as its effect becomes progressively and relatively less significant with the operation of other mediating mechanisms. Among these possible factors the "GPF", bradykinin and the kinin-forming enzyme kallikrein have been found to increase vascular permeability by means other than releasing histamine. Thus, it is suggested that the mediation of the delayed phase may depend on a mechanism similar to that discussed in other species (Spector and Willoughby, 1964c); i.e., on the formation of kinins by a "Globulin-kinin forming system", activated following injury. The duration of the increased vascular permeability induced by bradykinin for only about 15 min. in the sheep, suggests that any mediation by kinins (i.e., bradykinin) would require their continuous formation in this animal by the proposed "Globulin-kinin forming system". From studies on dog, Menkin (1936) found a permeability substance, in the later stages of pleural exudates, which he described as "leukotaxine", and considered to be polypeptide in nature. The nature of "leukotaxine" remains in doubt (see Spector, 1958; Wilhelm, 1965); it is possible that the permeability factor described by Menkin fits in with some aspect of the kinin-system. Similarly, Cullumbine and Rydon (1946) found a polypeptide

with permeability increasing activity in pleural exudate obtained in the goat.

An association of the delayed increase in vascular permeability in the present study with the onset of large-scale leucocytic emigration raises the possibility of polymorphonuclear neutrophiles contributing to the increased permeability indirectly by the lactic acid they may produce. Lactic acid increases vascular permeability on intradermal injection in the sheep. However, recent work by Logan and Wilhelm (1966a) indicates that the delayed permeability response to ultraviolet injury in the guinea-pig, rat and rabbit is independent of tissue leucocytosis. The possibility of a causal relationship between tissue leucocytosis and increased vascular permeability in the sheep remains to be investigated.

Studies on lymph-node-permeability factor (LNPF) carried out in laboratory animals indicate that it may be a mediator of the vascular changes of delayed hypersensitivity and auto-immune reactions (see Schild and Willoughby, 1967). Experiments on "LNPF" from sheep showed that its activity was unchanged on standing at room temperature for 24 hr. or on storage at $4^{o}C$ for two weeks. No loss of permeability activity occurred after dialysis, indicating that the activity in the extract was not due to histamine or other small molecules but due to a substance of larger molecular size. These results are similar to those obtained in the guinea-pig (Willoughby et al, 1962).

Mepyramine maleate suppressed for 2 - 3 hr. the increase in vascular permeability caused by "LNPF". This provides an indication that in the early stages the extract caused the release of histamine. The demonstrated in vitro release of histamine from sheep lung further supports this conclusion. This contrasts with findings in the rat, in which permeability-

increasing effect of LNPF is not suppressed by mepyramine, and in which LNPF shows no histamine-releasing activity *in vitro* (Willoughby *et al*, 1963). On the other hand, it has been shown that mepyramine antagonises LNPF in the guinea-pig (Boughton, 1965). The action of LNPF in sheep and guinea-pig appear to be similar in this respect and different from that in the rat.

LNPF has not been characterised as yet and how it releases histamine is not known. Recent work of Willoughby and Walters (1965) indicates that LNPF has many similarities with RNA. These workers found that in the rat intradermal injection of RNA increased vascular permeability. However, further studies revealed that the increase in permeability was not caused by RNA itself, but by a breakdown product of it, possibly an oligonucleoside. They excluded the possibility that the activity of RNA was due to its degradation into smaller breakdown products - nucleosides. This was based on the previous finding that nucleosides released histamine in the rat and that their activity in increasing permeability was antagonised by antihistamines (Moulton, *et al*, 1957; Spector and Willoughby, 1957a). Antihistamines had no effect on the permeability activity of either RNA or LNPF. Nucleosides have been found to release histamine in the sheep. If it is assumed that "LNPF" in the sheep also acts through its RNA content it may be postulated that, since histamine is released by "LNPF" in the sheep, it is broken down further than it is in the rat, i.e., at least to nucleosides. However, the failure to induce more than a very small increase in vascular permeability in the sheep by the injection of purified RNA (HP) does not support this idea. The RNA used was the same as that used by Willoughby and Walter (1965) in the rat. It is, however, derived from yeast which introduces another possible difficulty in comparing

results of the reaction of different species to the foreign substances. The failure of mepyramine to suppress the intradermal leakage of dye for longer than 2 - 3 hr. after injection of "LNPF" indicates that an increase in vascular permeability arises apparently through another mechanism, in the later stages.

The possible participation of the enzyme monoamine oxidase (MAO) in increasing vascular permeability in the inflammatory reaction in the sheep was also examined. This enzyme increases vascular permeability after turpentine and thermal injury in the rat by inactivating anti-inflammatory amines present in the tissues (Spector and Willoughby, 1960 b, c). There is an indication from the results obtained that in the sheep in the response to thermal, but not in turpentine injury, some local inactivation of anti-inflammatory amines may occur. In the rat, MAO inhibitors affect the increased permeability in response to both turpentine and thermal injury. However, the failure of MAO inhibitor to suppress pleural exudate formation in the sheep 2 hr. after turpentine injury does not seem to differ from the results reported in the rat. In the rat, Spector and Willoughby (1960b) did not record a significant reduction in the volume of pleural exudate in the first hour after turpentine administration. The effect of MAO inhibitor on the delayed permeability response to turpentine injury has not been investigated in the present work; it could profitably have been studied. The finding that in the sheep MAO inhibitor failed to suppress the permeability response that followed intradermal injection of histamine, 5-HT and bradykinin, is similar to that obtained in the rat (Spector and Willoughby, 1960b; 1965). It is suggested that the failure of MAO inhibitor to suppress the permeability response following intradermal injection of histamine may account for its failure to affect the increased vascular permeability after turpentine injury in the sheep, which is also mediated by histamine in the early stages.

This also suggests that the partial suppression by MAO inhibitor of the reaction to thermal injury is not due to interference with the early histamine phase, but to some effect on the later stages of the reaction.

Histological study of increased vascular permeability in the sheep revealed that in response to both turpentine and thermal injury, the permeability response was mainly venular; capillary involvement was minor. The results differ from those in the rat. In the rat both in turpentine (Hurley and Spector, 1965) and thermal injury (Wells and Miles, 1963; Cotran and Majno, 1964; Spector et al, 1965), although, the permeability response is mainly venular, significant involvement of capillaries has also been found. The weak capillary response in the sheep suggests that in this species, increased capillary permeability makes only a very minor contribution to formation of the inflammatory oedema.

Mepyramine maleate suppressed the carbon labelling of venules both in the skin and diaphragm in the histamine-mediated early phase in the turpentine injury. This supports the finding that histamine causes carbon leakage in venules (Majno et al, 1961; Hurley, 1963). The failure of mepyramine to suppress carbon labelling in the delayed phase is consistent with the concept that increased permeability in this phase is largely mediated by factors other than histamine. Suppression by salicylate of the carbon labelling in the venules in the delayed phase is consistent with similar suppression of pleural exudation by salicylate during this phase.

In the injury due to application of heat mepyramine suppressed the carbon labelling of venules at 15 min., and some suppression was seen up to one hour. In the rat, mepyramine fails to suppress the venular labelling in the reaction to thermal injury (Spector et al, 1965); this is thought to be due to a minor role of histamine in this reaction in the rat. In the sheep, histamine appears to be more important in thermal injury as con-

cluded earlier. Marked but incomplete suppression of carbon labelling by salicylate in thermal injury was first seen at 30 min. and persisted thereafter. This is consistent with the effect of salicylate on the inflammatory oedema in response to thermal injury in the sheep. The incomplete suppression of carbon labelling by salicylate may have been due to concurrent operation of other mechanisms in thermal injury in addition to the salicylate-sensitive one, for instance, inactivation of anti-inflammatory amines. The failure of mepyramine and salicylate to affect the minor capillary response both in turpentine and thermal injury, further supports the idea that permeability factors act only on the venules (Hurley and Spector, 1965).

Mepyramine maleate suppresses the dye-leakage (i.e., the increased vascular permeability) which normally follows the intradermal injection of turpentine, but fails to suppress the local 'flare' (erythema). Histological study with colloidal carbon technique confirmed this failure. Whereas carbon labelling of the venules was suppressed by mepyramine, the hyperaemia and dilatation of the vessels, which caused local 'flare', was not suppressed. It was further seen that combination of mepyramine-pretreatment with denervation, or denervation by itself also had no affect on the vascular hyperaemia and dilatation. These observations suggest that in the skin's response to turpentine, the hyperaemia is not due to the action of histamine but develops separately. Zweifach (1965) suggested, "The major features of the inflammatory process - vasodilatation, transudation of proteins, and cellular emigration --------- appear to develop separately -------". Logan and Wilhelm (1966a) found that whereas antihistamine suppressed the permeability response in ultraviolet injury it failed to affect the erythema. The observations made in the sheep support this latter finding.

Studies with denervation showed that it has no suppressive effect on the development of an increase in vascular permeability. This is similar to certain observations reported by other workers (see Chapman and Goodall, 1964). Some additional increase in the extent of blueing, i.e., increase in vascular permeability after denervation seems similar to the finding of Humphrey (1955a). Humphrey (1955a) found that denervation results in an increase in oedema formation in Arthus reaction in the rabbit.

The present study has revealed a delayed emigration of leucocytes in the inflammatory reaction in the sheep. The permeability increasing substances tested produced little immediate effect on leucocyte emigration. The results support similar findings obtained in the rat (Hurley and Spector, 1961b; Spector and Willoughby, 1964b). However, no significant leucocyte emigration was seen in the response to thermal injury even in the delayed phase in contrast to findings in the rat (Hurley and Spector, 1961b). In addition in the rat, the lymph node permeability factor (LNPF) produces an immediate large scale emigration of leucocytes (Spector and Willoughby, 1964b; Willoughby et al, 1964). In the sheep this was not obtained. It is clear that mode of action of LNPF differs between the rat and the sheep, at least in the early phases. The reasons for the difference are unknown.

Histamine, 5-HT and bradykinin increase vascular permeability almost immediately following their intradermal injections; and in all cases the permeability is restored to normal by the end of one hour. Leucocyte emigration occurs after this time. Similarly, saline which has no detectable effect on vascular permeability causes delayed emigration of leucocytes. Thus, increased vascular permeability to protein is dissociated in time from leucocytic emigration. This is supported by the results obtained with the colloidal carbon technique. It was found that emigration of leucocytes

apparently took place from venules that were not labelled with carbon and, presumably, therefore, were not abnormally permeable to protein. It has been shown that the two phenomena are similarly dissociated in the rat and leucocytes can emigrate from vessels which have a normal protein permeability (Hurley and Spector, 1961 a, b; Hurley, 1963). The present results suggest that permeability substances activated or released by injury do not exert much effect on leucocyte emigration and suggest the involvement of a separate endogenous mechanism controlling this. Hurley and Spector (1961 a, b) also concluded this from their work in the rat.

Mast cells appeared more numerous in the sheep skin following intradermal injection of turpentine and various permeability-increasing substances. Apart from an increase in mast cells in the mouse skin following local application of certain carcinogenic hydrocarbons (see Riley, 1959), no mention of increase in mast cells in response to injury or injection of permeability-increasing substances was found in the literature. It has been stated that mast cells are less evident in acute inflammation but are increased in chronic inflammatory processes (see Bloom, 1965). Regranulation of mast cells after degranulation has been reported. Sheldon and Buer (1960) found that 2 hr. after initial degranulation mast cells began to regranulate and after 12 hr. the number detectable was normal. The apparent fluctuations in the mast cell numbers in sheep skin may be due to their alternating degranulation and regranulation.

Mast cells can increase by mitosis (Hunt and Hunt, 1957; Allen, 1961; 1962; see Bloom, 1965). Since, no mitotic figures were seen in the present study, it suggests that increase in mast cells in the inflamed skin may not have been due to mitosis. The observation in several cases of the presence in a blood vessel of a polychromatic cell (presumably a basophil leucocyte), in

the sections of skin undergoing inflammatory changes but not in the normal skin, suggests that basophils in the blood may be a source for the increase in mast cells in the inflamed skin.

The relationship between histamine and eosinophilia is not clear. Histamine attracts eosinophiles in horse skin in as little as 10 µg doses (Archer, 1963). Such an eosinophilotactic effect has not been demonstrated in other species, and despite extensive studies, claims are disputed that histamine attracts eosinophiles to tissue sites, or to the blood stream from the marrow reserves (Vaughan, 1953; Litt, 1962). In the guinea-pig, histamine has little eosinophilotactic effect in doses of 0.1 to 100 µg (Litt, 1962). The present results indicate that histamine in high doses (i.e., in 100 µg amounts) attracts eosinophiles to the injection site in the sheep skin.

Many investigators have reported or proposed an antihistaminic function for eosinophiles, a proposal supported by detection of histamine-neutralising substances in extracts of eosinophil granules (Kovacs, 1950; Vercauteren and Peeters, 1952; Vercauteren, 1953; Broome and Archer, 1961). The present results could be taken to suggest that degranulation may play a role in the <u>in vivo</u> inactivation of histamine by eosinophiles. Degranulation of eosinophiles has been described before. Hirsch (1965) suggested that degranulation of eosinophiles which accompanies the ingestion of antigen-antibody precipitates (Archer and Hirsch, 1963; Sabesin, 1963) may lead to release and activation of enzymes, or other substances which destroy the immune aggregates. Although the present evidence is not adequate to confirm the inactivation of histamine by eosinophiles <u>in vivo</u>, it is suggested that sheep may prove useful in the study of anti-inflammatory role of eosinophiles.

The delayed migration of neutrophiles in the reversed passive Arthus reaction (RPA) following intradermal injection of antibody, is similar to

the late migration of cells seen with various permeability substances. However, in contrast to the cellular infiltration induced by them, in the RPA-reaction some mononuclear and eosinophilic infiltration of the tissues was seen in the early stages. Since the increase in vascular permeability in the RPA-reaction has been found to be mediated by histamine in the sheep and histamine produces little emigration of leucocytes in the early stages and then of neutrophiles, it does not appear that histamine was causing this early migration of mononuclear cells. Histamine also does not seem responsible for the eosinophil migration, since histamine in doses of 10 µg does not attract eosinophiles. Moreover, the area of blueing seen in the RPA-reaction is less than that produced by 10 µg of histamine, which makes it unlikely that histamine would have been released in amounts large enough to attract eosinophiles. The degranulation of eosinophiles noticed may have been associated with the ingestion of immune complexes by these cells, as reported by Archer and Hirsch (1963) and by Sabesin (1963). It seemed possible that immune complexes might have been responsible for both mononuclear and eosinophilic infiltration. However, further studies revealed that preformed insoluble precipitates, and also those allowed to be formed in vivo caused migration of only mononuclear cells in the early stages; eosinophiles were not seen in significant numbers.

Accumulation of neutrophiles in the vessel walls has been considered typical of the Arthus reaction and is believed to be due to the deposition of the immune reactants and host complement in the vessel wall, following which neutrophiles are attracted and accumulate in the vessel wall (see Cochrane, 1965). Marked accumulation of neutrophiles in the wall of the blood vessels was also noticed in the present study in the RPA reaction. This is not seen following the intradermal injection of turpentine and various

permeability-increasing substances although they all produce marked neutrophilic infiltration.

To conclude, the above discussion has shown marked species differences of the inflammatory reaction and some similarities. Whereas the inflammatory mechanism in the sheep seems to differ from that in laboratory animals, the results suggest a closer similarity between the sheep and the guinea-pig than between sheep and rat. The skin of sheep and guinea-pig shows a comparable response to histamine and in both species this is greater than that occurring in the rat. 5-HT is involved in the inflammatory response in the rat, but cannot be implicated in either the sheep or guinea-pig. The degree of involvement of histamine in the early phase of the response to thermal injury is comparable in the sheep and guinea-pig and perhaps greater than that in the rat. LNPF releases histamine on injection into the skin of sheep and guinea-pig, but does not do so in the rat. Finally, an indication of the release of histamine by mepyramine maleate injected into the skin has been obtained in sheep, and it has been suggested that this may occur in guinea-pig.

Despite the striking differences observed, however, the present study further suggests that a few generalisations on the inflammatory reaction may be made among these species, viz., sheep, rat and guinea-pig. A bi-phasic increase in the vascular permeability appears common to all. Whereas, histamine and/or 5-HT appear to mediate the early response, mediation of the late phase appears very complex. Also in the sheep, suppression of the early histamine phase does not seem to affect the development of the late phase of increased permeability. This supports the suggestion by Spector and Willoughby (1963a) and Wilhelm (1965) that although the histamine phase initiates the inflammatory response it does not sustain it.

Wilhelm (1965) has further suggested that the occurrence of the histamine phase may merely reflect the ease with which histamine is released in injured tissues.

It is apparent from the present study that there are clear differences between species in the inflammatory mechanism. While results obtained in one species may provide clues for another, inter-species differences may be wide and basic and it is not safe to assume that results obtained in one species necessarily apply to another. Extensive work is required to categorise the mechanism in domestic animals. This would provide a better understanding of the pathogenesis and pathology of diseases in them, and the information obtained may contribute towards a fuller understanding of the inflammatory reaction. It is also obvious that in spite of intense research, certain fundamental problems of the inflammatory process, namely - the way in which an endogenous permeability factor is released, how it actually produces an increase in vascular permeability, and the way in which its action is blocked by an antagonist - remain unknown. Studies on various species in this respect may contribute to obtaining the explanations. It is suggested that the sheep may prove a useful animal in exploring solutions for certain basic problems connected with the inflammatory reaction and that the experimental work presented in this thesis justifies this conclusion.

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Suppression of the Early Inflammatory Response in the Sheep by Strophanthin *G*

IT is well established in rats that the increased vascular permeability which follows the intrapleural injection of turpentine is due to the local release of histamine and is continued by the activation of other mechanisms[1,2]. Our experiments on sheep have shown a similar pattern of immediate histamine release and later continuation of the increased vascular permeability. Following the report by Judah, Ahmed and McLean[3] of the similarity of action between the antihistamine promethazine and the cardiac glycoside strophanthin *G*, strophanthin *G* was used as a possible suppressor of the early inflammatory changes in the sheep.

Romney Cheviot *X* lambs weighing about 20 kg were used: they were "blued" by intravenous injection of pontamine blue (25 mg/kg body weight of a 5 per cent solution in isotonic saline). Turpentine was used as the irritant to produce inflammation: the intradermal injection of 0·1 ml. of turpentine into the clipped back of "blued" sheep was followed by marked leakage of dye at the injection site. The size of the blue area varied from 3·5–5 cm in diameter at the end of 1 h. In "blued" sheep pretreated with the antihistamine mepyramine maleate (25 mg/kg body weight in 5 per cent solution, injected intramuscularly), the leakage of dye did not develop until 1 h after the intradermal injection of turpentine. Pretreatment of sheep with mepyramine maleate also suppressed exudate formation after the intrapleural injection of 0·5 ml. of turpentine into the right pleural space. The antihistamine treatment, however, failed to give complete suppression of exudate formation for longer than 2 h.

Histamine depletion using compound 48/80 gave similar results. Sheep were depleted of their stores of histamine by repeated injections of compound 48/80 using the method of Spector and Willoughby[1]; a 0·1 per cent w/v solution of compound 48/80 in saline was given intraperitoneally, morning and evening, in ten doses beginning with an evening dose. The dose used was 0·6 mg/kg body weight for the first eight injections and 1·2 mg/kg for the two final injections. Skin tests were carried out 2 h after the last injection. In sheep depleted of histamine both the early inflammatory changes in the skin and exudate formation in pleural cavity were suppressed. Compound 48/80, however, like mepyramine maleate, failed to suppress the inflammatory changes for longer than 1 h in the skin and 2 h in the pleural cavity. Pretreatment of sheep with strophanthin *G* (100 μg/kg body weight intra-

venously) suppressed the leakage of dye for 1 h after the intradermal injection of 0·1 ml. of turpentine, after which marked blueing took place. Pretreatment with strophanthin *G* also suppressed the formation of pleural exudate for 2 h after the intrapleural injection of turpentine. These results are similar to those observed with mepyramine maleate in the sheep.

These experiments show that a biphasic response of increased vascular permeability develops in the sheep after injury with turpentine, the first stage being inhibited by antihistamines. The antihistamine compound mepyramine maleate is a specific antagonist for histamine, and has not been shown to antagonize any other compound which increases capillary permeability[1,4–7]. In the sheep, the suppression of the early inflammatory changes by pretreatment with mepyramine maleate indicates that it is the local release of histamine which initiates the increased vascular permeability. The experiments with compound 48/80 lend further support to this view. Suppression of the early inflammatory changes in the skin and pleura by strophanthin *G* suggests that strophanthin *G* may be blocking the effect of local histamine release. It is thought that anti-histamines act by blocking the effects of histamine at tissue receptor sites, thereby preventing the normal physiological reactions to this substance[8,9]. The protective effects of antihistamines in preventing liver cell necrosis have been reported[10,12] to be due, in part, to their interaction with the system in the cell membrane which regulates active transport of sodium and potassium[11]. Both the antihistamine promethazine and strophanthin *G* have been reported to protect the rat liver against thioacetamide induced necrosis[3,10]. More recently, it has been reported that promethazine and strophanthin *G* show approximately equal activity in inhibiting potassium transport[3]. In the sheep the suppression by strophanthin *G* of the early inflammatory changes induced by histamine could be due to inhibition of the sodium/potassium ion transport mechanism of the capillary wall and this could, in return, suggest that part of the action of the antihistamine mepyramine maleate against histamine may be due to its action on the ion transport mechanism of the cell membrane.

Our results support the view put forward by Judah, Ahmed and McLean[11] that the antihistamines owe their effects to their interaction with that system in the cell membrane which regulates the active transport of sodium and potassium ions. The results suggest that strophanthin *G* may prove useful in the investigation of the inflammatory reaction, particularly histamine liberation and the mechanism of exudate formation, in the sheep.

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