BLOOD GLUCOSE LEVELS IN CATTLE IN RESPONSE TO DIFFERENT FORMULATIONS OF BETAMETHASONE

A THESIS PRESENTED IN PARTIAL FULFILMENT
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ABSTRACT

The hyperglycaemic response in adult cows to the subcutaneous injection of betamethasone at 0.04mg/kg body weight was studied using four different formulations of the drug. These were Betsopart and Betsolan, aqueous suspensions of betamethasone of 10mg/ml and 2mg/ml respectively and 5mg/ml and 2mg/ml preparations produced by diluting Betsopart with water immediately prior to injection. Four treatment groups and one control group each of four cows were bled daily for 12 days with the betamethasone being given on day 4. All trial blood samples were collected using fluoride-oxalate as anticoagulant which was shown to result in lower plasma glucose levels than in EDTA plasma or serum.

Daily plasma glucose concentrations were determined using 3 analytical methods. The alkaline ferricyanide method, as anticipated, gave higher glucose levels than the glucose oxidase method, and unexpectedly, the oxygen electrode method also overestimated the glucose concentration.

Despite differences in the analytical methods it was shown that the 10mg/ml Betsopart and the two 2mg/ml preparations (Betsolan and diluted Betsopart) gave similar peak plasma glucose concentrations while the 5mg/ml diluted Betsopart gave a significantly higher peak. The longevity of the hyperglycaemia was greatest in the 10mg/ml and 5mg/ml treated groups and less in the two 2mg/ml groups.

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CHAPTER 1

INTRODUCTION

The ability of betamethasone to raise blood glucose levels is well known, however the degree and longevity of the hyperglycaemia is not only dependent on the dose rate of the drug but also on the concentration of the drug in the injected preparation.

This study was carried out to compare the effect of four different formulations of betamethasone (10mg/ml, 5mg/ml and two of 2mg/ml) on blood glucose levels in cattle when administered at the same dose rate.

Blood glucose levels were measured using three commonly used analytical methods to investigate the correspondence between the methods. The effect of various anticoagulants on plasma glucose levels compared to serum glucose concentrations was also investigated as was the effect of time between blood collection and the separation of plasma or serum from the erythrocytes.

CHAPTER II

2.1 SUBJECT REVIEW

The outer portion of the adrenal gland, the adrenal cortex, produces a number of potent hormones all of which are steroid derivatives having the characteristic cyclopentanoperhydrophenanthrene nucleus. The most important ones are hydrocortisone (cortisol), corticosterone and aldosterone. These naturally occurring steroids, together with synthetic compounds having similar activity, are termed corticosteroids. Corticosteroids influence a multitude of physiological processes, including electrolyte balance and carbohydrate metabolism and are usually classified as mineralocorticoids (main effect on electrolyte metabolism) or glucocorticoids (marked effect on carbohydrate metabolism) respectively. The whole subject of steroid hormones is very wide, therefore, only glucocorticoids and their effect on carbohydrate metabolism will be emphasized here.

2.1.1 Chemistry of Corticosteroids

The chemistry of corticosteroids has been reviewed by many authors (Steele (1975), Grodsky (1977), Anon (1977) and McDonald (1977)).

The pregnane nucleus of corticosteroids contains four ring structures which are composed of twenty-one carbon atoms (Fig 1). Carbon-21 is always hydroxylated in both glucocorticoids and mineralocorticoids. A double bond between position four and five plus a ketone group at position three in the ring are necessary for typical corticosteroid activity (Fig 2). The ketone group at the twentieth carbon atom is also responsible for being biologically active and esterification of the hydroxyl group on carbon 21 prolongs the pharmacologic action of these steroids although the potency is reduced. Oxygenation of the eleventh carbon atom is very important for glucocorticoid activity and hydroxylation of the sixteenth and seventeenth carbon atoms further strengthens the glucocorticoid activity (Fig 3). In addition, fluorination of the sixth, ninth or twelfth carbon atoms increases the potency of glucocorticoids whereas methylation of the sixth or sixteenth carbon atom decreases the salt-retaining effects.

Most of today's synthetic corticosteroids for therapeutic use are based upon the knowledge of the chemistry of natural ones. Thus, introduction of a double bond between position one and two on the cortisone and hydrocortisone molecules produces prednisone and prednisolone respectively, the glucocorticoid potency being improved approximately fourfold (Fig 4 and 5). In the next step, the additions of a fluorine atom at position nine and an hydroxyl radical at position sixteen produce triamcinolone (Fig 6) whereas further improvement was achieved by replacing that hydroxyl radical at position sixteen with a methyl group to produce betamethasone (Fig 7), the resultant glucocorticoid potency being some thirty to forty times that of cortisone with negligible mineralocorticoid activity. Hence the mineralocorticoid and glucocorticoid activities of corticosteroids have been successfully separated and used for each specific role in electrolyte balance and carbohydrate metabolism.

Cortisol (hydrocortisone) and corticosterone (Fig 8) are the principal glucocorticoids of the adrenal cortex. Cortisol predominates in man, pig and dog whereas corticosterone predominates in the birds, rabbit, mouse, rats and other rodents. The ruminant is intermediate since its adrenals secrete both.

2.1.2 Physiological Effects and Mechanism of Action of Glucocorticoids

The physiological effects and mechanism of action of glucocorticoids has been reviewed by many authors (Frieden and Lipner (1971), Steele (1975), Grodsky (1977), Anon (1977), McDonald (1977) and Exton (1979).

Most of the metabolic action of adreno-cortical extracts have been attributed to the effect of the glucocorticoids.

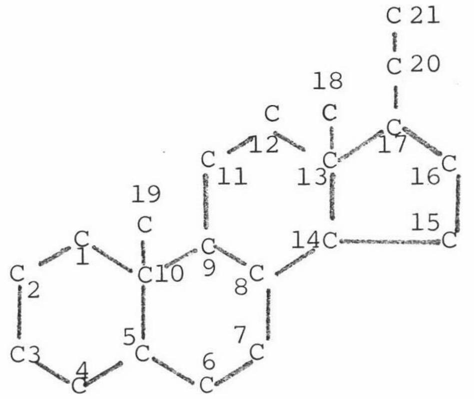


Figure 1: The pregnane nucleus

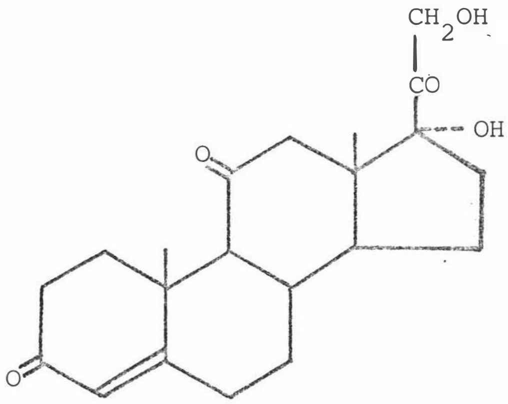


Figure 2: Cortisone

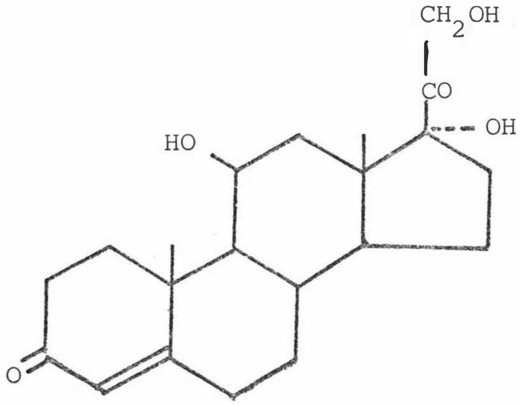


Figure 3: Cortisol (hydrocortisone)

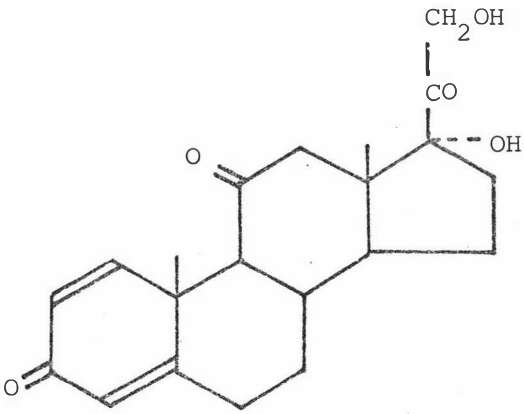


Figure 4: Prednisone

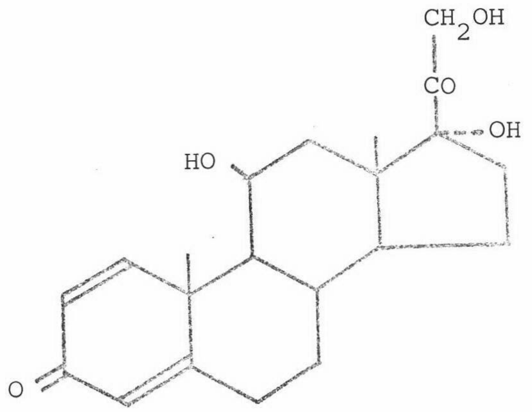


Figure 5: Prednisolone

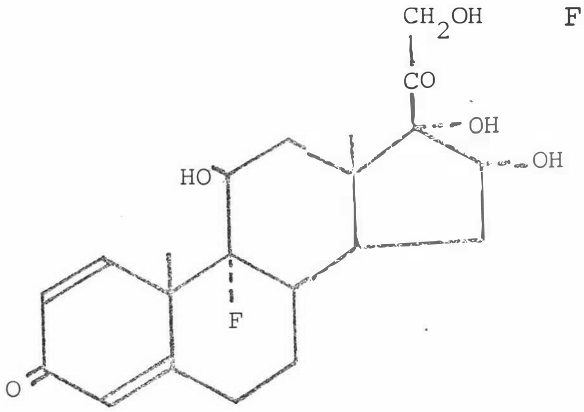


Figure 6: Triamcinolone

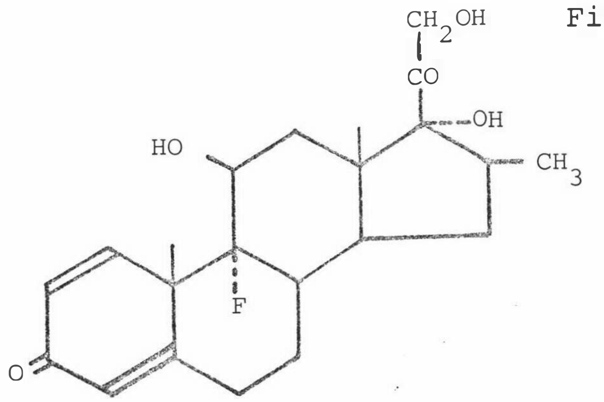


Figure 7: Betamethasone

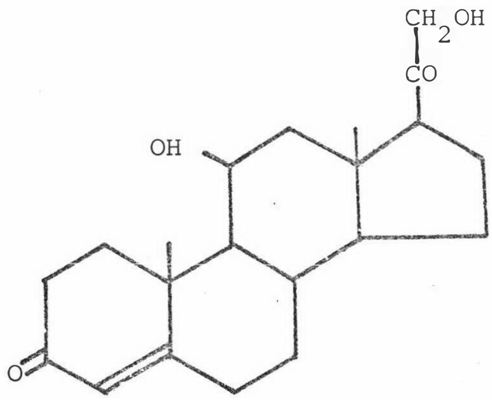


Figure 8: Corticosterone

Glucocorticoids show different physiological effects on the metabolism of various tissues. The general actions of glucocorticoids on carbohydrate, lipid and protein metabolism are shown in Figure 9. On most tissues, glucocorticoids induce catabolic actions, but in liver they produce anabolic effects. Generally glucocorticoids promote hepatic gluconeogenesis, increase protein degradation in the peripheral tissues (muscle, adipose and lymphoid tissue) and induce adipose tissue lipolysis. In the peripheral tissues glucose uptake, glycolysis, protein synthesis and fat synthesis are depressed. Glycerol from adipose tissue lipolysis and amino acids released from muscle, skin and connective tissue are good precursors for gluconeogenesis. In the ruminant propionic acid is the major source of glucose. The subsequent conversion of these substrates into glucose by the liver and, to a lesser extent, by the kidney is always accelerated by glucocorticoids. Another effect of glucocorticoid is to decrease the uptake and utilization of glucose by peripheral tissues, thus conserving circulating carbohydrate. This peripheral antagonism to insulin, similar to that of growth hormone, causes hyperglycaemia. In monogastric animals these effects cause an increase in liver glycogen whereas hyperglycaemic effects are much more marked in ruminants.

The metabolic functions of glucocorticoids on carbohydrate metabolism may be discussed in terms of acute and chronic phases (Fig 10). In the first phase, there is a rapid net transfer of amino acids from muscle to liver and kidney tissues, where pre-existing gluconeogenic enzyme systems convert amino acids into glucose. In the liver, glucocorticoids can induce the rapid biosynthesis of hepatic enzymes such as alanine-α-ketoglutarate, tyrosine transaminases, tryptophan oxygenase (tryptophan pyrrolase), ornithine decarboxylase, and glutamic-pyruvate transaminase which are involved in amino acids metabolism. These glucogenic enzymes make the carbon skeletons of numerous amino acids available for subsequent glucogenesis. (Drugs like actinomycin D, cycloheximide and puromycin prevent the transcription, translation and actions of these enzymes by glucocorticoids). There is also increased

FIGURE 9: The general actions of glucocorticoids on carbohydrate, lipid and protein metabolism.

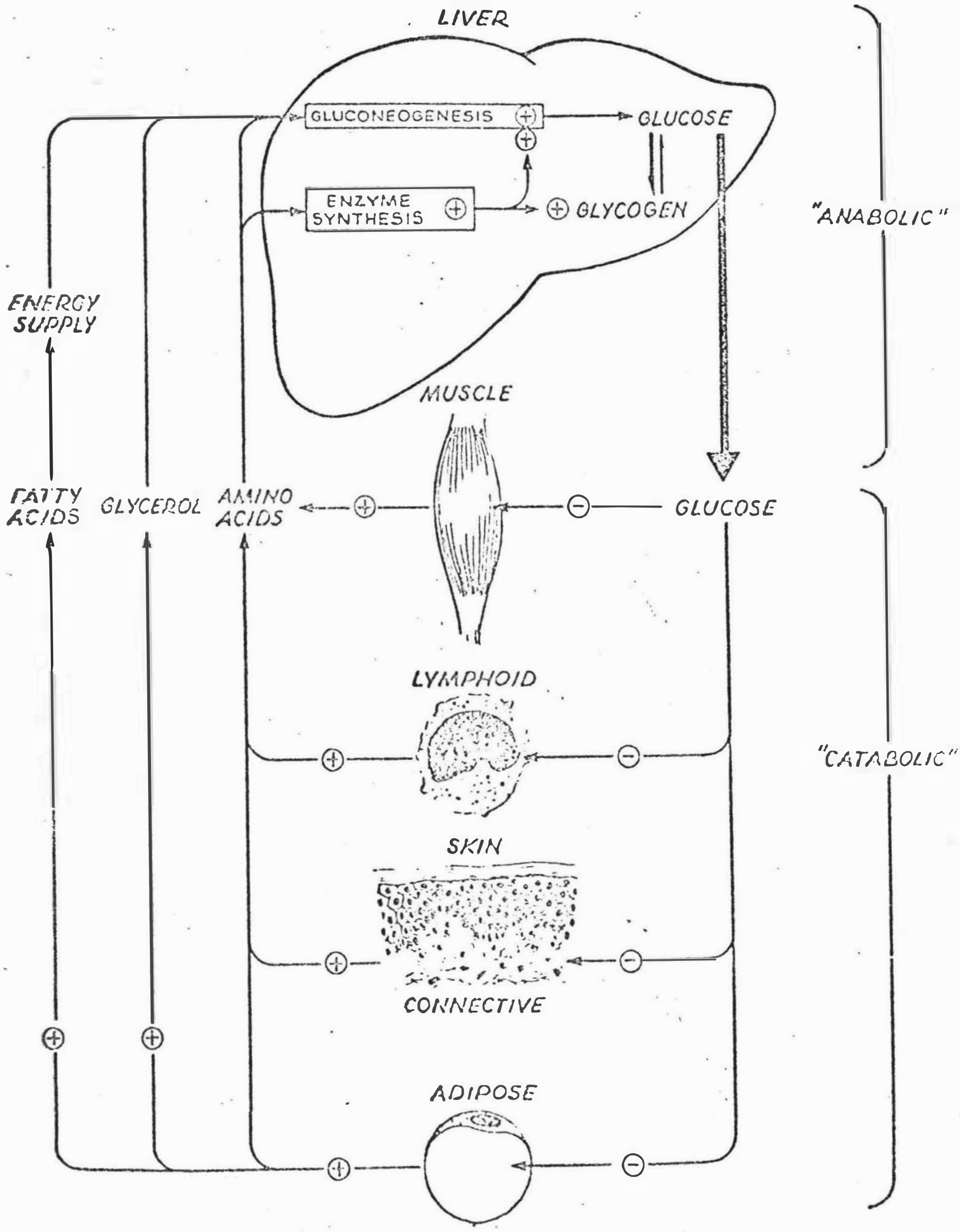
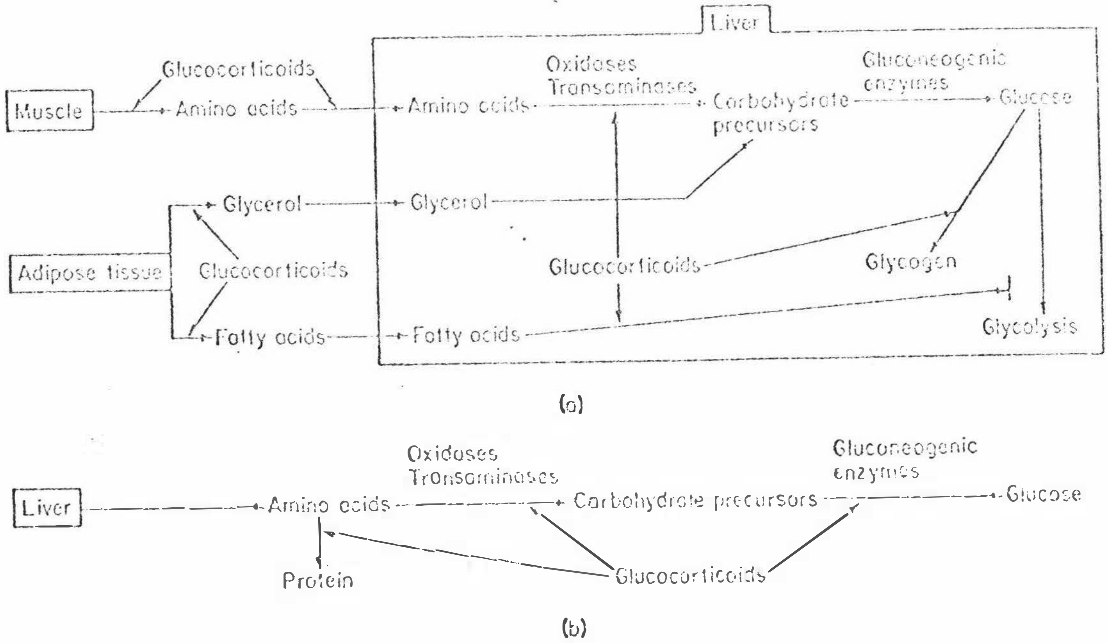


Figure 10: (a) Acute and (b) chronic effect of glucocorticoids on carbohydrate metabolism. The arrow ending in a bar (—→|) indicates an inhibitory effect. Unblocked arrows indicate stimulation.



release of fatty acids and glycerol from adipose tissue to the liver, where the glycerol can contribute to gluconeogenesis and where the increased fatty acid level inhibits key liver glycolytic enzymes (phosphofructokinase, pyruvate kinase, citrate synthase), thus reducing glucose breakdown. An increase in glycogen synthetase and pyruvate carboxylase activity contributes to an increase in liver glycogen (Fig 10a).

The most significant chronic effect of glucocorticoid action is the stimulation of biosynthesis of enzymes involved in glucogenesis that divert carboydrate precursors to glucose formation (Fig 10b). The key enzymes in the regulation of gluconeogenesis (pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose-1, 6-diphosphatase, and glucose-6-phosphatase) are also increased, possibly by the glucocorticoid stimulation of a functional genetic unit in the nucleus which controls their synthesis. At this molecular and genetic level insulin acts as a suppressor of these glucogenic enzymes, inducing glycogenic and glycolytic enzymes (Fig 11).

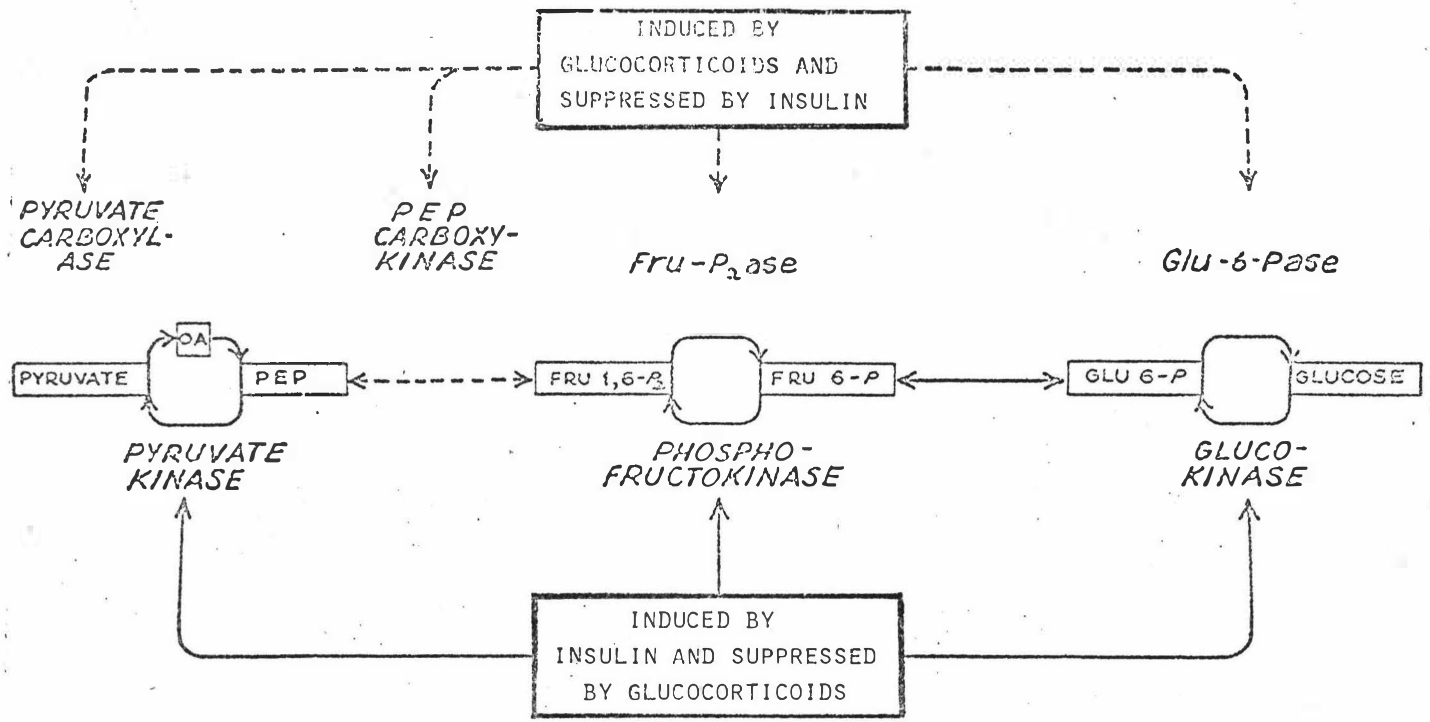
In the liver, glucocorticoids not only increase amino acid conversion to glucose but also incorporation of carbondioxide into glucose, via carbondioxide fixation, particularly at the level of pyruvate carboxylase. Thus glucocorticoids play a vital role in regulating carbohydrate metabolism. Other metabolites such as lactate and propionate can enter the gluconeogenic pathway through the tricarboxylic acid cycle.

In modifying enzyme activities, glucocorticoids act at the molecular level in a way similar to that of other steriod hormones on their target tissues. This mechanism involves modulation of DNA transcription into m RNA by a steroid-receptor complex formed in the cytoplasm (Fig 12).

Other important glucocorticoid actions are: -

(a) Musculo-skeletal system:
Glucocorticoids cause muscle wasting by increasing the mobilization of amino acids from muscle protein and by decreasing the uptake of amino acids by muscle fibres.

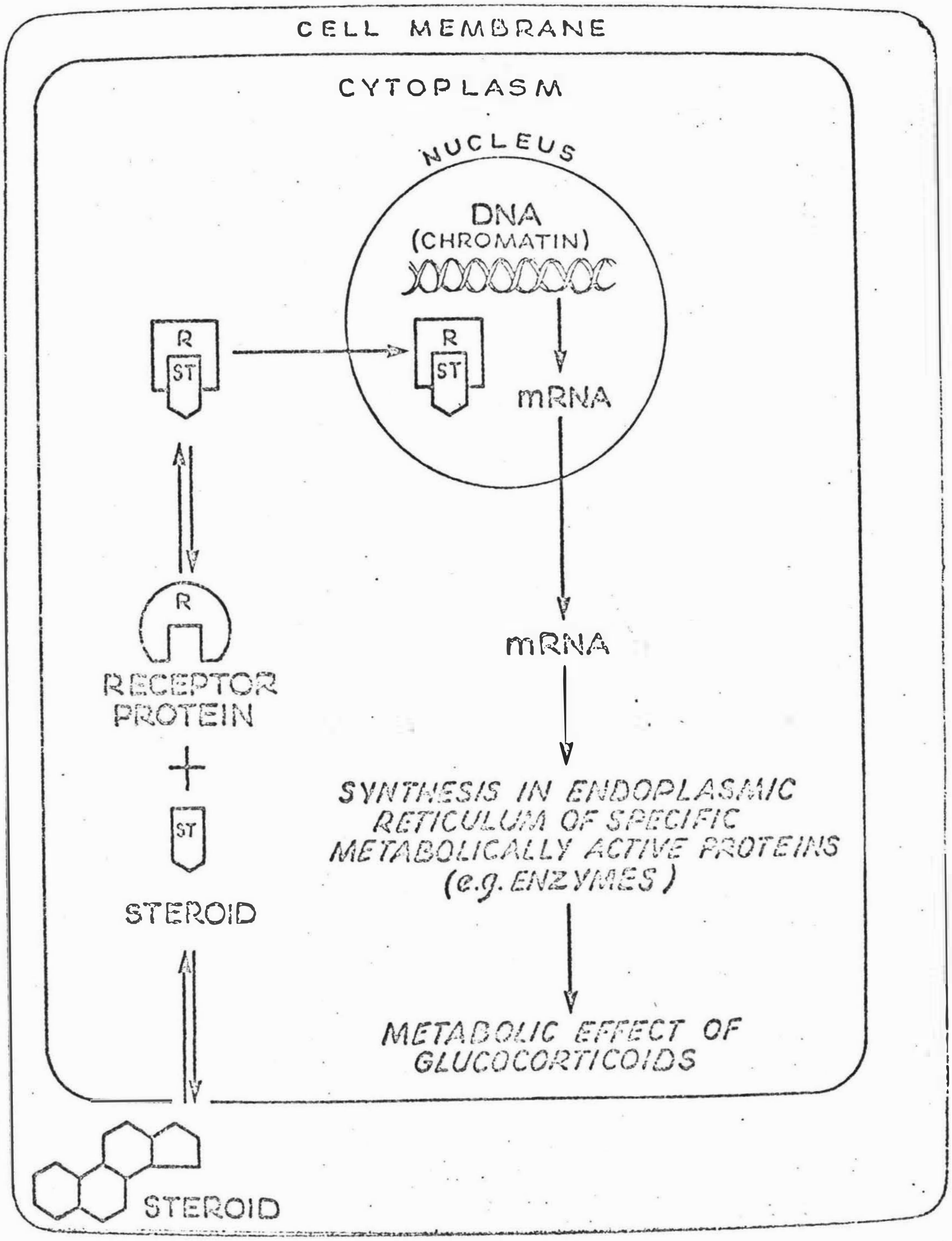
FIGURE 11: The actions of glucocorticoids and insulin on carbohydrate metabolism.



Abbreviations

OA = Oxaloacetate
PEP = Phosphoenolpyruvate
Fru 1,6-P_2 = Fructose 1,6-bisphosphate
Fru 6-P = Fructose 6-phosphate
Fru-P_2ase = Fructose bisphosphatase
Glu 6-P = Glucose 6-phosphate
Glu-6-Pase = Glucose-6-phosphatase

FIGURE 12: The cellular mechanism of action of glucocorticoids on their target tissues.



St = steroid; R = specific glucocorticoid receptor; the dissimilar shapes of R are intended to represent different conformations of this protein.

Increased catabolism of proteins leads to negative nitrogen balance and increased urinary elimination of nitrogen and uric acid. In the skeletal system, excess glucocorticoid causes impaired formation of cartilage, decreased bone formation and osteoporosis, decreased absorption of calcium from the gut (antagonism to vitamin D) and increased renal excretion of calcium and phosphorus.

(b) Immunological and anti-inflammatory effect:
Glucocorticoids have immuno-suppressive effects, since excess glucocorticoids cause lysis of lymphocytes and plasma cells with release of antibody and subsequently antibody levels are lowered.

Glucocorticoids also suppress the connective tissue response to injury, whether traumatic, anaphylactic or infective. They suppress the activity of fibroblasts and granuloma formation and prevent lysosomal proteolytic enzymes from escaping to damage surrounding cells.

(c) Hematopoietic system:
In some species glucocorticoid induce marked changes in the total and differential white blood cell counts (neutrophilic granulocytosis, eosinopenia and lymphop enia).

(d) Vascular system:
Glucocorticoids play an important role in maintenance of arteriolar tone and blood pressure and are necessary to sensitize the vascular system to the pressor action of epinephrine and norepinephrine (important in shock and stress).

(e) Endocrine system:
Excess glucocorticoid administration suppresses the production of adreno-cortico trophic hormone and/or the release of corticotropin releasing factor.

(f) Exocrine system:
The glucocorticoids may increase the secretion of pepsin and hydrochloric acid from the stomach.

(g) Water and electrolyte metabolism:
Glucocorticoids exert much smaller effects on water and electrolyte metabolism than the mineralo corticoids. Only excessive administration of cortisol causes sodium retention and kaliuresis.

(h) Cylic AMP:
In some tissues, the glucocorticoids decrease phosphodiesterase activity, and thus act similarly to cyclic AMP in increasing protein metabolism, lipolysis and gluconeogenesis although their primary action is at the nuclear site.

2.1.3 Application of Glucocorticoids in Veterinary Medicine

The corticosteroids are among the most important and widely used preparations in the pharmaceutical armamentarium. This great multiplicity of action and ability to influence a variety of metabolic processes justify their use in many clinical situations in which they may play a primary or supportive role. Corticosteroid substances with potent glucocorticoid activity are employed in several ways in veterinary therapy. They are widely used in the treatment of inflammatory disease, hypersensitivity reactions, some infectious conditions, shock and diseases due to impaired metabolism.

Application of glucocorticoids in treatment of animals was introduced by many veterinarians and other scientists since 1950's. Dye *et al* (1953) reported the use of cortisone in the treatment of ketosis in dairy cows and found that thirteen cases of postpartum ketosis in high-producing dairy cows were successfully treated with cortisone. Paterson (1957) used prednisolone in the treatment of bovine ketosis. From that time, corticoids were recognised for ketosis treatment. The therapeutic value of cortisone and adrenocorticotrophic hormones had been demonstrated by Shaw *et al* (1952, 1955), McAuliff *et al* (1954) and extended to hydrocortisone by Shaw *et al* (1954), and prednisolone by Morris and Hall (1956) and Shaw (1956). In 1959, Goetsch *et al* showed the effects of four synthetic corticosteroids on leukocytes, blood glucose and plasma sodium and potassium in the cow. They proved that prednisolone, 9 α-fluoro-hydrocortisone and 9.α fluoro-prednisolone were powerful in their ability to induce an elevation of blood glucose in the normal lactating cows. Neff *et al* (1960) found 9 α-fluoro-prednisolone acetate to be satisfactory for the treatment of bovine ketosis without affecting the milk production. Gruchy *et al* (1963) made an investigation of the value of five treatments for acetonaemia in Jersey cows and found that cortisone or ACTH treatment were the most suitable for the ketosis. Burns (1963) reported that the glucogenic effects produced by prednisolone and betamethasone were consistent with those observed in field cases of acetonaemia treated with those compounds. Thus the interest of many scientists in corticosteroids lead to a symposium held by the Veterinary Advisory Department, Glaxo Laboratories Limited, Greenford, Middlesex at London in 1971. In that symposium Austin (1971) reviewed the use of glucocorticoids by different routes for various indications; experiences on different species were discussed by the veterinarians.

Glucocorticoids are not only useful for ketosis, but are also useful for other treatments such as anti-inflammatory therapy where they are effective without regard to the aetiology. The mechanism of their anti-inflammatory activity has not been fully determined, but, may reduce hyperaemia, exudation, cellular infiltration, increased capillary permeability, endothedial swelling and release of vasoactive factors from plasma proteins (e.g. cortisol is used in collagen diseases such as rheumatoid arthritis).

It has long been known that if pregnant cows were treated with corticosteroids, they often aborted. Thus the corticosteroids were used in induced calving. It is postulated that the injection of corticosteroid into a cow in the last two weeks of pregnancy will stimulate the same response in the dam as cortisol production by the foetus towards the end of a normal pregnancy. Welch *et al* (1973) showed the induction of parturition in cattle with corticosteroids and proved that the milk production in cows following induced calving appeared to be about normal. Also it was shown that the infusion of ACTH or hydrocortisone into either the foetus or its dam result in premature parturition (Comline *et al* 1973).

There are contraindications to the use of ACTH or glucocorticoids (e.g. in late pregnancy, corneal ulcers, diabetes mellitus, hypertension, renal insufficiency and so on). Undesirable effects sometimes may occur during the therapeutic use of corticosteroids (e.g. marked sodium and water retention, polydipsia, polyuria, weakness and wasting of muscles, weakening of connective tissue and osteoporosis are seen in excess treatment with glucocorticoids).

The main objective of the present investigation was the study of the hyperglycaemic response to a synthetic glucocorticoid.

2.2 METHOD REVIEW

In this investigation, the following three methods for determination of plasma glucose were used to monitor the hyperglycaemic effect of betamethasone therapy.

2.2.1 Ferricyanide Reduction Method

Since the early years of the twentieth century, methods of blood sugar estimation were introduced by many scientists (Benedict 1911, Folin and Wu 1919, Oser and Karr 1926, Somogyi 1927). Their methods were based on the reducing properties of blood sugar which can react with any oxidant such as copper or picric acid in hot alkaline solutions. This reduction was coupled with a colour change and glucose concentration measured colorimetrically. Although the blood sugar values from those methods were not "true glucose" values, they were widely used by many laboratories at that time. The method using the alkaline ferricyanide to oxidize the glucose was introduced by Hagedorn and Jensen (1923), and Hawkins and van Slyke (1929). There were other colorimetric methods such as the *o*-toluidine method in which an aromatic primary amine, in glacial acetic acid solution, can react with glucose and yield a stable green colour which can be monitored at 625 nm. This method was introduced by Ek and Hultman (1957, 1959) and modified by Duboski (1961) and Hyvarin and Nikkila (1962).

The method used here was the ferricyanide method based on the method of Hoffman (1937). In principle, hot alkaline solutions of ferricyanide ion (yellow) can be reduced by glucose to ferrocyanide ion (colourless).

$$\mathrm{Fe^{III}\ (CN_6)^{3-}} \xrightarrow[\text{(glucose)}]{+e^-} \mathrm{Fe^{II}\ (CN_6)^{4-}}$$

(Ferricyanide-ferric ion) (Ferrocyanide-ferrous ion)

The decrease in colour of ferricyanide was measured at 420 nm by using a flow cuvette with 10 mm light path. The decrease in absorbance is proportional to the glucose concentration (inverse colorimetry).

Numerous modifications have been applied to this method by many scientists (Rappaport & Eichhorn (1950), Fingerhut *et al* (1963), Hunter 1967). This method was adapted for continuous-flow analysis by Johnson (1958) and now well-known as Technicon N.2.b method.

The alkaline ferricyanide method is a reliable method with good reproducibility and repeatability. The only defect is it measures not only glucose but also other reducing sugars. In spite of this limitation, the method is used widely in clinical laboratories.

2.2.2 Glucose Oxidase Enzymatic Method

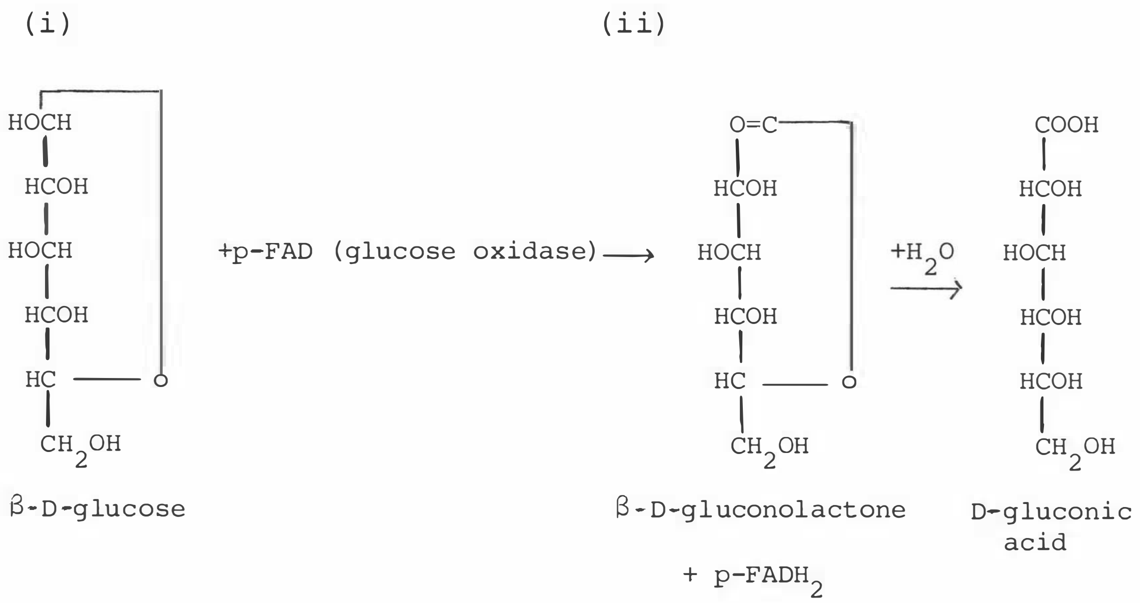
In 1928, Muller discovered a new enzyme in the culture of Aspergillus niger and Penicillium glaucum and named it glucose oxidase. He reported that the enzyme catalyses the oxidation of glucose to gluconic acid. Franke and Lorenz (1937) and Franke and Deffner (1939) succeeded in partial purification from Aspergillus niger and further identified hydrogen peroxide as a second product of the glucose oxidase reaction. Coulthard *et al* (1945) from Great Britain and Van Bruggen *et al* (1943) in the United States independently isolated glucose oxidase from the culture of Penicillium notatum and named it "Notatin" and "Penicillin B" respectively.

In 1948, Keilin and Hartree studied the enzyme extensively and reported the information relating to its assay and its composition as a flavoprotein. Since then the enzymatic oxidation of glucose became interesting to clinical chemists as the method is more specific for glucose than other methods. The first clinical method using glucose oxidase for the measurement of blood glucose was described by Froesch and

Renold in 1956. In the same year Keston introduced the coupled glucose oxidase-peroxidase enzyme system for the estimation of glucose in biological fluids by using the oxygen acceptor o-toluidine as a chromogen. Keston and Teller introduced o-dianisidine as a chromogen in 1956. Various modifications of this method have aimed at modifying the sensitivity and stability of the colour of the final product (Huggett and Nixon 1957, Saifer and Gerstenfeld 1958, McComb and Yushok 1958, Washko and Rice 1961, Getchell *et al* 1964).

The principle of this method is that glucose oxidase catalyses the oxidation of glucose to gluconic acid and hydrogen peroxide. The flavin-adenine dinucleotide (FAD), prosthetic group of the enzyme, is the initial hydrogen acceptor for the oxidation of glucose to gluconolactone and in turn the reduced FAD is reoxidized by molecular oxygen dissolved in the solution, the product of the reaction being hydrogen per oxide. In the presence of excess peroxidase and chromogen, the hydrogen peroxide is rapidly reduced to water and the chromogen oxidised to a coloured product (deep-pink) which can be monitored at 520 nm.

"Oxidation of glucose catalysed by glucose oxidase"



(iii) $p - FADH_2 + O_2 \longrightarrow p - FAD + H_2O_2$

(iv) O-Dianisidine + $H_2O_2 \xrightarrow[H_2SO_4]{\text{Peroxidase}}$ Oxidised o-dianisidine + H_2O
(deep-pink colour)

As this enzyme is specific for the β form of D glucose, some commercial preparations of glucose oxidase contains mutarotase enzyme which can transform the α forms to β form. The method is not inhibited by HCN, H_2S, HN_3, NaF or urethane (van Bruggen *et al* 1943), but uric acid, ascorbic acid, glutathione, cysteine, bilirubin, thymol and catechols can interfere with the final colour development (Fales 1963). Apart from these interferences, the method is reliable, reproducible and specific for glucose. Its specificity was shown by many investigations (Middleton and Griffiths 1957, Beach and Turner 1958, Kinglsey and Getchell 1960). This method was adapted for continuous-flow analysis since 1950's.

During the 1960's, other enzymatic assay methods specific for glucose were introduced by various scientists. Slein (1963) and Stark and Schmidt (1968) used hexokinase and glucose-6-phosphate dehydrogenase for blood glucose determination. In the presence of hexokinase and magnesium ions, glucose can be transformed to glucose-6-phosphate. In the presence of glucose-6-phosphate dehydrogenase, glucose-6-phosphate is oxidised to 6-phosphogluconate, thus reducing the coenzyme NADP to NADPH. The increase in absorbance due to NADPH is equivalent to the glucose originally present. This new yeast-enzyme method is simple and highly specific and therefore is still used as a reference method. Extensive investigations have demonstrated that this method is essentially free from the interferences because glucose-6-phosphate dehydrogenase is highly specific for the intermediate product glucose-6-phosphate (Wright *et al* 1971, Neeley 1972, Coburn and Carroll 1973). In 1974 Bondar and Mead showed that using glucose-6-phosphate dehydrogenase from Leuconostoc mesenteroides allowed NAD to be substituted for the more expensive and less stable $NADP^+$. The use of co-immobilized hexokinase/glucose-6-phosphate-dehydrogenase methods for glucose were introduced by Garber *et al* (1978) and Leon *et al* (1980).

2.2.3 Oxygen Electrode Method

In 1965, Kadish and Hall reported a new method for the continuous monitoring of blood glucose by measuring dissolved oxygen. The difference between the initial and final oxygen levels were detected by a polarographic oxygen-analyser which was attached to a recorder. Thus oxygen depletion was measured and interpreted as the glucose concentration in the original sample. In 1967, Updike and Hicks described a miniature electrical transducer: "The enzyme electrode" which was made by polymerizing a gelatinous membrane of immobilized enzyme over a polarographic oxygen-electrode. Thus the determination of glucose concentration by oxygen consumption method was gradually developed by further investigations (Kadish *et al* 1968, Chua and Tan 1978, Updike *et al* 1979, Sokol *et al* 1980). This method is not only highly specific for glucose, but also can avoid some of the problems associated with the previous method because it measures oxygen consumption instead of hydrogen peroxide formation. It was thus rapidly and widely applied in laboratories throughout the world. Because of its simplicity, reliability and reproducibility, it is well-known as one of the ideal methods.

The principle of an alternative oxygen electrode method is an amperometric electrode system which can respond to the hydrogen peroxide. Whenever hydrogen peroxide is produced by glucose oxidation, it diffuses towards the platinum anode of the sensor probe where it is reduced to produce a signal current. This current flow in the platinum anode circuit is completed by the silver cathode of the sensor probe. The serial reactions are as follows: -

$$\beta\text{-D-glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{Glucono-d-lactone} + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{platinum anode}} 2H^+ + O_2 + 2e^-$$

$$2AgCl + 2e^- \xrightarrow{\text{silver cathode}} 2\,Ag^o + 2Cl^-$$

At constant chloride concentration, the potential of this reaction is practically independent of current, so the silver electrode provides a stable reference potential. The silver chloride layer is regenerated spontaneously by atmospheric oxidation.

$$4\ Ag^{o} + 4Cl^{-} + O_2 + 2H_2O \longrightarrow 4\ AgCl + 4\ OH^{-}$$

Thus any change in local concentration of hydrogen peroxide causes a change in the current in the sensor probe electrodes and these signals are transformed to a voltage and shown on a digital display.

CHAPTER III

3.1 ORGANISATION OF THE TRIAL

The trial was conducted using twenty adult cows from the Massey Best farm. All the trial cows were free-grazing and non-lactating.

3.1.1 Grouping of the Cows

Twenty cows were divided into five groups of four, according to breed, age, body weight and condition score (Table 1). Each group contained Friesian and Jersey or Friesian and Friesian-Jersey cross cows. The total body weight of each group was adjusted to between 192.5 to 202 kg. Each group also contained one pregnant cow. The groups were thus matched as closely as possible.

3.1.2 Bleeding of the Cows

All the cows were bled daily for four days prior to and eight days after the injection of the drug. Every morning, between 9 a.m. and 10 a.m., blood samples were collected by jugular venepuncture into 10 ml fluoride-oxalate venoject(1) tubes, containing 20 mg potassium oxalate and 25 mg sodium fluoride.

3.1.3 ADMINISTRATION OF THE DRUG

Betsopart(2) or Betsolan(3) (0.04 mg/kg body weight) was injected subcutaneously in the neck after bleeding on the fourth day. The dilution of the drug with distilled water was made in the syringe just before the injection. The amount injected is shown in Table 1. The control group was injected

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(1) Venoject T-200PS VT-100FX
Jintan Terumo Co Ltd., Japan

(2) betamethasone aqueous suspension 10 mg/ml
Glaxo Laboratories (N.Z.) Ltd., Palmerston North

(3) betamethasone aqueous suspension 2 mg/ml
Glaxo Laboratories (N.Z.) Ltd., Palmerston North

TABLE I: BETAMETHASONE TRIAL PROTOCOL SHOWING THE GROUPING OF THE COWS BY BODY WEIGHT AND BREED, THE AMOUNT OF DRUG ADMINISTERED AND THE COMPOSITION OF THE INJECTED DOSE.

Groups	Cow No and breeds	Body Weight (kg)	mg of betamethasone	Volume injected (ml)	
1 Control	xb D.69	590.00		Distilled water 10.00ml	
	xb 659	530.50		"	
	F 204	490.50		"	
	F 77	380.00		"	
2 Standard Betsopart (10 mg/ml)				Drug	
	F 191	570.50	23.00	2.3 ml	
	F 10	530.00	21.20	2.12 "	
	F 16	520.50	21.00	2.10 "	
	J 2	390.00	15.60	1.56 "	
3 Standard Betsolan (2 mg/ml)	xb 41	500.00	20.00	10.00 ml	
	F 1	540.00	21.60	10.80 "	
	xb 14	480.00	19.20	9.60 "	
	F 76	420.50	17.00	8.50 "	
4 Diluted Betsopart (5 mg/ml)				Drug	Water
	F 100	500.00	20.00	2.0 ml	2.0 ml
	F 39	580.00	23.20	2.32	2.32
	F 43	470.00	18.80	1.88	1.88
	J 40	420.00	16.80	1.68	1.68
5 Diluted Betsopart (2 mg/ml)	F 80	510.00	20.40	2.04	8.16
	F 15	550.00	22.00	2.20	8.80
	J 78	430.00	17.20	1.72	6.88
	F 99	430.50	17.40	1.74	6.96

F = Friesian
J = Jersey
xb = Friesian/Jersey cow

with 10 ml of distilled water. Bleeding was continued through to the twelfth day.

3.2 COLLECTION AND PRESERVATION OF THE SAMPLES

Blood samples were taken at approximately the same time on each occasion to minimise the possible effect of diurnal changes in blood glucose level. The tubes were then centrifuged within one hour of bleeding (3500 r.p.m. for 15 minutes). The plasma was separated from the red blood cells, transferred to the labelled auto analyser cups, capped and stored frozen at -10°C.

3.3 ANALYSIS OF THE SAMPLES

Three assay methods were used to measure the plasma glucose level. A Chemlab(4) continuous-flow analyser was used for the alkaline-ferricyanide reduction method and the glucose-oxidase enzymatic method and a YSI Model 27 Glucose Analyser(5) was used for the oxygen electrode method. For precision control purposes, a control serum was analysed with every ten unknown samples. A stock standard glucose of 55.5mM in 0.2% benzoic acid was prepared and diluted with 0.2% benzoic acid to make working standard glucose solutions covering the range of 1.4mM to 8.4mM. The same set of six glucose standards were used for all three assay methods.

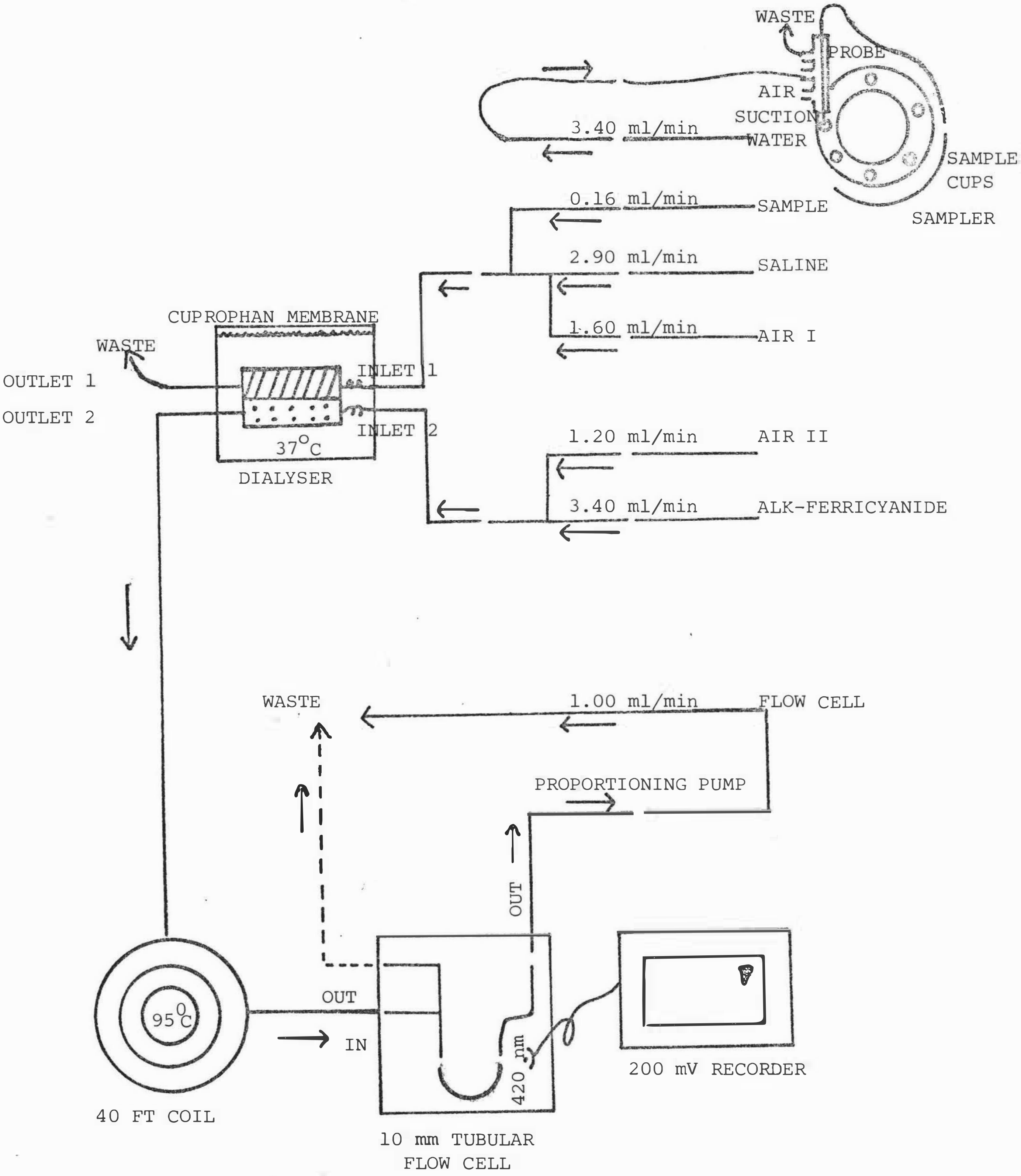
3.3.1 Alkaline Ferricyanide Reduction Method

The method used was based on the Technicon N.2b procedure which is inturn modified from a method by Hoffman (1937). The Chemlab modules used and the modified manifold for the proportioning pump, together with the flow-rates used are shown in Figure 13. The sampler was run at a rate of 40 samples per hour, with a 2:1 ratio of sample-wash time.

(4) Chemlab, England

(5) Yellow Springs Instrument Co. Inc., Yellow Springs, Ohio, U.S.A.

FIGURE 13: Chemlab-analyser modules and manifolds for Alkaline ferricyanide reduction method for plasma glucose.



3.3.1.1 Reagents

(a) Saline:
9 gm/l sodium chloride containing 0.5 ml/l 30% Brij-35 as a wetting agent.

(b) Alkaline potassium ferricyanide solution:
9 gm/l sodium chloride, 20 gm/l sodium carbonate, 0.25 gm/l potassium ferricyanide and 0.5 ml/l 30% Brij-35.
This was stored in a dark place at room temperature.

3.3.1.2 Colorimeter adjustment

Since increasing concentrations of glucose yield decreasing absorbances at 420 nm in this procedure, the flow cell was installed in the right hand beam of the colorimeter so that decreasing absorbance gave a positive (left-to-right) deflection of the recorder. The amount of light through the flow cell was adjusted to give almost a full scale reading from the high standard (8.4 mM), and the gain of the recorder used to set the reading of the reagent-only baseline near to zero on the chart.

3.3.1.3 Data processing : Ferricyanide method

A plot of peak chart reading versus concentration of glucose in the six standards (1.4 mM to 8.4 mM) was curvilinear and could not be linearised by simple transformation of the data. Consequently, the standard response in each batch of assays was fitted to a sixth-order polynomial:

$$\text{Concentration} = A + BR + CR^2 + DR^3 + ER^4 + FR^5$$

where R is the chart reading and A to F are constants. The coefficients A to F were computed on a Sharp PC 1211 pocket computer using a programme based on
programme P4-A1 of the Sharp PC 1211 Applications Manual. The concentrations of unknown were then computed from chart readings using this fitted curve.

3.3.2 Glucose Oxidase Enzyme Method

The following method was based on that of Rosevear *et al* (1969) modified for use with Chemlab modules and the available enzyme preparations. The method was glucose oxidase to catalyse the oxidation of glucose to gluconic acid and hydrogen peroxide. An excess of peroxidase (horseradish) is used to oxidise o-dianisidine with the peroxide generated, and sulphuric acid added to produce a deep-pink product from the chromogen, absorbing at 520 nm. The manifold used is shown in Figure 14.

3.3.2.1 Reagents

The same standards and control sera were used as in the ferricyanide reduction method.

(a) Diluent:
40 gm/l $MgSO_4.7H_2O$ in 800 ml distilled water containing 5 ml/l 20% Brij-35$^{(1)}$.

(b) Recipient:
16.1 gm/l Na_2SO_4 containing 10 ml of Triton x-100$^{(2)}$ per litre.

(c) Citrate buffer pH 5:
21.0 g/l citric acid monohydrate adjusted to pH 5 with 50% (w/v) NaOH.

(d) Enzyme-dye reagent prepared by mixing the following solutions:

(i) 800 mg of o-dianisidine dihydrochloride$^{(3)}$ in 50 ml of distilled water.

(ii) 700 mg of glucose oxidase enzyme$^{(4)}$ in 50 ml of distilled water.

(iii) 25 mg of peroxidase$^{(5)}$ in 25 ml water.

(1) Brij SP, Atlas Chemical Industries

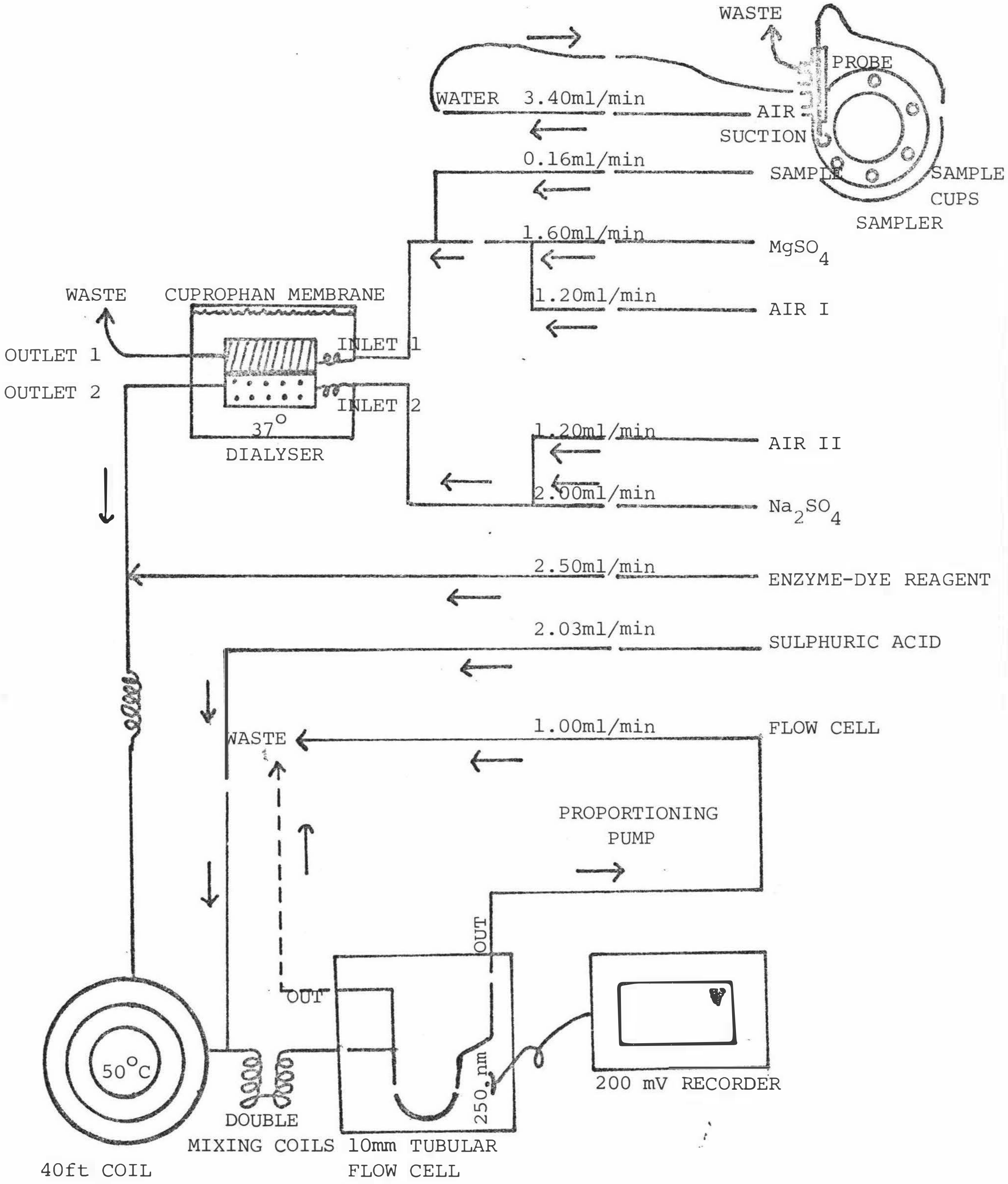
(2) Triton x-100, Rohm and Haas

(3) Sigma D.3252

(4) Sigma Type II G.6125

(5) Sigma Type I P.8125

FIGURE 14: Chemlab-analysier modules and manifold for glucose oxidase-peroxidase method for plasma glucose.



(iv) 500 ml of citrate buffer solution (pH 5) diluted with 400 ml glycerol.

To make one litre of enzyme-dye reagent, 50 ml glucose oxidase, 50 ml o.dianisidine and 25 ml peroxidase were mixed and diluted to one litre with glycerol-citrate buffer. The mixed reagent was then filtered through a sintered glass funnel and stored at 4°C until used.

(e) Sulphuric acid:
380 ml of analytical grade sulphuric acid added slowly to 684 ml distilled water with stirring and cooling.

3.3.2.2 Colorimeter adjustment

In this procedure increasing concentrations of glucose yielded increasing absorbances at 520 nm. The flow cell was installed in the usual left hand beam of the colorimeter, the amount of light through the flow cell was adjusted to zero chart reading with reagents only flowing, and the gain of the recorder was used to set the maximum chart reading for the highest concentration of standard glucose. Thus increasing absorbances gave left-to-right deflections of the recorder.

3.3.2.3 Data processing: Glucose oxidase method

Since a plot of peak chart reading verus concentration of glucose in standards was consistently linear up to 7 mM, it was possible to calculate the concentration of unknown by linear interpolation between standards. A Sharp Pc-1211 pocket computer was programmed to fit a least-squares regression to the readings of five standards (1.4 mM to 7.0 mM) and interpolate unknown readings from the regression. The computer programme was based on Programme P4-B12 of the Sharp PC 1211 Applications Manual.

3.3.3. Oxygen Electrode Method

The YSI Model 27 glucose analyser uses an amperometric method to measure hydrogen peroxide produced by the glucose oxidase reaction. Glucose from the sample diffuses through a polycarbonate membrane with 0.03 u pores which excludes colloids, and is oxidized by glucose oxidase immobilised with glutaraldehyde in a second membrane (Fig 15). Some of the generated hydrogen peroxide passes through a third membrane designed to exclude reducible molecules of molecular weight over 100, and reduction occurs at a platinum anode.

$$H_2O_2 \xrightarrow{\text{Pt: anode}} 2H^+ + O_2 + 2e^-$$

This anode is maintained at 0.7 v with respect to the reference silver/silver chloride electrode so that a current flows between these in proportion to the peroxide reduced. This current is converted to a digital voltage and displayed.

The model 27 takes a 25 ul sample, injected into the buffer in the electrode chamber when the operator is prompted by the instrument. The reading is displayed after approximately 60 seconds and each sample or standard takes about 2 minutes for analysis. The detailed procedure described in the Model 27 instruction manual was followed exactly in the present work. YSI buffer was used throughout, but the same glucose standards and precision control serum as used for other methods were used.

3.3.4 Precision Control

Precision control of the three analytical methods was carried out using Autonorm(1) control serum. The mean and standard deviation for the control serum was derived for each analytical method from a series of 20 analyses.

A control serum sample was run after every ten test samples and control serum results falling within ± 2.S.D. of the mean was taken as indicating that the method was in control (Fig 16, 17, 18). Results obtained when the control serum was out of the control limits were rejected and the analyses repeated.

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(1) Nyegaard & Co. A/S, Oslo, Norway

FIGURE 15: The sensor probe and the membrane of YSI Model 27 Analyser

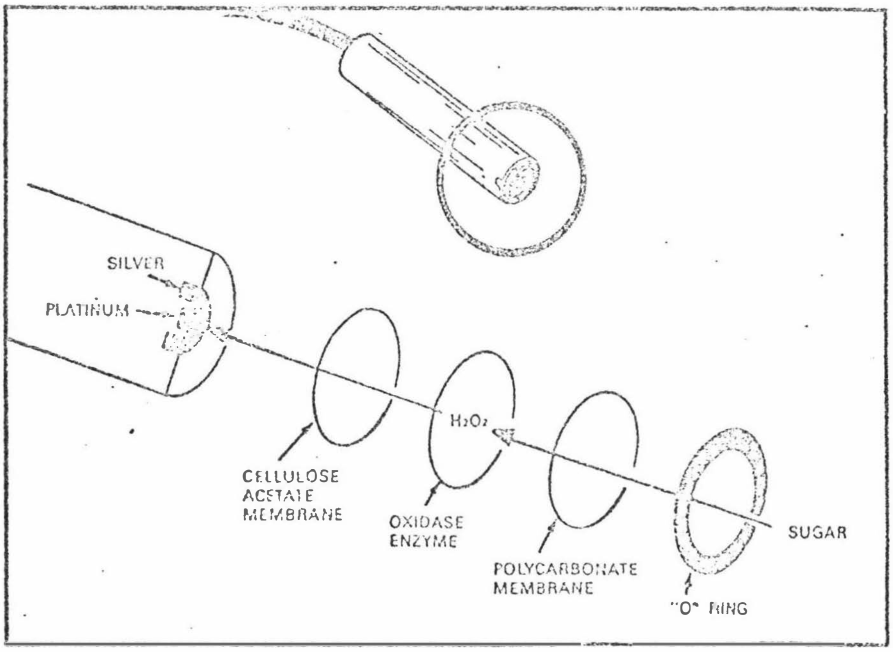


FIGURE 16: Determinations of glucose concentration in control serum (Ferricyanide Method)

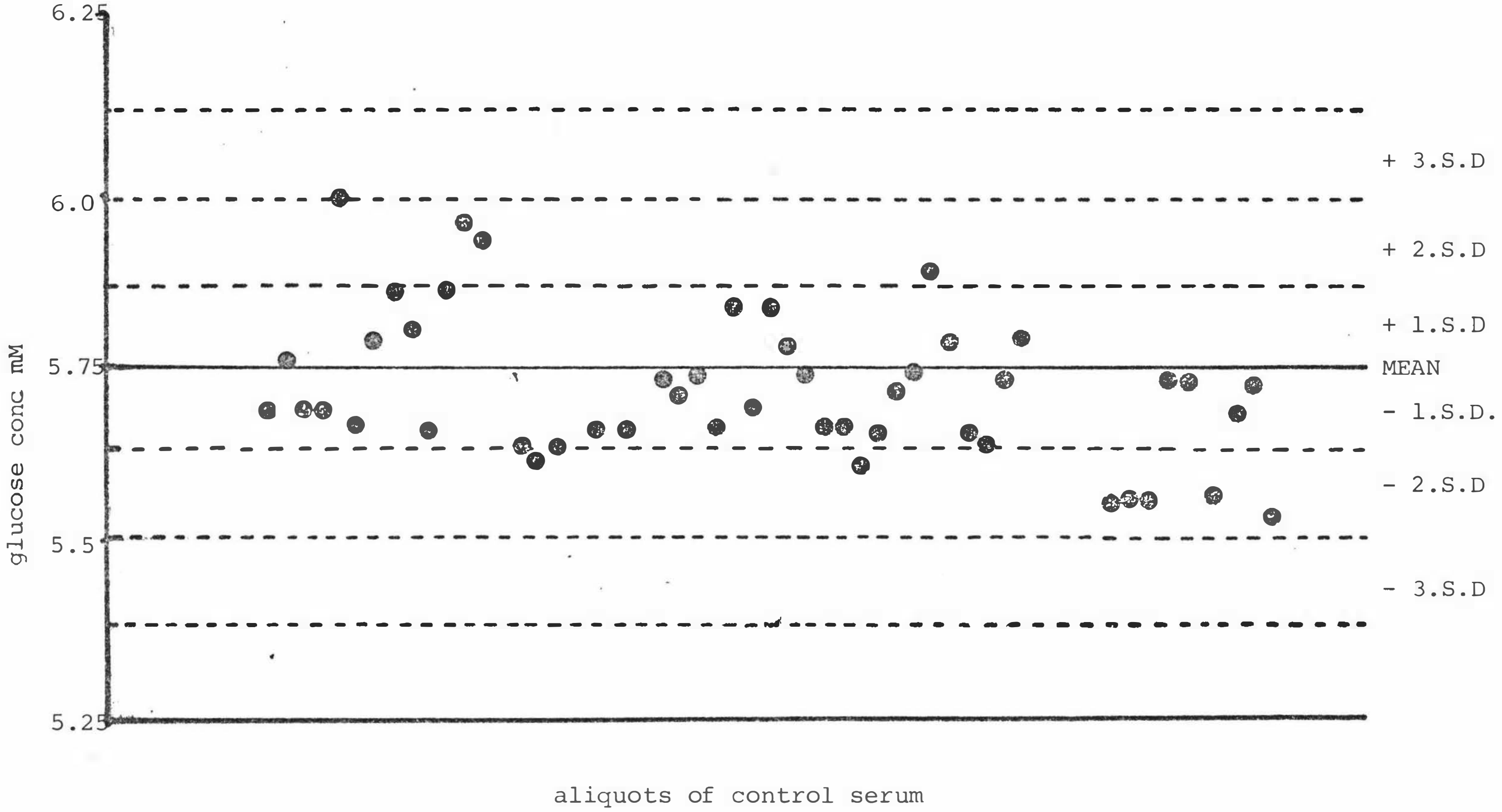


FIGURE 17: Determinations of Glucose concentration in control serum (Glucose Oxidase Method)

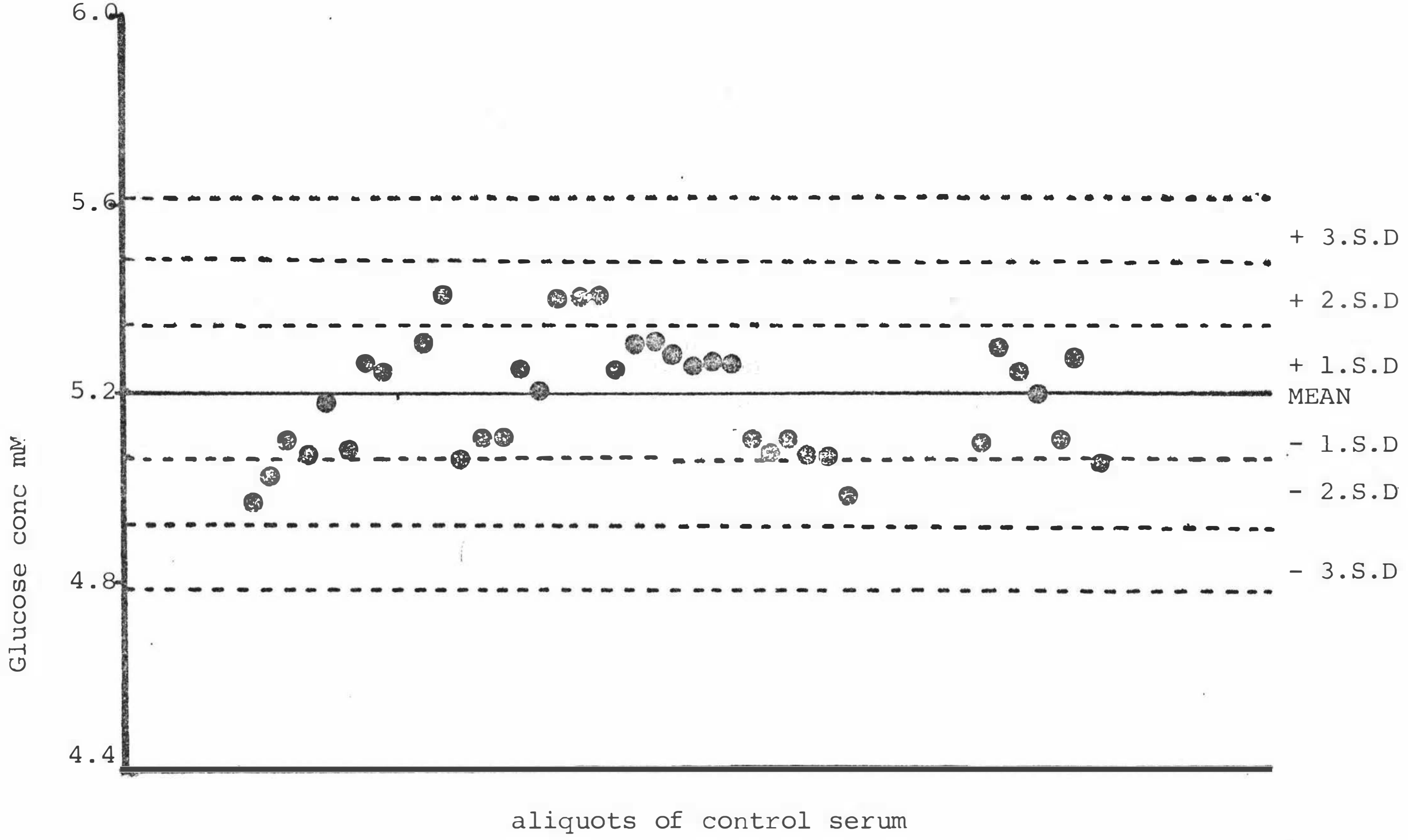
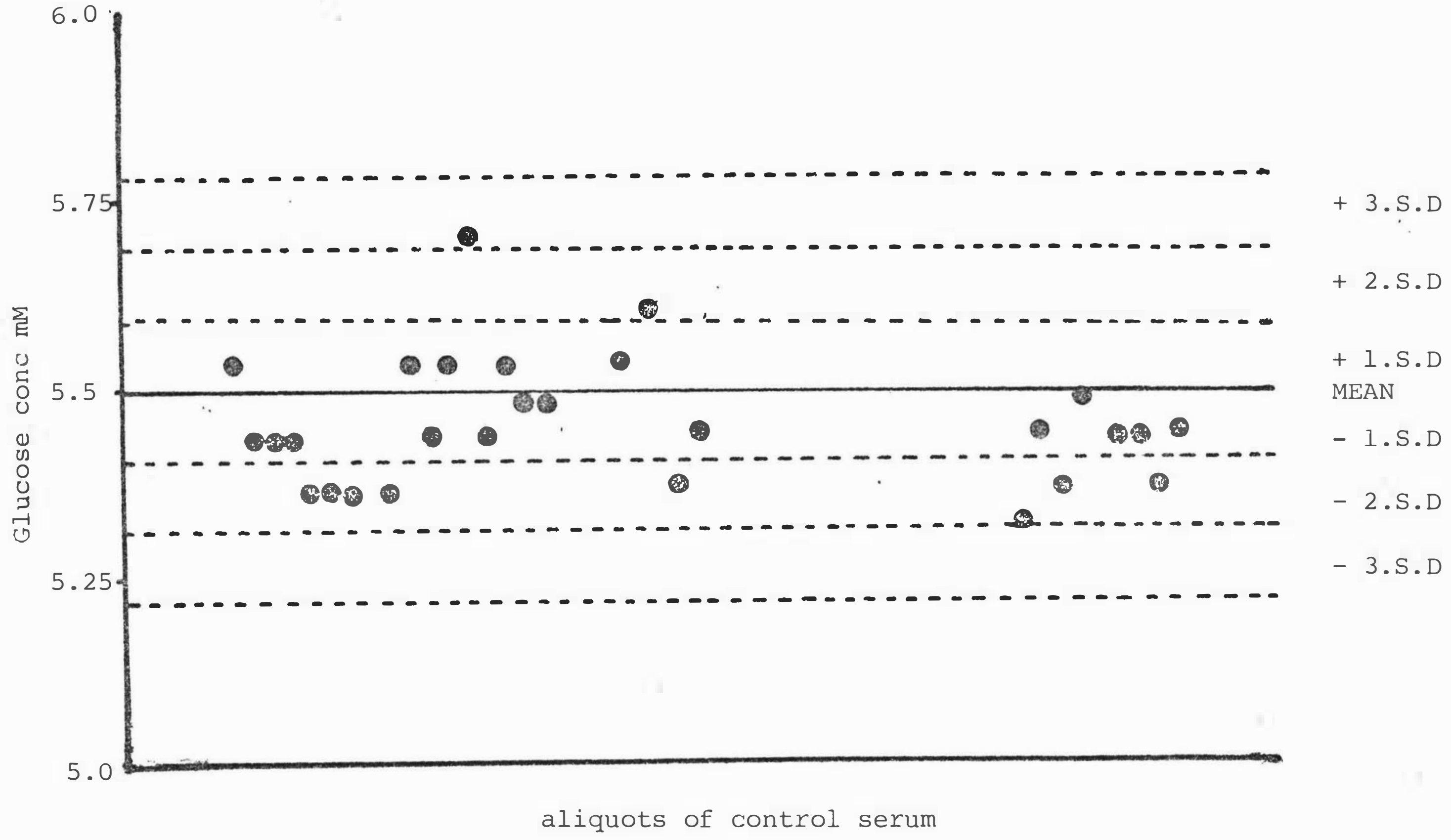


FIGURE 18: Determinations of Glucose concentration in Control Serum (Oxygen Electrode Method)



When all samples had been analysed by each of the three methods some results were found to be anomalous for one of the methods. These samples were centrifuged to remove any fibrin fragments and were reanalysed by the method concerned.

3.4 STATISTICAL METHODS

All statistical methods used are those of Bliss (1967). Linear regressions of the form $Y = a + bX$, with $Y = \log_{10}$ glucose concentration (mM) and X = time were fitted to the data for each cow. Day 0 corresponds to the first day after injection of the glucocorticoid, that is day 5 of the trial. As a first approximation, all samples following injection were included (day 0 to 7 inclusive). Estimates of the variance due to regression and of the residual variance of Y were obtained for subsets of the samples (days 0-6, 1-7, 1-6) and used to find the maximum linear range of the response curves for individual animals. A common linear range applicable to all animals was established and the variations in the regression coefficients for individual animals within each treatment group were examined by analyses of variance for the three methods of glucose determination.

Comparisons between treatment groups were made using estimates of the slope and intercept for the common linear regression for all cows in a group. The significance of differences between groups was assessed by constructing 95% confidence limits for each estimate.

The mean levels of glucose obtained with each of the three methods were compared for three sets of data. In the first set of data, which was obtained from repeated determinations of the precision control serum, differences between the means were assessed by simple t-tests. In the second set of data, obtained on the four days before treatments were applied, the effects of differences between cows were eliminated by using paired t-tests. In the third set of data, that used to assess the responses of the cows to the corticoid treatments, an analysis of variance was used to test the significance of differences between means. In this analysis the effect of variation between cows was excluded by using the mean squares within cows pooled over both treatment groups and method of glucose determination as the error mean square for the analysis of variance. The data had been transformed by taking logarithms to the base ten.

CHAPTER IV

4.1 INTRODUCTION

Before carrying out the betamethasone trial, some preliminary work was done to investigate the effect of various blood collection and handling techniques on the apparent plasma glucose levels. The results of these tests determined the methods used in the main part of the study.

4.2 THE EFFECT OF ANTICOAGULANTS ON PLASMA GLUCOSE LEVELS

Blood samples from two cows were collected into fluoride-oxalate venoject tubes,(1) EDTA venoject tubes,(2) and plain venoject tubes.(3) Plasma and serum glucose values were analysed by both Ferricyanide reduction and Glucose oxidase methods. Table 2 shows that by both methods of glucose analysis, serum and plasma glucose levels were very similar but fluoride-oxalate plasma had lower levels.

The effect of adding fluoride-oxalate anticoagulant to aqueous glucose solutions and serum was also evaluated by the same methods to determine whether fluoride-oxalate inhibits the analytical methods. Table 3 shows that the presence of fluoride and oxalate did not inhibit either analytical method applied to either aqueous glucose solutions or serum.

The effect of adding fluoride-oxalate to whole blood and EDTA plasma samples was investigated on samples from another cow to determine whether the fluoride-oxalate effect of lowering plasma glucose levels required the presence of blood cells. Table 4 shows that fluoride and oxalate only have the effect of lowering plasma glucose levels when added to whole blood.

(1) T.200 PS Vt * 100 FX
(2) T.200 Q VT * 100 NA
(3) T.200 VT * 100 P

TABLE 2: THE EFFECT OF ANTICOAGULANTS ON PLASMA GLUCOSE LEVELS

Cows	Anticoagulant	Plasma and serum glucose values (mM)	
		Ferricyanide Method	Glucose Oxidase Method
Cow No (1)	EDTA	3.68	3.64
	Fluoride-oxalate	3.25	3.24
	None (serum)	3.71	3.71
Cow No (2)	EDTA	4.31	3.98
	Fluoride-oxalate	3.75	3.64
	None (serum)	4.37	4.04

TABLE 3: THE EFFECT OF FLUORIDE-OXALATE ON AQUEOUS GLUCOSE SOLUTIONS AND SERUM

	Ferricyanide Method		Glucose Oxidase Method	
	Normal	Fluoride Added	Normal	Fluoride Added
Standard aqueous glucose solutions (mM)	8.40	8.51	8.40	8.32
	7.00	7.00	7.00	7.05
	5.60	5.49	5.60	5.91
	4.20	4.12	4.20	4.16
	2.80	2.60	2.80	2.68
	1.40	1.29	1.40	1.32
Serum Sample (mM)	3.85	3.92	3.73	3.72

TABLE 4: EFFECT OF ADDING FLUORIDE-OXALATE TO WHOLE BLOOD* AND EDTA PLASMA SAMPLES

Blood Samples of Cow No (4)	Plasma glucose values (mM)	
	Ferricyanide Method	Glucose oxidase Method
(1) Normal EDTA plasma	3.75	3.64
(2) EDTA plasma + fluoride oxalate	3.81	3.69
(3) Whole blood + EDTA + fluoride oxalate	3.42	3.30
(4) Whole blood + fluoride oxalate	3.47	3.36

* Whole blood samples centrifuged 15 minutes after addition of anticoagulant.

To further investigate whether the glucose lowering effect was due to oxalate or fluoride or both, two cows were used to obtain samples of whole blood with and without EDTA and also samples of serum and EDTA plasma. To aliquots of these samples were added either a mixture of fluoride and oxalate, sodium fluoride alone or potassium oxalate alone. All additions were at the rate equivalent to that produced by using venoject tubes.

Tables 5 and 6 show that the addition of sodium fluoride and potassium oxalate either alone or in combination do not lower the plasma or serum glucose levels more than about 1-4% unless they are added before the erythrocytes are separated in which case the decrease is about 5-10%. These results also show that sodium fluoride and potassium oxalate are each able to exert this glucose-lowering effect but that the effect is not cumulative in the case of the fluoride-oxalate combination.

4.3 THE EFFECT OF TIME BETWEEN BLOOD COLLECTION AND THE SEPARATION OF PLASMA OR SERUM FROM THE ERYTHROCYTES ON PLASMA AND SERUM GLUCOSE LEVELS.

To avoid the possible problems of glucose loss in stored blood samples, the effect of time between blood collection and the separation of plasma or serum from the erythrocytes was investigated on three cows using the same three types of venoject tubes. Figures 19, 20 and 21 show that by all three analytical methods, fluoride-oxalate plasma glucose levels, while lower than those of EDTA plasma or serum levels, are relatively more stable for up to three hours after collection.

4.4 BETAMETHASONE EXPERIMENT

The complete results for the trial are presented in Appendix I. The mean blood glucose levels for each experimental group for 12 days of the trial are shown in Tables 7, 8 and 9 and Figures 22, 23 and 24. All betamethasone treatments produced

TABLE 5: EFFECT OF SODIUM FLUORIDE AND POTASSIUM OXALATE ON PLASMA GLUCOSE LEVELS WHEN ADDED TO WHOLE BLOOD AND PLASMA CONTAINING EDTA.

All results are shown as percentage change in plasma glucose after addition of anticoagulants compared to EDTA plasma glucose level.

Analytical Methods / Anticoag-ulant Added	Ferricyanide		Glucose Oxidase		Oxygen Electrode	
	Whole Blood + EDTA	Plasma + EDTA	Whole Blood + EDTA	Plasma + EDTA	Whole Blood + EDTA	Plasma + EDTA
(1) Fluoride-oxalate	-7.79%	-1.21%	-9.74%	-3.06%	-6.96%	+0.25%
(2) Sodium fluoride	-9.99%	-1.65%	-11.78%	-2.94%	-9.85%	+0.56%
(3) Potassium oxalate	-5.66%	-1.28%	-5.15%	-1.59%	-5.54%	-0.34%

TABLE 6: EFFECT OF SODIUM FLUORIDE AND POTASSIUM OXALATE ON SERUM GLUCOSE LEVELS WHEN ADDED TO WHOLE BLOOD AND SERUM.

All results are shown as percentage change in serum glucose after addition of anticoagulants compared to serum glucose level.

Analytical Methods / Anticoag-ulant added	Ferricyanide		Glucose Oxidase		Oxygen Electrode	
	Whole Blood	Serum	Whole Blood	Serum	Whole Blood	Serum
(1) Fluoride-oxalate	-11.92%	-4.87%	-9.97%	-1.79%	-8.37%	-0.28%
(2) Sodium fluoride	- 6.15%	-1.72%	-9.69%	-1.71%	-8.06%	+0.97%
(3) Potassium oxalate	- 6.08%	-2.36%	-5.46%	-0.76%	-6.04%	+0.04%

FIGURE 19: The effect of time between blood collection and centrifugation on the stability of plasma and serum glucose concentrations. (Ferricyanide method)

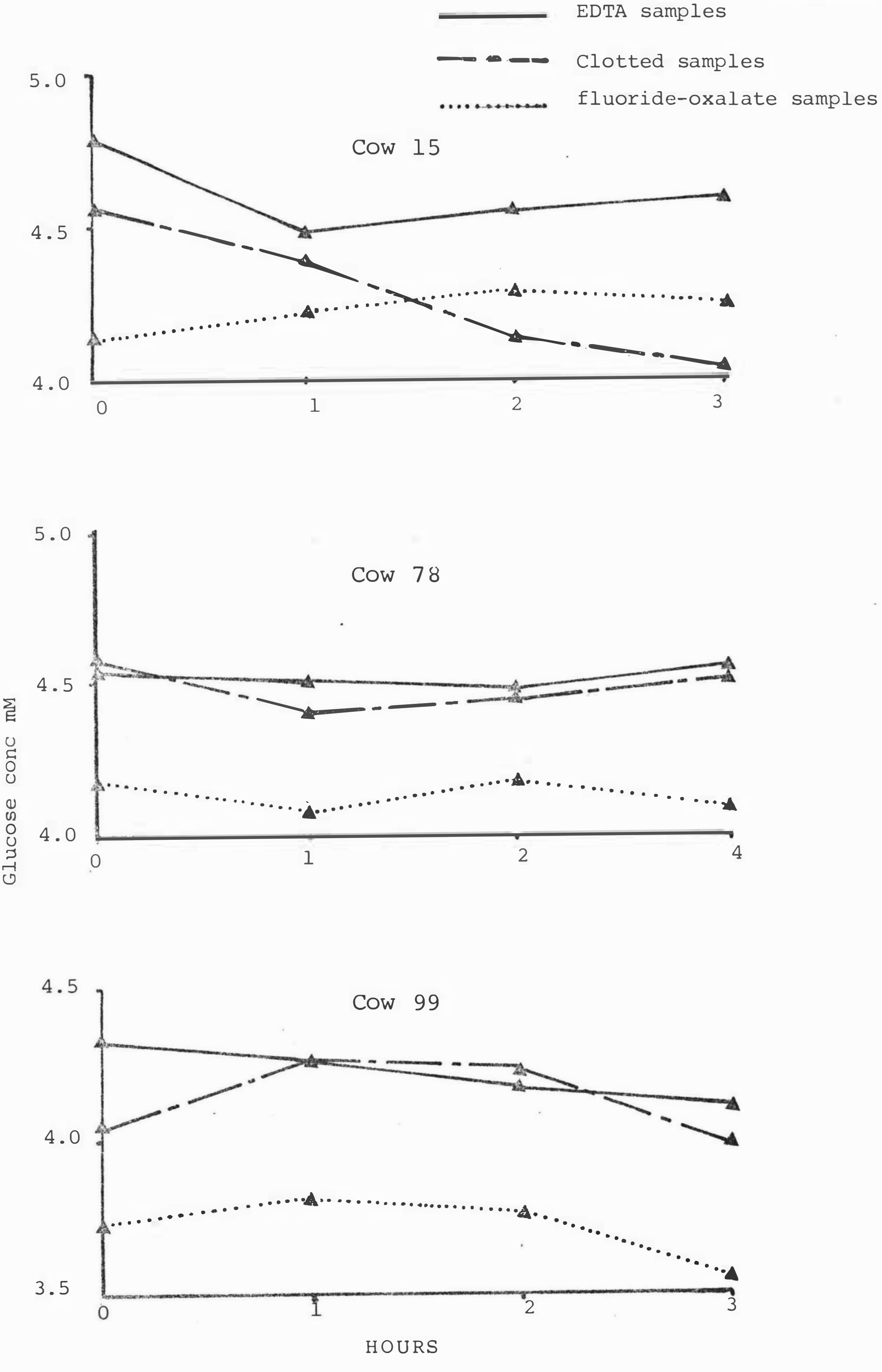


FIGURE 20: The effect of time between blood collection and centrifugation on the stability of plasma and serum glucose concentrations (Glucose oxidase method)

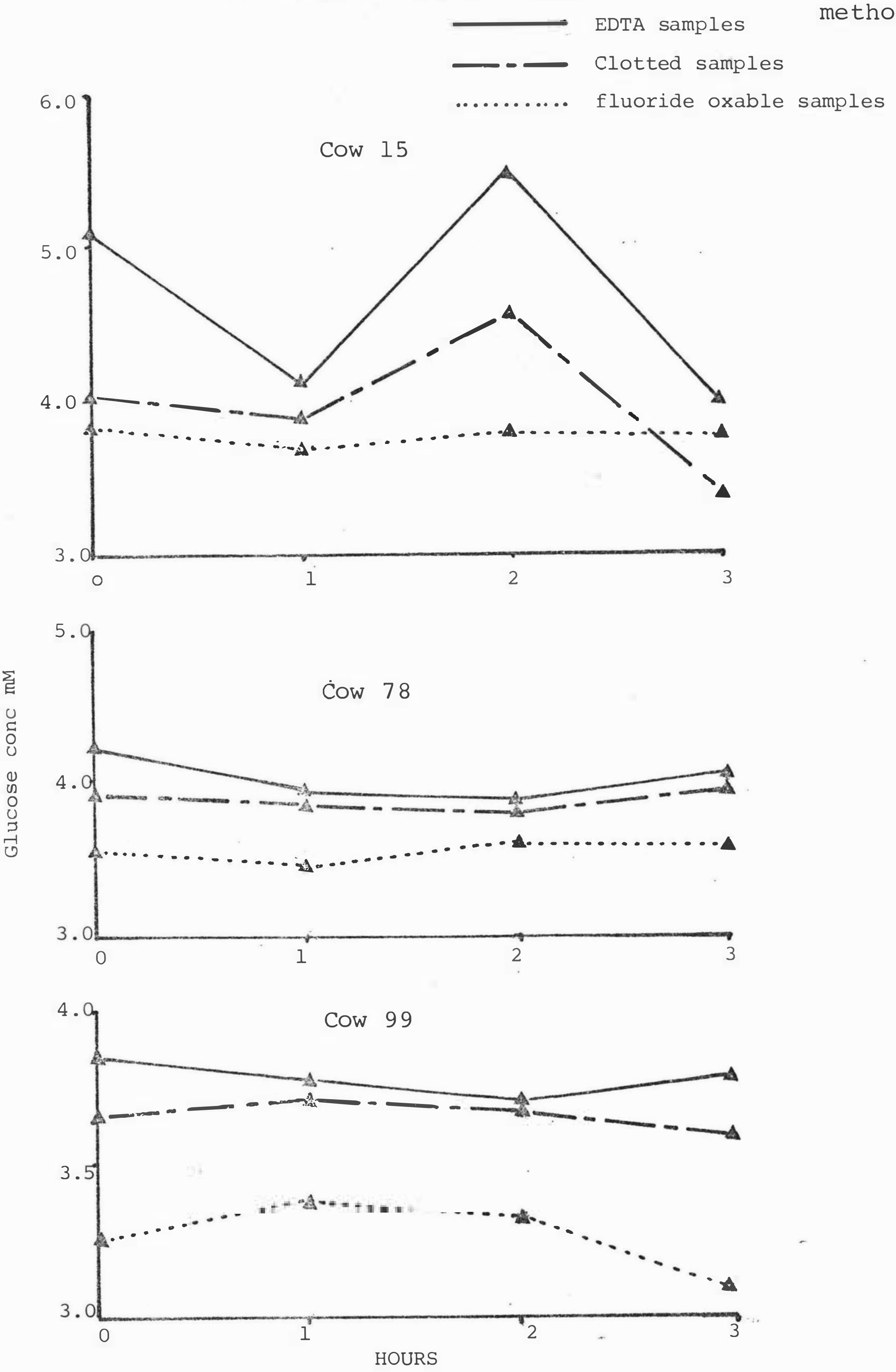


FIGURE 21: The effect of time between blood collection and centrifugation on the stability of plasma and serum glucose concentrations (Oxygen electrode method)

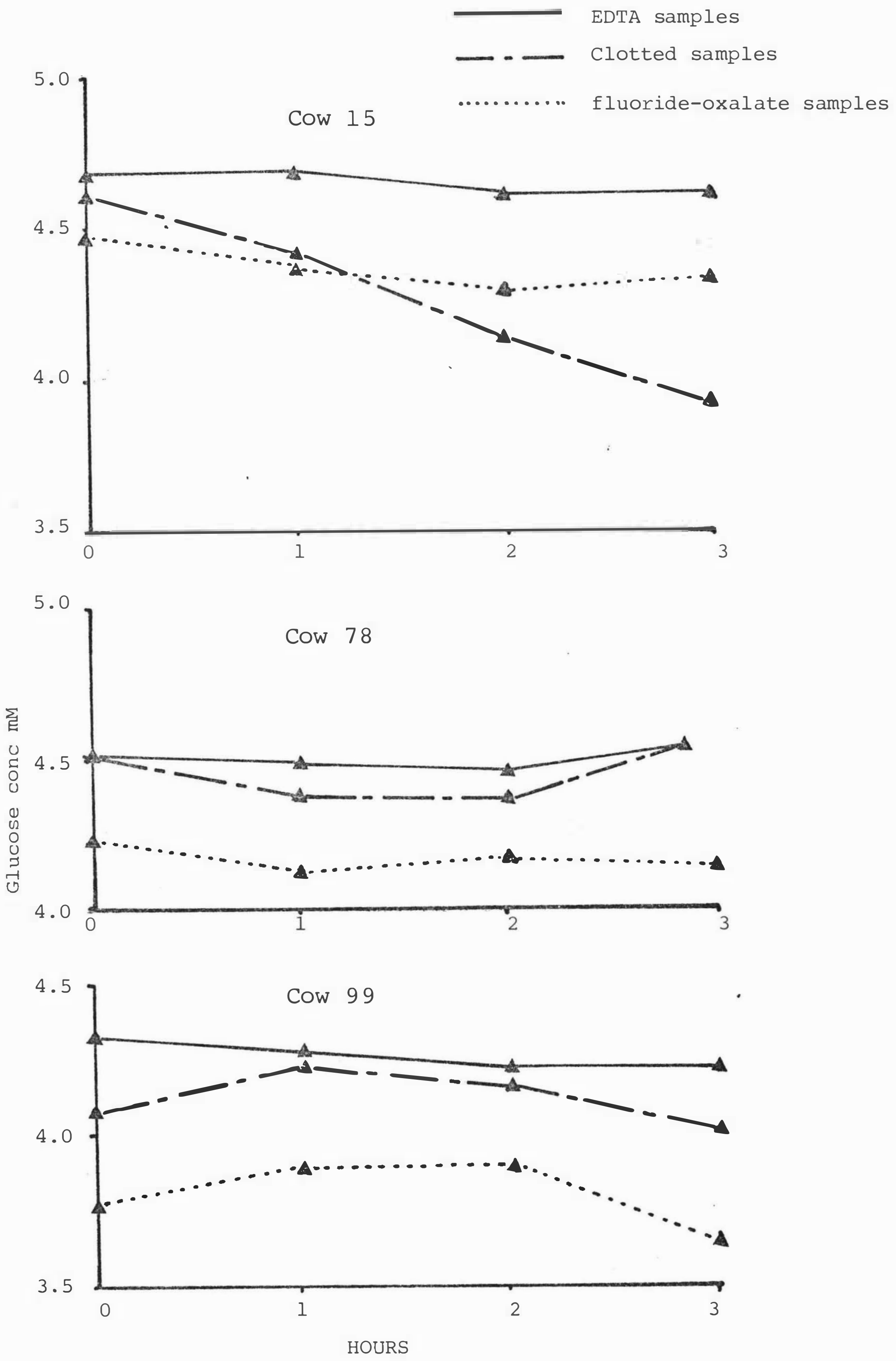


TABLE 7 : MEAN PLASMA GLUCOSE LEVELS OF TRIAL GROUPS (Ferricyanide Method)

Days	Group 1 Control	Group 2 Standard Betsopart	Group 3 Standard Betsolan	Group 4 Betsopart 5mg/ml	Group 5 Betsopart 2mg/ml
1	3.85	3.99	3.71	3.87	4.01
2	3.64	3.69	3.76	3.66	3.59
3	3.89	4.03	4.01	3.70	3.78
4	3.75	3.95	3.78	4.08	3.78
5	3.68	4.89	5.14	5.37	5.71
6	4.33	5.31	4.99	5.61	5.26
7	4.24	5.23	4.89	5.65	4.85
8	3.82	4.80	4.16	5.60	4.39
9	3.98	4.72	4.10	4.81	4.41
10	3.67	4.46	3.73	4.49	3.84
11	3.69	4.16	3.59	4.35	3.77
12	3.84	4.40	3.58	4.32	3.89

TABLE 8 : MEAN PLASMA GLUCOSE LEVELS OF TRIAL GROUPS (Glucose oxidase method)

Days	Group 1 Control	Group 2 Standard Betsopart	Group 3 Standard Betsolan	Group 4 Betsopart 5mg/ml	Group 5 Betsopart 2mg/ml
1	3.33	3.43	3.27	3.47	3.40
2	3.57	3.50	3.60	3.53	3.48
3	3.65	3.76	3.82	3.47	3.59
4	3.36	3.51	3.39	3.62	3.33
5	3.44	4.60	4.79	5.02	5.35
6	4.16	5.03	4.83	5.40	5.08
7	3.84	4.80	4.55	5.25	4.49
8	3.53	4.55	3.94	5.33	4.06
9	3.68	4.39	3.81	4.49	4.07
10	3.32	4.03	3.36	4.07	3.51
11	3.31	3.77	3.31	3.98	3.40
12	3.37	3.86	3.10	3.79	3.37

TABLE 9 : MEAN PLASMA GLUCOSE LEVELS OF TRIAL GROUPS (Oxygen electrode method)

Days	Group 1 Control	Group 2 Standard Betsopart	Group 3 Standard Betsolan	Group 4 Betsopart 5mg/ml	Group 5 Betsopart 2mg/ml
1	3.68	3.86	3.69	3.80	3.86
2	3.61	3.75	3.77	3.68	3.57
3	3.86	3.94	3.89	3.54	3.77
4	3.84	3.88	3.68	4.01	3.73
5	3.75	4.91	5.16	5.36	5.72
6	4.32	5.27	5.01	5.65	5.31
7	4.14	5.18	4.89	5.60	4.84
8	3.81	4.85	4.20	5.63	4.38
9	3.99	4.75	4.09	4.84	4.42
10	3.72	4.48	3.75	4.54	3.91
11	3.71	4.18	3.59	4.43	3.80
12	3.89	4.45	3.61	4.42	3.93

FIGURE 22: Mean Plasma Glucose levels of Trial Cows (Ferricyanide Method)

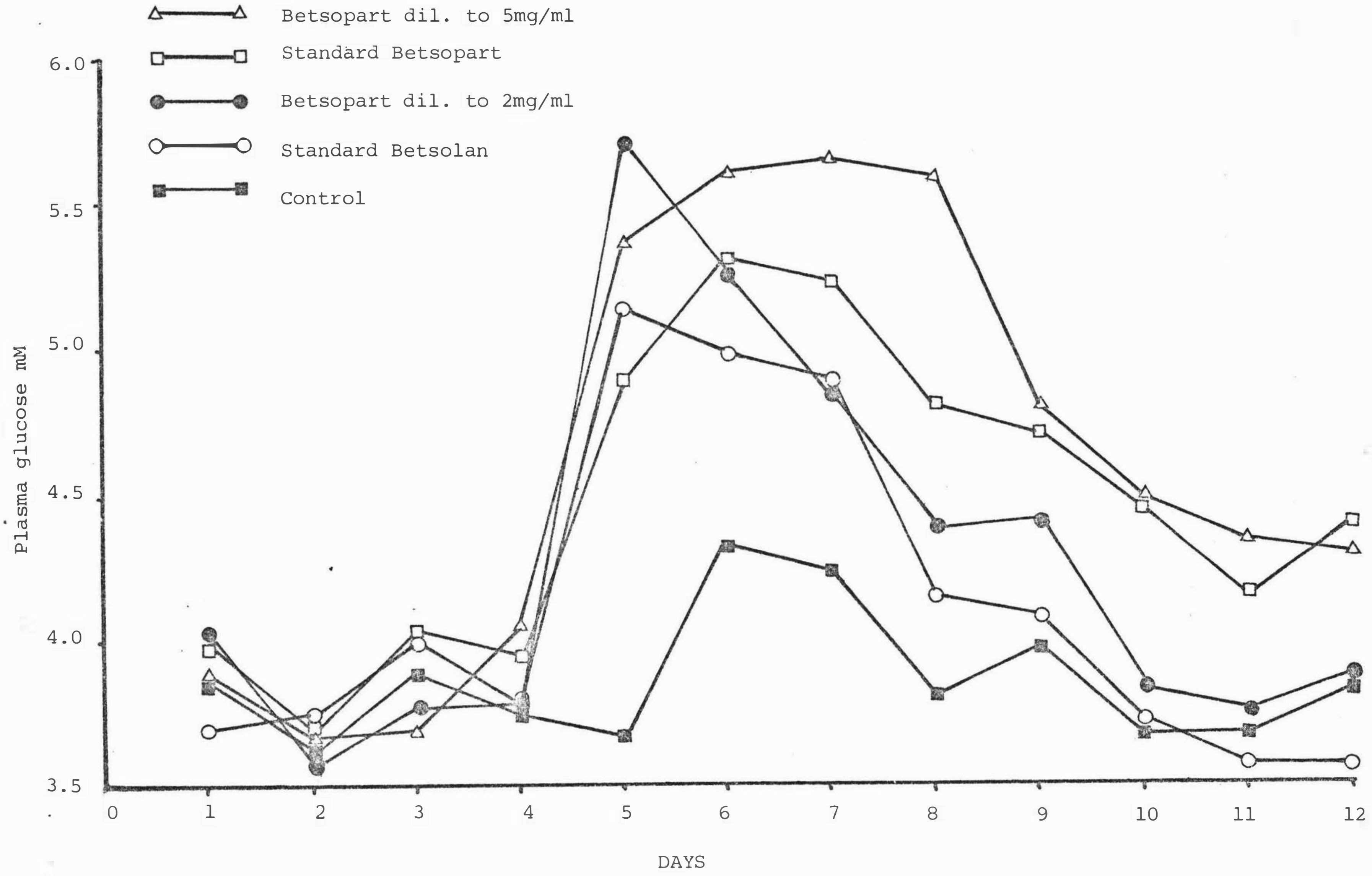


FIGURE 23: MEAN PLASMA GLUCOSE LEVELS OF TRIAL COWS (GLUCOSE OXIDASE METHOD)

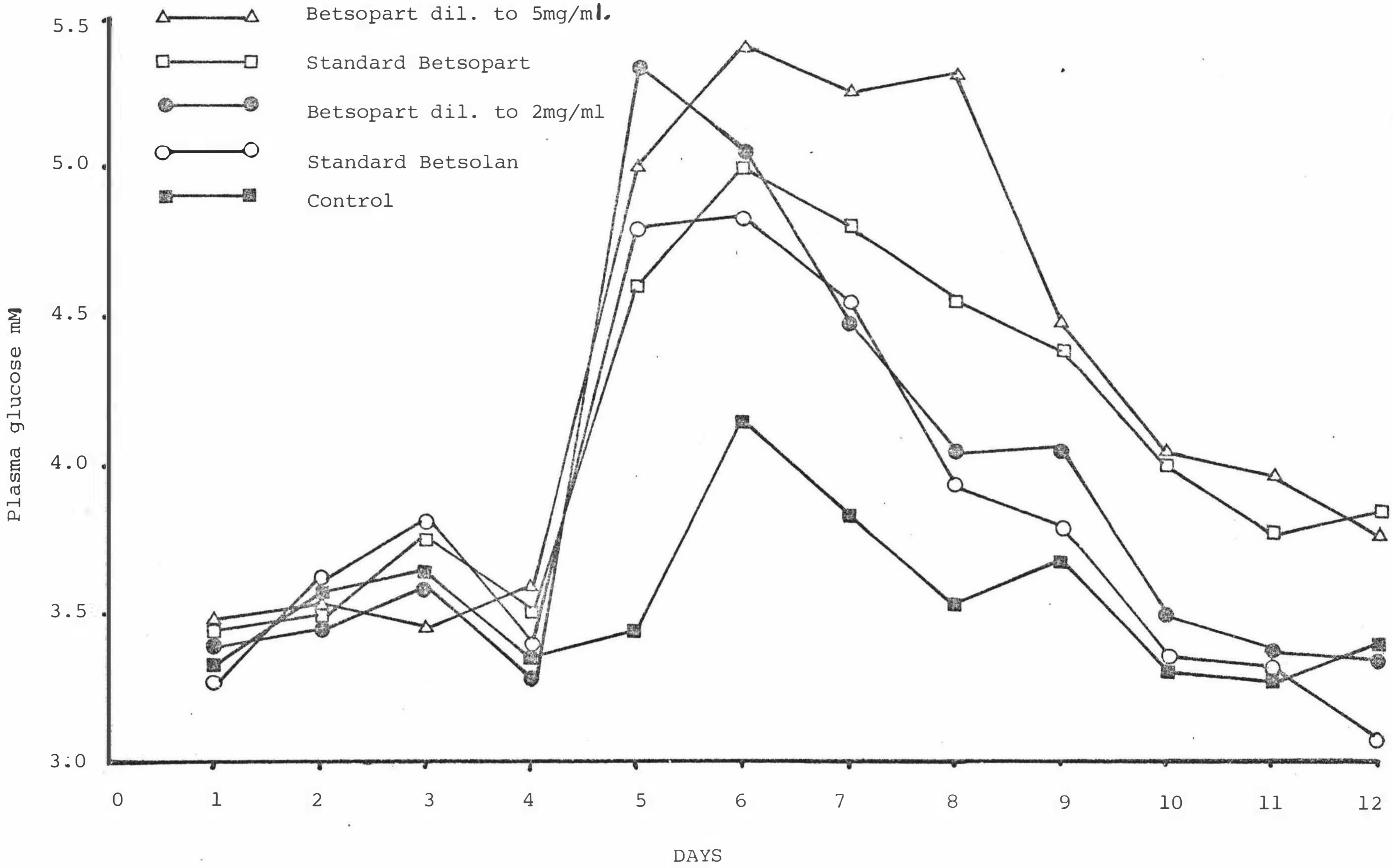
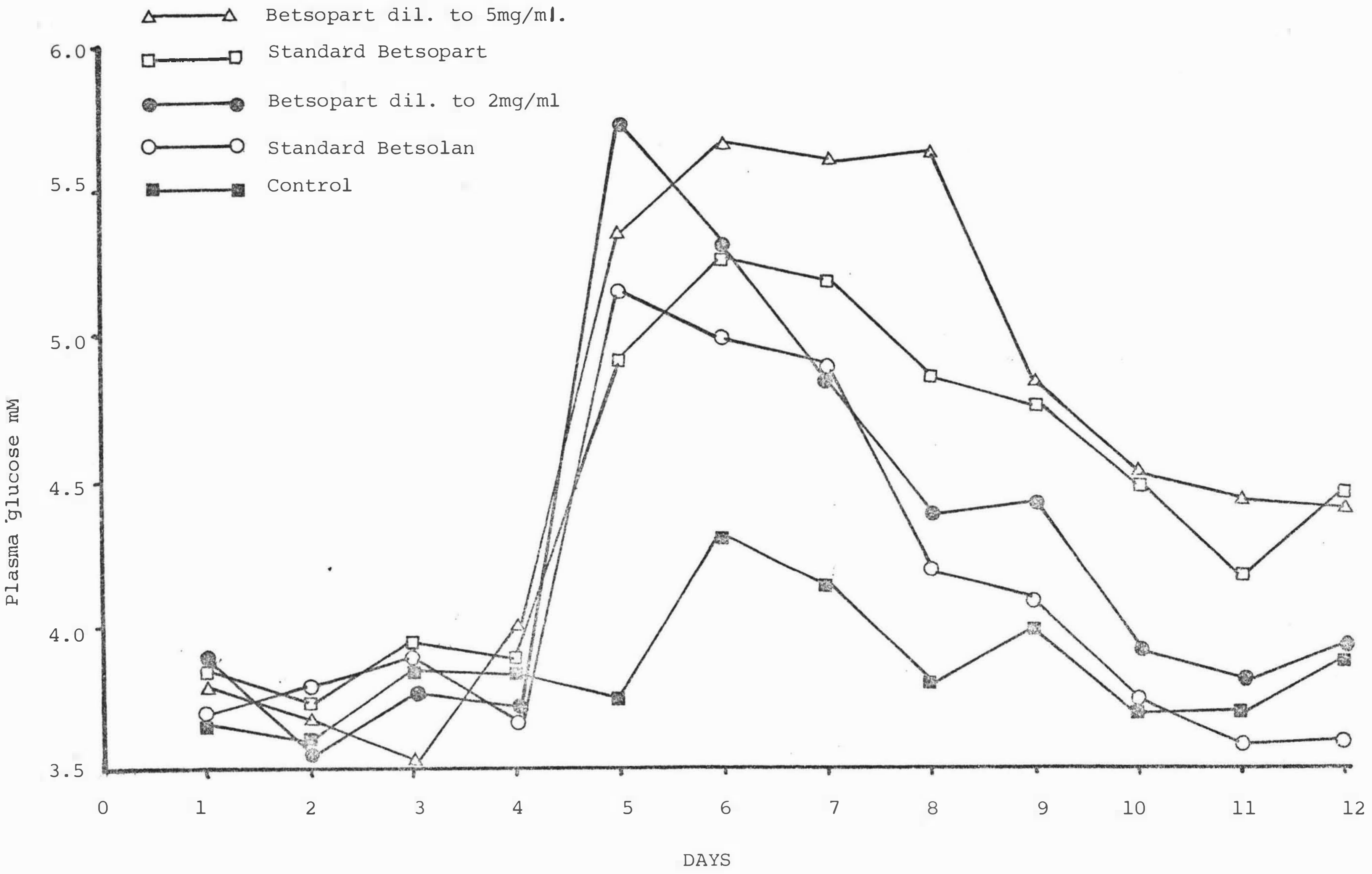


FIGURE 24: MEAN PLASMA GLUCOSE LEVELS OF TRIAL COWS (OXYGEN ELECTRODE METHOD)



a distinct hyperglycaemic effect following injection on day 4. The response for Betsolan and Betsopart diluted to a 2mg/ml appeared to be similar as anticipated, however the response of the 5mg/ml Betsopart was not intermediate between the Betsopart 10mg/ml and the two 2mg/ml formulations.

To compare the peak hyperglycaemic effects and the longevity of the hyperglycaemia in the treatment groups linear regression analyses for the five experimental groups were performed.

In all animals the range day 1 to day 7 (i.e. days 6-12 of the trial) was the maximum linear range and in the majority of animals the regression fitted over this range also had the lowest residual variance of Y (Appendix II). The variation in the estimates for the regression between animals within treatment groups was examined by analysis of variance (Table 10). With the exception of the control group the variance due to the common regression was a much larger component than the variation due to differences between the regressions for individual animals. Comparisons between treatment groups were therefore based on estimates of the slope and intercept of the common regression. These are summarized in Tables 11 and 12 and Figures 25, 26 and 27. Comparing the slopes of the linear regression lines, Betsolan and diluted Betsopart (2mg/ml) are similar in that they both show a significantly greater rate of fall in plasma glucose than the control group (Table 11, Figures 26 and 27). This difference is not significant in the results derived from the ferricyanide analysis because of the large 95% confidence interval for the control group (Table 11). Betsopart and diluted Betsopart 5mg/ml both produced a slow rate of fall of plasma glucose as shown by the fact that the slope of their regression lines is not significantly different from that of the control group. Only for the glucose oxidase analysed data was there a significant difference in the rate of fall of plasma glucose levels between the Betsolan and Betsopart groups (Table 11, Figure 25).

TABLE 10: ANALYSIS OF VARIANCE OF LOG GLUCOSE CONCENTRATIONS FOR EACH OF THE 5 TREATMENT GROUPS

		Glucose Oxidase		Oxygen Electr.		Ferricyanide	
	df	MS	F	MS	F	MS	F
CONTROL							
Within cows	24	.0023376		.0014857		.0046196	
Common regression (day 1-7)	1	.026220	20.18***	.0081324	6.79*	.023930	6.33*
Deviations from common regression	23	.0012993		.0011968		.0037800	
Differences between individual regressions	3	.0030579	2.96^+	.0029471	3.15*	.0095775	3.29*
Deviations from individual regressions	20	.0010355		.00093421		.0029104	
BETSOPART							
Within cows	24	.0028121		.017313		.0019571	
Common regression (day 1-7)	1	.053314	86.49***	.028261	48.90***	.032813	53.30***
Deviations from common regression	23	.00061638		.00057779		.00061563	
Differences between individual regressions	3	.00057644	<1	.00034263	<1	.00046309	<1
Deviations from individual regressions	20	.00062237		.00061307		.00063851	
BETSOLAN							
Within cows	24	.0048383		.0042382		.0043592	
Common regression (day 1-7)	1	.12088	134.11***	.077915	75.28***	.078892	70.52**
Deviations from common regression	23	.00090133		.0010349		.0011187	
Differences between individual regressions	3	.0017738	2.30	.0022745	2.68^+	.0025238	2.78^+
Deviations from individual regressions	20	.00077045		.00084899		.00090798	
BETSOPART (5mg/ml)							
Within cows	24	.0053885		.0035761		.0040013	
Common regression (day 1-7)	1	.092258	57.25***	.051455	34.43***	.059903	38.14***
Deviations from common regression	23	.0016115		.0014944		.0015708	
Differences between individual regressions	3	.0014881	<1	.0015036	1.01	.0015401	<1
Deviations from individual regressions	20	.0016301		.0014930		.0015753	
BETSOPART (2mg/ml)							
Within cows	24	.0050964		.0033128		.0035139	
Common regression (day 1-7)	1	.10230	117.57***	.060616	73.79***	.064730	75.94***
Deviations from common regression	23	.00087011		.00082144		.00085234	
Differences between individual regressions	3	.0013579	1.70	.0016070	2.28	.0018964	2.73^+
Deviations from individual regressions	20	.00079694		.00070361		.00069572	

+ $p < 0.1$ * $p < 0.05$ *** $p < 0.001$

TABLE 11: COMPARISON OF SLOPES AND (95% CONFIDENCE INTERVAL) OF THE REGRESSION LINES OF THE FIVE TREATMENT GROUPS

Treatment Group	Ferricyanide	Glucose oxidase	Oxygen electrode
Control	-.01462 -(.00260 to .02664)	-.01530 -(.00897 to .02163)	-.00852 -(.00176 to .01528)
Betsopart	-.01712 -(.01227 to .02197)	-.02182 -(.01694 to .02770)	-.01589 -(.01118 to .02060)
Betsolan	-.02654 -(.02000 to .03308)	-.03285* -(.02798 to .03872)+	-.02638* -(.02007 to .03267)
Betsopart 5mg/ml	-.02313 -(.01538 to .03088)	-.02870 -(.02085 to .03655)	-.02143 -(.01387 to .02899)
Betsopart 2mg/ml	-.02404 -(.01633 to .02975	-.03022* -(.02445 to .03599)	-.02326* -(.01766 to .02886)

* greater than control

\+ greater than standard Betsopart

TABLE 12: COMPARISON OF THE INTERCEPTS AND (95% CONFIDENCE INTERVAL) OF THE REGRESSION LINES AT DAY 0 (i.e. DAY 5 OF THE TRIAL) FOR THE 5 TREATMENT GROUPS

Treatment Group	Ferricyanide	Glucose oxidase	Oxygen electrode
Control intercept (95% confidence interval)	.6603 (.6378 to .6828)	.6150 (.6018 to .6282)	.6281 (.6154 to .6408)
Betsopart	.7401* (.7310 to .7492)	.7217* (.6880 to .7086)	.7363* (.7275 to .7451)
Betsolan	.7191* (.7069 to .7313)	.7096* (.6986 to .7206)	.7198* (.7080 to .7316)
Betsopart 5mg/ml	.7830*§ (.7695 to .7975)	.7714*§ (.7567 to .7861)	.7801*§ (.7660 to .7942)
Betsopart 2mg/ml	.7301* (.7194 to .7408)	.7165* (.7057 to .7273)	.7294* (.7189 to .7399)

* Greater than control

§ Greater than all other treated groups

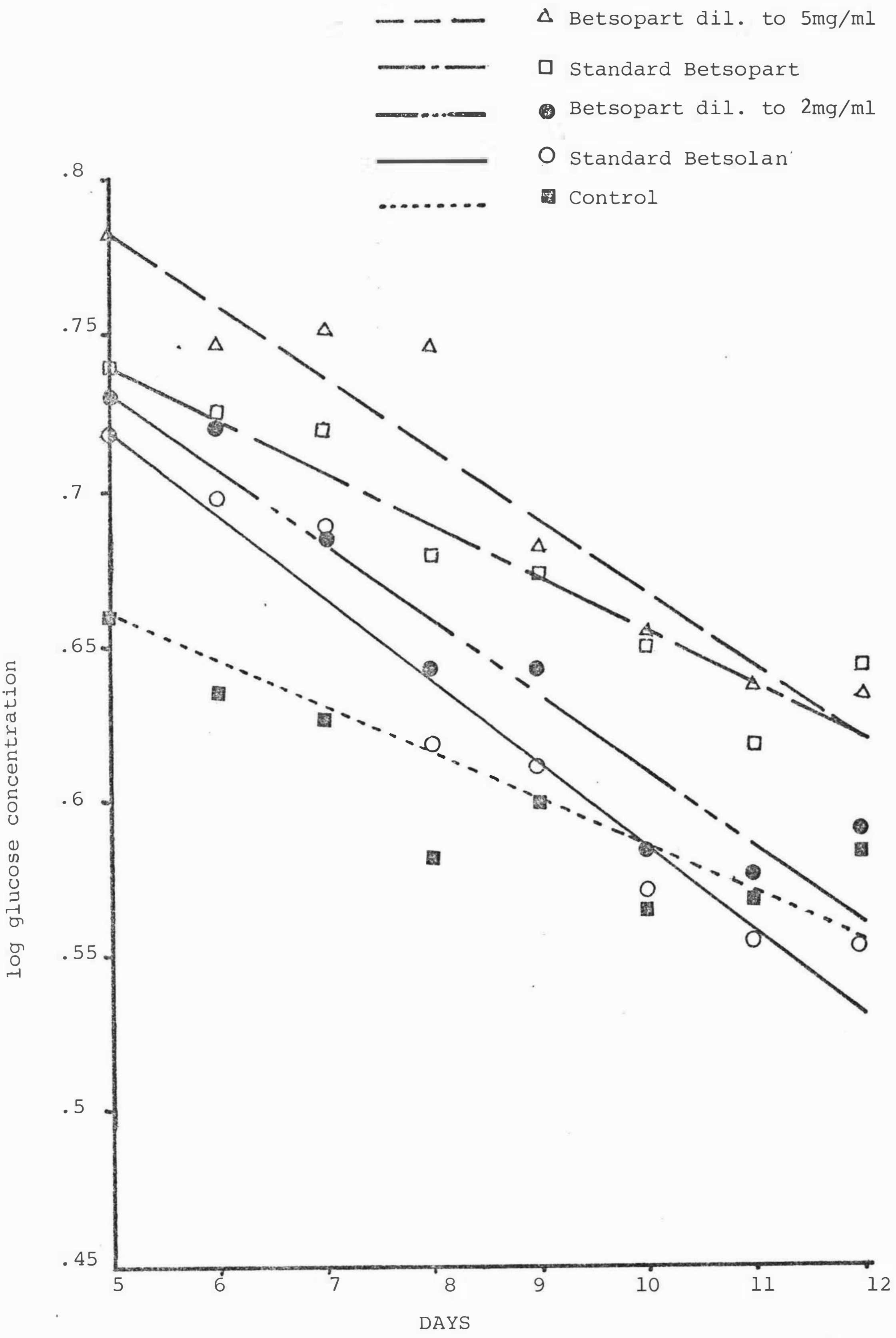


FIGURE 25: Statistically derived regression lines of the four betamethasone treatment groups and control group from day 5 to 12 (Ferricyanide method)

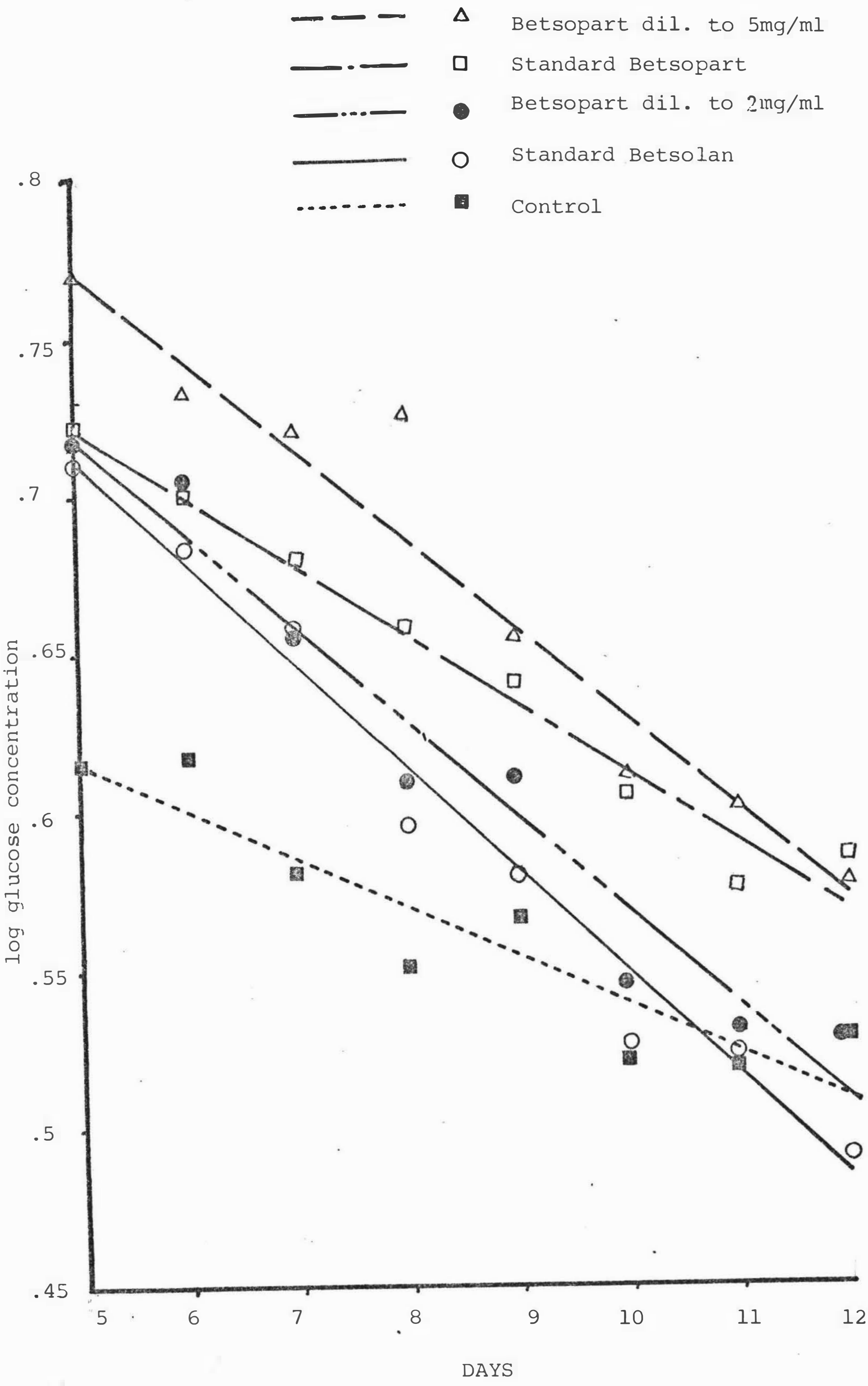


FIGURE 26: Statistically derived regression lines of the four betamethasone treatment groups and control group from day 5 to 12 (Glucose oxidase method)

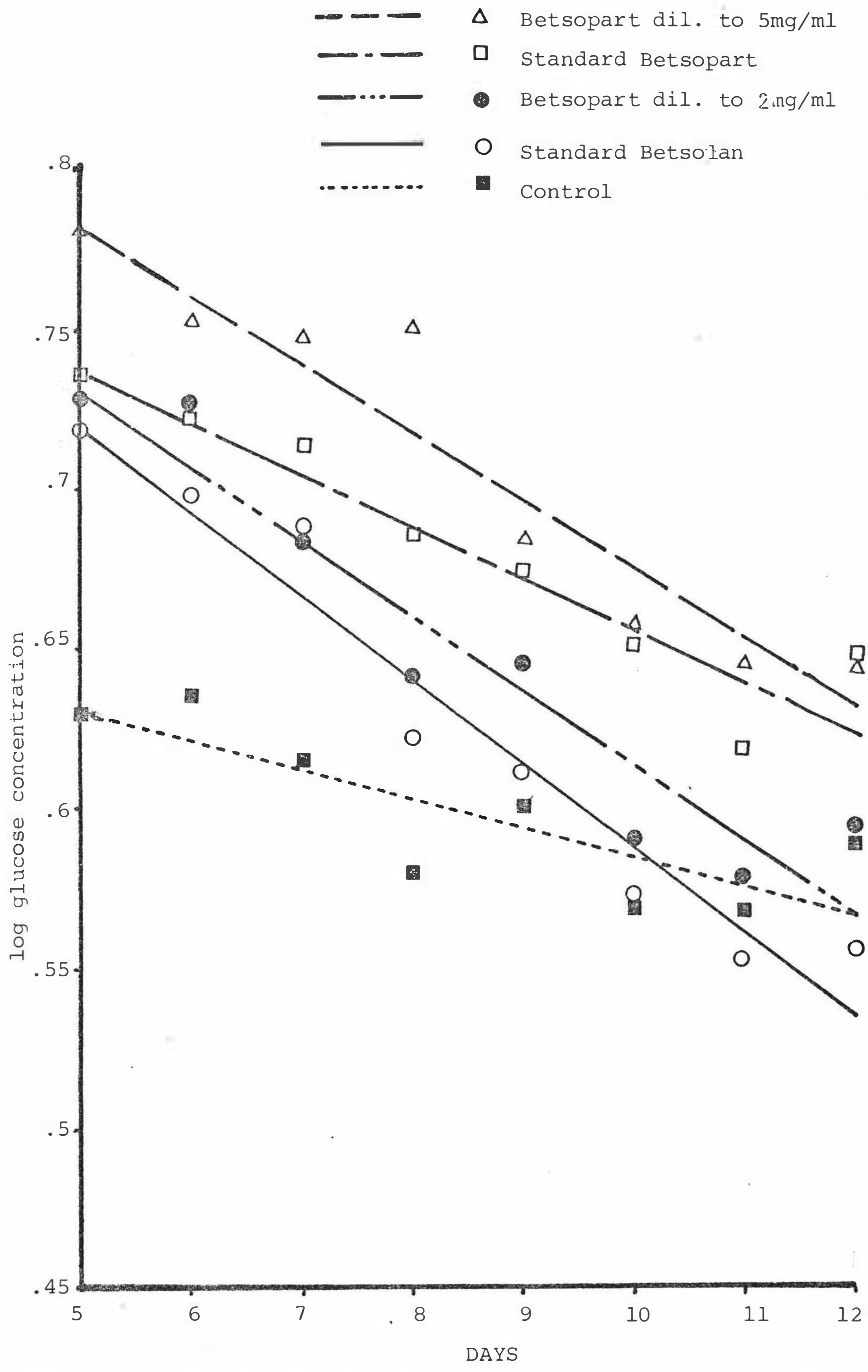


FIGURE 27: Statistically derived regression line of the four betamethasome treatment groups and control group from day 5 to 12 (Oxygen electrode method)

Table 12 and Figures 25, 26 and 27 compare the intercepts of the linear regression lines at day 0 (i.e. day 5 of the trial) as a way of comparing the estimated peak levels of plasma glucose. As anticipated these "peak" levels in all treated groups were significantly higher than in the controls. However the only significant difference between the betamethasone groups was that the diluted Betsopart 5mg/ml group showed a higher peak than all other groups.

4.5 COMPARISONS OF ANALYTICAL METHODS

Table 13 summarises the comparisons of the 3 analytical methods using the precision control serum data from Figure 16, 17 and 18. The t-test analyses reveal that both the ferricyanide and oxygen electrode methods give significantly higher results than the glucose oxidase method and that the ferricyanide method gives significantly higher results than the oxygen electrode.

Using the paired t-test on the results from the first 4 days of the betamethasone trial the same significant differences between methods were demonstrated (Table 14).

Using the trial data used in the regression analysis (i.e. day 6 to 12 of the trial) the analysis of variance within animals revealled a highly significant difference between the 3 methods ($p < 0.001$) which was entirely due to the difference between glucose oxidase and the other 2 methods. The mean square for the difference between the glucose electrode method and the ferricyanide method was very small and decidedly less than the residual (error) mean square (Table 15).

TABLE 13: COMPARISON OF THREE ANALYTICAL METHODS BY THE t-TEST USING THE PRECISION CONTROL SERUM DATA

Analytical Methods	N	Mean	S.D.	t. values and significance	
Ferricyanide	48	5.719	0.0148	$t = 20.69$ $p < 0.001$	
Glucose oxidase	38	5.203	0.1264	$t = 8.98$ $p < 0.001$	$t = 11.13$ $p < 0.001$
Oxygen electrode	27	5.455	0.0859		

TABLE 14: COMPARISON OF THE 3 ANALYTICAL METHODS BY THE PAIRED t TEST USING THE BETAMETHASONE TRIAL DATA FROM DAYS 1 TO 4

	Ferricyanide vs Glucose oxidase	Oxygen electrode vs Glucose oxidase	Ferricyanide vs Oxygen electrode
N	80	80	80
Mean difference	0.32226	0.26511	0.05715
S.D.	0.19222	0.17704	0.10558
t	14.99	13.39	4.84
p	< .001	< .001	<.001

TABLE 15: ANALYSIS OF VARIANCE (WITHIN COWS) OF THE DIFFERENCES BETWEEN METHODS USING THE BETAMETHASONE TRIAL DATA FROM DAYS 6 TO 12

Source of variation	df	Mean Squares	F ratio and significance
Between methods	2	.069382	15.7 $p < 0.001$
Glucose oxidase vs Oxygen electrode and Ferricyanide	1	.138758	31.4 $p < 0.001$
Oxygen electrode vs Ferricyanide	1	.000006	< 1 N.S.
Residual	360	.004421	

Notes: Data transformed by taking logarithms to base ten.

CHAPTER V

5.1 INTRODUCTION

In this investigation four betamethasone aqueous suspensions of different strength were administered to cows at the same dose rate per kg body weight to compare their different glucogenic effects. The main objective of the investigation was to see whether standard Betsopart (10mg/ml) diluted with distilled water for injection immediately prior to use to a concentration of 2mg/ml, would have the same effect as standard Betsolan (2mg/ml) and an intermediate effect when at an intermediate concentration of 5mg/ml.

5.2 EFFECT OF ANTICOAGULANTS

As the preservation of blood samples is crucial for the whole experiment, the effect of anticoagulants on plasma glucose levels and the effect of time between blood collection and the separation of plasma or serum from the erythrocytes on plasma and serum glucose levels were examined. Although some investigators (Van Bruggen *et al* 1943, Sunderman and Sunderman 1961, Lenz and Passannate 1970, Meites and Banrey 1973) reported that sodium fluoride has no inhibitory effect on the glucose oxidase enzymatic method and has negligible inhibitory effect on the alkaline ferricyanide reduction method (Passey *et al* 1974), some preliminary work was done to investigate its effect. Tables 3 and 4 show that fluoride-oxalate does not inhibit the analytical methods, but the fluoride-oxalate treated plasma glucose values were found to be lower than either EDTA plasma or serum samples (Table 2). Actually sodium fluoride has long been known as an antiglycolytic preservative (Aibara 1922, Major 1923, Sander 1923, Dennis and Beven 1924, Roe *et al* 1927, Hitchings *et al* 1938, Holmes 1940). To obtain better anticoagulant effect some authors (Sander 1923, Schwenkter 1927, Weissman and Klein 1958) reported the use of sodium fluoride with thymol and showed that the fluoride-thymol had no inhibitory effect on the glucose analysis and also proved that its function was solely inhibition of glycolysis.

Potassium oxalate is a common anticoagulant which produces insoluble complexes with calcium ions thus inhibiting coagulation. The immediate effect of potassium oxalate is a shrinkage of the erythrocytes and an alteration in the erythrocyte permeability (Eiseman 1927, Blitstein 1935). Warburg (1922) also showed that addition of oxalate to blood was analogous to the addition of an equivalent amount of hypertonic saline solution. Gaebler (1932-33) explained that potassium oxalate, by increasing the osmotic pressure of plasma, draws water from the erythrocytes, thus diluting plasma and concentrating the red blood cells. Boyd (1936) also showed that potassium oxalate added to defibrinated blood causes a shrinkage of the erythrocytes and an increase in their lipid content.

In 1923 Major introduced potassium fluoride as a preservative for blood. Fluorides are preservative through their inhibitory actions on certain enzymes, such as enolase (Tietz 1976), involved in glycolysis. Although high concentrations of fluoride cause significant water shift and tend to produce haemolysis, it appeared to be safe at lower concentrations used (John 1926). Roe *et al* (1927) reported the preservation of blood for chemical analysis by the use of sodium fluoride by using different concentrations for glucose stability for days. Major (1923), also showed that glucose and some other organic substances in fluoride-treated blood samples were stable for as long as ten days at room temperature if the blood is sterile. However, in this investigation, plasma glucose values in fluoride-oxalate treated blood samples were certainly lower than the other blood samples. The lowering effect is caused neither only by sodium fluoride nor by potassium oxalate but is due to the effect of both (Tables 5, 6).

Why the fluoride-oxalate plasma has the lower glucose value is still unexplained. Presumably the presence of fluoride and oxalate promotes the movement of glucose into the erythrocytes since the glucose lowering effect of these anticoagulants is only observed when the red cells are in contact with the plasma or serum (Tables 4, 5, 6).

5.3 EFFECT OF TIME BETWEEN BLEEDING AND CENTRIFUGATION

The effect of time between blood collection and separation of plasma or serum from erythrocytes was also investigated. From Figures 19, 20, 21 it can be seen that in all methods, fluoride-oxalate treated plasma gave the most stable glucose values up to three hours after collection. EDTA plasma and serum samples show somewhat less stability with time however there did not appear to be a constant decrease with time. Presumably with increased room temperatures there would have been decreased blood glucose levels. Herdt & Stevens (1981) showed that the rate of glucose catabolism is temperature dependent, with about 10 to 20% being destroyed per hour at body temperature and about one-half that amount being catabolised per hour at room temperature.

5.4 ANALYTICAL METHODS

From the statistical analyses of the three analytic methods, it can be seen that the glucose values of the plasma samples in the glucose oxidase method were significantly lower than those in the other two methods (Tables 13, 14, 15). This is as expected since the glucose oxidase enzyme is totally specific for glucose and does not detect other reducing sugars in the plasma (Middleton and Griffiths 1957, Beach and Turner 1958, Kinglsey and Getchell 1960, Fales *et al* 1961 and so on). The ferricyanide reduction method measures glucose as well as the other non-glucose reducing substances present in normal blood. These "saccharoids" (Benedict 1931) make the glucose values approximately 7% higher than the glucose oxidase method (Sunderman & Sunderman (1961), Pileggi and Szustkiewicz (1974)).

However, the oxygen electrode method, which is also specific for glucose, was found to give higher values than the glucose oxidase method. This means that the oxygen electrode method results are higher than they should be. This could be due to the presence of low molecular weight reducing substances in bovine plasma which were able to penetrate the cellulose acetate membrane in the sensor probe to be oxidised by the platinum anode and thus give erroneously high readings. Unfortunately a blank (i.e. non enzyme containing) membrane was not used in the instrument to check for the presence of such substances. In the results from the precision control serum and the trial samples before betamethasone injection, the ferricyanide method gave significantly higher results than the oxygen electrode method whereas there is no significant difference between these methods in rest of the trial samples (Tables 13, 14, 15). This is presumably due to the presence of reducing substances that can be detected by the ferricyanide method but which are of molecular weight high enough to be not detected by the oxygen electrode method. Apparently such reducing substances were not present after the injection of betamethasone.

5.5 THE BETAMETHASONE TRIAL

Many investigators have worked on the hyperglycaemic effect of glucocorticoids on normal cows, lactating cows and ketotic cows (Dye *et al* 1953, Shaw *et al* 1955, Holm 1957, Goetsch and Odell *et al* 1959, Bailey 1959, Neff *et al* 1960, Bassett 1963, Burns 1963, Braun *et al* 1970, Woollett and Evans 1971 and so on). Most of the hyperglycaemia investigations were for the treatment of pregnancy toxaemia and bovine ketosis. In the 1950's synthetic glucocorticoids were introduced to be used for the glucogenic effect on ketotic cows (Shaw *et al* 1954, 1955, McAuliff *et al* 1954, Morris and Hall 1956, Shaw 1956, Paterson 1957, Neff *et al* 1960, Burns 1963). Thus cortisone, adrenocorticotrophic hormone, hydrocortisone, prednisolone, betamethasone were introduced as effective synthetic glucocorticoids to raise the blood glucose level. In most of the investigations injection of glucocorticoids caused the elevation of blood glucose level within 24 hours after injection but returned to the pre-injection level by 72 and 96 hours depending on the dose of the drug. Thus cortisone and hydrocortisone were introduced by Hatziolas and Shaw (1950), adrenocorticotrophic hormone by Shaw *et al* (1954, 1955) & McAuliff *et al* (1954) prednisolone by Morris and Hall (1956) and Shaw (1956), fluorocortisone and fluprednisolone by Goetsch *et al* (1959), 9 α-fluoroprednisolone acetate by Neff *et al* (1960), betamethasone by Burns (1963) and they all agreed that 9 α-fluoro compounds are at least ten times as potent as their parent compounds and gave a 150% elevation of pretreatment levels at 24 hours after administration of the steroid. Longevity depends upon the dose of the steroid. Among these glucocorticoids, dexamethasone (16 α-methyl 9α-fluoro prednisolone) and betamethasone (16 β-methyl 9α-fluoro prednisolone) are accepted as the most potent synthetic glucocorticoids and have thirty and thirty-five times more potency respectively, compared to cortisol (Roberts 1963). Burns (1963) found that intramuscular injections of 20mg betamethasone suspension in lactating cows produced a greater and longer lasting hyperglycaemia than 100mg of prednisolone or prednisolone trimethyl acetate injected intramuscularly. Woollett and Evans (1971) reported the comparative glucogenic effect of corticosteroid in calves. They showed that intramuscular injections of betamethasone suspension at dose rates of 10 and 20mg gave similar peak glucose concentrations but the hyperglycaemia lasted up to 96 hours after the 20 mg dose but only 56 hours after 10 mg.

In this trial the dose rate was set at 0.04mg/kg body weight which meant that the dose per cow was about 20mg (Table 1). The object then was to compare the effect of different concentrations of betamethasone in the injected preparation and not the differences due to differing dose rates. In this experiment Standard Betsopart (10 mg/ml) was more long acting in its hyperglycaemic effect than either of the two

2mg/ml preparations in that only those two showed a significantly more rapid drop in glucose level than the controls (Table 11). However Standard Betsopart did not produce a glucose peak significantly different from that of the 2mg/ml preparations. In fact the intermediate concentration formulation of 5mg/ml produced a significantly higher peak than any of the other preparations (Table 12) and its effect was considerably long lasting in that its rate of fall from the peak glucose level was not significantly different from that of the Standard Betsopart (Table 11).

Since actual blood steroid levels were not measured in this experiment we do not know if the rate of absorption from the injection of the 5mg/ml preparation was intermediate between that of the 10mg/ml and 2mg/ml formulations. However if we assume that its rate of absorption was intermediate we can still explain why its net glucogenic effect was not "intermediate". If we assume that the rate of glucogenesis is a saturatable response to the steroid then there will be an optimum blood steroid concentration that will produce the maximum rate of glucogenesis. Higher steroid concentration would not produce any further increase in glucogenesis rate (Figure 28). The net effect on blood glucose concentration is the difference between glucogenesis and catabolism of glucose. Therefore the greatest net glucose response will be produced by a dose of steroid which maintains the blood steroid level at or about (S) optimum for as long as possible. If we assume that the greater the volume of injected material the faster the rate of absorption then we can represent the resultant blood steroid levels of the three different formulations as in Figure 29. The rapidly absorbed 2mg/ml preparation while producing a high steroid concentration would only produce maximum glucogenesis for a relatively short time. The most slowly absorbed 10mg/ml preparation while producing a long lasting blood steroid level may only briefly, if at all, reach (S) optimum so that the net difference between glucogenesis and glucose catabolism would result in a moderate blood glucose elevation. The 5mg/ml preparation with an intermediate rate of absorption

FIGURE 28: The theoretical relationship between blood steroid concentration and the rate of glucogenesis.

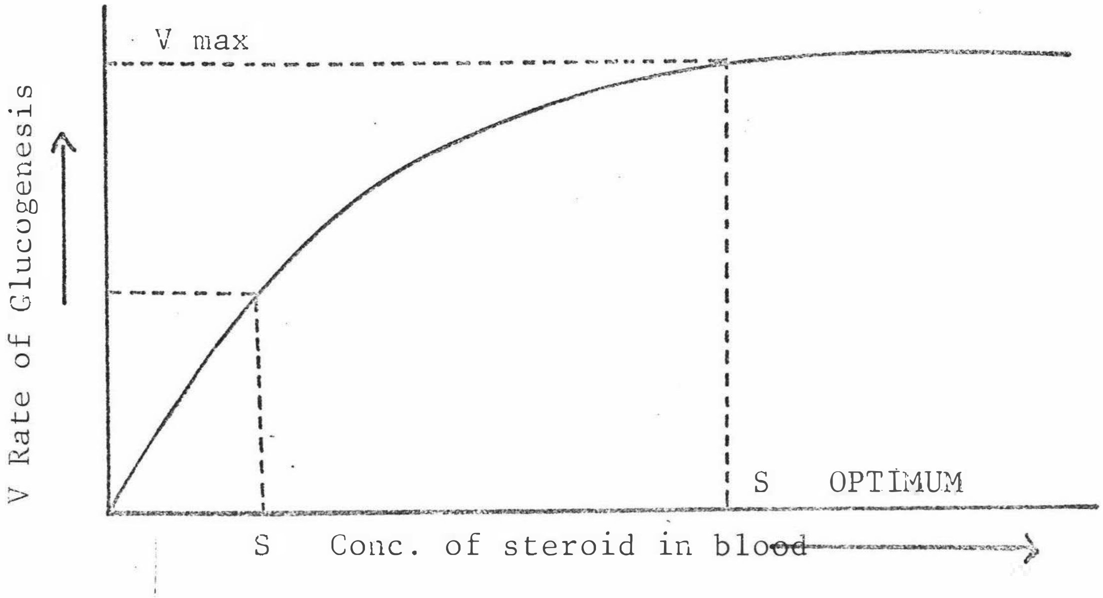
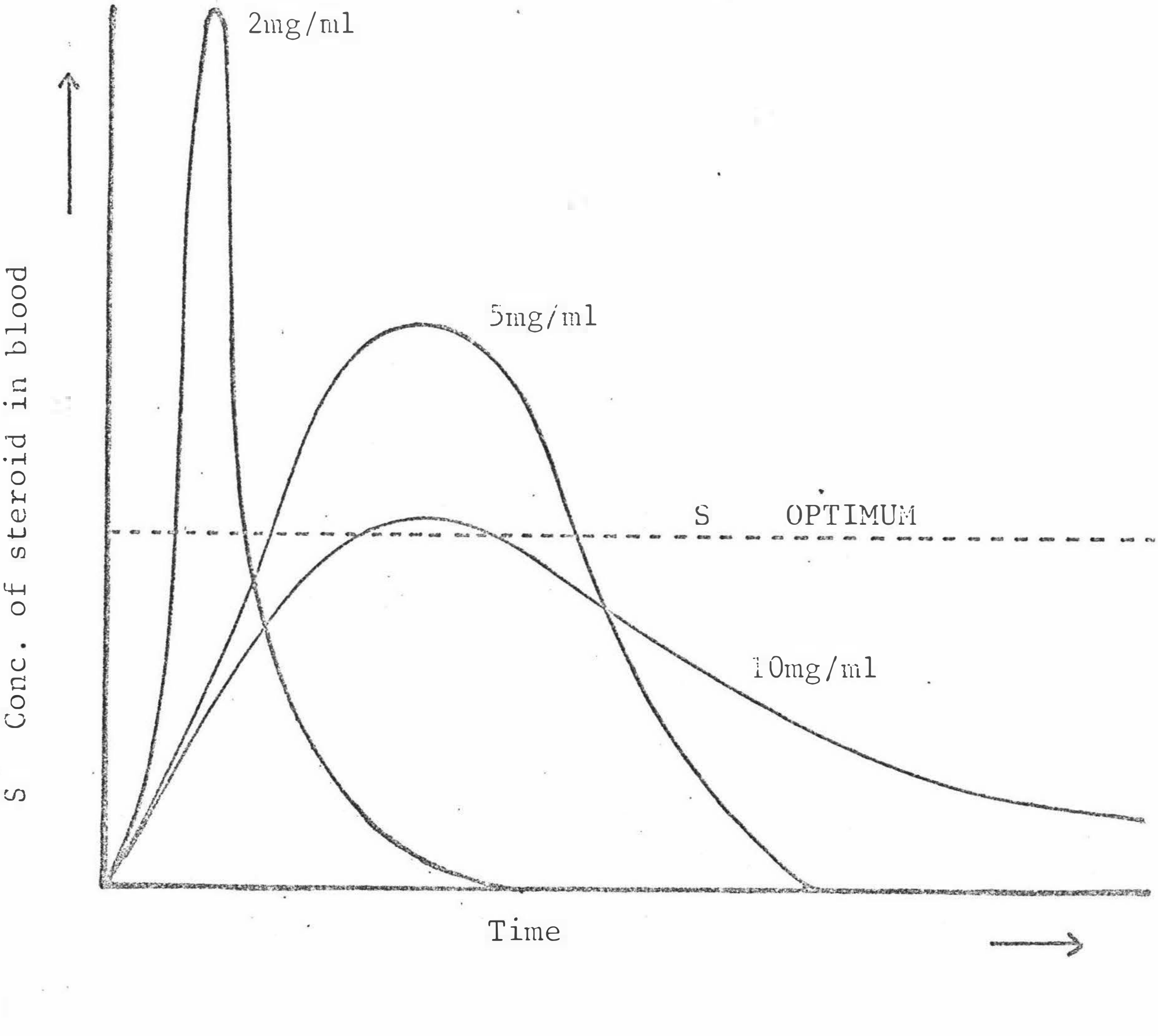


FIGURE 29: The theoretical relationship between the time course of blood steroid concentrations and the various concentrations of the injected steroid dose.



could maintain (S) optimum for longer and thus produce a much greater net rise in blood glucose.

5.6 CONCLUSION

This experimental work has shown that Betsolan (2mg/ml) and Betsopart diluted with water before injection to 2mg/ml when given at the same dose rate produce very similar hyperglycaemic responses in cows. The response observed with Betsopart diluted to 5mg/ml however has shown that the response to be expected from using formulations between 2 and 10mg/ml cannot be confidently predicted. Further trial work would be needed using formulations of 2, 4, 6, 8 and 10mg/ml betamethasone, all given at the same dose rate, preferably using more than 4 cows in each treatment group.

The mechanism of action of sodium fluoride and potassium oxalate in lowering the glucose concentration in plasma requires further investigation. The work reported here shows that it involves an interaction of the erythrocytes and the fluoride-oxalate anticoagulant but changes in intracellular and whole blood glucose level have not been investigated.

Comparison of the 3 analytical methods has shown yet again that the ferricyanide method over estimates the true glucose concentration. However the expected agreement between the glucose oxidase and oxygen electrode methods was not observed. A further anomaly was the absence of a difference between the ferricyanide and oxygen electrode method results in cows after betamethasone treatment whereas a difference was observed using other bovine serum and plasma. Further work to identify and quantitate non glucose reducing substances in bovine plasma is required to explain these anomalies.

APPENDIX 1a: BLOOD GLUCOSE CONCENTRATIONS FOR ALL COWS IN THE BETAMETHASONE TRIAL (FERRICYANIDE METHOD)

Days Cow Number	1	2	3	4	5	6	7	8	9	10	11	12
CONTROL												
D69	3.83	3.56	3.87	3.95	3.99	4.24	4.36	4.18	3.88	4.07	3.60	3.74
659	3.86	3.60	4.00	3.71	3.52	4.94	4.17	3.50	4.05	3.23	3.27	3.60
204	4.17	3.90	4.00	3.86	3.81	4.56	4.43	4.04	3.98	3.79	4.35	3.82
77	3.55	3.52	3.68	3.51	3.38	3.60	4.00	3.57	4.01	3.61	3.58	4.19
STD. BETSOPART												
191	3.64	3.39	4.45	3.97	5.16	5.48	5.49	4.77	4.99	5.10	4.58	4.57
10	3.83	3.57	3.68	3.78	4.75	4.79	5.22	5.10	4.53	4.06	4.12	4.38
16	4.86	3.92	4.10	4.15	5.34	5.57	5.63	5.10	5.03	4.70	4.24	4.70
2	3.66	3.88	3.91	3.88	4.33	5.41	4.60	4.24	4.31	3.97	3.69	3.95
STD. BETSOLAN												
41	3.70	3.84	3.49	3.94	5.05	4.86	4.90	4.12	4.03	3.90	4.01	3.37
1	3.60	4.02	4.00	3.74	4.34	4.71	4.23	3.79	3.72	3.26	3.36	3.58
14	3.60	3.39	4.80	3.52	6.16	5.91	5.15	4.24	4.18	3.73	3.25	3.51
76	3.96	3.79	3.75	3.91	5.00	4.48	5.29	4.50	4.46	4.05	3.74	3.86
BETSOPART 5mg/ml												
100	3.44	3.38	3.56	3.71	4.41	4.65	4.94	4.40	4.20	4.23	4.03	3.93
39	3.84	3.80	4.02	4.06	5.11	5.17	5.95	5.06	5.02	4.53	4.45	4.27
43	4.35	3.89	3.56	4.46	6.88	6.87	6.62	6.09	5.98	5.27	4.97	4.92
40	3.85	3.59	3.66	4.09	5.09	5.78	5.11	6.83	4.06	3.94	3.95	4.18
BETSOPART 2mg/ml												
80	3.61	3.57	3.82	3.71	6.08	5.21	4.64	4.08	4.05	3.65	3.50	3.43
15	4.03	3.86	3.87	3.87	6.26	5.73	5.35	5.03	4.92	4.25	3.64	4.29
78	4.42	3.30	3.75	3.74	4.79	5.08	4.65	4.32	4.24	3.60	3.78	3.52
99	3.97	3.61	3.69	3.80	5.70	5.02	4.77	4.14	4.42	3.87	4.18	4.34

APPENDIX 1b: BLOOD GLUCOSE CONCENTRATIONS FOR ALL COWS IN THE BETAMETHASONE TRIAL (GLUCOSE OXIDASE METHOD)

Days Cow Number	1	2	3	4	5	6	7	8	9	10	11	12
CONTROL												
D69	3.24	3.49	3.67	3.55	3.77	4.02	3.83	3.88	3.62	3.65	3.26	3.33
659	3.44	3.46	3.75	3.31	3.32	4.71	3.80	3.21	3.78	2.93	2.91	3.18
204	3.34	3.88	3.75	3.47	3.53	4.50	4.09	3.75	3.67	3.47	3.89	3.40
77	3.29	3.47	3.40	3.12	3.14	3.41	3.65	3.27	3.65	3.24	3.18	3.56
STD. BETSOPART												
191	3.25	3.33	4.17	3.67	4.91	5.39	5.08	4.52	4.70	4.68	4.21	3.97
10	3.16	3.28	3.47	3.27	4.44	4.44	4.81	4.81	4.23	3.71	3.77	3.92
16	4.12	3.79	3.78	3.71	5.07	5.22	5.17	4.85	4.74	4.24	3.82	4.11
2	3.20	3.63	3.62	3.37	4.01	5.08	4.15	4.01	3.88	3.50	3.26	3.44
STD. BETSOLAN												
41	3.15	3.69	3.31	3.65	4.85	4.84	4.63	3.90	3.70	3.47	3.56	2.85
1	3.10	3.83	3.79	3.26	4.10	4.62	3.89	3.56	3.52	2.96	3.08	3.18
14	2.91	3.22	4.58	3.12	5.50	5.54	4.83	4.01	3.89	3.30	3.21	3.06
76	3.91	3.66	3.62	3.55	4.74	4.31	4.86	4.30	4.12	3.71	3.40	3.29
BETSOPART 5mg/ml												
100	2.89	3.25	3.31	3.32	4.19	4.59	4.60	4.14	3.88	3.82	3.66	3.47
39	3.84	3.69	3.81	3.58	4.96	4.96	5.46	4.79	4.74	4.10	4.05	3.81
43	3.84	3.75	3.37	4.01	6.30	6.62	6.21	5.83	5.56	4.80	4.58	4.20
40	3.32	3.45	3.40	3.55	4.66	5.44	4.76	6.56	3.81	3.56	3.63	3.68
BETSOPART 2mg/ml												
80	3.02	3.49	3.60	3.30	5.66	5.08	4.22	3.62	3.72	3.29	3.11	2.99
15	3.49	3.68	3.75	3.34	6.00	5.53	5.05	4.78	4.57	4.08	3.31	3.81
78	3.85	3.28	3.52	3.28	4.36	4.89	4.18	4.01	3.91	3.25	3.37	3.02
99	3.25	3.47	3.48	3.40	5.38	4.81	4.54	3.83	4.08	3.42	3.82	3.65

APPENDIX 1c: BLOOD GLUCOSE CONCENTRATIONS FOR ALL COWS IN THE BETAMETHASONE TRIAL (OXYGEN ELECTRODE METHOD)

Days Cow Number	1	2	3	4	5	6	7	8	9	10	11	12
CONTROL												
D69	3.64	3.53	3.86	4.03	4.12	4.14	4.09	4.17	3.92	4.09	3.62	3.81
659	3.62	3.50	3.88	3.81	3.58	4.87	4.09	3.50	4.06	3.30	3.30	3.64
204	3.86	3.89	4.01	3.98	3.81	4.59	4.42	4.03	4.00	3.81	4.31	3.92
77	3.58	3.50	3.67	3.53	3.47	3.67	3.98	3.53	4.00	3.70	3.61	4.20
STD. BETSOPART												
191	3.58	3.44	4.44	4.06	5.24	5.52	5.49	4.79	5.04	5.15	4.65	4.59
10	3.62	3.64	3.53	3.61	4.76	4.82	5.18	5.12	4.62	4.12	4.09	4.45
16	4.50	3.92	4.03	4.09	5.35	5.43	5.52	5.18	5.07	4.70	4.31	4.70
2	3.75	3.98	3.75	3.77	4.28	5.32	4.52	4.31	4.26	3.95	3.67	4.032
STD. BETSOLAN												
41	3.66	3.84	3.36	3.81	5.18	4.84	5.01	4.14	3.98	3.89	3.96	3.44
1	3.62	4.09	3.86	3.61	4.31	4.76	4.20	3.84	3.75	3.28	3.36	3.58
14	3.49	3.53	4.62	3.44	6.08	5.94	5.15	4.31	4.23	3.72	3.30	3.58
76	3.98	3.61	3.73	3.86	5.07	4.48	5.18	4.51	4.40	4.12	3.72	3.81
BETSOPART 5mg/ml												
100	3.42	3.39	3.53	3.81	4.42	4.68	4.96	4.45	4.23	4.28	4.09	4.06
39	3.75	3.81	3.81	3.95	5.21	5.21	5.82	5.08	5.01	4.54	4.52	4.40
43	4.20	3.86	3.36	4.37	6.75	6.97	6.59	6.16	5.99	5.35	5.04	4.90
40	3.84	3.64	3.47	3.92	5.04	5.74	5.04	6.83	4.12	4.00	4.07	4.31
BETSOPART 2mg/ml												
80	3.53	3.56	3.81	3.75	6.10	5.26	4.56	4.00	4.03	3.70	3.51	3.56
15	3.98	3.75	3.75	3.67	6.27	5.85	5.41	5.07	4.96	4.34	3.70	4.34
78	4.14	3.33	3.78	3.75	4.79	5.15	4.54	4.31	4.28	3.67	3.81	3.56
99	3.78	3.64	3.72	3.75	5.71	4.96	4.84	4.14	4.40	3.92	4.20	4.28

APPENDIX II

GROUP	COW	RESIDUAL VARIANCES OF Y			
		Day 1-7	Day 0-7	Day 0-6	Day 1-6
		Actual*	Expressed relative to day 1-7		
CONTROL	D69	.0001702	2.36	1.25	2.67
	659	.0024936	1.91	0.90	2.26
	204	.0007372	2.56	1.25	2.87
	77	.0007412	1.10	0.91	1.21
BETSOLAN	41	.0006205	1.34	0.98	1.17
	1	.0011352	1.37	0.94	1.45
	14	.0005891	1.34	0.89	1.48
	76	.0007371	0.96	1.21	1.08
BETSOPART 10mg/ml	191	.0003294	2.15	1.11	2.10
	10	.0010209	1.13	1.18	1.36
	16	.0004036	1.78	0.98	2.12
	2	.0007356	3.01	0.89	3.62
BETSOPART 5mg/ml	100	.0001153	5.78	1.25	6.30
	39	.0005791	1.43	1.20	1.54
	43	.0001109	5.26	1.12	5.34
	40	.0057149	1.11	1.23	1.33
BETSOPART 2mg/ml	80	.0007055	1.04	1.06	0.98
	15	.0009871	0.92	.84	0.90
	78	.0004472	2.81	1.25	3.17
	99	.0010479	1.01	1.08	0.97

* Data has been transformed by taking logarithms (base 10) of the original glucose concentration (mM)

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