The Modification of Insulin

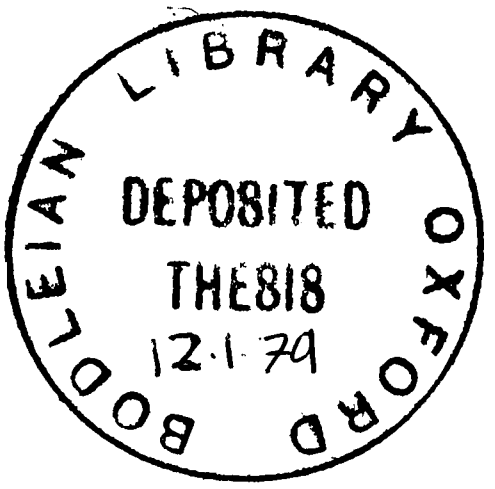
for

Therapeutic Purposes.

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To the First Three.

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Abbreviations

PZI	Protamine Zinc Insulin.
NPH	Neutral Protamine, Hagedorn. (insulin)
phe	phenylalanine.
gly	glycine.
lys	lysine.
DFP	diisopropylfluorophosphonate.
Dnp-	dinitrophenyl-
Dns-	dimethylaminonapthalenesulphonyl-
EDM-	1-ethyl-3(3-dimethylaminopropyl)-
CHM-	1-cyclohexyl-3(2-morpholinoethyl)-
Tris	Tris(hydroxymethyl)aminomethane.
BAWP	Chromatographic solvent containing n-butanol, acetic acid, water and pyridine.
BAW	Chromatographic solvent containing n-butanol, acetic acid and water.
Oct	Octanoyl (Capryloyl).
Non	Nonanoyl.
Seb	1,8-decanedioyl (Sebacoyl).
Aze	1,7-heptanedioyl (Azeloyl).
Pam	Hexadecanoyl (Palmitoyl).
Ste	Octadecanoyl (Stearoyl).
Ole	cis-9-Octadecenoyl (Oleoyl).

The modification of insulin for therapeutic purposes.

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First this thesis outlines the historical background and current status of diabetes therapeutics. This includes prolonged action preparations, oral hypoglycaemic agents, approaches to peroral insulin therapy and methods of insulin modification, with emphasis on derivatives of clinical interest.

Then the analytical methods are described. These include a novel method, using chromatography on silica-gel loaded paper, for analysing the state of acylation of water-insoluble insulin derivatives.

The next two chapters describe the synthesis of the acyl hydroxysuccinimidyl esters and the protected insulin derivatives, followed by the coupling, purification and analysis of the products. Six ($N^{\alpha B1}$)-acyl-insulin derivatives were synthesised, these were octanoyl, nonanoyl, stearoyl, oleoyl, 1,3-O-dipalmitoyl-2-O-azeloylglycerol ('triacylglycerol') and the mixed (nonanoyl/triacylglycerol) derivative. The corresponding six ($N^{\alpha B1}$,$N^{\epsilon B29}$)-di-acyl-insulin derivatives were synthesised. Three tri-acyl-insulin derivatives were synthesised, the acyl groups were octanoyl, stearoyl and triacylglycerol. One of the di-acyl-insulin derivatives was also synthesised using [1-^{14}C]-octanoate. Purification using partition chromatography on Sephadex-LH20 and Phenyl-Sepharose was briefly investigated. Also in these chapters the synthesis of a Ficoll-insulin complex coupled via a Schiff's base reaction is described.

In the final chapters the results of intravenous and peroral administration assays of these derivatives, using rabbits, are described and discussed. By intravenous administration the mono-acyl-insulin and Ficoll insulin derivatives had reduced activity of normal duration. The di-acyl-insulin derivatives had reduced, but prolonged activity. This was supported by the use of the radio-labelled di-acyl-insulin derivative which had a half-time in the blood of about 25 minutes.

By peroral administration, only the tri-(triacylglycerol)-insulin derivative had hypoglycaemic activity. The efficiency of this derivative was 6% of an equivalent dose of insulin.

A brief toxicological examination was performed on several of the rabbits used. No adverse effects attributable to these insulin derivatives were found.

An appendix describes some unrelated work on a fluorescent amino-acid for use in protein semisynthesis.

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Chapter 1

Introduction

1A History of Diabetes

Diabetes or 'pisseynge disease' has been identified as a clinical entity for at least 3 to 4 thousand years. The early Egyptian, Indian and Chinese medical literature identified both polyuria and glycosuria as presenting symptoms, as well as weight loss and lethargy. Though they tended to confuse what is now known as diabetes mellitus with other renal and hormonal disorders presenting with similar symptoms, they knew that it was a wasting disease which was frequently fatal. (Papaspyros, 1952). Thomas Willis (1621-1675) differentiated between the insipidus and mellitus forms of the disease and proposed some dietary cures (Allan, 1953). Many clinicians at this time tended to favour dietary restrictions as beneficial towards the alleviation of the symptoms of the disease, reflecting the current concern about dietary control of the disease. Others proposed high carbohydrate diets, "due to the loss of so much sugar in the urine", no doubt producing a high mortality among their patients. In the nineteenth century a great deal of work was performed on the role of the pancreas in diabetes. Mering and Minkowski showed that the loss of the pancreas led to the onset of symptoms that were identical to diabetes. Vassale showed that the islet tissue previously described by Langerhans, was the active part of the pancreas, the loss of which caused diabetes. Many attempts to produce an active extract from the pancreas were attempted but were unsuccessful until the experiments of Paulesco in Europe, and Banting and Best in Canada. Despite the success of these workers in controlling experimental diabetes in animals, it was not until the successful treatment of a human diabetic by Banting, Best, Campbell,

Collip and Fletcher (Banting et al, 1922) that the pancreatic extract, originally named 'isletin' and later 'insulin' could be considered an effective cure for diabetes. The treatment of a juvenile diabetic suffering from hyperglycaemia and metabolic ketosis with the pancreatic extracts led to near normoglycaemia, abolished glycosuria and acetonuria, and metabolic studies showed evidence for increased carbohydrate utilization.

The preparation of insulin was quickly taken over by commercial interests and the large scale extraction of the hormone and its purification by crystallization was carried out. Two disadvantages of insulin therapy were soon found, firstly that the hormone could not be administered by mouth (it was shown to be a medium-sized protein in 1926 by Abel) and secondly that the soluble insulin preparations were of very short duration of effect, necessitating several daily subcutaneous injections. Work started almost immediately on attempts to produce insulin derivatives or preparations which could be administered orally and which had a prolonged hypoglycaemic effect in vivo.

1B Preparations of Insulin with Prolonged Activity

1Bi Protamine-Insulin Complexes

The first major breakthrough in the production of long-acting insulins, was the observation by two groups of workers that protamine affected the solubility of insulin. Due to the excess of carboxyl over amino and other positively charged groups the isoelectric pH of insulin is 5.35. It has a low solubility in aqueous solution between pH 4.5 and 6.0. Hagedorn et al (1936) and Scott and Fisher (Fisher & Scott, 1936, and Scott & Fisher, 1936) describe experiments showing that the isoelectric pH of a mixture of insulin and protamine (the latter

having an isoelectric pH of about 12) is about 7.0 to 7.5. As long as an adequate quantity of zinc ion is present, an insoluble amorphous suspension of insulin, which has a long duration of action in vivo (Root et al, 1936), can be made. Scott and Fisher's preparation called 'Protamine Zinc Insulin' (PZI) was a stable preparation at a pH of between 6.9 and 7.4, with a zinc content of 4 g% and a protamine content of 25 g%. This preparation, although better than soluble insulin, was rather too retarded in effect and a single daily dose tended to lead to matitutinal hyperglycaemia and nocturnal hypoglycaemia. However a mixed regime of one injection of soluble insulin and one of protamine insulin was more effective than three or four injections of soluble insulin per day, especially for brittle diabetics.

The preparation of Hagedorn was similar to PZI, but was less stable (due to a lower zinc content) and was made up freshly, by the addition of sterile phosphate buffer to a soluble insulin and protamine preparation, before injection.

Both protamine insulins suffered from two further disadvantages. As there was an excess of both zinc and protamine in the solution, a biphasically-acting mixture could not be prepared by adding soluble insulin to the protamine-insulin preparation - an unknown quantity of the soluble insulin would be converted to protamine-insulin. Furthermore the local irritant action of the excess of zinc and protamine caused the injections to be more painful than those of soluble insulin.

Hagedorn and co-workers then studied the complexing of zinc insulin with protamine, to find the concentrations needed to give the optimal ratio of zinc insulin to protamine (which they called the isophane ratio). This improved insulin preparation is

microcrystalline, has a 1:1 protamine to insulin ratio (about 8 g% of protamine) and contains 0.36 g% of zinc (approximately one atom of zinc to three of insulin monomer molecules) and was called 'Isophane' or 'Neutral Protamine, Hagedorn' (NPH) insulin. (Hagedorn, 1946 and Krayenbuhl & Rosenberg, 1946). The conversion of the initial amorphous precipitate to a crystalline suspension also relied on the presence of a phenol (preferably cresol) present in the crystallization mixture. It is a more stable and less troublesome preparation than the earlier protamine-insulin preparations. It is interesting to note that the prolongation of activity of Isophane insulin is independent of the crystal size of the preparation, and virtually independent of the dose, which suggests that the dissociation of the protamine-insulin complex is the rate limiting factor in the solubilization of the insulin. This is emphasized by the fact that crystalline and amorphous insulins made by Scott and Fisher (1936) were without prolonged activity.

1Bii Globin-Insulin Complexes

The prolonged action of globin insulin is very similar conceptually to protamine insulin. It is a protein-insulin complex in which the rate of dissociation of the complex is the rate-limiting factor in insulin release. It was described later than protamine insulin and did not come into general use until after the war (Reiner et al, 1939 and Bauman, 1939). The suspension has an acid pH (3.0 to 3.5) and the prolongation of activity is rather less than that for protamine insulin. Glucose homeostasis is better maintained than by PZI for the first six hours post-injection. The maximum time of action was about 12 hours and it is suitable for use as a twice daily injection for brittle diabetics.

Another advantage of this preparation, although expensive to

realize, is that human globin can be used, thus reducing doubts about the immunological safety of these mixed non-human-protein mixtures. It is less unusual for globin to be found in the extracellular fluid and in the blood stream than protamine, and this may further reduce immunological problems.

1Biii Other complexing agents

In the early history of the development of the clinical use of insulin, many different substances were used in an attempt to prolong the activity of insulin. For example yeast extracts, lipids, cholesterol, tannin and basic ferric chloride were all used as vehicles or as complexing agents in attempts to alter the duration of activity of insulin. Various methods of administration were also tried in order to achieve this same end. Administration by inunction or endonasal instillation were thought to be effective, though none of these methods was used to any extent in clinical practice. (Jensen, 1938a).

The most important insulin preparation of this type is 'Surfen' insulin developed by workers at Hoechst A.G. (Umber et al, 1938). This derivative, used mainly on the Continent, is a complex of insulin with a urea (1,3-bis(4-amino-2-methyl-6-quinolyl) urea). This has an intermediate prolongation of activity (Schlichtkrull et al, 1975).

A variety of insulin derivatives covalently coupled to macromolecules have been synthesised and shown to have prolonged activity or a reduced rate of degradation or both of these properties. These include insulin coupled to dextrans (by CNBr activation) (Suzuki et al, 1972), to Sepharose (Cuatrecasas, 1969 and Oka & Topper, 1974), to polyacrylate (Kramer & Lehmann, 1972) and to a polyvinylpyrrolidine and crotonic acid copolymer (Kropachev et al,

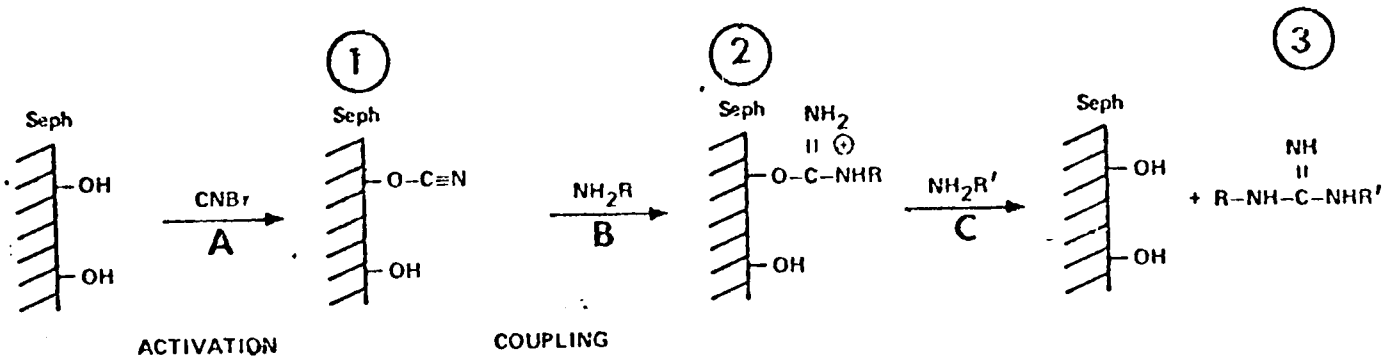


Figure 1.1 Activation of Sepharose (Seph), coupling with amines to form Sepharose-bound isoureas and release of substituted guanidines (from Wilchek et al, 1975).

1972). Generally these derivatives have a reduced activity (10-80% of soluble insulin) and are coupled by only one amino group (usually the ϵ-amino group of lysine B29 or the α-amino group of phenylalanine B1). The derivative of Oka and Topper is claimed to have a higher activity than soluble insulin, though these results are in in vitro assays.

Others have described similar derivatives with a normal or increased rate of degradation. Canfield et al (1972) describe a polyalanyl derivative coupled mainly at the glycine A1 and the phenylalanine B1 amino-groups, which has a rapid clearance from the liver and kidney. Armstrong et al (1972) describe an insulin coupled to dextran (by CNBr activation) which has a reduced duration of action, compared with soluble insulin. Care has to be taken in the interpretation of some of these results, as doubt has been cast on the stability of the insulin-Sepharose bond produced by cyanogen bromide activation. Davidson et al (1973) have described experiments showing that there is a steady release of about 80% of the total insulin bound to the Sepharose, and that this release accounts for the insulin activity of these preparations. Kolb et al (1975) proposed that this material is not native insulin, but that it has been modified in some fashion. Wilchek et al (1975), propose a mechanism for the release of insulin from these complexes, by the action of amino-group nucleophiles on the susceptible bond (see Fig.1.1). The products are substituted guanidines. As the substituting nucleophile can be a serum protein, a 'high molecular weight' insulin coupled to this protein can be produced.

1Biv Crystalline Preparation

These preparations were developed by Schlichtkrull and co-workers (Schlichtkrull, 1957 and

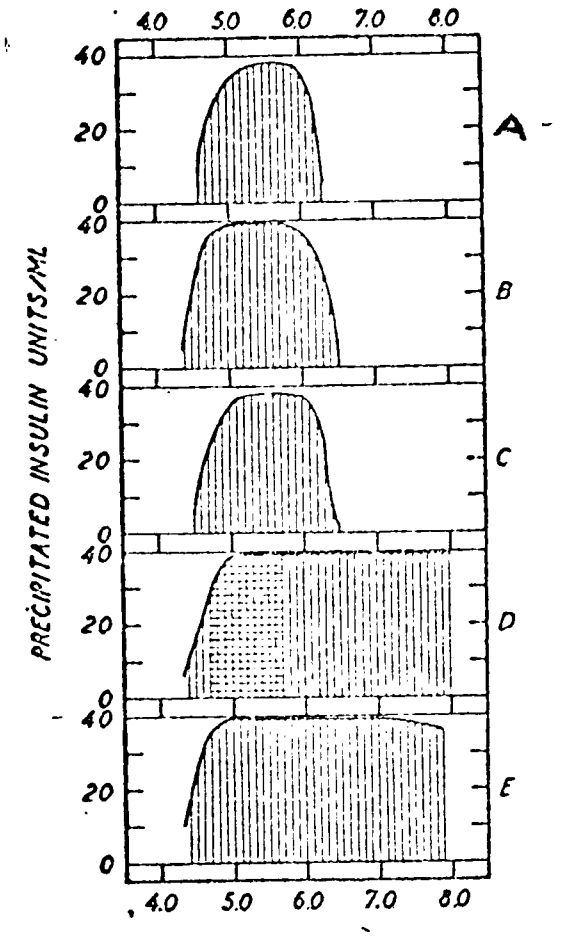


Figure 1.2 Precipitation zone of insulin (40 U/ml) in: A, 0.01M sodium phosphate B, 0.01M sodium phosphate with 2 mg zinc (as chloride)/ 1000 U; C, 0.01M sodium acetate; D, 0.01M sodium acetate with 2mg zinc (as chloride)/100C U; E, 0.01M sodium phosphate with 2 mg zinc (as chloride)/1000 U and 8.5 mg protamine/1000 U (from Schlichtkrull, 1957).

Hallas-Møller et al, 1952a,b). They found that when buffers other than those containing phosphate were used for measuring the variations of insulin solubility with pH, the precipitation zone extended to a higher pH. In the presence or absence of zinc in sodium acetate buffer the precipitation zone extends above pH 7 (see Fig.1.2). By varying the pH, the concentration of insulin and of zinc it was possible to produce crystalline and amorphous forms of insulin, stable at neutral pH, with varying particle or crystal size. The prolonged effect of these derivatives is dependent on crystal size, and thus the rate of dissolution of the crystals is the limiting step in the response, this being proportional to the surface area:dose ratio. These preparations are described as 'Lente' insulins and there are three main types with different rates of activity; 'Semi-Lente', 'Lente' and 'Ultra-Lente'. 'Lente' insulin is a mixture of 'Semi-Lente', an amorphous zinc-insulin suspension and 'Ultra-Lente', a crystalline zinc-insulin suspension. An early description of the use of these types of insulin for diabetics with various diurnal blood sugar profiles is described by Hallas-Møller et al (1952b). Since then extensive work on the nature of crystalline forms of insulin has been performed (Schlichtkrull, 1956, and Blundell et al, 1971).

These insulin preparations have several advantages over earlier long-acting insulins and are used extensively for newly diagnosed diabetics. These preparations have no added proteinaceous components, reducing immunological complications, which can be reduced still further if monocomponent insulin is used for their preparation. The suspensions are stable and in a neutral non-toxic buffer, which helps reduce the discomfort of injection. Mixed preparations can be made, containing two or more components with

different times of onset and duration of activity. These preparations (such as Rapitard) simplify the clinicians' job in the treatment of all the varied diurnal blood glucose profiles that are found in diabetics. Long-acting preparations, although disliked by some, are attaining a greater importance as more clinicians are attempting to produce a basal level of insulin by replacement therapy both in cases of coma (Page et al, 1974) and in treatment of mild diabetes (Holman et al, 1976; Pickup et al, 1978).

1C Studies on the Peroral Administration of Insulin

1Ci Introduction

The literature on the experimental and clinical use of insulin administered perorally, rectally and endonasally is fraught with pitfalls. Much of the earlier literature draws conclusions from experiments which were inadequately controlled or the number of experiments were insufficient for statistical analysis, or this analysis was not performed. Also when an insulin preparation is instilled into the gut after some operative procedure, there is always a danger that the insulin may be absorbed into the blood stream via cut tissues. This is especially a problem when large doses are given. This interpretation must be considered when it is observed that an increased dose does not lead to an increased response.

1Cii Early Studies

Attempts at peroral or intestinal administration followed closely on the discovery of insulin. For example Hachen & Mills (1923) described experiments involving instillation of about 10 U/kg of insulin alone, into the intestines of dogs and rabbits. Blood glucose depression was observed in some of these experiments. However they could discern no effect in man

when insulin was given orally or by stomach tube. Salen (1924) describes experiments using large quantities of alcohol or olive oil (both good hypoglycaemic agents at the large doses used) as a vehicle for insulin. The positive results he achieved, without any control experiments, are meaningless.

One tireless worker in this field, J. R. Murlin, published papers, with others, from 1927 to 1941. (Murlin & Hawley, 1927; Eaton & Murlin, 1933; Daggs et al, 1937; Murlin et al, 1937; Young et al, 1939; Sealock et al, 1939; Murlin et al, 1940 and Driver & Murlin, 1941). Many of the earlier papers only describe the effect of insulin on respiratory quotient or urinary glucose to nitrogen ratio, without blood glucose measurements. Blood serum, sodium bicarbonate and calcium lactate were the agents he used in early experiments. Then after a study of various resorcinols, hexyl resorcinol was chosen as a suitable vehicle. He described results in dogs and man showing blood glucose depression when large insulin doses (10–50 U/kg) were given by mouth or stomach tube in the presence of a 0.25% hexyl resorcinol solution. But a clinical study in 1940 (Murlin et al, 1940) was not very convincing. He also did experiments with compounds, proposed by other workers to be effective in promoting oral insulin action, and concluded that they were, in fact, ineffective. These compounds were saponin, the dyes, malachite green and trypan red, and various detergents, alcohols and esters.

A great many compounds were tested in this period and proposed as promoters of intestinal insulin absorption. Bollman and Mann (1931) saw no effect of insulin administered with blood, blood serum or dilute hydrochloric acid in exposed duodenal loops of dogs at 100–200 U per animal (in contrast to the results of Murlin and others), Walton & Basset (1932) saw no effect with hexyl resorcinol

or toluene (in contrast to Murlin) but did find an effect with saponin, lactic acid or ethanol. Campbell & Morgan (1933) produced an insulin-lecithin complex, believed to be protease resistant, which was however, ineffective. Major (1938) described the use of a solid insulin-pinacol complex which was active between 4 and 9 U/kg in depancreatised and normal dogs. Wilson et al (1938) demonstrated no effect of insulin alone by stomach tube (at up to several hundred units per animal), but produced a partial papain digest of insulin which was effective orally at about 5–10 U/kg. Cutting & Robson (1941) described clinical trials in man with insulin tablets containing 500 U insulin and 500 mg quinine in gelatine and cellulose enteric capsules. In three cases out of ten, there was a reduction in glycosuria, in three others however these tablets (up to three, three times a day) produced severe tinnitus and cinchonism.

Perhaps some of the best clinical results of this period were produced by Lasch (Lasch & Schönbrunner, 1938 and Lasch, 1951). He describes the use of trypan red and malachite green as acidic and basic dyes, which bind to insulin and protect it from degradation in the gut, and the use of saponin to promote enteral absorption. He describes several cases, mainly elderly, maturity-onset diabetics, where peroral insulin as tablets mixed with these dyes was effective in controlling blood glucose levels and glycosuria for long periods. Unfortunately the dyes and saponin are irritants not well tolerated orally. In the later paper he describes the separation of an ultra-filterable insulin which is more effective than normal insulin when given alone. In all these results, the doses given were between two and five times the dose which the patients had previously been taking subcutaneously. The sulphur and nitrogen content of this latter preparation was very different from insulin and so its chemical

nature is rather obscure.

Hanzlik and Cutting (1941) give an exhaustive survey of all the compounds claimed to promote the absorption of insulin, in man or animals, after peroral administration. Acids, astringents, dyes, irritants, alcohols and surfactants head the list; presumably damage to the gastric mucosa is the major mode of action in most of these cases.

1Ciii Recent Studies

With some exceptions (e.g. the astonishing study of Galloway and Root (1972), in which large, nauseating, doses of detergent were administered to promote insulin absorption) most of the more recent studies in this field have been more considerate of medical ethics and scientific method. These experiments were helped by technical improvements notably the ability to label insulin with radioactive markers, and the vast improvements in the sensitivity of methods of insulin assay.

In infant animals, insulin alone can be absorbed from the gut, but this is part of a general effect found only in neonates which allows the passage of whole proteins through the gut. It is due to a lack of tight junctions between the mucosal cells and the absence of gut protease secretion. (Mosinger et al, 1959 and Kelly, 1960).

Some of the technical difficulties of assays of insulin administered perorally are shown in the papers of Speth and Christian (1963) who claimed substantial blood glucose depression in rabbits with insulin administered at low doses (0.3-3 U/kg) into gastric, jejunal or ileal fistulae; whereas Tocus et al (1965), who performed the same experiment using catheterisation rather than an operative exposure of the gut did not see an absorption at doses of up to 50 U/kg. They proposed that the results of Speth and Christian were

due to the absorption of insulin from cut tissue surfaces exposed by the operative procedure.

D'Addabbo et al (1967) and Shichiri et al (1972, 1973) showed that soluble insulin alone could be absorbed from the gut; over a short time period between 0.1 and 10% of the total dose given would be absorbed, depending on the site. They also demonstrated that the stomach is the main site of insulin destruction. Thus if peroral administration is to be effective, the insulin must be protected from protease digestion and must be modified to improve its absorption in the gut. Crane & Luntz (1968) performed an experiment which supports these results. In a totally pancreatomised man, soluble insulin (100 U/kg) administered via a Ryles tube positioned in the upper jejunum was effective in reducing blood glucose for at least 6 hours.

1Civ Studies using Anti-Protease containing Preparations

Some of the early experimenters claimed that the substances that they used to promote insulin activity after peroral administration were effective due to a reduction in the rate of insulin degradation. Harned and Nash (1932) were some of the few to use a specific antiprotease. They prepared an antiprotease from Ascaris Lumbricoides, which protected insulin from degradation by a crude pancreatic extract. In normal dogs the insulin-antiprotease complex was ineffective at doses of up to 15 U/kg, in pancreatomised dogs they describe a reduction in glycosuria and azoturia without significant hypoglycaemia. Laskowski et al (1958) and Danforth & Moore (1959) described experiments in rats using soybean trypsin inhibitor (1.2 mg/kg), pancreatic trypsin inhibitor (0.25-0.6 mg/kg), diisopropylfluorophosphonate (DFP) (0.5 U/kg) and DFP with indole-3-acetate. Soybean trypsin inhibitor

was found to be ineffective, but the other inhibitors were found to be effective with insulin doses of between 40 and 250 U/kg. Haberland et al (1966) using Trasylol (bovine pancreatic trypsin inhibitor) at 200,000 U per rat, describe a linear dose-response curve to insulin at doses of between 25 and 40 U/kg. All these experiments were performed on anaesthetized rats, injecting the drugs into exposed portions of the intestine. Shichiri et al (1971) showed that insulin bound non-covalently to β-napthyl-azo-polystyrene beads was protected against chymotryptic or peptic hydrolysis. When this complex was administered by stomach tube into rabbits, a strong hypoglycaemic effect was noted at doses of between 20 and 150 U/kg.

Although these experiments are convincing, they all suffer from the disadvantage that very high doses have to be given (at least 10 and up to several hundred units per kilogramme) compared with normal therapeutic doses of 0.25 to 2 U/kg in man when given subcutaneously. And the extensive use of protease inhibitors, acting for up to 6 to 8 hours per dose is liable to cause gastric disorders and malabsorption.

1Cv Studies involving the Encapsulation of Insulin

Two approaches using encapsulated insulin have been studied. Engel et al, (1968) and Shichiri et al (1974, 1975) describe the use of insulin emulsified into a "water-in-oil-in-water" emulsion. This preparation was effective in lowering blood glucose in rats, rabbits or gerbils at doses of 50 to 150 U/kg. Shichiri performed a long term study treating alloxan-diabetised rats with doses of 50 to 100 U/100 g three times a day for up to 14 days. There was a general reduction in glycosuria compared with controls and about 50% of the animals

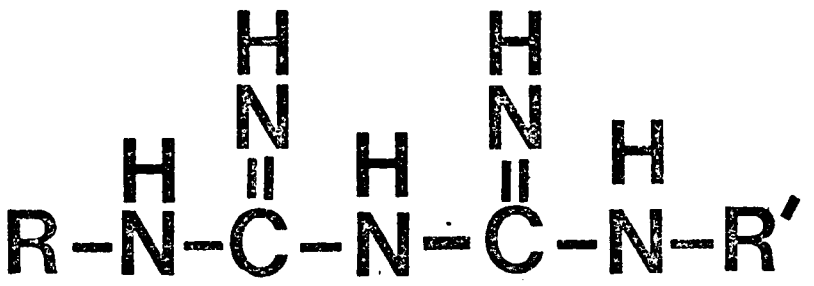
showed reduced blood glucose levels.

The doses used in these studies are still very high. Better results were seen by Patel & Ryman (1976, 1977) and Gregoriadis and Dapergolas (1976) when insulin entrapped in liposomes (Gregoriadis, 1976a,b) was used. Patel & Ryman showed that liposome-entrapped insulin was effective, perorally, at doses of 30 to 70 U/kg in streptozocin-diabetised rats, but ineffective in normal rats. Gregoriadis & Dapergolas have demonstrated significant blood glucose depression in normal and streptozocin-diabetised rats at doses as low as about 2 U/kg (Gregoriadis, personal communication). The choice of lipids for the liposomes is critical for this work, as the rate of hydrolysis of liposomes by pancreatic phospholipases depends on the transition temperature of the phospholipids used (Op den Kamp et al,1971). One drawback in these studies is the fact that liposomes alone can exert a hypo- or hyperglycaemic effect (Bruny et al, 1976 and Patel & Ryman, 1977) and so careful control experiments must be performed. However, the results of this work are very encouraging.

1Cvi Other Non-Parenteral Routes of Administration

As well as peroral administration a variety of other non-parenteral routes of administration have been tried with varying results. Low doses can be absorbed efficiently sublingually with the aid of a vasodilator (Earle, 1971). Endonasal aerosol delivery also appears to be effective in some cases at doses only a little above subcutaneous levels (Jenson, 1938a; Faelli, 1954; Wigley et al, 1971 and Lee, 1975). Langner (1939) also claimed clinical efficacy of insulin administered per rectum, in suppositories, in several patients.

a



b

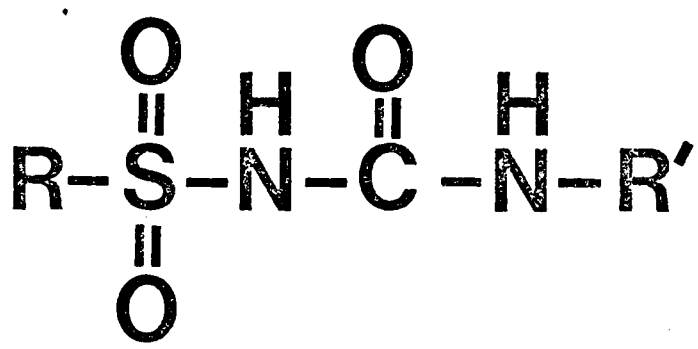


Figure 1.3 General structural formulae of biguanides (a) and sulphonylureas (b). R, R', various side chains.

1D Oral Hypoglycaemic Agents

1Di Introduction

As well as a search for oral preparations of insulin, a search for other oral hypoglycaemic agents has been pursued by many workers. Hypoglycaemia can be induced metabolically; amino-acids, free fatty acids and alcohol for example are all hypoglycaemic agents (Efendic et al, 1974; McDaniel et al, 1973; Fajans et al, 1967; Cahill, 1971, and Carrol & Nestell, 1972) when given orally, these effects are due to a feed-back on hepatic glycolysis and gluconeogenesis via gut hormones and catabolic intermediates of the fatty acids, etc.

A large variety of plant and animal extracts were proposed as potent hypoglycaemic agents at pharmacological levels, for example, 'myrtillin' extracted from blueberries was supposed to be efficacious in maturity-onset diabetes. As shown by Watanabe (1918) guanidine and its derivatives are effective as hypoglycaemic agents. They are, however, toxic at pharmacologically effective levels. (Jensen, 1938b).

1Dii Sulphonylureas and Biguanides

The breakthrough in the development of clinically useful oral hypoglycaemic agents came in the 1950's when two types of compound appeared. They were the sulphonylureas and the biguanides (see Fig.1.3). The acute toxicity was overcome and these products came into general use. (Kirtley et al, 1956; Cox et al, 1956; American Diabetic Association, 1957, and Williams et al, 1958). The mode of action of these agents is not fully understood, despite extensive research (Colwell et al, 1972; Pek et al, 1972; Grodsky et al, 1977, and Fain, 1977 and references therein). To summarize, it is believed that biguanides act to potentiate insulin action, increasing the extent of cellular uptake

of glucose, induced by whatever amount of insulin the patient's pancreas can produce. They also act by decreasing the uptake of glucose by the gut. Sulphonylureas appear to stimulate insulin release from the B cells, although they decrease net protein synthesis in them.

Recently there has been a decline in the use of these agents. This is due partly to a high initial failure rate, coupled with a significant steady rate of secondary failures after prolonged use. The University Group Diabetic Program studies have shown that there is a significantly increased risk of cardiac disease in oral hypoglycaemic agent treated diabetics compared with those treated by insulin or diet alone, although other studies contradict this. There is also a 5% incidence of other side-effects, mainly gastrointestinal and haematological, along with the severe problem of refractory hypoglycaemia, induced by overdose of these drugs or concurrent disease. (Shen & Bressler, 1977; and University Group Diabetic Program, 1970a,b, 1971a,b, 1975, 1976).

1Diii Dietary Adducts

Although strict dietary control has been one of the corner-stones of diabetes prophylaxis, some recent studies have shed a new light on this aspect. Trowell (1976) and Burkitt et al (1974) have been leading proponents of the theory that one major causative agent in diabetes (and other diseases) is the lack of dietary fibre in Western, refined and processed foods. Studies by Jenkins et al (1976) and Miranda and Horwitz (1977) have tended to support this hypothesis. The addition of various fibre additives (guar gum, pectin, etc.) to the diet of both insulin-dependent and insulin-independent diabetics has led to a significant improvement in glucose tolerance, reduction in blood-glucose levels and in some

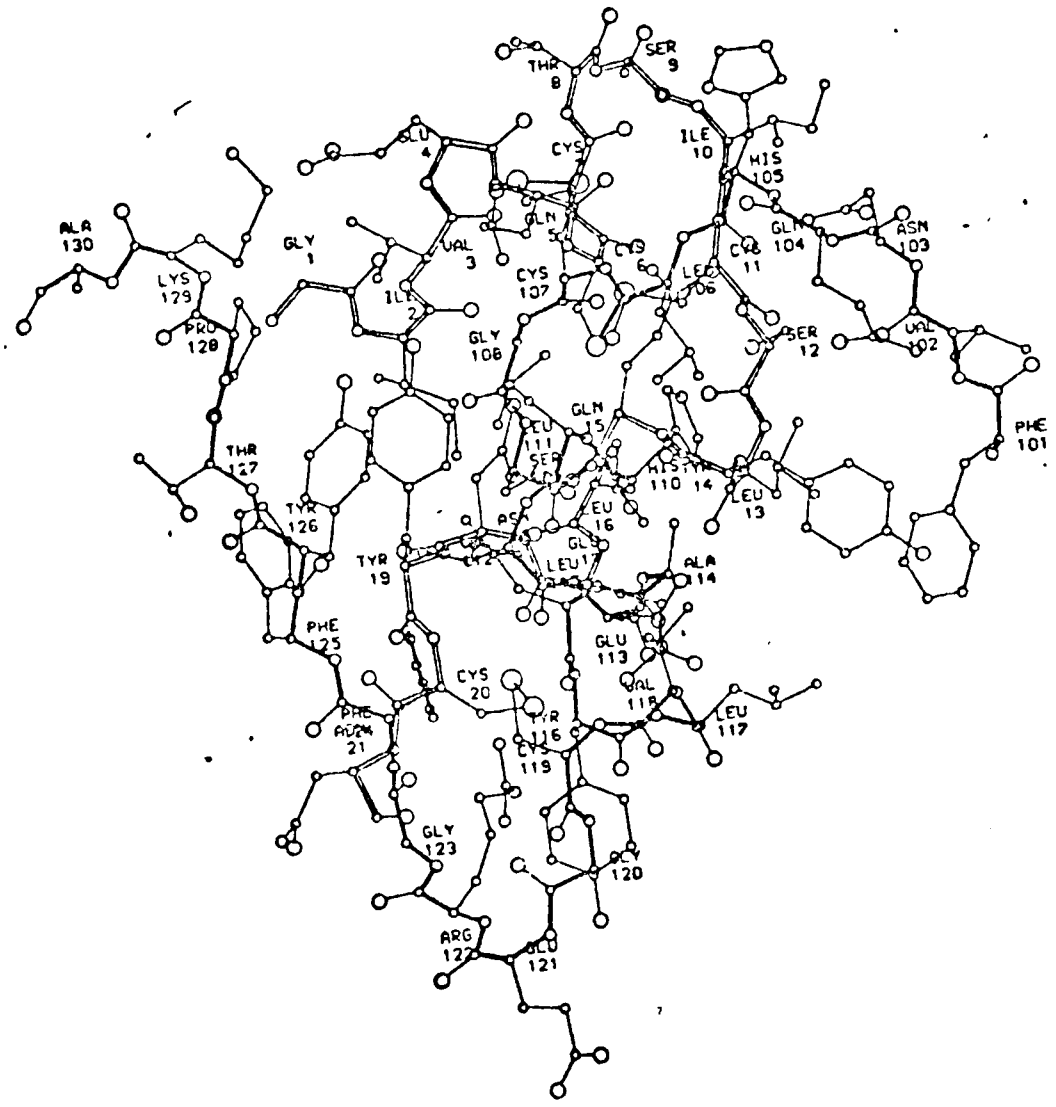


Figure 1.4 Tertiary structure of porcine 2-zinc insulin monomer.

cases a reduced insulin requirement.

1E Some Current Aspects of Insulin Research

1Ei Introduction

Because of the importance of insulin both chemically, clinically and commercially, it is one of the most extensively studied proteins. The scope of this field extends from X-ray crystallographic analysis, which is being pushed to higher and higher resolution and to a wider and wider range of species variants, (insulin-like activity has been found in invertebrates, Kramer et al (1977) and Gomih and Grillo (1976)), through the analysis of insulin and metabolic action at the cellular and tissue level; up to the full scale clinical investigation into the role of insulin, other hormones, diet etc. in the aetiology, treatment and incidence of complications of diabetes. Consequently in this section I only wish to mention a few aspects of insulin and diabetes research, either because of their relevance to insulin structure and function, or the treatment of diabetes in relation to the work in this thesis.

The tertiary structure of insulin has been described (Blundell et al, 1971) (see Fig.1.4) and recently with the help of chemical modification studies a model of the receptor binding region of insulin has been proposed (Pullen et al, 1976) (Fig.1.5). This knowledge is invaluable in planning the modification of insulin for therapeutic purposes. As most side-chain modifiers in this region appear to dramatically reduce insulin activity, such modifications ought to be avoided. The amino-termini are at different distances from this region, allowing a modulation of activity if they are selectively modified. It is interesting to note that the centre of the proposed receptor binding region is the section of the B chain from residues B22 to B26, and it is peptides corresponding to this

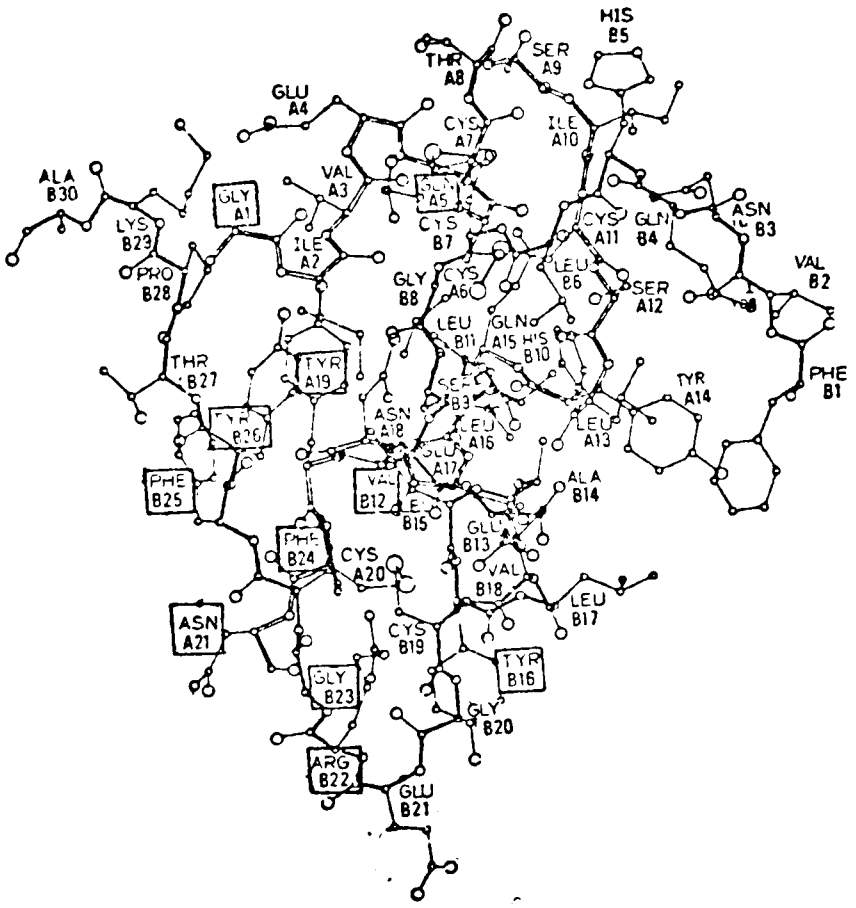


Figure 1.5 Receptor binding region of insulin, residues involved in receptor binding are outlined (from Pullen et al. 1976).

sequence which have an apparent insulin-like activity (Weitzel et al, 1973).

The discovery of insulin-like activity helps to complicate the role of insulin in glucose homeostasis. Substances that seem to be other hormones with hypoglycaemic activity and insulin-protein complexes have been detected by some assay methods (Batchelor, 1967), the role of these activities in normal hormonal regulation is not fully understood. Proinsulin and preproinsulin (Yalow & Berson, 1973 and Steiner, 1977) are found in the blood of normal patients at a few per cent of the insulin concentration and most preparations of crystalline insulin for therapeutic use, contain these species, as well as ethyl and des-amido insulin produced as contaminants in the extraction procedure (Steiner et al, 1968).

Perhaps one of the most exciting developments, is the realization that the pancreatic islets are a far more sophisticated system than was previously thought. The innervation of the islets is probably as important as metabolite levels in controlling hormone release (Woods & Porte, 1975 and Smith & Porte, 1976) and direct interactions between the cells composing the islets are important in controlling relative hormone levels (Unger & Orci, 1976). A newly recognized polypeptide involved in the response of the body to feeding has been recently discovered (Floyd et al, 1977), and cells producing pancreatic-like hormones have been discovered in the gut (Sasaki et al, 1974). A better understanding of these new developments may lead to new and improved pharmacological agents for controlling diabetes.

1Eii Receptors and Degradation

The primary interaction of insulin with tissues leading to the changes in metabolite levels is

beginning to be understood. Originally it was believed that insulin interacted directly with membranes, selectively altering membrane permeability to sugars and metabolites and that this change in substrate levels caused the change in intracellular enzyme activity (Stadie, 1957). In order to accommodate the enormous amplification effect (1 mole of insulin causes the utilization of 10^6 moles of glucose (Levine, 1961)), it was proposed that insulin acts directly on the glucose transport system. Although there is no doubt that insulin can interact with lipid altering the membrane permeability (Perry et al, 1971 and Birdi, 1976), it is now generally believed that the main action of insulin is dependent on its binding to specific cell-surface receptors. The main supportive evidence is the finding that binding ability (K_{diss}) of insulin and insulin analogues to receptor preparations or whole cells is proportional to the activity of the insulin or analogues in those cells or tissues, (Pilkis & Park, 1974; Cuatrecasas et al, 1975; Roth et al, 1975; and de Meyts et al, 1976) and that certain diabetic states (e.g. in obesity) are due to an altered insulin-receptor-binding ability (Olefsky, 1976). Despite this evidence there is still support for a direct, non-receptor-mediated role of insulin in cells. This is mainly due to the absence of evidence for a second messenger system, to support the receptor hypothesis, the occurrence of strong insulin binding to cytoplasmic constituents and the knowledge that other peptide hormones (Insulin-like Growth Factor and prolactin) enter their target cells (Goldfine, 1977). Freeze-fracture studies have shown a change in the surface structure of adipocytes incubated with insulin, this may be due to a direct, non-receptor-mediated interaction with the membrane (Carpenter et al, 1976).

There is now evidence that receptor binding leads to insulin

degradation (Terris & Steiner, 1974; Terris & Steiner, 1975 and Steiner, 1977). This is probably a different activity than the glutathione-requiring protein found by Katzen and Stetten (1962) and extensively studied by Varandani and co-workers (Varandani, 1972 and Chandler & Varandani, 1976) and the protease studied by Izzo et al (1972) and Kitabchi (Kitabchi et al, 1972 and Burghen et al, 1972).

The glutathione-requiring enzyme is probably not important in the degradation of insulin in normal circumstances, and the fact that it has a high Km and is found in the pancreas at a high concentration suggests that it may even be involved in disulphide bond making, rather than breaking. The protease has a Km which is close to the physiological concentrations of insulin in body fluid. However it is a cytoplasmic enzyme and circulating insulin will not be exposed to it. The liver has a massive over-capacity for proteolytic insulin degradation, with a maximum rate of about 200 µg (about 5 Units) of insulin per gramme of wet tissue per minute. (Langner et al, 1971). The insulin degrading activity (glutathione-independent) described by Wisher et al (1977) found associated with liver membranes may be more important and perhaps related to the receptor-binding-degradative activity described by Terris and Steiner. The liver has other effects on insulin, a major one being the production of a high molecular weight product, with insulin-like activity (Lenti & Pagano, 1972). Besides the liver, the kidney is the other major site of insulin degradation.

Although only about 10-30 mU of insulin are excreted per day in man, about 6-8 U/day are consumed by the kidneys. About 20% of a peripherally injected dose being sequestered by the kidney within the first 15 minutes of injection, despite the fact that the role of

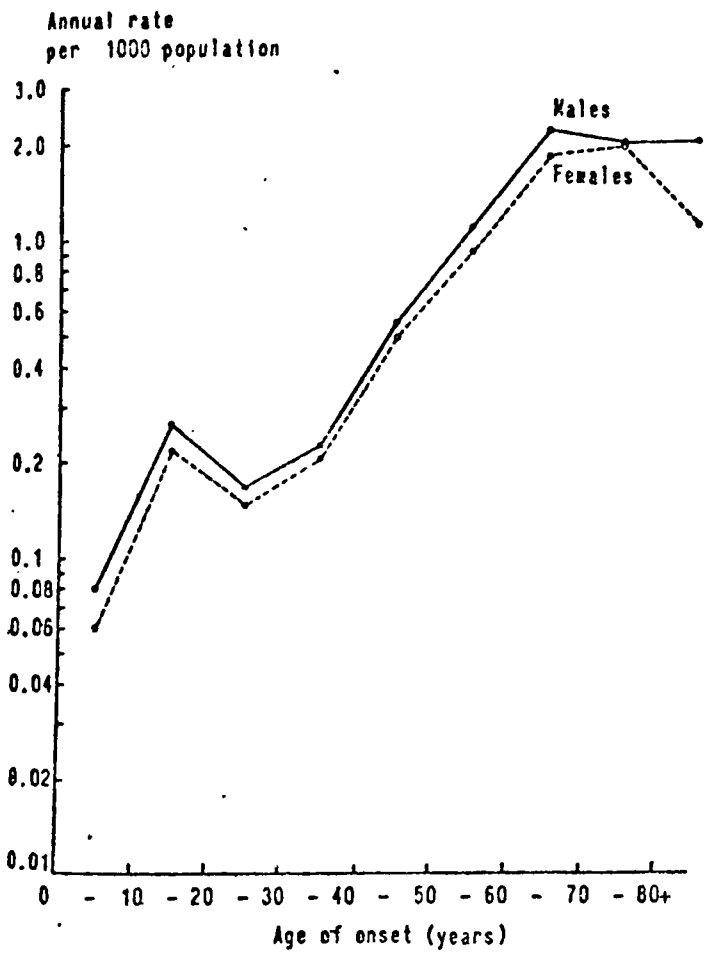


Figure 1.6 Age standardised incidence of diabetes mellitus in Edinburgh, 1969-1971 (from A. Al Kafajei, Ph. D. Thesis, University of Edinburgh, 1974).

the kidney in insulin-induced glucose homeostasis is slight. (Chamberlain & Stimmler, 1967 and Rubenstein, 1975).

One method of producing a very long-acting insulin is to reduce its degradation. Whether or not protection from degradation can be produced without loss of activity is a problem to be overcome. One possible mechanism is explored in this thesis, using low activity, heavily modified insulin derivatives, which may be 'activated' by enzymatic removal of the protecting groups in the body.

1Eiii Clinical Aspects

Clinically, diabetes is a complex syndrome. There are many ill-defined factors involved in the aetiology of the disease, and at least two types of diabetes have been proposed (Ganda & Soeldner, 1977). However dividing the disease into two types has been described as a 'polite fiction' since the incidence is a function of age (Jarrett, 1977) (see Fig.1.6). This is coupled with the difficulties in defining the disease. A broad range of syndromes are seen, varying from normal through degrees of asymptomatic and symptomatic glucose intolerance (chemical and pre-diabetes) to the overt diabetic state where the patients may suffer from mild easily controllable hyperglycaemia to a severe ketosis-prone form of the disease (Jarret & Keen, 1975). The incidence and types of complication are very variable (Ellenberg, 1976), and the value of rigid control on the progress of these complications, though supported by many (Bloodworth & Engerman, 1973 and Job et al, 1976), is challenged by others (Siperstein et al, 1977).

All this means that the development of insulin analogues may only be chemically useful for a small proportion of diabetes. Furthermore the realization that hormonal changes (primarily changes

in glucagon levels, Unger & Orci, 1976) other than insulin lack are involved in the development of hyperglycaemia, had led to clinical efforts towards the use of a 'total hormone' adjustment in diabetes (Gerich et al, 1977). Improvement of insulin by modification for therapy must only be seen as a temporary means until total hormone replacement by artificial pancreas or pancreas transplantation (Tze et al, 1976; Bessman & Schulz, 1972; Albisser et al, 1974; Leonard et al, 1973; Lazarow et al, 1973; Lacy & Bollinger, 1977 and Lazarus, personal communication) becomes a clinical reality. Consequently such modifications of the insulin molecule must be simple and technologically undemanding.

1F Insulin Assay Methods

1Fi Physical Methods

There are many methods to assay for insulin. For general reviews see Dorfman, 1962; Cauwenberge and Franchimani, 1970; Stewart, 1974. Physical methods, such as paper chromatography (Fenton, 1959), electrophoresis (Light & Simpson, 1956 and Gutman et al, 1972) and polarographic and spectrometric methods (Stewart, 1974) have been used and some are still used to some extent (Ruckert & Schone, 1969 and Laszlone & Gabor, 1971). Although these methods can distinguish between insulin and 'ineffective peptides' or protamines, etc., they can give no measure of potency of insulin analogues or the pharmacokinetics of these analogues.

1Fii In-Vivo Biological Methods

The quantitation of insulin by its hypoglycaemic effects in experimental animals (usually rabbits) is probably one of the most extensively used potency assay. (Lacey, 1941). This method is recommended in many Pharmacopoeia. A variety of precautions have to be taken, as many factors

(anaesthetics, trauma, exercise, heparin and feeding) can affect the results, especially when the nadir of the depression is used as the measurement of response (Carrol & Nestall, 1972; Thorp, 1944; Nijjar & Perry, 1971 and Tjalve et al, 1974). This method is fairly insensitive, in rabbits the limit of sensitivity being about 0.05 to 0.1 unit, in rats or mice proportionally less, about 1 milli-unit. However accurate results can be obtained in this method with four to six rabbits, using a cross-over test. Mice can be used in order to detect quantities down to a few milli-units, by using the incidence of convulsions as the response. Then fairly large numbers of animals are required (20 to 100) for probability analysis. The sensitivity of hypoglycaemic bioassays can be reduced to about 50 microunits (in rats) if 'de-endocrinised' animals are used; these pancreatomised, hypophysectomised, adrenolectomised, decerebrate animals are much more sensitive to insulin, no longer having any hormonal or nervous compensatory systems. This is a technically difficult assay, used before the development of immunoassay or the cell stimulation methods. Normal animals are most sensitive to bolus intravenous injections of insulin, the onset of hypoglycaemia being more rapid and greater than when insulin is administered intramuscularly or subcutaneously (Guerra & Kitabchi, 1976).

Other methods utilize the specific effects of insulin on various target tissues. The increase in glycogen deposition in the diaphragm consequent to intraperitoneal injection was one of the first of these methods. (Groen et al, 1952 and Vallance-Owen & Hurlock, 1954). This method was sensitive to about 100 micro-units, though with a poor index of precision. Soon the incorporation of radiolabelled fats or glucose was used in these assays as a more easily measurable parameter. The best tissues are the diaphragm and epididymis adipose

pads, when the insulin and label are injected intraperitoneally (Rafaelson et al, 1965).

1Fiii In-Vitro Biological Methods

Because of the success of these later in-vivo experiments, similar experiments were performed using isolated tissues or cell suspensions and incubating them with insulin or insulin analogues and a labelled metabolite. The main methods used for assay are uptake of labelled glucose, glycogen synthesis or labelled glycine uptake measured in isolated mouse or rat diaphragm, this method is sensitive to about 20 μU of insulin per ml (Manchester et al, 1959 and Wiberg & Jessup, 1961); and conversion of labelled glucose into fat or labelled carbon dioxide release from rat or mouse epididymal fat pads or isolated epididymal fat pad adipose cells (Glieman, 1966; Moody & Glieman, 1968; Glieman & Moody, 1974). The assays with fat pads are sensitive down to about 50 μU/ml but the isolated fat cell assay is sensitive down to about 5 μU/ml.

All these methods suffer from various disadvantages. There can be a large day-to-day variability in the tissue and cell preparations, leading to variations in accuracy and sensitivity. Various pharmacological agents, and even dilution, can interfere with the assay, producing spurious 'insulin-like activity' (Gundersen & Lin, 1965).

1Fiv Radio-Immuno-Assay Methods

In these methods, insulin is bound specifically to anti-insulin antibodies, and then it is separated from bound insulin using various techniques: electrophoresis (Yalow & Bersen, 1960), a second antibody (Morgan & Lazarow, 1963 and Welborn & Fraser, 1965), dextran-coated charcoal (Keane et al, 1968) and other adsorbents. A known amount of radiolabelled insulin is

incorporated into the incubation mix and this is competed off using various concentrations of unlabelled insulin, with either the unknown, or a known sample to provide a standard. It can be used with very small samples (Tiernan et al, 1968) and is sensitive to less than 0.1 μU per ml of insulin.

None of these methods described in the previous two paragraphs has any value however in assays for prolonged activity.

1Fv Pharmacokinetic Assay Methods

Rasio and co-workers (1967) showed that in anephric patients loss of blood insulin after intravenous injection was slow, and there was an equilibration of the vascular and lymphatic compartments within about fifteen minutes post-injection. Sherwin et al (1974) and Pilo et al (1976) have described models that attempt to describe the pharmacokinetics of insulin, and they have performed studies with ^{131}I-labelled insulin as a tracer to support these models. One result of these experiments is that portal vein administration leads to a much higher clearance of insulin (due to hepatic clearance) than peripheral vein administration. There is reasonable doubt as to whether the clearance of radio-iodinated insulin is a valid tracer for insulin, and the studies by Sonksen, Jones and co-workers are more acceptable. (Sonksen et al, 1973; Tompkins et al, 1975; Jones et al, 1976; Jones et al, 1976 and Ellis et al, 1977). They measure the effect of various doses of insulin and insulin derivatives administered by constant rate infusion. They find that many derivatives modified at the A1 and B29 amino group are cleared more slowly than insulin, and that the apparently high activity of these derivatives in vivo compared with in vitro is due to the reduced rate of degradation. They state that derivatives modified at the B1 residue amino-group

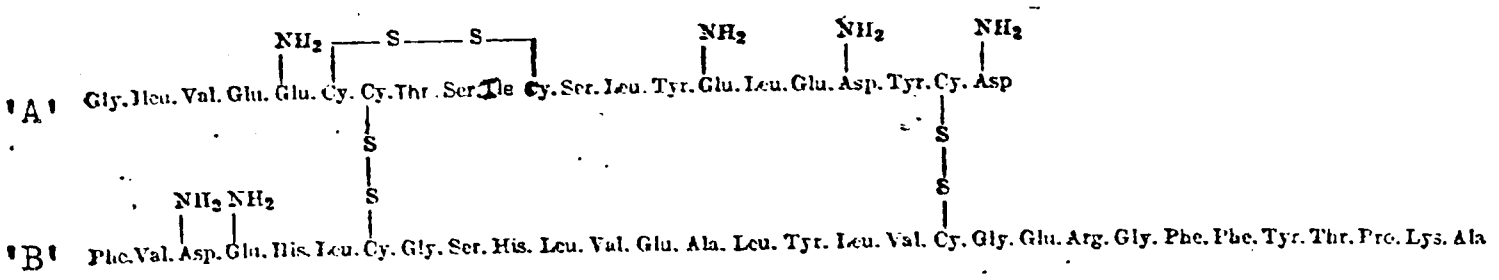


Figure 1.7 Amino-acid sequence of porcine insulin, showing the disulphide bonds.

did not differ in any way from insulin.

1G Modification of Insulin

1Gi Introduction

Attempts at chemical and enzymic modifications of insulin in order to determine the part or parts of the molecule responsible for hormonal activity were started shortly after its discovery. More recently total- and semi-synthetic methods towards the production of modified and normal insulins have been performed. Charles and Scott (1931) showed that extensive methylation (about 6 methoxy group per molecule) or ethylation, reduced the biological potency to about 10%, and that reactivation by saponification with 0.085N sodium hydroxide, led to only a partial restoration of activity. The Fraenkel-Conrats (1950) review the results of chemical modification studies up to that time. They conclude that partial derivatisation of most of the reactive groups (amino, carboxyl, aromatic and aliphatic hydroxyl, guanidino and imidazole) led to a moderate reduction in potency. Reduction of the disulphide bridges or total derivatisation of the other group leads to substantial loss of activity. The drawback of most of the work in this period (Kaiser et al, 1953) is that until the sequence was determined by Sanger and co-workers in 1955 (see Fig.1.7), and the structure in the crystal was determined at the beginning of this decade, by Hodgkin and co-workers (see Fig.1.4) much of the work used to propose a mechanism for insulin action was hampered by a lack of specific knowledge of the interrelationship of the amino-acid residues. Now very many insulin derivatives with specific modifications have been synthesized, and the effects of the modification on the potency of these derivatives have been useful in assisting the identification of receptor and immunoglobulin binding sites (Glieman & Gammeltoft,

1974; Freychet et al, 1974; Zahn et al, 1972; Rager & Buss, 1975 and Brandenburg et al, 1973).

Chemical modification has involved derivatisation of a reactive group or groups, replacing certain reactive groups (mainly the disulphide, amido or carboxyl groups) or lengthening or shortening the peptide chains at amino or carboxyl termini. Enzymic modifications are endo- or exo-protease digestion to remove amino acids or peptides from amino and carboxyl termini, partial synthetic methods involve the use of chemical and enzymatic removal and replacement of amino-acids to effect a specific replacement (Offord & DiBello, 1978). Total synthesis, either by fragment condensation or the stepwise method, is also used to effect the specific replacement of one or several residues (Bodanski et al, 1976). For clinical use, a few chemically derivatised insulins have been used, and there has been a limited use of chemically or semi-synthetically radiolabelled insulins for pharmacokinetic studies in man (Genuth, 1972 and Navalesi et al, 1978) as well as in animals (Berger et al, in press and Berger et al, in press).

1Gii Modifications to the Amino Groups

The Fraenkel-Conrats first showed that the activity of insulin was fairly insensitive to acetylation. More recent work with a variety of acylating groups has shown that the activity of acyl-insulin derivatives depends on which of the amino-groups is modified. When acylation by diketene to produce acetoacetyl insulins was performed, $N^{\alpha A1}$-mono-, $N^{\alpha B1}$-mono-, $N^{\alpha A1}$,$N^{\alpha B1}$-di and $N^{\alpha A1}$,$N^{\alpha B1}$,$N^{\epsilon B29}$-tri-acetoacetyl insulins all have approximately the same activity (50% of native) in the mouse convulsion assay. (Lindsay & Shall, 1969). The smaller acetyl group has no effect on activity in the B1 or B29 positions, but reduces

activity by about 10% in mouse convulsion or 70% in fat-cell-binding or lipogenesis assay, in the A1 position (Lindsay & Shall, 1971 and Glieman & Gammeltoft, 1974). As previously mentioned this difference in potency is explained by reduced degradation leading to higher concentrations in the in-vivo assays compared with in vitro assays (Kaplan et al, 1976 and Jones et al, 1976). Similarly the equally small trifluoroacetyl, carbamyl and methylcarbamyl groups have no effect on potency when in the B1 or B29 positions, and a small effect in the A1 position (Lindsay et al, 1972 and Levy & Paselk, 1973).

$N^{\alpha B1}$-mono-carbamyl-insulin has a reduced potency by immunoassay suggesting that the B1 amino group is an antigenic determinant (Lindsay et al, 1972; Levy & Paselk, 1973; Glieman & Gammeltoft, 1974; Levy, 1973; Geiger et al, 1975; Geiger et al, 1971 and Africa & Carpenter, 1968). One effect of acylation is to reduce the dimerisation of insulin, this is most marked in A1 derivatives (Massey & Smyth, 1972). Larger groups such as Boc-, Msc-, succinyl- and phenylthiocarbamyl lead to a marked decrease in activity in A1 derivatives (to about 10-45% of normal), a slight decrease in activity in B1 or B29 derivatives (to 70-80% of normal), though B1, B29 bis-derivatives have a greater reduction in activity.

Cross-linked insulin analogues synthesized by the reaction of bifunctional reagents have been produced by various workers. When dicarboxylic acid active-esters are used the major product is A1, B29 cross-linked insulin, irrespective of chain-length of the ester.

Very short or very long cross linking agents reduce the biological potency to about 30-50% of normal, and reduce the potency in the fat-cell assay to around 10% or less. However when the cross-link is about the same size as the A1-B29 separation (about 10 Å in the crystal), the potency in an in-vivo assay is high, though

fat cell assay potency is still very low.

Cross-linking of the A1 and B1 amino-groups does not occur as readily at A1, B29 cross-links and potency by in-vivo or in-vitro assays are very low. Probably because, even if the cross-link does not distort the structure, it would have to traverse the proposed receptor binding site (Lindsay, 1971; Brandenburg, 1972; Geiger & Obermeier, 1973; Freychet et al, 1974 and Glieman & Gammeltoft, 1974).

One of the main purposes of cross-linking insulin is to attempt to produce analogues of pro-insulin, which would have an enhanced yield of insulin after combination of the chains and oxidation of the sulphydryl groups to reform the disulphide bonds. A variety of removable cross-links agents have been shown to be effective in this role (2,7-diaminosuberic acid, carboxyl-bis-(1-methionine) and bis-(oxycarbonylethylsulphone). (Brandenburg et al, 1973; Geiger & Obermeier, 1973; Wollmer et al, 1974; Busse & Carpenter, 1974a,b; Busse et al, 1974 and Obermeier & Geiger, 1975).

1Giii Substitutions at the Amino Termini

Removal of residues from the amino termini of the A chain, leads to an inactivation of the insulin, des-Gly^{A1}-insulin has between 10 and 25% potency 'in vivo', whereas des-(Gly-Ile-Val-Glu)$^{A1-4}$-insulin is less than 1% active by mouse convulsion assay (Brandenburg et al, 1975; Katsoyannis & Zalut, 1972). Replacement of the A1 glycine by β-alanine (Geiger et al, 1976); D-alanine (Geiger et al, 1975) or leucine (Krail et al, 1975) leads to an approximate halving of biological activity, replacement with glutamic acid, lysine, proline or valine however leads to a reduction in fat cell potency to less than 10% of normal. However replacement of the A1 glycine by D-lysine produces a fully active product (Saunders, 1978). Studies on these lower

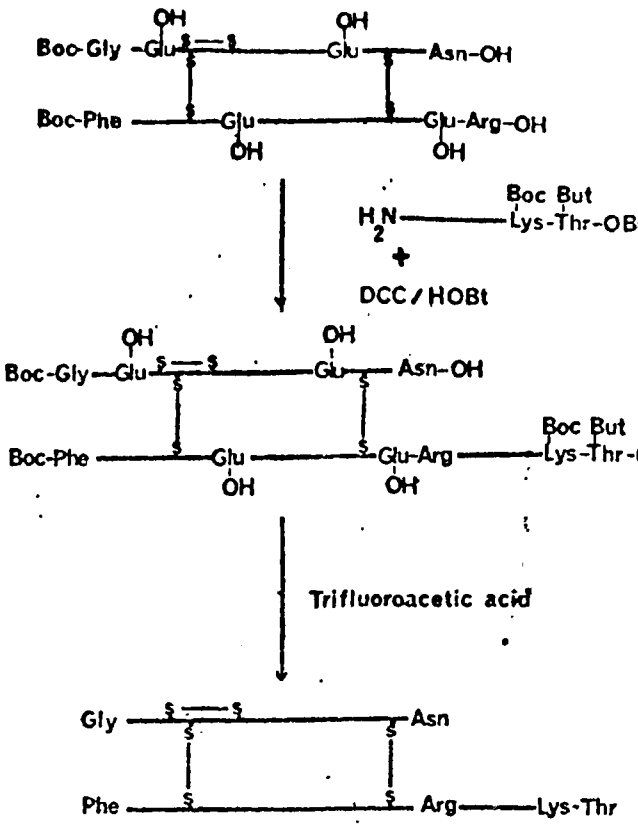
activity analogues suggest that there is an alteration in the structure.

Removal of residues from the amino-terminus of the B chain leads to much less loss of activity. Des-Phe^{B1}-insulin and des-(Phe-Val)$^{B1-2}$-insulin are fully potent by rabbit blood sugar depression, and virtually so by fat-cell assay. Once the third and fourth residues are removed there is a reduction in activity to 70 and 40% respectively. Once the fifth and sixth residues are removed there is a drop in activity to about 10%. The histidine, B5, is involved in zinc binding and this may explain the loss in activity when residues B4 and B5 are removed (Kerp et al, 1974 and Weiman, 1977). Replacement of the B1 residue with alanine, valine, tyrosine, various fluorophenylalanines or iodotyrosine, reduces potency to between 30 and 65% (Saunders & Offord, 1977 and Krail et al, 1971). (3,5-diiodo-Tyr^{B1}) insulin synthesized by Geiger and Teetz, proved to be identical to normal insulin, when assayed by the method of Sonksen and co-workers (Ellis et al, 1977).

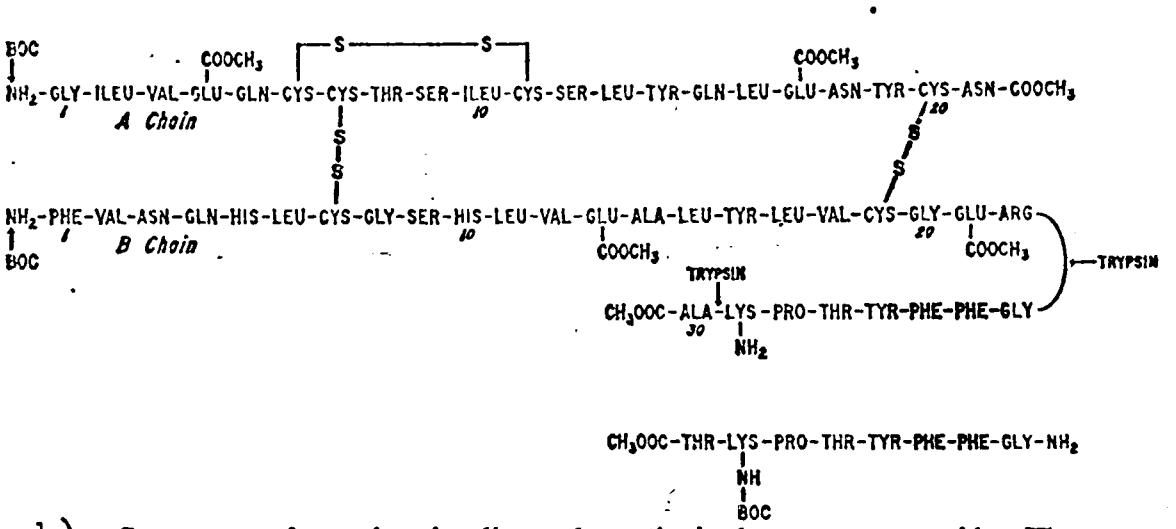
1Giv Modifications for Clinical use

Very few modified insulin analogues have found their way into clinical use. Probably the most extensively used has been the sulphated insulin, prepared by Glendenning et al (1947), and used to treat insulin resistance in some patients (Moloney et al, 1964 and Little & Arnott, 1965). Katsilambros (1972a,b) describes the use of 'phenylated' and 'formalized' insulin for the desensitization of insulin-resistant patients. The chemical nature of these modifications was not characterized, however.

It is believed that the use of human insulin rather than bovine or porcine insulin would reduce the immunological problems



a) Reaction scheme for the synthesis of human insulin from $N^{\alpha A1}, N^{\alpha B1}$-di-Boc-desoctapeptide-(B23-30)-insulin without carboxyl protection.



b) Structures of porcine insulin and synthetic human octapeptide. The upper structure represents the amino acid sequence of porcine insulin with its six carboxyl groups esterified. Digestion with trypsin rapidly cleaves the molecule at the positions shown. After isolation of the resultant desoctapeptide insulin and reversible blocking of its two free amino groups with BOC azide as indicated by the arrows, the material can be coupled to blocked synthetic human octapeptide, the lower structure. Subsequent removal of protecting groups gives human insulin.

Figure 1.8 Methods for the semisynthesis of human insulin from porcine insulin, a) from Obermeier & Geiger (1976) and b) from Ruttenberg (1972).

seen in some patients. Successful total synthetic routes to human insulin are possible but are very complex and unlikely to be viable commercially (Sieber et al, 1974). Semisynthetic routes from porcine insulin are also feasible (see Fig.1.8), and despite the unsubstantiated claims of Ruttenberg (1972), pure material is only produced in about 15% yield at best (Obermeier & Geiger, 1976). This is unlikely to be a satisfactory commercial proposition.

The radiolabelled insulins produced by Teetz et al, (1976) and Halban and Offord (1975), should prove useful for tracer studies in understanding the fate of injected insulin in normal and diabetic men.

1H Scope of this Thesis

This work is based on a desire to synthesize a long-acting insulin, with improved glucose homeostasis, and to attempt to modify insulin in order to make a peroral administration effective.

Prolonged-action insulin preparations are not novel, as has already been outlined. However, in these studies it is proposed to synthesize insulin derivatives which are normally of low activity but are potentially reactivatable.

Coupling a diacylglycerol via a short chain dicarboxylic acid to an insulin amino group (or groups) (see Fig.3.2) could potentially produce a derivative which could be activated by enzymes such as lipoprotein lipase. This enzyme has an elevated activity after a meal and it is at this time that insulin secretion is needed to maintain glucose homeostasis.

At the same time an attempt was made to synthesize an insulin derivative coupled as a substituted imine to aldehyde groups on water-soluble polysaccharides. It was hoped that the lability of

these bonds would lead to a reactivatable insulin derivative.

As some of the early triacylglycerol-insulin derivatives were found to be simpler acyl derivatives synthesized in error, the effect of varying chain length on the prolonged activity of fatty acyl substituted insulin was measured. A radiolabelled fatty acyl insulin analogue was made, in an attempt to observe the pharmacokinetics of these derivatives.

Most of the derivatives were assayed for activity when administered perorally. It was believed that given a sufficient degree of acylation, the insulin might be protected from gut proteases and be sufficiently hydrophobic to be absorbed more efficiently from the intestine than normal insulin.

The acylation of the A1 amino group leads to the greatest loss of potency and of the B1 amino group to the least loss of potency. In order to synthesize acyl-insulin derivatives of the highest potency, all the mono-acyl derivatives synthesized were B1 derivatives and all the di-acyl derivatives synthesized were B1,B29 derivatives. Only when tri-acyl derivatives were synthesized was the A1 amino-acid residue modified.

The assays for potency and prolongation of activity were performed in rabbits. The derivatives were administered intravenously in order to be better able to follow the kinetics of the drug effect in the blood, rather than the mobilization from a subcutaneous site as well. A few simple biological examinations of the rabbits were made to see if they had been adversely affected by these drugs. Post-mortem examinations were performed on four of the animals, with a histological examination of the tissues from two of these, and the animals were examined for the presence of serum antibodies to insulin.

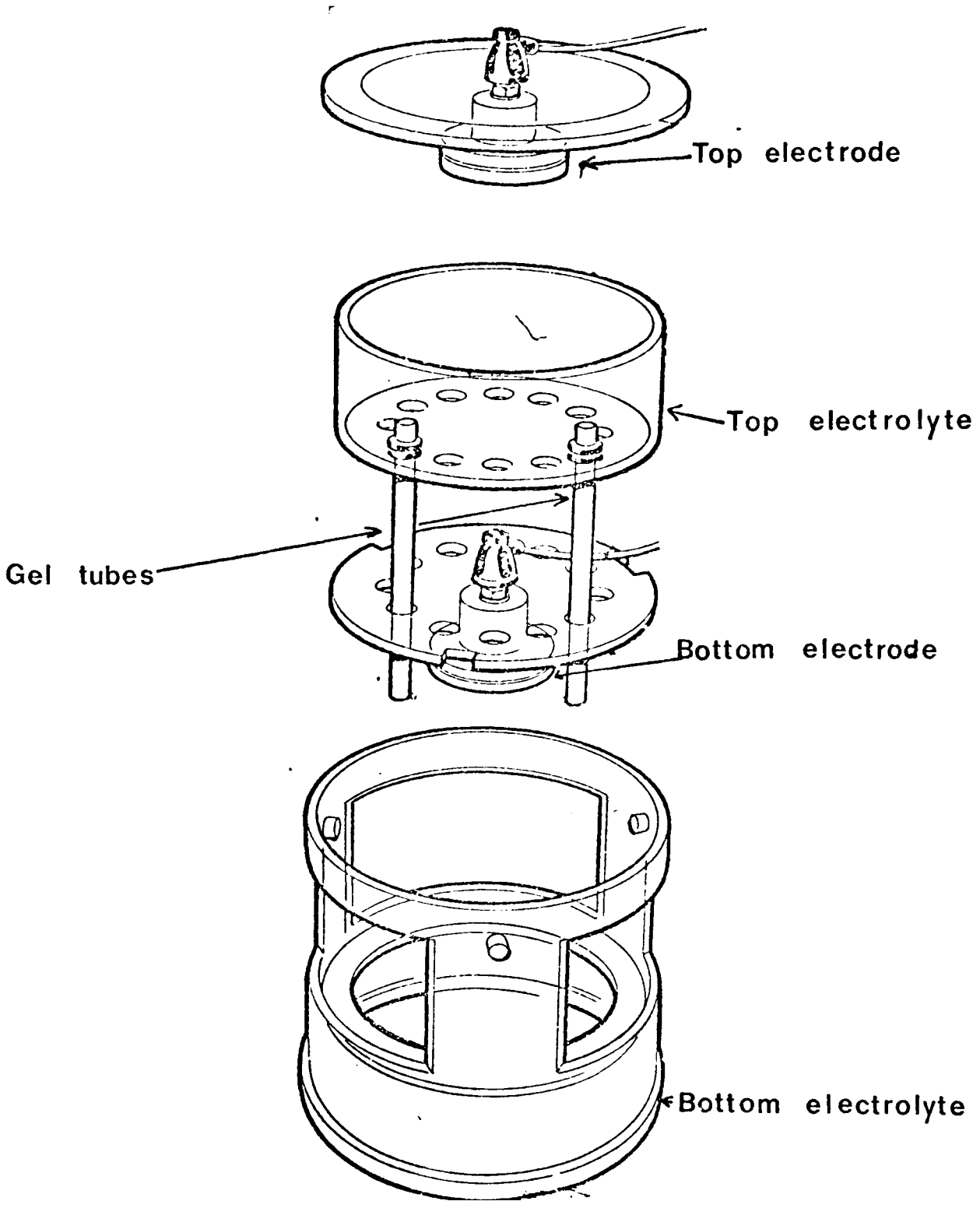


Figure 2.1 Electrofocussing apparatus, from manufacturers literature.

Chapter 2

Analytical Methods

2A Introduction

This chapter describes the physical and chemical methods used to analyse the progress of the syntheses described in chapters 3 and 4 and used to determine the purity of the products of each stage of the syntheses. This chapter also describes the biological assay methods used for the insulin derivatives and the toxicological methods the results of which are described in chapter 5. Several experiments, which were performed in order to check the reliability or sensitivity of the assay methods, are included in this chapter. The reasons for some of these experiments and the reaction methods for the synthesis of some of the intermediate products mentioned in this chapter, are more fully described or explained in subsequent chapters.

2B Methods for the Analysis of Modifications to Insulin

2Bi Polyacrylamide-Gel Electrofocussing

This technique is used to analyse modifications to the insulin molecule that involve changes in the overall charge of the molecule (Wrigley, 1968; Borras & Offord, 1970; Saunders & Offord, 1977). The method involves the polymerisation of an acrylamide solution containing the protein sample and a carrier ampholyte in urea solution in a Perspex tube, to form a gel. This tube is then placed in the apparatus (see Fig.2.1) between two compartments: one, the anode compartment, containing 0.2% phosphoric acid and the other, the cathode compartment, containing 2% triethylamine. The carrier ampholytes (Ampholine, LKB Pharmacia, Sweden) are artificial co-polymers of di-amino and di-carboxylic amino acids of a specified size range.

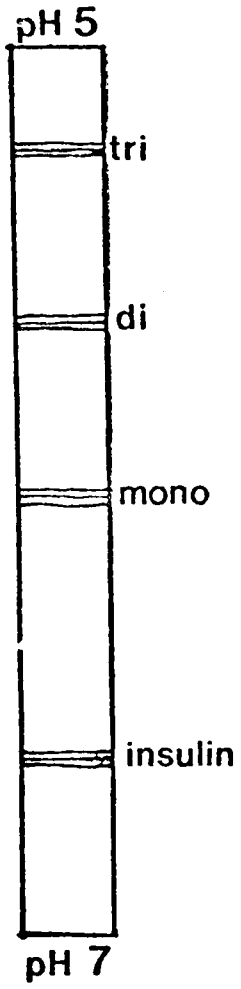


Figure 2.2 Positions of insulin and mono-, di- and tri-Boc-insulin on electrofocussing using pH 5-7 Ampholyne.

When a potential gradient is applied across the ampholyte-containing gel, the charged ampholyte molecules migrate towards the electrode of opposite sign until they are discharged by the inward diffusion of the electrolyte solutions or by ampholyte molecules with a higher net charge. Thus the ampholytes form a gradient of apparent pH depending on the ratio of carboxyl to amino groups in the ampholyte molecules. The insulin sample simultaneously moves with the ampholyte until it appears as a band on the pH gradient, where it has no net charge and it no longer migrates due to the applied potential gradient. This pH corresponds to the isoelectric point of the protein. The changes in the isoelectric point of insulin when it is acylated, esterified or deamidated etc., allow the separation of insulin derivatives by this method. The most suitable pH range was found to be 5 to 7 for the separation of insulin and mono- and di-acyl-insulin derivatives. Fig.2.2 shows the positions of these derivatives on gel electrofocussing using pH 5 to 7 Ampholine. From time to time intermediate bands were observed, these could be due to chemical changes in the insulin molecule or to local perturbations in the ampholyte gradient if the sample is overloaded.

The details of the analysis are as follows: Stock solutions were prepared. A) 0.952 g of N,N'-methylenebisacrylamide and 35.7 g acrylamide were made up to 1 l with water and the solution filtered and stored in a stoppered bottle at $4^{\circ}C$, in the dark. B) 1 ml of 1,1,3,3-tetramethyl guanidine was made up to 10 ml with water and kept in a sealed vial at $4^{\circ}C$ in the dark. An 8M solution of urea in water was prepared, filtered through macerated filter paper under vacuum, stored at room temperature in a stoppered vessel, and immediately before use, it was passed through a mixed bed resin (MB3, BDH Chemicals Ltd.) to remove ammonium isocyanate.

A sample of insulin, 150–200 μg, was dissolved in 1 ml of 8M urea, then 0.5 ml of the gel mixture was added, this gel mixture contained 0.315 ml of solution A, 0.01 ml of solution B, 0.0625 ml of 40% Ampholines, pH 5–7, and 0.125 ml of water. This solution was made up in bulk for as many tubes as were to be focussed, immediately before use. The samples were then degassed in 125 x 16 mm glass tubes, in a desiccator on a water pump, for 15 minutes. During this time the gel tubes (75 x 4.5 mm, Perspex) were prepared by sealing one end with two layers of Nescofilm. They were then placed, with the open end uppermost, vertically in the carrier. Fifty microlitres of a fresh 50 mg/ml solution of potassium persulphate was added to each degassed sample, this was mixed in carefully without bubbling or frothing. The samples were then transferred to the gel tubes, without the inclusion of air bubbles. They were then left for 30 minutes at room temperature to gel. The film seals on the bottom of the tubes were punctured in several places with a fine needle. The carrier and tubes were transferred to the apparatus, the top and bottom electrolyte solutions were added and the electrodes were fitted. Potential was supplied by a constant voltage power pack and was increased in 50-volt steps at about 10-minute intervals, until 300 volts was reached. The apparatus was then left for about 3 hours. The current was not allowed to exceed 2 mA per tube and the focussing was performed at 4°C. On completion of the focussing, the tubes were removed and the gels were separated from the tube walls using a fine syringe needle. The gels were gently expelled into tubes containing 5% aqueous trichloroacetic acid. Within a short time the acid caused the precipitation of the protein bands. Permanent records of the gels were made by optical densitometry after standing overnight at 4°C in the trichloroacetic

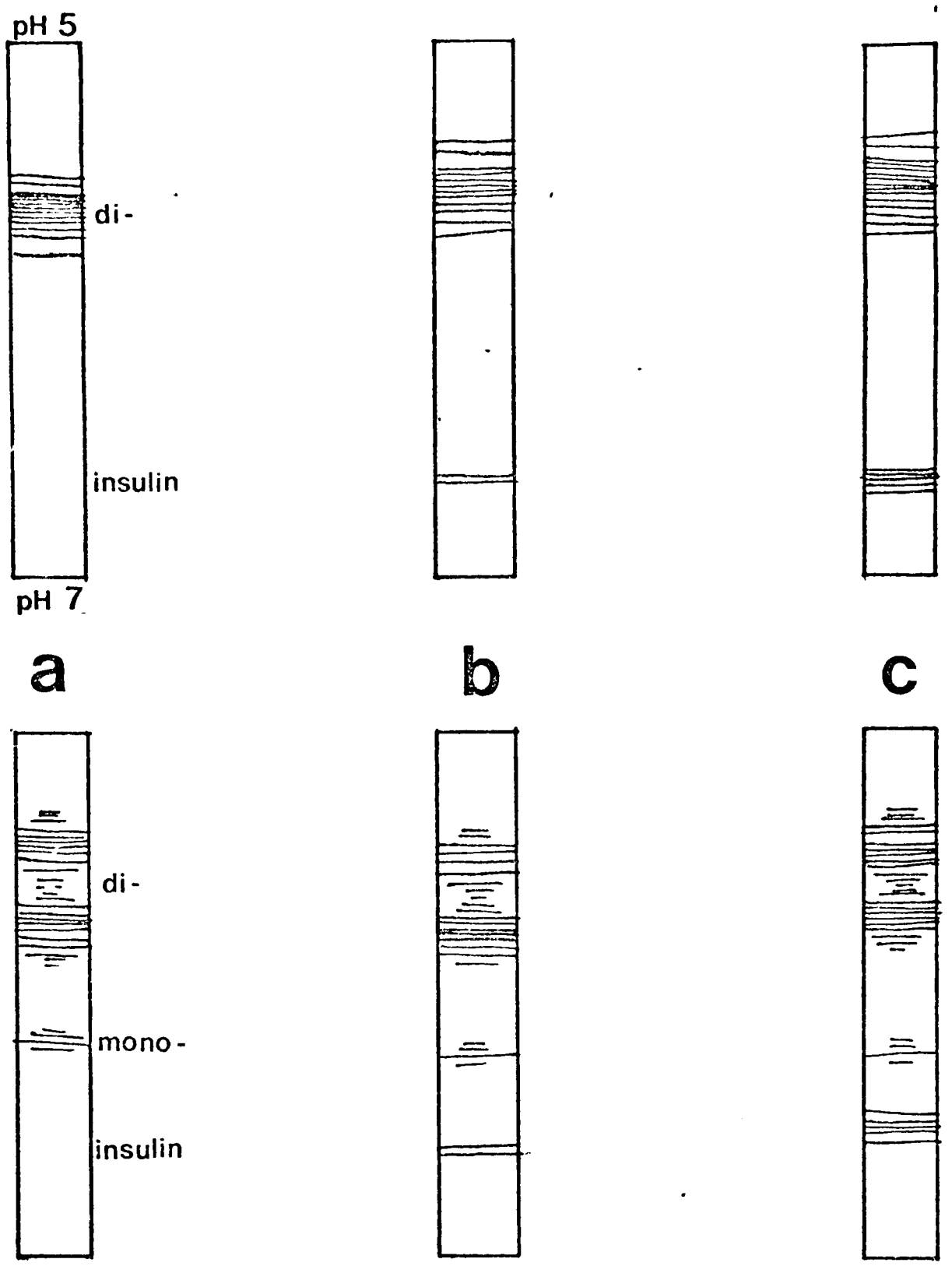


Figure 2.3 Electrocussing gels from experiment to determine the sensitivity of the method: upper, di-nonanoyl-insulin; lower, di-stearoyl-insulin with: a, 1%; b, 3% and c, 10% normal insulin added.

acid solution.

This method was suitable for analysis of the N-tert-butyloxycarbonyl derivatives of insulin, but was less so for the acyl- or the (triacylglycerol)-derivatives. Those modified with the larger acyl groups were sparingly soluble in the urea solution and gel mixture and did not focus at all. The less heavily acylated derivatives with short chain-length acyl groups did electrofocus but the bands were broad and diffuse and were not entirely satisfactory.

An experiment was performed to determine the sensitivity of this method for the detection of contaminating insulin in di-acyl-insulin derivatives. Samples of N^{B1}, N^{B29}-di-nonanoyl-insulin and N^{B1}, N^{B29}-di-stearoyl-insulin (prepared as described in chapters 3 and 4) were dissolved in dimethylsulphoxide and water was added to make them about 50% aqueous. The concentration of insulin in these solutions was ascertained by measuring the O.D. at 278 nm with 50% dimethylsulphoxide as reference. Aliquots containing 200 µg of protein were withdrawn and contaminated with aliquots, from a solution of insulin, to provide samples with 1, 3 and 10% insulin in the di-acyl-insulin. These were diluted with water and lyophilised. A small drop of dimethylsulphoxide was added and then these samples were electrofocussed. No band corresponding to insulin was seen from the 1% contaminated sample, a very faint band not registered by the optical densitometer was seen with the 3%-insulin-containing gel which faded after a further 24 hours in trichloroacetic acid. A dense stable band was seen with the 10%-insulin-containing sample. This was true for both derivatives. The bands corresponding to the di-acyl-insulins were broad and diffuse, covering 15-20 mm of the gel length, at about the correct position (see Fig.2.3).

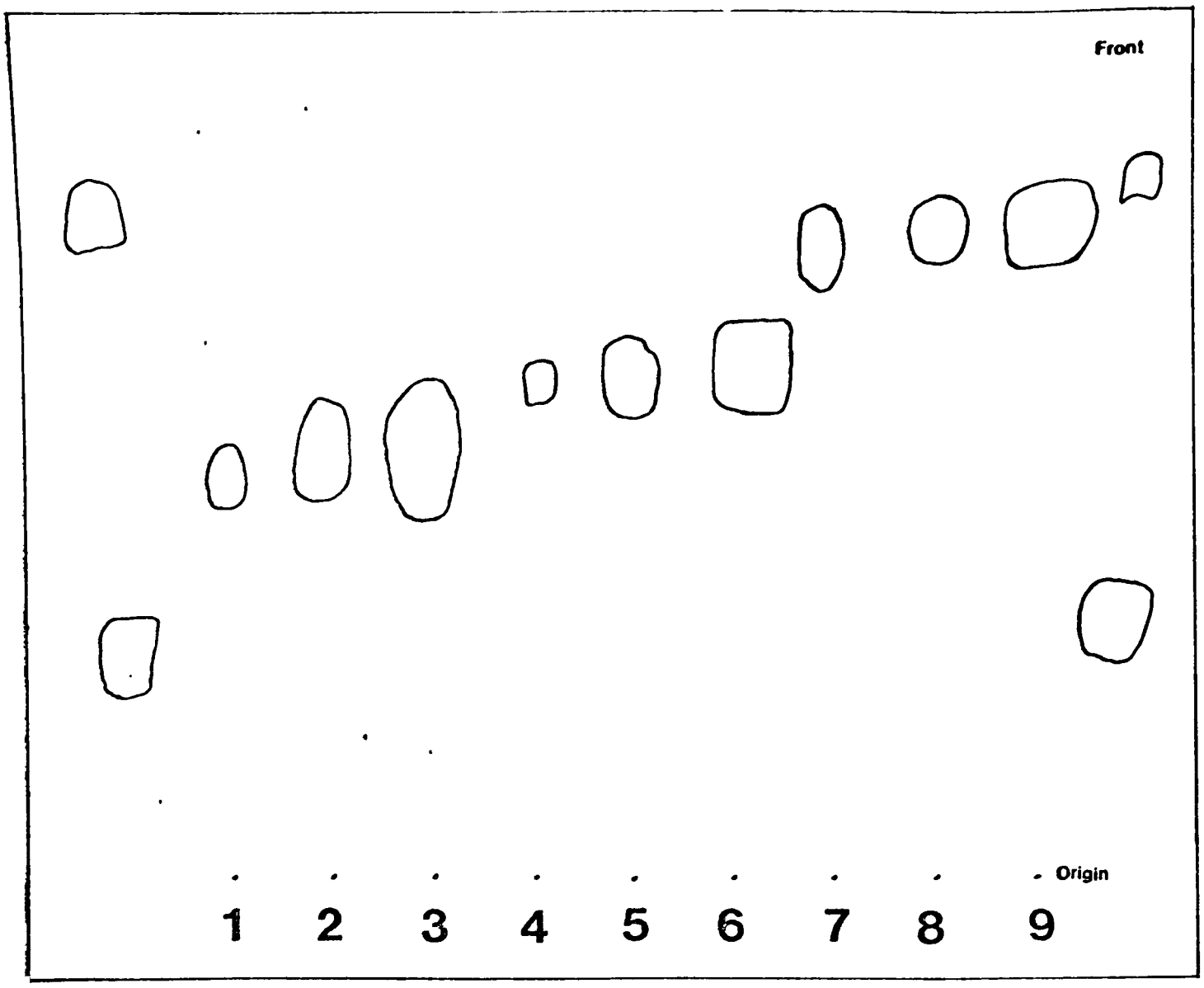


Figure 2.4 Tracing of SG-81 chromatogram showing variation in R_f with loading: 1,2,3 normal insulin; 4,5,6, mono-Boc-insulin and 7,8,9, di-Boc-insulin at: 1,4,7, 30 ug; 2,5,8, 100 ug and 3,6,9, 300 ug loading.

2Bi. Chromatography on Silica-Gel-Loaded Paper

As isoelectric focussing was unsuitable for the analysis of the degree of acylation of the acyl-insulin derivatives, an alternative method was sought. Analysis of insulin or its separated A and B chains, in various forms, has been performed by electrophoresis on paper, on cellulose acetate, on starch and on polyacrylamide gels, or by thin-layer chromatography on silica gel. (Stewart, 1974; Brandenburg et al, 1972; Arx & Faupel (1976)).

In 1951, Hall et al described the use of descending paper chromatography as an assay for serum proteins. The eluants used were aqueous sugar or hydroxy-acid solutions with Tween. However the results were somewhat confusing. Grodsky et al (1956) and Fenton (1959) describe the use of paper-chromatographic analysis of insulin on Whatman No. 1 paper, using non-aqueous eluants based mainly on butanol and organic acids. These methods separated insulin from 'damaged' insulin which had a low or zero R_f in these systems. The insulin spots tended to streak badly, however. At about the same time Porter (1953) showed that insulin could be purified by reversed-phase chromatography on Super-Cel powder as a solid support and Cellosolve:aqueous phosphate mixtures as stationary and mobile phases. In 1966, Katsoyannis described the chromatography of insulin A-chain sulphonate on paper using BAWP as eluant. In 1971, Perry et al showed that insulin and insulin-phospholipid complexes could be separated by an organic solvent on Whatman No. 1 paper. Marinetti (1964) describes the use of silica-gel-loaded paper (SG-81 paper, Whatman) for the analysis of phospholipids. I decided to try this paper for the chromatography of insulin and its acyl derivatives. A 2 l beaker without a lip, 22 cm tall was used as a

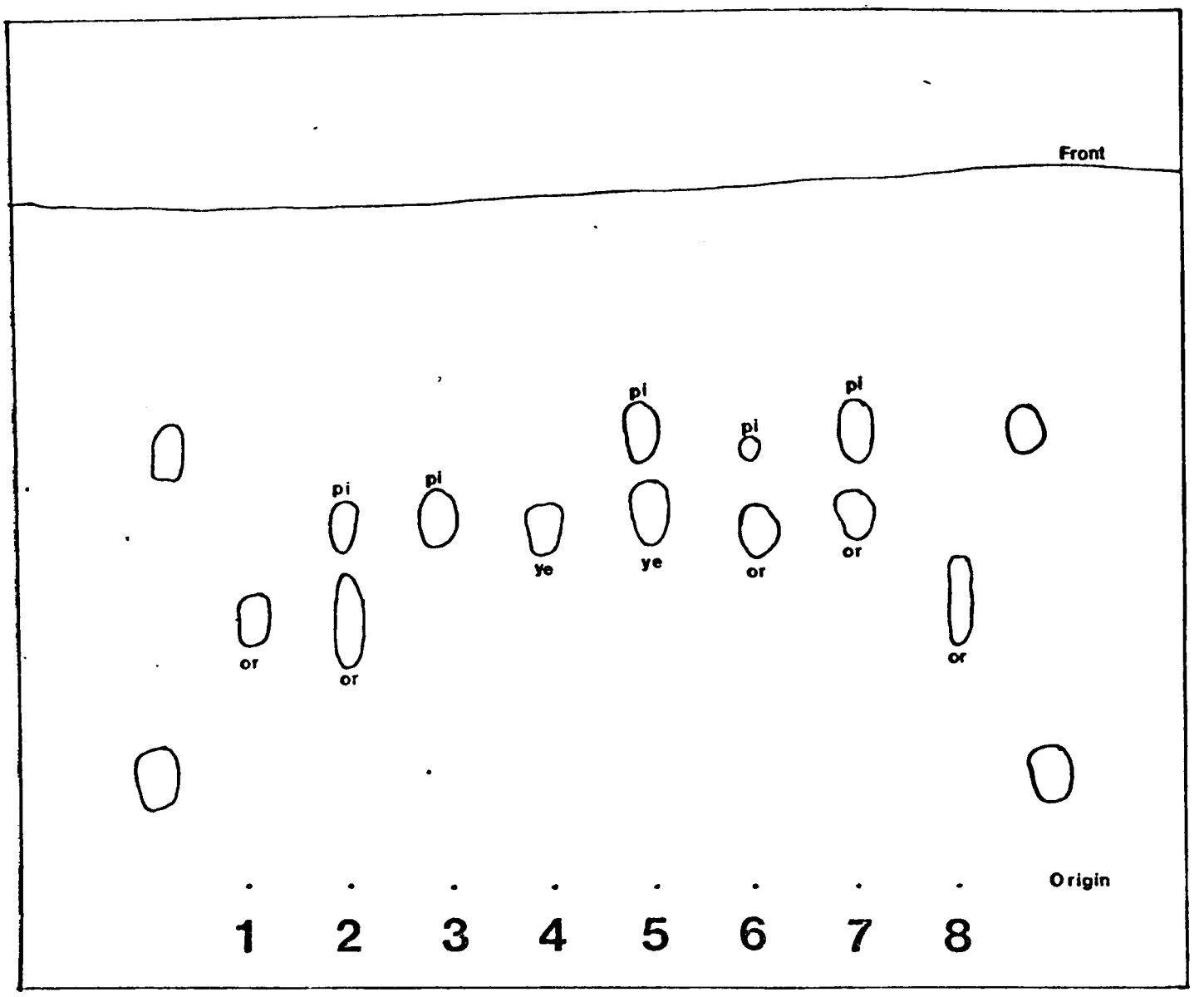


Figure 2.5 Tracing of SG-81 chromatogram showing insulin and various Boc-insulin derivatives: 1,8, insulin; 2, insulin and $N^{\alpha A1}$-Boc-insulin; 3, $N^{\alpha A1}$-Boc-insulin; 4, $N^{\epsilon B29}$-Boc-insulin; 5,6,7, $N^{\epsilon B29}$-Boc- and $N^{\alpha A1}$,$N^{\epsilon B29}$-di-Boc-insulin (three different mixtures). Colour of Ninhydrin/cadmium staining shown adjacent to each spot; pi, pink; or, orange and ye, yellow.

chromatography tank. It was lined with filter paper moistened with eluant, and eluant to a depth of 1 cm was poured in the bottom. 18 by 23 cm sheets of SG-81 paper were cut and the origin marked at 2 cm from the longest side. The samples were spotted at 2 cm intervals along this line. Standard dye mixtures (Xylene-Cyanol-FF, Orange G, Acid Fuchsin, ϵ-Dnp-L-lysine, L-aspartic acid, L-lysine, L-histidine and L-tyrosine ethyl ester) were applied at the edge, the paper was made into a tube and the edges butted together by clips. It was put into the chromatography tank and developed for about 4-5 hours. The paper, after drying, could be stained by any common technique. Usually the following were performed. Firstly, the paper was stained with ninhydrin/cadmium acetate (Offord, 1969) to show free amino- groups, followed by staining for tyrosine (Offord, 1969) to demonstrate protein, the paper was then washed with water or 5% bicarbonate solution to remove excess acid and finally stained with 0.2% 2,7-dichlorofluorescein in 90% ethanol. Some loss of insulin was observed after washing, but the lipids and acyl-insulin derivatives were largely unaffected. Under u.v. light, lipids appear as yellow spots, protein as deep red and lipophilic insulin as an intermediate orange colour.

Initially both BAWP (n-butanol: glacial acetic acid:water: pyridine, 30:6:12:10, by vol.) and BAW (n-butanol:glacial acetic acid: water, 75:25:8, by vol.) were used. With BAWP as eluant the R_f values of insulin and tri-acyl-insulin were about 0.40 and 0.85 respectively, with BAW as eluant these values were about 0.0 and 0.30 respectively. BAWP was a better eluant as it gave a wider range of R_fs of the chosen derivatives. BAW also tended to cause streaking of the spots. The effect of sample loading was then measured, samples of 30, 100 and 300 μg of insulin and mono- and di-Boc-insulin were

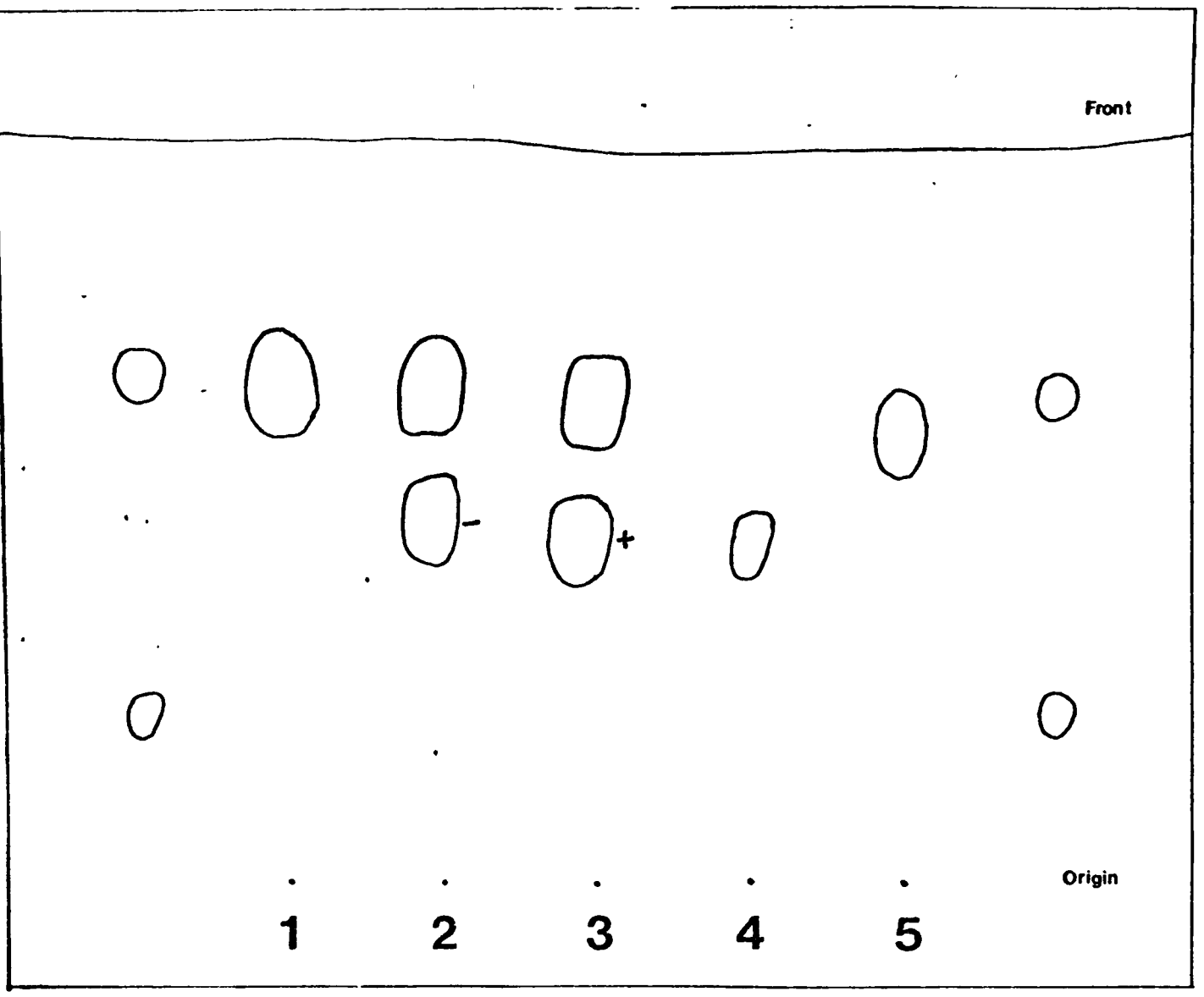


Figure 2.6 Tracing of SG-81 chromatogram showing the sensitivity of the method: 1,2,3, di-nonanoyl-insulin with 1, 1%; 2, 3% and 3, 10% insulin; 4, insulin standard and 5, di-nonanoyl-insulin standard.

chromatographed in BAWP for 16 hours. There was a small increase in R_f value with loading for the proteins from 30 to 100 μg of sample, but the R_f values of the 100 and 300 μg samples were the same (see Fig.2.4). If the chromatogram was allowed to run overnight, there was a better separation of insulin and mono- and di-acyl-insulin derivatives, however the di- and tri-acyl-insulin derivatives then overlapped, furthermore the spots became diffuse, reducing sensitivity.

The method used in this thesis was: the eluant was BAWP (see above), the sample loading was 200 ± 50 μg dissolved in 5 to 10 microlitres of eluant, applied onto a 6 to 8 mm diameter spot, elution was ascending, in the tank described above, at room temperature, then the chromatogram was dried and stained as described above (see Fig.2.5). Recently Lutz (1975) has described a similar method for the separation of basic peptides with molecular weights ranging from 1200 to 1800.

By performing an experiment identical to that performed for isoelectric focussing, the sensitivity of the technique with mixtures of di-acyl-insulin derivatives and insulin was measured. A 1% contamination was not detected with tyrosine staining but a 3 and 10% contamination was detected (see Fig.2.6).

An experiment was performed to determine whether contamination of the insulin sample with fat affected the method. Approximately 200 μg of nonanoic acid and 200 μg of 1,2-dipalmitoylglycerol were added to 200 μg samples of insulin and di-Boc-insulin. On chromatography of these samples, there was no difference in R_f or apparent staining when compared with uncontaminated insulin preparations (see Fig.2.7).

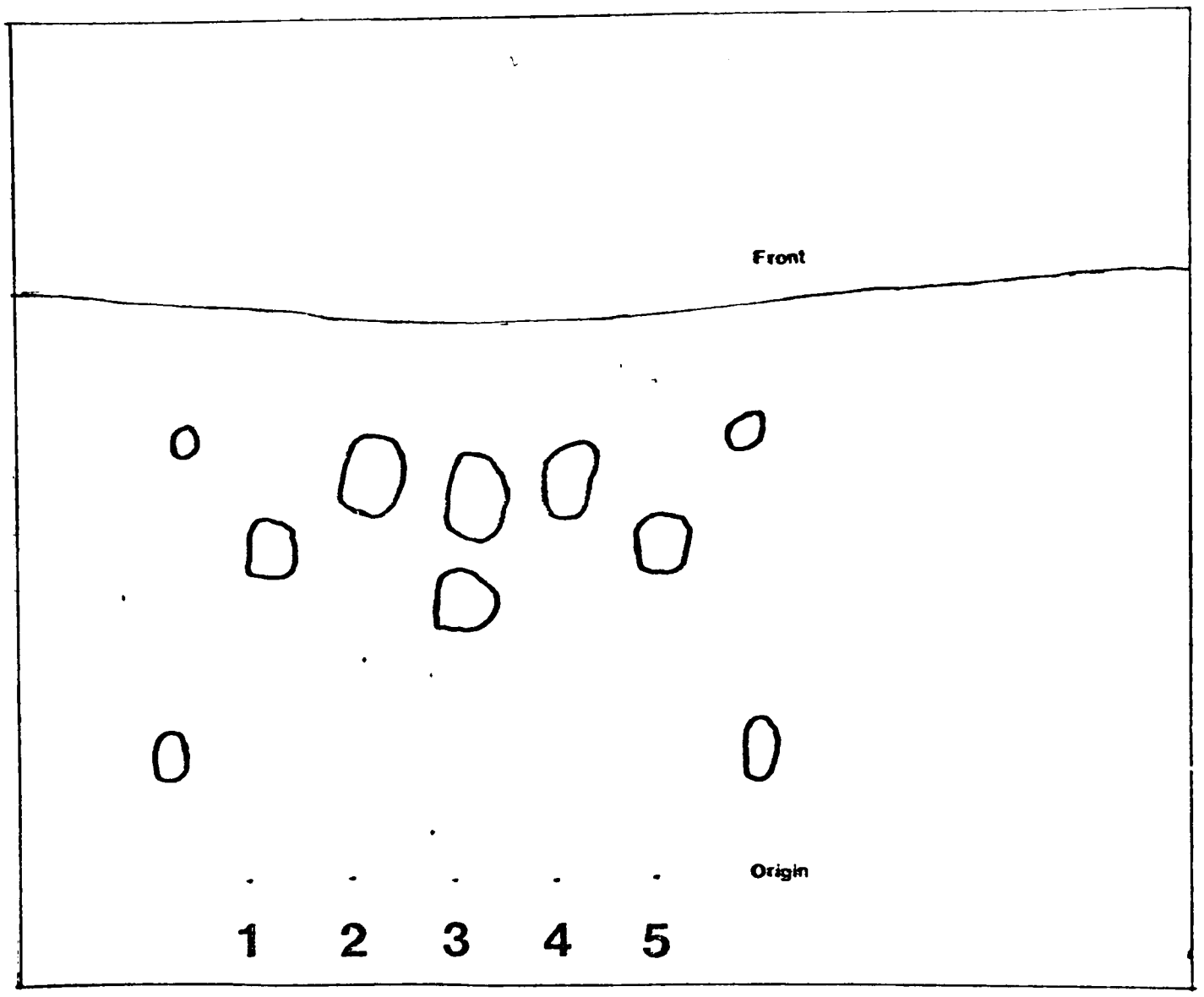


Figure 2.7 Tracing of SG-81 chromatogram showing the effect of lipid on R_f: 1,5, insulin standards; 2.4, di-Boc-insulin; 3, insulin and di-Boc-insulin with 200 µg of nonanoic acid and 1,2-dipalmitoylglycerol.

2Biii Determination of Amino Groups

Of the various methods available for end-group analysis the method of Gray (1967) as modified by Woods and Wang (1967) was used. 0.5 mg of insulin or its derivative was dissolved in 2 to 3 drops of 5% aqueous sodium bicarbonate and 1 to 2 drops of 20 mg/ml dimethylaminonaphthalenesulphonyl chloride in acetone was added. This was mixed well and incubated at 37°C for between 1 to 3 hours. If the insulin derivative was insoluble in this mixture it was finely ground with a spatula tip and shaken at intervals during the incubation to maintain a fine suspension. The insulin was precipitated by adding about 20 to 30 drops of acetone, centrifuged and then washed once with acetone and once with ether and then carefully blown dry in a stream of nitrogen. Two drops of 6M hydrochloric acid were then added, the tube was sealed in a flame and the mixture was hydrolysed overnight at 108°C. The next morning the tube was opened and the acid was dried off in a desiccator attached to a vacuum pump. About 10 µl of water-saturated ethyl acetate was used to dissolve the hydrolysed sample and this was spotted in small aliquots at the origin (1 cm from each edge) on a double sided polyamide layer plate cut to 5 x 5 cm. (Chen-Ching Trading Ltd., from BDH Chemicals Ltd.). A standard mixture was spotted on the back of the plate. The plate was developed in two dimensions, first using 1.5% formic acid, drying well and then using toluene:acetic acid (9:1, v/v). The spots were observed under a u.v. lamp. A plate showing the Dns-amino acids of interest is shown in Fig.2.8.

Using a method identical to that used for the previous two methods, the sensitivity of this technique with mixtures of di-acyl-insulin and insulin was measured. A 1% contamination was

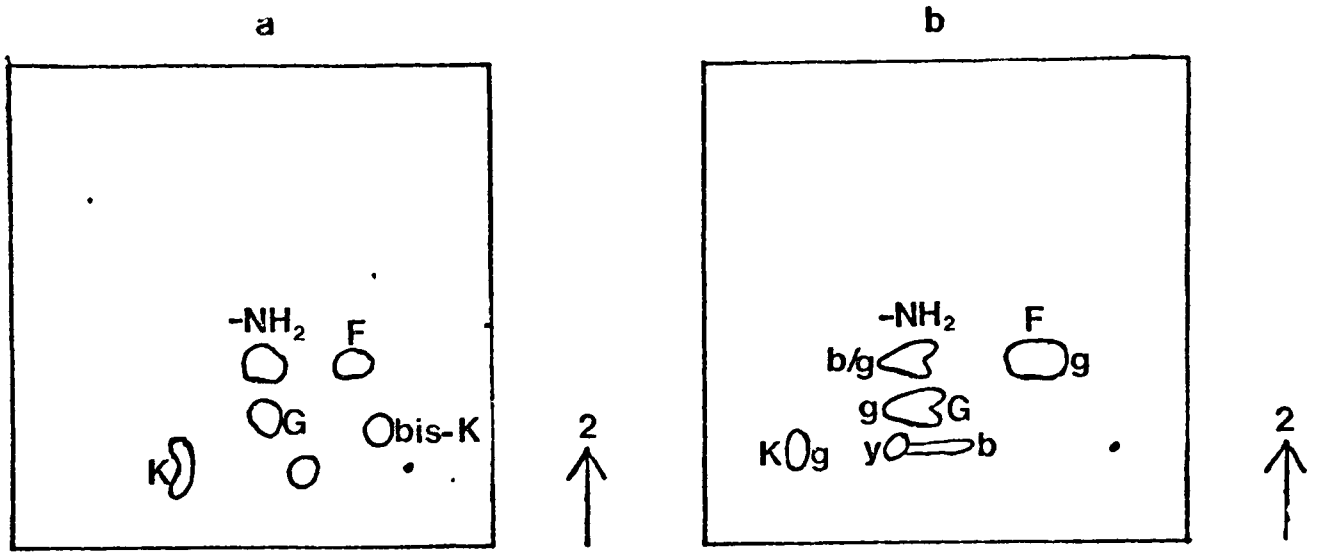


Figure 2.8 Two-dimensional chromatograms of various Dns-amino acids on polyamide layers: a, standard mixture; b, insulin. F, Dns-phenylalanine; K, Dns-lysine, G, Dns-glycine; $-NH_2$, Dns-amide; g, green fluorescence; b, blue fluorescence; y, yellow fluorescence; b/g, blue/green fluorescence.

not detected, but 3% was, the diagnostic spot being that of Dns-phenylalanine.

Similarly, an experiment was performed to see if fat had any effect on the method, no apparent difference in the intensities of the Dns-amino-acid spots and the ratio of the spot intensities was detected when 1,2-dipalmitoylglycerol and nonanoic acid were added, compared with uncontaminated samples.

End-group analysis by deamination of the protein by nitrous acid, and difference amino-acid analysis (Levy & Paselk, 1973) was attempted but was unsuccessful due mainly to the insolubility, in acid solution, of most of the acyl-insulin derivatives used.

2C Methods for Analysis of Lipids

2Ci Thin Layer Chromatography

Most routine analysis of lipids was performed by thin layer-chromatography. A wide variety of methods are available to separate lipid classes, and to distinguish between acyl groups of different chain length and degree of saturation within the same lipid class (Skipski et al, 1969; Marinetti, 1964; Morris, 1964; Kaufmann & Makus, 1960 and Kaufmann et al, 1961).

For routine analysis of reactions involving glycerol-esters and fatty acids the well tried system using silica-gel coated plates (Machery-Nagel HR, Camlab, Nuffield Road, Cambridge), was used (Skipski et al, 1969). Depending on the lipid to be analysed, between 50 and 250 µg of it was applied, in 2 to 4 µl of chloroform or chloroform:ether (1:1, v/v), to a point about 6 to 8 mm from the edge of a 5 x 5 cm plate. The plates were supported in a metal holder and were developed by ascending chromatography in a small glass vessel (6 x 6 x 10 cm), which had been pre-equilibrated with eluant and which

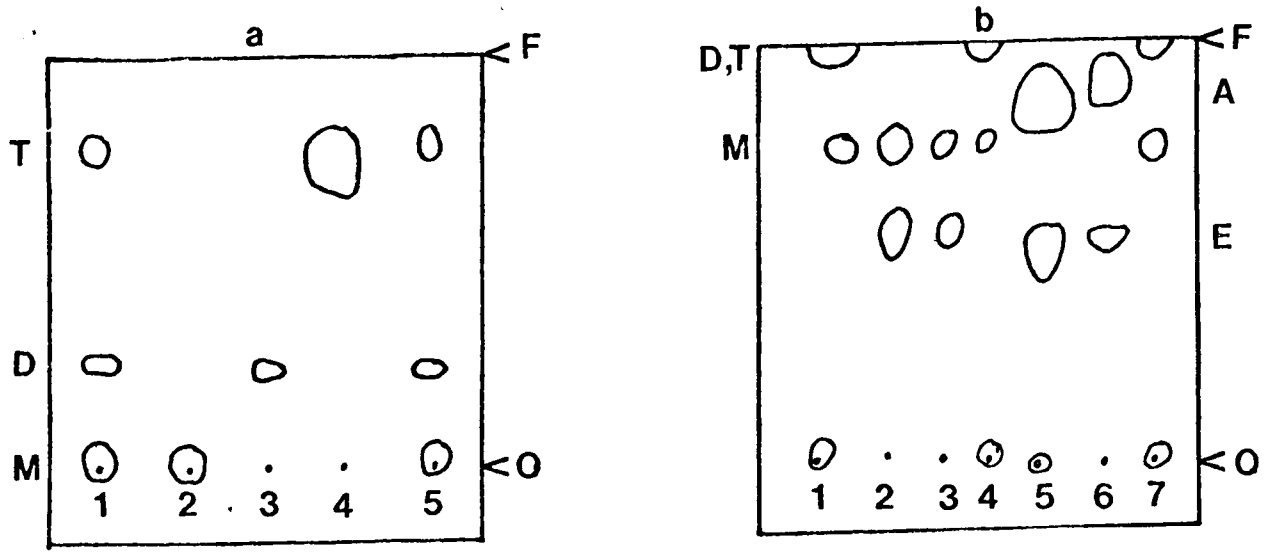


Figure 2.9 Tracings of silica-gel chromatograms: a, ether solvent, b, pyridine solvent. For both a and b: T, triacylglycerol; D, diacylglycerol; M, monoacylglycerol; F, solvent front and O, origin; for a: 1,5, mixture of mono-, di- and tri- acylglycerols; 2, monoacylglycerol; 3, diacylglycerol; 4,triacylglycerol; for b: 1,4,7, mixture of mon-, di- and tri- acylglycerols; 2,3, mixture of 1,3-di-palmitoyl-2-azeloylglycerol and its N-hydroxysuccinimidyl ester; 5,6, mixture of nonanoic acid and its N-hydoxysuccinimidyl ester; A, acids; E, esters.

was fitted with a lid. The eluant used was diethyl ether:petroleum ether (boiling range 60–80°C):glacial acetic acid, (10:90:1, by vol.) and it is referred to as 'the ether solvent' (see Fig.2.9a).

This system was not satisfactory for the separation of 1,3-O-dipalmitoyl-2-O-azeloylglycerol from its hydroxysuccinimidyl ester, as both compounds had the same low R_f in this system. As these compounds are probably more nearly related in charge and hydrophobic properties to glycerophospholipids, methods used in the chromatographic analysis of these compounds were considered (Marinetti, 1964).

A series of solvents containing heptane and di-isobutylketone were tried, with SG-81 paper or silica-gel plates as the solid support. Using a range of mixtures of these solvents, the 1,3-O-dipalmitoyl-2-O-azeloylglycerol and its ester either had a very high or low R_f often with streaking. The addition of 1% acetic acid to these solvents reduced the streaking but did not affect the R_f values. Two other solvents, 1) di-isobutylketone:glacial acetic acid:water (40:20:3, by vol.) 2) di-isobutylketone:pyridine:water (54:40:6, by vol.) (referred to as 'the pyridine solvent') were tried on both SG-81 and silica gel plates. The acid-containing solvent, 1), caused streaking but the pyridine-containing solvent, 2), did not. Furthermore there was a good separation between 1,3-O-dipalmitoyl-2-O-azeloylglycerol (R_f 0.8) and its N-hydroxysuccinimidyl ester (R_f 0.5). Potential impurities from the conversion of the acid to its ester are N-hydroxysuccinimide, the carbodiimide and the urea derived from it. The former two were shown to have R_f values of 0.0 in this system and to be very weakly staining. The urea had an R_f of 0.8 and was strongly staining (see Fig.2.9b).

Amido black (0.1%) in 7% acetic acid was the most generally used stain for lipids on silica gel plates. The plates were dipped in the stain for 10 to 20 seconds and then excess stain washed off in running tap water for 10 to 20 minutes.

2,7-dichlorofluorescein (0.2%) in 95% ethanol, although unsatisfactory for silica-gel plates, was the preferred stain for SG-81 papers. The papers were dipped (or occasionally sprayed) with the stain, air-dried for a few minutes and the spots observed under u.v. light. The neutral lipid spots were yellow/orange.

The short chain fatty acids and their N-hydroxysuccinimidyl esters did not stain readily with either of these reagents. Bromothymol blue (0.4% in 10mM NaOH) was used, acting as an acid/base indicator more than an adsorption stain. The acids appeared as yellow spots, and the esters as dark blue spots, on a pale blue background.

Phosphomolybdic acid was tried as a general staining reagent but it was found to be less effective than those used.

2Cii Gas-chromatography coupled to Mass-spectrometry

This technique was used to answer some questions that were raised during some unsuccessful attempts to synthesise the triacylglycerol active ester, as described in chapter 3, section Aii. Firstly, what was the chemical identity of the products of periodate oxidation of oleic acid? And secondly, what was the cause of resistance to oxidation of the oleic acid by periodate?

All triacylglycerols were hydrolysed to glycerol and free acid, and the acids were analysed as their methyl esters (Ackmann, 1969 and McClosky, 1969).

Oleoyl chloride (40 ul) was treated with 60 ul of quinoline and

40 ul of methanol and left for 1 hour at 37°C. The reaction mixture was dissolved in 1 ml of 1M sulphuric acid and extracted with 1 ml of chloroform. The chloroform extract was reduced to about 50-100 microlitres by rotary evaporation and used for gas-chromatography coupled to mass-spectrometry.

Triacylglycerols were hydrolysed before methylation. 1 mg of lipid was dissolved in 2 ml of 2M potassium hydroxide, in 50% aqueous ethanol, sealed in a tube and incubated at 108°C for 1 hour. 1 ml of water was added and the unsaponified lipid was removed by extracting twice with 3 ml of petroleum ether (60-80°C boiling fraction). The aqueous fraction was acidified with sulphuric acid to pH 1.5 and extracted 3 times with 3 ml of petroleum ether. The collected ether fractions were pooled and washed 3 or 4 times with water until neutral, and then dried, over anhydrous magnesium sulphate, overnight.

The dry ether fractions were then filtered, dried by rotary-evaporation and then methylated by the addition of 1 ml of 5M methanolic hydrogen chloride for 16 hours at room temperature. The methyl esters were rotary-evaporated to dryness and stored at 4°C under nitrogen in a sealed flask until used.

A minimum volume of chloroform or dichloromethane was used to dissolve the sample for injection into the g.l.c. inlet and the volume injected adjusted to give maximum sensitivity without overloading. The instrument used is the prototype version of the Micro mass 30 mass-spectrometer (VG-Organic Ltd., Altrincham, WA14 5TL, U.K.). It is a 90°, 30 cm radius magnetic-sector instrument which has a slit height of 5 mm which gives high transmission. Its mass range extends to 1500 at the maximum accelerating voltage of 5 kV. It was operated at low resolution for gas-chromatography. The dual electron ionization/chemical ionization source is fitted

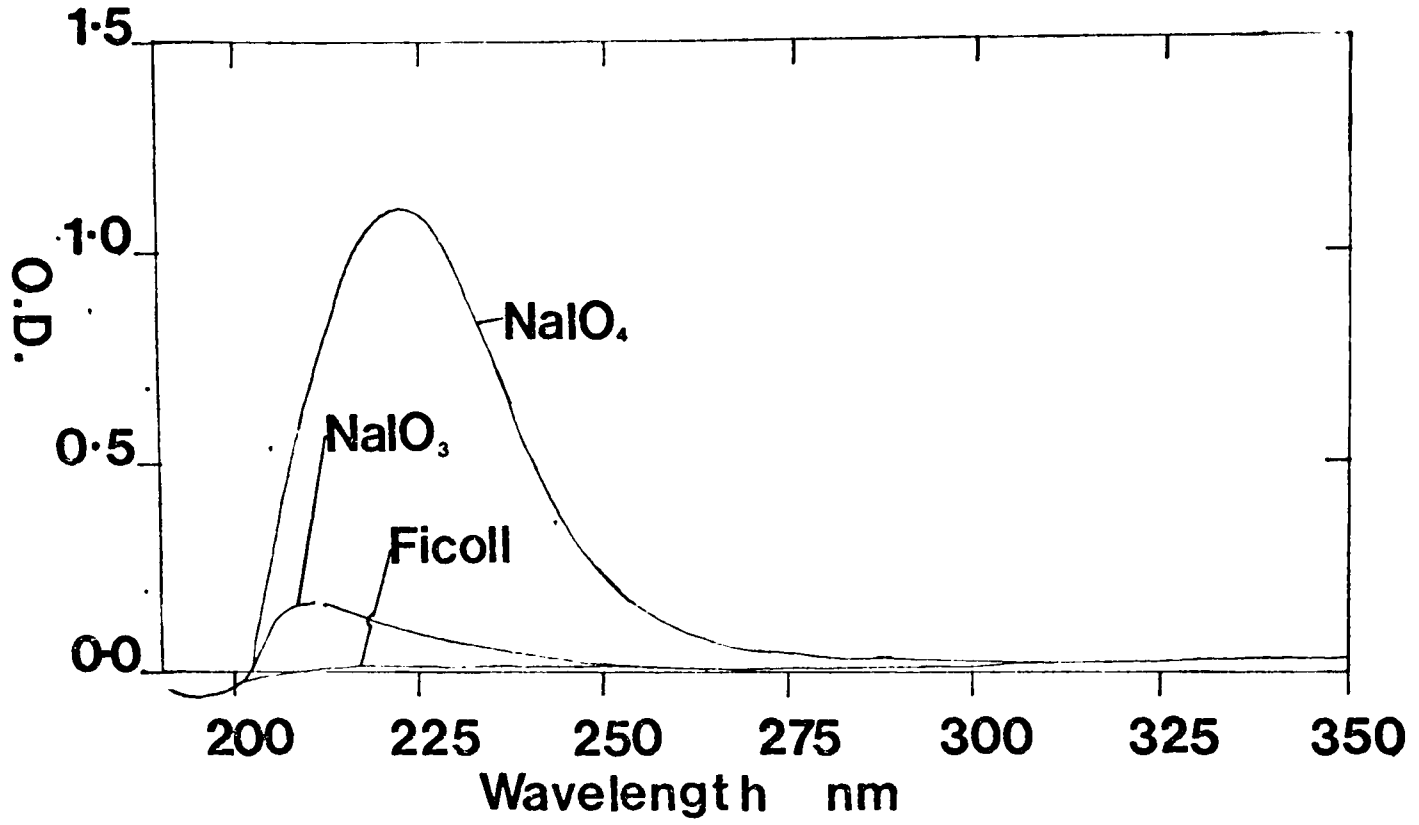


Figure 2.10 Spectra of sodium periodate and sodium iodate, both at 100 μM and Ficoll-400 at 1 mM.

with a 15 cm diffusion pump, while the analyser is differentially pumped by a 10 cm pump. This combination permits a gas flow of at least 15 atm. ml/min helium to enter the source without any form of molecular separator, before significant degradation of resolution begins. The short interface tube is glass-lined throughout its length. With 2 mm i.d. packed columns a simple T-splitter was used to send 5 ml of gas per minute to the source. For chemical ionization, a narrower source slit is moved into position with a micrometer. The reagent gas (isobutane) is led through a capillary restriction to enter the block co-axial with the probe, the tip of which is inserted into its aperture in the block during chemical ionization working also.

2D Methods for Carbohydrate Analysis

2Di Periodate Consumption

The progress of the oxidation of carbohydrates by periodate can be followed by various titrimetric and chemical methods (Guthrie, 1962), but it is most easily followed by measuring the change in optical density at 222.5 nm (Guthrie, 1962; Aspinall & Ferrier, 1957 and Dixon & Lipkin, 1954). The extinction coefficient of periodate at this wavelength is about $10^4 M^{-1} cm^{-1}$ over the pH range 3 to 6 at 25°C (Crouthamel et al, 1951). The usual concentration of periodate for the oxidation of carbohydrates is about 20mM in pH 5.0 phosphate buffer, so a dilution of this solution by 200 times would give a solution of 100 μm which has an optical density of about 1 at 222.5 nm in a 10 cm light path. Iodate at the same concentration had an optical density of about 0.1 and Ficoll 400 had an optical density of about 0.05 at a concentration of 1mM (see Fig.2.10).

In practice, a 20 μl sample was taken from a reaction mixture,

diluted into 4 ml of water and its optical density was measured over the range from 200 to 300 nm. Water was in a matched cell in the reference sample position of the spectrophotometer. One hundred per cent reduction of the periodate was considered to have occurred when the optical density fell to about 0.15 (the combined O.D. of the resulting iodate and Ficoll in the sample).

2Dii Colorimetric Determination of Aldehyde Groups

In order to measure the amount of free aldehyde groups after periodate oxidation of a sample of Ficoll, the colorimetric method involving the formation of a coloured hydrazone by the reaction of the carbonyl compound with 2,4-dinitrophenylhydrazine (Critchfield, 1963 and see Fig.2.11) was used. Carbonyl-free methanol was prepared by refluxing 1 l of A.R. grade methanol with 3 g of 2,4-dinitrophenylhydrazine and 1 ml of 12M hydrochloric acid for 3 hours and then the fraction boiling at 65°C was collected. The 2,4-dinitrophenylhydrazine reagent was 1 mg/ml of the hydrazine in carbonyl free methanol:8% aqueous hydrochloric acid (1:1, v/v), it was stored in a stoppered flask at 4°C. The pyridine stabilizer was A.R. grade pyridine:water, (80:20, v/v). Potassium hydroxide solution, 33%, in carbonyl free methanol was also prepared.

An aliquot of the reaction mixture for analysis, containing about 0.5 micromoles of carbonyl groups, was made up to 1 ml with carbonyl free methanol. It must not contain more than 0.1 ml of water, or phase separation occurs in the final assay mixture which interferes with the spectrophotometric measurements. To this, 1 ml of the dinitrophenylhydrazine reagent was added, mixed thoroughly and incubated at room temperature for 30 minutes. Five ml of pyridine stabilizer was added, followed by 1 ml of methanolic

potassium hydroxide. The optical density of this solution at 480 nm was measured after a further 10 minutes. Appropriate blank assays have to be performed and a standard curve using A.R. acetone diluted into carbonyl-free methanol was constructed.

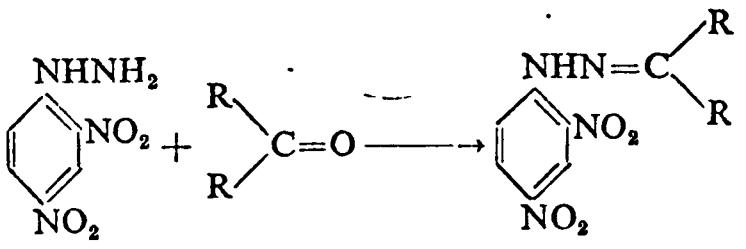
2E Biological Assays for Insulin

2Ei Introduction

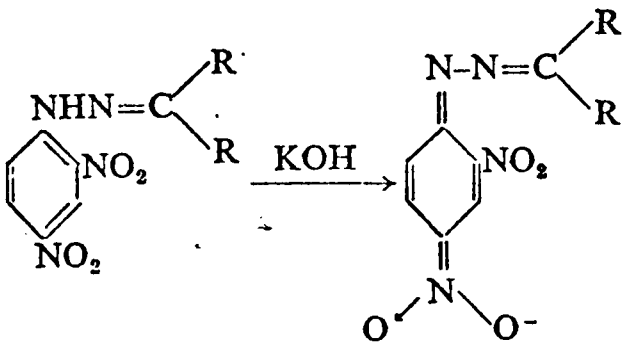
In order to assay for prolongation of insulin activity, none of the in vitro methods described in chapter 1 section Eii are suitable. One of the symptoms of diabetes is hyperglycaemia, thus the hypoglycaemic activity of the insulin derivatives under test is one of the best assays. Both the U.S. and British Pharmacopoeias describe assays involving the measurement of insulin-induced hypoglycaemia. (U.S. Pharmacopoeia, 1974 and British Pharmacopoeia, 1973). A better method than either of these methods is the constant-infusion technique described by Sonksen and co-workers (Sonksen et al, 1973; Jones et al, 1976 and Ellis et al, 1977). A good experimental design for the assay for the potency and prolongation of activity of modified insulins involves a four point cross-over assay with at least five animals in each group, analysing the results by a point-by-point statistical comparison at various selected time intervals. (Finney, 1955; Lacey, 1941; British Pharmacopoeia, 1973; and the U.S. Pharmacopoeia, 1974).

For the assays described in this thesis, the following general experimental design was followed. New Zealand White rabbits were used (weights between 3 and 5 kg). Bolus injections of insulin were performed intravenously, injecting at a rate of 1 to 2 ml/min. Although many workers use subcutaneous injections, the animals are more sensitive to intravenous administration (Guerra & Kitabchi, 1970). Intravenous administration of the drug helps to separate

2,4-dinitrophenylhydrazine is the most commonly used reagent for determining carbonyl compounds and the reaction described here is based on the reaction of the carbonyl compound to form the corresponding hydrazone:



Potassium hydroxide is then added to produce a wine-red color, presumably due to a resonating quinoidal ion:



The color is measured spectrophotometrically at 480 mμ.

Figure 2.11 Method for the determination of aldehyde groups, from Critchfield (1963).

effects due to a reduced rate of degradation of the drug from those due to a reduced rate of mobilization of the drug. The assays were intended as screening tests for the derivatives made. The significance proposed for the results was solely a means of identifying derivatives worthy of a more detailed study. This is true of the assays for prolonged activity, as well as those for activity by peroral administration. As facilities were limited, each derivative was tested on only three to eight animals. For the assays by intravenous administration controls were not performed equally with every test assay. The same, small group of animals was used. Regular, random retesting of the animals with blank controls and standard insulin preparations was performed in order to accumulate a body of control and standard data. Within the limits of the size of the overall testing programme there was no statistically significant change in the animals' responses to standard insulin preparations with change in weight, age, or time of year.

The effect of various factors on blood glucose levels was considered. Heparin was administered intravenously in order to prevent clotting of the blood samples. One effect of heparin is to stimulate serum-lipoprotein-lipase activity, (Cherkes & Gordon, 1959 and Elkeles, 1974) this could depress blood glucose by the release of fatty acids. Similarly heparin promotes the release of 'insulin-like activity' from blood serum (Gunderson & Lin, 1965). These effects must have been slight or had a net zero effect as blank controls with or without heparin showed no significant differences. Local anaesthesia with xylocaine was used to reduce trauma during blood sampling, and Tjalve et al (1974) have shown that procaine promotes insulin secretion from the pancreas. However the maximum concentration from local application is unlikely to be more than 1%

of the concentration indicated by Tjalve's studies to be maximally effective. Again controls showed no apparent effect. Aqueous dimethylsulphoxide was the usual solvent for the insulins for injection. Dimethylsulphoxide has a wide range of pharmacological effects, especially increasing membrane permeability to low molecular weight compounds (Wood, 1975). The maximum dose of dimethylsulphoxide given as a vehicle is about 0.1 g/kg and the more usual level was about 0.03 g/kg of body weight or less. This is a low dose of dimethylsulphoxide even for intravenous administration and controls showed that there was no significant effect on blood glucose levels alone, nor did it potentiate the effects of insulin at these doses. The intravenous infusion of lipids causes a depression of blood glucose (Guisard & Debry, 1972 and Carrol & Nestell, 1972) but only at levels of about 200 mg/kg. Traces of fat in the insulin derivatives are unlikely to exceed tenths of a milligramme per dose, and so would be unlikely to cause hypoglycaemia by this mechanism. Trauma and exercise cause hyperglycaemia in rabbits (Nijjar & Perry, 1971 and Thorp, 1944). Care was taken to ensure the minimum pain whilst taking blood samples and making injections (hence the use of xylocaine local anaesthesia), and throughout the experiments the animals were kept in a restraint box (similar to that described in Nash et al (1966)). After the first few experiments the animals became accustomed to the procedure and rarely became agitated. Fasting of the animals was not deliberately performed. The animals would be fed on the day prior to the experiment, and normally, having consumed that day's food before 8 p.m. would then not be fed the next morning before the experiment, nor during it. To have fasted each animal for one day before the experiment would have led to them being fasted for three days when

they were used on consecutive days for cross-over purposes. Parker and Yamamoto (1976), have shown that there is little difference between short and long fasts on the glucose tolerance tests in man.

I considered that a normally fed animal would be a better model for these studies than a fasted animal, and in fact this procedure is recommended by the U.S. Pharmacopoeia.

The animals were maintained in metal cages in a temperature- and humidity-controlled room. They were fed on a standard laboratory diet (Labsure diet R14, Rank-Hovis-Mcdougall, Poole, England) 240 g per day and water as required. The health of the colony was constantly monitored, any cases of 'snuffles' were treated with Terramycin, 200 mg for three days. Regular dips in Tetmosol (I.C.I. Ltd.) were performed to combat mite infestation. The animals were first used at age 6 to 9 months when they weighed about 3 kg, the stable adult weight was normally 4.5 to 5.0 kg. Out of the colony of 11, two rabbits died during the experimental series. Both deaths were found on post-mortem examination to have been caused by pneumonia.

2Eii Assays by Intravenous Administration

Blood samples were taken by venepuncture (using a 23 or 25 gauge needle) and diluted into uranyl acetate (for manual analysis) or collected in tubes containing fluoride (Teklab Ltd.) (for auto-analysis). Friction or radiant heat from an electric light bulb was used to promote blood flow. Very rarely, xylene was used. Bacteriostasis was ensured by swabbing with 70% ethanol or isopropanol. The ears were shaved and Vaseline or Hemo-Lube (Merck and Dade, Berne, Switzerland) applied to assist the flow of blood and inhibit clotting. The posterior peripheral ear vein was used (Gay, 1965 and Hume, 1972). Half an

hour prior to the start of an experiment Xylotox-Extra anaesthetic cream (raspberry flavour, Willows-Francis, Westhoughton, Bolton, Yorks.) was applied, and it was reapplied at hourly intervals during the course of the experiment. Then an intravenous injection of heparin (Pularin injection, Evans Medical, Speke, Liverpool) at about 500 U/kg was made. One or two zero time blood samples were taken and as soon as possible the test solution was injected. Before administration, the insulin solutions were sterilized by filtration. Dimethylsulphoxide-containing solutions were sterilized using regenerated cellulose filters (No. 11607, 0.2 um pore size, Sartorius GMBh, Gottingen, West Germany) and other solutions were sterilized using cellulose nitrate filters (No. 11307, 0.2 um pore, Sartorius). If the concentration of the solution to be injected was less than 0.2 mg/ml, it was sterilized at a concentration greater than 0.2 mg/ml and diluted to the appropriate concentration using sterile diluent. Usually a volume of 0.5 ml was used and it was injected over 15 to 45 seconds. For most of these experiments blood samples were taken at 5 minute intervals up to 30 minutes, at 10 minute intervals up to 1 hour and at 20 minute intervals up to 3 hours. For some of the earlier experiments other sampling intervals than this were used.

For the experiments using the radiolabelled-insulin derivative ($N^{\alpha B1}$,$N^{\epsilon B29}$-di-[1-^{14}C]-nonanoyl-insulin), a slightly modified procedure was performed. The preparation of the animal was as above. The solution for injection contained about 2 x 10^6 c.p.m. of radioactivity, which corresponded to an insulin dose of about 20 to 25 µg/kg of body weight. Blood samples were taken at the following times post-injection: 3, 7, 10, 15, 20, 25, 35, 45 and 60 minutes. Samples were one ml and were collected into heparinised tubes, and

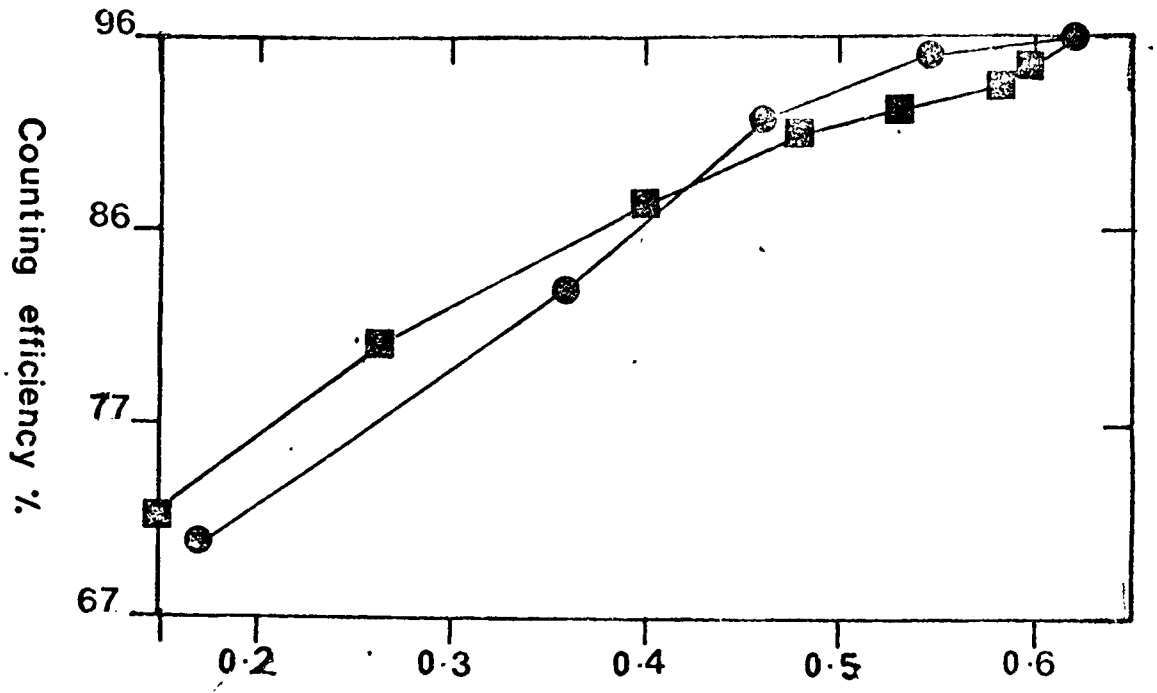


Figure 2.13 Quenching curve for the quenching of ^{14}C by chloroform (squares) and haemoglobin (circles).

were immediately centrifuged. The plasma was separated, and the erythrocytes were washed with 0.4 ml of 0.9% saline and centrifuged again. The saline washing and the plasma were combined. To this mixture, 0.5 ml of chloroform was added, it was vigorously mixed for 30 seconds and then centrifuged to separate the phases. The chloroform phase and the aqueous layer plus precipitated protein were separated, and separately added to scintillation vials containing 10 ml of scintillant. (Biofluor, New England Nuclear).

Appropriate quenching curves for chloroform or blood protein had been determined (see Fig.2.13), using the external-standard-ratio facility of the scintillation counter (Beckmann, model No. LS 230). The samples were counted, with measurement of the external standard ratio. The measured counts were then corrected for quenching. The separation of the erythrocytes is necessary as they cause massive quenching at even very low concentrations.

2Eiii Assays by Peroral Administration

The preparation of the animal was the same as for the assays by intravenous administration. The test samples were dissolved in a suitable vehicle, usually 50% tributyrylglycerol:dimethylsulphoxide by volume, then injected into a No. 6 soft gelatin capsule (gift from R. P. Scherer Ltd.) and administered in a dosing gun as described by Rudolph and Smolen (1970). This was found to be a very easy and effective technique. A blood sample was taken before oral dosing and at hourly intervals up to 6 hours (excluding the 5 hour sample). These assays were performed as paired tests. A control and experimental assay was performed on day one. On the second day a similar control and experimental assay were performed on the same pair of rabbits, but changing them around.

2Eiv Blood Glucose Determination

For the earlier insulin assays the blood glucose analysis was performed manually, later analyses were performed on a Technicon-type auto-analyser system. In both methods whole blood glucose was determined.

For the manual assays the following stock solutions were prepared. The enzyme-buffer solution contained 2.752 g disodium hydrogen phosphate dodecahydrate, 1.817 g sodium dihydrogen phosphate dihydrate, 375 U horse-radish peroxidase (Sigma London, Type II) and 2,500 U of glucose oxidase (Sigma London, Type V) made up to 250 ml with distilled water, the pH was adjusted to 6.9 ± 0.1 and two drops of chloroform added as a preservative. This solution was stable at $4^{o}C$ for up to 1 month. Ortho-dianisidine was used as chromogen. The crude dye (BDH Chemicals Ltd.) was purified by extraction with activated charcoal under reflux for 30 minutes in a 12.5% solution in acetone:water, (1:3, v/v), after filtration, the filtrate was allowed to crystallize overnight at $4^{o}C$. One gramme of recrystallized material was dried, dissolved in 20 ml of warm water, 0.85 ml of 12M hydrochloric acid was added and this was crystallized by the slow addition of 100 ml of cold acetone, stirring vigorously. The immediate crystalline precipitate of the hydrochloride was filtered, washed once with acetone, twice with ether and dried in a desiccator over potassium hydroxide pellets. A stock solution of this was made by dissolving 50 mg of the hydrochloride in 10 ml of water. When stored in the dark at $4^{o}C$ this is stable for 6 months. A glucose standard was prepared by drying A.R. grade glucose overnight in a desiccator in the presence of sodium hydroxide pellets and concentrated sulphuric acid, under vacuum. Twenty-five milligrammes of this was then dissolved in 250 ml of 1.75% perchloric acid

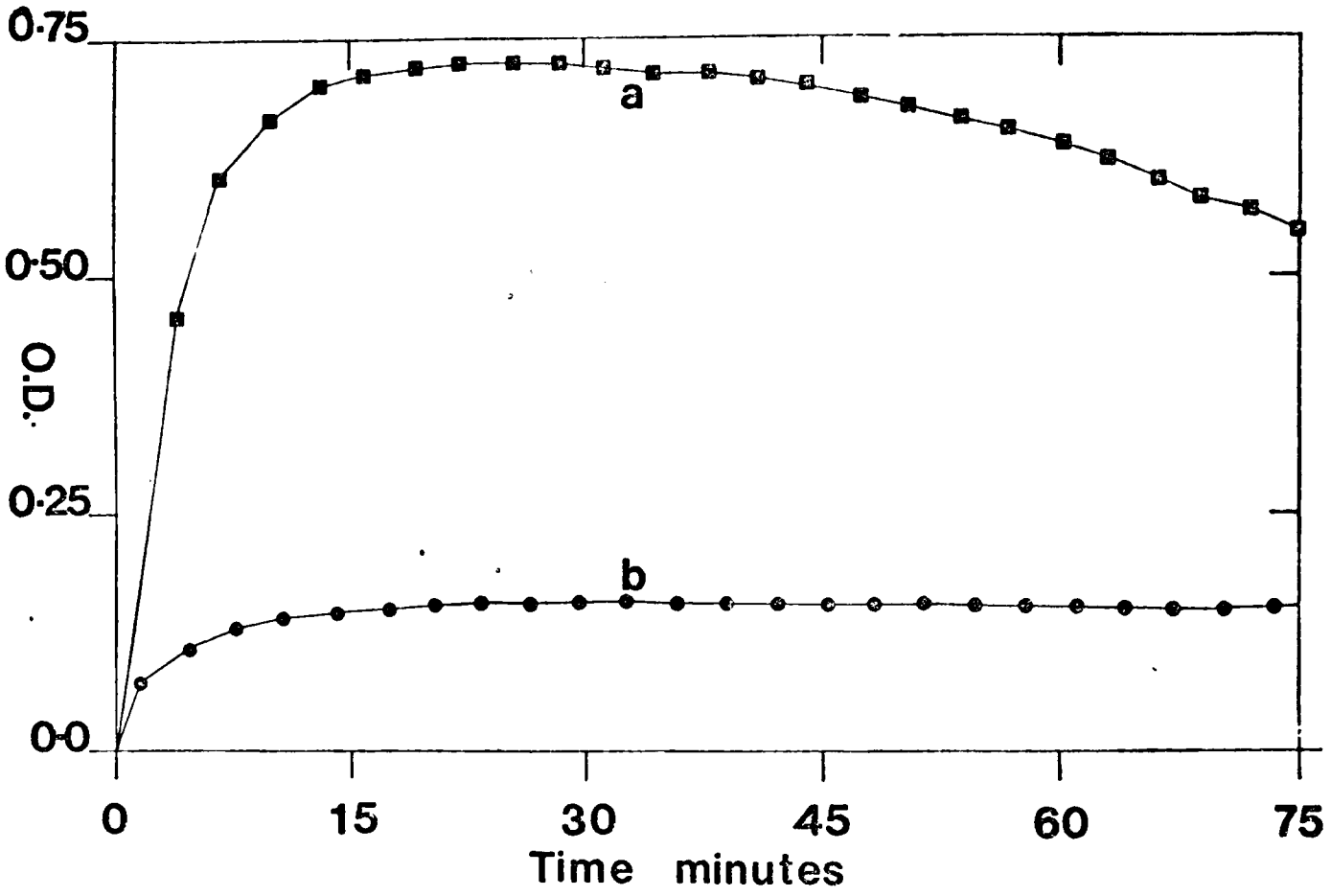


Figure 2.12 Kinetics of colour development in manual glucose analysis measuring O.D. at 450 nm; a, 2.75 mM glucose; b, 0.55 mM glucose.

solution. The working enzyme-buffer-chromogen reagent contained 0.5 ml of stock chromogen solution in 50 ml of stock enzyme buffer, and was used within 12 hours of preparation. Ten microlitre blood samples were withdrawn into plain glass capillary tubes, in duplicate, and each was immediately diluted into 90 microlitres of a uranyl acetate solution, 0.16% in 0.9% saline. All the samples were stored at 4°C until assayed together, at the end of the experiment. The diluted samples were centrifuged on a bench centrifuge, two or three samples of 20 ul were taken from the supernatant and each was added to 0.5 ml of the working reagent. After mixing well the solution was left at room temperature for 30 minutes and the optical density at 450 nm was measured. A solvent blank and a 0.55mM glucose standard (equal to 5.5mM glucose in an undiluted blood sample) was performed with each set of blood assays. Each preparation of stock solution was checked by performing a standard curve with glucose solutions of 0.137 to 1.1mM glucose.

At the outset a kinetic assay following the development of colour was performed for 0.55 and 2.75mM glucose solutions (see Fig.2.12). At room temperature it was found that the 0.55mM solution achieved 99% of its maximal O.D. within 21 minutes of mixing, and faded by less than 3% from the maximum by 75 minutes from mixing. More fading was observed with the 2.75mM standard, it remained between 98 and 100% of the maximal O.D. from 21 to 39 minutes of mixing, and faded (mainly by precipitation of chromogen) to 91% in 55 minutes. No blood glucose values greater than 0.82mM (after dilution) were observed. The absorption maximum of the o-dianisidine was very broad and any convenient wavelength between 425 and 500 nm could be monitored without loss in sensitivity of the assay.

As each blood sample was taken in duplicate (occasionally in

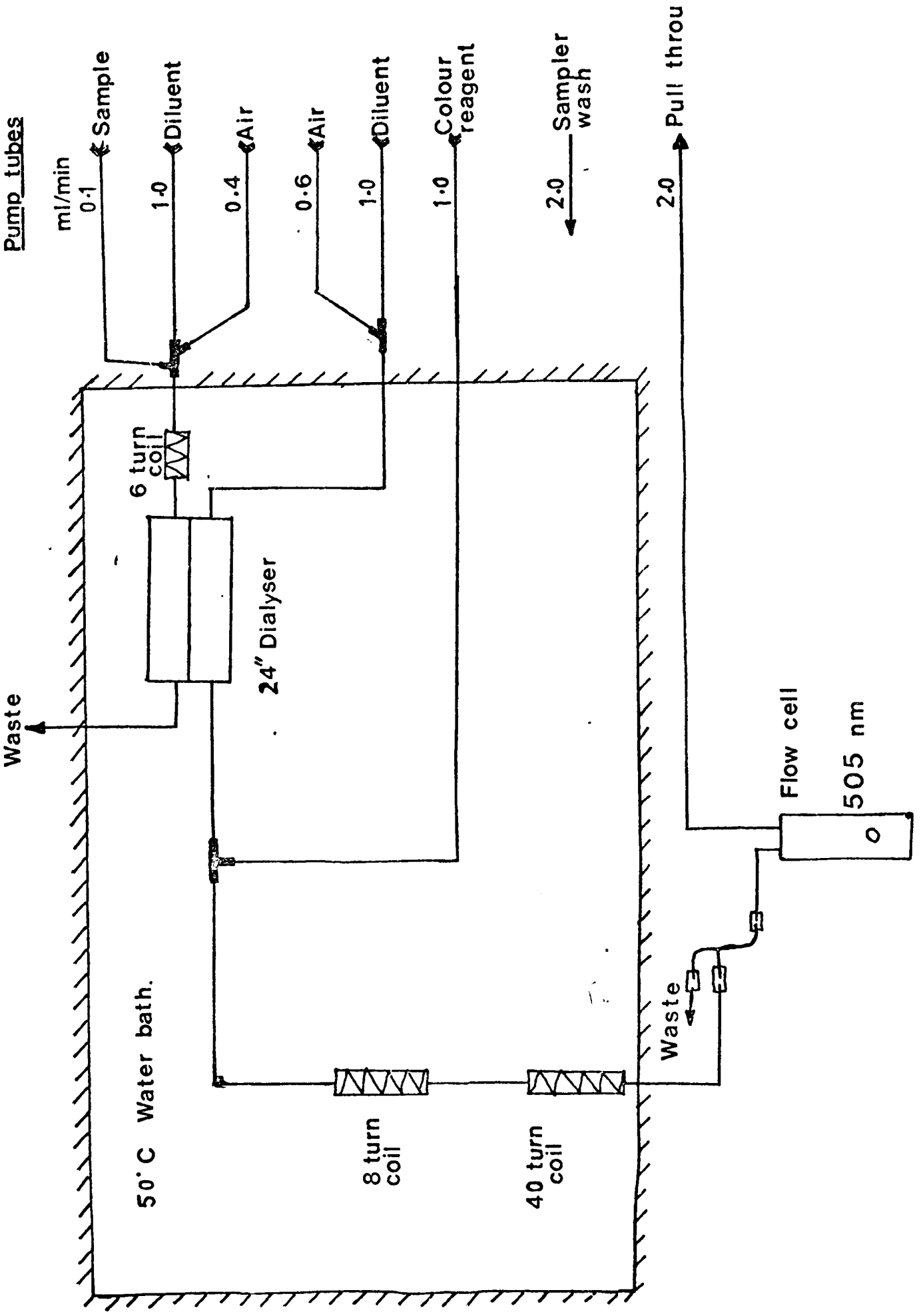


Figure 2.14 Blood-glucose auto-analyser manifold diagram.

triplicate) and each sample assayed in duplicate or triplicate, each time point was the average of four to nine assays. The standard error of the means of these replicates was usually ± 3 to 4%.

The auto-analyser method was a modification of the standard procedure, to give fairly high sensitivity with low variance. The manifold is as shown in Fig.2.14. At first the water bath was maintained at 37°C but was soon elevated to 50°C which led to an approximate doubling in sensitivity. The pump had a variable speed control and was adjusted so that a grey/grey colour coded tube, I.D. 1.3 mm, of nominal flow rate of 1 ml/min had an actual flow rate of 0.75 ml/min. To increase sensitivity higher concentrations of enzymes and chromogen (4-aminophenazone and phenol) were used than is normal (Rosevear et al, 1969). To prevent cross contamination of samples, a 1:1, sample:wash ratio was used rather than the more normal 2 or 3 to 1; and the detergent in the buffers (Triton X-100) was at a concentration of 1% (v/v) rather than the normal 0.25% (v/v) in order to reduce trailing. The tubes and dialysis membranes were changed at monthly intervals or sooner if irregularities in peak shape were seen. No pulse suppressors were used.

Stock solutions were prepared and stored at room temperature. Stock phosphate buffer consisted of 40 g of disodium hydrogen phosphate, 40 g of potassium dihydrogen phosphate and 8 g of sodium azide made up to 2 l with water. Stock saline was 18% aqueous solution. Preservative was 2% phenol in water. The working reagents were prepared, and stored at 4°C in the dark. The donor and recipient reagents contained 125 ml of stock phosphate buffer, 25 ml stock saline, 25 ml of a freshly prepared solution of 0.5% 4-aminophenazone in water and 5 ml of Triton X-100, made up to 500 ml with water and the pH was adjusted to 7.0 ± 0.1 with 3M sodium hydroxide solution.

Figure 2.15 Histological Procedures

Fix	Zenker 4d	Helly 4d	Ca formol 4d
		Postchrome 7d	Postchrome 7d
	Wash 4h	Wash 4h	Wash 24h
Dehydrate	70% EtOH 6h		12.5% gelatine 24h
	90% EtOH 20h		25% gelatine 24h
	Absolute EtOH 2,3,3h		
	Toluene 20h		
	Paraffin wax 1,1,1h		
Embed	Paraffin wax		25% gelatine-formol
Section	8–12 u	4–8 u	Tissue-Tek 6–16 u
Rehydrate	2x Xylene 2min		Water 5–10min
	2x Abs. EtOH 2min		70% EtOH
	90% EtOH dip		
	70% EtOH I_2 4min		
	70% EtOH		
	Hypo 2min		
	Water 5min		
Stain	Haematal 8 8–10min	70% EtOH dip	Sudan IV 5–10min
	Water rinse	90% EtOH dip	70% EtOH rinse
		Abs. EtOH 2min	Water rinse
		Aniline fuchsin	
		Heat 2–3min	
		Water rinse	
Differentiate	Biebrich Scarlet 20–30s	Picric acid 5–15min	Haematal 8 5–8min
	Water wash	Water wash	Water wash
Mount	70% EtOH dip		Gum Arabic
	90% EtOH dip		
	Abs. EtOH dip, 2min		
	Xylene dip, 2min		
	Canada Balsam		

The colour reagent contained 125 ml of stock phosphate buffer, 5 ml of stock phenol solution, 750 U of peroxidase (about 3 mg), 7,500 U of glucose oxidase (in about 5 ml) made up to 500 ml with water, pH 7.0 ± 0.1. The wash reagent was 1% (v/v) Triton X-100 in water. The sample rate was 8 per hour and the sample:wash ratio was 1:1 at a pumping speed of 0.75 of nominal (65 on the pump dial). Blood samples of 0.3 to 0.5 ml were taken, and 0.5 ml sample cups were used on the sampler. The sampler head was fitted with a stirrer to prevent settling of the blood samples before analysis. This method had a variance of about 1%, was sensitive down to about 0.3mM and was linear over the range 0.0 to at least 11.0mM.

2F Toxicological Assays

2Fi Post-mortem Examination

Two animals died during the course of these studies. A further two were killed immediately prior to a planned post-mortem examination.

The abdominal cavity was first opened and the gut, liver, spleen and inguinal lymph nodes were examined. The kidneys and adrenal glands were dissected free of fat and inspected externally and after sectioning. The thorax was then opened and the heart and lungs were examined. In two cases the aorta was dissected free from the heart to the diaphragm and cut open to examine the interior surface.

Tracheal swabs were taken from the animals that died spontaneously, and these were cultured and examined by the staff of the University Veterinary Laboratory, (Laboratory of Physiology, Parks Road, Oxford).

2Fii Histological Examination

During the gross-post-mortem examination on two animals, portions of tissue were removed for

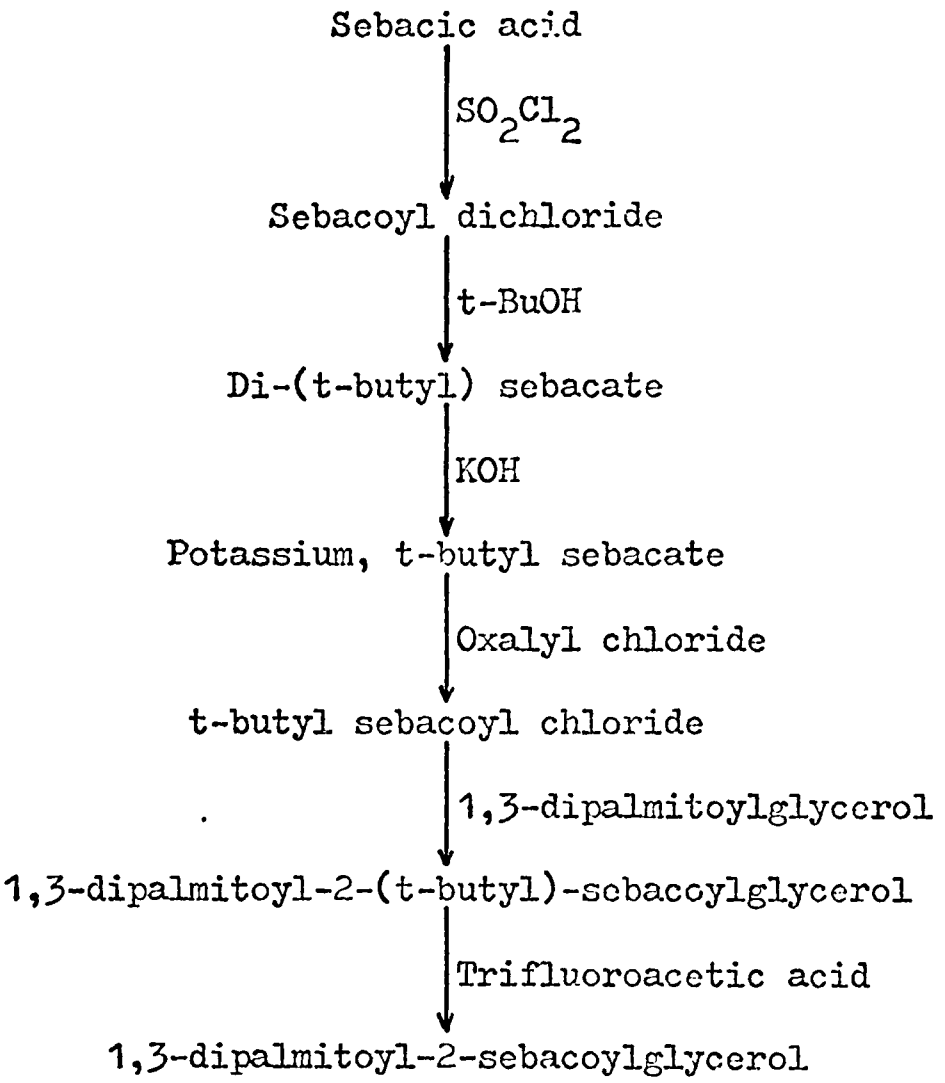


Figure 3.1 Reaction pathway for the synthesis of 1,3-O-dipalmitoyl-2-O-sebacoylglycerol.

microscopical examination. All the fixatives were freshly prepared and the glutaraldehyde fixative was kept on ice during use. For electron-microscopic examination samples smaller than a 2 mm cube were cut and fixed immediately in 2.5% glutaraldehyde in 5% sucrose and cacodylate buffer, pH 7.2. Tissue samples for light microscopic examination were usually slightly larger and were fixed in Zenker's solution, Helly's solution or calcium formol fixative. Samples from the lung, liver, kidney, heart, aorta and spleen were taken.

All the samples for light microscopy were fixed for four days with several changes of fixative. Those fixed in Zenker's or Helly's fluid were then washed in tap water and embedded in paraffin wax. Those fixed in calcium formol were post-chromed, washed in tap water and embedded in gelatine. The tissues fixed in Zenker's fluid were sectioned on a Cambridge rocker microtome at 8 to 12 microns thickness and those fixed in Helly's fluid were sectioned at 4 to 6 microns. Freeze-sectioning of the gelatine blocks was unsuccessful due to chipping of the blocks; the tissues were dissected from the gelatine, remounted in Tissue-Tek (R.A. Lamb, London), flash frozen in liquid nitrogen and freeze-sectioned on a Jung rotary microtome in a Bright cabinet at 12 to 16 microns, for the harder tissues and 6 to 8 microns for the softer ones.

The tissues for electron-microscopic examination were post-fixed in 1% osmium tetroxide, dehydrated, embedded in Araldite and stained with Reynolds lead citrate and uranyl acetate, after sectioning at 75 nm.

The tissue sections fixed in Zenker's solution were stained with Haematal 8 or a two-bath iron-haematoxylin stain, differentiating with Biebrich scarlet, and then mounted in Canada Balsam. The tissues fixed in Helly's fluid were stained with aniline-fuchsin,

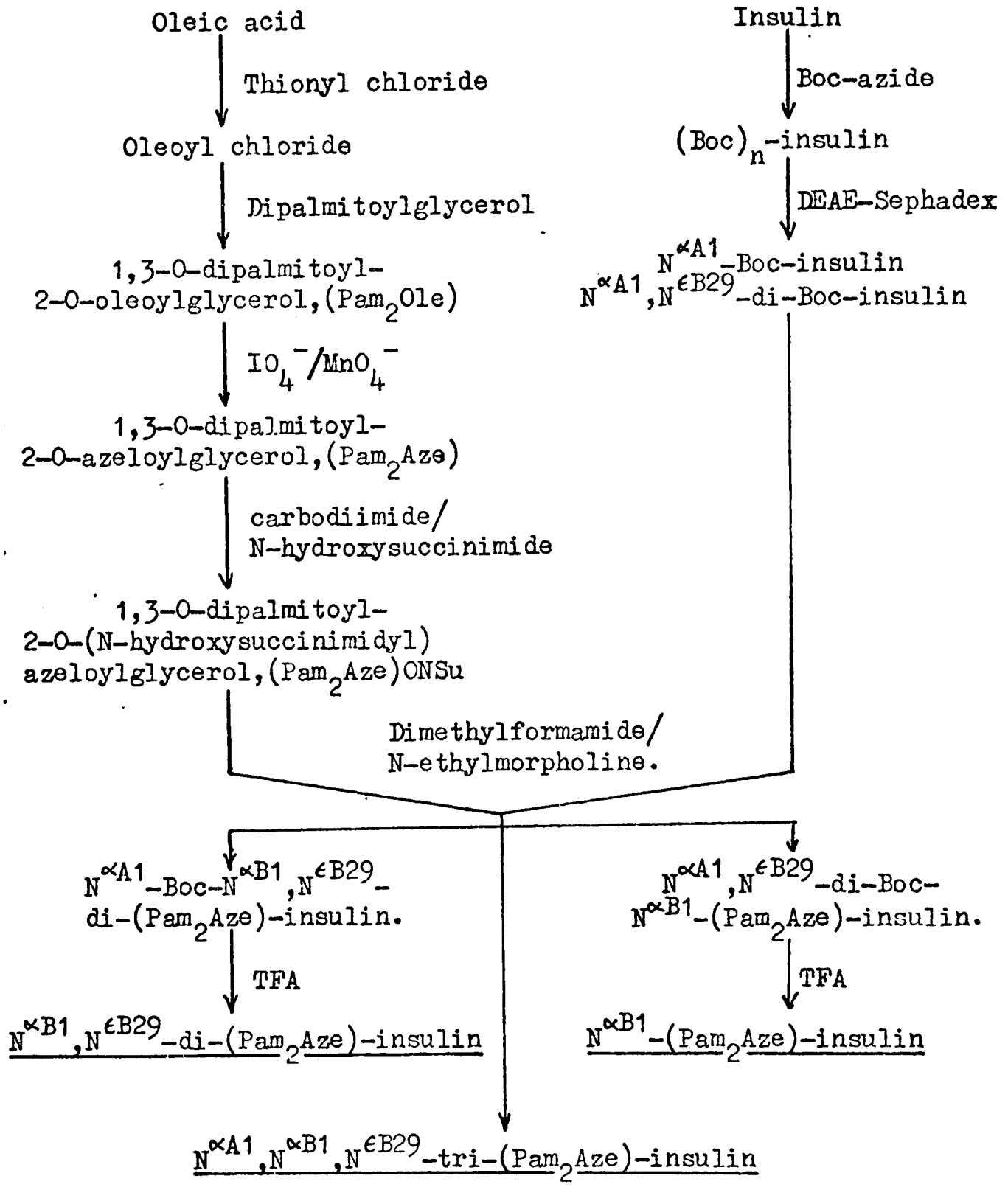


Figure 3.2 Reaction pathway for the synthesis of triacylglycerol derivatives of insulin.

differentiating with picric acid and mounted in Canada Balsam. Tissues fixed in calcium formol were stained in Sudan IV and lightly counter-stained with haematal 8 or iron-haematoxylin and mounted in acacia gum.

For further details see Fig.2.15 and Drury & Wallington, 1956 and Sumner and Sumner, 1969.

2Fiii Immuno-Assays

Blood serum from the rabbits was examined for anti-insulin antibodies by gel double diffusion. (Brown, 1967). Plates were prepared as follows, a 10 x 10 cm glass plate was degreased by washing in chloroform and ethanol, dipped in 0.5% Oxoid agar No. 1, in water and dried at 37°C. The main agarose layer was 9 ml of a 1% solution of agarose (Miles-Seravac, England) in a buffer containing 50 ml of phosphate-buffered saline (Dulbecco 'A'), 2.5 ml of 1M glycine, and 0.5 ml of 1% (w/v) thimerosal in water, pH 7.4. This solution was warmed to 37°C to melt it, poured over the level plate and left to set. Then a hexagonal pattern of holes was cut using a 2 mm diameter cutter. On one plate there were set up serum dilutions (1:1 to 1:64) against insulin (2 mg/ml); and insulin dilutions (2 mg/ml) (diluted 1:1 to 1:64) against undiluted serum. 4 µl of solution were applied to each well. The prepared plate was left at 4°C in a sealed tank containing moist paper tissue, and was inspected daily for up to 7 days. If no precipitation lines were observed after this time, the plate was washed, during 3 days, with many changes in phosphate-buffered saline, dried at 37°C and then stained with 1% Coomassie Blue in 7% acetic acid in aqueous methanol, destaining in 7% acetic acid.

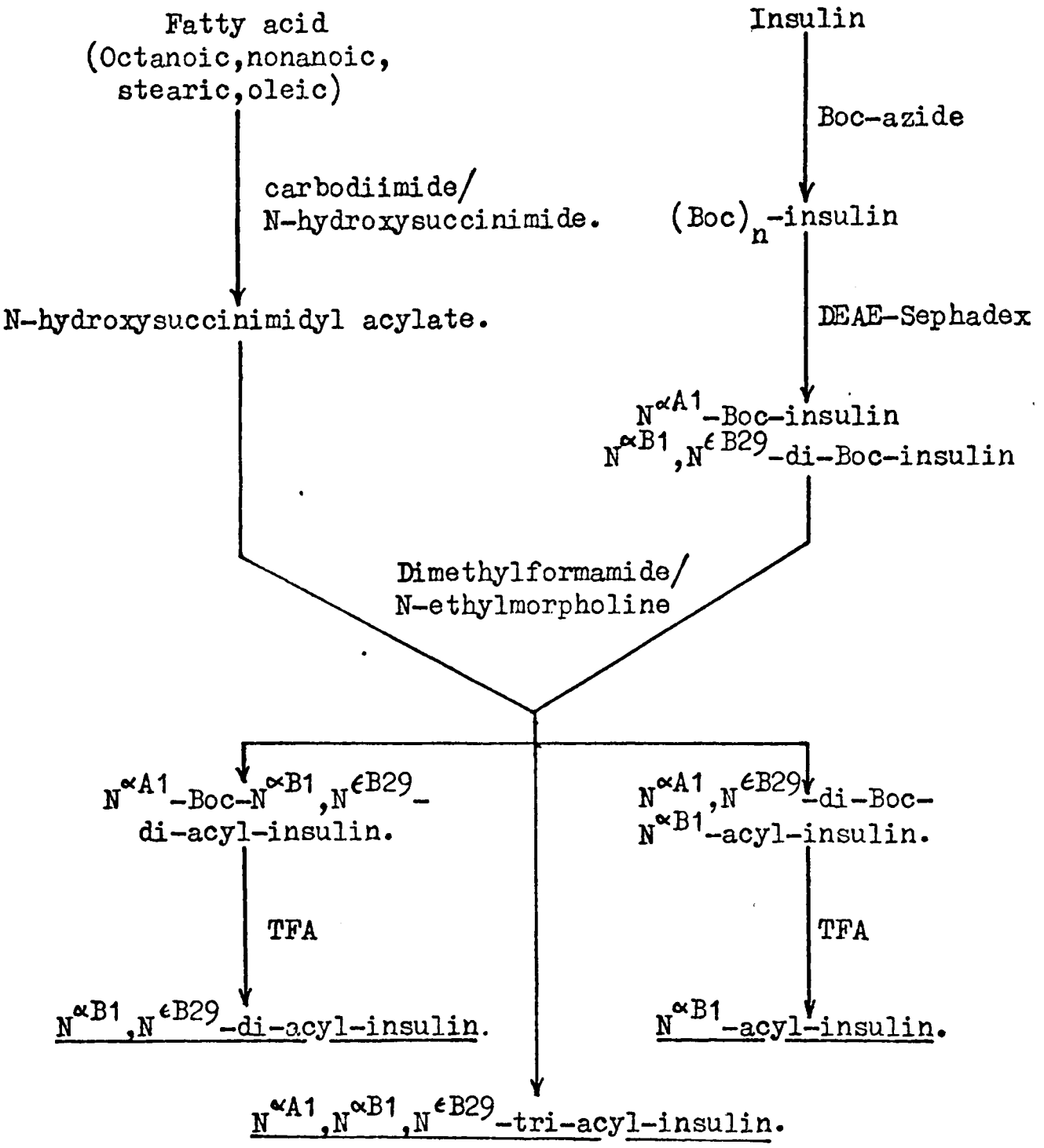


Figure 3.3 Reaction pathway for the synthesis of acyl-insulin derivatives.

Chapter 3

Organic Syntheses

3A Introduction

In order to attach a triacylglycerol molecule to insulin via an amide linkage, one of the fatty acids, attached to the glyceryl moiety, must have a second carboxylic-acid group at the end distal to the glycerol. At first, an attempt was made to use sebacic acid, by following the route shown in Fig.3.1. Using the method of Backer and Homann (1939) this route was followed as far as the synthesis of the half potassium salt, half t-butyl ester of sebacic acid. It was abandoned at this stage when a simpler and more elegant route presented itself (Fig.3.2). An acylglycerol could be synthesised containing at least one molecule of an acyl-enoic acid (Malkin & Bevan, 1957). Using the method of Rudloff (1956) the unsaturated bond can be oxidized, producing a free carboxylic acid group at the end of the now shortened acyl chain. An N-hydroxysuccinimidyl ester of this acid can be made, using the method now common in peptide chemistry (Andersen et al, 1964) and this ester can then be coupled to the partially protected insulin derivatives.

At a later stage when it was realized that the investigation of fatty acyl substituents on the insulin might be of value, N-hydroxysuccinimidyl esters of various fatty acids were made and coupled by similar methods (see Fig.3.3).

An attempt was made to synthesise a Schiffs-base derivative between the amino-groups of insulin and the aldehyde groups produced by the oxidation of Ficoll, using a method similar to that described by Pasternak et al (1976) for the synthesis of a derivative of naloxone. Periodic acid (Jackson & Hudson, 1937 and Heath & Roseman,

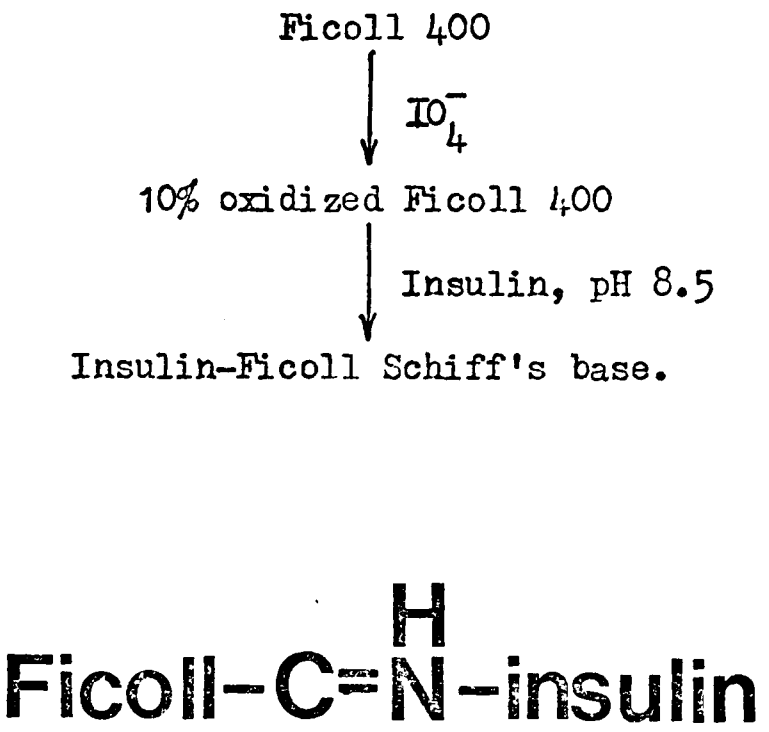


Figure 3.4 Reaction pathway for the synthesis of the Ficoll-insulin derivative.

1963) or lead tetra-acetate (Jackson, 1944 and Wolf & Weijlard, 1963) have been used to oxidize sugars, starches and celluloses but periodic acid is best in aqueous solution. Jackson (1944) and Jackson and Hudson (1938) have shown that the main product under mild conditions, from starch or cellulose, is the di-aldehyde produced by oxidation of the only glycol group in each sugar residue, chain cleavage does not occur. Under suitable conditions the product of limited oxidation of Ficoll might be expected to form a labile Schiffs base with the free amino groups of insulin (see Fig.3.4).

3B Synthesis of 1,3-O-dipalmitoyl-2-O-(N-hydroxysuccinimidyl)-azeloylglycerol

3Bi Synthesis of Oleoyl Chloride

Some of the oleoyl chloride used was purchased from Sigma London Co., however most of it was synthesised using a method similar to that described by Allen et al (1963) and Bauer (1946).

7.6 g of oxalyl chloride dissolved in 12.5 ml of toluene was poured into a 100 ml single-necked round-bottomed flask and a long column packed with glass wool was attached. A separating funnel with a side-arm by-passing the valve was attached to the top of this packed column and was filled with 14.1 g of oleic acid dissolved in 12.5 ml of toluene. The top was stoppered with a drying tube containing sodium hydroxide pellets. The flask was heated until the toluene and oxalyl chloride mixture was gently refluxing up the column, and then the oleic acid and toluene mixture was allowed to drip through the column at a rate of 0.3 ml/min. The mixture was refluxed for a further hour. The separating funnel was removed and a condenser and side-arm fitted, and the toluene was distilled off under water-pump vacuum at about 25°C. The condenser was removed and

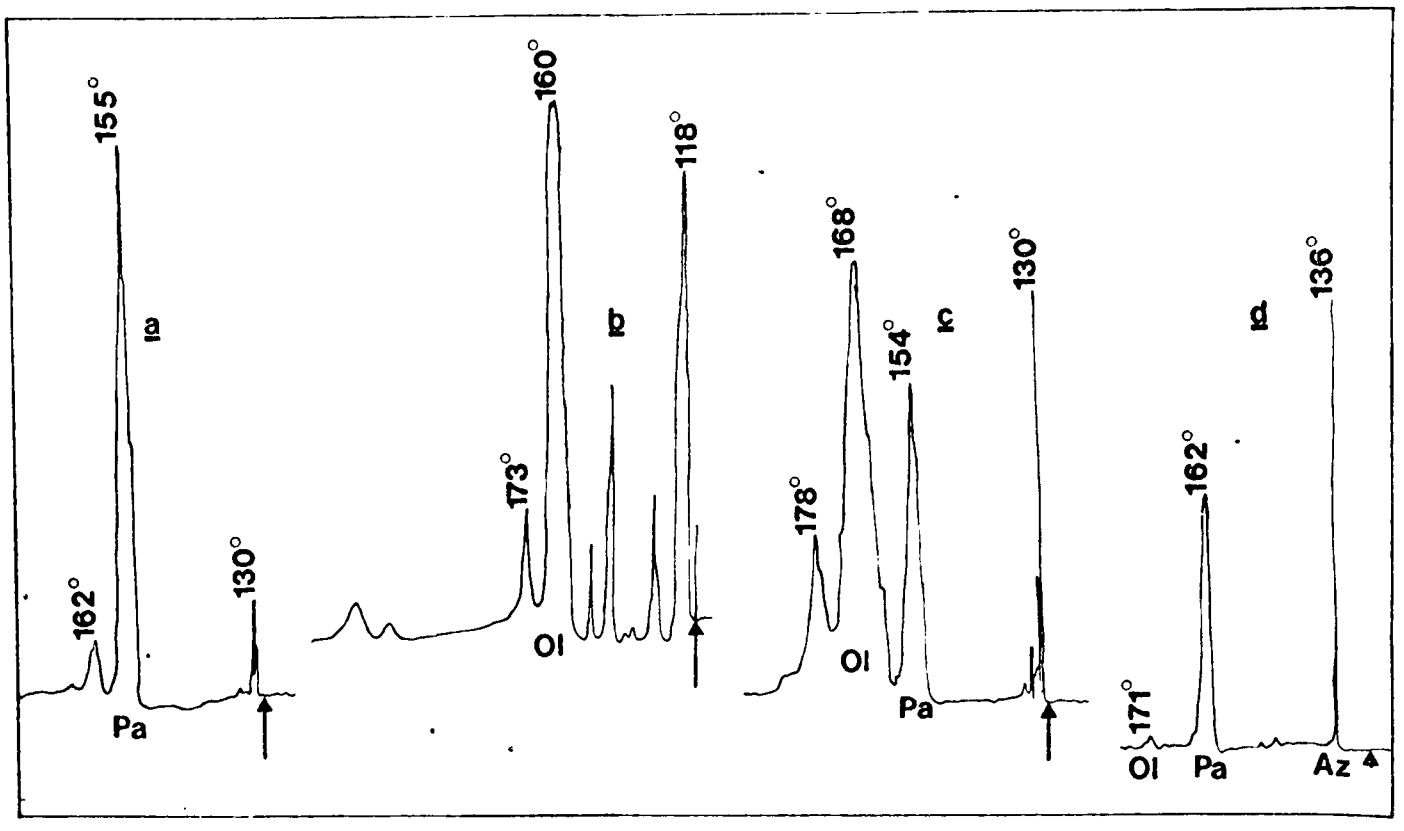


Figure 3.5 Tracings of gas-chromatograms of methyl esters of fatty acids; a, from hydrolysed 1,3-dipalmitoylglycerol; b, from oleoyl chloride; c, from 1,3-dipalmitoyl-2-oleoylglycerol and d, from 1,3-dipalmitoyl-2-azeloylglycerol. Temperatures are those of the column at the time the peak elutes; Pa, methyl palmitate; Ol, methyl oleate; Az, dimethyl azelate. All assignments of peaks confirmed by mass-spectrometry.

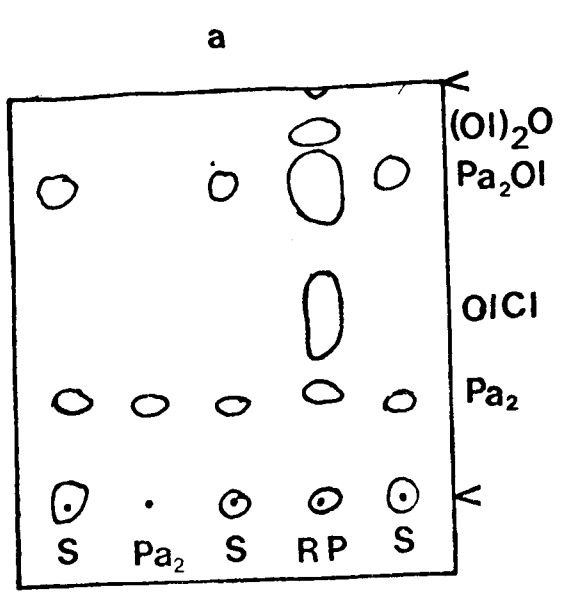
the column was stoppered and left overnight for the product to drain into the flask.

The product was then twice redistilled at reduced pressure of about 1 to 2 mmHg at 170 to 180°C. The final product was water-clear and there was only one spot of R_f 0.4 when it was chromatographed on silica-gel plates with the ether solvent (described in chapter 2). This was stored at 4°C in the dark. After storage for a few months, some of it had decomposed producing oleic anhydride (as shown by silica gel t.l.c., R_f 0.95) and presumably chlorine or hydrogen chloride. These could chlorinate the double bond and when oxidation was unsuccessful the oleoyl chloride was checked by gas-chromatography coupled to mass-spectrometry, to see whether this had occurred. The main chromatogram peak was shown to be methyl oleate and there were minor peaks corresponding to methyl palmitate, and the methyl esters of various related fatty acids (see Fig.3.5). The only chromatogram peaks at high retention times were small, the mass spectra were not those of fatty acids, chlorinated or not, but contained ions characteristic of silicones and were probably derived from contamination of the oleoyl chloride during reduced pressure distillation. Thus there was no evidence of double-bond substitution of the oleic acid, while stored as oleoyl chloride, prior to use.

3Bii Synthesis of 1,3-O-dipalmitoyl-2-O-oleoylglycerol

Whilst testing for the best conditions for this reaction, 1,2-O-distearoylglycerol (Sigma London Co.) was sometimes used as starting material, but for the actual preparation of derivatives 1,3-O-dipalmitoylglycerol (Sigma London Co.) was used.

500 mg of diacylglycerol was suspended in 1 ml of redistilled quinoline, (SLR quinoline, BDH Ltd., was distilled under water pump



Key: S - Acylglycerol standards
Su - Supernatant
Xt - Crystals
Pa_2 - Dipalmitoylglycerol
Pa_2Ol - Dipalmitoyloleoylglycerol
$(Ol)_2O$ - Oleoyl anhydride
OlCl - Oleoyl chloride
RP - Reaction product

Figure 3.6 Tracings of silica-gel chromatograms of the synthesis and purification of 1,3-dipalmitoyl-2-oleoylglycerol, all using the ether solvent; a, starting material and reaction product; b, first recrystallisation supernatant and crystals; c, second; d, third and e, last recrystallisations. Xt, crystals and Su supernatant.

vacuum, at 110 to 112°C, the clear colourless product was stored at 4°C in a tightly-sealed dark-glass bottle for up to 3 months) and a solution of 1 g (about 1.2 ml) of oleoyl chloride in 2 ml of chloroform was added. This was gently warmed at 37°C to dissolve all the diacylglycerol. The reaction mixture often turned a deep red or orange colour within the first half hour of reaction. It was left several days at room temperature, taking samples for analysis by silica-gel t.l.c. When the oleoyl chloride was not fresh, the reaction was sometimes rather slow, and further oleoyl chloride (1 g) would be added after 2 or 3 days of reaction. The reaction was usually complete (greater than 90% of triacylglycerol) within 3 to 7 days. The 1,2-O-diacylglycerol reacted faster than the 1,3-O-diacylglycerol. The whole reaction mixture was dissolved in 50 ml of chloroform, (or occasionally chloroform/ether, (1:1, v/v)) and washed twice with 50 ml of 1M sulphuric acid, and then 2 to 4 times with 50 ml of water until the pH of the aqueous washing was about 5. Sodium chloride was used to break any emulsion formed in the last one or two washings. The organic layer was dried overnight with anhydrous magnesium sulphate. The magnesium sulphate was filtered off, it was then washed with a few ml of chloroform and the combined chloroform extracts were rotary-evaporated to dryness. The product was dissolved in about 4 to 5 ml of warm ethanol and left to recrystallize at 4°C. Excess solvent was removed by filtration under reduced pressure in a Buchner funnel, firmly pressing the solvent out of the cake of product. This was repeated for between two and four times until the product was white, and little or no diacylglycerol was detected on silica-gel t.l.c. (see Fig.3.6). The yield varied between 60 and 80% of theoretical, depending to some extent on the number of recrystallizations that had been performed.

3Biii Oxidation of 1,3-O-dipalmitoyl-2-O-oleoylglycerol using Periodate and Permanganate

Several preliminary experiments were performed using the conditions of Rudloff (1956) with dioleoylglycerol as substrate, following the reaction by the change in periodate absorption at 222 nm. To get meaningful results it was necessary to soak all the glassware used in the experiments with the periodate and permanganate oxidant mixture for 60 minutes prior to washing with doubly-distilled water, that had been distilled from potassium permanganate. Using Rudloff's conditions, 80 to 90% of the reaction occurred within the first two hours at 37°C, and the reaction had achieved 100 ± 5% reaction after 24 hours (as measured by periodate consumption). The product of the oxidation of the dioleoylglycerol had an R_f of about 0.1 on silica-gel t.l.c. using the ether solvent and no spot corresponding to the starting material (R_f 0.3) was seen.

For the preparative-scale reactions, the solvents (water and tert-butanol) were freshly redistilled from potassium permanganate (about 7.5% w/v) and the glassware for the synthesis was washed in the oxidant solution or saturated potassium permanganate solution. 400 mg (0.45 mmol) of 1,3-O-dipalmitoyl-2-oleoylglycerol was dissolved in 216 ml of tert-butanol and to this 144 ml of water containing 1.54 g of sodium periodate (a 16-fold excess over carbon-carbon double bonds, 7.2 mmol), 120 mg of potassium permanganate (a 1.6-fold excess, 0.72 mmol) and 90 mg of potassium carbonate (for a final concentration of 0.25 mg/ml) was added. The pH of this mixture is about 8.0 to 8.5, and the butanol was 60% of the total volume. The reaction was left for 16 hours at 37°C and then rotary-evaporated to remove the butanol and much of the water,

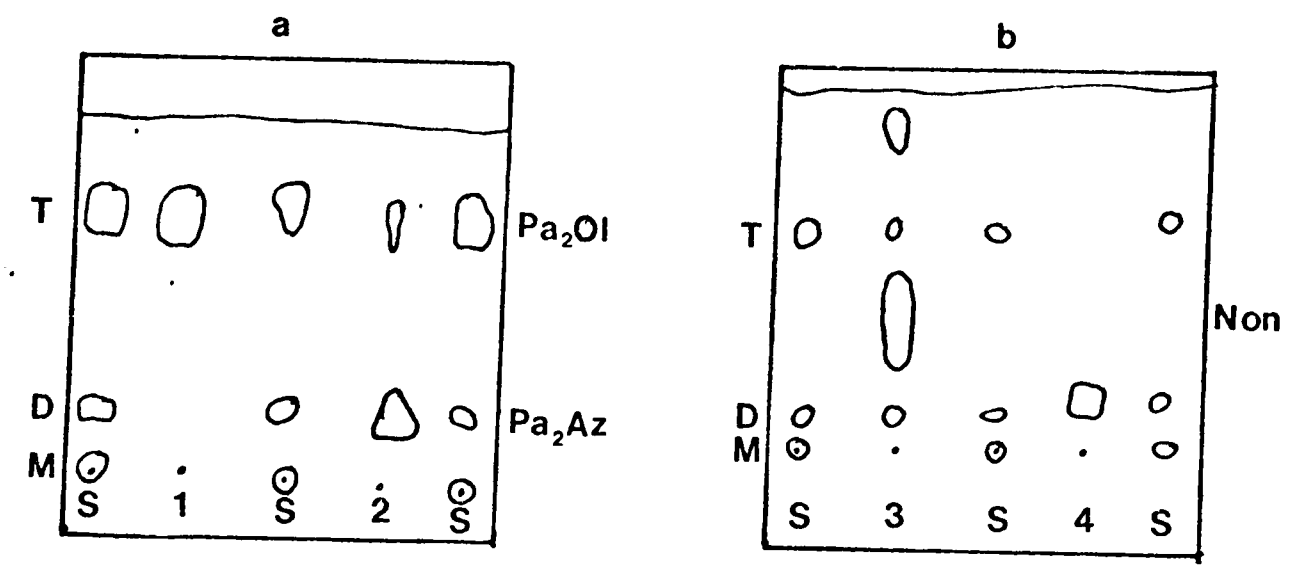


Figure 3.7 Tracings of silica-gel chromatograms of the oxidation of 1,3-dipalmitoyl-2-oleoylglycerol: a, staining with amido-black showing: 1, starting material (Pa_2Ol) and 2, product (Pa_2Az); b, staining with bromothymol blue showing: 3, nonanoic acid (Non) in the initial product and 4, pure 1,3-dipalmitoyl-2-azeloyl-glycerol after purification; S, acylglycerol standards mixture.

reducing the volume to between 50 and 80 ml. It was diluted to about 100 ml with water, acidified to between pH 1.5 and 2 with dilute sulphuric acid and then extracted twice with 100 ml of chloroform. The pooled chloroform extracts were washed three to five times with water until the water washings were colourless and had a pH of about 5. Sodium chloride was added if necessary to break any emulsions that formed. The chloroform extract was dried with anhydrous magnesium sulphate overnight, filtered free of the magnesium sulphate and then rotary-evaporated to dryness.

The product usually contained up to 5% of unoxidized triacylglycerol, and attempts were made to purify it, as the protonated acid or as the triethylamine salt, by crystallization from ether, chloroform or ethanol, but without success. As it is unlikely that this trace of triacylglycerol would affect later reactions no other attempts at purification were made. The R_f of the oxidized triacylglycerol on silica gel t.l.c. using the ether solvent was about 0.2 (see Fig.3.7).

However the nonanoic acid cleaved from the oleoyl chain would compete in the subsequent esterification and couplings. By oversight this was allowed to occur in the synthesis of the first batch of triacylglycerol derivatives of insulin, and, in fact, mixed nonanoic acid and triacylglycerol derivatives were made. For later batches, the nonanoic acid was removed from the oxidized product. This was accomplished by reduced-pressure sublimation. The product was placed in a thick-walled Pyrex test-tube fitted with a side-arm and a ground glass joint at the top. A cold finger filled with solid CO_2 and acetone was fitted in the ground glass joint and the side arm connected to a water pump. The tube was heated gently in a sand bath to about 125°C whilst under reduced pressure to remove

traces of volatile solvents and any included gas. An oil pump was connected in place of the water pump, and heating was continued until a white solid began to sublime on the cold finger. At intervals, the vacuum was released and the cold finger washed into a vial with a few ml of chloroform, until no further sublimate appeared. The residue was analysed by silica gel t.l.c. using the ether solvent and staining with 2,7-dichlorofluorescein, or amido-black with only very slight destaining. This showed that there was very little or no nonanoic acid left in the triacylglycerol. After the first sublimation later t.l.c. analyses of the products were stained with bromothymol blue, which was more sensitive to the short chain acids.

The product of sublimation contained some materials of R_f 0.0 by t.l.c. analysis, this was removed by recrystallization of the product from ethanol. The overall yield was between 40 and 60% of the theoretical yield.

3Biv Synthesis of 1,3-O-dipalmitoyl-2-O-(N-hydroxysuccinimidyl)-azeloylglycerol

The first synthesis of a, N-hydroxysuccinimidyl ester of 1,3-O-dipalmitoyl-2-O-azeloylglycerol used N,N'-dicyclohexylcarbodiimide. However the solubility properties of the ester produced, the urea derived from the carbodiimide and the carbodiimide were very similar and they could not be separated. The presence of carbodiimide and its urea in the next stage of coupling the active ester to insulin could be harmful to the protein by causing undesired side reactions. Should any carbodiimide survive it would be a toxic contaminant in the final product.

Two alternative carbodiimides were tried, these were 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDM carbodiimide) and

1-cyclohexyl-3(2-morpholinoethyl)carbodiimide-methoxy-p-toluenesulphonate (CHM carbodiimide). Under mildly acidic conditions they are soluble in water due to the presence of the quaternary nitrogen atom. 20 micromoles of each carbodiimide (CHM carbodiimide, 8.5 mg and EDM carbodiimide, 3.8 mg) were placed in separate hard glass tubes followed by 2.3 mg of N-hydroxysuccinimide (20 µmole), 14.8 mg of 1,3-dipalmitoyl-2-azeloylglycerol (20 µmole) and 0.1 ml of dimethylformamide. The tubes were degassed under vacuum, flushed with nitrogen several times and finally sealed under nitrogen at low pressure. They were heated at 108°C for 90 minutes, cooled and opened. The dimethylformamide was removed overnight, under reduced pressure in a desiccator over sodium hydroxide. The dried product was a dirty brown oily material, this was triturated with 2 to 3 ml of 10mM hydrochloric acid and almost immediately it became a white fluffy wax-like solid, this was centrifuged and the supernatant decanted. The washing was repeated. The product was dissolved in about 1 ml of ethanol and 5 µl samples taken for t.l.c. analysis on silica-gel plates using the ether solvent. A new spot, believed to be the active ester was seen, with an R_f of about 0.1 in this system. With EDM carbodiimide as the carbodiimide, the yield was about 70% and with CHM carbodiimide it was about 50%.

For preparative-scale synthesis, 110 mg of 1,3-O-dipalmitoyl-2-O-azeloylglycerol (150 µmol) 20.7 mg of N-hydroxysuccinimide (180 µmol) and 34.6 mg of EDM carbodiimide (180 µmol) were dissolved in 0.75 ml of dimethylformamide, apparent aqueous pH of 5.0 to 5.5, in a hard glass tube. The tube was degassed, flushed with nitrogen and sealed. The mixture was heated at 108°C for 2 to 4 hours, cooled and opened. The mixture was dried in a desiccator under reduced pressure. It was then washed twice

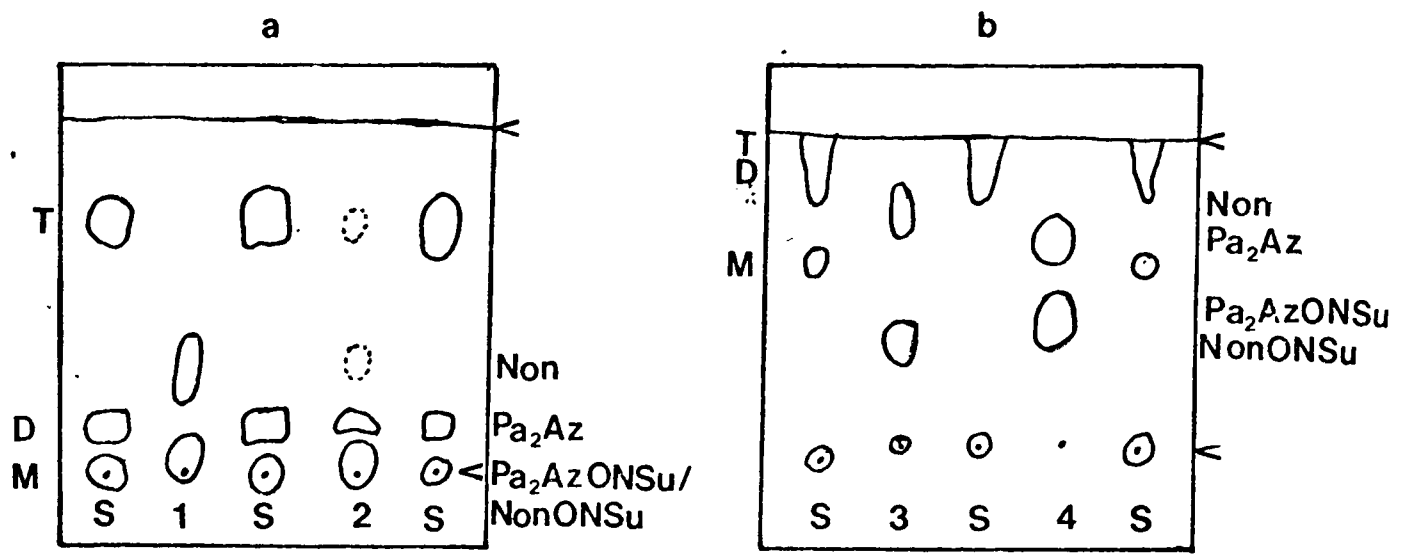


Figure 3.8 Tracings of silica-gel chromatograms of the synthesis of N-hydroxysuccinimidyl esters (ONSu) of nonanoic acid (Non) and 1,3-dipalmitoyl-2-azeloylglycerol: a, using the ether solvent b, using the pyridine solvent; 1,3, nonanoic acid and its ester; 2,4, 1,3-dipalmitoyl-2-azeloylglycerol and its ester; S, acylglycerol standards mixture.

with 2 to 3 ml of 10mM hydrochloric acid and 3 or 4 times with the same volume of water until the water washings were about pH 5. The solid product was dried and stored at 4°C until used. This material was characterized by a new spot appearing on silica-gel t.l.c. analysis. Using the ether solvent, this new spot had an R_f of about 0.1 and using the pyridine solvent (see chapter 2), it had an R_f of about 0.5 (see Fig.3.8). By comparison of the staining intensities of the spots on the chromatograms the ester was usually contaminated with about 30% of unreacted 1,3-O-dipalmitoyl-2-O-azeloylglycerol. Attempts were made to purify the product by crystallization from ethyl acetate, chloroform and ether mixtures or ethanol, but these were unsuccessful. The yield was about 90% of this 70% pure product.

3C Synthesis of the N-hydroxysuccinimidyl Esters of Fatty Acids

The reaction conditions for this synthesis were similar for all the fatty acids used. To one millimole of fatty acid (174 µl of octanoic acid, 311 µl of oleic acid, 144 mg of octanoic acid or 284 of stearic acid) in a thick-walled Pyrex tube, 1.2 mmol of N-hydroxysuccinimide (138 mg), 1.2 mmol of EDM carbodiimide (230 mg) and 5 ml of dimethylformamide was added. The mixture was warmed gently and agitated to dissolve all the solids. It was degassed under vacuum and flushed with nitrogen, sealed under nitrogen at low pressure and then left to react at 108°C. The mixtures containing the short chain acids (octanoic and nonanoic) were usually left for 2 to 4 hours; those containing the longer chain acids (oleic and stearic) were left overnight (16 to 20 hours). The tube was cooled, opened and the dimethylformamide removed under reduced pressure. The brownish oil that remained was triturated with a few ml of dilute hydrochloric acid, pH 2. The oil became a

white waxy material, which was centrifuged and the supernatant was discarded. It was washed once more with acid and 3 or 4 times with water until the pH of the water was about 5. To break up the emulsions that occasionally formed, solid sodium chloride was added to the water washes. The product from oleic acid did not form a solid, but an oil which floated on centrifugation. The aqueous layer in this case was removed with a pipette. All the esters had an R_f of about 0.1, on silica-gel t.l.c. using the ether solvent, a result which was apparently independent of chain length. The acids had an R_f of 0.4 to 0.6, increasing with chain length. In the pyridine solvent the esters had R_f values of 0.5 and the acids had R_f values of about 0.8 (see Fig.3.8). Staining with Amido-Black showed an apparent 90 to 100% purity for the short chain acids. This is probably an overestimate due to the fact that the esters are much more strongly staining than the acids. Using bromothymol blue a purity of 70 to 80% was estimated. The long chain acids had a purity of 60 to 70%. The overall yield varying from preparation to preparation, was about 50 to 80%. It was not related to the fatty acid used. The N-hydroxysuccinimidyl esters of acetic and butyric acid were made by this method but not used for coupling.

Some preparations by the method of Lindsay and Shall (1971) were performed. One millimole of fatty acid (174 µl of nonanoic acid, 144 mg of octanoic acid or 284 mg of stearic acid) was dissolved in 0.2 ml of dichloroethane, 115 mg of N-hydroxysuccinimide in 0.2 ml of dioxan and then 206 mg of 1,3-dicyclohexylcarbodiimide in a further 0.2 ml of dioxan was added. This was stirred overnight at 4°C, filtered in a Buchner filter and washed twice with 0.5 to 1 ml of dichloroethane. The filtrate was transferred to a small round-bottomed flask and reduced to about 0.5 ml by rotary-evaporation.

One or two ml of light petroleum (60 to 80°C boiling fraction) was added and the product was filtered. The purity by this method was poor, rarely as much as 50%. In fact from nonanoic acid, the product instead of being a white waxy solid, was an oil, which had to be separated by centrifugation at the final stage. One of the major contaminants as identified by silica gel t.l.c. was 1,3-dicyclohexyl urea.

3D Synthesis of N-hydroxysuccinimidyl-[1-^{14}C]-octanoate

The octanoic acid was supplied by the Radiochemical Centre, Amersham as the sodium salt and had a specific activity of 31.8 Ci/mol. The salt had to be converted first into the free acid before active-ester synthesis was possible.

The freeze-dried solid (250 μCi or 7.85 μmol), was dissolved in 0.1 ml of 3M sodium hydroxide, then 0.15 ml of 3M hydrochloric acid was added. This solution was transferred into a 75 x 8 mm glass test-tube. It was then extracted with three aliquots of 0.25 ml of chloroform, washing the original tube with the chloroform first. Small samples were taken from the combined chloroform extracts and the aqueous fraction and the radioactivity measured by scintillation counting. (Scintillant was 10 ml of Bio-Fluor, New England Nuclear, counting in a Beckmann model LS230 scintillation counter using the makers' preset discriminator settings for ^{14}C). 99% of the radioactivity was found to be in the chloroform and less than 0.5% in the aqueous phase. The chloroform was evaporated off by blowing carefully with a steady stream of nitrogen.

N-hydroxysuccinimide and EDM carbodiimide were dissolved in freshly redistilled dimethylformamide so that when appropriate aliquots were added, 0.903 mg of N-hydroxysuccinimide and 1.51 mg of

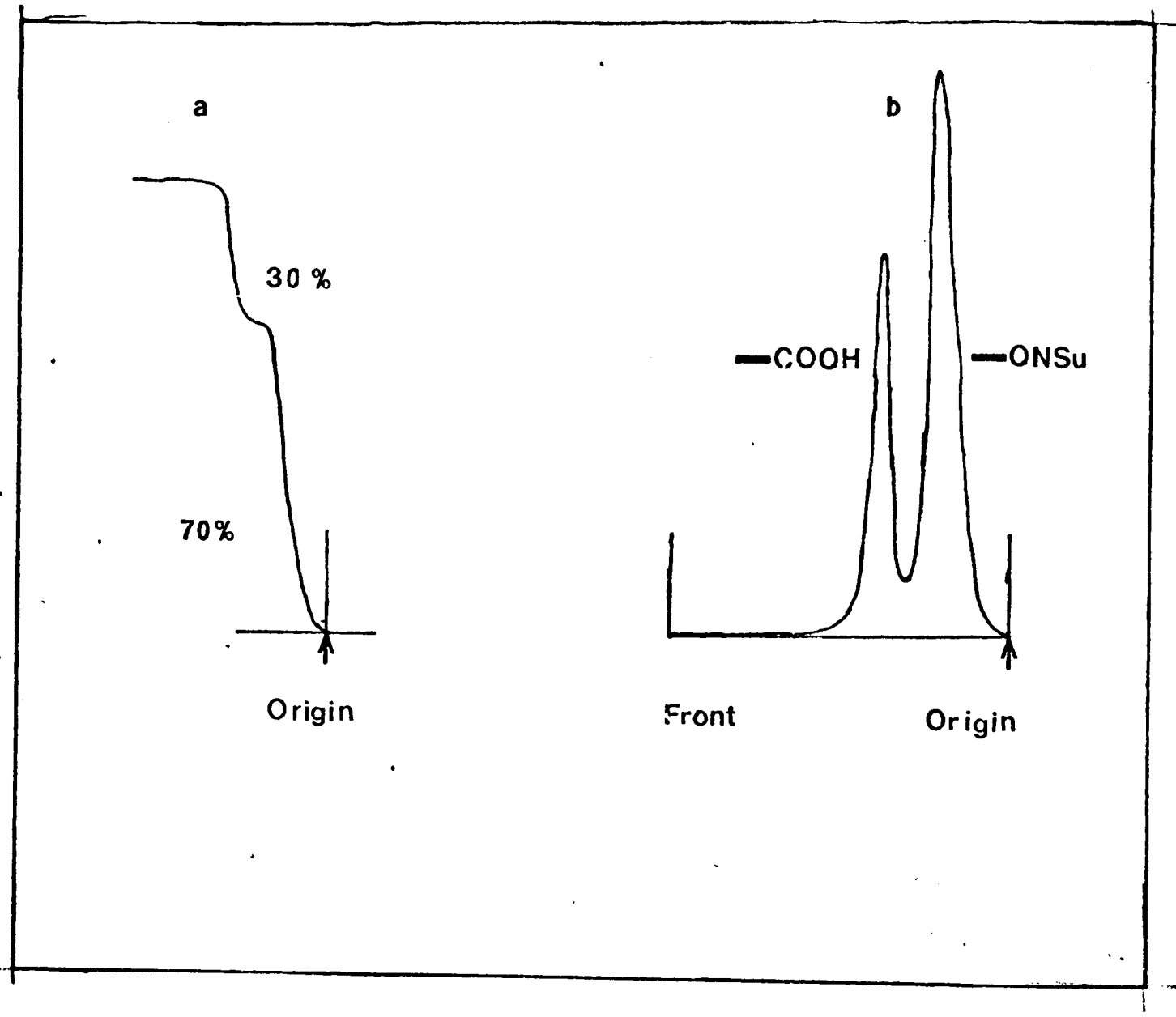


Figure 3.9 Radiochromatogram scanner trace of silica-gel chromatogram of N-hydroxysuccinimidyl [1-^{14}C]octanoate using the ether solvent: a, integrated scan; b, normal scan; -COOH, octanoic acid and -ONSu, N-hydroxysuccinimidyl ester.

EDM carbodiimide were added to the octanoic acid, in a final volume of 43 µl of solution. The tube was sealed and left for 2.5 or 4 hours at 108°C. (The final yield of the 4-hour-incubated preparation was virtually 100%, that of the 2.5-hour-incubated preparation was 70%.) The tube was cooled, opened and the dimethylformamide removed in vacuo, at 0.1 torr for 1 hour. The tube was then left overnight in a sealed exhausted desiccator, containing fresh sodium hydroxide pellets and concentrated sulphuric acid.

The residue was dissolved in 0.25 ml of 0.1M hydrochloric acid, the tube was washed with a further 0.25 ml of 0.1M hydrochloric acid. The combined 0.5 ml of acidic solution was extracted four times with 0.25 ml of water-saturated ethyl acetate, which was first used to rinse out the original reaction tube. The phases were separated by centrifugation if necessary. The ethyl acetate extract was washed twice with water. The radioactivity of an aliquot was measured by scintillation counting, and an aliquot (containing about 2.5×10^5 c.p.m.) was run on silica-gel plates using the ether solvent and scanned in a Bertholdt radiochromatogram scanner Model LB2723 (see Fig.3.9). About 2% of the radioactivity remained in the aqueous extracts and between 72% and 80% of the radioactivity remained in the ethyl-acetate extract. The rest was probably lost by adhesion to tubes and pipettes. The ethyl acetate was evaporated with a stream of nitrogen. The dried product was used for coupling to protein without further purification.

3E Limited Periodate Oxidation of Ficoll

Some preliminary experiments using both Ficoll and starch were performed. It was assumed that both substances contained about 15% by weight of water, making the monomer molecular weight 186. Initially, starch or

Ficoll 400 (approximately 10 mg in 8 ml of 0.02 m phosphate buffer, pH 3 to 6, concentration of carbohydrate monomer about 6.7mM) was reacted with a three-fold molar excess of sodium periodate (concentration 20mM, 21 mg in 8 ml) at room temperature. Twenty µl aliquots were taken at intervals for up to 72 hours and diluted 1 in 200 into water and the O.D. at 223 nm measured. By the change in absorbance the reaction was 80 to 90% complete in all cases after between 1 and 3 hours and complete after 48 hours. 20 µl samples of the reaction mixture were taken after 90 hours diluted into carbonyl free methanol and the content of aldehyde groups assayed by the hydrazone method previously described in chapter 2. For the reactions between pH 4 and 6 the content of aldehyde groups was between 94 and 118% of that theoretically expected, however for the reaction at pH 3 there were about twice as many aldehyde groups as expected. With the excess of reagent and low pH it is possible that hydrolysis of the polymer followed by over-oxidation occurred.

On a preparative scale, in order to produce Ficoll with 10% of the glycol groups oxidized, 100 mg of Ficoll was dissolved in 2.65 ml of 0.02M sodium periodate in 0.02M phosphate buffer. This is one tenth of the periodate required to oxidize all the glycol groups in the Ficoll. All the periodate was consumed within 5 minutes at room temperature, but the reaction mixture was left a further 24 hours. Excess periodate and iodate was precipitated with barium chloride solution and the excess barium removed by addition of sodium carbonate. All the precipitates were removed by centrifugation. On assaying for aldehyde groups, 39% of the theoretically expected yield was found i.e. 3.9% of total. This low result could be explained by two factors. First of all, the terminal hexoses of the carbohydrate chain could be preferentially oxidized.

These produce only one free aldehyde group for each molecule of periodate consumed, and although they only constitute about 0.5% of the number of monomers in the average molecule, this is equivalent to one-twentieth of the 10% of the monomers to be oxidized. Secondly the product of limited oxidation is more likely to maintain some structure compared with the product of complete oxidation of the carbohydrate chain. In the case of limited oxidation, the two aldehyde groups in each monomer will be close together and only one may be able to react with the hydrazine. The carbonyl-group standard was acetone, which is completely unhindered in this respect, and is perhaps not a suitable standard for comparison with these hindered molecules.

Chapter 4

Biochemical Syntheses

4A Synthesis of t-Boc-Insulin Derivatives

4Ai Reaction Conditions

The conditions for the synthesis of $N^{\alpha A1}, N^{\epsilon B29}$-di-tert-butyloxycarbonyl insulin are essentially those described by Geiger (Geiger et al, 1971) except that the dimethylformamide is replaced by methanol. To synthesise $N^{\alpha A1}$-t-Boc-insulin, the modification described by Saunders (Saunders & Offord, 1977) was used.

The di-Boc-derivative was prepared as follows. 250 mg of insulin were suspended in 10 ml of 5% aqueous sodium bicarbonate, and whilst stirring vigorously, 40 ml of methanol was slowly added to this. Any slight precipitate of bicarbonate was ignored. The reaction was started by adding 0.625 ml of tert-butyloxycarbonylazide (about a one-hundred-fold excess) and the mixture was stirred at room temperature for 16 to 18 hours. The methanol and much of the water was removed by rotary-evaporation until the volume was reduced to about 5 ml. 1M HCl was added drop by drop with stirring, until a copious white precipitate appeared, due to the isoelectric precipitation of the protein. This precipitate was centrifuged, and washed two or three times with small volumes of water to remove salt. The moist pellet was lyophilised.

The mono-Boc-derivative was prepared in an identical manner except that 5% aqueous triethylamine, adjusted to a pH of 9.3 with concentrated aqueous hydrochloric acid, was used rather than the 5% sodium bicarbonate and the reaction was left for 4 hours rather than 16.

For some of the later preparations, di-tert-butyl-dicarbonate

(Fluka A.G., West Germany) was used in place of t-Boc-azide. In this case, for the production of the di-Boc-insulin, 27 µl of reagent was used for the same amount of insulin (3-fold excess) and it was reacted for 3 hours; and for the synthesis of the mono-Boc-insulin, 9 µl of reagent was used (1-fold excess) reacting for 3 hours. Otherwise the conditions and work-up were identical.

4Aii Purification by Ion-Exchange Chromatography

The purification of insulin derivatives by ion-exchange chromatography on DEAE-Sephadex has been described by Bromer and Chance (1967) and by Africa and Carpenter (1968) and is now widely used.

The same method was used to purify both mono- and di-Boc-insulin preparations. The aqueous starting buffer contained 7M urea, 0.2M Tris and 50mM sodium chloride and the pH was adjusted to 8.40 ± 0.02 with 3M HCl, the conductivity being about 5.8 x 10^{-3} $ohm^{-1}cm^{-1}$. Fourteen grammes of DEAE-Sephadex A-25 (Fine Grade) (Pharmacia Ltd.) were suspended in 250 ml of buffer, and stirred for 30 minutes, the pH was readjusted to 8.40 with solid Tris base. After the suspension had been allowed to settle for 20 to 30 minutes the supernatant containing the fines was decanted off. This process of resuspension and decantation of the fines was repeated a further two or three times. The gel was poured into a column, 2.6 x 15 cm, volume 75 ml, and then buffer was pumped through at 100 ml/h for 4 to 12 hours until the pH and conductivity of the buffer entering and leaving the column were identical. This equilibration was timed to be complete when the insulin derivatives were ready for separation, the column once equilibrated was not allowed to stand for any length of time due to deterioration of the buffer by formation of cyanate ions. Since insulin which has been isoelectrically precipitated is moderately

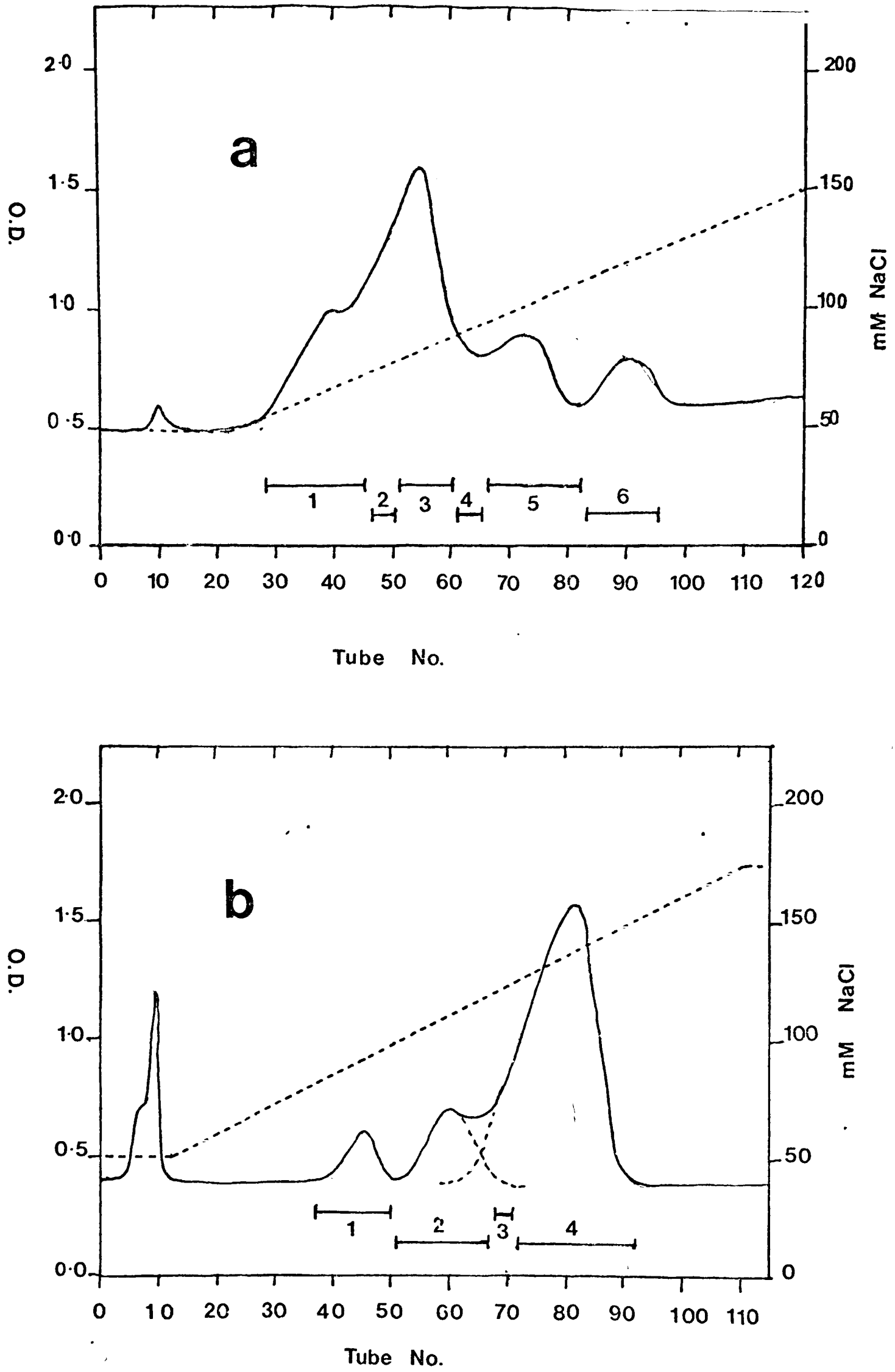


Figure 4.1 Separation of Boc-insulin preparations on DEAE-Sephadex: continuous line, O.D. of eluate at 280 nm; dotted line, salt concentration of eluate. Pooled fractions are identified as: a, mono-Boc-insulin preparation: 1, insulin; 3, $N^{\alpha A1}$-Boc-insulin; 5, $N^{\epsilon B29}$-Boc-insulin; 6, $N^{\alpha A1}$,$N^{\epsilon B29}$-di-Boc-insulin; b, di-Boc-insulin preparation: 1, $N^{\alpha A1}$-Boc-insulin; 2, $N^{\epsilon B29}$- Boc-insulin; 4, $N^{\alpha A1}$,$N^{\epsilon B29}$-di-Boc-insulin.

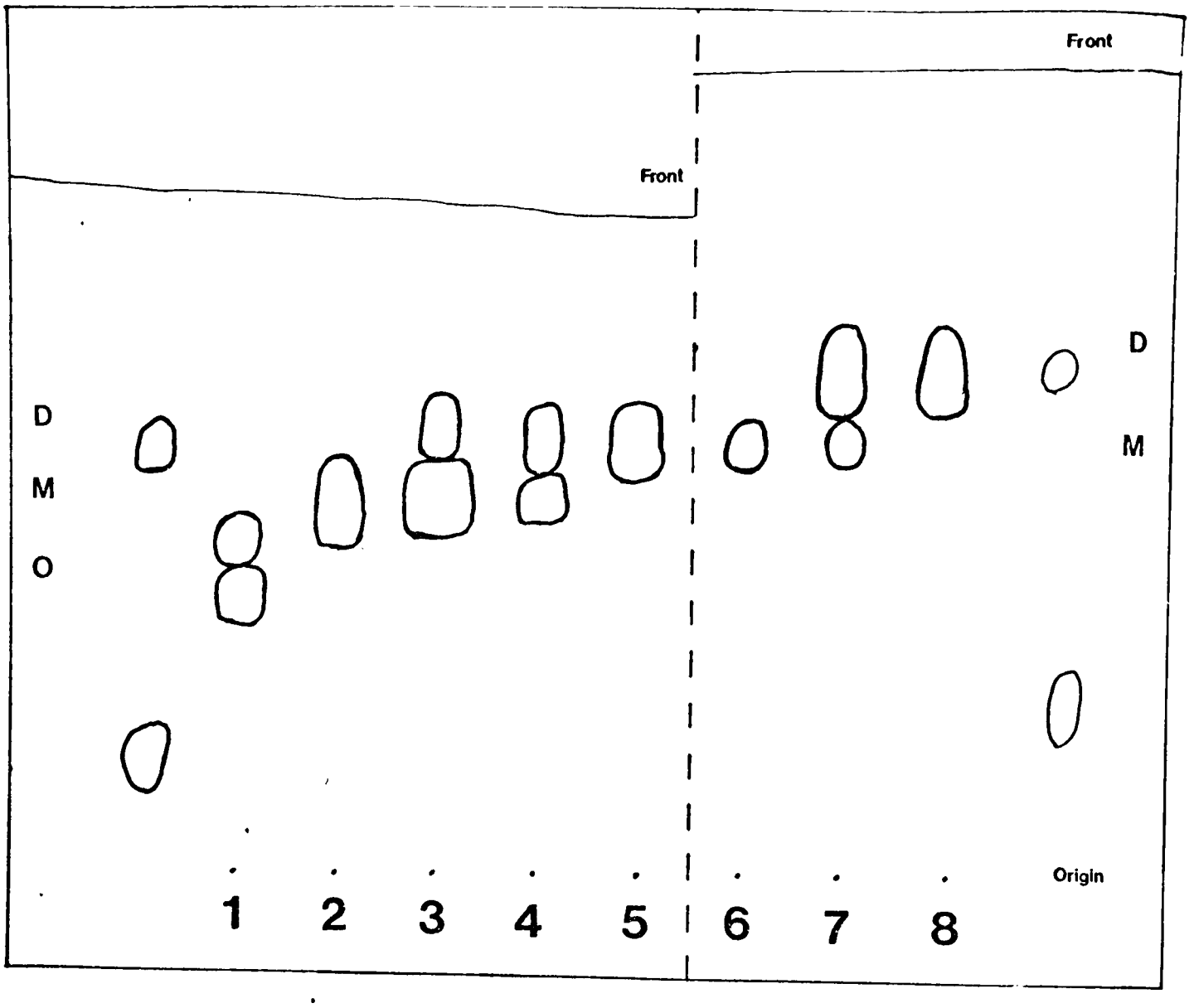
acid, a 30 ml sample of the buffer did not have its pH adjusted to 8.40 until after the insulin derivatives were dissolved in it. The sample was then loaded onto the column at a flow rate of 70 ml/h. Between 10 and 20 fractions (fraction size 10 ml) would be collected before starting a linear salt gradient of one litre, rising from 50mM NaCl to 200mM NaCl, in the same concentration of urea and Tris buffer, at pH 8.40.

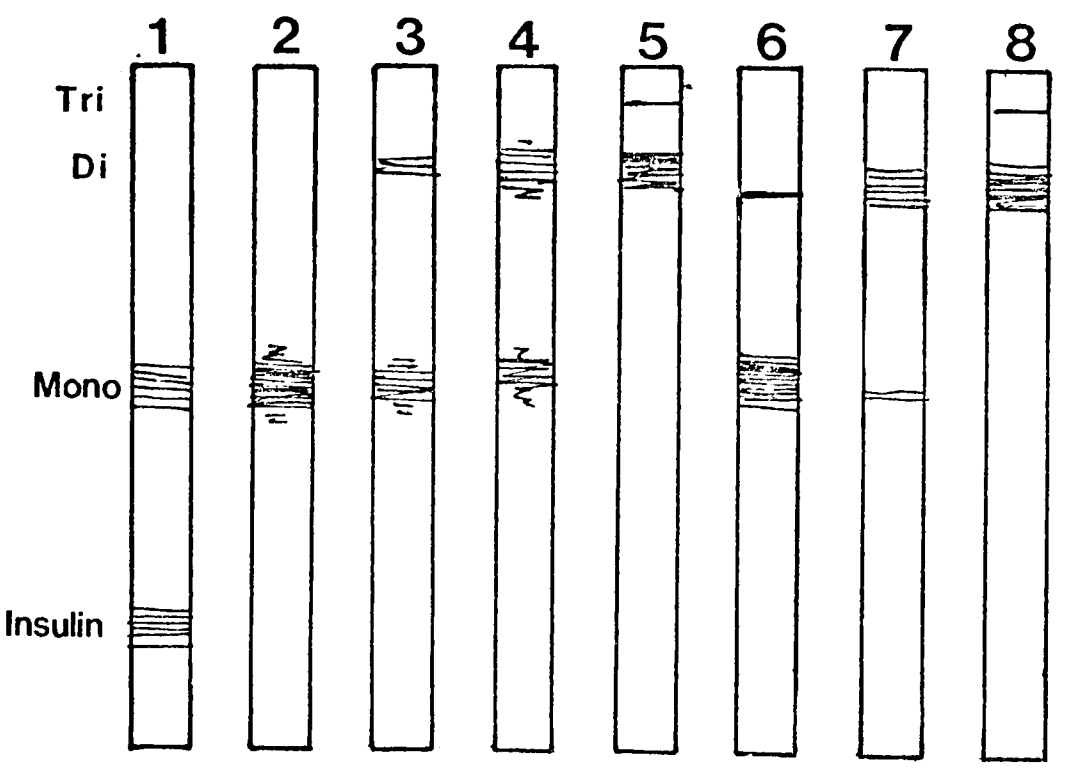
Some average separations are shown in Fig.4.1. Fractions would be separated to exclude 'valleys' between peaks in order to reduce the cross-contamination of products. This cuts down slightly on yield but improves purity. The pooled fractions were adjusted to pH 4.5 to 5.0 with 6M HCl. As a simple means to determine the insulin content of each of the pooled fractions the volume and O.D. at 278 nm of each was measured. The fractions were then dialysed against at least 4 changes of 20 l of distilled water over 24 hours and then lyophilised. The purity of the fractions was determined by isoelectric focussing, SG-81 paper chromatography and end-group analysis by dansylation (see Fig.4.2).

The yield from a preparation of mono-Boc-insulin was 25 to 35% of the desired product of a purity of 95 to 100%. From a preparation of di-Boc-insulin the yield was 40 to 50% of 90 to 95% purity.

4B Coupling of N-hydroxysuccinimidyl Esters to the t-Boc-Insulin Derivatives

Hydroxysuccinimidyl esters have been widely used for the acylation of insulin and other proteins. They have been used to couple both amino acids (Levy & Carpenter, 1967) and other compounds of use in modification studies (Lindsay & Shall, 1971 and Kaplan et al, 1976) to the amino groups of proteins. They have also been used to attach protecting groups to proteins


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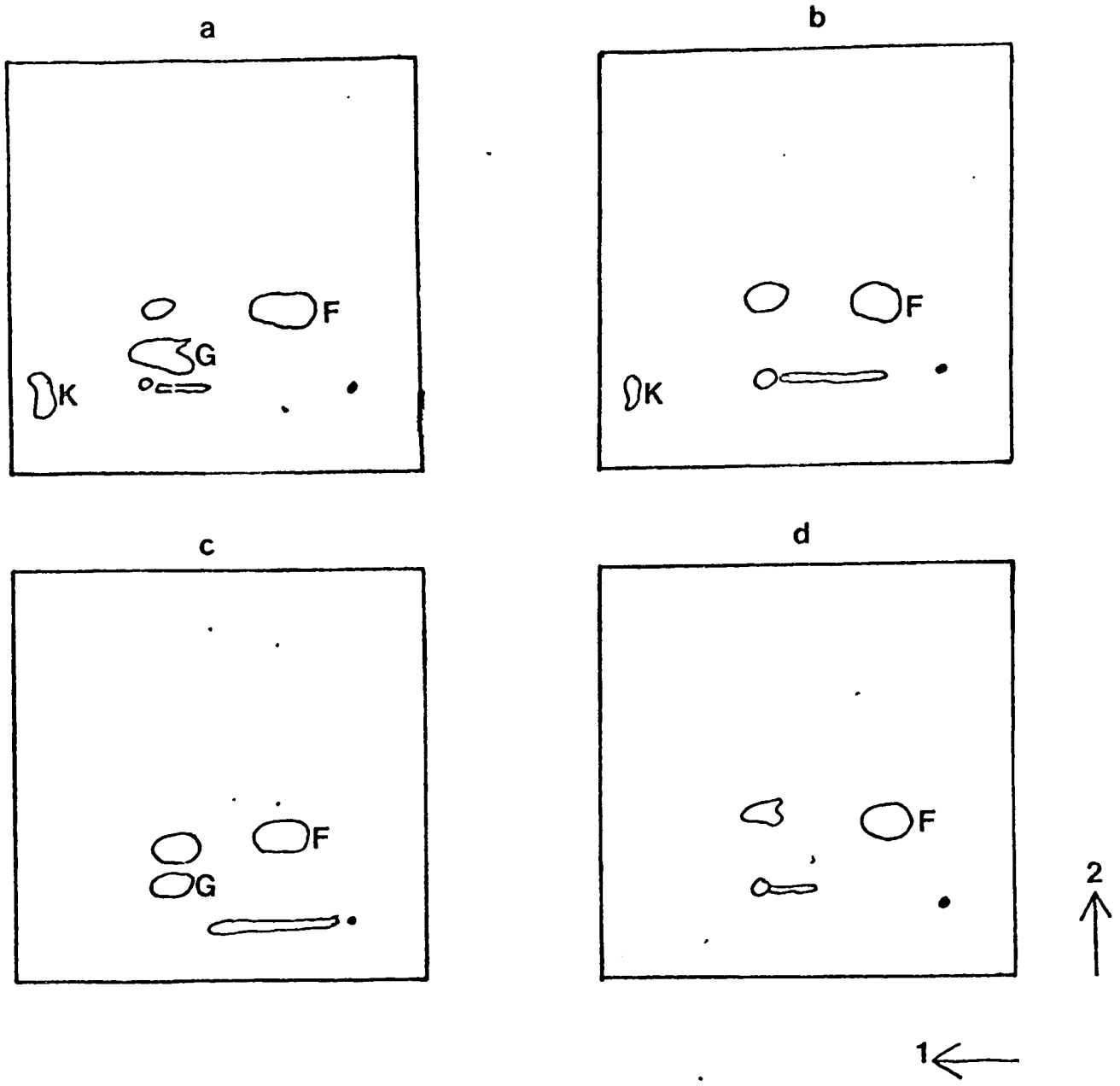


Figure 4.2 Analyses of separations of Boc-insulin derivatives. Dansyls: a, insulin; b, $N^{\alpha A1}$-Boc-insulin (Fig. 4.1a, peak 3); c, $N^{\epsilon B29}$-Boc-insulin (Fig. 4.1a, peak 5); d, $N^{\alpha A1}$,$N^{\epsilon B29}$-di-Boc-insulin (Fig. 4.1b, peak 4).. SG-81 chromatogram tracing: 1 to 5, from Fig. 4.1a, peaks 1,3,4,5,6 respectively and 6 to 8, from Fig. 4.1b, peaks 1,2,4 respectively.. Gel electrofocussing, numbering as for SG-81 chromatography.

(Obermeier & Geiger, 1975 and Geiger et al, 1975). Aqueous solvents are not suitable for the present work as the fatty acyl esters are only slightly water-soluble at best. Nor is dimethylsulphoxide suitable as it promotes coupling of reagents to the A1 and B29 amino groups (Brandenburg et al, 1972). In dimethylformamide the reactivity of coupling reagents to the B1 amino group is high (Levy & Carpenter, 1967) and so this solvent was used for the couplings.

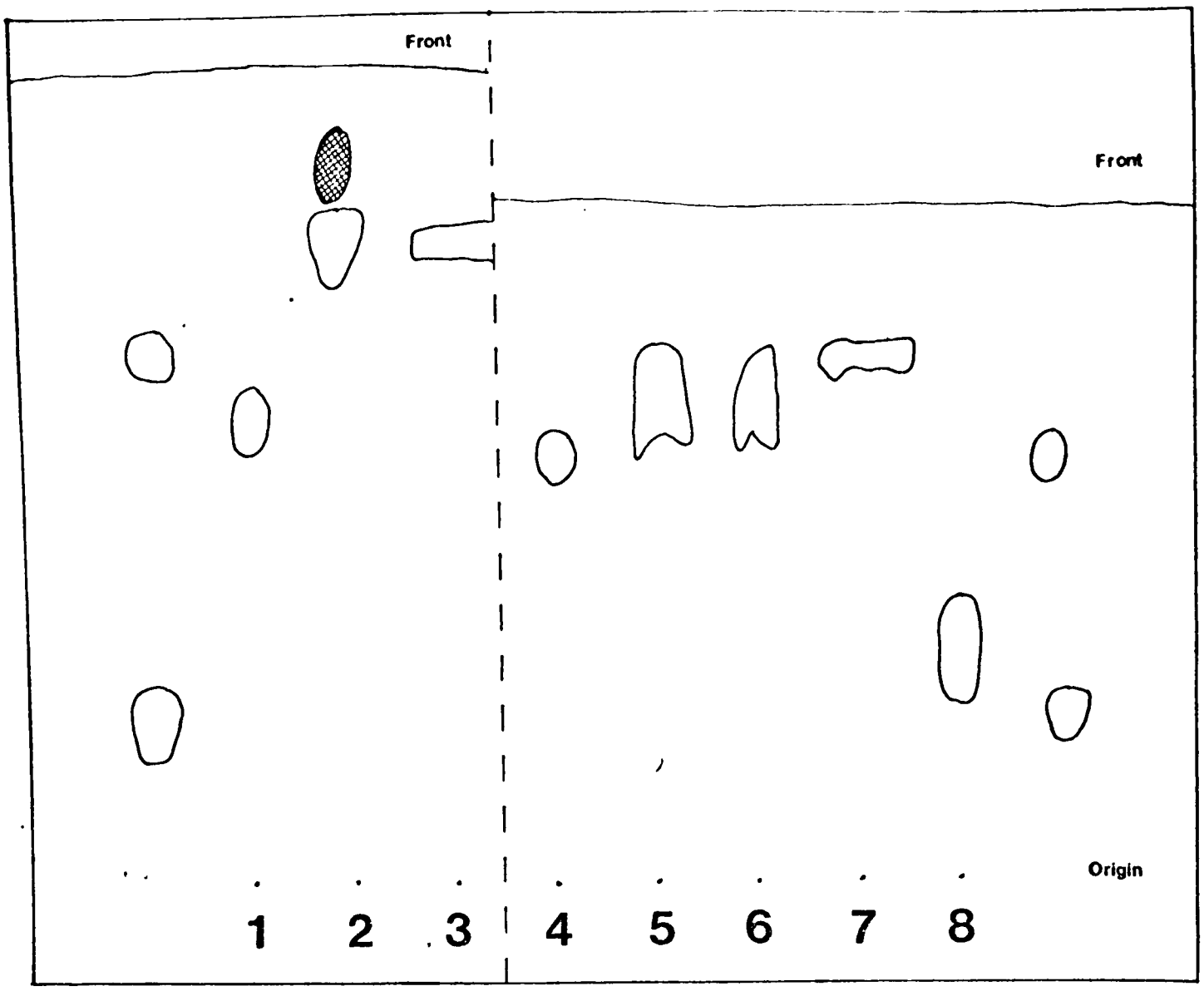
In order to test hydroxysuccinimidyl ester preparations, trial couplings were frequently performed before a large amount of protected insulin was committed to the reaction. Both insulin and the tetrapeptide, H_2-Ser-Pro-Phe-Arg(NO_2)-OBz, were used as the amino component for these tests. The quoted excesses of ester over amino component takes into account, firstly the fact that the purity of the esters varies from about 50% to 90% and secondly, that depending on the degree of protection of the insulin, there are one, two, or three free amino groups per insulin molecule. "An n-fold excess" therefore means the excess of available active ester in relation to the free amino groups.

Test couplings were performed in dimethylformamide with one to two micromoles of tetrapeptide or one quarter to one half of a micromole of insulin, with a two- to six-fold excess of active ester, at an amino-component concentration of 5mM (tetrapeptide) or 1mM (insulin). N-ethylmorpholine was added as base until the pH was 9.0 to 9.5 (purple/grey on moist pH 8–10 paper). The reaction was incubated at 37°C and samples of 50 nmol were taken at intervals and analysed by chromatography on SG-81 paper. Disappearance with time of material staining with ninhydrin/cadmium acetate (tetrapeptide) or increase of R_f of material staining for tyrosine (insulin) were used to assess the progress of the reaction. The reaction of the

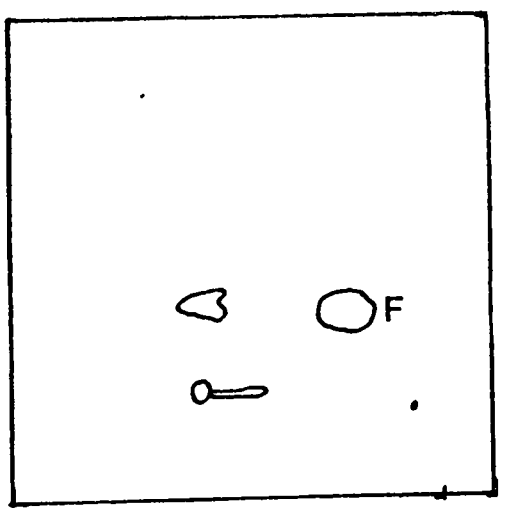
triacylglycerol hydroxysuccinimidyl ester with the tetrapeptide was rapid, being complete within 1 to 2 hours, when a satisfactory preparation of ester was used. With short-chain acyl esters the reaction was even faster, taking 10 to 20 minutes. If insulin was used as the amino component, the reaction was slower, taking about 20 hours with fatty-acyl esters and 3 to 4 days with triacylglycerol esters.

After the success of trial couplings using small (2 to 4 fold) excesses of ester over amino component, several large scale preparations using these quantities were performed. Detailed analysis (isoelectric focussing, SG-81 paper chromatography and dansylation) showed that the reaction was usually only about 80% complete. However further experiments showed that larger excesses (7 to 10 times over amino groups) led to essentially complete reactions.

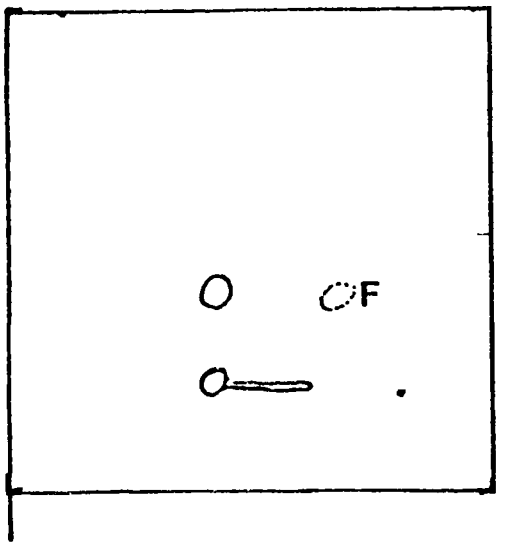
Apart from the different number of amino groups affecting the amount of ester used, the conditions for the coupling of insulin, $N^{\alpha A1}$-Boc-insulin, or $N^{\alpha A1}$,$N^{\epsilon B29}$-di-Boc-insulin were identical. The insulin was dissolved in dimethylformamide at a concentration of 1mM, usually with 2 to 6 μmol of insulin per preparation. The ester was added and stirred, warming to up to 37°C if necessary, to dissolve it. A ten-fold excess of ester was used for stearoyl, oleoyl and octanoyl esters, a seven to ten-fold excess was used for the nonanoyl ester and a three to six-fold excess was used for the triacylglycerol esters. The pH was adjusted to 9.0 to 9.5 (purple/grey on moist pH 8-10 paper). With the fatty acyl esters the reaction was generally complete within 20 hours at 37°C, but the reaction with the triacylglycerol esters had to be left for two to four days for maximal yield. Even then up to 10% of free-amino groups, particularly from phenylalanine B1 were detected by dansylation. The progress of



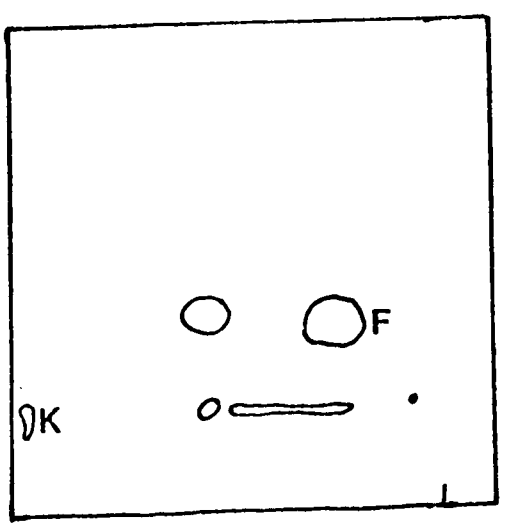
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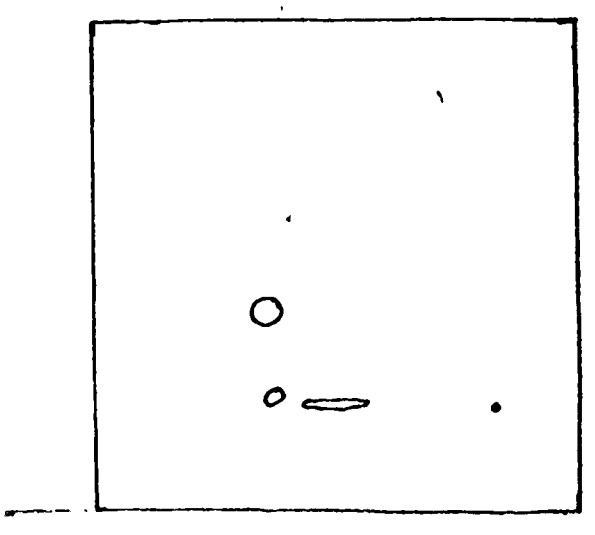
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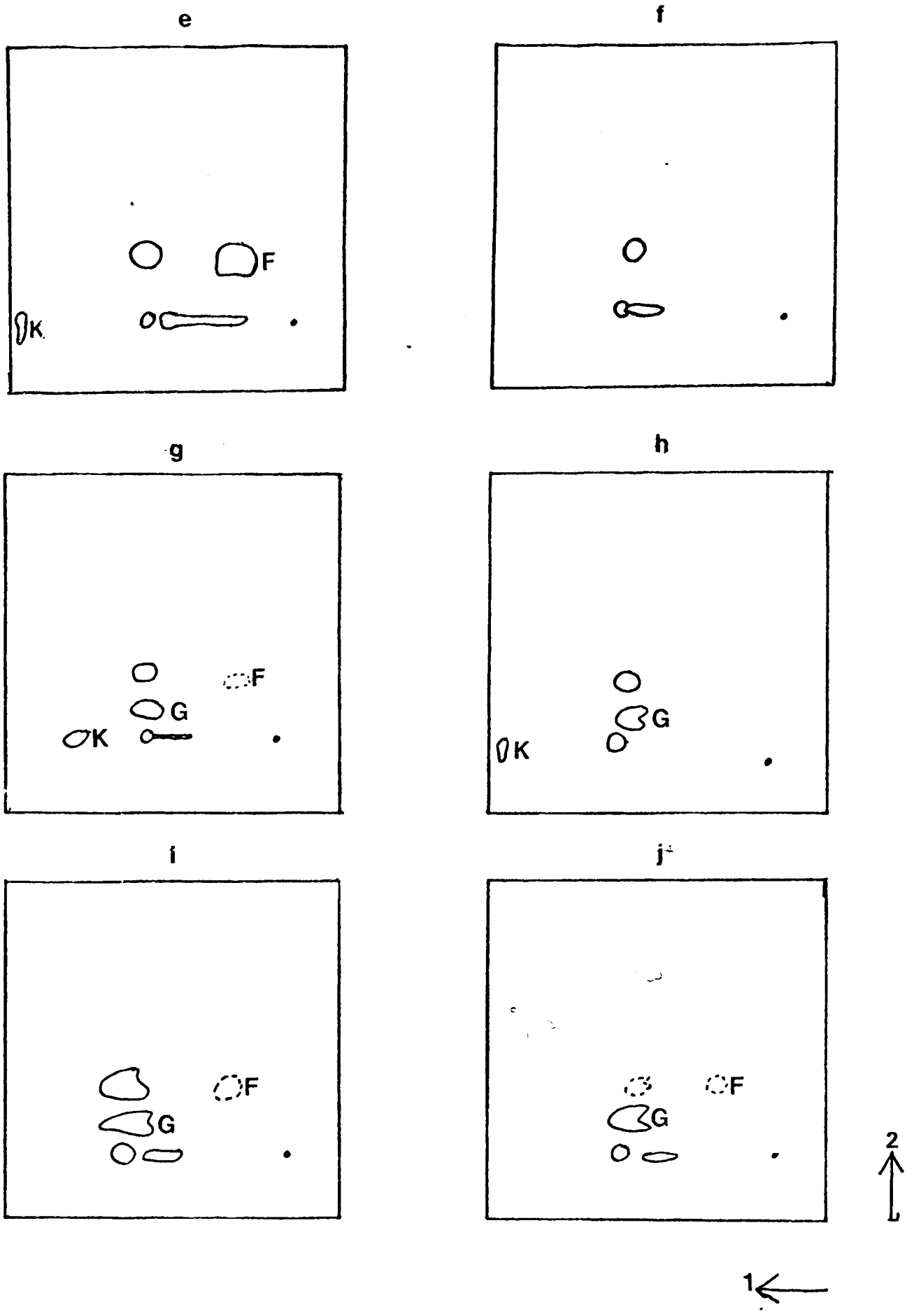


Figure 4.3 Analyses of couplings and deprotection of acyl esters to Boc-insulin derivatives.

SG-81 chromatograph tracing: 1, $N^{\alpha A1}$-Boc-insulin; 4, $N^{\alpha A1}$,$N^{\epsilon B29}$-di-Boc-insulin; 8, insulin; 2,3,5,6,7, mono-Boc-insulin coupled with: 2, 1,3-dipalmitoyl-2-azeloylglycerol-ONSu; 3, nonanoyl-ONSu; 5, stearoyl-ONSu; 6, octanoyl-ONSu and 7, oleoyl-ONSu. Dansyls: a, di-Boc-insulin; c,e, $N^{\alpha A1}$-Boc-insulin; b, di-Boc-insulin coupled with 1,3-dipalmitoyl-2-azeloylglycerol-ONSu; d, mono-Boc-insulin coupled with stearoyl-ONSu; f, mono-Boc-insulin coupled with octanoyl-ONSu; g-j, coupled and deprotected derivatives: g, N^{B1}-(1,3-dipalmitoyl-2-azeloylglycerol)-insulin; h, $N^{\alpha B1}$-nonanoyl-insulin; i, $N^{\alpha B1}$,$N^{\epsilon B29}$-di-oleoyl-insulin; j, $N^{\alpha B1}$;$N^{\epsilon B29}$-di-octanoyl-insulin.

the reaction was monitored by withdrawing a sample (20 to 30 microlitres of 1mM insulin solution), precipitating it and washing it with about 1 to 2 ml of diethyl ether, evaporating the ether and then analysing by chromatography on SG-81 paper. Completion of the reaction was indicated by disappearance of the spot corresponding to the starting material and appearance of a spot corresponding to tri-acyl-insulin (R_f 0.85) which stained weakly, if at all, with ninhydrin/cadmium acetate and strongly with the stain for tyrosine. The reactions, when complete, were stopped by precipitation with ten volumes of diethyl ether and then the precipitate was washed two or three times with ether. The ether washings contained up to 10% of the total protein from couplings of triacylglycerol to insulin or mono-Boc-insulin, obviously this extremely hydrophobic compound is quite soluble in dimethylformamide:ether, (10%, v/v). The ether washings also contain most of the excess lipid. A proportion (10% at most and often less than 5%) of the precipitate is not protein. The ether-washed product was dried and samples taken for analysis by dansylation and chromatography on SG-81 paper. The protected lipophilic insulins were not at all suitable for analysis by isoelectrofocussing because of their insolubility.

Generally the results of these acylations were good. In particular the short-chain acyl esters gave a product of high purity with no detectable free amine groups by dansylation or spots of low R_f on SG-81 paper chromatography. The purity was probably better than 97%, as 3% is the limit of sensitivity of these analytical methods (see Fig.4.3). The products from acylation with long-chain esters, usually contained detectable amounts of partially acylated products, the free amino group usually being phenylalanine the purity was less than 97% (see Fig.4.3). The products of reaction with

triacylglycerol esters were the least pure, often about 10% of free B1 phenylalanine amino groups could be detected and the corresponding amount of incompletely acylated product was seen by SG-81 paper chromatography (see Fig.4.3).

4C Deprotection

Anhydrous acid at low temperature is the best reagent for the removal of tert-butyloxycarbonyl groups from the amino groups of proteins with the minimum of side reactions. Anhydrous trifluoroacetic acid was used and it was always redistilled immediately before use.

The protected, coupled insulin was dissolved in trifluoroacetic acid at a concentration of 1mM, at $4^{o}C$. Some of the more hydrophobic derivatives were slow to dissolve taking 15 to 20 minutes with stirring. To ensure complete reaction the solution was left, after the last of the insulin had completely dissolved, for 45 minutes at $4^{o}C$. The reaction was stopped by precipitation of the insulin with ten volumes of cold diethyl ether and the precipitate was washed two or three times more with ether. None of the derivatives were soluble in the trifluoroacetic acid and ether mixture, but usually the last remaining traces of lipid from the coupling stage were removed by these ether washings. The ether moist pellet was dried by gently blowing with a stream of nitrogen, followed by standing overnight in a desiccator, over sodium hydroxide pellets and concentrated sulphuric acid, under reduced pressure.

4D Synthesis of $N^{\alpha B1}, N^{\epsilon B29}$-di-[1-^{14}C]-Octanoyl-Insulin

The coupling of N-hydroxysuccinimidyl-[1-^{14}C]-octanoate to insulin was performed under the same conditions as the coupling of the unlabelled esters. The overall yield of the ester was about 50% of the starting

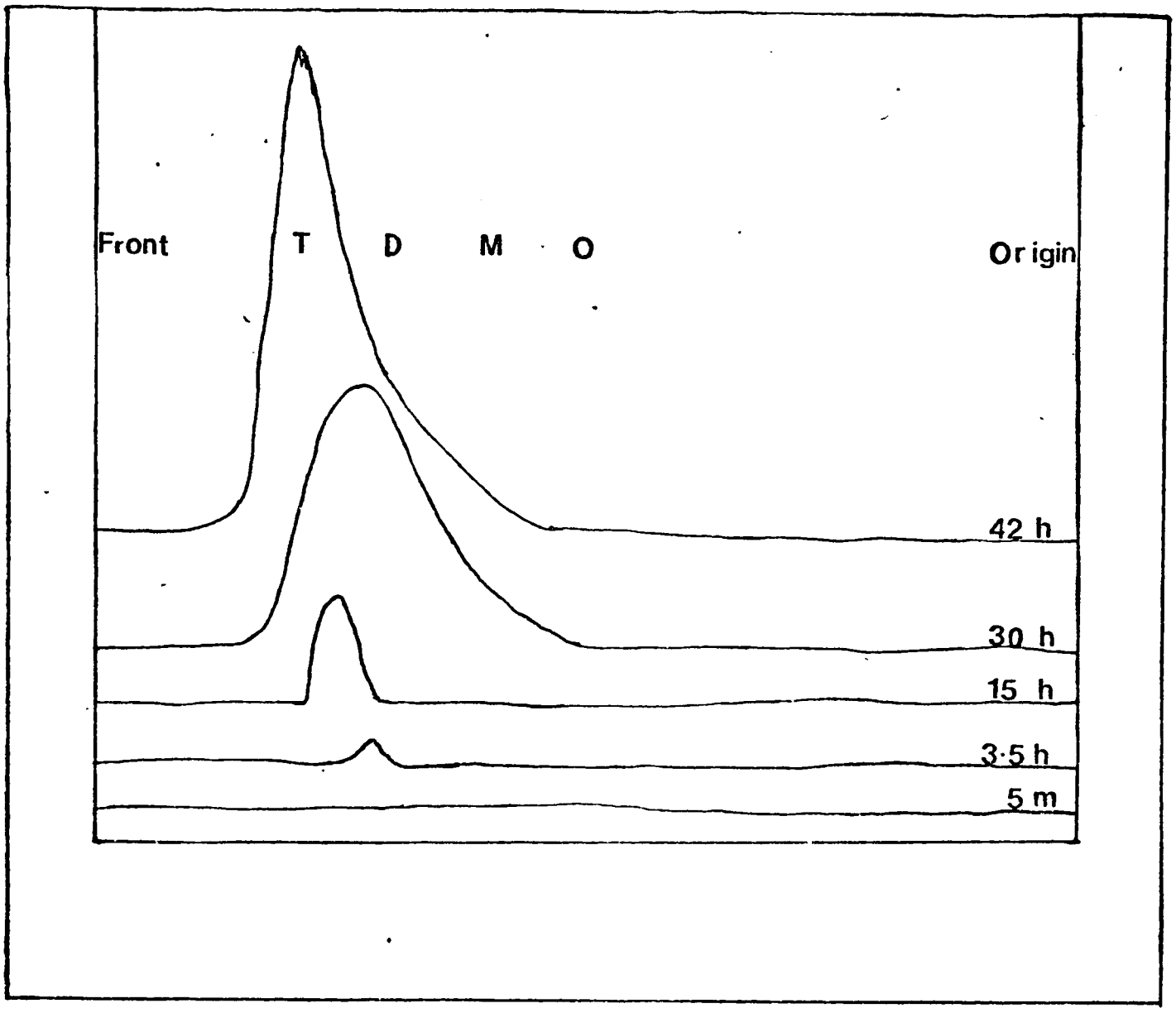


Figure 4.4. Radiochromatogram scanner trace of SG-81 chromatogram of coupling of N-hydroxysuccinimidyl [1-^{14}C]-octanoate with N$^{\varepsilon A1}$-Boc-insulin. R_f of: T, triacylinsulin; D, di-acyl-insulin; M,mono-acyl-insulin and O, insulin.

quantity i.e. 3.9 μmole (125 μCi). 1.4 mg of Boc-insulin (0.25 μmole) was used as amino component. Thus there was almost an eight-fold excess of ester over amino groups. The concentration of insulin was 1mM in dimethylformamide, and 80 microlitres of N-ethylmorpholine per ml was added to adjust the pH to 9.0 to 9.5 as measured on moist pH 8–10 paper. Samples containing 150 μg of protein were taken at 5 minutes, 3.5, 15, 30 and 42 hours. These were precipitated with ether and dried carefully. The samples were chromatographed on SG-81 paper using BAWP as eluant and then examined by the use of the Bertholdt radiochromatogram scanner followed by ninhydrin/cadmium acetate staining and tyrosine staining (Fig. 4.4). No free amino groups were detected on the 42 hour sample, though about 10% of the radioactivity had the R_f value of di-acyl-insulin. The reaction mixture was left a further 16 hours, then precipitated with ten volumes of cold diethyl ether and then washed twice with ether. The dried product was dissolved in 0.5 ml of freshly redistilled anhydrous trifluoroacetic acid and left at 4°C for 45 minutes. The protein was again precipitated with ten volumes of cold ether and washed twice with ether. It was dried and then dissolved in dimethylsulphoxide-saline (1:9, v/v), and sterilized by filtration, ready for use. The optical density and radioactivity of this solution were measured and the specific activity was shown to be 65.8 Ci/mol; the theoretical value for 2 moles of [1-^{14}C]-octanoate per mole of insulin is 63.6 Ci/mol.

4E Purification of Acyl-Insulin Derivatives

4Ei Introduction

The required degree of purification of the acyl-insulin derivatives after coupling and deprotection, depends to some extent on the evaluation of the results of the assays for hypoglycaemic potency and prolonged activity. For any derivative of

degree of acylation n, a derivative of degree of acylation n + 1 is likely to be the less active of the two, and if it is present as a contaminant at only a few per cent, it is unlikely to interfere with the interpretation of the assay. These over acylated products would be formed from the contamination of an n-Boc-insulin with traces of an (n-1)-Boc-insulin. More worrying is the contamination of a derivative with a less acylated product. Di-Boc-insulin usually contains about 2 to 4% of tri-Boc-insulin, deprotection of this and of di-Boc-insulin which had not reacted in the coupling step, leads to the contamination of mono-acyl-insulin with insulin. When the acyl group was derived from short or long chain fatty acids, this contamination varied from undetectable (less than 3%) to just detectable (3% and greater). When the acyl group was the triacylglycerol one, the contamination was always greater than 3% and up to 10%.

To remove traces of insulin from a mono-acyl-insulin preparation, it was washed two or three times with 2 to 3 ml of water which was made just alkaline (pH 8 to 9) by the addition of a drop of dilute ammonia. Dansylation of dried down residues of these alkaline extracts showed the presence of all the amino groups of insulin (phe, gly and lys). SG-81 paper chromatography demonstrated the presence of insulin as well as two other spots staining with ninhydrin/cadmium acetate, which were identified as Tris (R_f 0.3) and urea (R_f 0.7, staining whiter than the background). One of the first di-(triacylglycerol)-insulin preparations was washed twice with water, thrice with methanol, twice with chloroform-methanol (1:1, v/v) and twice with chloroform. All the organic washings contained decreasing amounts of lipid. The chloroform/methanol washings contained traces of protein, in the form of the

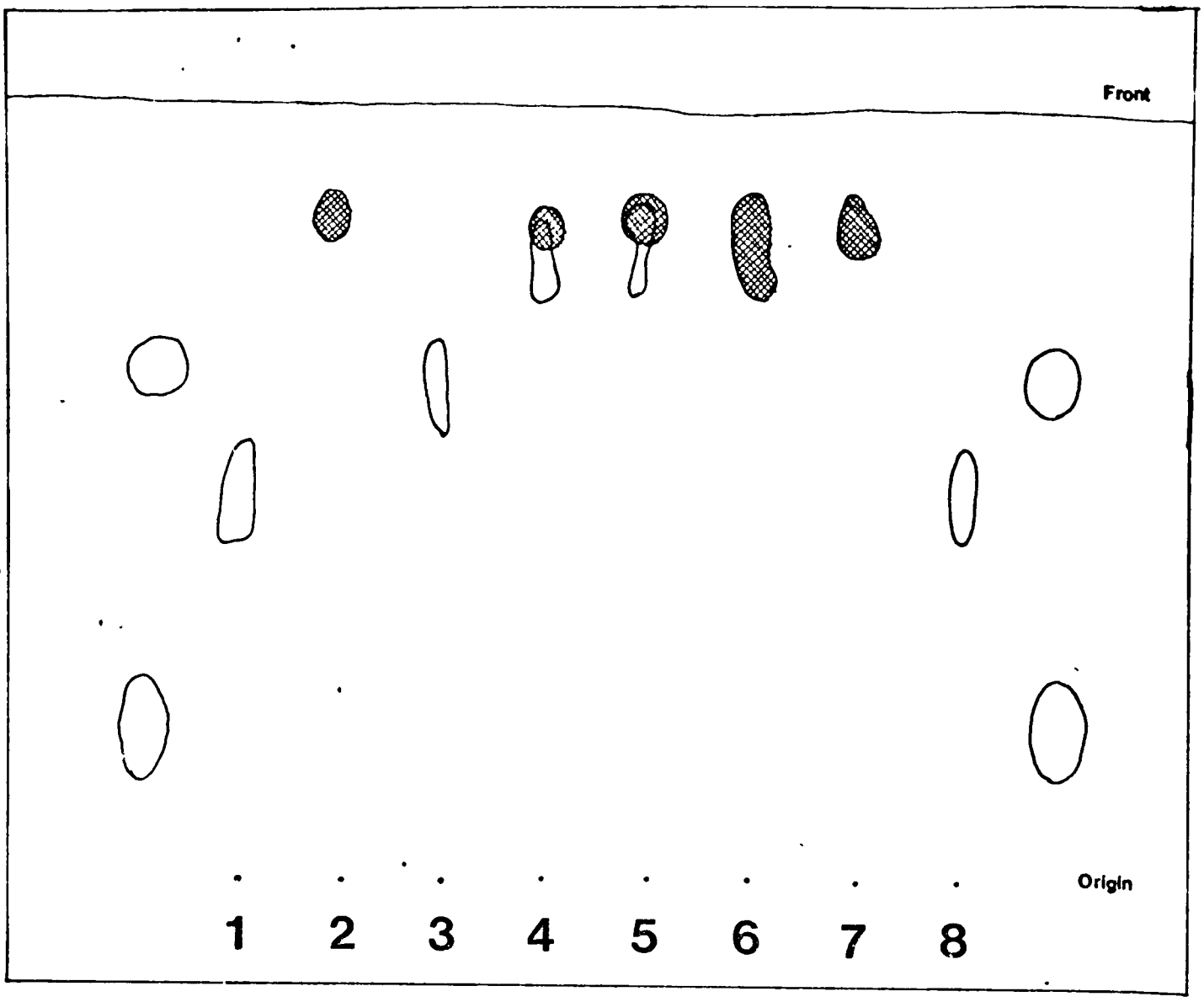


Figure 4.5 SG-81 chromatogram tracing of solvent washings of $N^{\alpha B1}$,$N^{\epsilon B29}$-di-(1,3-dipalmitoyl-azeloylglycerol)-insulin: 1,8, insulin; 2, lipid standard; 3, aqueous extract; 4, methanolic extract; 5, methanol: chloroform extract; 6, chloroform extract; 7, residual solid. hatched spots stain with 2,7-dichlorofluorescein only, open spots stain with ninhydrin/cadmium or tyrosine stain as well.

di-(triacylglycerol)-insulin derivative. This product had an R_f of 0.7 on SG-81 paper chromatography and an amino terminus of glycine as determined by dansylation (see Fig.4.5).

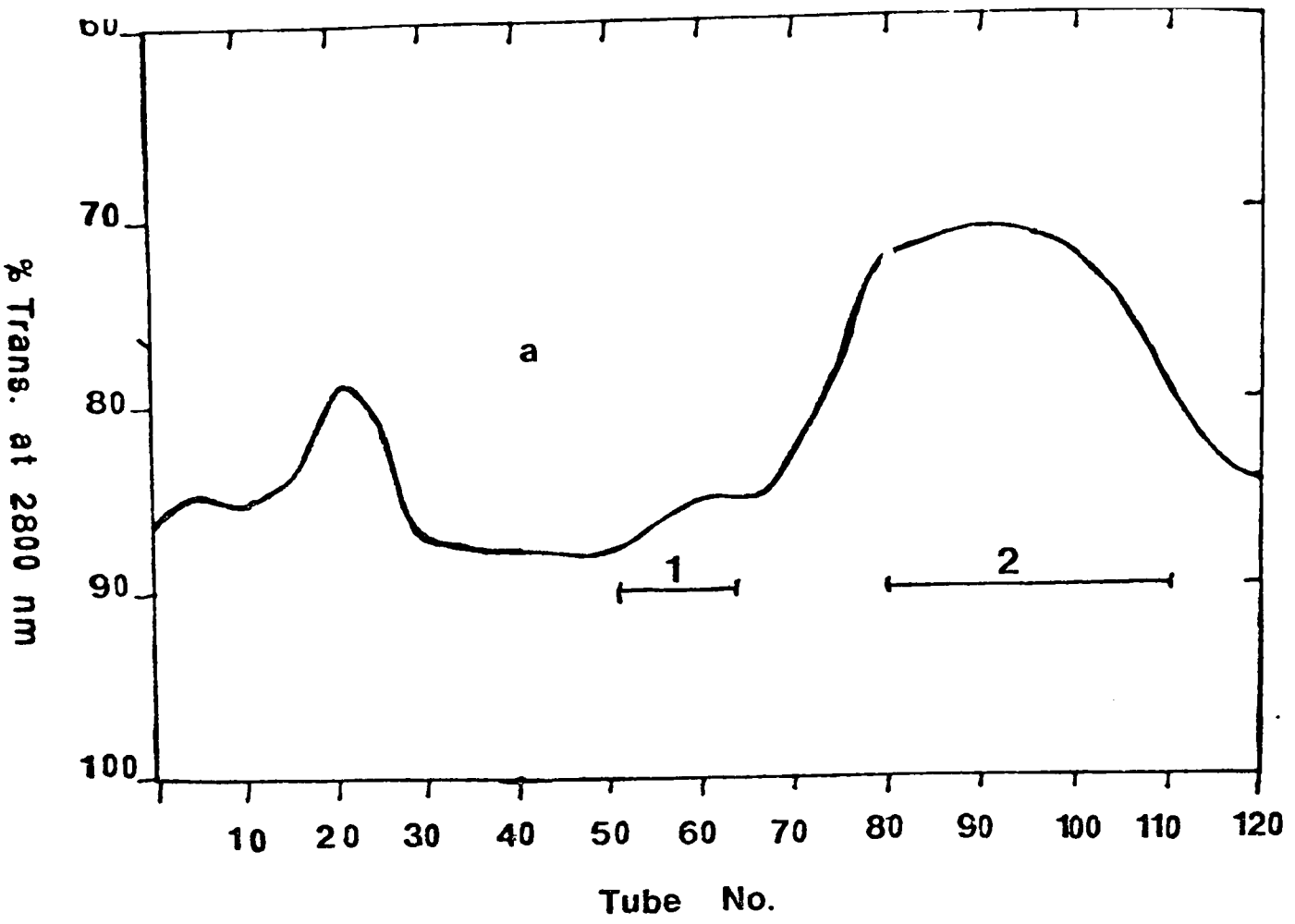
Diethyl ether washing of the precipitate after deprotection was generally sufficient to extract most of the excess lipid in the preparation. Sometimes an extra wash with chloroform was performed if the sample was still contaminated with lipid, as shown by SG-81 paper chromatography. (Lipids stained with 2,7-dichlorofluorescein and had an R_f of 0.9, using BAWP as eluant.)

Generally the reactions proceeded to give a high yield of the desired products and the washing procedures described above were sufficient to remove impurities.

4Eii Partition Chromatography on Sephadex-LH20 or Phenyl-Sepharose-CL-4B

Two of the preparations using N-hydroxysuccinimidyl nonanoate were unsuccessful and partition chromatography (Geiger and Obermeier, 1973) was used to purify the products.

The first preparation was of $N^{\alpha B1}$,$N^{\epsilon B29}$-di-nonanoyl-insulin from mono-$N^{\alpha A1}$-Boc-insulin. After deprotection, the di-acyl-insulin was found to be contaminated with about 20% mono-acyl-insulin. A quantity of Sephadex-LH20 (Pharmacia Ltd.) which had previously been used for chromatography, was washed once with water, once with dimethylformamide, once with acetone, three times with water and then lyophilised, in order to remove any contamination from previous use. Fifteen grammes of this powder were stirred for 30 minutes with 19 ml of the upper phase from water:n-butanol:acetic acid (10:2:1, by volume) and then suspended in 80 ml of the lower phase from the same mixture, which was used as eluant, and stirred for a further 3 hours.



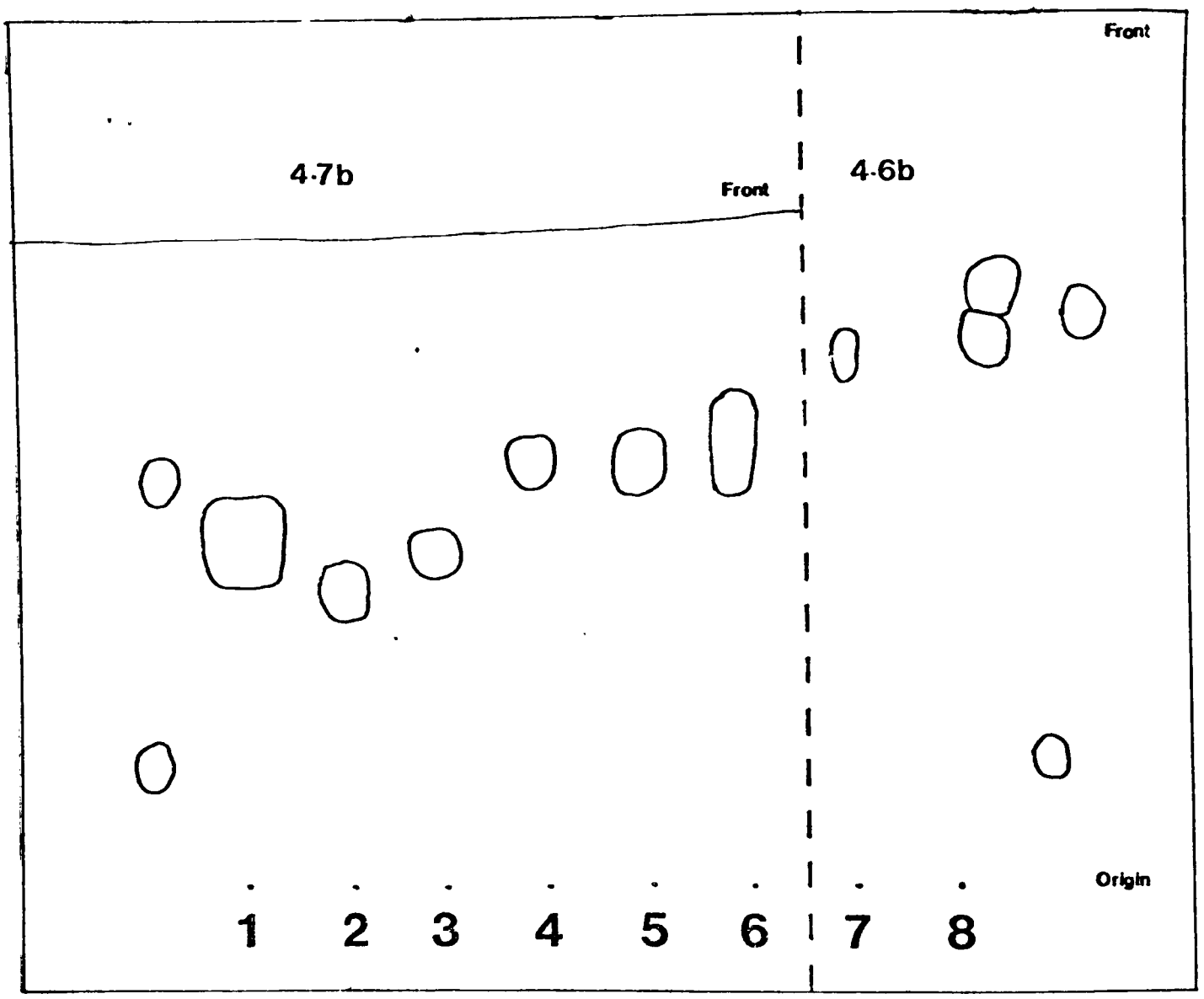


Figure 4.6 Separation of nonanoyl-insulin on Sephadex-LH20: a, trace of transmittance of eluate at 280 nm; b, SG-81 chromatogram of products; 7, fraction 1 and 8, fraction 2.

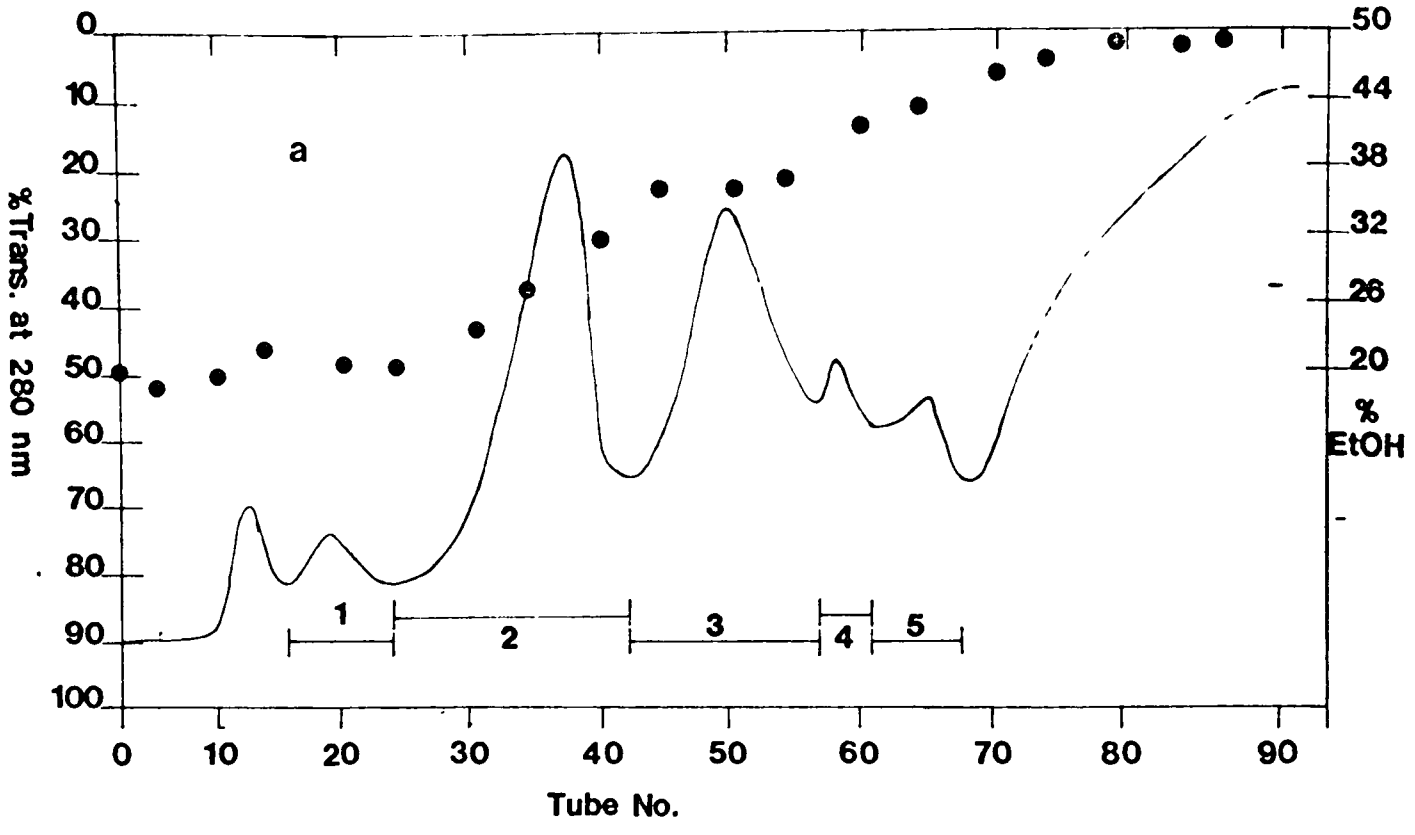


Figure 4.7 Separation of nonanoyl-insulin on Phenyl-Sepharose: a, trace of transmittance of eluate at 280 nm, continuous line; percentage of ethanol; b, SG-81 chromatogram of products: 1, mono-Boc-insulin; 2, fraction 1; 3, fraction 2; 4, fraction 3; 5, fraction 4; 6, fraction 5.

This was packed into a column, 1.3 x 30 cm, (volume 40 ml) and the eluant was pumped through at 140 ml per hour for 6 hours and then 40 ml per hour for a further 4 hours. The protein sample (10 mg) was dissolved in 1 ml of eluant and loaded onto the column. The column was eluted at 20 ml per hour and 150 fractions of about 0.7 ml were collected. The separation was poor (see Fig.4.6) but dividing the peaks as shown gave a yield of about 6 mg of di-nonanoyl-insulin with no detectable mono-nonanoyl-insulin as assayed by SG-81 paper chromatography.

The second preparation was worse, containing a 1:1 mixture of mono- and di-nonanoyl-insulin. Phenyl-Sepharose-CL-4B 40 ml was suspended in 60 ml of 1% aqueous ammonium bicarbonate and packed in a 1.3 x 30 cm column (volume 40 ml). It was equilibrated for 2 hours, at a flow rate of 40 ml per hour, with an eluant of 1% ammonium bicarbonate in 20% aqueous ethanol, pH 7.85 and conductivity 7.7×10^{-3} $ohm^{-1}cm^{-1}$. 75 mg of protein was loaded in 12 ml of this eluant and 20 fractions of 4.8 ml were collected with this starting eluant. A linear gradient was then set up with 150 ml of starting eluant and 150 ml of 50% aqueous ethanol as final eluant. The separation by this method was good and several peaks were found. On analysis by SG-81 paper chromatography and dansylation, peak 1 was found to be insulin, peak 2 was a mixture of $N^{\alpha A1}$- and $N^{\epsilon B29}$-mono-nonanoyl-insulin and peak 3 was the desired di-nonanoyl-insulin. Total yield of all species from the column was 59% (see Fig.4.7).

4F Synthesis of Ficoll-Insulin Derivative

To an aqueous 10%-oxidized-Ficoll solution containing 43 μM aldehyde groups, 1.1 μmole of insulin (3.3 μmol of amino groups) was added, the pH was

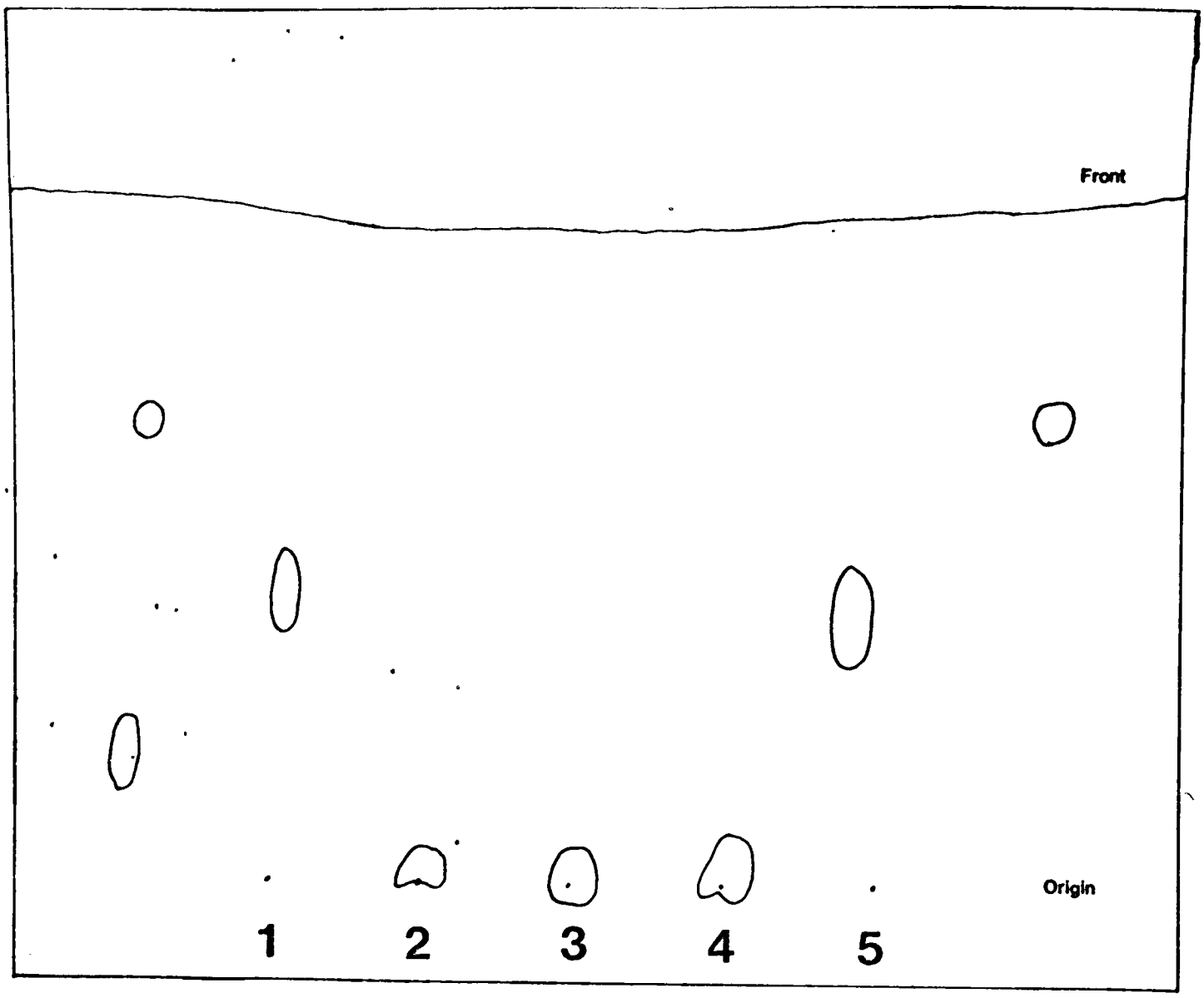


Figure 4.8 SG-81 chromatogram of Ficoll-insulin: 1,5, insulin; 2,30 ug; 3, 100 ug and 4, 300 ug of insulin in Ficoll-insulin sample.

adjusted to 9.7 with sodium carbonate and this was left for 16 hours at 37°C. The solution was dialysed against three changes of 5 l of water, to remove the carbonate, and lyophilised. A sample of this product had an R_f of 0.0 on SG–81 paper chromatography, staining with ninhydrin/cadmium acetate and for tyrosine (see Fig.4.8). No further purification was performed. Dansylation showed the presence of phenylalanine and glycine amino groups and very little or no lysine.

Chapter 5

Results

5A Introduction

The results of the biological assays of the modified-insulin derivatives have been examined with two null hypotheses in mind.

In the case of the assays by intravenous administration the null hypothesis was that there is no statistically significant difference between the blood-glucose-depression curves from the assays of normal insulin and from the assays of the modified-insulin derivatives over the latter half of the assays, i.e. from the time when the curves of the normal-insulin assays have returned to the initial blood-glucose values.

In the case of the assays by peroral administration, the null hypothesis tested was that there was no difference at any time after the start of the experiment between the normal-insulin assay curves and the modified-insulin assay curves.

Three statistical methods were used to test these hypotheses. They were the Student's 't'-test and Fischer's analysis of variance used on the whole data set together, and the Student's 't'-test used on paired sets of data.

For the assays by intravenous administration, the control set of data for the statistical examination was, initially, all of the assay curves of normal insulin that had been performed. After a preliminary examination, it was found that there was apparently no statistically significant difference between the assay curves for normal insulin and those of modified insulin. This was due to the presence in the control set of a few assay curves corresponding to doses which produced a very substantial blood-glucose depression

and produced a prolongation of activity due to overdosing. These made the variance at the later time points very large. As it would be wrong to compare assays of modified-insulin derivatives which gave small blood glucose depressions with assays of normal insulin which gave a prolongation of activity due to overdosing, these were excluded from the control set. Two other standard-insulin assay curves were excluded, in both cases because of difficulty in blood sampling during the experiment, it was modified in a way that might possibly invalidate the results. There was also a set of blank assay curves, in which only dimethylsulphoxide/saline (at various concentrations) was injected.

Using the paired Student's 't'-test, assay curves of the modified-insulin derivatives from each individual rabbit were compared with assay curves of normal insulin from the same rabbit. This method helps to reduce error due to the variation from animal to animal.

All these methods give broadly similar results, which helps to increase confidence in them. The paired 't'-tests produced results in which the significance levels were greater. This is to be expected if the difference between the animals was a major contribution to the overall variance.

For the assays by oral administration, the entire set of assay curves of normal insulin was used as the control set. Also there was a set of blank assay curves.

A preliminary examination of the results from the control set showed that for either the intravenous or the oral assays, there was no significant difference between the blank assay curves and the normal-insulin assay curves, over the times of interest. Nor was there any difference between the control assays before and after the

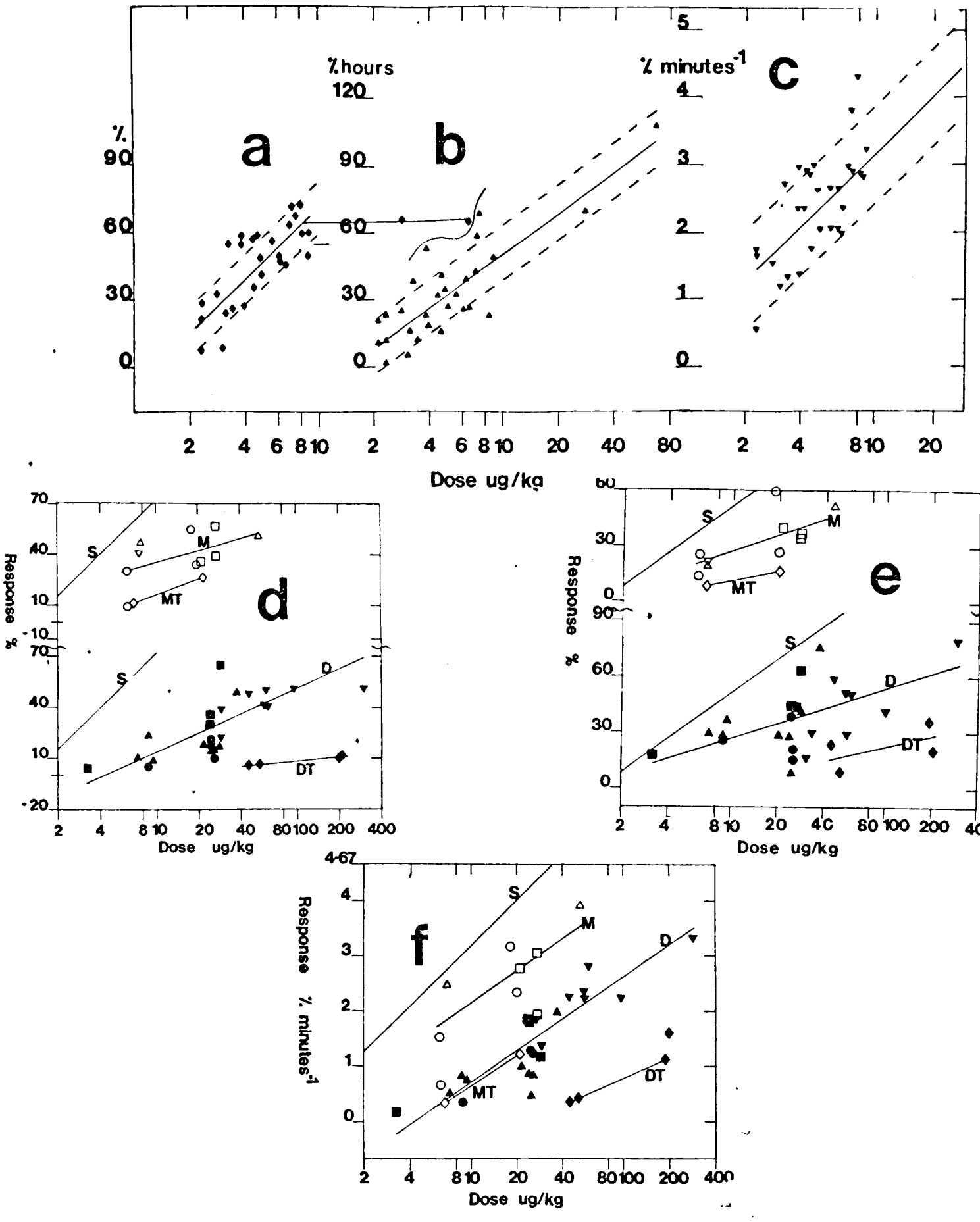


Figure 5.1 Log(dose)-response curves of normal and modified-insulin bioassays: Normal insulin assays as measured by a, nadir of blood-glucose depression; b, time-integrated blood-glucose depression and c initial rate of blood-glucose depression. Modified insulin assays as measured by d, nadir of blood glucose depression; e, time-integrated blood-glucose depression and f, initial rate of blood-glucose depression. Regression lines of: S, normal insulin; M, mono-acyl-insulin derivatives; MT, mono-(triacylglycerol)-insulin derivative; D, di-acyl-insulin derivative and DT, di-(triacylglycerol)-insulin derivative. Acyl groups: o ● ,octanoyl; ▲ nonanoyl; □ ■ stearoyl; ▽ ▼ nonanoyl/triacyl-glycerol and ◊ ◆ triacylglycerol; open characters for mono-acyl derivatives and closed characters for di-acyl derivatives.

commencement of use of the blood-glucose auto-analyser for blood-glucose analysis, and the slight differences in experimental procedure caused by its use.

It should be emphasized that all this work is regarded as a preliminary, screening test. Any highly significant indication of success would have been firmly established by the assays. But, at the outset, it seemed more likely that the tests would serve only to indicate which derivatives should be used for more extensive testing, in larger groups of both normal and diabetic animals. This was found to be the case.

All the values of 'P' given on the diagrams are those derived from the paired comparisons using the Students 't'-test.

5B Assays by Intravenous Administration

5Bi Potency of Normal Insulin

First of all, it was necessary to construct the log(dose)-response curve, in order to find the range of response that is within the linear portion of the curve. It is necessary to avoid very high doses (from the end of the linear part of the log(dose)-response curve to the dose at which convulsions and death occur), because in this dose range even normal insulin gives an apparent prolongation of activity. Although the half-time of insulin in the body is short (about 5 to 10 minutes) the massive non-physiological doses in this range are such that even after many half-times, the concentration of the drug still exceeds the concentration needed to produce a measurable effect. The ratio of the lethal dose to the effective dose is quite large, so that large overdoses can be tolerated without fatal consequences.

The response of the rabbit to insulin administration is usually measured by the nadir of the blood-glucose depression curve (which

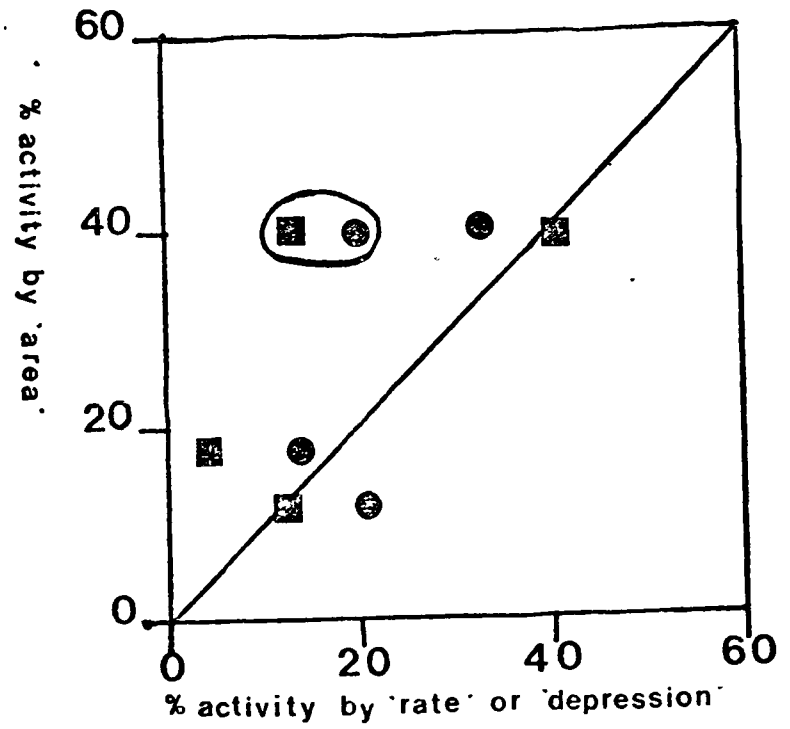


Figure 5.2 Graph of activity of acyl-insulin derivatives as measured by 'area' measurement compared with activities as measured by 'rate' (squares) or 'depression' (circles). Encircled points are those for di-acyl-insulin derivatives.

will be called 'depression' from now on), or by the initial rate of depression of the blood glucose concentration (which will be called 'rate' from now on). However when comparing the response of normal insulin with that of a modified-insulin derivative which has prolonged action, the time integrated blood-glucose depression, i.e. the area between the blood-glucose depression curve and a blank assay curve (which will be called 'area' from now on) is a better measure of the response. Consequently the correlation between log(dose) and response, as measured by these three methods, was calculated (see Fig.5.1). The correlation coefficients for all the log(dose)-response curves are about 0.8 ((r = 0.84, n = 27 (area); r = 0.80, n = 29 (rate) and r = 0.76, n = 27 (depression)), which are all statistically significant at better than the 0.1% level. The residual deviation of the curves is 12.3 (area); 35.1 (rate) and 11.4 (depression), with indices of precision of 2.6, 7.0 and 2.0 respectively. All the log(dose)-response have values of zero response at a dose of about 1.4 ug/kg body weight. The 'depression' curve levels out at a value of about 65% to 70% (i.e. the actual blood glucose concentration will not fall below 1.0 to 1.5mM until convulsions and possibly death occur). The 'area' and 'rate' curves are linear over the whole measured range up to the highest measured response at a dose of 65 μg (about 1.6 U) per kg of body weight.

5Bii Potency and Prolonged Activity of Modified-Insulin Derivatives

Five $N^{\alpha B1}$-acyl-insulin derivatives were tested. The acyl groups modifying the insulin were octanoyl, nonanoyl, stearoyl, 1,3-O-dipalmitoyl-2-O-azeloylglycerol and mixed nonanoyl/triacylglycerol. Five $N^{\alpha B1}$,$N^{\epsilon B29}$-di-acyl-insulin derivatives were tested, the same acyl groups being used.

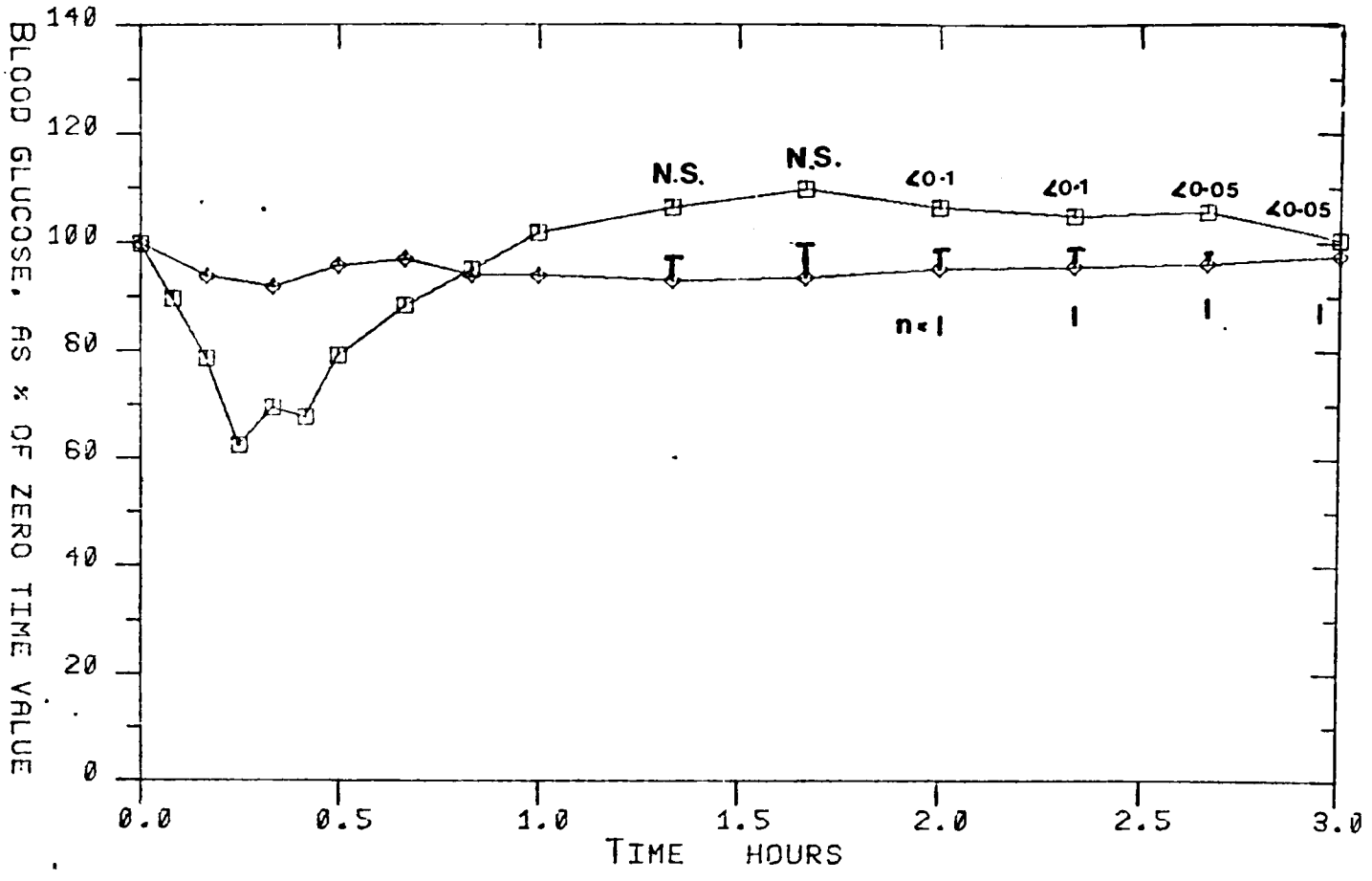


FIGURE 5.3E I.V. INSULIN ASSAY.
□ INSULIN STANDARD, ◇ ($N^{\alpha B1}$,$N^{\epsilon B29}$)-DI-OCTANOYL-INSULIN.
P AND DEGREE OF FREEDOM AT EACH POINT AFTER 60 MINUTES.

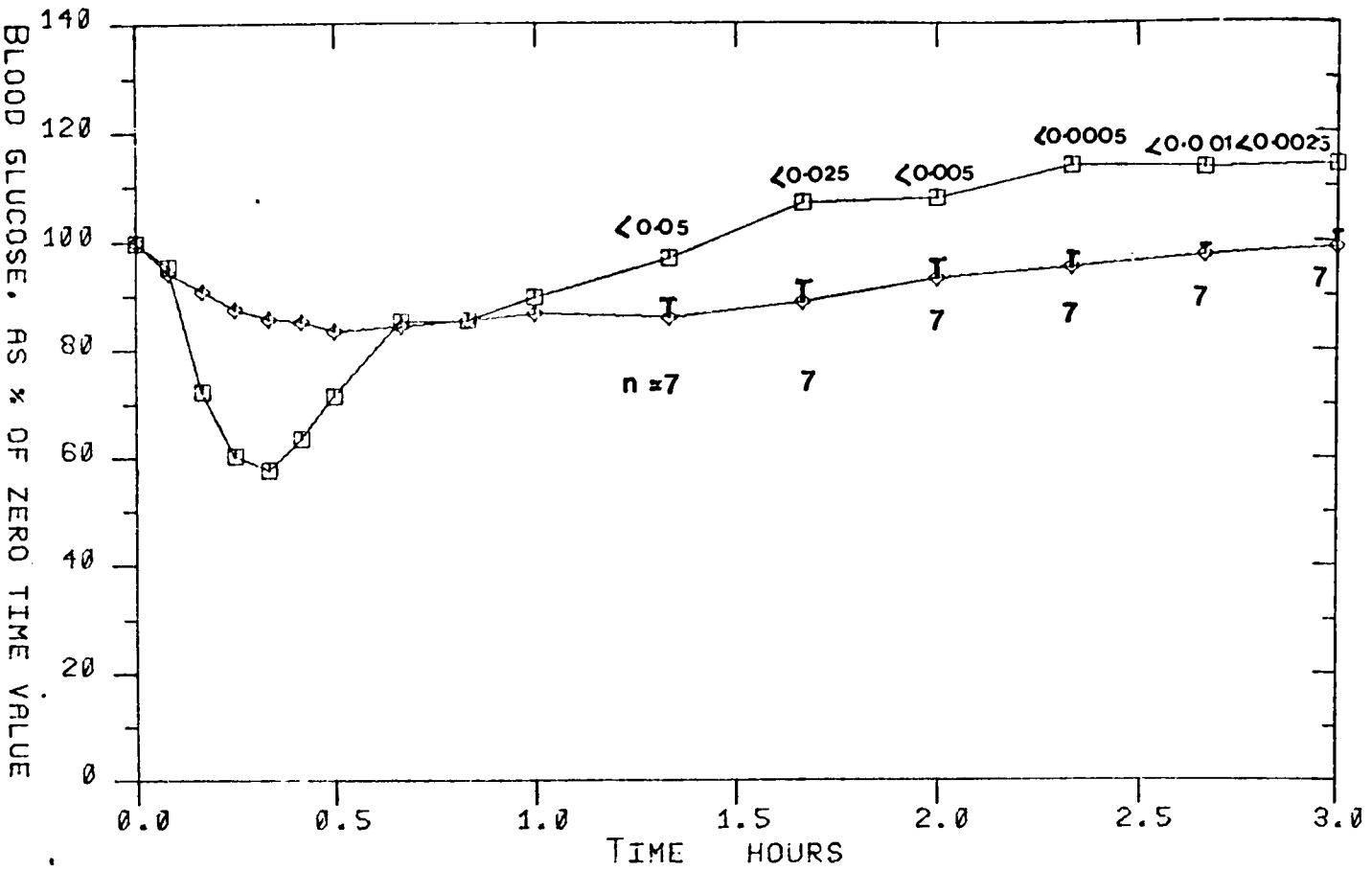


FIGURE 5.3C I.V. INSULIN ASSAY.
□ INSULIN STANDARD, ◇ ($N^{\alpha B1}$,$N^{\epsilon B29}$)-DI-NONANOYL-INSULIN.
P AND DEGREE OF FREEDOM AT EACH POINT AFTER 80 MINUTES.

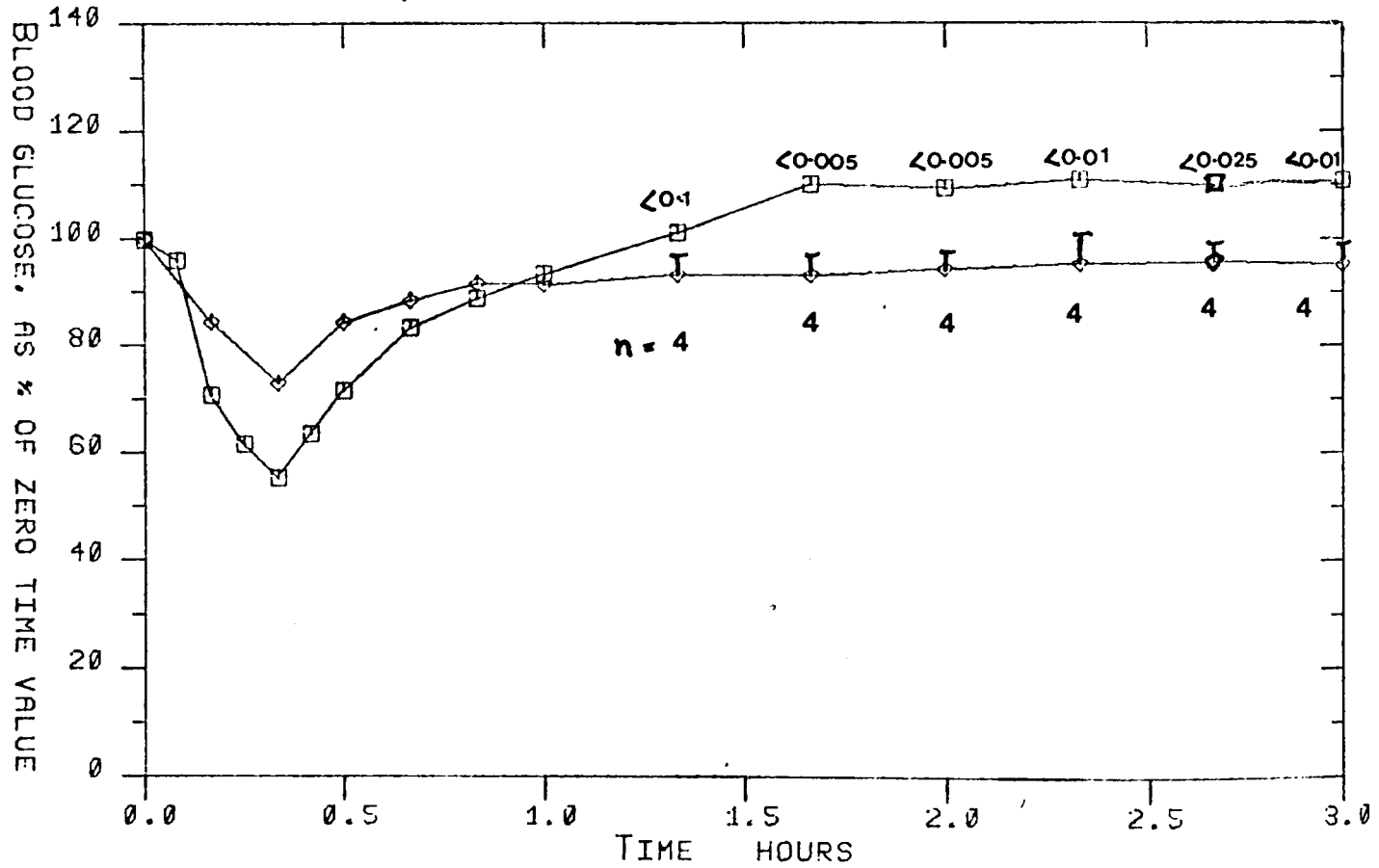


FIGURE 5.3D I.V. INSULIN ASSAY.
□ INSULIN STANDARD, ◇ ($N^{\alpha B1}$,$N^{\epsilon B29}$)-DI-STEAROYL-INSULIN.
P AND DEGREE OF FREEDOM AT EACH POINT AFTER 60 MINUTES.

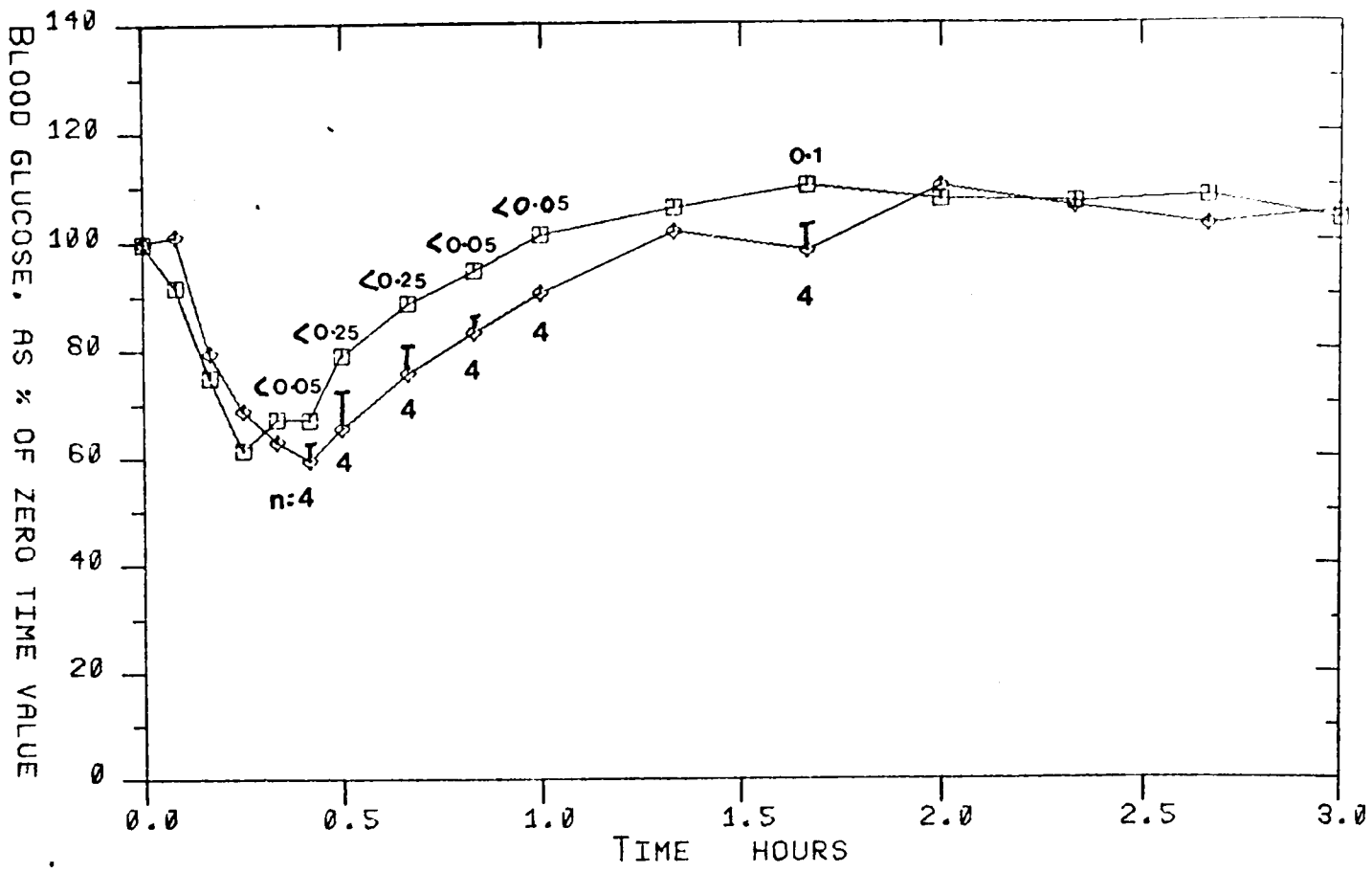


FIGURE 5.3A I.V. INSULIN ASSAY.
□ INSULIN STANDARD, ◇ ($N^{\alpha B1}$,$N^{\epsilon B29}$)-DI-(TRIACYLGLYCEROL/NONANOYL)-INSULIN.
P AND DEGREE OF FREEDOM FOR EACH POINT AFTER 30 MINUTES

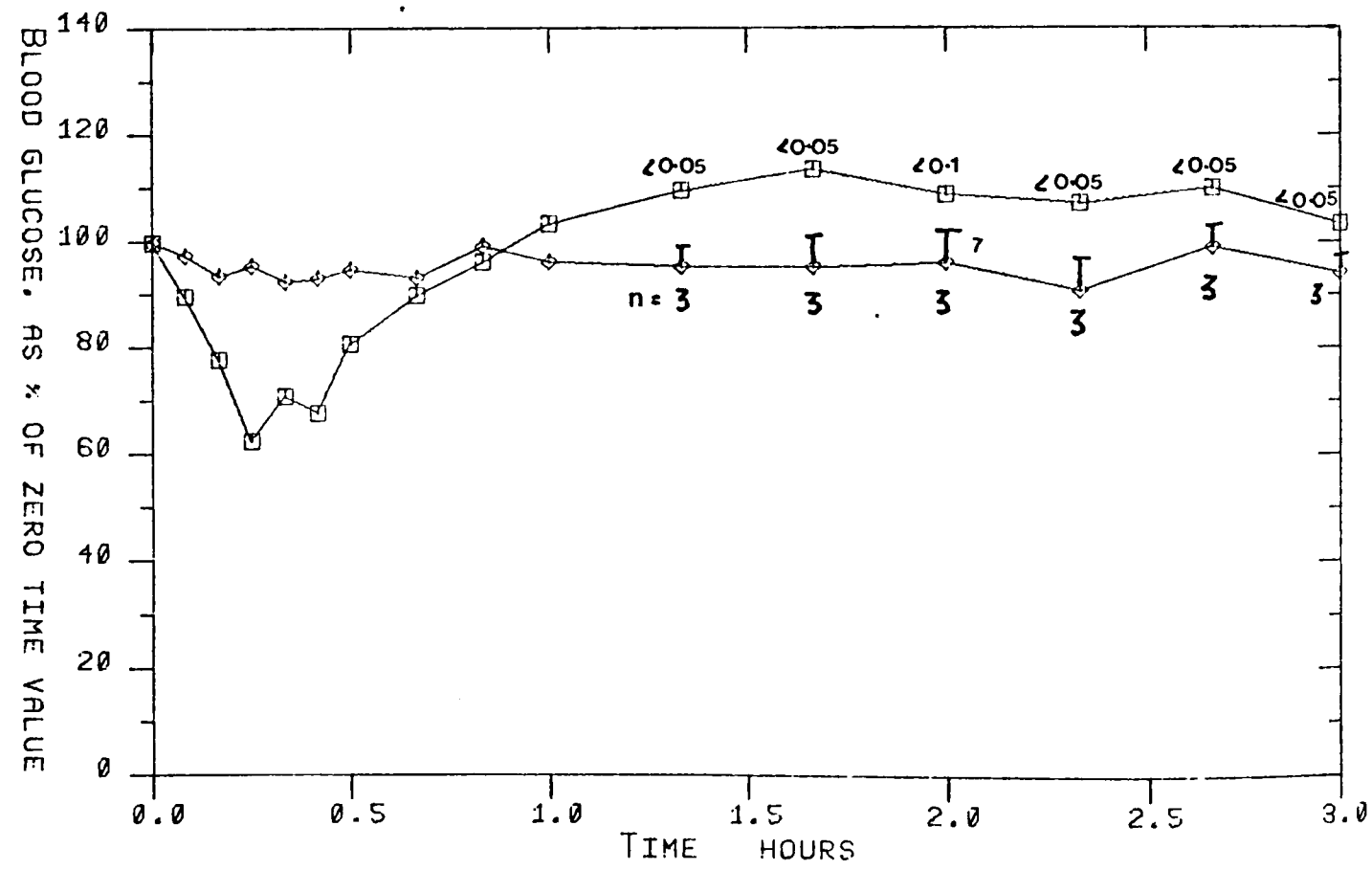


FIGURE 5.3B I.V. INSULIN ASSAY.
□ INSULIN STANDARD, ◇ ($N^{\alpha B1}$,$N^{\epsilon B29}$)-DI-(TRIACYLGLCEROL)-INSULIN.
P AND DEGREE OF FREEDOM AT EACH POINT AFTER 60 MINUTES

When the log(dose)-response curves for these derivatives are compared with the controls, they are significantly different. When the response is measured by 'depression', the slopes of the log(dose)-response curve of the mono- and di-acyl-insulin and the mono-(triacylglycerol)-insulin derivatives are about one half, and the slope of the log(dose)-response curve of the di-(triacylglycerol)-insulin derivative is about one tenth of the slope of the log(dose)-response curve of normal insulin. When measured by 'area', the slopes for all the modified insulin derivatives are between about one half and one third of the control slope. This means that it is not possible to calculate a figure for the activity of the modified insulin derivatives, using these criteria, as this calculation depends on the log(dose)-response curves of the derivatives being parallel with the log(dose)-response curve of normal insulin. The measured dose ranges of the modified-insulin and normal-insulin assays overlap at about 10 μg/kg. A very approximate estimate of the activities can be gained by comparing the dose of normal insulin needed to give the same response as a dose of 10 μg/kg of modified insulin. By this criterion, when measured by depression, the mono-acyl-insulin derivatives have 33% activity (95% fiducial limits 26% to 45%, n = 9), the mono-(triacylglycerol)-insulin derivative has 21% activity (n = 1), the di-acyl-insulin derivatives have 20% activity (17% to 25%, n = 23) and the di(triacylglycerol)-insulin derivative has 13% activity (9% to 19%, n = 3). When measured by 'area', the mono-acyl-insulin derivatives have 40% activity (30% to 55%, n = 9), the mono-(triacylglycerol)-insulin derivative has 12% activity (n = 1), the di-acyl-insulin derivatives have 40% activity (32% to 49%, n = 23) and the di-(triacylglycerol)-insulin derivative has 18%

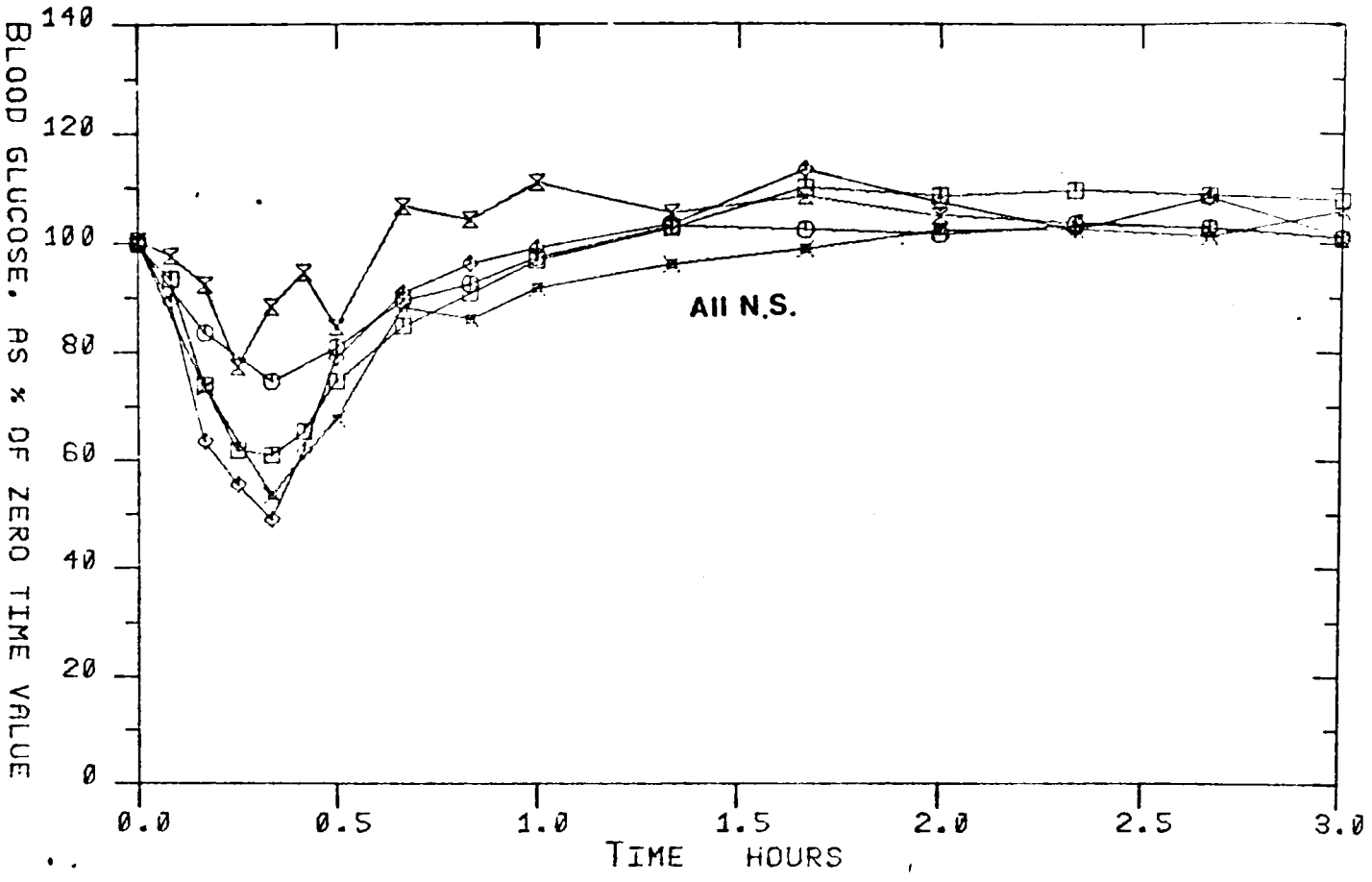


FIGURE 5.4 I.V. INSULIN ASSAYS

□ INSULIN STANDARD, ($N^{\alpha B1}$)-MONO-ACYL-INSULINS: ○ OCTANOYL, ◇ NONANOYL, ✕ STEAROYL, ⧖ TRIACYLGLYCEROL,

P AND DEGREE OF FREEDOM AT EACH POINT AFTER 60 MINUTES

activity (9% to 40%, n = 3). (Figure 5.1 d,e.)

Using the rate of blood glucose depression as response, the log(dose)-response curves are more nearly parallel. The slopes of the curves only differ by about 25%, from the log(dose)-response curve of normal insulin. The activities by this criterion are 41% (27% to 65%, n = 8) for the mono-acyl-insulin derivatives, 13% (11% to 15%, n = 22) for the di-acyl-insulin derivatives and 12% (n = 1) for the mono-(triacylglycerol)-insulin derivative and 4% (2% to 6%, n = 3) for the di-(triacylglycerol)-insulin derivative.(Fig 5.1f)

To summarize, the activities as measured by initial response (i.e. maximal depression or rate of depression of blood glucose) approximately correlate with each other (see Fig.5.2). However when the overall response (i.e. the time integrated depression of blood glucose) is measured, the di-acyl-insulin derivatives are anomalous, with a higher activity than would be expected by comparison with the other responses. This is the first piece of evidence suggesting that these derivatives have a prolonged activity in vivo.

When the blood glucose depression curves are considered this effect is substantiated. Generally, with the normal-insulin assay curves the blood-glucose concentration returns to the initial level between 60 and 100 minutes after the administration of insulin. Consequently from this time until the end of the experiment if the blood-glucose concentration for the normal-insulin and modified-insulin assay curves are compared, any significant differences will imply that the modified insulin will exert a significantly, prolonged, hypoglycaemic effect.

When the results are compared in this method using Student's 't' test and analysis of variance on the whole set of controls and with a Student's 't' test on paired data, it can be seen that the

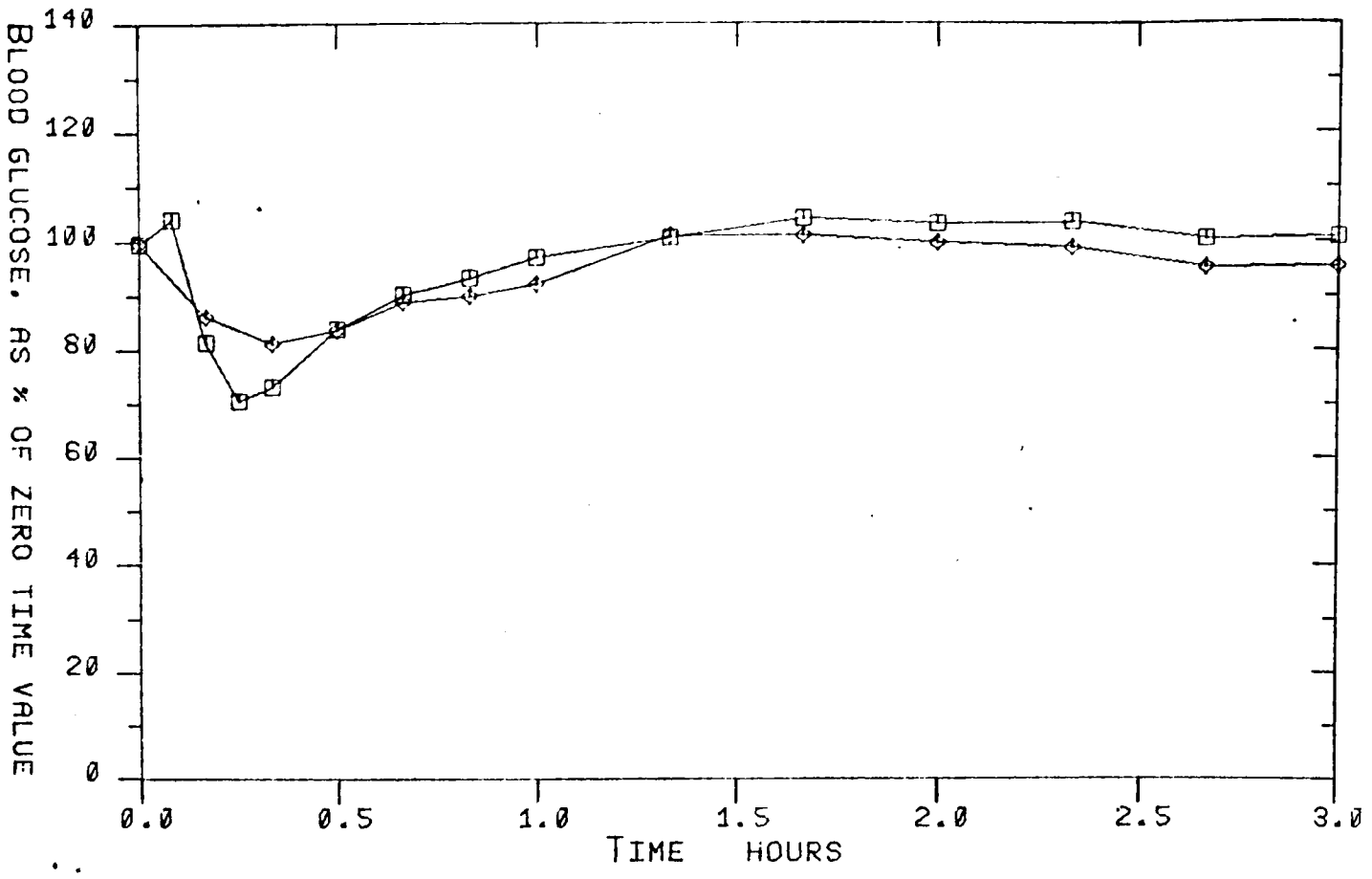


FIGURE 5.5 I.V. INSULIN ASSAY.
INSULIN STANDARD, OXIDISED-FICOLL INSULIN.
ALL DIFFERENCES ARE NOT SIGNIFICANT. N=4.

di-acyl-insulin derivatives are significantly better at lowering blood glucose for a longer period than normal insulin (see Fig. 5.3a,b,c,d). In all cases the last four or six time points have blood glucose concentrations significantly lower, when compared by the paired test. The results when analysed by the other statistical methods are nearly as good, the variance between animals (which is partly excluded in the paired test) leading to lower significance levels.

The blood-glucose levels produced by all of the mono-acyl-insulin derivatives and the di-(triacylglycerol)-insulin derivatives are not significantly lower than the levels for standard insulin at these times (see Fig.5.4).

The blood-glucose curves produced by the di-(triacylglycerol)-insulin derivative was not significantly altered by the concomitant administration of heparin by intravenous injection. When the animal was fed after the administration of the di-(triacylglycerol)-insulin or the experiment was performed with the animal in a fed state, the blood glucose levels were not significantly different from those of a fasted assay curve. The blood-glucose assay curves produced by the mono-(triacylglycerol)-insulin derivatives were not distinguishable from the short chain mono-acyl-insulin derivatives, apart from the lower activity.

The log(dose)-response of the Ficoll-insulin derivative, gave a potency of about 5% as measured by all the three responses. There was no significant prolonged activity, the blood sugar curves being indistinguishable from those of normal-insulin (see Fig.5.5).

5Biii Pharmacokinetics of Radioactively-Labelled Insulin Derivative

First of all some experiments were performed

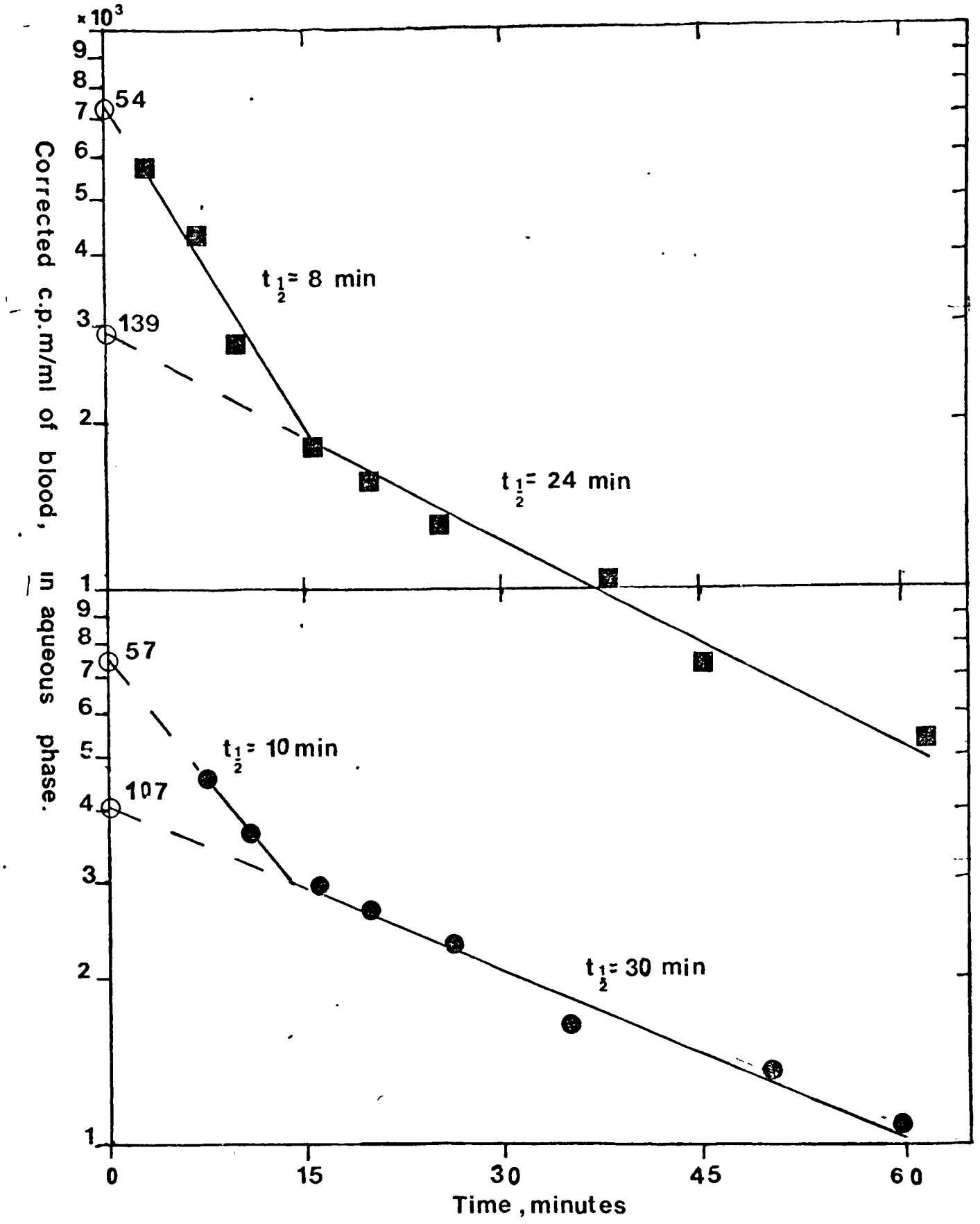


Figure 5.6 Kinetics of loss of radioactivity from the blood after injection of about 2×10^6 c.p.m. of $N^{\alpha B1}, N^{\epsilon B29}$-di- 1-^{14}C -nonanoyl -insulin into two rabbits.

by extracting 1 ml of whole blood or blood serum with 0.5 ml of chloroform. These showed that the chloroform extracted greater than 95% of an added aliquot of [1-^{14}C]-octanoate and less than 5% of an added aliquot of $N^{\alpha B1}$,$N^{\epsilon B29}$-di-[1-^{14}C]-octanoyl-insulin. Thus one can be sure that label in the plasma after extraction, probably represents insulin derivative, or its proteinaceous degradation products rather than fatty acid.

The results of the two experiments performed were very similar (see Fig.5.6). The amount of radioactivity in the chloroform phase was low, just above background, and constant with time. In the plasma there is a biphasic loss of label. The first rapid phase has a half-time of 8 to 10 minutes, and extrapolation to zero time gives a distribution volume of 54 to 57 ml/kg. The second slower decay has a half-time of 24 to 30 minutes and the intercept corresponds to a distribution volume of 107 to 139 ml/kg.

5C Assays by Peroral Administration

The assay method used to measure the effect of the insulin derivatives on peroral administration is not the most sensitive possible. The method of instilling the preparation, in solution, into surgically-exposed loops of intestine or stomach, would be better to compare the rates of absorption of normal and modified insulin. This method is however rather pointless if the derivatives when administered perorally are going to be destroyed by gastric or duodenal proteases, before they can reach the intestine for absorption. An artificially-diabetic animal is probably a better model. As it has no exogenous insulin, any insulin that is absorbed is more likely to reduce hyperglycaemia.

But in order to be able to compare any positive results from this method of administration with the intravenously administered

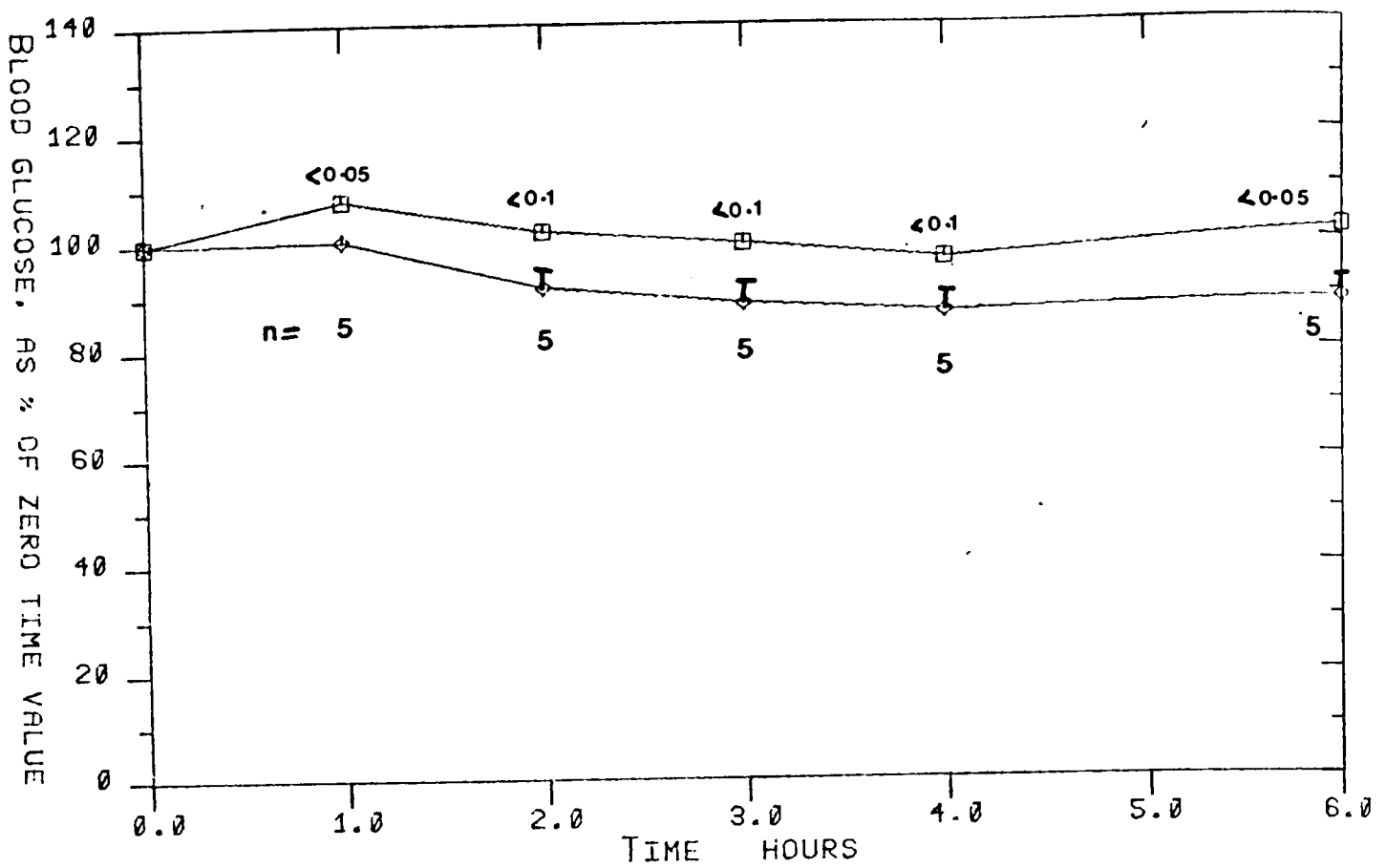


FIGURE 5.7A PERORAL INSULIN ASSAY.
□ INSULIN STANDARD, ◇ ($N^{\alpha B1}$)-MONO-NONANOYL-INSULIN.
P. AND DEGREE OF FREEDOM AT EACH POINT

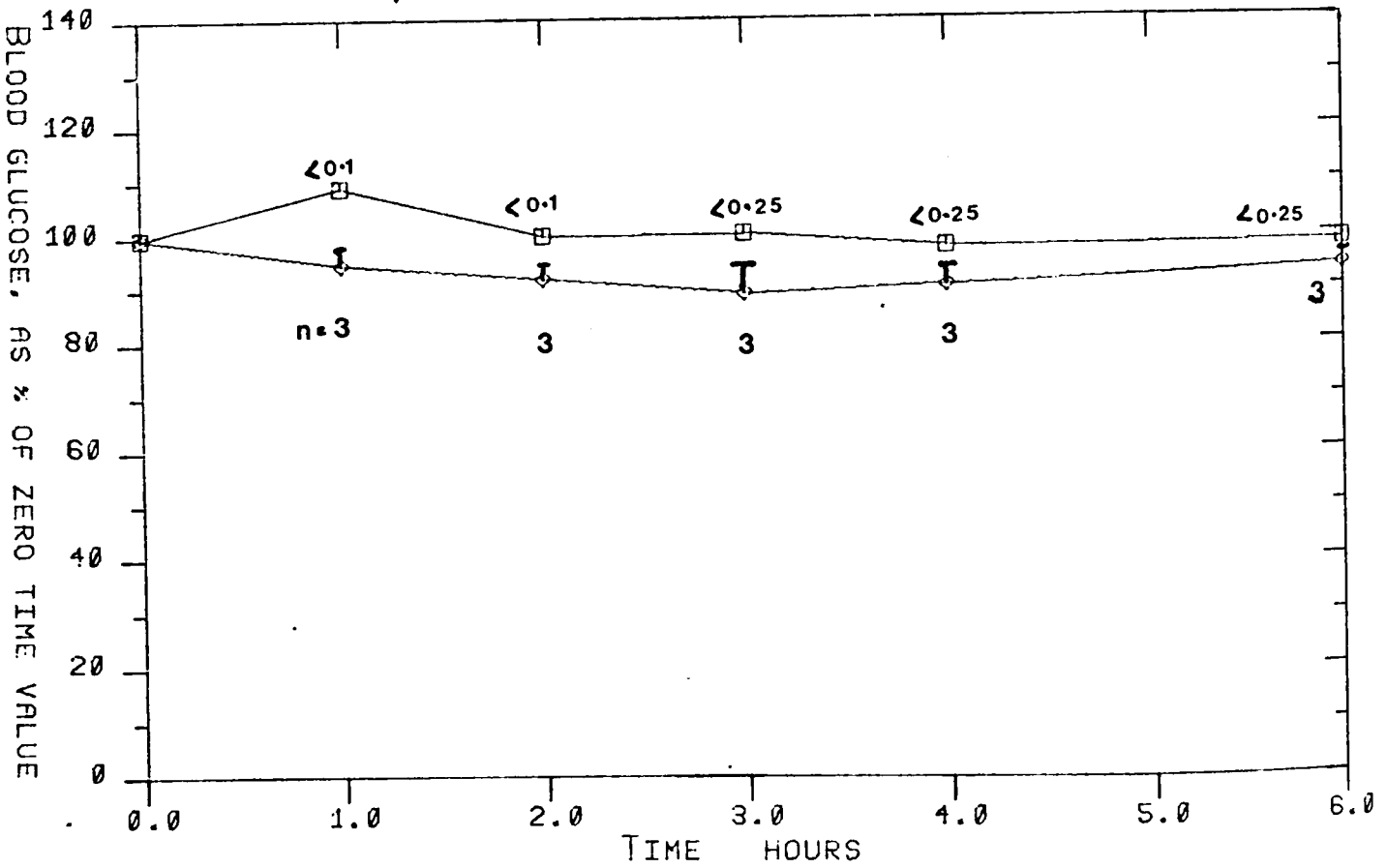


FIGURE 5.7B PERORAL INSULIN ASSAY.
□ INSULIN STANDARD, ◇ ($N^{\alpha B1}$,$N^{\epsilon E29}$)-DI-OCTANOYL-INULIN.
P AND DEGREE OF FREEDOM AT EACH POINT

standards, it was decided to use normal rabbits. An increase in blood insulin levels of up to a hundred microunits per ml may be necessary to cause a substantial blood glucose depression in these circumstances. Small depressions of blood glucose are much more likely, and thus paired cross-over tests were performed in order to be able to reliably interpret the results. This is borne out by the fact that the statistically significant results produced by paired analyses become non-significant when the whole population of controls are used as a comparison and the variance between animals is introduced. The results are calculated as follows. The raw data from the analyses was normalized so that the zero-time blood-glucose concentration was called 100%. Then the difference between control and experiment for each animal was calculated for each time. When more than one control was performed on an animal at different times, for different experiments, all these controls were used in the calculation of the differences.

Five $N^{\alpha B1}$-acyl-insulin derivatives were tested, the acyl groups used were octanoyl, nonanoyl, oleoyl, stearoyl and 1,3-O-dipalmitoyl-2-O-azeloylglycerol. No statistically significant effect was observed for any of these analogues except a slight effect for the nonanoyl derivative. The P values for each time point for this derivative are mostly about 0.1, which when taken alone is only slightly significant. However the cumulative probability of five out of five independent events, each of probability 0.1, occurring is less than 0.0001. The mean blood glucose depression produced is about 10% with the nadir of 13% at the six hour point (see Fig.5.7a).

A similar result is found for the di-acyl-insulin analogues. The modified amino groups are the B1 and B29 ones, and the same acylating groups were used: octanoyl, nonanoyl, stearoyl, oleoyl and

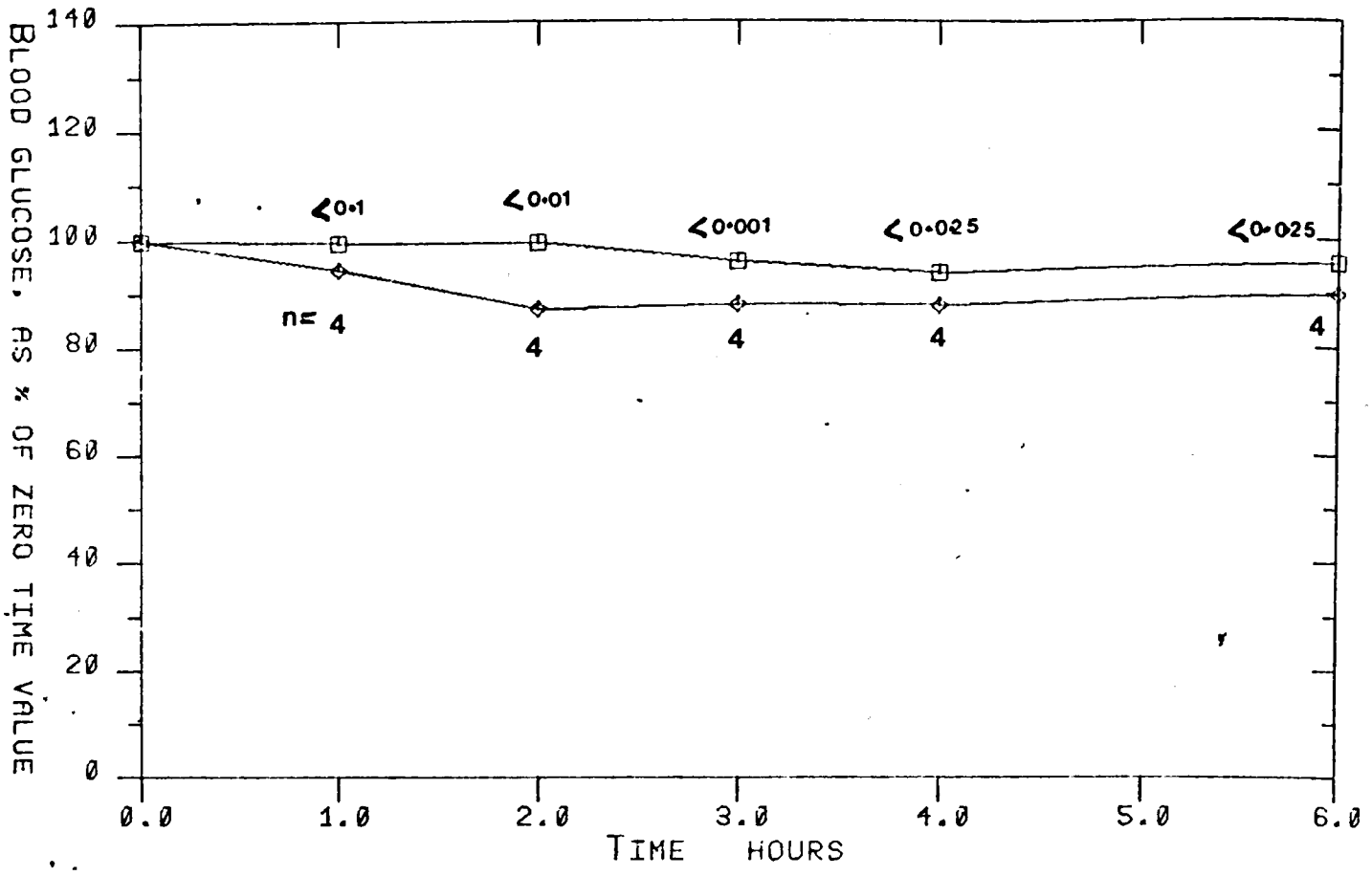


FIGURE 5.8A PERORAL INSULIN ASSAY.
□ INSULIN STANDARD, ◇ ($N^{\alpha A1}$,$N^{\alpha B1}$,$N^{\epsilon B29}$)-TRI-T RIACYL-GLYCEROL)-INSULIN.
P AND DEGREE OF FREEDOM AT EACH POINT

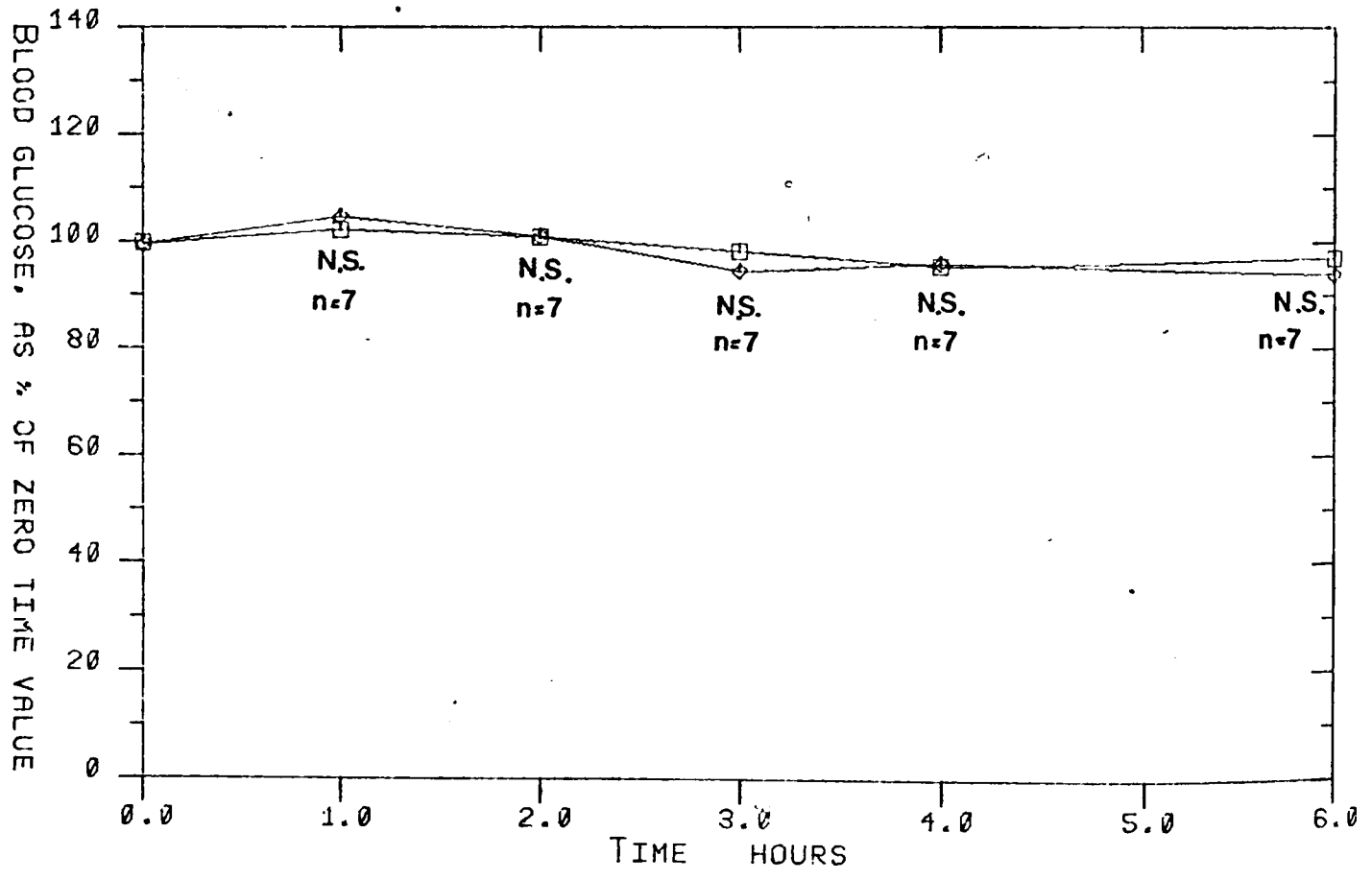


FIGURE 5.8B PERORAL INSULIN ASSAY.
□ INSULIN STANDARD, ◇ ($N^{\alpha B1}$,$N^{\epsilon B29}$)-DI-NONANOYL-I SULIN
P AND DEGREE OF FREEDOM AT EACH POINT

1,3-O-dipalmitoyl-2-O-azeloylglycerol. Again one of the derivatives, di-octanoyl-insulin, caused a slightly significant blood glucose depression. It was most marked after one hour (depression of 14%, P = 0.05) and steadily decreased throughout the experiment. The P values are higher than in the previous case (generally less than 0.25), however this still gives a cumulative probability of about 0.001 (see Fig.5.7b). By the same criterion mono-stearoyl-insulin raised the blood glucose level slightly significantly. The blood glucose elevation was slight, between 3 and 10%.

There was no relationship between dose and response with these derivatives, the doses were fairly high, 0.5 to 2.0 mg/kg of body weight, corresponding to an unmodified insulin dose of about 12 to 50 U/kg.

Three $N^{\alpha A1}, N^{\alpha B1}, N^{\epsilon B29}$ tri-acyl-insulin derivatives were tested, the acyl groups were octanoyl, stearoyl and 1,3-O-dipalmitoyl-2-O-azeloylglycerol. The tri-(triacylglycerol)-insulin derivative caused a blood glucose depression which was very significantly different from the control. The blood glucose depression varied from 5 to 12%, with P increasing from less than 0.1 at 1 hour, to less than 0.001 at three hours and declining to less than 0.025 after six hours (see Fig.5.8). As only a small amount of the derivative was synthesised, the doses used were low. The dose varied between 0.15 and 0.4 mg/kg, which related to the insulin content is about 3 to 9 U/kg. Though this derivative is unlikely to have more than 5% of the activity of an equivalent amount of normal insulin. To produce a 12% blood glucose depression by intravenous administration, a dose of about 0.05 U/kg would be needed, i.e. 1% of the perorally administered dose. And to produce the same time-integrated blood-glucose depression a dose of about 0.20 U/kg

would be required, i.e. 4% of the perorally administered dose. There was no significant hypoglycaemic effect with doses of up to 8 U/kg of tri-octanoyl-insulin or tri-stearoyl-insulin.

5D Toxicology

5Di Post-Mortem Pathology

Four animals were examined. Two of which had been found dead, and the other two were killed, one by cervical dislocation and one by injection of barbiturate.

In the case of the animals that had been found dead, the examination was delayed and post-mortem degenerative changes had occurred. However no obvious lesions were observed in the heart, liver and spleen. In both cases, one or both of the lungs was partially congested, with necrosis at the lower borders. The outer surface of both kidneys in these animals was pitted, and on dissection, the kidneys were found to be partially necrosed. This suggests pneumonia as the cause of death. These animals had been prone to outbreaks of respiratory disease. One of the other animals had a thickening of the tissue along the sternum. This had been diagnosed as being of traumatic origin due to a postural defect caused by ulceration of the hind hocks. On examination, this tissue was about 11 cm long, by 2 cm wide and 8 to 10 mm thick. It was white, firm and continuous with the skin. All the other tissues of this and of the other killed animal were normal.

The animals that had died of pneumonia had lost up to 1.5 kg in weight over the previous 4 to 6 weeks, and were both less than 3 kg in weight. The infection was resistant to Terramycin treatment. The livers of the diseased animals were about two thirds the weight of those of the healthy animals, though the kidneys were nearly twice the weight. All the healthy animals were moderately obese.

5Dii Histology

Micrographs of the tissues from the healthy animals are shown in Fig.5.9 (light micrographs) and Fig.5.10 (electron micrographs).

The light micrographs are of moderate quality. Due to my lack of experience, knife marks, section tearing and irregular staining occurred. Despite this, the results of the best sections are quite typical of normal healthy tissue. No undue deposition of fat can be seen. In the aorta particularly, no arteriosclerotic plaque can be observed. The kidney tissue from one of the animals that died showed signs of damage (see Fig.5.11). Distorted cells and nuclei were present, though there was no sign of an infective agent. The tissue from the chest of the animal with the thickening was shown to be of an oedematous, fibrous nature (see Fig.5.12). This confirmed the diagnosis and excluded the possibility of neoplasm.

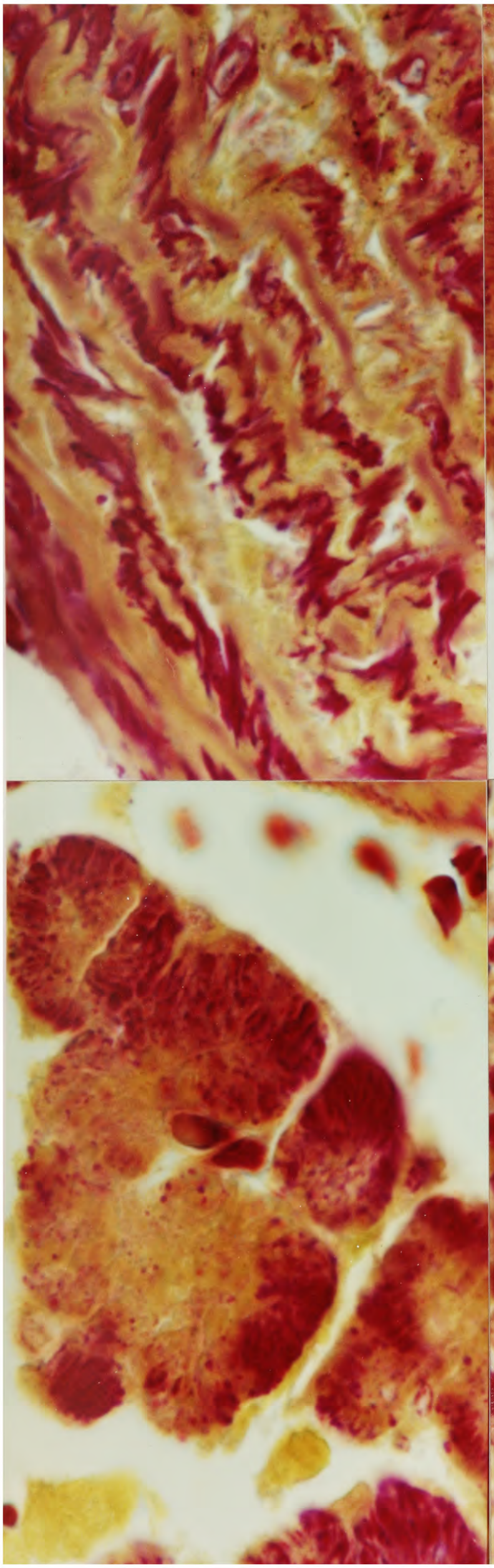
The electron micrographs of the tissue from the healthy animal confirmed the normality of the tissues.

5Diii Immunology

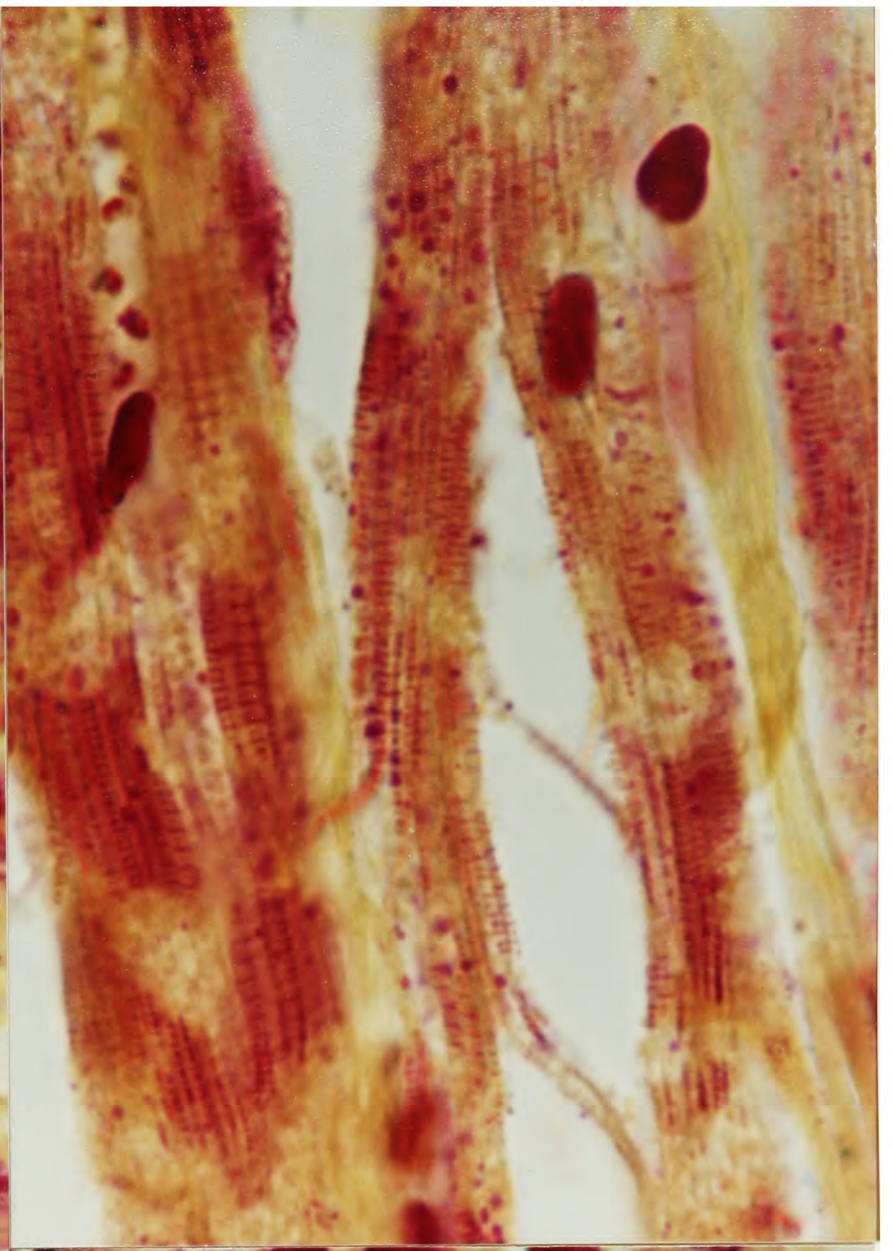
Serum from seven animals was assayed by Ouchterlony double-diffusion. Assays with a range of dilutions of serum against insulin and against di-nonanoyl-insulin or di-stearoyl-insulin were performed. No precipitin lines were observed in any case, even after washing and staining with amido-black.

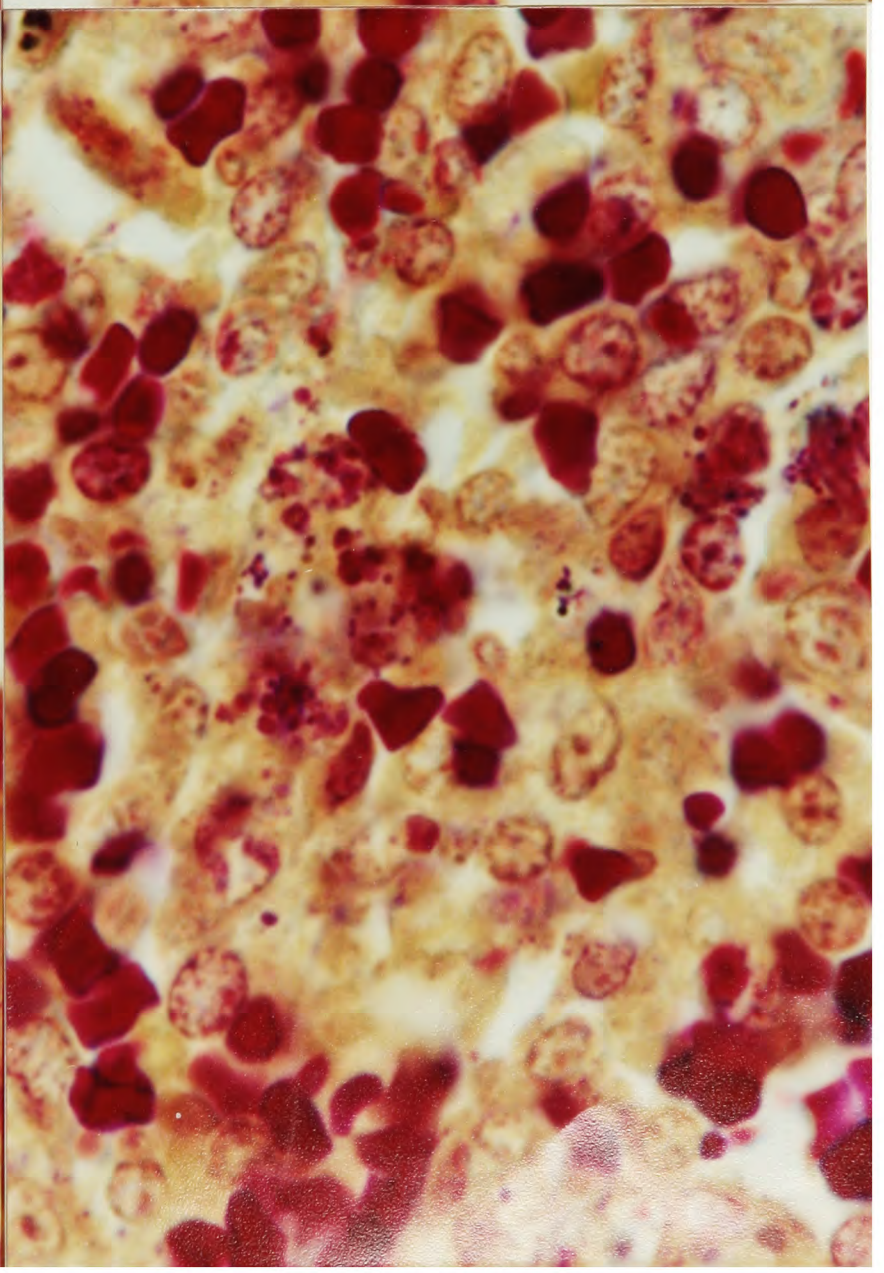
Figure 5.9 Light micrographs of normal rabbit tissues.
a-h, stained with Altmann stain, x 1,400:
a, aorta; b, heart, L.S.; c, heart, T.S.; d, spleen; e, liver;
f, lung; g, kidney (capsule); h, kidney (tubule);
i-p, stained with Haematal 8 and Biebrich scarlet except for l,
Altmann stain and k and p, iron haematein, x 140:
i, aorta; j, heart; k, heart; l, spleen; m, liver; n, lung;
o, kidney; p, kidney;
q-s, stained with Sudan IV , x 140:
q, aorta; r, liver and s, heart.

a



b



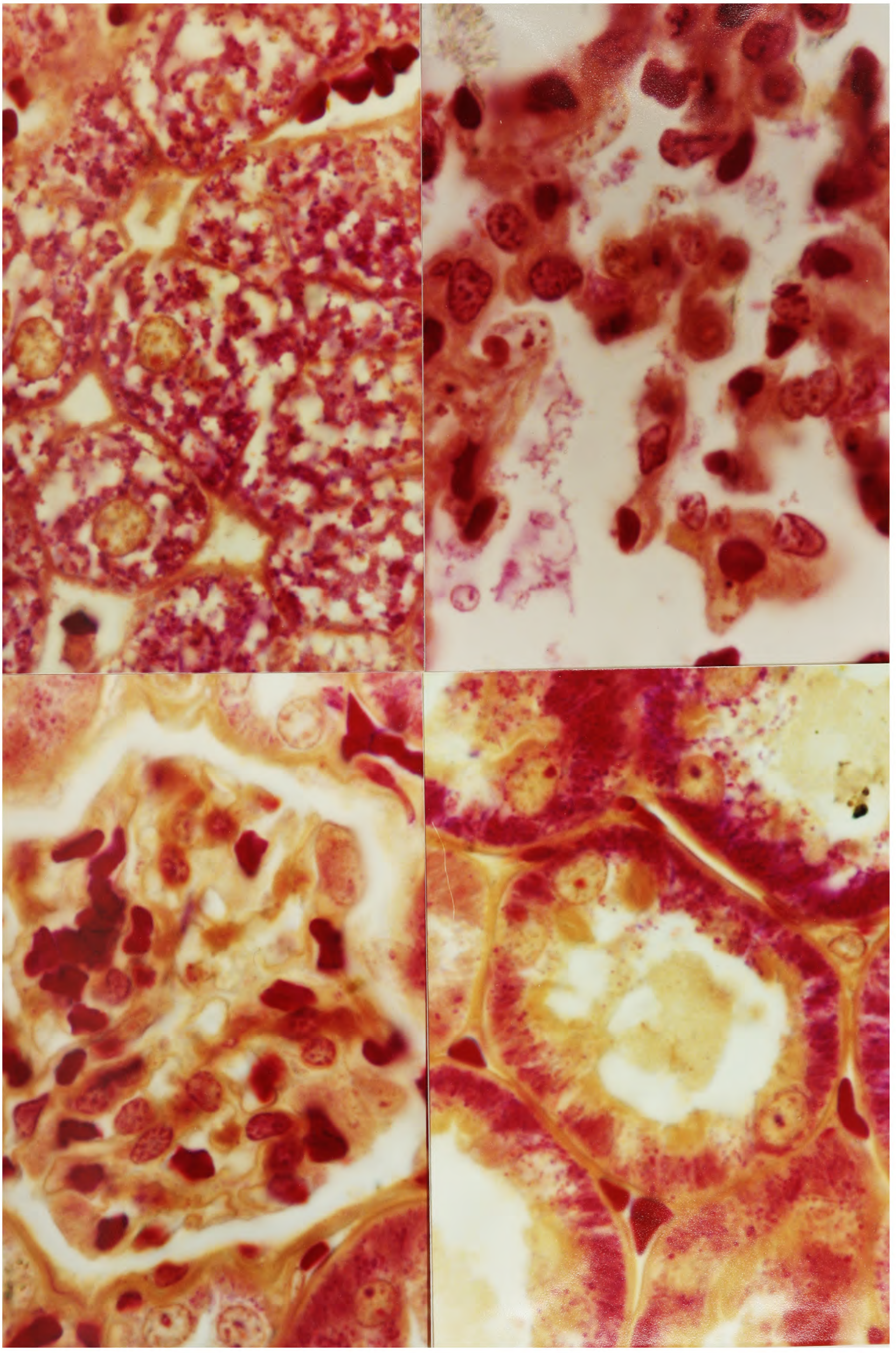


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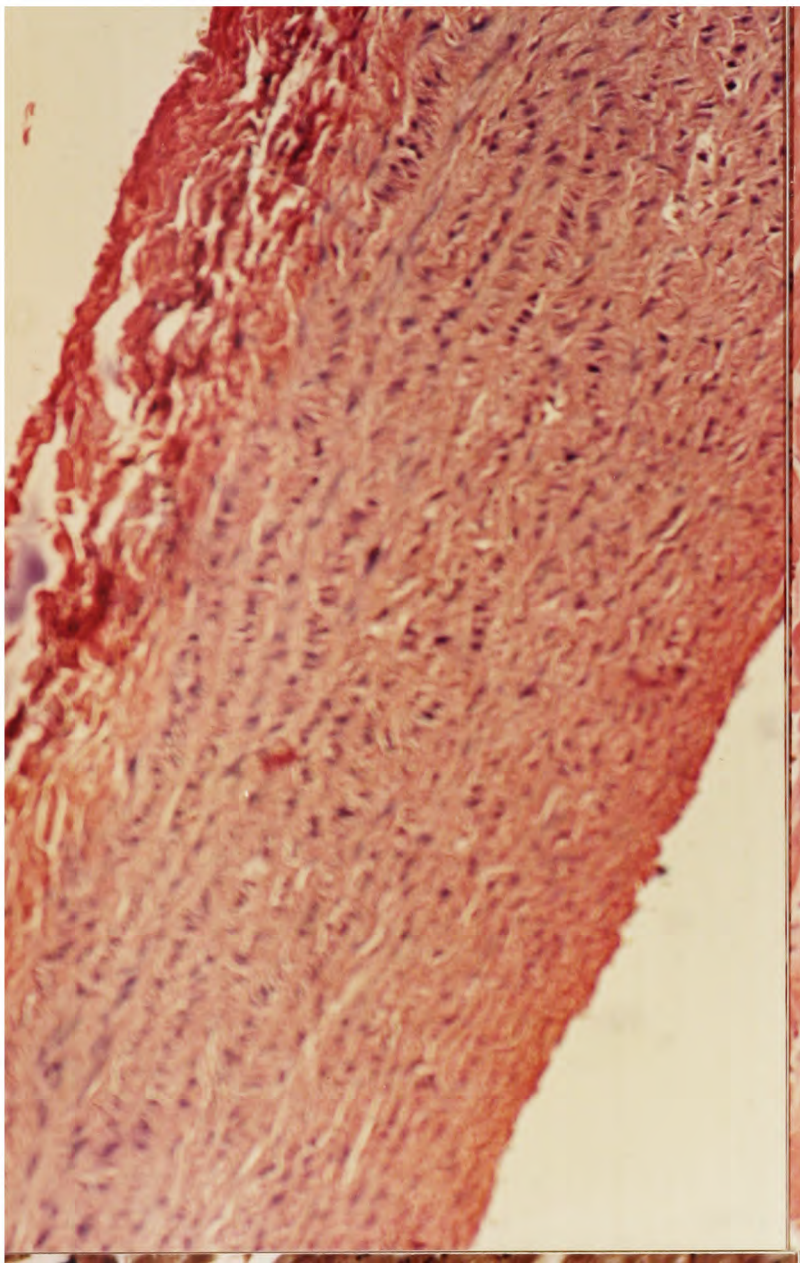
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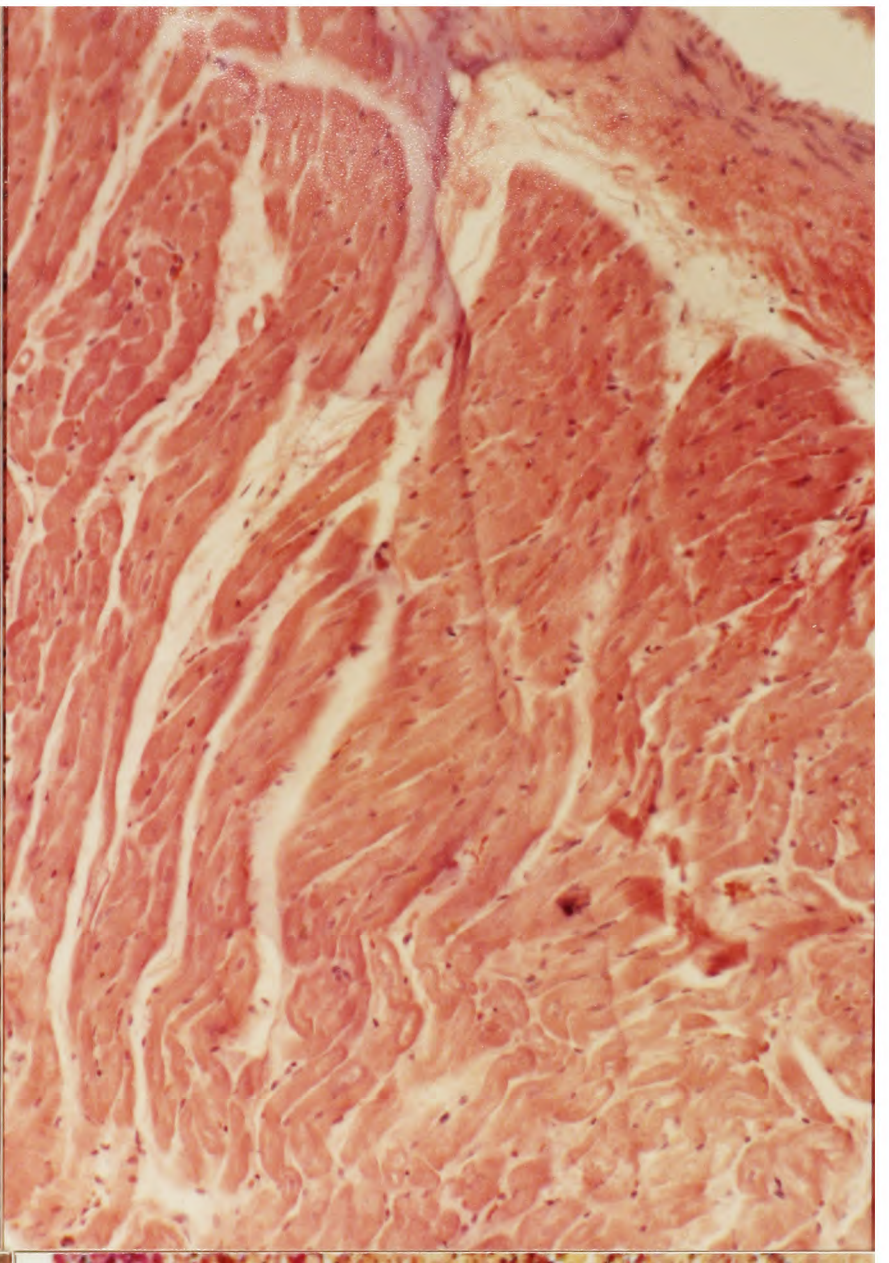
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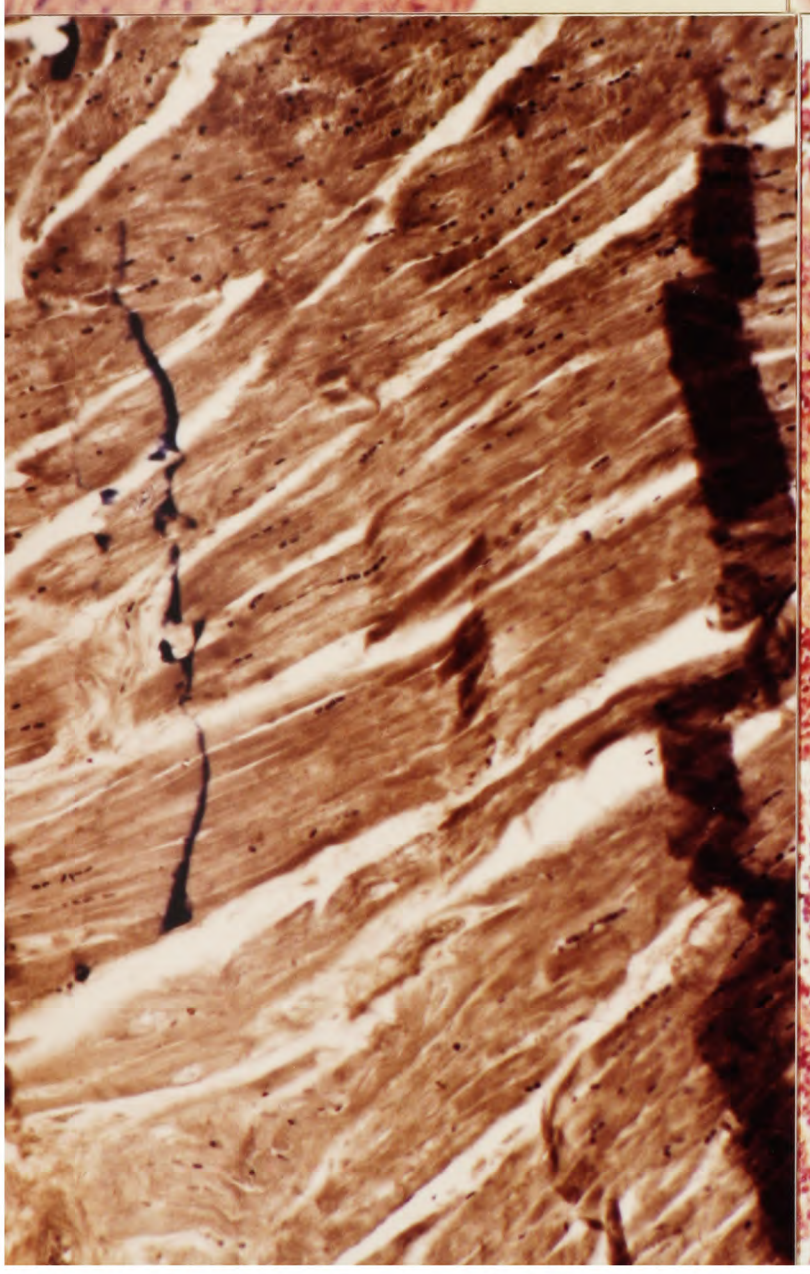
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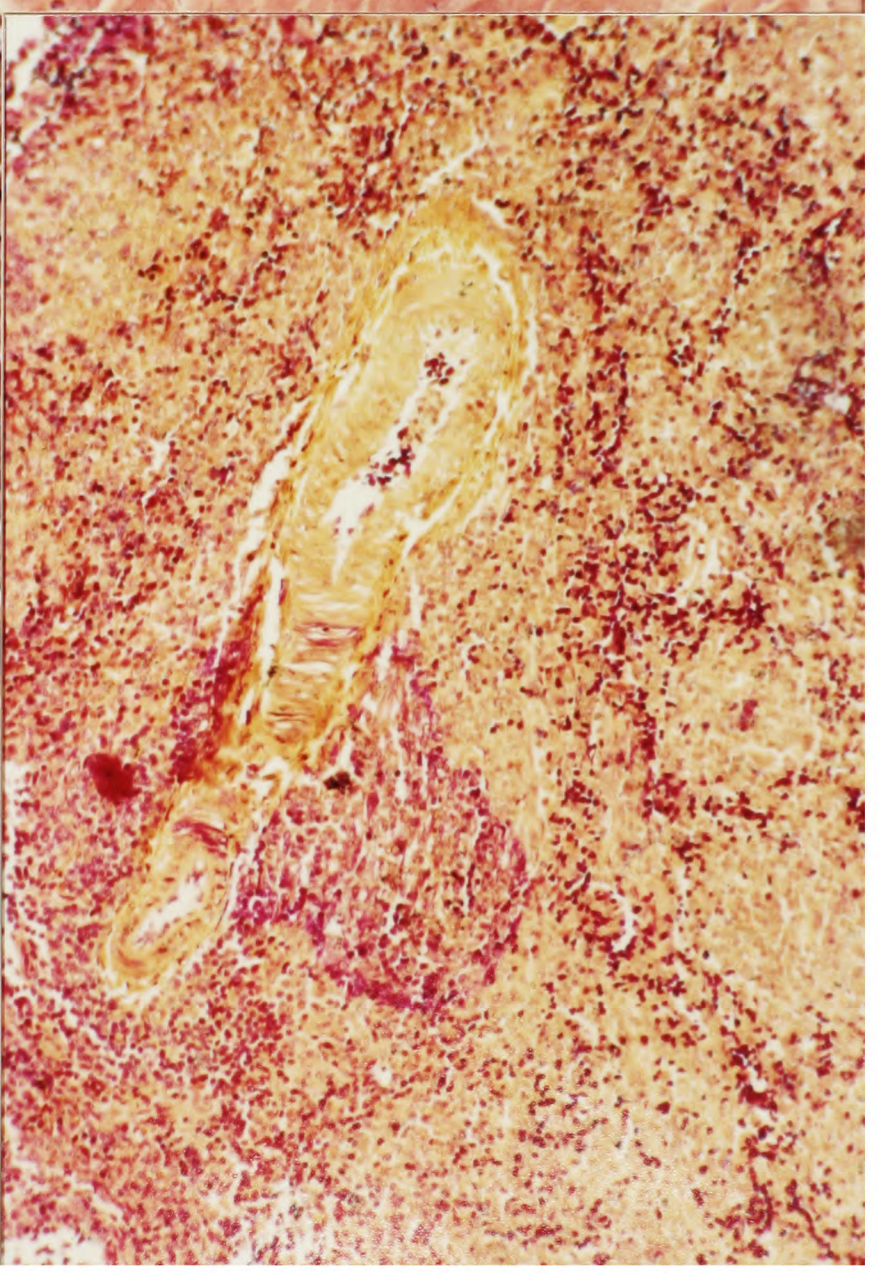
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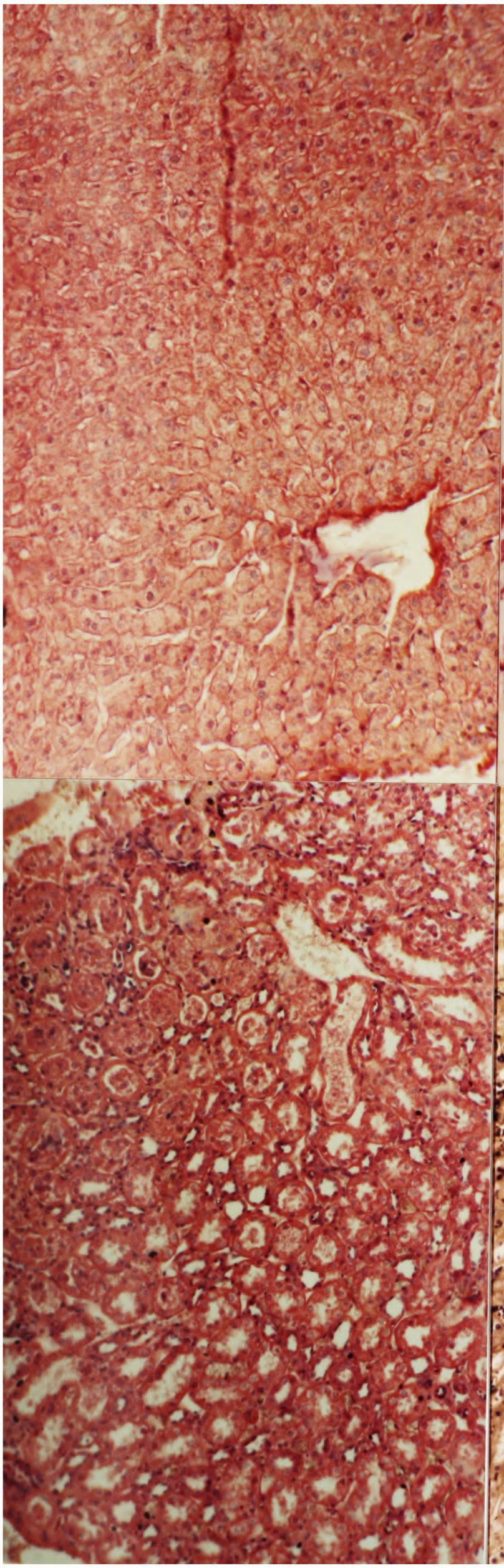




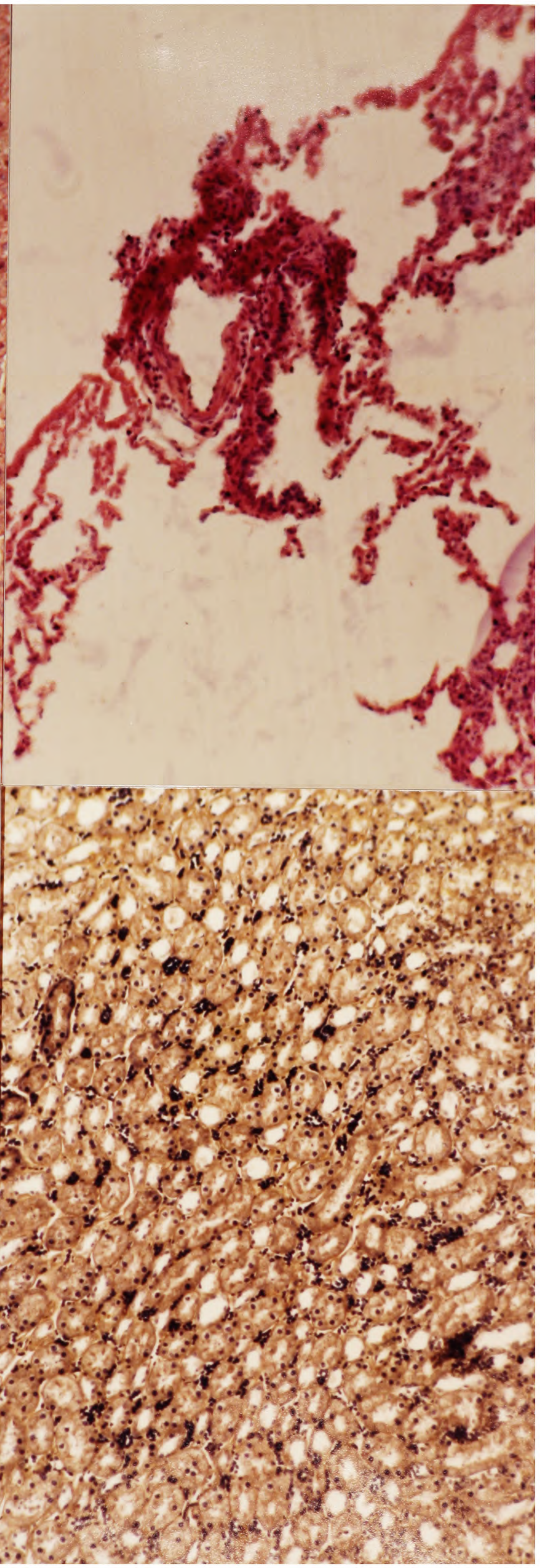
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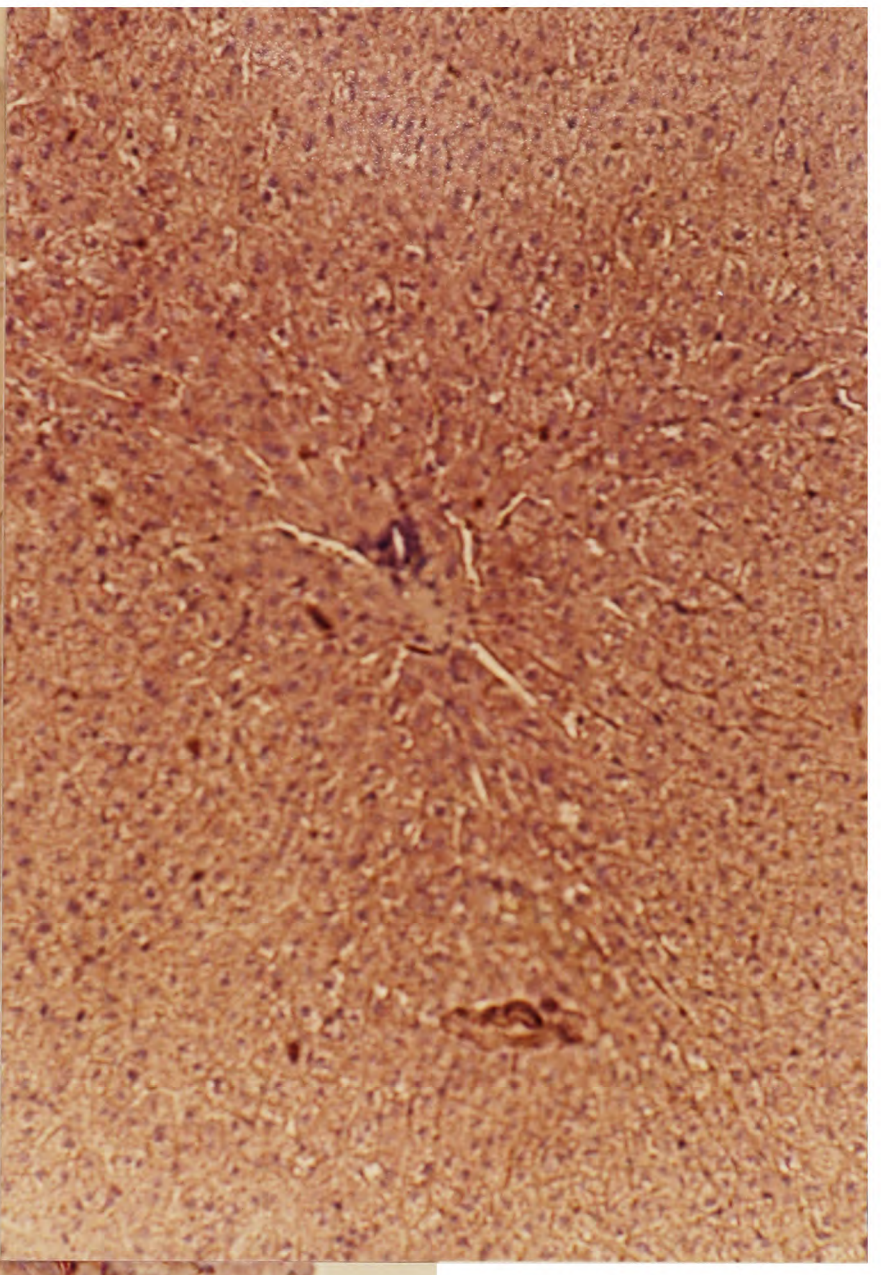
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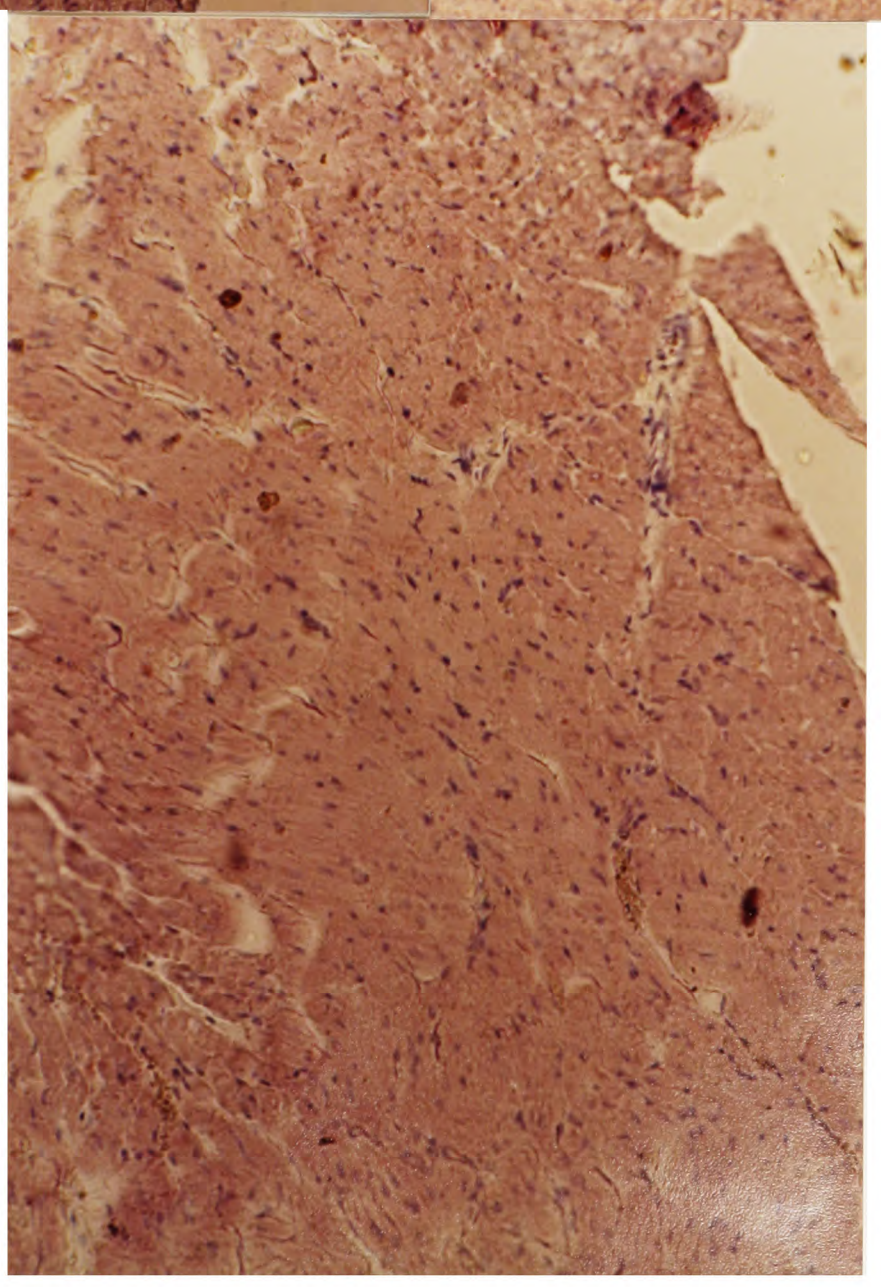
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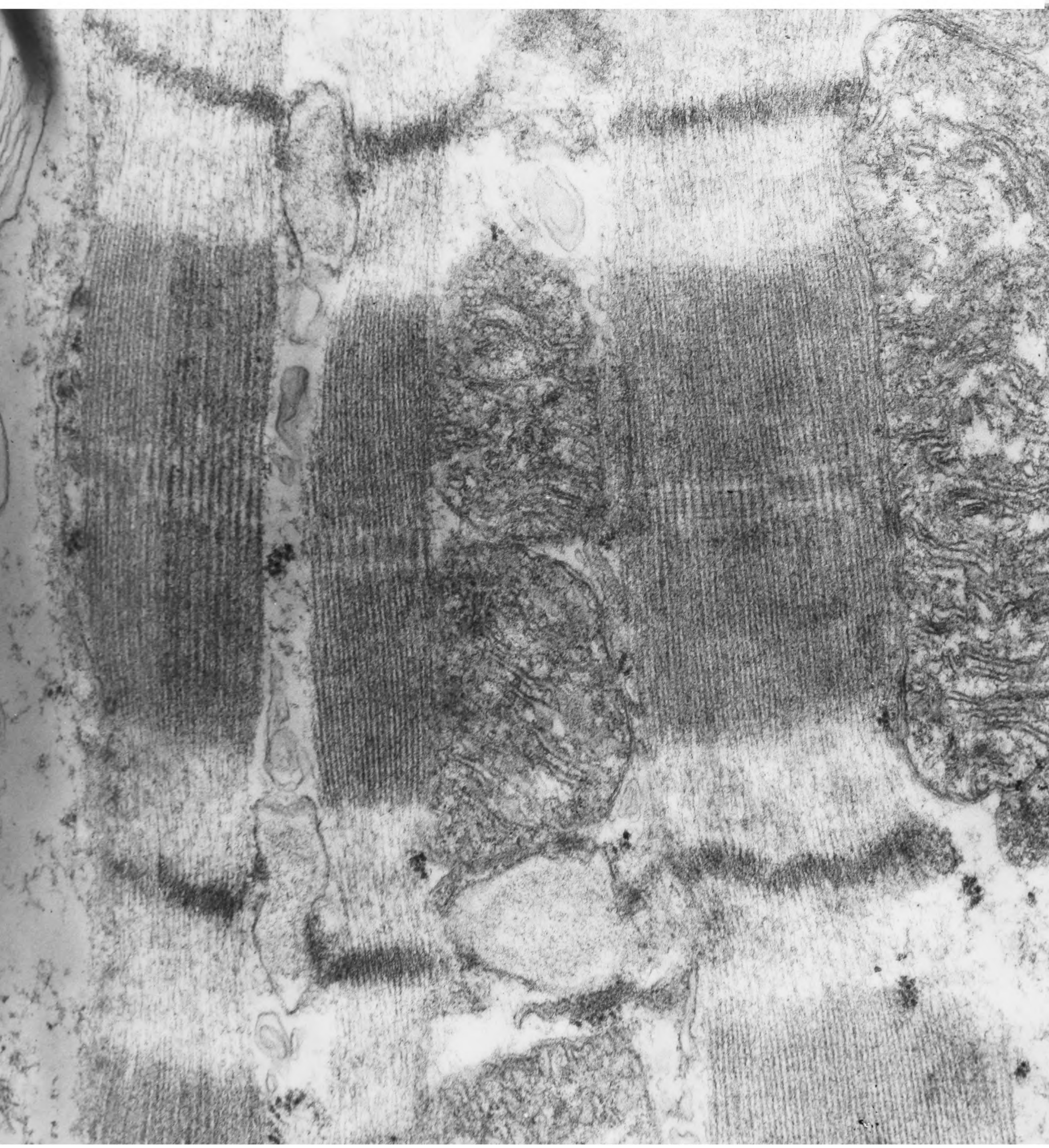
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Figure 5.10 Electron micrographs of normal rabbit tissues.
All stained with uranyl acetate and lead citrate. a, aorta, x 10,000;
b, heart, x 47,500; c, heart, x 25,000; d, spleen, x 25,000;
e, spleen, x 10,000; f, liver, x 25,000; g, liver, x 17,750;
h, lung, x 25,000; i, lung, x 6,300; j, kidney, x, 25,000; and
k, kidney, x 10,000.

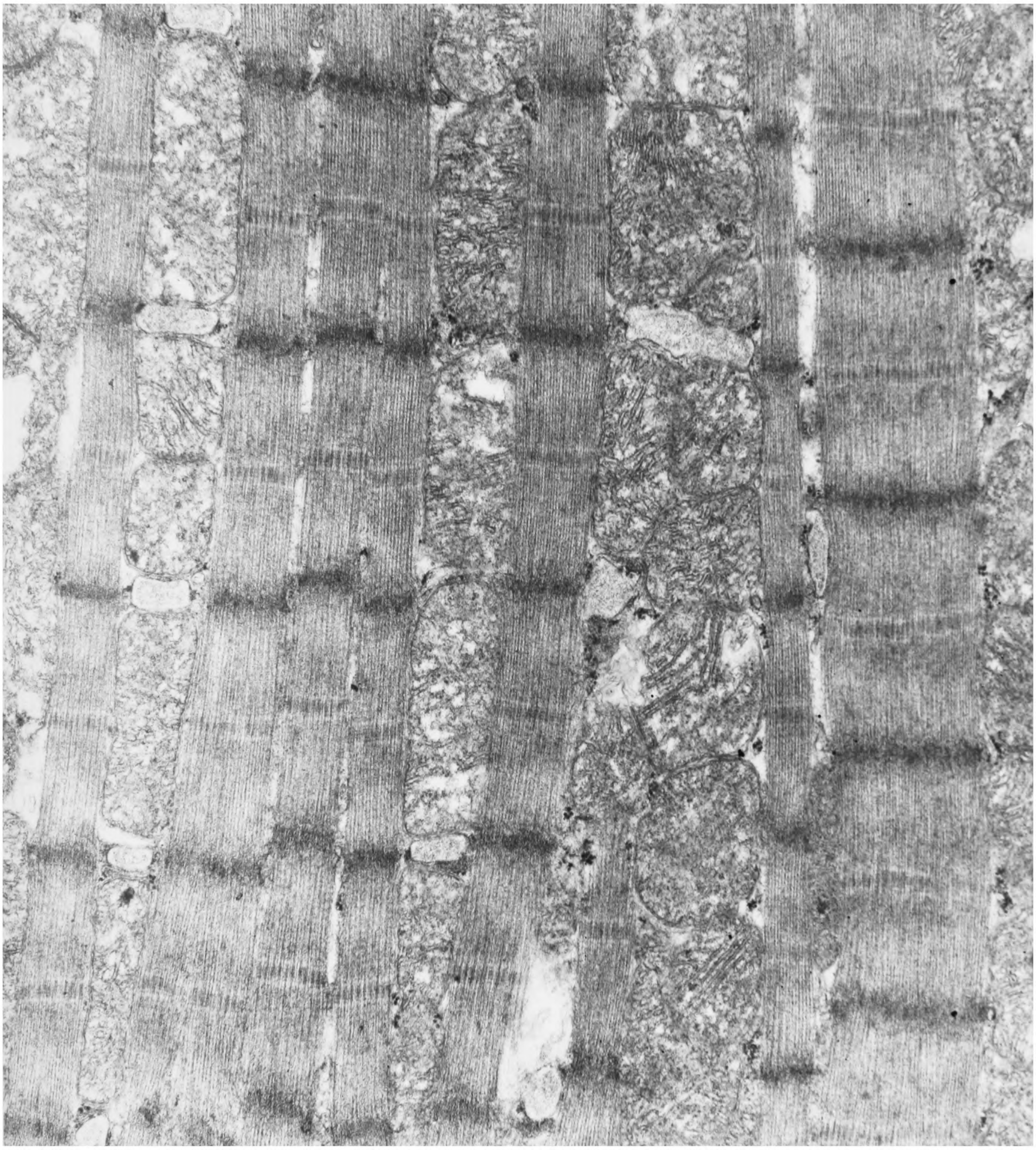
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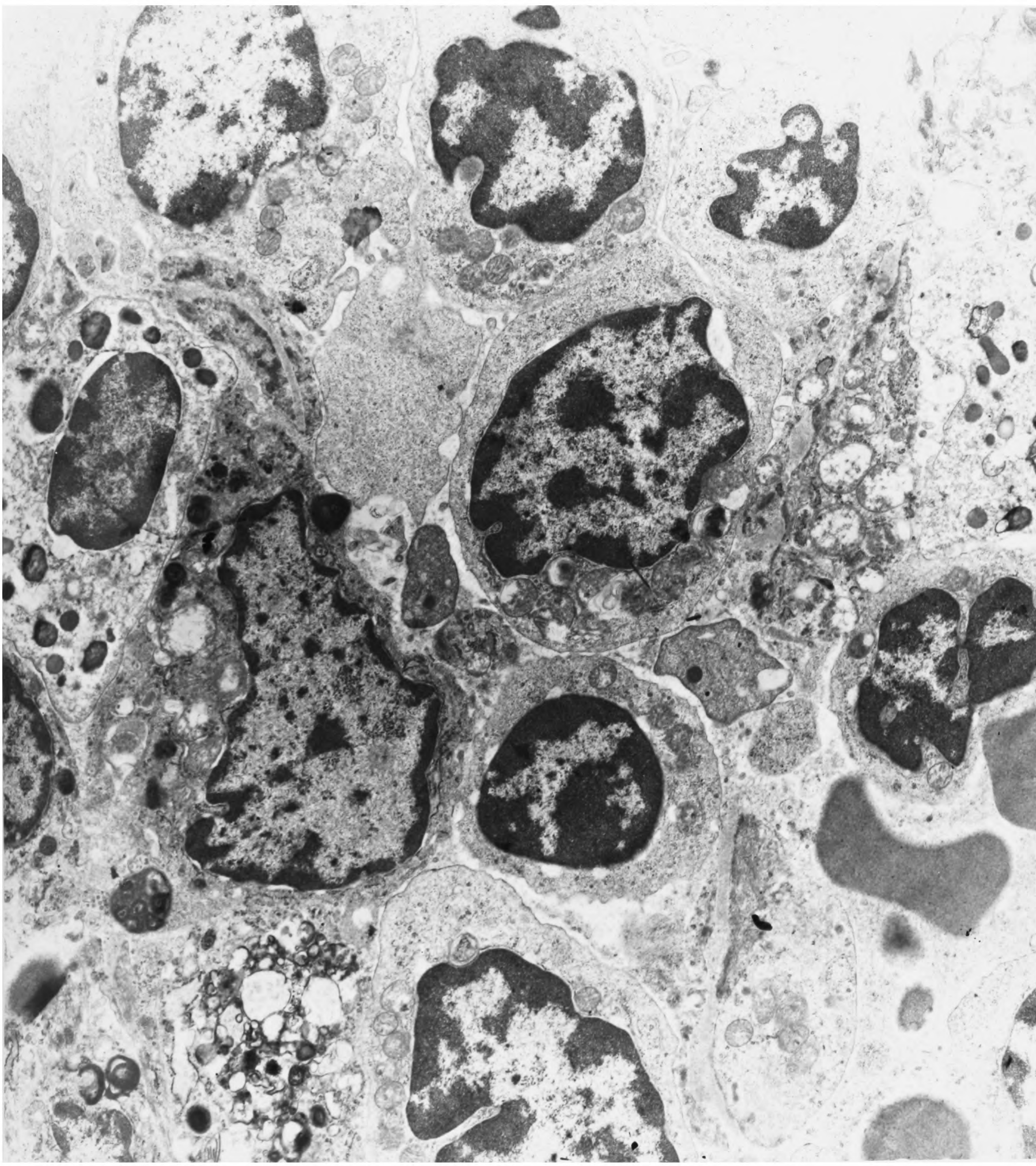
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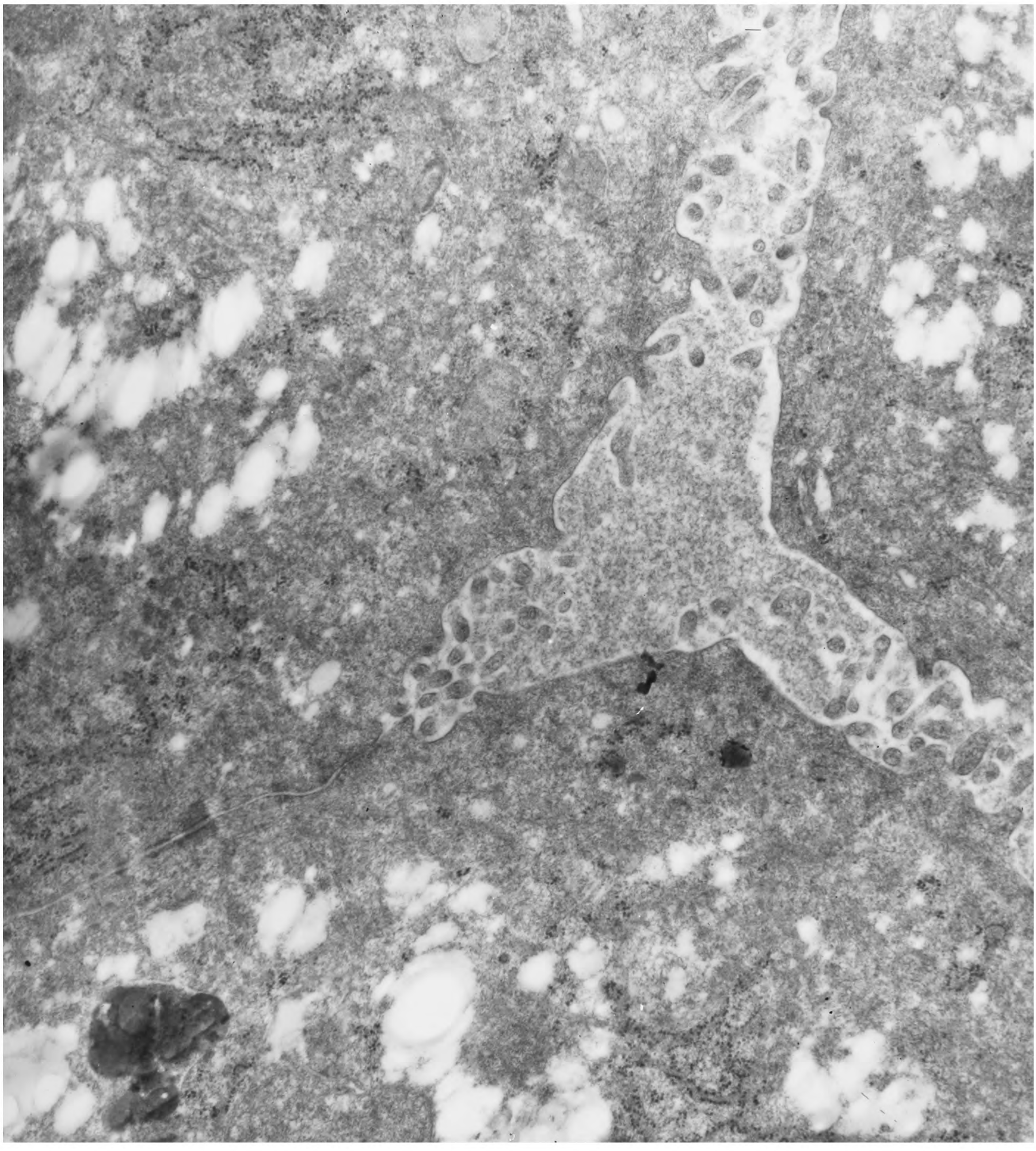
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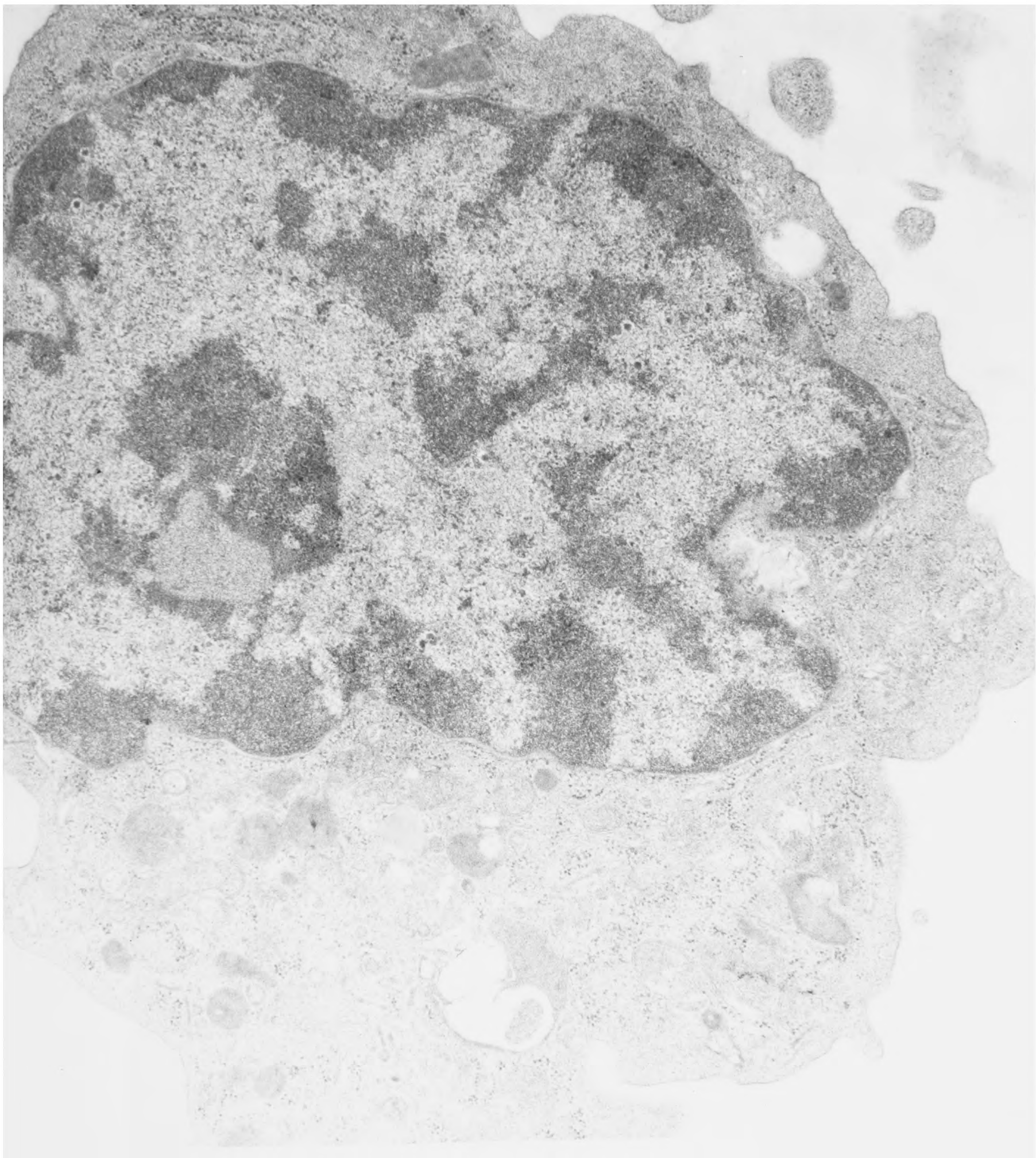
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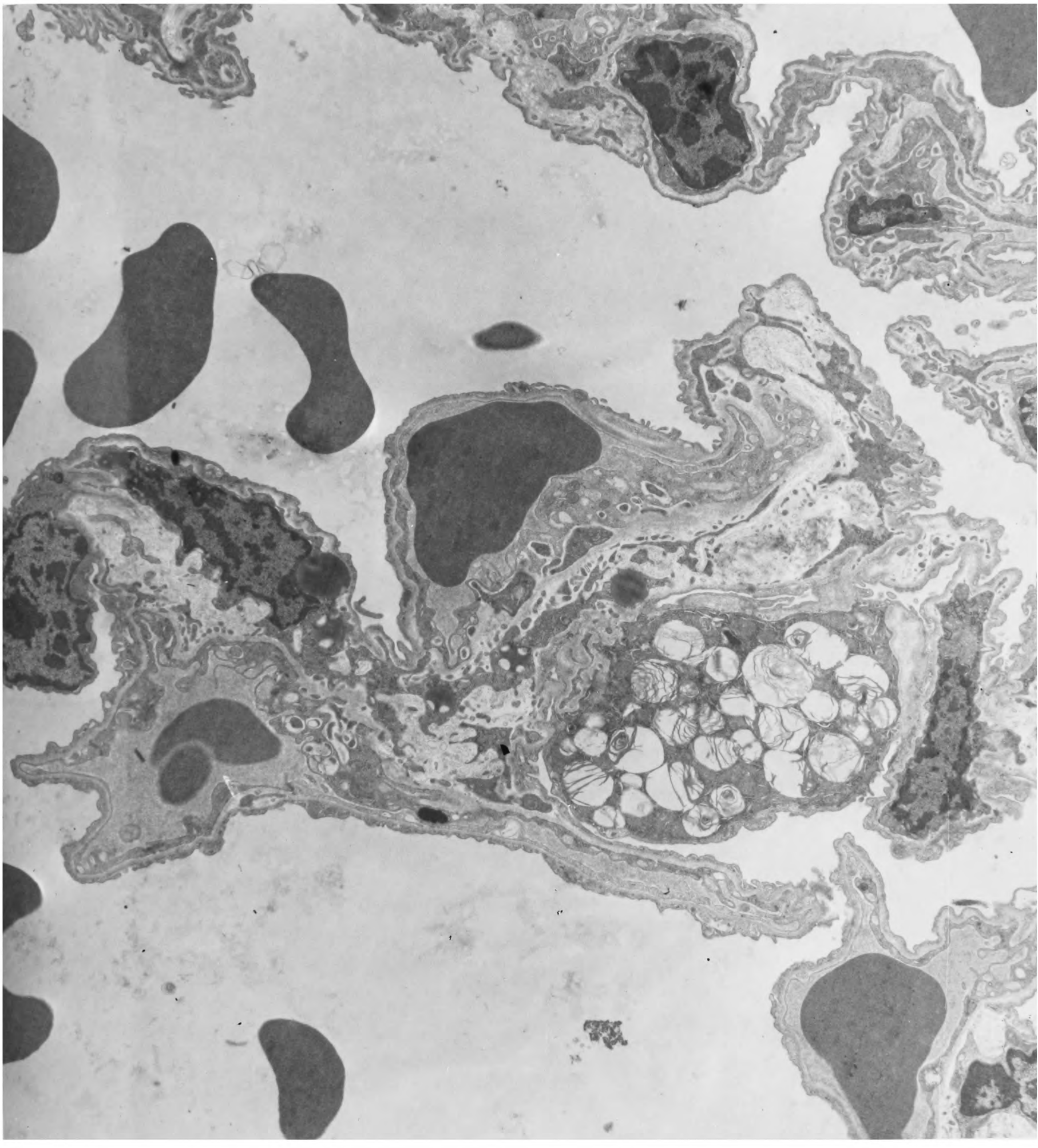
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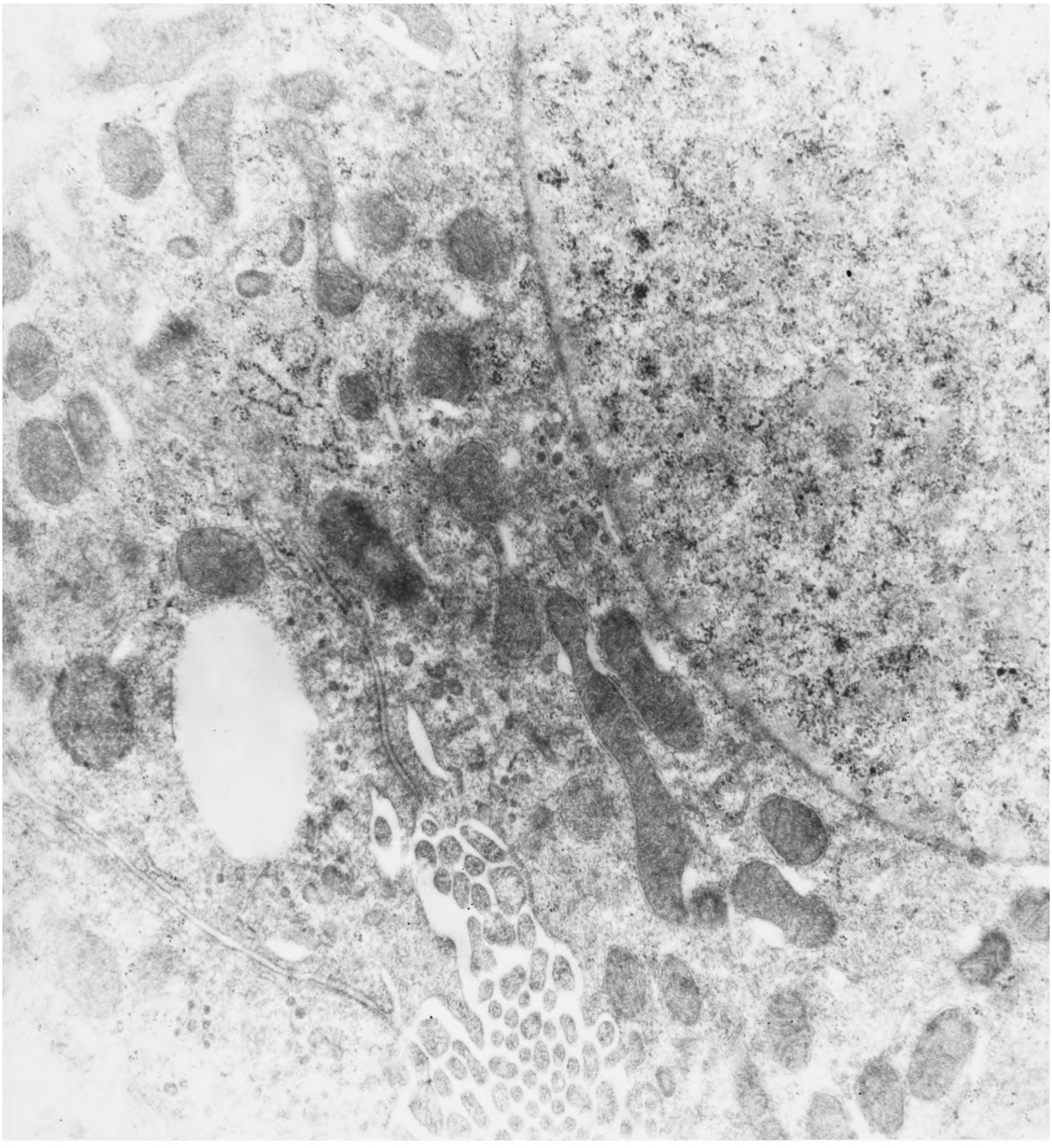
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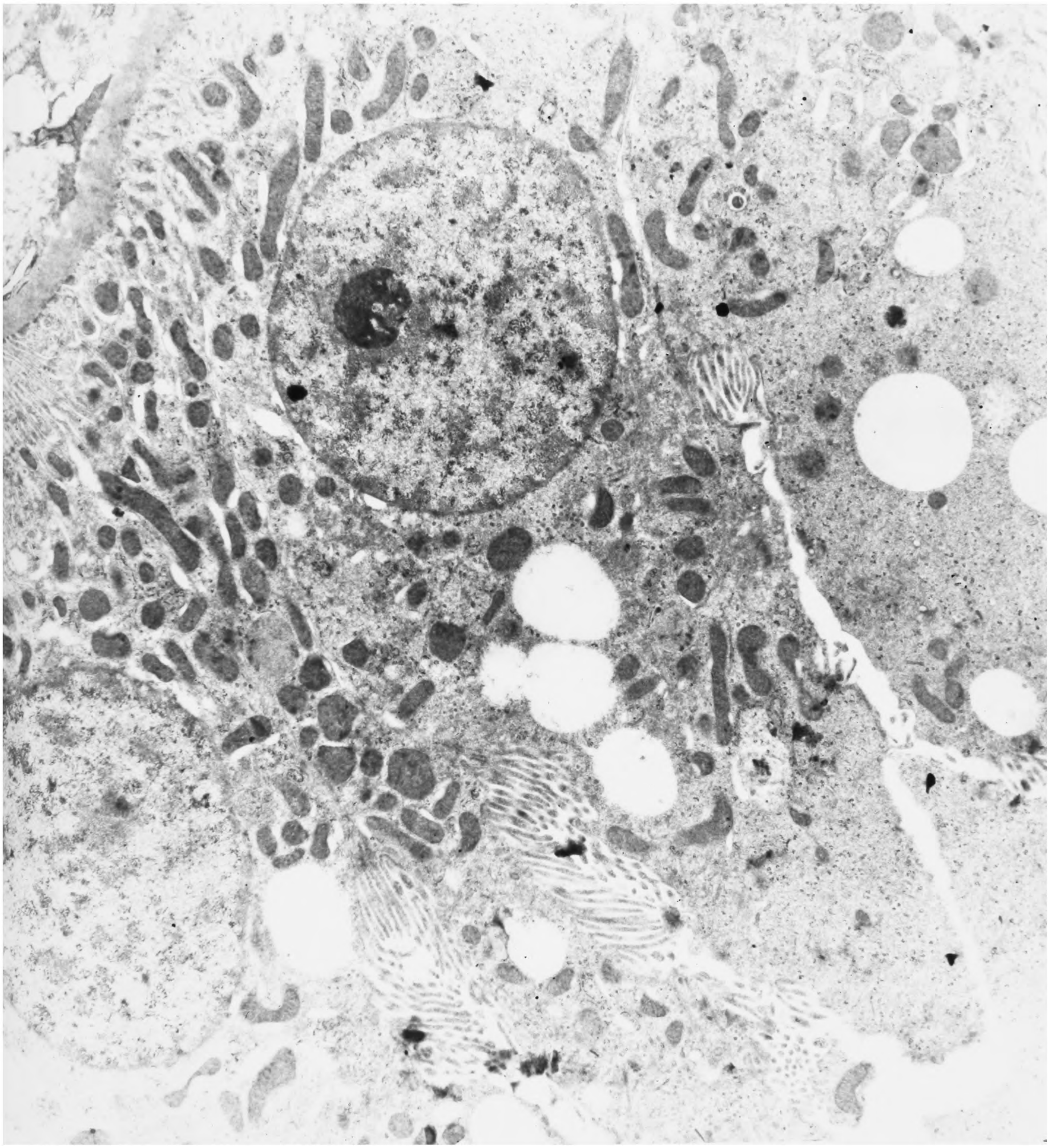
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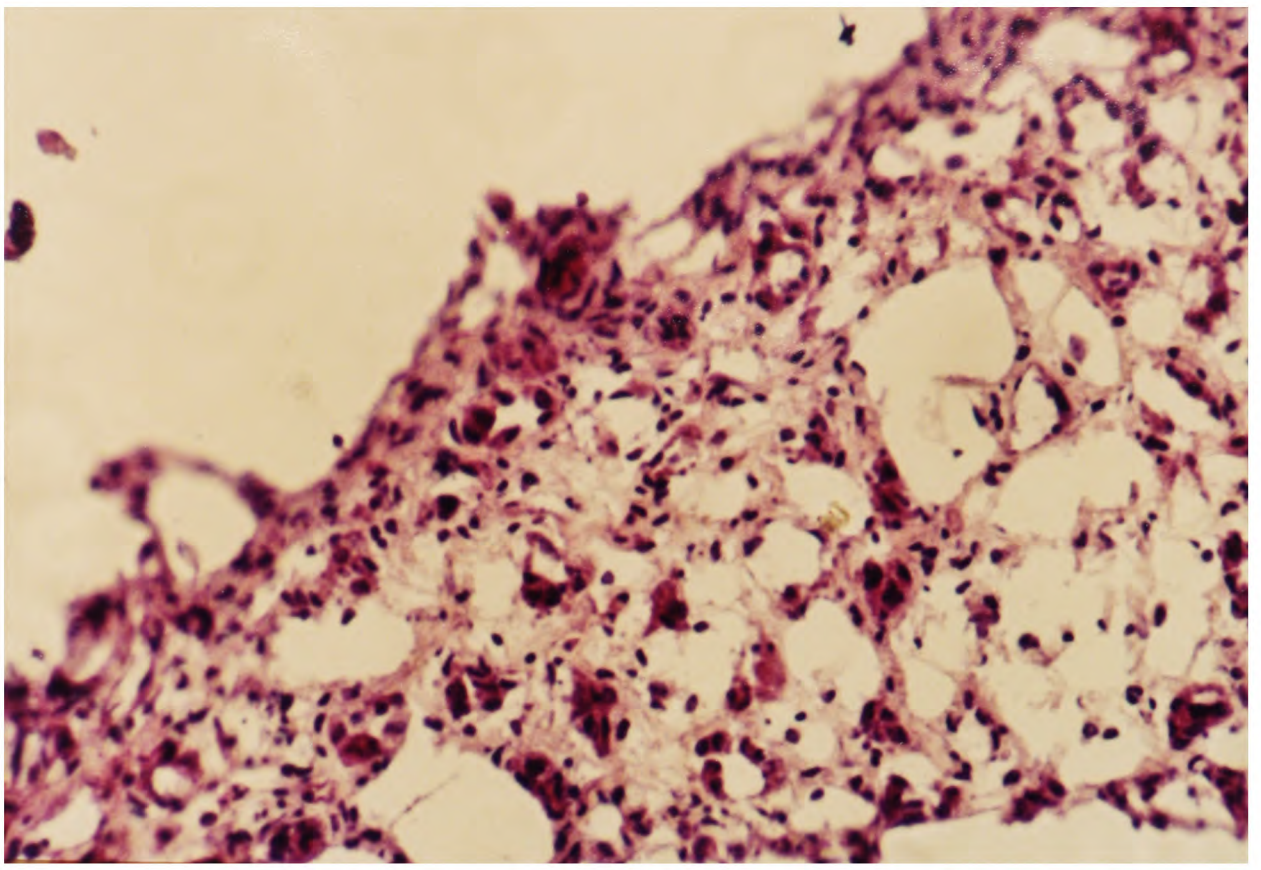
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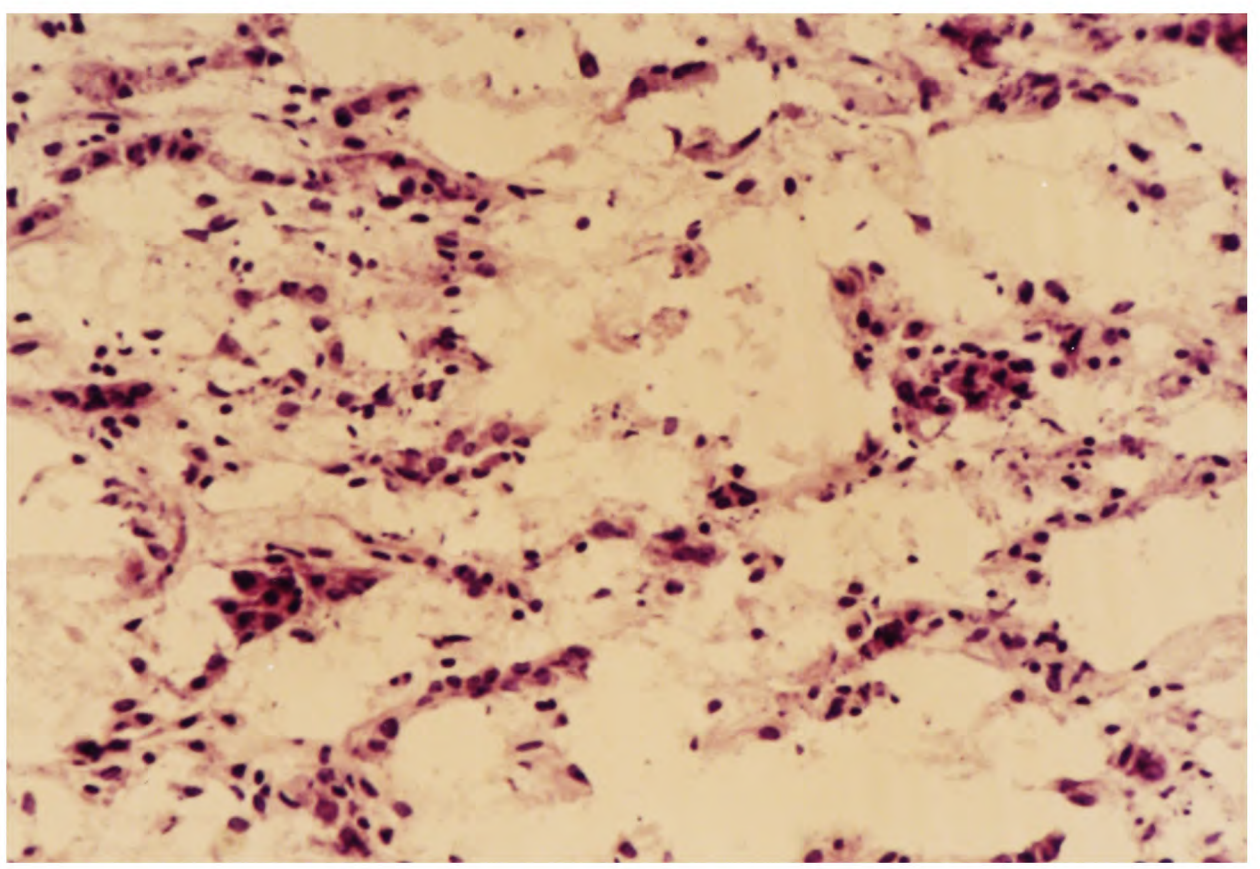


Figure 5.11 Light micrographs of kidney from diseased rabbit. Stained with Haematal 8 and Biebrich Scarlet, x 280: a, T.S. and b, L.S.

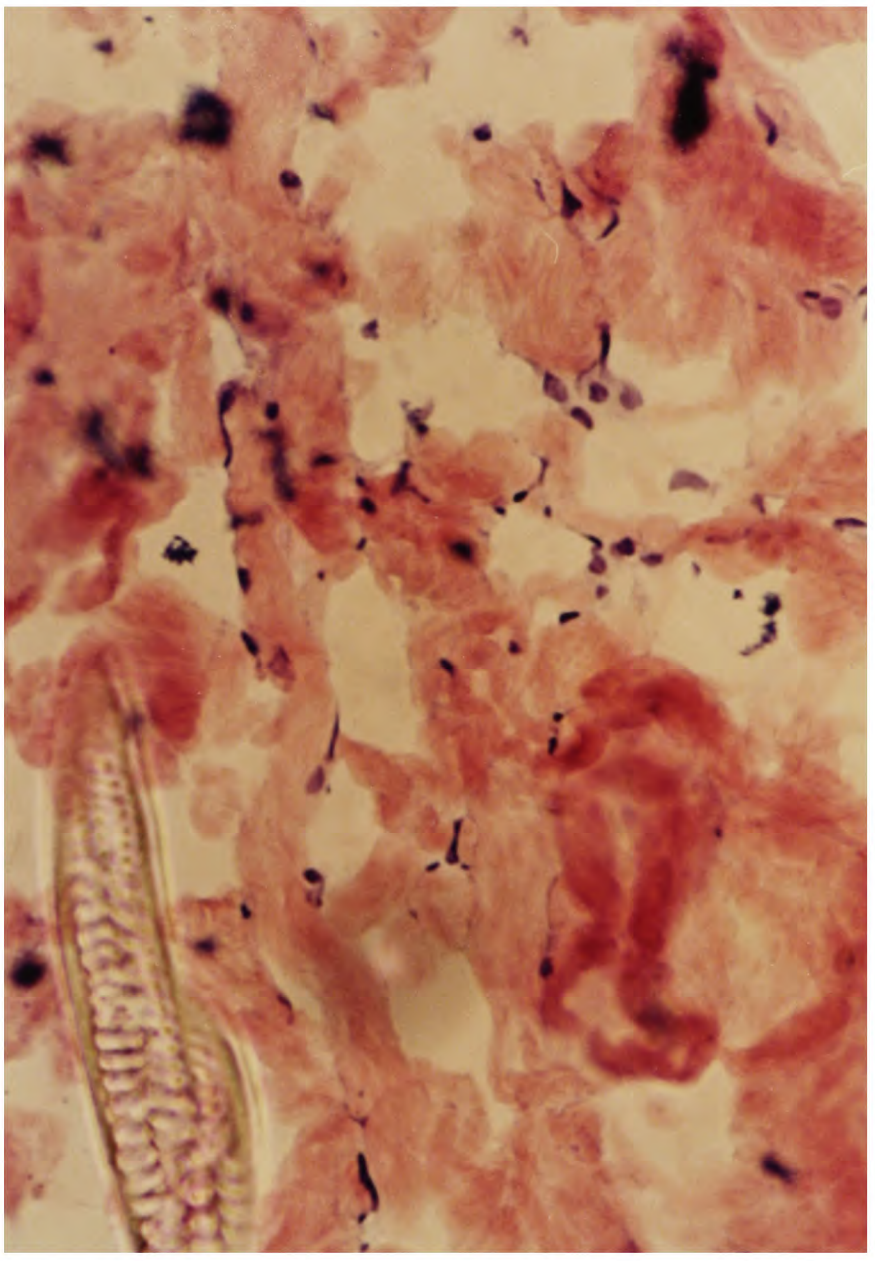


Figure 5.12 Light micrograph of chest wall of rabbit with 'sore hock' syndrome. Stained with Haematal 8 and Biebrich scarlet, x 140.

Chapter 6

Discussion

6A Introduction

This work has consisted of an attack on two problems of the use of insulin in the clinical treatment of diabetes: firstly the search for modifications that would give a derivative with a more physiological mode of action, and the search for a perorally active preparation of insulin.

The use of a triacylglycerol derivative of insulin as a potentially reactivatable preparation, whose reactivation is dependent on the post-prandial elevation of serum-enzyme activities was examined. It was discovered that the majority of the prolonged activity of the first preparation of one of these derivatives was due to the accidental acylation of the insulin with nonanoic acid (a by-product of the reaction pathway), rather than by the triacylglycerol. Consequently a variety of fatty-acyl derivatives of insulin were then studied. One of these was labelled with carbon-14 and the pharmacokinetics of the label in the blood was measured.

Another potentially reactivatable insulin derivative was synthesised and tested. It was a compound of partially oxidized Ficoll coupled to insulin via the formation of a Schiff's base.

It was also believed that an insulin derivative, with covalently bound lipid, might be resistant to gut protease attack and be more readily absorbed from the gut, due to its increased lipophilicity. This would make it a potentially useful perorally administrable insulin preparation. All the acyl- and triacylglycerol-insulins were tested for hypoglycaemic activity when administered perorally.

Finally, a toxicological examination was performed on four of the rabbits used in testing the insulin derivatives. A selection of tissues from two of these was processed and examined by light and electron microscopy. Blood serum from seven of these animals was tested by Ouchterlony double-diffusion for the presence of antibodies to insulin and to insulin derivatives.

6B Assays by Intravenous Administration

6Bi Potency of Derivatives

The hypoglycaemic response of an animal to insulin is difficult to measure reliably. Often the maximal blood glucose depression is used. In a normal animal, all the other hormonal responses to changing blood glucose and insulin levels are active in maintaining glucose homeostasis and minimizing the effects of exogenous insulin. The importance of these responses can be seen by comparing the sensitivity of normal animals to that of 'de-endocrinised' animals used in insulin assays. The normal animals are at least 10 times less sensitive. Furthermore there is evidence to support the belief that the restoration of blood glucose levels is dependent on the rate of change of blood glucose concentrations, rather than the absolute change in levels. Consequently the restorative response to a large insulin dose, inducing rapid, and marked hypoglycaemia will be different from the restorative response to a large dose of an insulin derivative with a slower induction of hypoglycaemia, but a prolonged hypoglycaemic effect. The fasting blood glucose and minimum attainable blood glucose (before convulsions, coma and death occur) are variable from day to day. This leads to at least a 20% to 30% variation in the maximum response. This is a reason for normalizing the data. Furthermore as it is unlikely that a fixed-time sampling plan will sample at the exact

time that the blood glucose concentration reaches its nadir (which is a variable time, dependent on the dose), there is a further error in measuring the response by blood glucose depression.

Measuring the initial rate of fall of the blood-glucose levels is probably a better method. The first few minutes of the blood glucose depression are relatively unaffected by other hormonal responses which take several minutes to manifest themselves. The rate measurement is also less affected by high doses, as it will be less limited by the maximal achievable blood glucose depression. Thus a log(dose)-response curve can be extended to higher doses. It is possibly a better measure of the initial binding of insulin to the receptive tissues and of their response, not lessened by degradation of the hormone and the effect of antagonistic hormones, than the measurement of the response by maximal blood-glucose depression which will be affected by a change in the rate of degradation of the hormone. The maximal hypoglycaemic response to normal insulin is not achieved until the concentration of the hormone in the blood has been substantially reduced by degradation.

The rate of fall of the blood-glucose concentration is not a simple function, even theoretically, because at least two distinct, major compartments (the liver and the peripheral tissues) are responding differently to the administered insulin. In practice there are problems in measuring the rate of fall of the blcod-glucose concentration. After injection, the time of the onset of the response varies from three to six minutes. Depending on the dose, the initial rapid phase of the blood-glucose depression may last a further five to fifteen minutes. In order to be able to measure accurately the initial rate, these two times (the time of onset and of duration of the response) ought to be measured. It is not easy to

make an unbiased assessment of these times when a blood-sampling plan with fixed time intervals is used. The mean slope of the line between the zero and the twenty minute blood glucose levels was measured. If the zero or twenty minute sample varied significantly from this slope, they would be excluded from the measurement. Despite these difficulties this method of measuring the response was found to be better than the measurement of the nadir of the blood-glucose depression. The log(dose)-response curve of the normal-insulin assays as measured by the initial rate of blood-glucose depression had a better index of precision and correlation coefficient than the curve as measured by the maximal blood-glucose depression.

Neither of these methods for the measurement of the response is well suited to the comparison between insulin and its derivatives which may have possible differences in degradation rates or rates of mobilization from depots. A variation in the rates of degradation or mobilization of the hormone will lead to differences in its blood concentration even when the initial doses are equal.

Ideally the constant-rate-infusion method described by Jones and Sonksen is the best for this type of analysis. The blood-insulin and blood-glucose levels are measured simultaneously, whilst insulin is infused at a series of increasing concentrations. The hypoglycaemic response is calculated and related to the steady-state blood concentration of the normal or modified insulin that was administered. When using a bolus injection, as I have done, the method of choice for the determination of the response is the measurement of the time-integrated blood-glucose depression. This approach will accommodate the measurement of the responses of long-acting modified-insulin and rapid-acting normal-insulin. One practical problem is to compensate for the overall rise or fall in

the blood-glucose concentration caused by factors other than the administered insulin. Although the initial and final blood-glucose levels were usually about the same, they could vary by up to twenty percent. A blank-control blood-glucose assay curve (the average of six experiments involving the administration of only 10% or 30% dimethylsulphoxide/saline) was used as an adjustable baseline for the insulin-assay curves in which the initial and final blood-glucose levels varied by more than 5%.

Despite these considerations, with one exception, the log(dose)-response curves give very similar results. Only in the case of the response as measured by the initial rate of blood-glucose depression were the log(dose)-response curves of normal insulin and the modified-insulins approximately parallel. Too much reliance must not be placed on the log(dose)-response curves of the other two types (maximal blood-glucose depression and time-integrated blood-glucose depression). All the $N^{\alpha B1}$-acyl-insulin derivatives were shown to have an activity of about 40% related to insulin. These derivatives are less active than the $N^{\alpha B1}$-acyl-insulin derivatives described by others (acetyl-100% activity by mouse convulsion assay (Lindsay & Shall, 1971; acetoacetyl-50% activity by mouse convulsion assay (Lindsay & Shall, 1969); carbamyl and methylthiocarbamyl-100% by mouse convulsion assay (Lindsay et al, 1972) and t-Boc-90% (Levy, 1973)). It might be expected that modification with the more bulky triacylglycerol group would lead to a different activity from other acyl-insulin derivatives. The activity of these derivatives is about 20% compared with normal insulin. The B1-phenylalanine residue is about 20 Å from the edge of the receptor-binding region and so larger modifying groups will reduce activity by interfering more with binding than smaller

modifying groups. However these di-acyl-insulin derivatives are much more active than the A1-B1, or B1-B29 cross-linked insulin derivatives (Freychet et al, 1974 and Gliemann & Gammeltoft, 1974). This suggests that, in this case, the size of the acyl groups is not so important in reducing the activity of the derivatives as the fact that the amino-groups are cross-linked, which can cause a structural distortion.

A similar result is found with the di-acyl-insulin derivatives. When the blood-glucose depression or initial rate of blood-glucose depression is measured as the response, the activity of the triacylglycerol-insulin derivative is about 10% while the activity of the simple acyl-insulin derivative is about 17%. However when the time-integrated response is measured the simple di-acyl-insulin derivatives have a much higher activity - about 40% of standard insulin. By contrast the value for the activity of the di-(triacylglycerol)-insulin derivative is unchanged when this method of calculation is used. As has been previously mentioned this is explained by the fact that the simple di-acyl-insulin derivatives have a prolonged action, and therefore you would not expect a correlation between activity as measured by an initial response compared with activity as measured by a time-integrated response.

6Bii Prolonged Activity

The results show that one class of the insulin derivatives had prolonged activity. These were the $N^{\alpha B1},N^{\epsilon B29}$-di-acyl-insulins. None of the mono-acyl-insulin, the mono- or di-(triacylglycerol)-insulin or Ficoll insulin derivatives have blood glucose curves different from normal insulin (once adjustment for different activities have been allowed for). This difference in activity leads to an effective doubling of time of

activity of the derivative, compared with equally effective doses of normal insulin.

This effect may be due either to a decreased rate of degradation (as suggested by the work of Sonksen and co-workers or Kaplan and co-workers), or to a decreased rate of mobilization of the insulin from a depot. The latter could occur, if the insulin derivative (which is poorly soluble and administered in 30% dimethylsulphoxide/saline) was precipitated when mixed with the blood on administration. A simple in vitro experiment was performed to examine this possibility. Di-nonanoyl-insulin was made up in 30% dimethylsulphoxide/saline, as if for injection, at about 500 ug/ml. (The usual concentration administered was between 60 and 600 ug/ml). Half a millilitre of this was injected into 10 ml of stirred blood serum, which had been separated from freshly drawn rabbit blood. No opalescence could be detected by eye, or by spectrophotometric measurement at 500 nm. If excess of the dimethylsulphoxide solution was added, precipitation of protein occurred. This could be shown by an appropriate control to be due entirely to the effect of dimethylsulphoxide on the serum.

It is apparent that a depot of insoluble, lipophilic insulin does not form. However the derivative may bind more strongly to serum proteins or blood vessel surfaces to form a cryptic depot. The use of the radioactively labelled di-octanoyl insulin does not support this hypothesis. The serum-soluble fraction (assumed to be the labelled, undamaged insulin) disappears from the serum with two half-times. The first, rapid phase (half-time 8 to 10 minutes) corresponds to the distribution of the insulin from the blood to the 'insulin distribution-space', which includes the blood volume, the lymphatic volume and some of the extracellular fluid spaces. The extension of

this decay to zero time gives a value equal to that expected if the label was instantaneously distributed around the whole blood volume (54 to 57 ml/kg). The second phase has a slower half-time (24 to 30 minutes) and when extrapolated to zero time corresponds to a total insulin distribution space of 107 and 139 ml/kg which is about to the same value as that found by other workers for soluble insulin, proinsulin and other labelled insulin derivatives. Thus the lipophilic insulin is not being sequestered by any organ but remaining in the body fluids. Whether it is reversibly bound in an inactive form too or has a reduced rate of degradation cannot be decided from this experiment. The prolonged activity is due to the reduced clearance rate (half time is about 27 minutes, compared with the 5 to 10 minutes expected for normal insulin).

The activity of the di-(triacylglycerol)-insulin derivative was unaffected by feeding the animal after the administration of the drug. This result was also borne out by some in vitro experiments performed in this laboratory (A. J. Furth, unpublished experiments). This insulin derivative was not deprotected by the action of pancreatic lipase, gastric lipase, post-heparin plasma or liver acetone-powder. The pancreatic lipase and liver acetone powder contained sufficient protease to lead to the removal of the (B22-30) octapeptide. This evidence, as well as the negative in vivo results suggests that the triacylglycerol attached to insulin cannot act as a substrate for normal lipases.

6C Assays by Peroral Administration

The slight hypoglycaemic activity of the di-octanoyl-insulin and the mono-nonanoyl-insulin, can most probably be discarded as chance events. In fact the major contribution to the hypoglycaemic activity of these two derivatives

is due to one animal which showed a marked hypoglycaemia (up to 20%) in the experiments. This animal was used only in the assay of these two derivatives. Either this animal is unduly sensitive to perorally administered insulin, or more likely, it was affected by other elements of the experiment. For five out of five events, each of low probability, the cumulative probability of them occurring is very low, but this assumes that the individual time points are independent events. If the blood-glucose concentration at one time point is low due to circumstances other than the random variance of glucose assay etc., it is likely that these factors will contribute to a lowering of the blood glucose at adjacent time points. Also on a teleological basis it is unlikely that di-octanoyl-insulin and mono-nonanoyl-insulin have a hypoglycaemic effect, whilst the closely related homologues, di-nonanoyl and mono-octanoyl-insulin are completely without effect.

For tri-(triacylglycerol)-insulin the evidence is much more convincing. Not only is the significance of the individual points very high, there is a steady intensification and reduction in the hypoglycaemic effect, which would be expected if it was caused by an agent with a limited duration of activity rather than a chance decline due to other factors. And finally there is a tendency towards an increased dose leading to an increased effect. (The hypoglycaemic effect increases 1.7-fold with a 3.0-fold increase in the dose.)

The doses used in these assays (3 to 9 U/kg) are lower than those for almost any of the other analogues assayed by peroral administration (8 to 30 U/kg).

Though no rigorous comparison can be made between species and between diabetic and non-diabetic subjects, the hypoglycaemic effect of this derivative would suggest that a dose of at least 10 times

that given by injection would be needed to effect any substantial blood glucose depression in human diabetics. This coupled with a likely loss of some of the insulin during synthesis makes the future use of this derivative doubtful.

6D Silica-gel-loaded Paper Chromatography

During the course of these studies, a simple method for measuring the modification of insulin was developed. It is not intended to replace the more precise methods currently in use. However it is cheap, simple and fairly rapid in use, making it a useful complementary technique.

The method involves the ascending chromatography of the protein on silica gel loaded paper (SG-81 paper, Whatman Ltd.). Including preparation and staining the total analysis time is about 5 hours. This compares favourably with methods used to determine charge changes such as gel electrofocussing (total time about 6 hours, or longer to stain weak bands), or paper or cellulose acetate electrophoresis after sulphitolysis (about 4 hours) and with end group determinations, such as dansylation or deamination, both of which take at least a day before results are produced.

Another useful point which commends the technique is as follows. If ninhydrin/cadmium acetate stain is used, it can help to confirm the free amino groups of the insulin derivative. As glycine stains yellow, and phenylalanine and lysine both stain pink, then $N^{\alpha A1}$-acyl-insulin stains pink, whereas $N^{\alpha B1}$,$N^{\epsilon B29}$-di-acyl-insulin stains yellow. So the synthetic sequence from insulin, to $N^{\alpha A1}$-mono-Boc-insulin, to $N^{\alpha A1}$-mono-Boc-$N^{\alpha B1}$,$N^{\epsilon B29}$-di-acyl-insulin and deprotection to $N^{\alpha B1}$,$N^{\epsilon B29}$-di-acyl-insulin, as well as producing mobility changes, leads to colour changes from orange to pink, to colourless and to yellow.

Various simple precautions need to be taken to ensure good results. The mobility varies slightly with loading and the use of suitable standards at the same loading as the unknown is advised. Mixtures of proteins or contamination of the sample with traces of salt, urea or lipid do not appear to affect the separation. The performance of control experiments is recommended for any new use. The sample is best loaded in a spot of about 6 to 10 mm in diameter, diffusion is small and the spot remains quite discrete. Loading the sample onto a smaller spot leads to the production of a streak. In fact it is satisfactory to load the sample as a one cm streak, at right angles to the eluant flow, and after development virtually square spots are produced. Ascending chromatography is used because descending chromatography, though faster, causes streaking.

6E Toxicology

The animals examined were those that had been used most extensively during the testing of the insulin derivatives. None of the animals had had regular daily injections of the derivatives for any period. During the two-and-a-half year period in which the assays were performed, there were seven testing periods of approximately six to eight weeks duration. In these periods any animal would have about three or four assays performed on it, one or two of which would be insulin standards. Consequently the toxicological results only demonstrate that there are no obvious, serious effects due to this very limited administration programme. Dimethylsulphoxide has been shown to have acute side-effects consequent on intravenous injection, especially in the liver and kidney (Brown et al, 1963), but no effects attributable to the action of dimethylsulphoxide were detected. The aorta was examined with some care. No sign of a arteriosclerotic plaque was observed by eye

or microscopically. The rather unusual 'lipophilic' nature of the insulin derivatives could possibly have induced plaque formation, especially when injected in the presence of dimethylsulphoxide.

The double-diffusion immunoassays were negative. However this result does not exclude the existence of antibodies in one or more of these rabbits. Insulin is not very satisfactory as an antigen in this sort of assay. However the lower solubility of the acyl-insulins might improve the chances of precipitation of the antigen/antibody complex. A negative result, was however, also observed with the use of di-nonanoyl- and di-stearoyl-insulin as antigen in the assay.

There is no reason to believe that the deaths that occurred were caused by the insulin derivatives administered. Pneumonia is a common cause of death in laboratory animals. The 'sore hocks' syndrome was also common amongst our rabbits and was due to the poor design of the cage floors. Only in the case described in chapter 5 did the syndrome lead to more serious effects.

6F Prospects

The encouraging results achieved in the present work lead to proposals for future work on similar lines.

One necessary task is to test more thoroughly the tri-(triacylglycerol)-insulin derivative which showed the most promising results when assayed by peroral administration. It would also be valuable to test related derivatives. When administered perorally the $N^{\alpha B1}$-mono- and $N^{\alpha B1},N^{\epsilon B29}$-di-(triacylglycerol)-insulin derivatives were shown to be inactive. However other triacylglycerol-insulin derivatives ($N^{\alpha A1}$- or $N^{\epsilon B29}$-mono-, $N^{\alpha A1},N^{\alpha B1}$- or $N^{\alpha A1},N^{\epsilon B29}$-di-) may prove to have the same or better bioavailability with an increased hypoglycaemic potency.

Another approach to the problem of making a

perorally-administrable insulin preparation is the use of liposomes. As mentioned in chapter one, liposome-entrapped insulin is moderately effective as a peroral hypoglycaemic agent in experimental animals. The loss of insulin by this method is probably due to enzymatic and mechanical damage to the liposomes in the gut. Rupture of the liposomes permits protease attack on the insulin. The entrapment of a lipophilic insulin derivative in liposomes may be better than the use of normal insulin because lipophilic insulin is likely to become absorbed into or onto the liposome membranes. Even when the liposomes are ruptured, lipophilic insulin is less susceptible to protease attack than is normal insulin. The exact nature of the lipid modifications needed for this application is likely to be different from the modification described in this present work. For example, charged lipids, like those used in the liposomes, may be needed to modify the insulin.

Some clinicians object to the concept of an peroral insulin preparation, as they feel that accurate dosing will be difficult and that relatively minor gastric disorders will severely affect the therapeutic control. Although these are valid objections, they are not insuperable. Many other drugs which need to be given in very accurate doses are administered perorally. For example, therapy with anti-coagulants, cardiac glycosides and anti-cholinesterases can lead to cerebrovascular accident or cardiac failure if the drug effect is reduced or augmented by accident or another concurrent disease. Secondly, I do not propose that such oral hypoglycaemic agents would necessarily become widely used. The expense, in terms of the insulin required and the manufacturing costs, would probably act as a limiting factor. However these preparations would be beneficial to those diabetics for whom the parenteral administration of insulin is

a problem. Brittle diabetics could reduce the number of daily injections needed by the partial replacement of injected insulin by an oral preparation, this treatment could also possibly improve the diabetic control of these patients. If a peroral insulin preparation was used elderly and blind diabetics would no longer be troubled with accidental misinjection etc. A peroral preparation would also be of benefit to those diabetics with psychological problems associated with the injection of insulin. Finally there is no reason why a carefully manufactured preparation should suffer from any more variation in potency and bioavailability than is found for other drug preparations. The changes in sensitivity to insulin due to infection or a change in life-style leading to hypo- or hyperglycaemic shock, occur as readily with parenterally administered insulin now as they would be likely to with perorally administered insulin.

The development of an *in vivo* reactivatable long-acting insulin derivative was unsuccessful. In fact it became side-tracked into the study of the acyl-insulin derivatives whose prolonged activity proved to be due to an altered rate of degradation. However it is possible that other triacylglycerol-insulin derivatives can be reactivated *in vivo*. As well as altering the fatty acids involved, alternative methods of attaching the modifying group should be considered.

Insulin preparations that have prolonged activity, although clinically useful, are very abnormal physiologically. Most of the available prolonged-action insulin preparations may have a duration of hypoglycaemic effect of up to 24 hours, with a peak effect from about 2 to about 6 hours post-injection. They are usually administered as a single dose in the morning. The peak hypoglycaemic effect then occurs during the day, when meals (a little and often) are eaten. The effect is reduced at night when there is no food

intake. This contrasts sharply with the natural supply of insulin in a normal person, which peaks for about 2 hours in response to each meal. The large fluctuations in glycaemia caused by prolonged-action insulin therapy in diabetes is probably a major cause of the long-term complications of the disease. In attempts to reduce the severity of diabetic complications the development of an insulin derivative, the prolonged action of which is linked to the changes in blood-glucose, is an important possibility for a more physiologically-acting drug.

6G Summary

Three different types of lipophilic insulin derivative were synthesised ($N^{\alpha B1}$-mono, $N^{\alpha B1}$,$N^{\epsilon B29}$-di-, $N^{\alpha A1}$,$N^{\alpha B1}$,$N^{\epsilon B29}$-tri-) with five different acyl groups (octanoyl, nonanoyl, stearoyl, oleoyl and 1,3-dipalmitoyl-2-O-azeloylglycerol), though one early batch of the triacylglycerol derivatives was probably a mixture of nonanoyl and triacylglycerol acylating groups. These were tested for prolonged hypoglycaemic activity when administered intravenously and for any hypoglycaemic activity when administered orally.

Of these the $N^{\alpha B1}$,$N^{\epsilon B29}$-di-acyl-insulin derivatives, as a class, had prolonged activity. The main reason for this prolonged activity was shown by the use of a radioactively-labelled derivative to be due to a prolonged half-time of the derivative in the blood. There was no evidence for the deprotection of any derivatives in the blood as had been hoped for at the outset of the work.

The tri-(triacylglycerol)-insulin derivative was shown to be active when administered perorally at fairly low doses, in comparison with normal insulin controls.

A different type of derivative, the Schiff-base coupled Ficoll-insulin, was shown to have reduced activity and no prolonged

activity.

A superficial toxicological survey showed that these derivatives had no observable, deleterious side-effects.

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Appendix

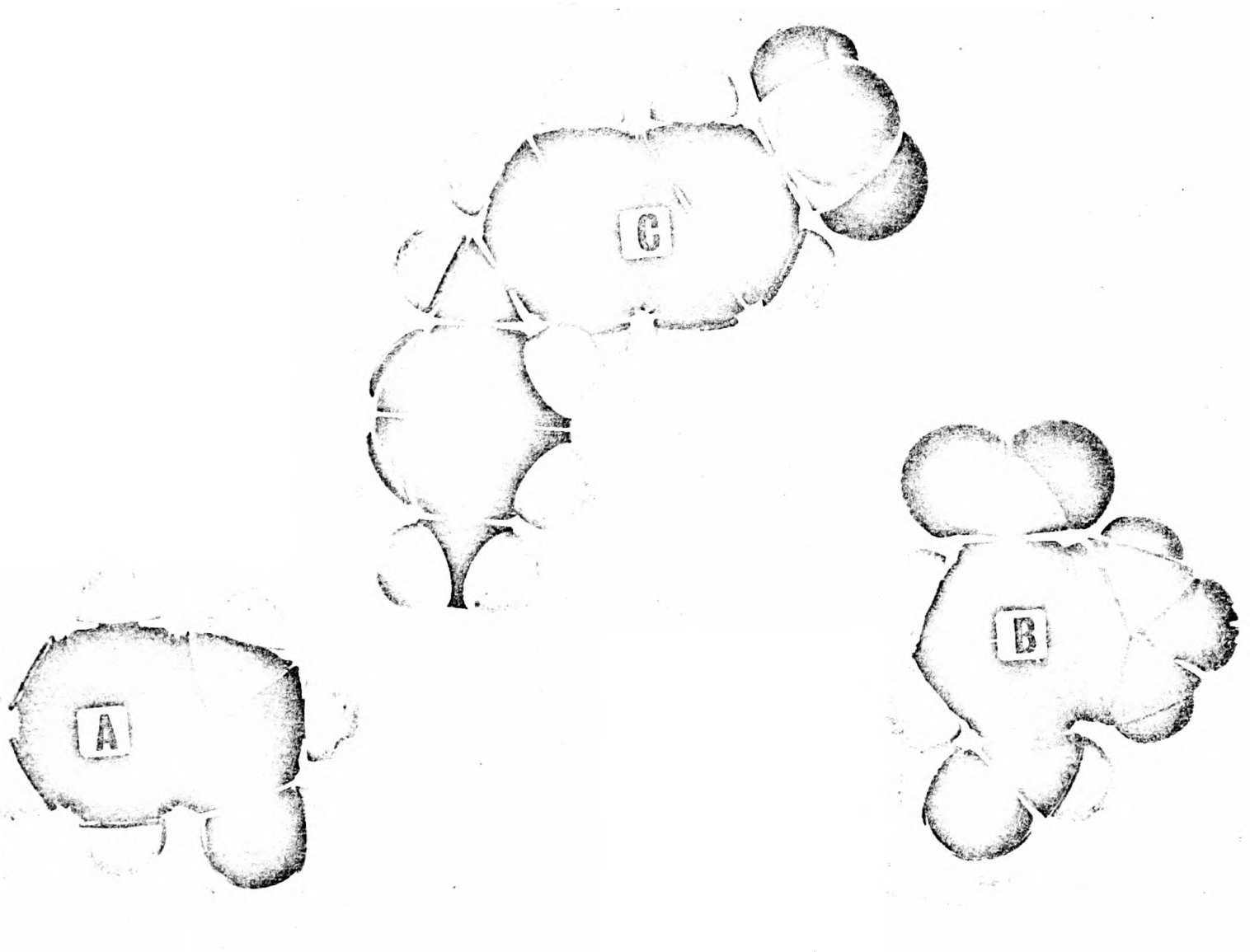
This describes some unrelated work on the development of a novel fluorescent amino-acid for use in protein semisynthesis. It includes a copy of a preliminary published report, Moore, R.R. (1978) in "Semisynthetic Peptides and Proteins", (Offord, R.E. and DiBello, C. eds.), Academic Press, London and New York, pp. 367-371, and a copy of a paper which is to be submitted for publication.

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One of the main problems with methods of studying protein molecules in solution is the ambiguity or imprecision in the interpretation of the results. Changes in the NMR or fluorescent spectra, for example, can usually only be interpreted in terms of a general change of shape in the molecule. Semisynthetic methods are ideal for the insertion of specific labels into known positions in proteins, and from these modified macromolecules useful information may be obtained. For instance, the introduction of semisynthetic ^{13}C labels has been performed by Saunders & Offord (1972), and a radio-label by Halban & Offord (1975). Fluorescence spectroscopy can be a valuable method for supplying information about changes in molecular orientation and structure (by studies on the depolarisation of fluorescence) and about protein–protein and protein–ligand interactions (by fluorescence enhancement spectroscopy). There are, however, drawbacks to many of the fluorescent molecules used. The natural fluorescence of the amino acids, tyrosine, tryptophan or phenylalanine; or of the coenzymes NADH, FADH, or pyridoxal phosphate has been used but often these natural fluorophores may not be in the most convenient site, and if there is more than one fluorophore, the overlapping fluorescent spectra can produce confusing results. Extrinsic fluorophores have been coupled, covalently or non-covalently, to proteins in a non-specific manner, often with several fluorphores being bound to unspecitied sites in the macromolecule. Also the molecules used, such as: ANS, 1-anilino-8-naphthalene sulphonate; TNS, 2-para-toluidinyl-6-naphthalenesulphonate; DNS, 1-dimethyl-amino-6-naphthalene sulphonamide and others, are large, containing two or three aromatic rings and five or more hetero-atoms. These reagents may cause unacceptable perturbaticns when bound to the protein under study.

I have chosen to use the compound of Ghosh and Whitehouse (1968), 1-nitro-4-chlorobenz-2,3-furazan (Nbf-chloride, Fig. 1). This compound has advantages over these other fluorescent molecules as it is small, it contains only two aromatic rings. It has an activated chlorine atom


C
A
B

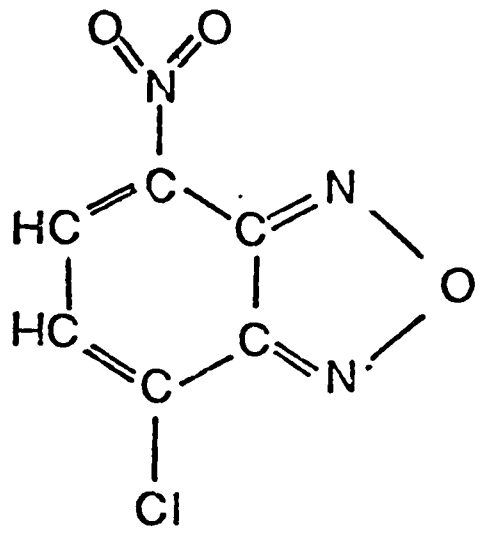


Fig. 1. Structural formula of 1-nitro-4-chlorobenz-2,3-furazan.

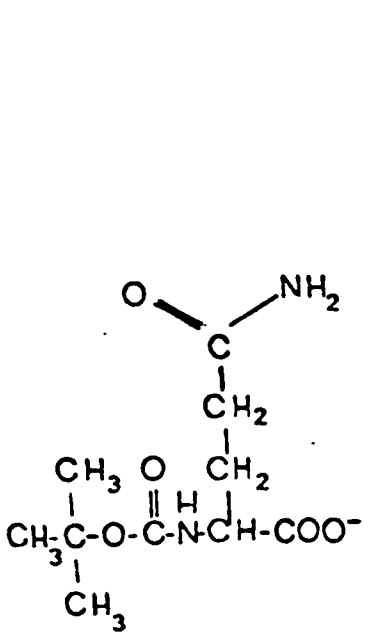
which can react with amino or thiol groups and thus it may be attached to the side chain of a suitable amino acid to form one that is fluorescently labelled. If one of the lower homologues of lysine (L-α,β-diaminopropionic acid or L-α,γ-diaminobutyric acid) is used, a molecule is produced which can be shown by model building to be about the same size as the larger amino acids such as tryptophan (Fig. 2).

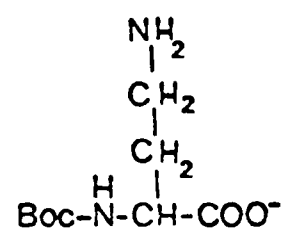
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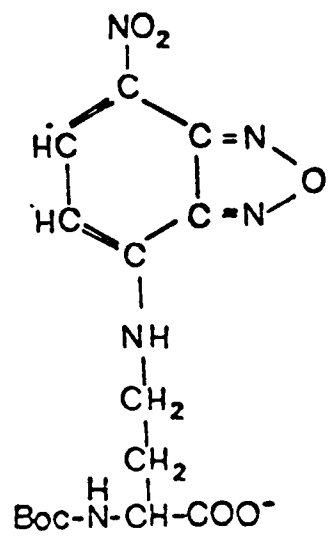
Fig. 2. Comparison of N^{γ}-Nbf-diaminobutyric acid with tryptophan and TNS using space-filling models. A: Tryptophan (side-chain only); B: N^{γ}-Nbf-diaminobutyric acid (side-chain only); C: 2-p-toluidinyl-6-naphthalene sulphonate.

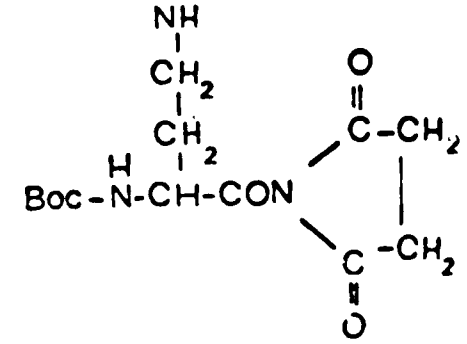
This fluorescently labelled amino acid is smaller than ANS or TNS and when a residue in the native protein is semisynthetically replaced by it, there is a minimal increase in bulk. There are likely to be sites that are sufficiently free from constraint for substitution by this molecule not to cause any serious structural perturbation in the protein into which it has been incorporated.

This fluorescent label has been synthesised and incorporated into proteins as shown in Fig. 3. The first reaction is best carried out with the α-amino group of the









See facing page

Fig. 3. Scheme of the synthesis of N^{α}-Boc-N^{γ}-Nbf-diaminobutyric acid-N-hydroxysuccinimidyl ester from N-Boc-glutamine.

starting material protected before the side-chain amino group is formed, thus achieving differential protection between the two. Usually the starting material used was Boc-glutamine* (although Boc-asparagine has been used as well to produce the α,β-diamino-propionic acid derivative). The side-chain amino group was produced by a Hoffman amide degradation, as was done by Rudinger *et al.* (1960) on the *p*-toluenesulphonyl derivative of glutamine and asparagine. The 3M NaOH used in this reaction could possibly have caused racemisation but it will be shown below that it does not. The product of this first step, formed in high yield, was desalted on AG11A8 ion retardation resin (Bio-Rad Laboratories Ltd., 27 Holmesdale Rd., Bromley, Kent, U.K.); and recrystallised from methanol/acetone. The newly generated γ-amino group was then reacted with Nbf-chloride, in mildly basic semi-aqueous conditions. The product formed in moderate yield from this reaction was purified by solvent extraction and by column chromatography on a polycaprolactam column with ethyl acetate, methanol, and acetic acid as eluant. This product was then converted into an active ester in good yield. The N-hydroxysuccinimidyl ester was made using dicyclohexylcarbodiimide and was purified by recrystallisation from ethyl acetate.

All the crystallised products had the correct elemental composition (within 0.4% of theoretical) and sharp melting points. The N^{α}-Boc-N^{γ}-Nbf-amino acid was an oil and so could not be characterised in this way. A sample of the N^{α}-Boc-diamino acid from the Hoffman degradation was deprotected with anhydrous trifluoroacetic acid, to form the free α,γ-diamino acid. After recrystallisation this product was shown to have the same optical rotation and

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*Boc = *tert*butyloxycarbonyl; Nbf = 1-nitrobenz-2,3-furazan-4-yl.

elemental composition as an authentic sample, suggesting that no racemisation had occurred in the Hoffman degradation (this result was also found by Rudinger for the *p*-toluenesulphonyl derivatives). The diamino acids and their N^{α}-Boc derivatives have the expected mobility on paper electrophoresis at pH 1.9, 3,5, and 6.5. The N^{α}-Boc-N^{γ}-

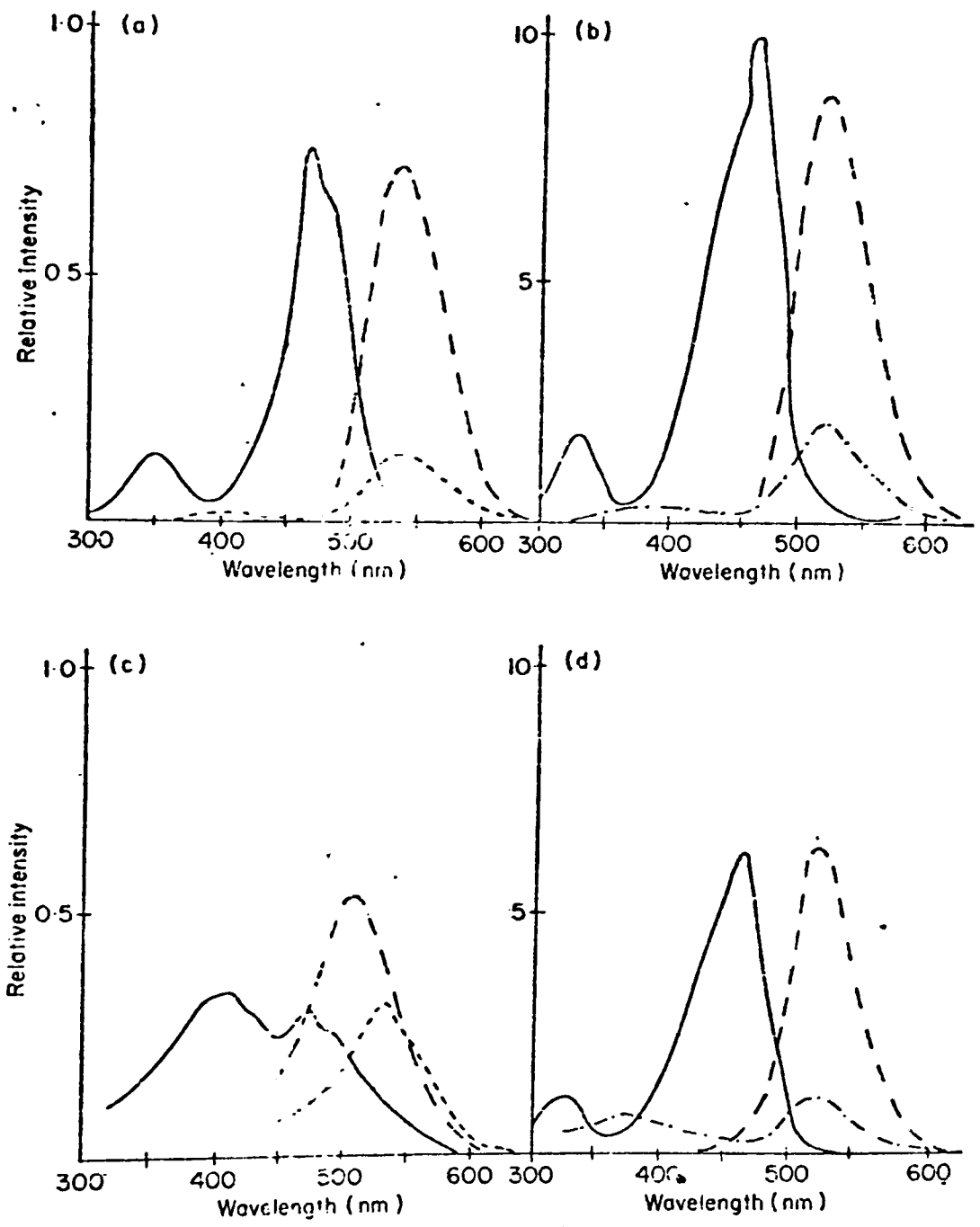


Fig. 4. Fluorescent spectra of N^{α}-Boc-N^{γ}-Nbf-diaminobutyric acid and its N-hydroxysuccinimidyl ester. Spectra of acid (a,b) and ester (c,d). Solvents were water (a,c) or ethyl acetate (b,d) all at 10 g/ml ——— Excitation spectra. ----- Emission spectrum for excitation at 470 nm. ····· Emission spectrum for excitation at 350 nm. —·—·— Emission spectrum for excitation at 320 nm.

Nbf-diamino acid and its active ester gave single spots on three thin-layer chromatography systems. Full details will be reported elsewhere.

The active ester has been coupled to a fragment of bradykinin; a truncated CBr fragment of cytochrome *c*, replacing residue 82; and onto the B1 position of insulin. Coupling was performed with a 7-to-10-fold excess of active ester over amino groups, in DMF at 1μ mol/ml of amino groups, with N-ethylmorpholine as base, at an apparent pH of 9 (as described by Rees & Offord, 1976).

Excitation and emission spectroscopy were performed on an Aminco-Bowman spectrofluorimeter. The excitation peaks at 350 and 470 nm and the emission peak at 540 nm (Fig. 4) are at a much higher wavelength than those of extrinsic fluorphores. The spectra of the free acid and of the active ester are very similar. There is a marked difference in the quantum yield in aqueous and hydrophobic solutions.

Thus, when this molecule is incorporated into proteins it is a potentially valuable tool for binding and energy-transfer studies using fluorescence enhancement. For example, it could give information about the role of the group of aromatic residues found adjacent to the haem crevice in cytochrome *c* if incorporated into a suitable place in this molecule.

Acknowledgements

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Rudinger, J., Poduska, K. and Zaoral, M. (1960). *Coll. Cz. Chem. Comm.* **25**, 2022-2030.
Saunders, D.J. and Offord, R.E. (1972). *FEBS Letters* **26**, 286-288.

Discussion

Laskowski: Are your amino-acid esters hydrolysed by chymotrypsin, as tryptophan esters are?

Moore: I haven.t examined that.

Carpender: Is the residue stable to acid hydrolysis?

Moore: It does not seem to survive 24 hours in 6N HCl. It leaves some ill-defined peaks on the analyser trace.

Hagenmaier: What is its fluorescent yield compared to that of the dansyl group?

Moore: About the same.

An amino acid with a fluorescent side chain for use in protein semisynthesis

by

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Running Title: Fluorescent amino acid

Synopsis

1. This paper describes the preparation of N^{α}-Boc-N^{γ}-(1-nitrobenz-2,3-furazan-4-yl)-L-α,γ-diaminobutyric acid hydroxysuccinimido ester*. 2. We describe the chemical characterization of this compound and of the intermediates leading to its synthesis. 3. We discuss the use of the compound for the semisynthesis of fluorescently labelled protein analogues. In contrast to experiments in which large fluorescent chromophores are bound to existing side chains, the net increase in the bulk of the protein that is produced when a residue is substituted by the use of our compound can be quite small. 4. The excitation and emission spectra are favourable for structural studies on proteins containing the amino acid.

*Footnote:

Boc = tert-butyloxycarbonyl

SCHEME 1

N^{α}-Boc-L-glutamine (I)

↓ Hoffman amide degradation

N^{α}-Boc-L-α,γ-diaminobutyric acid (II)

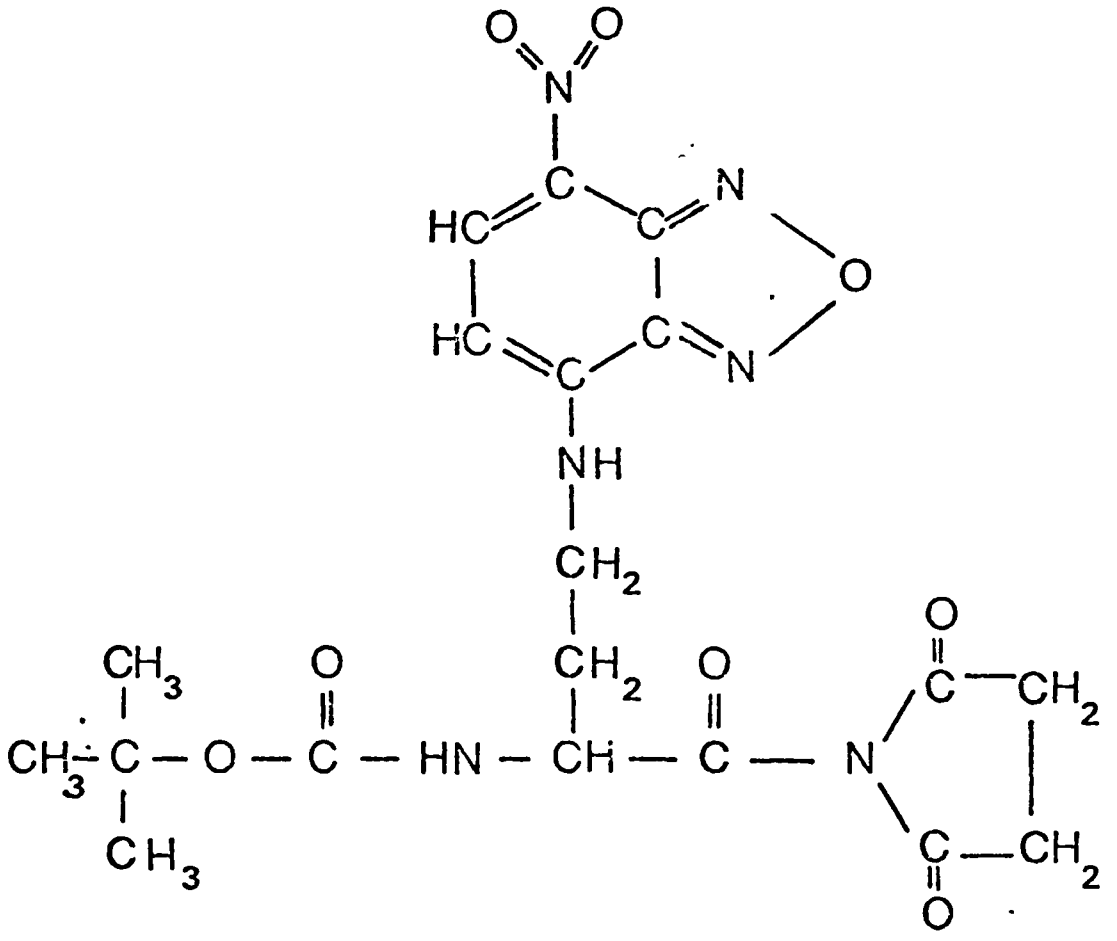
↓ Arylation with 1-nitro-4-chlorobenz-2,3-furazan

N^{α}-Boc-N^{γ}-(1-nitrobenz-2,3-furazan-4-yl)-L-α,γ-diaminobutyric acid (I

↓ N,N -dicyclohexylcarbodiimide.
N-hydroxysuccinimide.

N^{α}-Boc-N^{γ}-(1-nitrobenz-2,3-furazan-4-yl)-L-α,γ-diaminobutyric acid N-hydroxysuccinimido ester (IV)

Scheme 1. The reaction pathway leading to the desired product.



Structural Formula

Introduction

An amino acid that has a fluorescent side chain, and that can be semi-synthetically introduced into proteins (e.g. by the methods described by Rees and Offord, 1976 or by Saunders and Offord, 1977), would be useful in the study of the solution properties of proteins. The design of such a molecule is affected by the following considerations: Firstly, it should be as near in size as possible to the natural amino acid that it is to replace; secondly, it must be a true α-amino acid, so that it can be incorporated into a polypeptide chain in the normal way; thirdly, the excitation and emission peaks of the molecule should be at different wavelengths from those of the natural fluorophores of proteins.

In order to fulfil these requirements we have used 1-nitro-4-chlorobenz-2,3-furazan (Ghosh and Whitehouse, 1968) in the reaction pathway shown in scheme 1. Compound (IV), when purified, is ready for immediate use in semisynthesis. Its structural formula is

(See facing page)

We have also started with Boc-asparagine and obtained a product which is shorter in its side chain by one $-CH_2-$ group (R. R. Moore and R. E. Offord, unpublished experiments).

The first step, which resembles the reaction carried out by Rudinger et al (1952) on N^{α}- p-toluenesulphonyl glutamine, has the advantage that it produces the diamino acid with its α-amino group already protected. Although the strongly alkaline conditions used in this step might have been expected to lead to racemization, Rudinger et al (1952) found that racemization did not occur with the N^{α}- p-toluenesulphonyl glutamine and we have been unable to detect it with the Boc- compound (see below).

Experimental and Results

1. Preparation of N^{α}-Boc-L-α,γ-diaminobutyric acid (II)

1.0 ml of 2 M Boc-L-glutamine (Sigma London, Norbiton Station Yard,

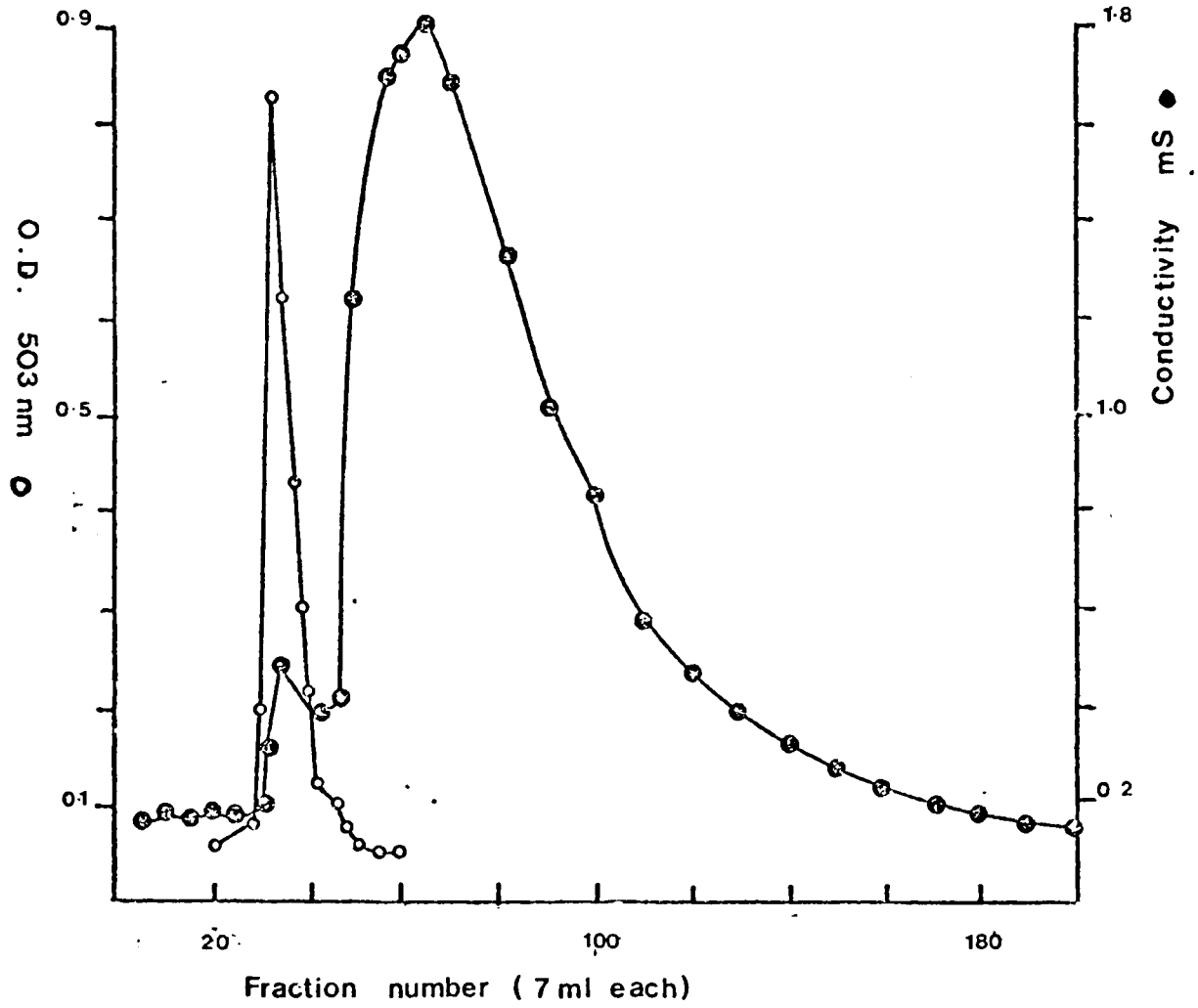


Fig. 1 Desalting of N^{α}-Boc-L-α,γ-diaminobutyric acid on resin AG11A8

Specific electrical conductivity (•) in mS and O.D. of ninhydrin/amino-acid complex at 503 nm (o) eluted in 1 ml of water/ethanol (1:19, v/v) from a paper electropherogram. The column details are given in the text.

Kingston-upon-Thames, Surrey, England KT2 7BH) and 2.5 ml of 0.8 M Br_2 solution, both in 3 M sodium hydroxide solution, were mixed at 4°C and left for a few minutes until the yellow colour was discharged. The solution was then heated at 75°C for 30 minutes. After cooling, the reaction mixture was neutralized with 3 M hydrochloric acid solution. It is essential to add the acid in very small increments, and to stir vigorously, in order to prevent the Boc group being removed by locally high concentrations of HCl. The neutral solution was then diluted with water until approximately 10 mg/ml in amino acid and desalted on AG11A8 resin (Bio-Rad Laboratories Ltd., 27 Holmesdale Road, Bromley, Kent).

450 g of resin was made into a slurry in water and poured into a column 90 cm by 2.5 cm. The maximum loading of this column, consistent with good resolution, is about 400 mg of amino acid as a 1% solution (w/v), in up to 3% (w/v) salt solution. The amino-acid solution was loaded on the column and eluted with water at a flow rate of 360 ± 120 ml/h. The eluate was collected in 7 ml fractions. The presence of amino acid in a fraction was detected by staining a paper electropherogram run at pH 1.9 with ninhydrin/cadmium acetate (all paper methods are as described in Gonzalez and Offord, 1971). Salt was detected by measuring the electrical conductivity of the eluate. The fractions that eluted between 210 and 290 ml contained amino acid and were salt-free. These tubes were pooled and lyophilised (Fig. 1).

Two batches of the resin appeared to be contaminated with strongly basic material. The pH of the column effluent was greater than 11, rather than the value of 8.5 as specified by the manufacturers. The pH could not be brought below 10 despite repeated washings and cycling through the regeneration procedure recommended by the manufacturers (Bio-Rad Bulletin 1005). The effluent from the column in this condition smelt strongly of amine, but did not leave a visible residue on evaporation.

The dried amino acid was dissolved in the minimum volume of warm methanol, and crystallized by the addition of acetone. The crystals were filtered, washed once with acetone and once with ether, and dried. The N^{α}-Boc-L-α,γ-diaminobutyric acid (II) had a melting point 220-222°C (decomp.), $[\alpha]_D^{20}$ 7.8 (water, C = 1.0), (Found C 49.5%, H 8.3%, N 12.8%; $C_9H_{18}N_2O_4$ requires C 49.5%, H 8.2%, N 12.9%).

2. Preparation of L-α,γ-diaminobutyric acid

100 mg of (II) was dissolved in 10 ml of anhydrous trifluoroacetic acid and left at 4°C for 30 minutes. The trifluoroacetic acid was removed by rotary evaporation at room temperature. The resulting oil was dissolved in aqueous 6 M hydrochloric acid and then rotary-evaporated to dryness. The product was twice recrystallized from hot aqueous ethanol. The α,γ-diaminobutyric acid had m.p. 126-128°C, $[\alpha]_D^{20}$ + 22.7 ± 2° (c 1.0 in 5 M HCl). Found C 31.3%, H 7.0%, N 17.7%. Calcd. for $C_4H_{10}N_2O_2 \cdot 1$ HCl : C 31.3%, H 7.0%, N 18.1%). Shou-Cheng et al (1952) gave a value of +24.2 for the specific optical rotation (c = 2.5 in 5 M HCl) for L-α,γ-diaminobutyric acid, which had been obtained enzymatically. We were therefore unable to detect any racemization resulted from the alkali treatment of the Hoffman degradation.

3. Preparation of N^{α}-Boc-N^{γ}-(1-nitrobenz-2,3-furazan-4-yl)-L-α,γ-diaminobutyric acid (III)

Equal volumes of a 2 mg/ml solution of (II) in 90% (v/v) aqueous ethanol and a 2 mg/ml solution of 1-nitro-4-chlorobenz-2,3-furazan in absolute ethanol, were mixed together. 5% of this total volume of ethanol saturated with sodium acetate trihydrate was then added. The mixture was heated at 70°C for 6h and then rotary evaporated to dryness.

A settled volume of 800 ml of dry polycaprolactam powder (described as "Ultramid-pulver für chromatographische Zwecke" No. 934K299, manufactured by Badische Anilin und Soda Fabrik AG, Ludwigshafen am Rhein, West Germany; a gift from Dr. P. C. J. Brunet of this department), was made into a slurry

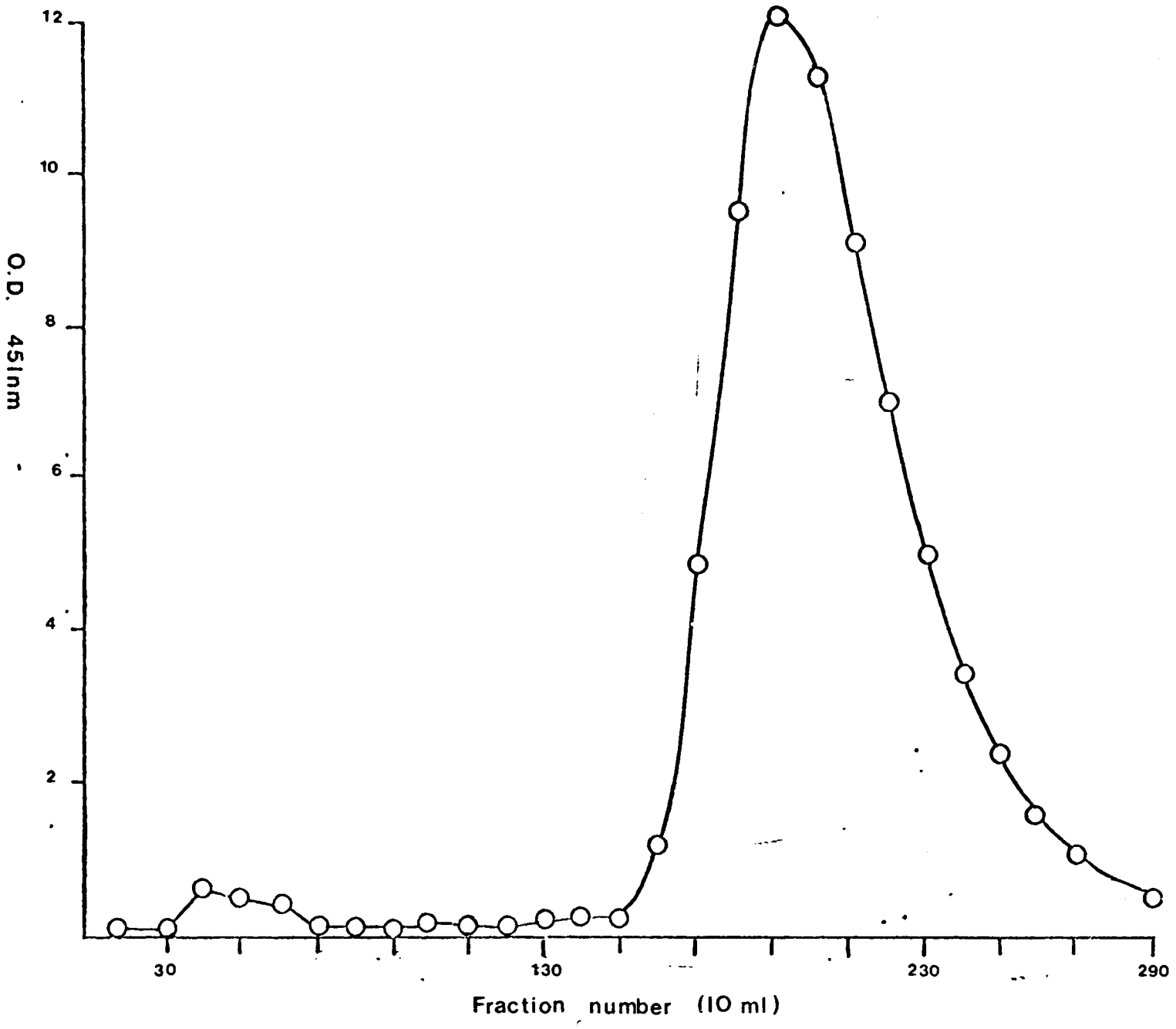


Fig. 2 Purification of N^{α}-Boc-N^{γ}-Nbf-L-α,γ-diaminobutyric acid on a polycaprolactam column.

O.D. of column eluate at 451 nm (o). The column details are given in text.

in one litre of eluant (ethyl acetate/acetic acid/methanol, 200:10:10 (by vol.)) and poured into a 75 x 5 cm column. The column was washed with a further one litre of eluant. The maximum loading of this column consistent with good resolution is about 200 mg of sample dissolved in 10 ml of eluant. This quantity of the crude compound (III) was separated on the column at its maximum flow rate (approximately 400 ml/h). The eluant was monitored at a wavelength of 450 nm (Fig. 2). The major peak, eluting between 1500 and 2700 ml, was collected. The pooled fractions were washed repeatedly with water until the aqueous washings were neutral. The ethyl acetate layer was dried with anhydrous $MgSO_4$ overnight, filtered and rotary-evaporated to an oil. Attempts at crystallization were unsuccessful.

On t.l.c. on polyamide layer plates (British Drug Houses, Atherstone, Warks., the material gave a single spot of Rf 0.9 in ethyl acetate/methanol/acetic acid, 200:10:10 (by vol.), of Rf 0.25 in toluene/acetic acid, 9:1 (v/v) and of Rf 0.0 in 1.5% (v/v) formic acid.

4. Preparation of the N-hydroxysuccinimido ester of N^{α}-Boc-N^{γ}-(1-nitrobenz-2,3-furazan-4-yl)-L-α,γ-diaminobutyric acid (IV)

22 μmoles of N,N'-dicyclohexylcarbodiimide and 22 μmoles of N-hydroxysuccinimide were dissolved in 0.1 ml each of ethyl acetate. 20 μmoles of (III) were dissolved in the hydroxysuccinimide solution and then the carbodiimide solution was added. This mixture was left at 4°C for 16h, during which time a precipitate formed. The reaction mixture was centrifuged, the supernatant washed once with an equal volume of 5% (w/v) sodium carbonate solution and twice with saturated sodium chloride solution. The ethyl acetate solution was dried overnight over anhydrous magnesium sulphate, filtered and left in an open dish to crystallize.

The crystalline compound (IV) gave a single spot of Rf 0.95 in ethyl acetate/methanol/acetic acid, 200:10:10 (by vol.) and in toluene/acetic acid, 9:1 (v/v) on t.l.c. on polyamide layer plates, melting point 200-202°C (decomp.), $[\alpha]_D^{20}$ + 83.8 (ethyl acetate, c = 1.05) (Found C 45.4%, H 4.9%,

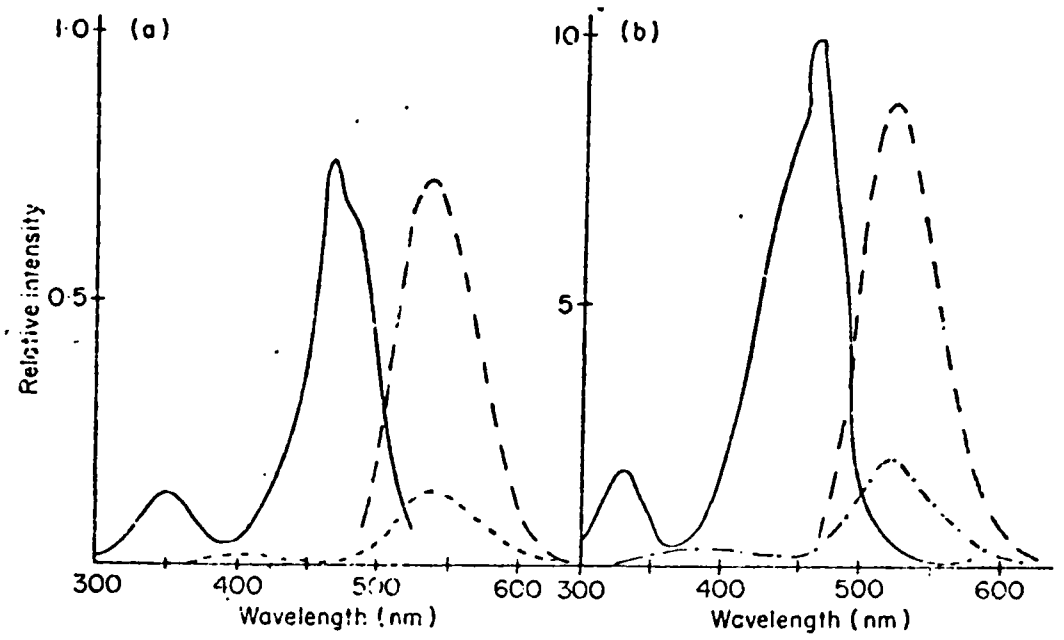


Fig. 3. Fluorescent spectra of N^{α}-Boc-N^{γ}-Nbf-diaminobutyric acid and its N-hydroxysuccinimidyl ester. Spectra of acid (a,b) and ester (c,d). Solvents were water (a,c) or ethyl acetate (b,d) all at 10 g/ml. ——— Excitation spectra. ----- Emission spectrum for excitation at 470 nm. ····· Emission spectrum for excitation at 350 nm. —·—·— Emission spectrum for excitation at 320 nm.

N 16.4%, $C_{19}H_{22}N_6O_9$. 1.5 H_2O requires C 45.2%, H 4.9%, N 16.6%). The crystals, once desiccated at room temperature, were hygroscopic. They decomposed in a heated desiccator on attempting to dry them and elemental analyses suggested an even higher degree of hydration if a sample was left to stand at room temperature in an open vessel.

Discussion

Model building shows that the fluorescent amino-acid residue is little bigger than tryptophan. Both are fused ring systems containing a five- and a six-membered ring, and the furazan ring is not greatly different in size from the pyrrole ring. The aliphatic parts of the side-chains differ by only one $-CH_2-$ group and even this difference could be eliminated by starting with Boc-asparagine. If there were to be steric problems they would be more likely to arise from the aromatic parts, in particular the $-NO_2$ of the nitrobenzfurazanyl group, but there are likely to be many sites in proteins that will accept the substitution of a residue by our compound.

Preliminary couplings of this fluorescently labelled amino acid to a synthetic peptide, Ser-Pro-Phe-Arg(NO_2)-benzyl ester, to a peptide derived from horse-heart cytochrome c by CNBr cleavage and Edman degradation ($N^{\varepsilon 86,87,88,98,99}$-penta acetimidyl-cytochrome c -(82-104)-docosapeptide) and to $N^{\alpha A1,\varepsilon B29}$-bis carbobenzoxymethionyl-des-Phe^{B1}-insulin have been successfully performed (R. R. Moore, unpublished experiments).

The fluorescent emission and excitation spectra are shown in Fig. 3. There is a ten-fold increase in quantum yield on changing from aqueous to a hydrophobic solvent. This property will be of use if the molecule is used to investigate changes in the degree of polarity in the local environment of the side chain.

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