~~STRUCTURAL~~ STUDIES ~~ON~~ IN GLYCOPROTEIN STRUCTURE

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ABSTRACT

In studies on the carbohydrate-protein linkage in glycoproteins it is desirable to prepare glycopeptides containing only the amino acid to which the carbohydrate is attached. This has been achieved in the case of ovalbumin by a combination of enzymic digestion and gel filtration.

An estimate of the molecular weight, derived from the molar extinction of the benzyloxycarbonyl derivative, was compatible with previous suggestions that the glycopeptide was composed of five residues of mannose, three residues of N-acetylglucosamine and one residue of aspartic acid.

Formation of the phenylthiohydantoin of the glycopeptide demonstrated that the carbohydrate was linked through the β-carboxyl group of the aspartic acid.

Unequivocal evidence to show that the carbohydrate-protein linkage was that of an N-glycoside was obtained by the reduction of the methylated N-benzyloxycarbonyl glycopeptide with lithium aluminium hydride to convert the substituted amide bond of the linkage to a secondary amine. Dinitrophenylation followed by hydrolysis yielded a substance chromatographically identical with di- DNP-4-amino-2-methylaminobutane-1-ol. A method of synthesis was devised for the last mentioned compound which is the substance expected from the reduction of an N-benzyloxycarbonyl asparagine substituted at the amide group. A closely similar reduction of

3,4,6-tri-O-acetyl-2-acetamido-1-(L-β-aspartamido)-1,2-dideoxy-β-D-glucose, the model compound of the proposed linkage in the glycopeptide, gave results in agreement with those obtained from the glycopeptide. The synthesis and a few properties of a number of compounds related to diaminobutanol are also described.

Potentiometric and spectrophotometric titration of the glycopeptide showed the presence of an ionising group, in the high alkaline range, that was assigned to the imino group in the carbohydrate-protein linkage.

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LIST OF ABBREVIATIONS

Substance	Abbreviation
Glycopeptide prepared by Pronase digestion.	Glycopeptide-P
Glycopeptide prepared by Pronase-carboxy-peptidase digestion.	Glycopeptide-PC
Glycopeptide prepared by papain digestion.	Glycopeptide-PA
Glycopeptide prepared by papain-Pronase digestion.	Glycopeptide-PA-P
Benzyloxycarbonyl group.	ebzo-
Lithium aluminium hydride.	LAH
4-amino-2-methylaminobutane-1-ol.	AMAB or FII
2-acetamido-1-(L-β-aspartamido)-1,2-dideoxy-β-D-glucose.	AADG

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Historical

The viscosity of many biological secretions has probably been noticed since time immemorial. The word "mucus" that is used today is unchanged from the Latin and in its early usage referred to the nasal secretions. The word "jelly" is from the Middle English "gele" and was specially applied to the alga Nostoc, and slime is from an Old English word "slim" which was used in 1471 to refer to the same alga, which was thought to be "star jelly" or the remains of a fallen star. The word "albumen" derives from the Latin "album" and by 1599 was used in England to refer to the egg white.

It was not until more recent times that any scientific interest was taken in these compounds. At the beginning of the nineteenth century John Bostock (1805) performed some remarkable experiments with what he called the primary Animal Fluids. He distinguished between albumen, jelly and mucus by several chemical and physical methods. He denatured egg white at 100° and found 4.5 per cent uncoagulable, and in a later paper (1806) identified this as mucus because it was precipitated by lead acetate. He also observed that ovalbumin resisted putrefaction whereas mucus did not. Using similar methods he investigated cerebrospinal fluid and found mucus, a little albumen and a minute amount of sodium chloride; in pericardial

fluid he found 5.5 per cent albumen, 2.0 per cent mucus and 0.5 per cent sodium chloride; and in saliva he found 0.8 per cent albumen, 1.1 per cent mucus and 0.1 per cent sodium chloride.

The nature of these substances was the subject of investigation by several workers towards the end of the nineteenth century. Eichholz (1898) states that "the idea of sugar being contained within the proteid molecule can be traced back as far as Berzelius", but he claims it was Krukenberg (1885) who first definitely put forth the view that proteins contain a carbohydrate group as part of the molecule and based his opinion on the fact that they would reduce alkaline solutions of cupric oxide on boiling.

However it seems that twenty years earlier Eichwald (1865) was able to isolate a mucoprotein from Helix pomatia which, on acid hydrolysis, yielded a substance which reduced Fehling's solution.

In 1888 Hammarsten showed that in the case of the submaxillary gland mucins carbohydrate and protein are chemically linked, and in 1896 Krawkow (quoted by Eichholz, 1898) obtained osazones having a melting point of 182-185° from egg white, fibrin and serum albumin. Eichholz (1898) separated the egg white proteins ovomucin, ovomucoid and ovalbumin and after acid hydrolysis prepared osazones from them. He treated serum globulin similarly and obtained an osazone but he was unable to prepare the same derivative from serum albumin. In the same year Seeman (1898) prepared crystalline

"chitosamine" (glucosamine) hydrochloride from ovalbumin, and in 1901 this was repeated by Muller.

The quantitative estimations of these workers were less reliable than their qualitative work often because pure material was not available, but Eichholz (1898) found 5 per cent carbohydrate in ovalbumin and Osborne and Campbell (1900) after recrystallising the same protein six times obtained figures of between 3 and 5 per cent. These values agree well with modern estimates.

The next two decades saw little progress and when he reviewed the subject in 1925 Levene said "the most conservative conclusion is that the glycosidic structure of ovalbumin requires reinvestigation."

It was not until Neuberger (1938) looked more carefully at this molecule that convincing evidence for a covalent linkage between carbohydrate and protein was presented. He found that repeated crystallisation, ultrafiltration and denaturation failed to alter the carbohydrate composition of the protein. Prolonged incubation with trypsin followed by a process of acetylation, extraction and deacetylation yielded a glycopeptide having a molecular weight of about 1200. On the basis of the molecular weight of ovalbumin, which at that time was believed to be 40,000, the carbohydrate moeity was estimated to contain two molecules of glucosamine and four molecules of mannose. Acetyl estimations indicated that the glucosamine was N-acetylated and it was clear

that the polysaccharide was present as one unit. Hydrolysis showed the presence of an unidentified nitrogenous component that Neuberger suggested could be involved in the carbohydrate-protein linkage.

Since 1938 many investigators have studied a wide variety of glycoproteins from many sources and in several cases covalent bonding between carbohydrate and protein has been demonstrated. As more and more glycoproteins have been discovered so it has become necessary to define and classify them. In the section that follows the question of classification is briefly reviewed and an attempt is made to give a new definition for this group of macromolecules.

Definition, classification and distribution of the glycoproteins

In the past a number of alternative definitions and classifications have been proposed in order to divide up the whole group of substance in which carbohydrate and polypeptide are associated with each other. None of these has been entirely satisfactory and some unnecessarily arbitrary divisions have been made.

Probably the first attempt at classification was that of Schafer (1898) who divided the group of compounds he called proteids into i) mucins, ii) mucoids, iii) hyalogens and iv) phosphoglucoproteids.

In class i) he included colloid viscous substances of acidic nature, soluble in alkalis and precipitable by acetic acid. On acid hydrolysis they yielded a substance reducing Fehling's solution

and they were found in salivary and other mucous glands. Class ii) differed in being unprecipitable from alkaline solution by acetic acid and included substances such as chondromucoid, cornea mucoid, pseudomucin from cysts and ovomucoid. With regard to class iii) there was some doubt as to whether they should be separated from class ii) since they came from the same animal sources. Class iv) referred to the nucleoproteins.

A later attempt was that of Levene (1925) who distinguished mucoproteins, which were precipitated by dilute acid in the cold and contained chondroitin and mucoitin sulphates, from glucosido-proteins which behaved like normal proteins but contained a carbo-hydrate group. He included in the mucoproteins all those substances that were viscous in solution, soluble in weak alkali and insoluble in water. He thought that the sulphated sugars were present in all members of this group.

Twenty years later Meyer (1945) limited his discussion to compounds containing hexosamine, dividing them into three groups, the mucopolysaccharides, the mucoids and the glycoproteins. The first group was defined as polysaccharides which contain hexosamine as one component, the second was defined as mucopolysaccharides containing more than 4% hexosamine, in firm combination with a peptide and the third was defined as proteins containing carbo-hydrate with less than 4% hexosamine. This has proved an unsatis-factory division since the principal aim of any classification

is to group together things with fundamental similarities and to separate those with fundamental differences, and a purely arbitrary hexosamine content was unlikely to fulfil these requirements.

Bettelheim-Jevons (1958) has reviewed these compounds and has described two classes, those containing ester sulphate in which the bond between carbohydrate and protein was relatively weak and those containing hexosamine, hexose and sometimes sialic acid but not hexuronic acid or ester sulphate and in which the linkage was firm. The first class he called acid amino polysaccharides and the second he called mucoproteins and mucopolysaccharides, drawing no clear distinction between these two subgroups. This system was unsatisfactory in that it grouped together substances so fundamentally unlike as bacterial cell walls and γ-globulin. He defined the prefix 'muco-' as meaning "protein or peptide firmly linked to a particular type of carbohydrate" and not to "physical properties such as viscosity".

In contrast Spiro (1963) in his brief but useful review defines 'muco-' as meaning the presence of amino sugars and goes on to distinguish between the mucopolysaccharides which contain hexuronic acids and ester sulphate and the glycoproteins which contain only hexosamine, hexose and sometimes sialic acid.

Gottschalk (1965) in a later review has defined glycoproteins as "conjugated proteins containing as prosthetic group one or more heterosaccharides with a relatively low number of sugar residues,

lacking a serially repeating unit and bound covalently to the polypeptide chain", so separating them from all other protein-carbohydrate complexes. This is a more satisfactory definition in that it is more precise and less arbitrary than earlier attempts. Nevertheless, blood group substances and the related mucins must be classed as glycoproteins by this definition, although in many respects they have little in common with most other members of the group. In contrast to the others they contain about twice as much carbohydrate as any other glycoprotein, the polypeptide has a restricted variety of amino acids and they seem to possess some of the heterogeneity of the polysaccharides. If these facts are a reflection of an underlying fundamental difference then they should be classified separately from the other glycoproteins of Gottschalk's definition.

In the present thesis an alternative definition is proposed that is based upon the probable mechanism of biosynthesis of the particular compound. Whereas precise experimental evidence to justify a definition on these grounds is scanty, enough is known about the structure and biosynthesis of proteins and carbohydrates as separate compounds to allow certain inferences to be made.

Definition

The glycoproteins are a group of proteins linked by covalent bonds to carbohydrate, which may be present as a single or as multiple units not showing the polydispersity of the polysaccharides but having a definite monosaccharide sequence and the whole mol-

ecule possessing a precise primary, secondary and tertiary structure.

It is intended to imply in this definition that the basic precisely ordered structure of the proteins as determined by the generally accepted mechanism of synthesis on the ribosome is retained and upon this framework, at a stage that is at present unknown, is built up a carbohydrate superstructure of comparably precise form. It may be that further work will show that the blood group and related substances that were quoted as examples above are included in this definition but it seems possible that they may be distinguished by essential biosynthetic differences.

The classification of the glycoproteins

The glycoproteins are very widely distributed in nature and have been described in a large number of species ranging from micro-organisms to man.

The classification proposed here is on the basis of function as far as the present state of knowledge allows. There are some cases in which a particular glycoprotein could equally well be placed in more than one category, as for instance with ceruloplasmin which is both the copper transporting protein of the serum and has enzyme activity.

The examples given in table 1 do not claim to be a comprehensive list but merely cover the more commonly encountered substances.

Table 1

Classification of the glycoproteins

BIOLOGICALLY ACTIVE		
Enzymes	Ribonuclease B	Plummer and Hirs (1964)
	Ceruloplasmin	Schulze (1957)
	β-amylase of Aspergillus oryzae	Tsugita and Akabori (1959)
	Serum cholinesterase	Surgenor and Ellis (1954)
Hormones	Follicle stimulating hormone	Li and Harris (1952)
	Interstitial cell stimulating hormone	Li, Simpson and Evans (1942)
	Thyroglobulin	Brand, Kassel and Heidelberger (1939)
	Thyrotropin	Fels, Simpson and Evans (1955)
	Pregnant mare's serum gonadotropin	Bourillon and Got (1957)
	Human chorionic gonadotropin	Gurin, Bachman and Wilson (1939)
	Erythropoetin	Rambach, Shaw, Cooper and Alt (1958)

Table 1 continued

Antibodies	γ-globulins	Pappenheimer, Lundgren and Williams (1940)
Transporting substances	Ceruloplasmin	Schulze (1957)
	Transferrin	Koechlin (1952)
	Haptoglobins	Jayle and Abdellatif (1946)
Blood clotting substances	Prothrombin	Lorand, Alkjaersig and Seegers (1953)
	Fibrinogen	Conaden and Stanier (1952)
	Platelet cofactor 1	Seegers, Landaburn and Fenichel (1957)
STRUCTURAL AND PROTECTIVE		
	Collagen	Bowes and Moss (1951)
	Reticulin	
	Basement membranes	Mainly from histochemical studies
	Descmet's membrane	
	Lens capsule	

Table 1 continued

	Mucus of the gastrointestinal tract	Graham and Gottschalk (1960)
	Mucus of genitourinary tract	Tamm and Horsfall (1950) Gibbons (1959)
	Glycoproteins from molluscs	Eichwald (1865)
NUTRITIONAL AND MISCELLANEOUS		
	Ovalbumin	Neuberger (1938)
	Ovomucoid	Morner (1894)
	Ovomucin	Young (1937)
	Casein	Johannesen and Svennerholm (1956)
	Intrinsic factor	Latner and Mc Evoy-Bowe (1953)
	B_{12} binding factor	Holdsworth (1961)
	Avidin	Fraenkel-Conrat, Snell and Ducay (1952)
	Glycoproteins of serum	

Preparation and purification of glycoproteins

A commonly employed principle has been to utilise the resistance to precipitation shown by many glycoproteins in conditions that precipitate proteins. In what is perhaps the earliest report of the preparation of ovalbumin, Hofmeister (1890) used half saturated ammonium sulphate to obtain a crystalline substance and eight years later Hopkins and Pinkus (1898) improved this method by adding acetic acid to the ovalbumin-ammonium sulphate solution until precipitation had just begun. Although the product was crystalline, conalbumin, the iron binding protein was still present (Fevold, 1951). Osborne (1899) further improved the method by using hydrochloric acid in the place of acetic acid and went on (Osborne and Campbell, 1900) to show that on the basis of the amount of osasone formed, ovalbumin contained 3-5% carbohydrate. A similar method but employing sodium sulphate, in order to avoid the addition of ammonium ion, has been described (Kekwick and Cannan, 1936) and Warner (1954) has worked out a scheme for separating all the proteins of egg white by precipitation, acidification and dialysis. More recently Schmid (1954) has used the same principle in the preparation of α_1-acid glycoprotein, removing the majority of the other plasma proteins by precipitation with ethanol and fractionating the remaining solution by selective precipitation with divalent metal ions. The product was eventually isolated as the crystalline lead salt. A similar method was used

by Spiro (1960) in the isolation of fetuin from foetal calf serum.

In preparing human chorionic gonadotropin Gurin, Bachman and Wilson (1939) adsorbed the protein on to benzoic acid and in the isolation of prothrombin Laki et al (1954) utilised adsorption on to magnesium hydroxide. Similarly Fraenkel-Conrat, Snell and Ducay (1952) prepared avidin, the biotin binding principle from egg white, by adsorbing it on bentonite and eluting with di-potassium phosphate.

Acetone has been used as a precipitant by Gross and Cole (1940) in the purification of pregnant mare's serum gonadotropin and by Ciereszko (1945) in the preparation of ceruloplasmin.

In an earlier preparation of fetuin Deutsch (1954) has precipitated most of the plasma proteins with 5% trichloroacetic acid (pH 3.5) and then fractionating, first with ethanol and then with 50% saturated ammonium sulphate, achieving by these means an electrophoretically pure protein.

Cohen and Freeman (1960) have described three methods of preparing γ-globulins. In one case he used zone electrophoresis on a cellulose column, in another case he used dialysis against a phosphate buffer gradient and in another case precipitating three times with 16% sodium sulphate, followed by chromatography on DEAE cellulose. DEAE cellulose has also been used by Spiro and Spiro (1963) in the purification of thyroglobulin and by Rambach et al (1958) in the isolation of erythropoetin.

Schmid et al (1960) have separated α_1-acid glycoprotein, an α_2-glycoprotein and serum albumin, using both artificial mixtures and naturally occurring samples, by chromatography on an ion exchange column packed with a resin possessing carboxyl groups, and eluting, in different experiments with citrate and phosphate buffers at various pH's.

The glycoproteins of the submaxillary gland have been prepared by utilising the property first described by Hammarsten (1888) of being precipitated by acetic acid in the cold. Thus Hlix (1936) obtained a preparation from bovine submaxillary gland containing two electrophoretically distinct glycoproteins by repeated precipitation from acid solution. An electrophoretically homogeneous glycoprotein was prepared from the same source by water extraction of the tissue, removal of material soluble at pH 4 from the extract followed by precipitation with methanol in the presence of barium acetate (Curtain and Pye, 1955; Murphy and Gottschalk, 1961).

Tsuiki, Hashimoto and Pigman (1961) have described the preparation of bovine submaxillary gland mucin by precipitation with cetyltrimethylammonium bromide and a later fractional alcohol precipitation from solutions in 50% aqueous calcium chloride.

Odin (1955) has used the method of Hammarsten in the preparation of glycoprotein from ovine submaxillary gland. A procedure yielding a product of higher carbohydrate content involved water extraction of dehydrated tissues followed by fractional precipitation

with ethanol (Mc Crea, 1953).

More recently gel filtration with cross linked dextran (Sephadex) or a combination of gel filtration and ion exchange (DEAE- and CM-Sephadex) has proved valuable in the isolation and purification of ceruloplasmin (Hansen,1963; Broman and Kjellin, 1964), prothrombin (Lanchantin and Friedman, 1963; Mammen and Ramien, 1962), urinary γ-globulins (Cornillot et al, 1963) and in the fractionation of the serum proteins (Hana and Styk, 1962; Gelotte et al, 1962; Flodin and Killander, 1962).

In the present thesis the ovalbumin was prepared from eggs, from what was believed to be a fairly homogeneous strain of hen, by the method of Warner (1954).

THE PREPARATION OF GLYCOPEPTIDES

THE PREPARATION OF GLYCOPEPTIDES

INTRODUCTION

Structural studies on glycoproteins have three main aims, i) to elucidate the amino acid sequence of the polypeptide chain and to determine the position of the carbohydrate group or groups upon it, ii) to elucidate the sequence of sugars in the oligosaccharide unit or units and iii) to investigate the types of linkage joining carbohydrate to polypeptide.

These aims have necessitated the development of methods for preparing glycopeptides possessing certain characteristics. Most commonly, as for ii) and iii), the object has been to prepare a glycopeptide with the oligosaccharide intact but with all amino acids removed apart from the one involved in the carbohydrate-protein linkage. This ideal has proved difficult to achieve because it appears that in most cases the last three or four amino acids are very resistant to cleavage by the proteolytic agents that are available.

In other cases it is desirable to have a glycopeptide that still contains several amino acids as this may assist in locating its position in the polypeptide chain as in i) mentioned above or it may facilitate its separation from the other products of proteolysis either by charge differences or by size differences, or it may aid in the distinction between different glycopeptides so giving some indication of the number of carbohydrate units present in a particular glycoprotein.

Early attempts to obtain polysaccharides from glycoproteins involved the use of alkaline hydrolysis (Rimington, 1929 and Frankel, and Jellinek, 1927) in the belief that the glycosidic bonds in the carbohydrate would be sufficiently resistant to be left intact whilst the peptide bonds would be split. However this hope was shown to be unjustified when Neuberger (1938) demonstrated that in ovalbumin, treatment with normal sodium hydroxide at 100° for 16 hr destroyed appreciable amounts of glucosamine.

It is now fairly clear that conventional chemical methods of cleaving peptide bonds are unlikely to be satisfactory in the preparation of glycopeptides with the required characteristics since they are insufficiently selective to avoid damage to the carbohydrate.

In his 1938 paper Neuberger introduced the use of proteolytic enzymes for the preparation of glycopeptides. In experiments with ovalbumin he digested the protein with trypsin for 4 - 6 weeks at pH 7.8 and separated the products first by precipitating the basic amino acids with phosphotungstic acid, and secondly by N-acetylating the remaining amino acids with ketene and extracting them into chloroform. The glycopeptide so prepared gave a negative biuret reaction and specific tests for tyrosine, tryptophan, histidine, arginine and cysteine were negative. There was no free reducing group but reducing sugars, estimated to be two thirds fermentable sugar (mannose) and one third amino sugar (glucosamine) were released.

In later preparations a variety of proteolytic enzymes have been

used to digest the polypeptide chain, but in several cases a marked resistance to hydrolysis has been reported. Thus Yamashina (1956) and Popenoe and Drew (1957) found α_1-acid glycoprotein to be poorly broken down by trypsin, papain or carboxypeptidase.

It was later found that removal of N-acetylneuraminic acid allows enzymic digestion to occur (Schmid, Bencze, Nussbaumer and Wehrmiller, 1959 and Kaniyama and Schmid, 1962, a and b). Similarly Weinfeld and Tunis (1960) found the same protein showed limited cleavage by pepsin. However Bourillon, Got and Meyer (1963) have shown that pleuromucoid, a protein very similar to the α_1-acid glycoprotein of serum is readily susceptible to digestion by a number of enzymes. In studies on ovalbumin Cunningham, Muenke and Muenke (1957) and Muenke and Cunningham (1961) and in work with human γ-globulin Rosevear and Smith (1961) have also noticed the unusual resistance of peptide bonds in the region of carbohydrate prosthetic groups.

In continuing the work on ovalbumin Neuberger modified the method described in 1958 by first digesting the heat denatured protein with pepsin for 96 hr at 37° and pH 2.8, then incubating with a mixture of trypsin and chymotrypsin at pH 7.8 for 24 hr and finally using a mould protease from Aspergillus oryzae (Johansen, Marshall and Neuberger, 1958 and Johansen, Marshall and Neuberger, 1961). A rather similar procedure has been described by Jevons (1958) but in both these cases amino acids other than that involved in the linkage remain in the preparation, although when the method of Johansen et al (1961) was followed by digestion with

carboxypeptidase for three days only traces of amino acids other than aspartic acid were found. Nuenke and Cunningham (1961) using essentially the same techniques have come to similar conclusions. It is of interest that in spite these frequent reports of resistance to hydrolysis, Cunningham, Nuenke and Strayhorn (1957) have shown that of the thirty-five lysine and arginine residues in ovalbumin, 33 ± 2 of the bonds involving those amino acids are split by the action of trypsin as estimated by the release of carboxyl groups. It was concluded from the composition of these glycopeptides that the amino acid involved in the protein-carbohydrate linkage was aspartic acid. Moreover it was postulated that the unidentified nitrogenous component observed by Neuberger (1938) was associated with this linkage probably in the form of a N-glycosidic bond between the β-carboxyl of the aspartic acid and an N-acetylglucosamine residue.

In more recent investigations a new proteolytic enzyme "Pronase-P" has been used, (Nomoto and Narahashi, 1959 a, b, c and d and Nomoto, Narahashi and Murakami, 1960 a and b). This protease has proved very useful because of its broad specificity. However there is some evidence that the activity of different batches of Pronase are somewhat variable (R. D. Marshall, Personal communication). Thus in most cases it is estimated that about 33% of the peptide bonds of ovalbumin are split (Fletcher, Marks, Marshall and Neuberger, 1963) but in other experiments a figure of 17% has been obtained. Cunningham (personal communication) has also noticed a similar variability in the enzymic activity of Pronase.

However it should be pointed out that the extent of degradation in terms of number of peptide bonds split is not necessarily a good indication that a suitable glycopeptide has been prepared, since only two peptide bonds, those on each side of the prosthetic group, have to be cleaved in order to achieve this object. Since there is, as yet, no enzyme available that is specific for peptide bonds adjacent to amino acids involved in carbohydrate linkages it is, in general, desirable to effect an extensive degradation.

Pronase has been used either alone or in combination with other enzymes to digest a wide variety of glycoproteins. Thus it has been used to prepare glycopeptides from transferrin (Jamieson, 1963), the β-amylase of Aspergillus oryzae (Tsugita and Akaborin, 1959), thyroglobulin (Spiro and Spiro, 1963), α_1-acid glycoprotein (Eylar, 1962 and Winzler, 1964), γ-globulin (Rosevear and Smith, 1961), bovine submaxillary gland mucin (Hashimoto, Tsuiki, Hisisawa and Pigman, 1963), ovomucoid (Marks, Marshall, Neuberger and Papkoff, 1962 and Montgomery and Wu, 1963), ovalbumin (Yamashina and Makino, 1962 and Fletcher et al, 1963 a) bovine submaxillary gland mucin (Bhavanandan, Buddecke, Carubelli and Gottschalk, 1964) and ovine interstitial cell stimulating hormone (Papkoff, 1963).

In the case of fetuin, Spiro (1962) has used a combination of papain and a protease from bacillus subtilis (Nagarse), a combination of trypsin and chymotrypsin, and pepsin. He found that papain split approximately 40% of the peptide bonds, this extensive digestion occurring with the protein in the native state and, in contrast to

α_1-acid glycoprotein, in the presence of its high content of sialic acid.

An observation pertinent to the experimental work about to be described was made by Lee and Montgomery (1962) who digested ovalbumin with papain in attempts to prepare a glycopeptide containing only aspartic acid. It has been established by a number of groups of workers that the amino acid sequence in the vicinity of the carbohydrate is as shown below.
(Johansen et al. 1961; Lee and Montgomery, 1962; Clamp and Hough, 1965)

Carbohydrate
|
- Glu - Lys - Tyr - Asp - Leu - Thr - Ser -

It would appear the the action of papain is to completely cleave the asp-leu bond but is without effect on the tyr-asp bond. The significance of this in relation to the specificity of Pronase will be discussed after the experimental section.

Having achieved a satisfactory enzymic digestion there remains the problem of separating the glycopeptide from the other degradation products. A wide variety of methods involving fractional precipitation, electrophoresis, paper chromatography and ion exchange chromatography have been used. In particular the introduction of dextran gels as a means of ultrafiltration has proved to be of great value. Porath and Flodin (1959) described the separation of protein salt mixtures by filtration through grains of crosslinked polysaccharide (Sephadex) and shortly afterwards it was shown by Konigsberg and Hill

(1960) that the same method could be used to separate the peptides derived from the tryptic digestion of the α- chain of human haemoglobin. Since the separation depends upon a difference in molecular size it is necessary if the method is to be applied to the purification of glycopeptides, to chose a means of digestion that produces peptides significantly smaller (or larger) than the glycopeptide. The first application to the purification of glycopeptides was by Colobert and Dirheimer (1961) who separated lysozyme from a mixture of glycopeptides derived from Micrococcus lysodeiktieus. Lee and Montgomery (1962) and Marks et al (1962) extended the method to the purification of the ovalbumin glycopeptides obtained by digestion of the protein with papain and carboxypeptidase in the first case and with Pronase in the second. Fletcher et al (1963 a) further modified the prodedure to achieve a glycopeptide containing only a single amino acid.

Other glycopeptides prepared with the aid of gel filtration include ovine submaxillary gland glycoprotein (Bhavanandan et al, 1964 and Carubelli, Bhavanandan and Gottschalk, 1965), ovomucoid (Marks et al, 1962), transferrin (Jamieson, 1963), α_1-acid glycoprotein (Popence and Mendelsohn, 1963) and rabbit γ-globulin (Nolan and Smith, 1962).

On the experimental work about to be described the object has been to prepare a glycopeptide containing aspartic acid as the only amino acid. Two methods have been used, firstly digestion with Pronase-p, followed by digestion with carboxypeptidase (after carbobenzoxylation of the glycopeptide formed by Pronase) and secondly digestion with

papain followed by Pronase.

EXPERIMENTAL.

METHOD 1

Digestion with Pronase

Crystalline ovalbumin (50 gm) prepared by the method of Warner (1952), was dissolved in 100 ml of 0.15M calcium chloride solution. 371 ml of water was added and the pH adjusted to 8.5. Digestion was commenced by the addition of 1.0 gm of Pronase-P (Kingsley and Keith (Chemicals) Ltd., Rex House, 36, King William Street, London, E.C.4.) and the mixture incubated at 37°. At zero time 0.1 ml of the digestion mixture was withdrawn and estimated by the ninhydrin method of Moore and Stein (1948). Similar portions were taken at hourly intervals for six hours and at 48 and 72 hr. The pH was checked at intervals and adjusted to 8.5 by the addition of N NaOH. A further 0.5 gm Pronase was added after 24 hr and again after 72, 120 and 168 hr. (In later preparations this time was reduced to 24 hr since very little further degradation occurs after that time). The course of the reaction is shown in fig. 2 and table 2.

Ninhydrin Method.

Reagents - Citrate buffer

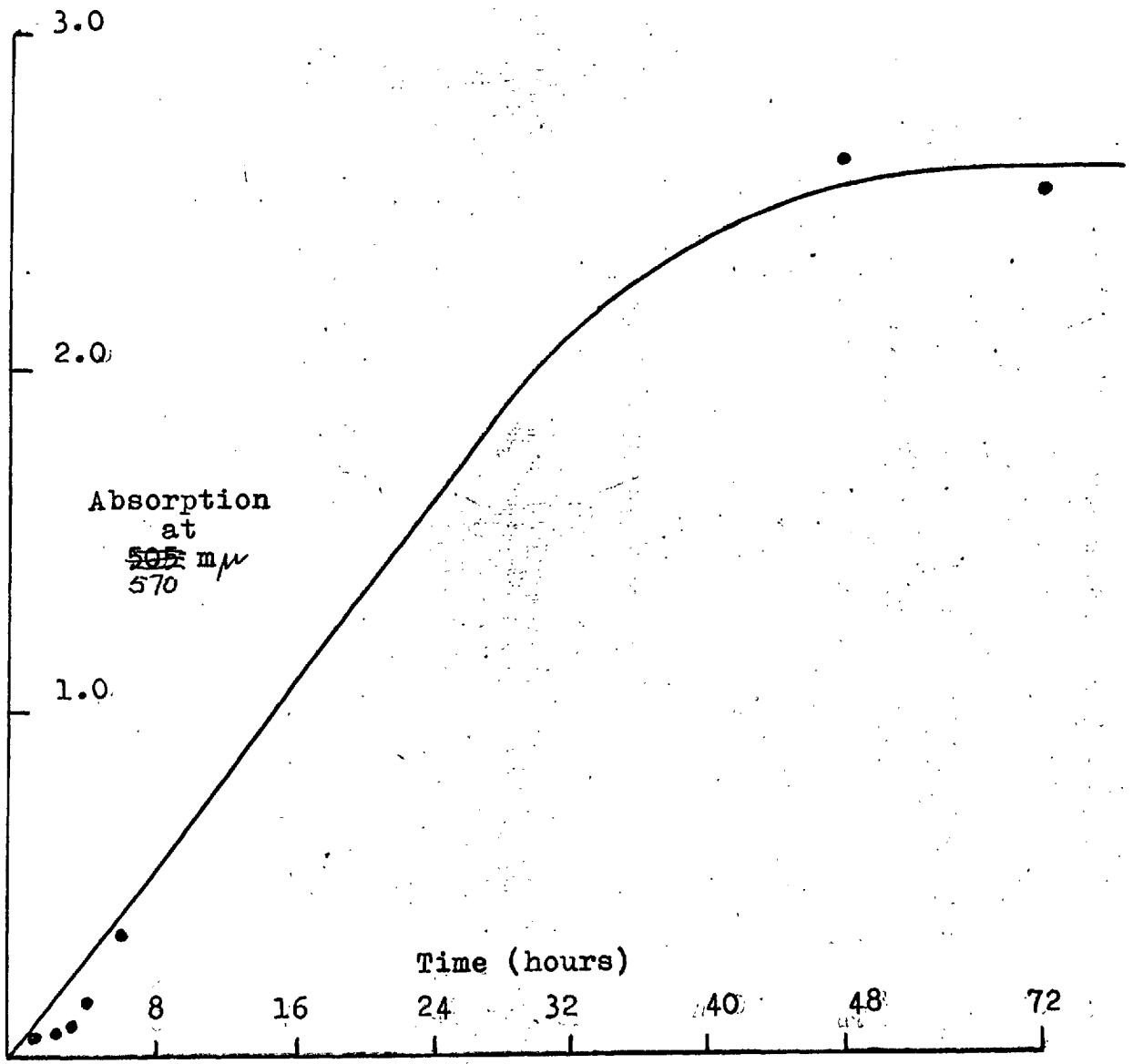
Citric acid (21.008 gm) + N NaOH (200 ml) was diluted to 1,000 ml with water and the pH was adjusted to 5.0 ± 0.1.

Ninhydrin solution

(i) Stannous chloride (0.80 gm) was dissolved in citrate buffer (500 ml).

(ii) Ninhydrin (20.0 gm) was dissolved in methyl cellosolve (500 ml).

Fig. 2



Digestion of ovalbumin with Pronase-P

Table 2

Digestion of ovalbumin with Pronase-P

Time hr	$E_{570\ m\mu}$	Time hr	$E_{570\ m\mu}$
0	0.01	5	0.19
1	0.07	6	0.36
2	0.05	48	2.62
3	0.07	72	2.52
4	1.14		

Table 3

Mannose estimations of fractions from Sephadex G-25 of Pronase-P digest

Tube no.	$E_{505\ m\mu}$	Tube no.	$E_{505\ m\mu}$
20	0.17	32	1.7
23	0.225	33	0.75
25	0.063	34	0.35
26	0.20	35	0.25
27	0.15	38	0.12
28	0.225	41	0.08
29	0.41	44	0.11
30	0.52	47	0.095
31	0.67		

Equal volumes of (i) and (ii) were mixed just prior to use. The solution to be estimated (1.0 ml) was mixed with 1.0 ml of the ninhydrin reagent and placed in a boiling water bath for 15 min. After cooling for 15 min dilutions were made as necessary by the addition of 50% ethanol. The colour produced was read at 570 mμ

The digestion mixture (520 ml) was then centrifuged at 1800 rev/min for 30 min, the supernatant was poured off and evaporated to 335 ml on a rotary evaporator at 40°.

Ethanol (1340 ml) was added to give a final concentration of 80% and the mixture left to stand at 4° for 48 hr. The supernatant was decanted and the precipitate dissolved in 35 ml 0.1 N acetic acid, a small amount of insoluble material being removed by centrifugation. The solution was applied to a column of Sephadex G-25 which was prepared as follows.

Preparation of column.

Sephadex G-25 (100 gm) was suspended in 1 litre of water, allowed to stand overnight and poured as a slurry into a column 34.5 x 4 cm which was washed by allowing 0.1 N acetic acid to run through for 6 hr.

Fractionation on Sephadex G-25.

The column was eluted with 0.1 N acetic acid and 25 ml fractions were collected. The fractions were estimated for hexose by the orcinol-sulphuric acid method (Johanson et al, 1960) as described in the following paragraphs, and for free amino groups by the ninhydrin method described previously.

The orcinol-sulphuric acid method for the estimation of hexoses

The reagent consisted of a mixture of 7.5 volumes of 60% sulphuric

acid and 1.0 volume of 1.6% orcinol in water. The reagents were cooled to 4-5° before mixing. 1.0 ml of a solution containing 100-500 μg of hexose was mixed with 8.5 ml of the reagent and heated in a reflux tube for 15 min at 80°. The tubes were cooled and read in the spectrophotometer at 505 mμ.

Hexose estimations were performed on every third tube from 20-59 and repeated on every tube from 25-35. Ninhydrin estimations were performed on every other tube from 15-71. The results are shown in tables 3 and 4 and fig 3.

Tubes 26-35 inclusive were pooled and estimated for mannose. The results are shown in table 5 and fig 4.

The total volume of the solution of glycopeptide was 250 ml and reading from the standard curve it is seen that the estimation indicates a mannose concentration of 190 μg per 0.1 ml. The total yield in terms of mannose is therefore 475 mg. If a mannose content of 2% for ovalbumin is taken, then 50 gm would give a theoretical yield of 1.0 gm. The yield in the present experiment is therefore 47.5%.

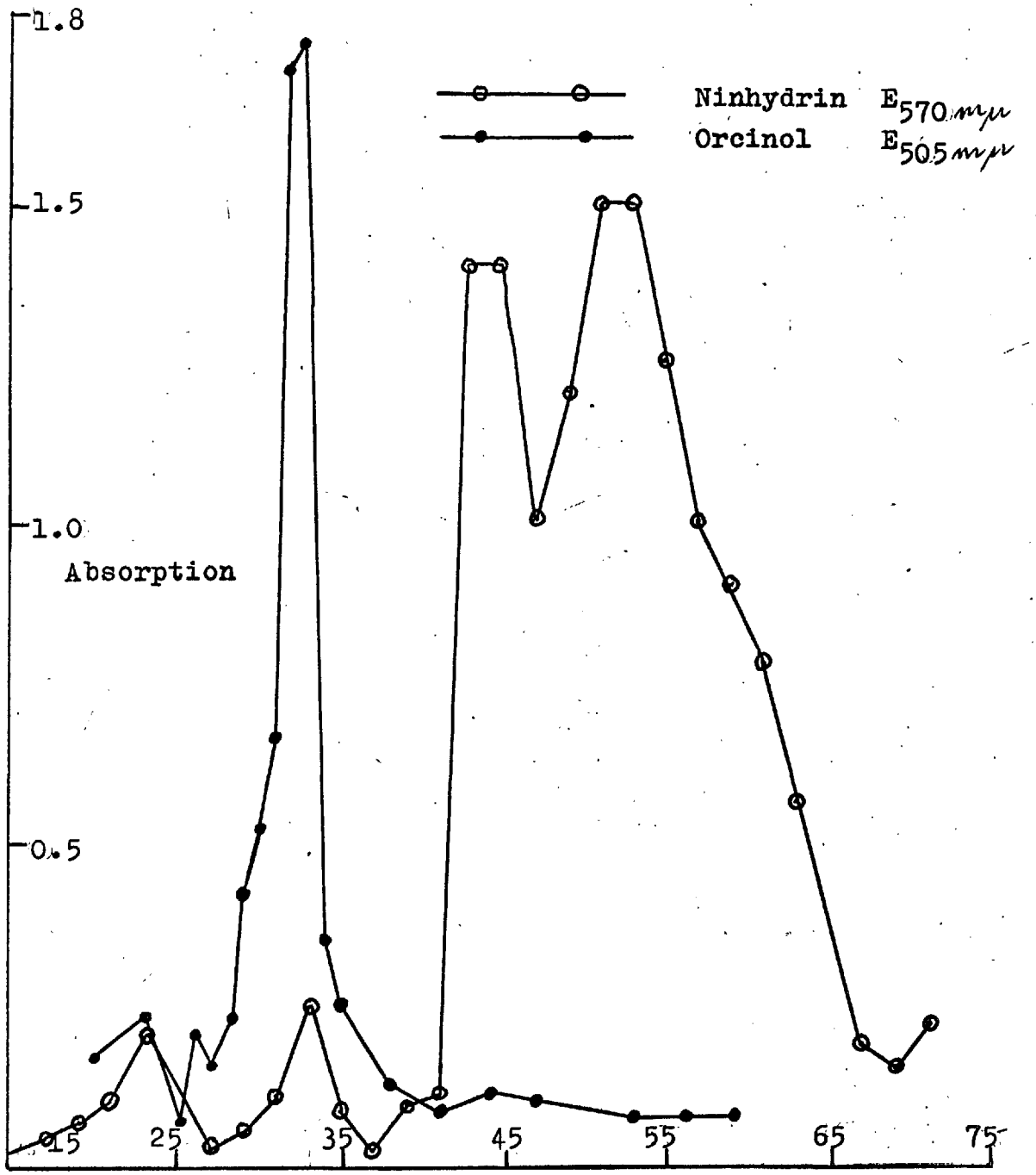
Amino acid analysis of the glycopeptide.

Glycopeptide solution (1.41 ml; equivalent to 3.0 μmoles of glycopeptide) was evaporated to dryness and 4.0 ml of constant boiling hydrochloric acid added. The tube was sealed under a vacuum and heated at 110° for 18 hr.

The hydrochloric acid was removed by evaporation and the products of hydrolysis taken up in 3.0 ml of water (1 μmole/ml).

Amino acid analysis was carried out by Moore and Stein chromatography

Fig.3



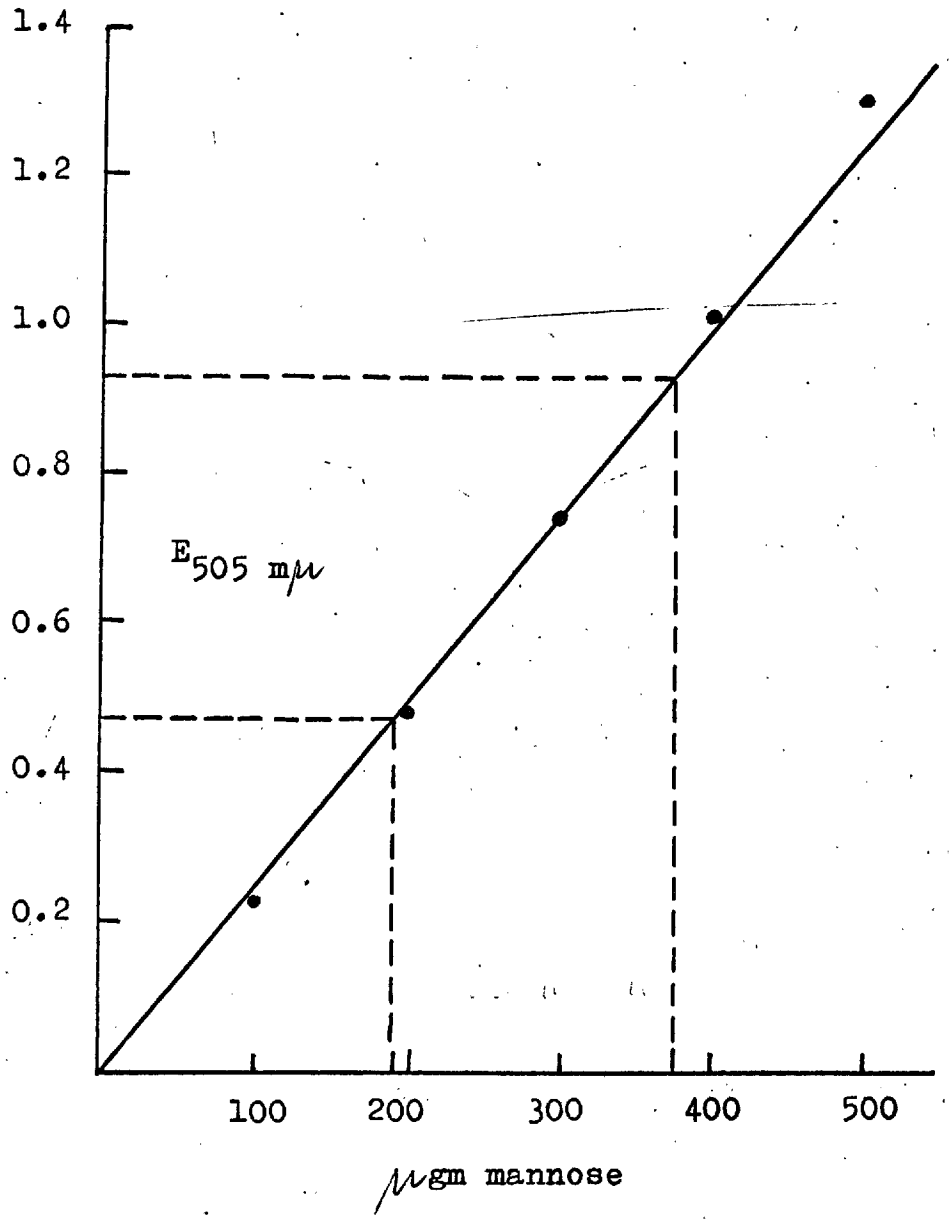
Fractionation of glycopeptide P on Sephadex G-25

Table 4

Ninhydrin estimations of fractions from Pronase-P digest on Sephadex G-25

Tube no.	$E_{570\ m\mu}$	Tube no.	$E_{570\ m\mu}$
15	0.08	45	1.4
17	0.045	47	1.0
19	0.065	49	1.2
21	0.10	51	1.5
23	0.20	53	1.5
25	0.10	55	1.25
27	0.02	57	1.0
29	0.05	59	0.90
31	0.105	61	0.78
33	0.25	63	0.56
35	0.08	65	-
37	0.02	67	0.185
39	0.09	69	0.15
41	0.105	71	0.220
43	1.4		

Fig. 4



Mannose estimation of glycopeptide P

Table 5

Mannose estimation of pooled glycopeptide

µgm mannose	E_{505} mµ	Mean	Corr. for blank
Blank	0.037	0.0395	
Blank	0.042		
100	0.265	0.262	0.223
100	0.260		
200	0.50	0.52	0.481
200	0.54		
300	0.78	0.78	0.741
300	0.78		
400	1.05	1.05	1.011
400	1.05		
500	1.35	1.35	1.311
500	1.35		
0.1 ml glyco.	0.44	0.47	0.441
0.1 ml glyco.	0.50		
0.2 ml glyco.	0.95	0.93	0.891
0.2 ml glyco.	0.92		

with a Technicon Autoanalyser using an adaptation of the cyanide-ninhydrin method (Yemm and Cocking, 1955) for detection and eluting the column with a gradient of citrate buffer controlled by an Autograd (9-glass chambered gradient device developed by the National Cancer Institute, National Institutes of Health, U.S. Department of Public Health, Bethesda, Maryland). See fig. 5 and table 6.

The glycopeptide was also examined in the Autoanalyser prior to hydrolysis using similar conditions to those described. See fig. 6.

Carbobenzoxylation of the glycopeptide

This was carried out by the modified Schotten-Baumann procedure of Greenstein and Winitz (1961).

The pooled glycopeptide solution was evaporated to dryness at 45° leaving a yellowish hygroscopic material which was taken up in 10 ml of water, transferred to a small vessel and again evaporated to dryness.

The residue was dissolved in 4.9 ml of water and 984 mg of sodium bicarbonate was added. Benzyloxycarbonyl chloride (1.08 ml) was added and the mixture shaken at 37° for 1 hr when a further 1.08 ml of benzyloxycarbonyl chloride was added and shaking continued at 37° for 24 hr.

Undissolved sodium bicarbonate was removed by centrifugation, washed with cold water, and the washings added to the main solution which was extracted three times with 20 ml portions of ether to remove excess benzyloxycarbonyl chloride.

Ninhydrin estimations were performed on the solution thus obtained and showed that no free amino groups could be detected.

Digestion with carboxypeptidase A

The benzyloxycarbonyl glycopeptide was brought to pH 7.5 by the

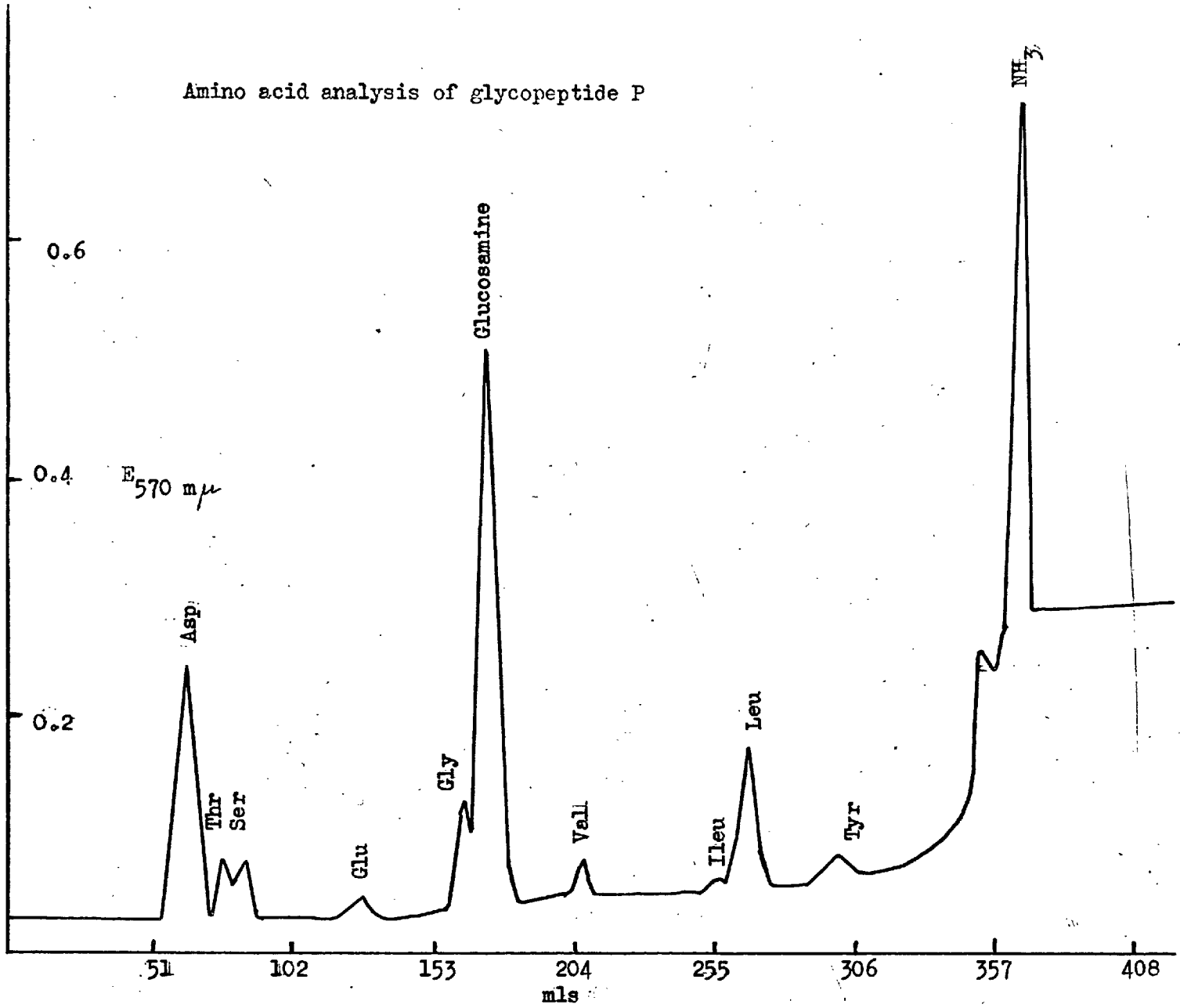

Amino acid analysis of glycopeptide P
0.6
0.4
0.2
$E_{570\ m\mu}$
Asp
Thr
Ser
Glu
Gly
Glucosamine
Val
Ileu
Leu
Tyr
NH_3
51
102
153
204
255
306
357
408
mls

FIG. 5

Table 6

Amino acid analysis of glycopeptide prepared by digestion with Pronase-P

Amino acid	Micromoles
Aspartic acid	1.0
Threonine	0.23
Serine	0.17
Glutamic acid	0.08
Glycine	0.32
Valine	0.11
Isoleucine	0.05
Leucine	0.51
Tyrosine	0.07
Arginine	0.37
Glucosamine	1.89

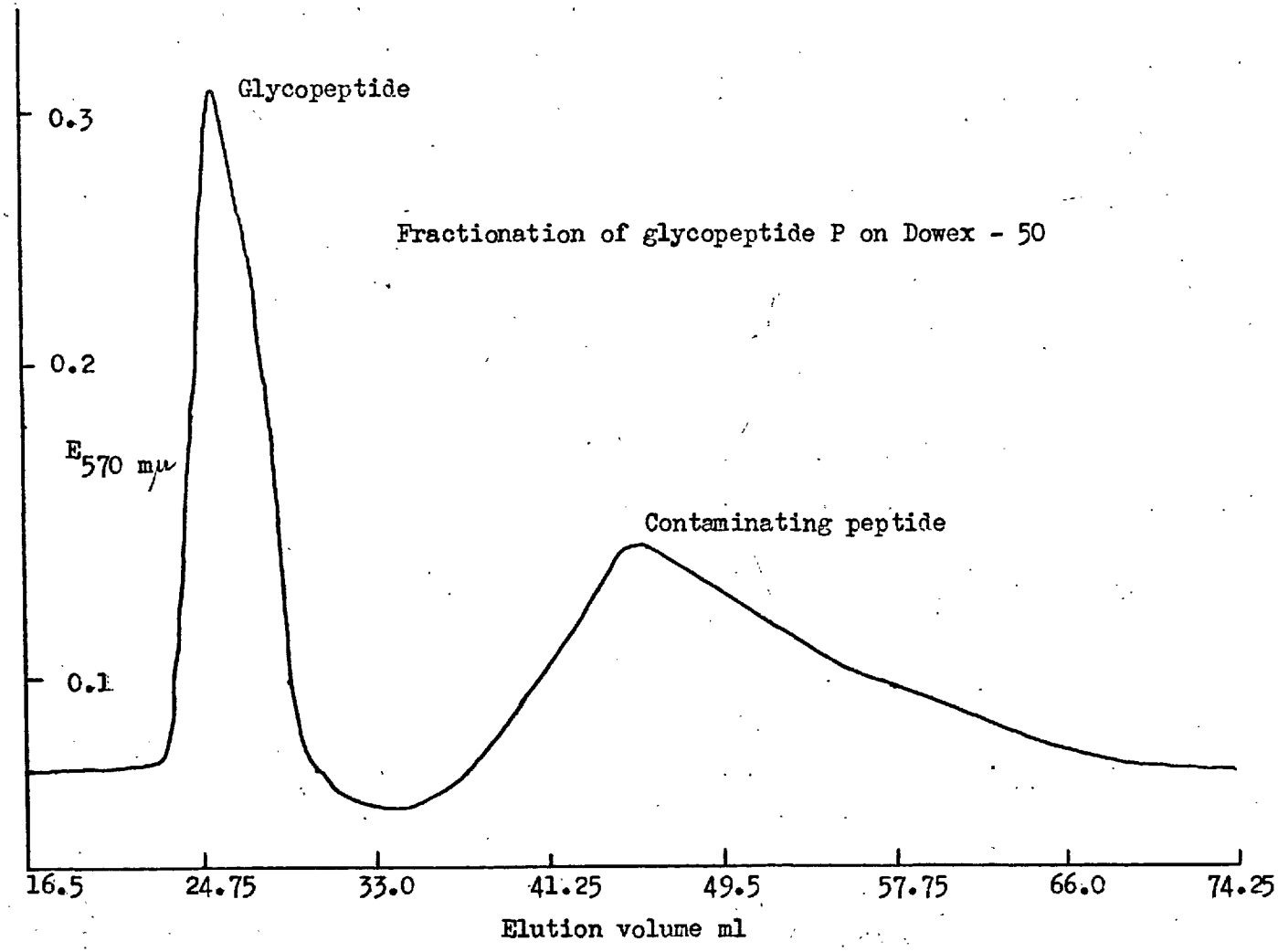

Glycopeptide
Fractionation of glycopeptide P on Dowex - 50
0.3
0.2
$E_{570\ m\mu}$
0.1
Contaminating peptide
16.5
24.75
33.0
41.25
49.5
57.75
66.0
74.25
Elution volume ml

Fig. 6

by the addition of N HCl. Carboxypeptidase A (47.5 mg) was added and the mixture incubated at 37° for 24 hr.

A portion was withdrawn at zero time and another after 24 hr fo ninhydrin estimations which indicated that the carboxypeptidase had resulted in a 27% increase in ninhydrin colour. The results are shown in table 7 and fig 7

Purification of the benzyloxycarbonyl glycopeptide after digestion.

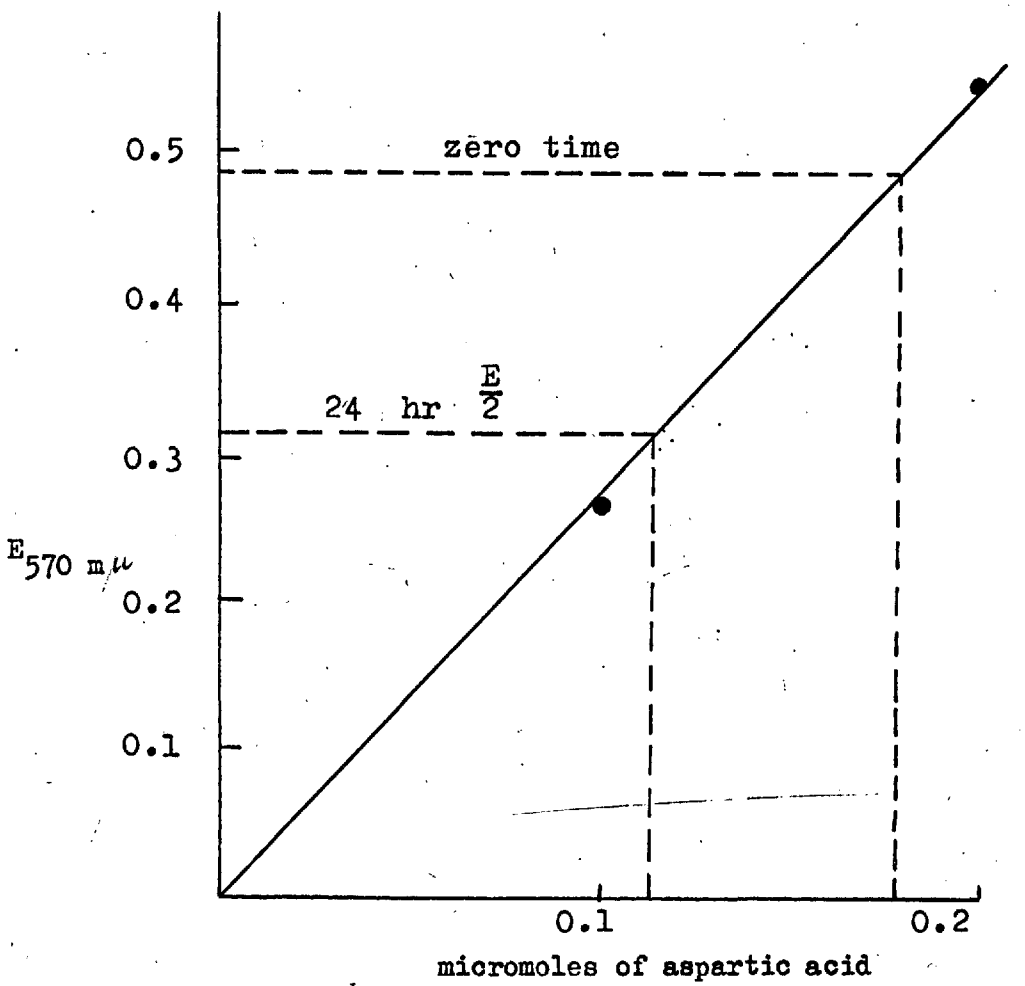
The solution of glycopeptide was evaporated to dryness and the residue dissolved in 15 ml of 0.1 N acetic acid and applied to a column of Sephadex G-25 prepared as describe above. The column was eluted with 0.1 N acetic acid and 10 ml fractions were collected. Every fifth tube from 50-100 was estimated for mannose and every fifth tube from 50-145 was estimated by the ninhydrin method. The results are shown in table 8&9 and fig 8

Tubes 55-85 were pooled and estimated for mannose which indicated a concentration of 1.00 mg/ml, corresponding to a total of 320 mg of mannose. The yield for this stage of the preparation was therefore approximately 70%. See table 10 and fig. 9

Aminoacid analysis of the glycopeptide.

Glycopeptide solution (1.82 ml; 2.0 μmoles) was evaporated to dryness and dissolved in 4.0 ml of constant boiling hydrochloric acid. The tube was evacuted, sealed and heated at 110° for 18 hr. Aminoacid analysis was performed as described previously. The results are shown in table 11 and fig 10

Fig.7



Carboxypeptidase digestion of glycopeptide P

Table 7

	$E_{570\ m\mu}$.	Corr. for blank
Blank	0.105	
0.1 ml of 1 mM aspartic acid	0.370	0.265
0.2 ml of 1 mM aspartic acid	0.658	0.553
0 hours	0.588	0.483
24 hours	0.720	0.615

Purification of glycopeptide PC on Sephadex-G25

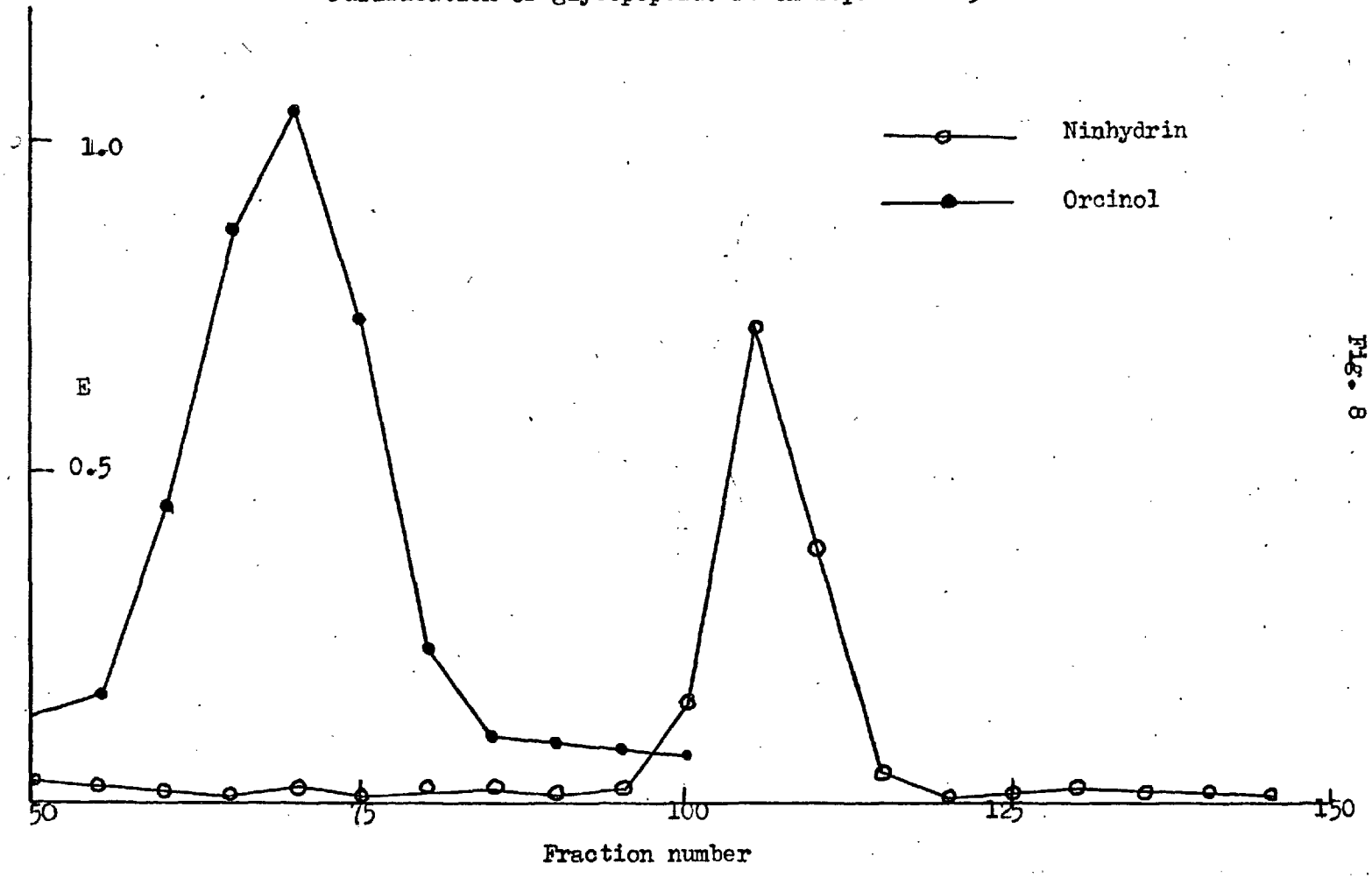


Fig. 8

Table 8

Mannose estimations of fractions from Pronase-carboxy-peptidase digest on Sephadex G-25

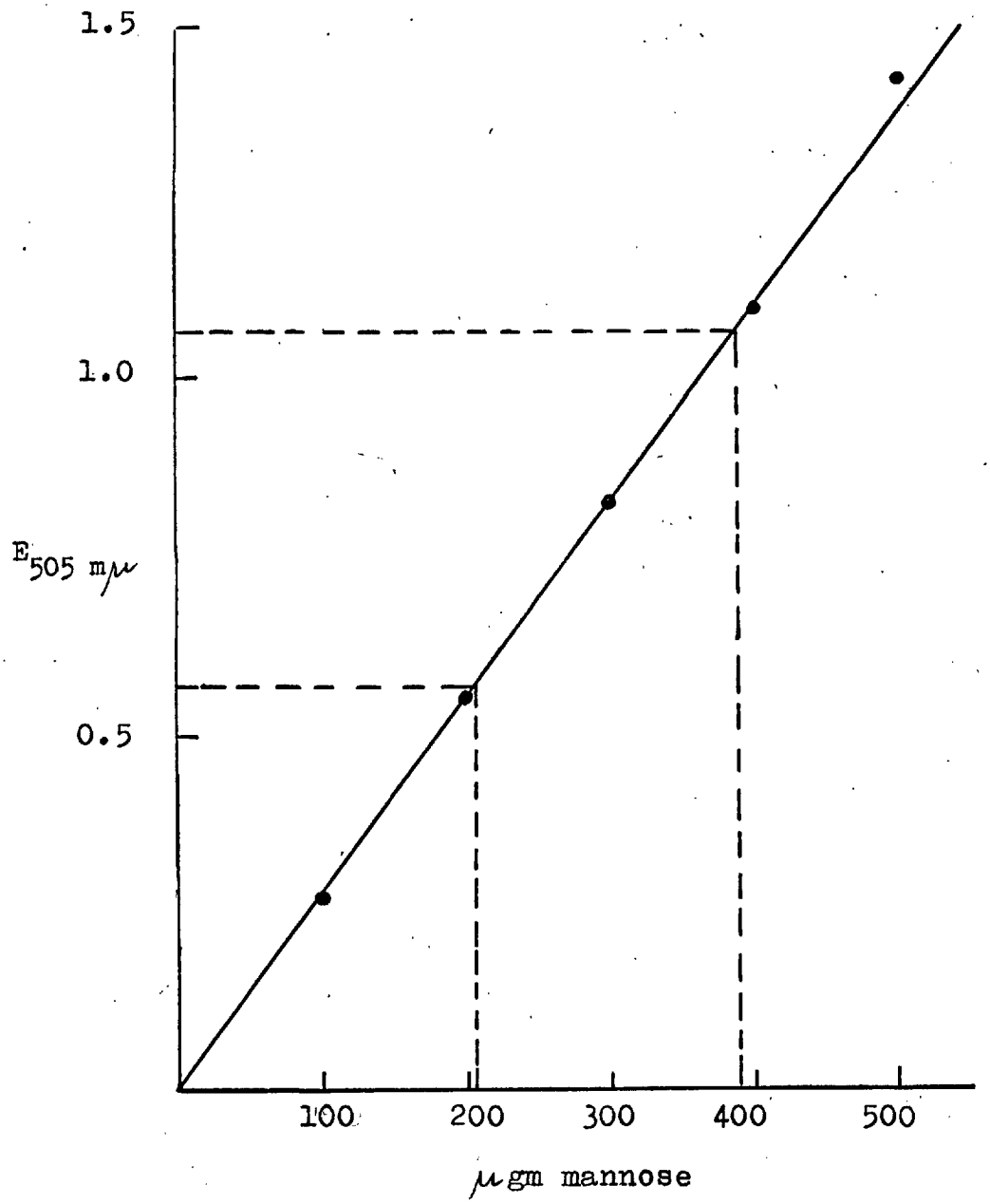
Tube no.	$E_{505\ m\mu}$	Tube no.	$E_{505\ m\mu}$
50	0.134	80	0.272
55	0.163	85	0.144
60	0.447	90	0.130
65	0.910	95	0.120
70	1.04	100	0.115
75	0.775		

Table 9

Ninhydrin estimations of fractions from Pronase-carboxy-peptidase digest on Sephadex G-25

Tube no.	$E_{570\ m\mu}$	Tube no.	$E_{570\ m\mu}$
50	0.032	100	0.153
55	0.026	105	0.723
60	0.020	110	0.383
65	0.009	115	0.134
70	0.017	120	0
75	0.006	125	0.013
80	0.009	130	0.016
85	0.017	135	0.007
90	0.006	140	0.007

Fig. 9



Mannose estimation of glycopeptide PC

Table 10

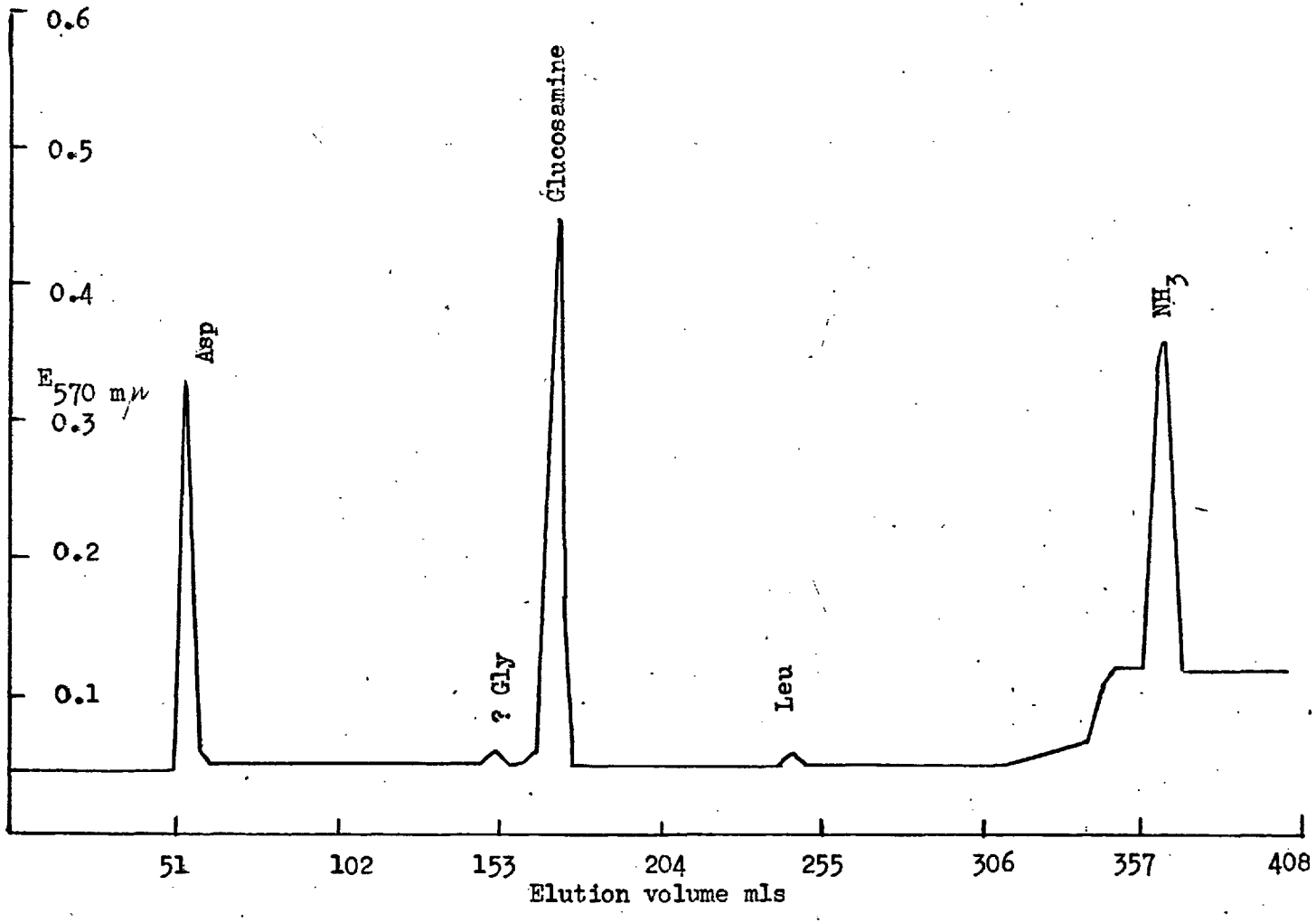
Mannose estimation of pooled glycopeptide PC

	$E_{505\ m\mu}$	Corr. for blank
Blank	0.028	
100 μgm mannose	0.295	0.267
200 μgm "	0.581	0.553
300 μgm "	0.855	0.827
400 μgm "	1.13	1.10
500 μgm "	1.46	1.43
0.2 ml glyco.	0.594	0.566
0.4 ml glyco.	1.10	1.07

Table 11

Amino acid analysis of glycopeptide PC

Amino acid	Micromoles
Aspartic acid	1.0
? Glycine	0.028
Leucine	0.029
Glucosamine	1.74



Amino acid analysis of glycopeptide PC

Fig. 10

METHOD 2

Digestion with papain.

Crystalline ovalbumin (5.0 gm; prepared as in the previous experiment) was dissolved in 200 ml of water, the pH adjusted to 7.5 and the protein denatured by heating at 80-82° for 15 min. The solution was cooled and 22.0 ml of 0.5 M phosphate buffer pH 7.5 was added making a final concentration of 0.05 M with respect to phosphate. Sodium versenate (837 mg) and 0.172 gm (0.155 ml) of 2-mercaptoethanol were added. The pH was adjusted to 7.5 with N NaOH.

Papain (15 mg) was added and the mixture incubated at 60° for 3 hr.

A portion was taken at zero time and at hourly intervals thereafter and estimated by the ninhydrin method. The results are shown in table 12and fig 11

The papain was inactivated by bringing the digest to pH 3.5 by the addition of HCl and heating at 75° for 30 min. The mixture was cooled and the pH brought to 8.5 with NaOH.

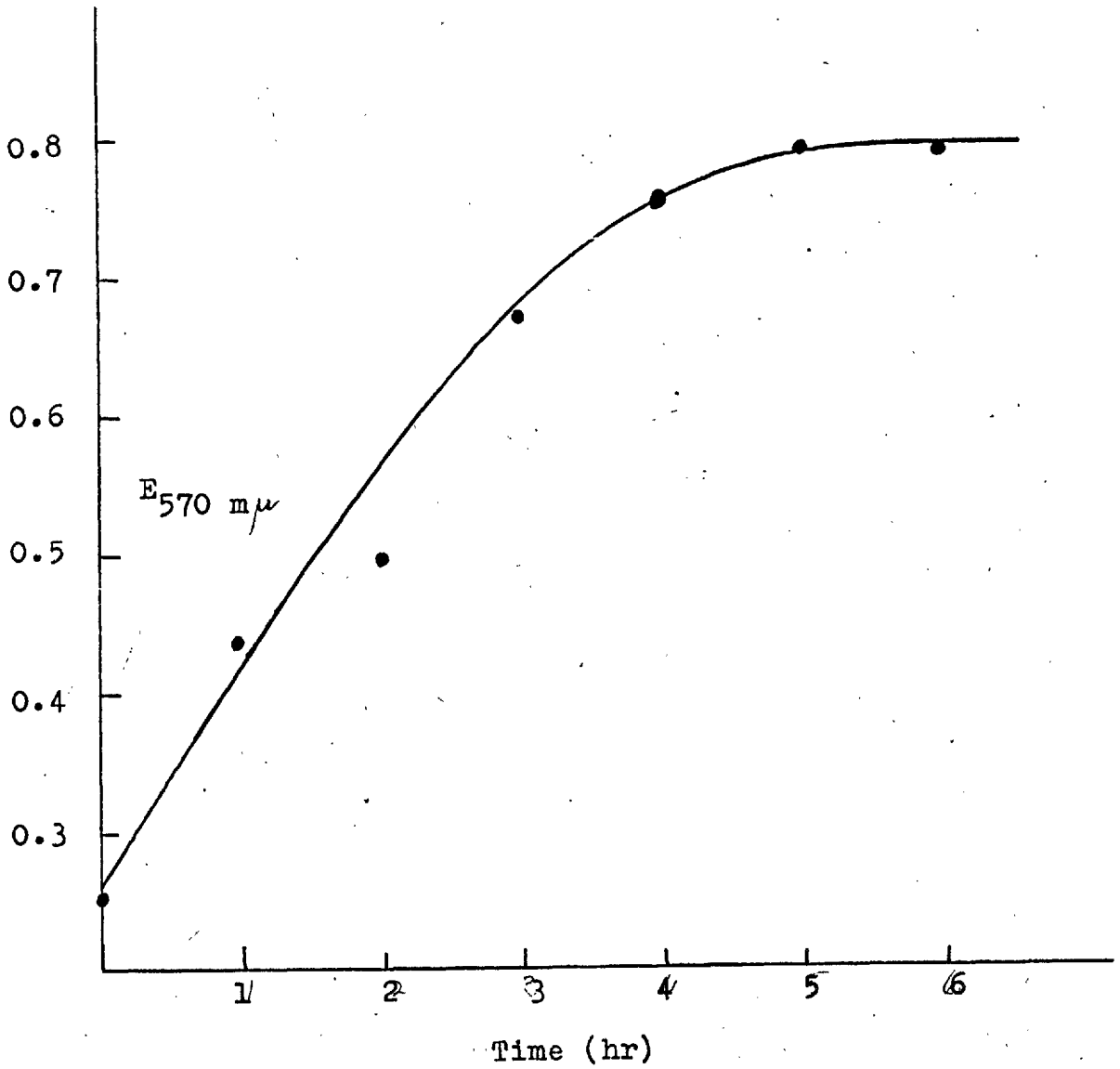
Digestion with Pronase-P.

A small precipitate had formed during papain digestion which was removed by centrifugation. Calcium chloride (1.53 gm) was added to a concentration of 0.015 M, taking into account the uptake of the sodium versenate and the 2-mercaptoethanol added before the first digesti on.

Pronase-P (100 mg) was added and the mixture incubated at 37°, portions being taken for ninhydrin estimation as before. The pH was kept constant at 8.5 by the addition of NaOH.

After 24hr a further 100 mg of Pronase was added and incubation continued

Fig. 11



Papain digestion of ovalbumin

Table 12

Papain digestion of ovalbumin (ninhydrin colour)

Time (hr)	$E_{570\ m\mu}$
0	0.240
1	0.434
2	0.495
3	0.664
4	0.756
5	0.790
6	0.785

Table 13

Pronase digestion of glycopeptide PA (ninhydrin)

Time (hr)	$E_{570\ m\mu}$
0	0.470
1	0.495
2	0.380
3	0.502
4	0.598
5	0.540
6	0.606
24	0.648
48	0.700
144	0.635

for another 24 hr. This was followed by leaving the mixture at room temperature for 96 hr. The results are shown in table 13 and fig 12

A precipitate had formed during the incubation which was removed by centrifugation and the supernatant was evaporated to dryness at 33-35°. The residue was dissolved in 30 ml of water, ethanol (120 ml) was added and the mixture left at 4° for 48 hr. A sticky yellow precipitate had formed from which the supernatant was removed by decantation.

Purification on Sephadex G-25

The precipitate was dissolved in 15 ml 0.1 N acetic acid and applied to a column of Sephadex G-25 prepared as described previously. The column was eluted with 0.01 N acetic acid and 10 ml fractions were collected. Alternate tubes were analysed for mannose and ninhydrin colour. The results are shown in table14 and fig 13

Tubes 16-23 were pooled and estimated for mannose which indicated a concentration of 1.075/mg/ml and corresponded to an 85% recovery from the starting material. See table 15 and fig. 14

Aminoacid analysis

The glycopeptide (3.0 μmoles) was hydrolysed and analysed as described previously. The results are shown in table16 and fig15

Ultraviolet spectrum of glycopeptide

Assuming a molecular weight of 1600 for the glycopeptide prepared and described above a solution of 1.5 mM concentration in water was made up from a weighed quantity of the material. The ultraviolet spectrum was recorded with the Unicam SP 700 and is shown in fig 16

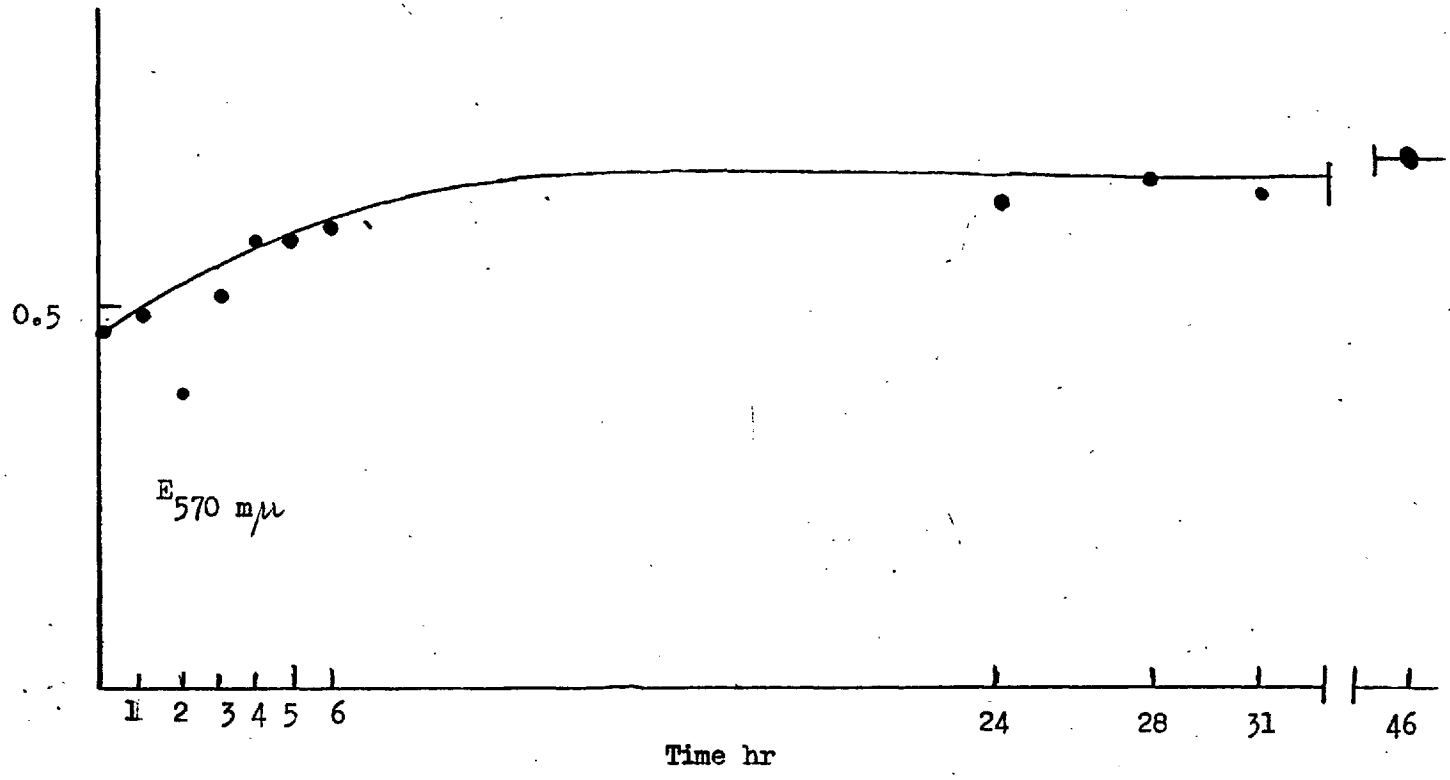


Fig. 12

Pronase digestion of glycopeptide PA

Table 14

Fractionation of glycopeptide PA-P on Sephadex G-25 (ninhydrin)

Tube number	$E_{570\ m\mu}$	Tube number	$E_{570\ m\mu}$
10	0.050	30	0.590
12	0.025	32	0.525
14	0.025	34	0.510
16	0.010	36	0.264
18	0.093	38	0.118
20	0.046	40	0.012
22	0.058	42	0.017
24	0.153	44	0.017
26.	0.330	46	0
28	0.480		

Fractionation of glycopeptide PA-P on Sephadex G-25 (orcinol)

Tube number	$E_{505\ m\mu}$	Tube number	$E_{505\ m\mu}$
10	0.041	22	0.281
12	0.048	24	0.126
14	0.098	26	0.069
16	0.081	28	0.049
18	0.376	30	0.062
20	0.510	32	0.042

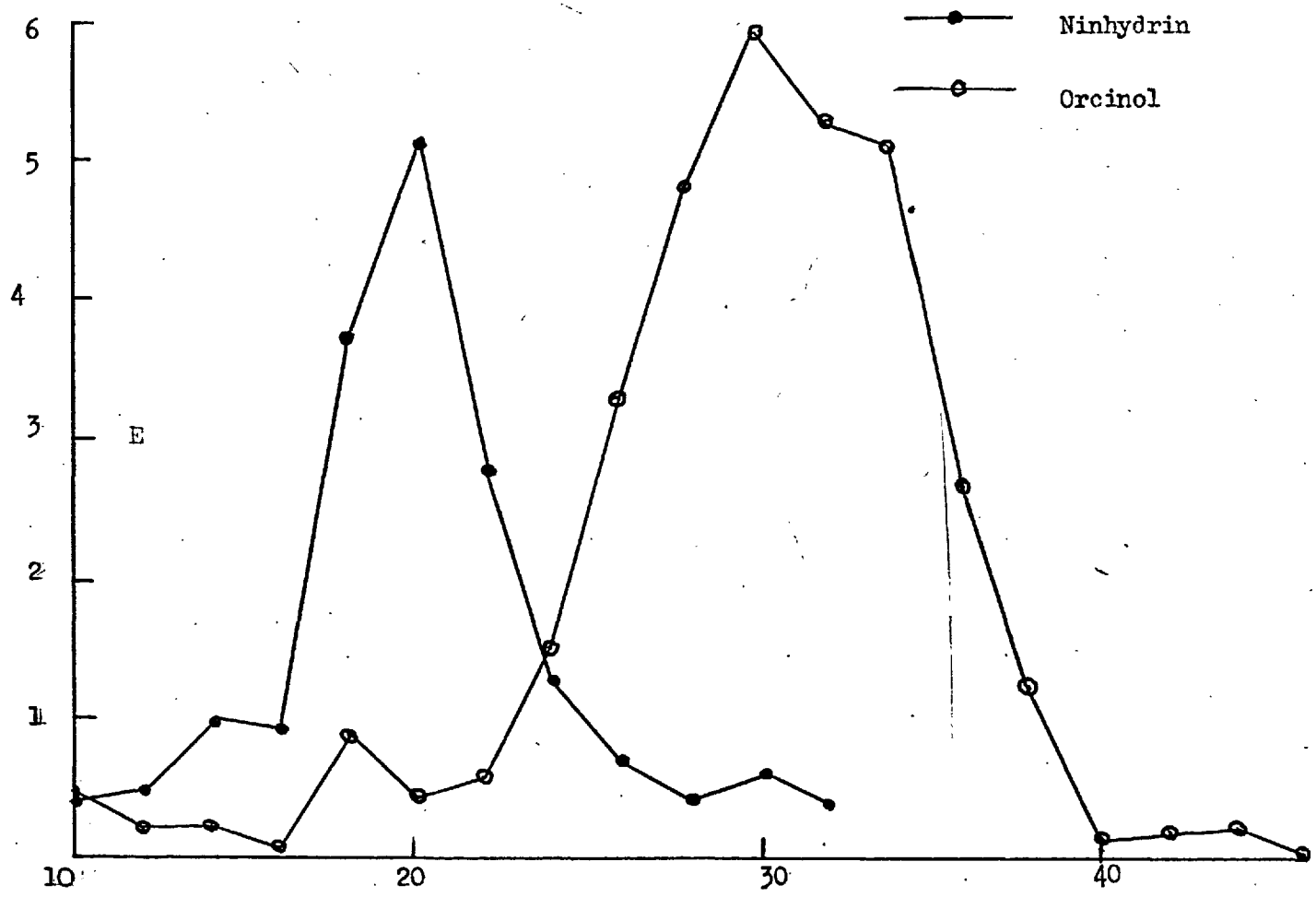
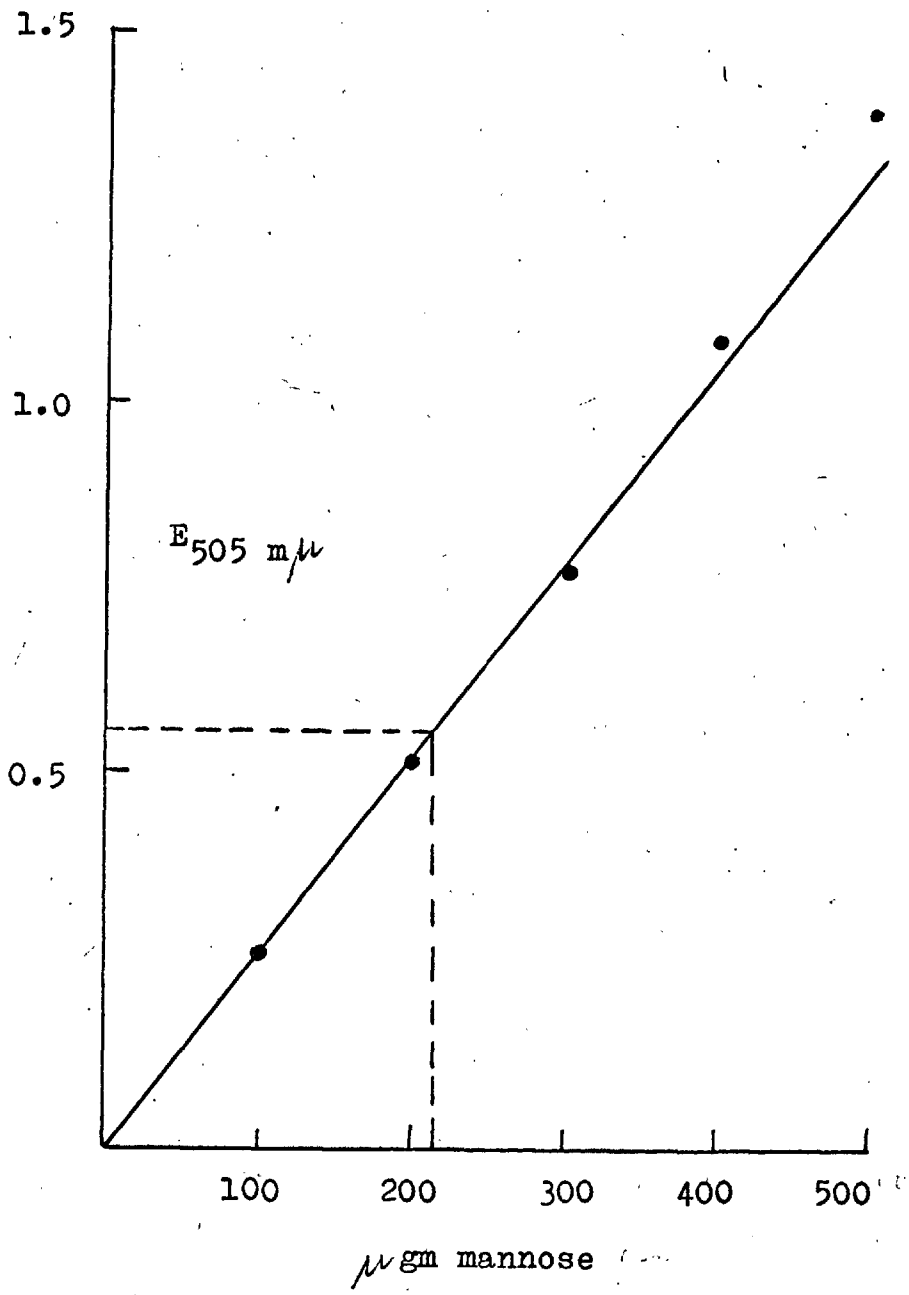


Fig. 13

Purification of glycopeptide PA-P on Sephadex G-25

Fig. 14



Mannose estimation of glycopeptide PA-P

Table 15

Mannose estimation of pooled glycopeptide PA-P

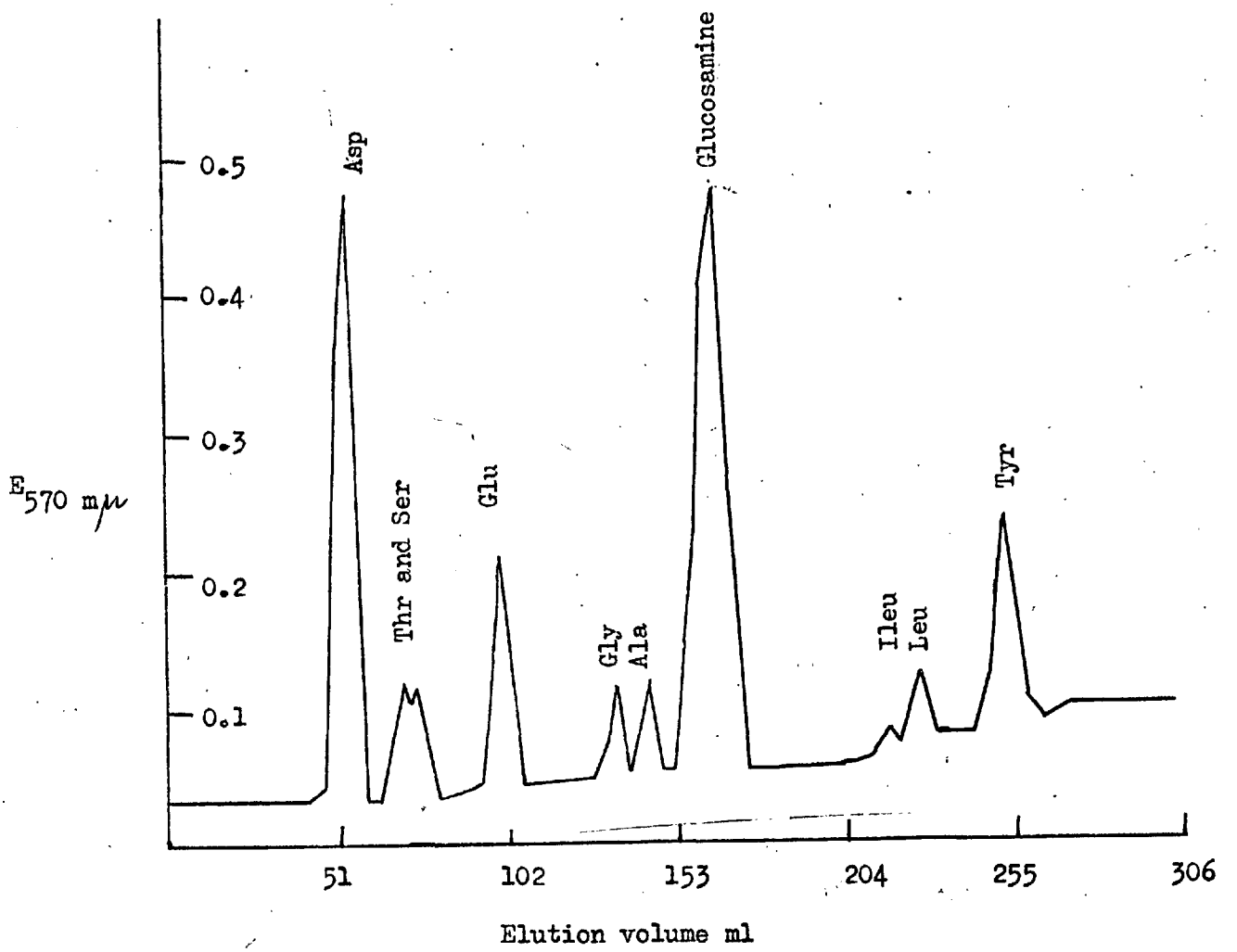
	$E_{505\ m\mu}$	Corr. for blank
Blank	0.044	
100 gm mannose	0.300	0.256
200 gm "	0.554	0.510
300 gm "	0.816	0.772
400 gm "	1.086	1.086
500 gm "	1.45	1.406
0.2 ml glyco.	0.600	0.556
0.2 ml glyco.	0.608	0.564

Table 16

Amino acid analysis of glycopeptide PA-P

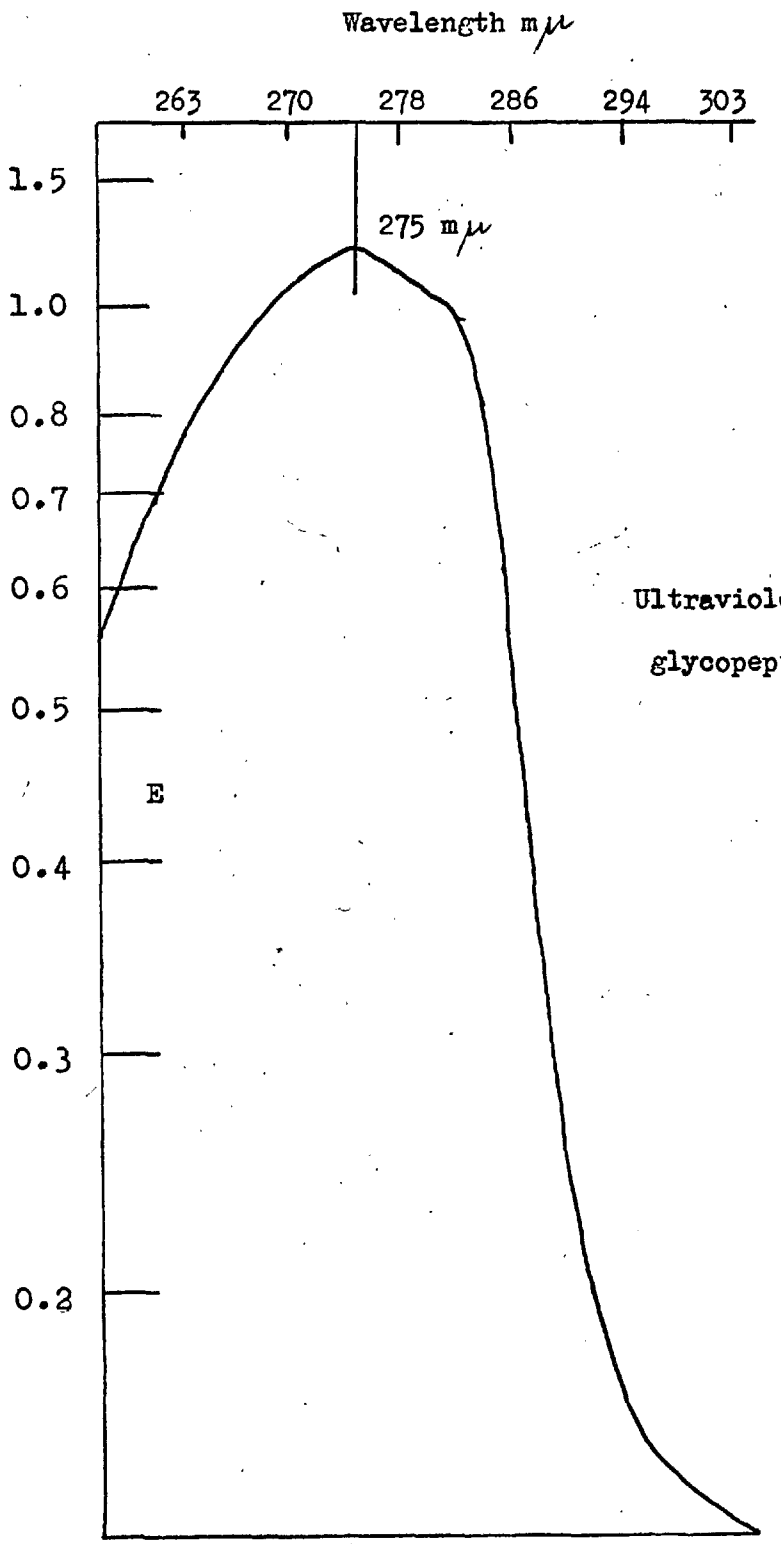
Amino acid	Micromoles
Aspartic acid	1.0
Threonine	0.155
Serine	0.14
Glutamic acid	0.33
Glycine	0.12
Isoleucine	0.045
Leucine	0.13
Tyrosine	0.52
Alanine	0.14
Glucosamine	1.62

Fig. 15



Amino acid analysis of glycopeptide PA-P

Fig. 16



Ultraviolet spectrum of glycopeptide PA-P

RESULTS AND DISCUSSION

METHOD 1.

Pronase digestion

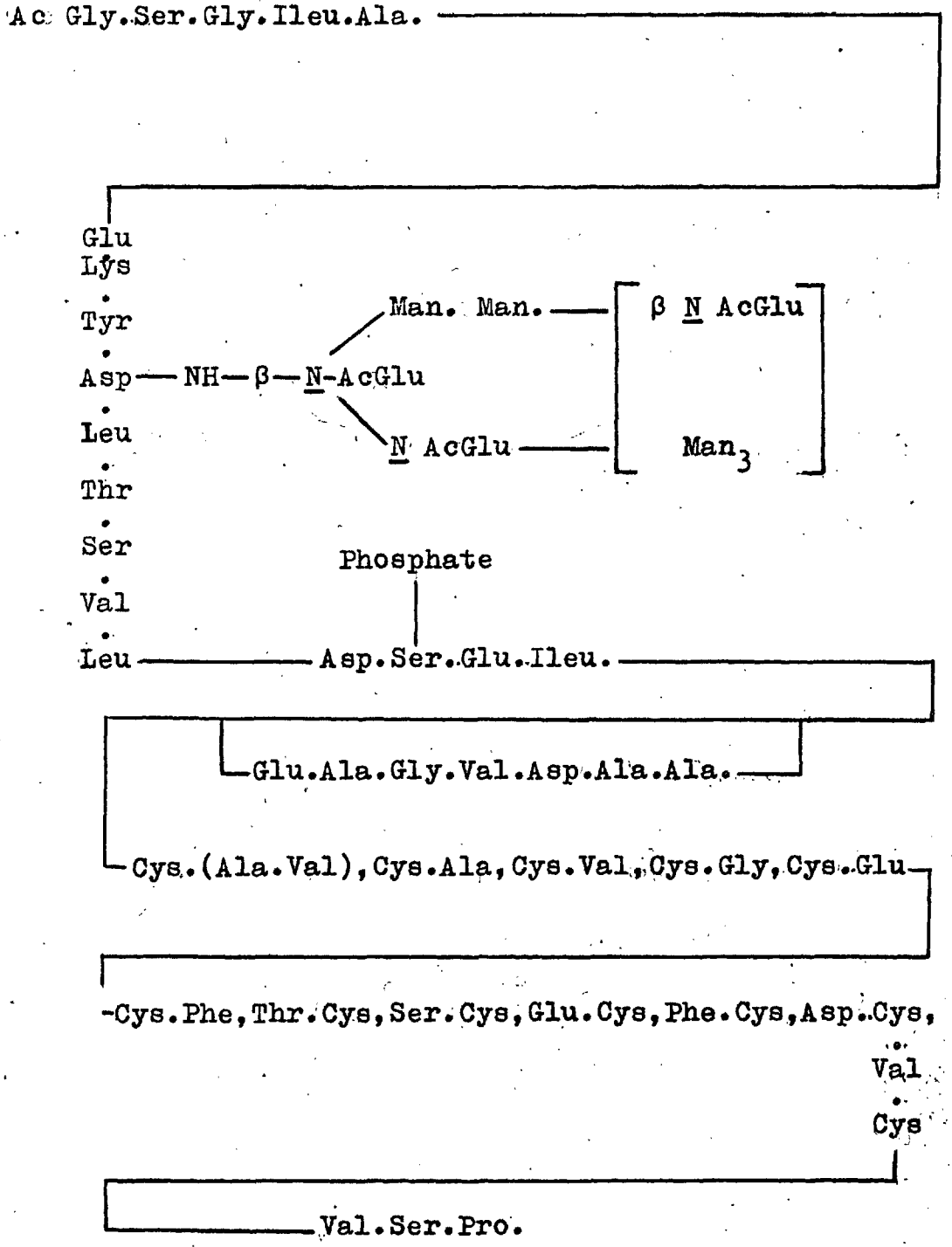
It will be seen from inspection of fig 2 that maximal digestion by Pronase is attained after approximately 24 hr and in later preparations incubation was limited to this time. The most extensive degradation in terms of number of peptide bonds split, as estimated by the ninhydrin method, is that achieved by the prolonged use of pepsin (Fletcher et al, 1963) but amino acid analysis shows that even when pepsin treatment is followed by trypsin and chymotrypsin the amino acids leucine, serine and threonine are still present in the glycopeptide.

Amino acid analysis of the glycopeptide prepared by Pronase digestion alone (estimated 33% of peptide bonds split) indicated the presence of significant amounts of threonine, serine, glycine, valine, leucine and arginine and small amounts of glutamic acid, isoleucine and tyrosine (see table 2). From fig 1 it will be seen that the amino acid sequence in the region of the carbohydrate does not include the amino acids glycine arginine, glutamic acid and isoleucine, it was therefore concluded that these might be present in a contaminating peptide of approximately the same molecular weight glycopeptide.

Analysis of the glycopeptide preparation before hydrolysis indicates two peaks, one corresponding with the position known by previous experiments (Dr. R. D. Marshall, personal communication) to be characteristic of the glycopeptide and another less sharp peak, slightly

Fig. 1

N-terminus The ovalbumin molecule



C-terminus

(from Marshall and Neuberger from Gottschalk, 1965)

more retarded than the glycopeptide. From the amino acid analysis it can be seen that the molar composition of the peptide could be $(arg)_4(gly)_3(ileu)(glu)$, which would have a molecular weight of 1200 and so would not be expected to be separated from the glycopeptide on Sephadex G-25. Consideration of fig 1 and table 2 shows that Pronase has virtually completely split the tyr-asp bond but has only partially split the asp-leu bond, the leu-thr bond and the thr-ser bond. It is known (Fletcher et al, 1963a) that a glycopeptide of this composition is completely resistant to hydrolysis by pepsin, trypsin, chymotrypsin, papain and a streptomyces protease. Johansen et al (1961) have shown that although carboxypeptidase releases some ser, thr and leu the resultant glycopeptide still contains significant amounts of these amino acids. Kaverzneva and Bogdanov (1961) and Nuenke and Cunningham (1961) have reported similar findings.

Carboxypeptidase digestion.

Bogdanov, Kaverzneva and Tsi De-Fan (1961) indicated that the N-benzyloxycarbonyl derivative of such a glycopeptide would render it susceptible to the action of carboxypeptidase. Treatment of the glycopeptide prepared by pepsin, trypsin and chymotrypsin digestion in this way yielded a glycopeptide with only trace amounts of amino acids other than aspartic acid (Fletcher et al, 1963a). Similar treatment of the Pronase glycopeptide has removed all amino acids other than aspartic acid leaving only 0.03 mole of leucine.

The satisfactory separations achieved with Sephadex G-25 in these experiments suggest that gel filtration has a wide application in the

preparation of glycopeptides providing that the other products of digestion are significantly different in size. Thus a glycopeptide of molecular weight approximately 1500 is completely separated from di- and tri-peptides and free amino acids. However the presence, as described above, of a contaminating peptide of comparable size to the glycopeptide, shows that amino acid analyses indicating several amino acids other than the one expected to be involved in the linkage require some caution in their interpretation.

METHOD 2

Papain digestion

In the preparation of glycopeptides from ovalbumin by papain digestion Lee and Montgomery (1962) have shown that the asp-leu bond (see fig 1) is completely cleaved whereas the tyr-asp bond is resistant. Since Pronase apparently possesses the converse specificity it seemed reasonable to try a combination of the two enzymes to achieve the preparation of a glycopeptide containing only aspartic acid.

The findings of Lee and Montgomery were confirmed by the present experiments but it appears from the amino acid analysis that the tyr-asp bond left in this glycopeptide by the papain digestion is made resistant to Pronase by the absence of amino acids on the carboxyl side of aspartic acid. Then Pronase is acting on the whole glycoprotein it seems possible that the converse situation occurs, that is, the rate of hydrolysis of the asp-leu bond and when all the tyr-asp bonds have been cleaved the remaining asp-leu bonds become resistant. This

would account for the glycopeptides containing fractional molar quantities of leucine or tyrosine.

Ultraviolet spectrum of the papain-pronase glycopeptide.

Fig[16] showing the ultraviolet spectrum of the papain-pronase glycopeptide confirms the presence of tyrosine by the appearance of a maximum at 276 mμ. An experimentally determined value for the molar extinction of tyrosine at this wavelength was 1380 from which it was calculated that a 1.5 mM solution of glycopeptide was 0.72 mM in tyrosine per mole of glycopeptide. This value is in good agreement with the value of 0.52 moles given by amino acid analysis.

Table 17 shows the composition of some glycopeptides that have been prepared from ovalbumin.

Table 17

The composition of some glycopeptides

References	Hexose	Hexosamine	Tyr	Asp	Leu	Thr	Ser	Val	Ala	Gly	Glu	Lys
Jevons, 1958.	4	2	-	1	1	-	-	-	-	-	-	-
Johansen et al, 1961.	5	3	0.2	0.99	0.78	0.35	0.32	-	-	-	-	-
Nuenke and Cunningham, 1961.	5	2	0.9	1.0	1.84	0.92	0.93	0.65	-	-	-	-
Kaverznova and Chi Tieh-fang 1961.*	5	3	-	1.0	1.6	0.5	0.4	-	0.2	0.3	-	-
Lee and Montgomery, 1962.	6	3	0.93	1.03	0.05	0.05	0.05	-	0,08	0.05	0.11	0.1
Fletcher et al, 1963.	5	3	-	1.01	0.07	-	0.11	-	0.04	-	0.04	-
Present studies.	5	3	-	1.0	0.03	-	-	-	-	-	-	-
Fletcher et al, 1963 and present study.	5	3	0.53	1.0	0.13	0.15	0.14	-	0.14	0.12	0.33	+
Clamp and Hough, 1965.	5	3	0.6	1.0	0.7	0.9	0.7	-	-	-	-	-

* Biokhimiya, 26 420.

DETERMINATION OF THE MOLECULAR WEIGHT OF THE OVALBUMIN GLYCOPEPTIDE

DETERMINATION OF THE MOLECULAR WEIGHT OF THE OVALBUMIN GLYCOPEPTIDE

INTRODUCTION

The molecular weight of a glycopeptide from ovalbumin (Neuberger, 1939) was estimated by an isopiestic method involving bringing solutions of the glycopeptide into osmotic equilibrium with a solution of KCl of known concentration. A value of about 1200 was obtained.

Using a titration method Kaverzneva and Bogdanov (1962) found a value of 1500 for a glycopeptide from the same source and by the Ramsay depression of freezing point micro-method Clamp and Hough (1965) have estimated the molecular weight of a glycopeptide, containing appreciable amounts of amino acids other than aspartic acid, to be 1660±300.

In the present study the ultraviolet absorption of a substituted glycopeptide (Fletcher, Marshall and Neuberger, 1963b) was used to estimate the molecular weight. It was desirable to select as chromophoric substituent a group which was sufficiently isolated to be unaffected by changes in the rest of the molecule. This would enable a molar extinction to be calculated from other known similarly substituted compounds, with reasonable confidence that no serious error would be involved in using this figure for subsequent calculations.

It was also desirable that the substitution should be achieved under mild conditions that would be unlikely to alter the glycopeptide structure, and also that the product should be stable at ordinary temperatures and under the conditions necessary for ultraviolet examination.

The chromophore should also absorb at a wavelength well separated from the main amide bond absorption of the unsubstituted glycopeptide, but on the other hand it should not have an absorption likely to interfere with the estimation of mannose by the orcinolsulphuric acid method as this procedure was necessary to match the concentrations of the substituted and unsubstituted glycopeptides.

An accurate estimation of the molecular weight seemed desirable in view of some doubt as to the carbohydrate composition of the glycopeptide expressed by some workers (Lee and Montgomery, 1961; Nuenke and Cunningham, 1961, Horman and Gollwitzer, 1962 and Jevons, 1956). However these estimates have been based on colourimetric methods which may be non-specific and should be interpreted with some caution. A radio-isotope dilution method (Francois, Marshall and Neuberger, 1962) which avoids this disadvantage indicated that the glycopeptide contained five residues of mannose and three residues of N-acetyl-glucosamine. Although in the present study the orcinol-sulphuric acid method has been used, this has only been done in order to match the solution in sample and reference cells with respect to mannose, and not to obtain an absolute value.

It seemed that the benzyloxycarbonyl group would meet the necessary requirements outlined above, the $-CH_2OCO-$ group acting as a "spacer" between the chromophoric benzene ring and the glycopeptide, so minimising the possibility of interactions between the two components that might cause an undetected alteration in the molar extinction.

Five compounds linked with the benzyloxycarbonyl group were studied,

i) 1-β-(N-benzyloxycarbonyl-L-β-aspartamido)-1-deoxy-D-glucose (preparation by Dr. G. S. Marks; Marks and Neuberger, 1961), ii) N-benzyloxycarbonyl-glucosamine, iii) N-benzyloxycarbonyl-α-methylglucosaminide, iv) N-benzyloxycarbonyl-α-amino-β-hydroxyadipic acid (preparation of Laver, Neuberger and Scott, 1959) and v) N-benzyloxycarbonyl aspartic-β- semialdehyde (kindly given by Dr. G. H. Tait, see Neuberger and Tait, 1962).

The ultraviolet spectra were recorded with a Unicam SP 700 using 1 cm Suprasil cells. Molar extinctions were calculated for maxima at 252 mμ, 254 mμ, and 267 mμ.

EXPERIMENTAL

Molar extinction of the benzyloxycarbonyl group.

Between 5 and 10 mg each of the benzyloxycarbonyl derivatives was weighed accurately to a hundredth of a milligram, dissolved and made up to 10.0 ml in 30% ethanol.

Fig. 17 shows the spectrum of 1-β-(N-benzyloxycarbonyl-L-β-aspartamido)-1-deoxy-D-glucose which was typical of all the substances examined. The recordings were made with 30% ethanol in the reference cell. Table 18 shows the molar extinctions of each substance and the mean value of each maximum.

Molecular weight estimation of the glycopeptide.

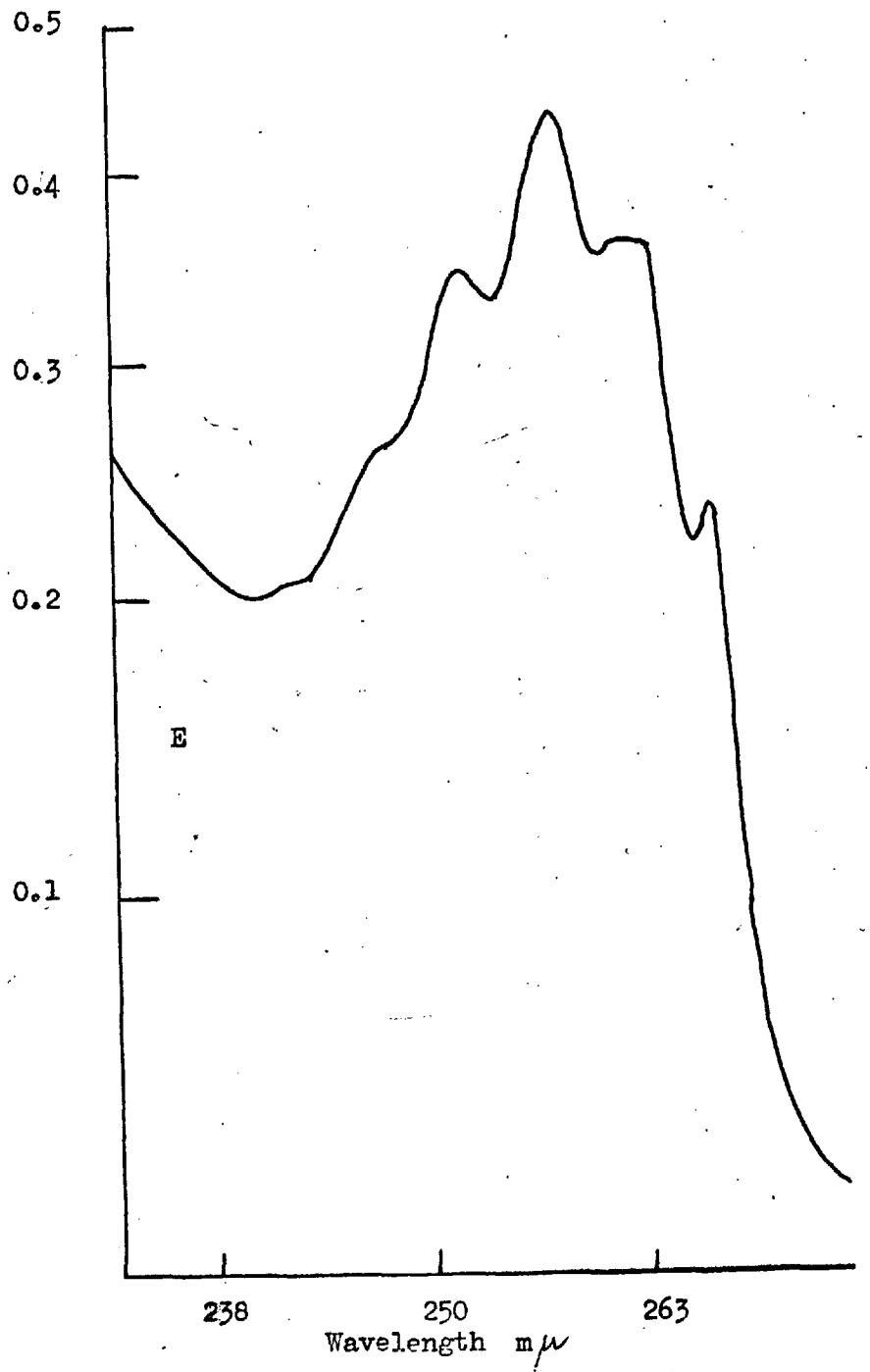
The benxyloxycarbonyl glycopeptide used in these studies was prepared by method 1 described in the previous section and ninhydrin estimations showed that carbobenzoxylation was complete.

Table 18

Molar extinctions of benzyloxycarbonyl compounds

Substance	252 mμ	254 mμ	267 mμ
Cbzo methylglucosaminide	168	217	114
Cbzo glucosamine	166	213	115
Cbzo α-amino-β-hydroxyadipic acid	173	206	115
Cbzo aspartic-β-semialdehyde	168	209	119
Cbzo aspartylglucosylamine	167	202	119
Mean	168	209	116

Fig. 17



Ultraviolet spectrum of 1-β-(N-benzyloxycarbonyl-L-β-aspartamido)-1-deoxy-D-glucose

Removal of the benzyloxycarbonyl group.

The method employed was by hydrogenation in the presence of palladium black.

Benzyloxycarbonyl glycopeptide corresponding to 320 mg of mannose was dissolved in 25 ml of 20% acetic acid and 20 mg of palladium black added. The mixture was stirred and hydrogen was passed over the surface for 4 hr. Palladium black was removed by filtration and the solution evaporated to dryness. The residue was taken up in water and a pure white fluffy product obtained by freeze-drying.

The ultraviolet spectrum of the product showed de-carbobenzoxylation to be incomplete so the above procedure was repeated to give a glycopeptide free of the substituent.

Dry weight of the glycopeptide.

Benzyloxycarbonyl glycopeptide (18.975 mg) was heated at 80° for 4 hr in vacuo. The weight loss was 2.00 mg.

Spectrum of the benzyloxycarbonyl glycopeptide.

Benzyloxycarbonyl glycopeptide (35.43 mg) was dissolved in water and made up to 10.0 ml.

Glycopeptide (23.61 mg) was dissolved in water and made up to 5.00 ml.

Benzyloxycarbonyl glycopeptide solution (3.50 ml) was made up to 5.00 ml with ethanol (30% in ethanol) and a volume (2.36 ml) of glycopeptide solution containing the same amount of mannose on the basis of an orcinol-sulphuric acid estimation, was similarly made up to 5.0 ml in 30% ethanol. Thus with benzyloxycarbonyl glycopeptide in the sample cell and glycopeptide in the reference cell the observed spectrum would

be a measure of the benzyloxycarbonyl group present. Fig. 18 shows the spectrum of the benzyloxycarbonyl glycopeptide.

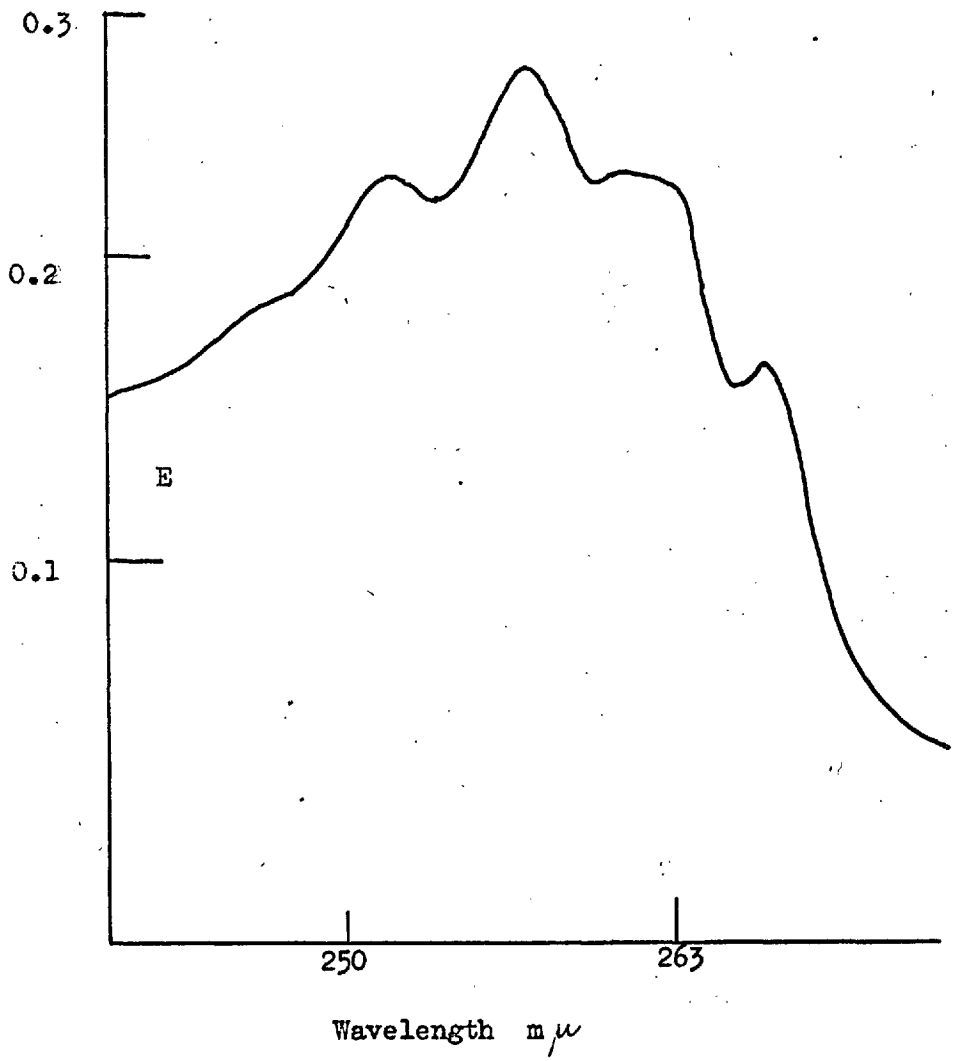
RESULTS AND DISCUSSION

The spectrum of the benzyloxycarbonyl group shows well defined maxima at 252 mμ, 254 mμ and 267 mμ, and also a plateau at about 262 mμ. Molar extinctions for the maxima are shown in table 18. The individual values for each of the maxima are seen to be satisfactorily similar between different compounds in spite of considerable differences in structure. It therefore seemed justifiable to use mean values in calculations on the glycopeptides.

The form of the benzyoxycarbonyl glycopeptide spectrum is closely similar to that of the reference compounds, and using the extinctions referred to above and the known concentration of the benzyloxycarbonyl glycopeptide on a weight basis, values of 1710 (252 mμ), 1750 (254 mμ) and 1660 (267 mμ) for the molecular weight were obtained. The mean of these figures when corrected for moisture and the benzyloxycarbonyl group gives a molecular weight of 1570 for the glycopeptide.

The molecular weight required for a glycopeptide composed of five mannose three N-acetylglucosamine and one asparagine residues is 1551. The figure obtained in this experiment is consistent with this structure and it seems unlikely that this glycopeptide contains a sixth residue of mannose.

Fig. 18



Ultraviolet spectrum of benzyloxycarbonyl glycopeptide

DEMONSTRATION OF A β-ASPARTYL LINKAGE WITH THE CARBOHYDRATE COMPONENT

DEMONSTRATION OF A β-ASPARTYL LINKAGE WITH THE CARBOHYDRATE COMPONENT

INTRODUCTION

Evidence supporting the hypothesis that in ovalbumin the carbohydrate component is linked (as an N-glycoside) to the β-carboxyl group of an aspartic acid residue has been largely indirect. Thus the resistance of this bond to cleavage by enzymes that acton peptide bonds is very suggestive and a linkage through the α-carboxyl group would necessitate a peptide bond with the adjacent amino acid through the β-carboxyl group unless the aspartic acid was C-terminal.

Edman (1950) and others (Fraenkel-Conrat and Harris, 1954) have developed a technique for the determination of the amino acid sequence in peptides involving the reaction of the free terminal amino group with phenylisothiocyanate to form the phenylthiocarbamyl (PTC) derivative. In acid conditions cyclisation occurs with the carboxyl group that is in peptide linkage, cleaving the peptide bond with the formation of the phenylthiohydantoin (PTH) derivative of the terminal amino acid.

It seemed that direct evidence for a β-aspartyl linkage might be obtained by the application of this method. Thus if it were possible to prepare a substance in which the PTH derivative of aspartic acid were still linked to the carbohydrate, it is clear that the α-carboxyl group of aspartic acid would not be involved in the bond since if it were the cyclisation reaction would have resulted in its cleavage and the liberation of PTH-aspartic acid. Furthermore, if subsequent hydrolysis yielded PTH-aspartic acid the β-linkage would be confirmed.

EXPERIMENTAL

PTH-aspartic acid and other PTH-amino acids for use as chromatographic markers were kindly supplied by Dr. E. Papkoff.

PTH-asparagine was prepared by two methods, i) was a modification of the method of Fraenkel-Conrat, Harris and Levy (1955) and ii) a modification of the method of Konigsberg and Hill (1962).

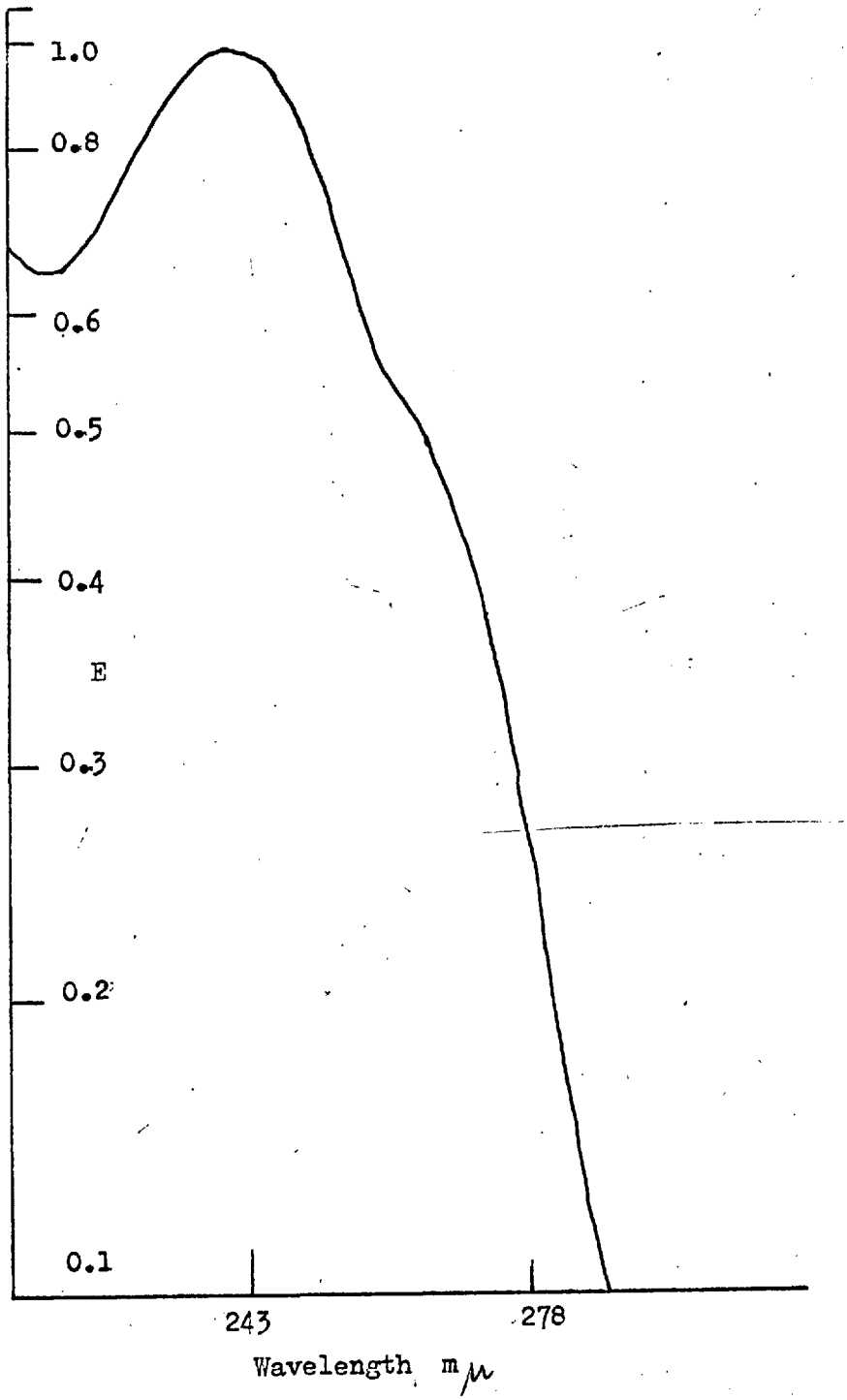
Method i). Formation of PTC-derivative of asparagine.

Asparagine (2.0 gm); supplied by BDH was dissolved in 70ml of 50:50 dioxan-water. Sodium bicarbonate (4-5 gm) and redistilled phenylisothiocyanate (2.0 ml) were added and the mixture shaken at 40° for 2.5 hr. Excess phenylisothiocyanate was removed by extracting seven times with benzene. The mixture was neutralised with hydrochloric acid and phenylthiocarbamyl-asparagine precipitated out. The precipitate was extracted into ethyl acetate and dried over anhydrous sodium sulphate. Evaporation of the ethyl acetate yielded 2.2 gm of pale yellow crystals. The ultraviolet spectrum of this material showed a maximum at 252 mμ which is characteristic of PTC compounds, (Fig. 19).

Formation of the PTH-derivative.

PTC-asparagine (0.5 gm) was shaken with 20 ml of 2N HCl prepared from constant boiling HCl at 40° for 1 hr. A white insoluble substance formed which was extracted into ethyl acetate and dried over anhydrous sodium sulphate. The ethyl acetate was removed by evaporation and the residue dissolved in a small volume of hot 50% ethanol. Crystals of PTH-asparagine formed on cooling. Recrystallisation was achieved from ethanol-water giving a substance with melting point 234°-236° (Edman, 1950, gives 234°).

Fig. 19



Spectrum of PTC-asparagine

Method ii). The PTC compound was formed as in i).

Formation of PTH compound.

PTC asparagine (0.5 gm) was dissolved in 4-5 ml of trifluoroacetic acid (BDH) and left at room temperature for 4 hr. The solution was evaporated to dryness leaving a pale yellow solid residue which was dissolved in a small volume of 50% ethanol and allowed to crystallise at 4°. The material was recrystallised twice from 50% ethanol to give a substance with melting point 233-236°. The ultraviolet spectrum was recorded on a Unicam SP 700 and is shown in fig. 20.

Preparation of the PTH-glycopeptide.

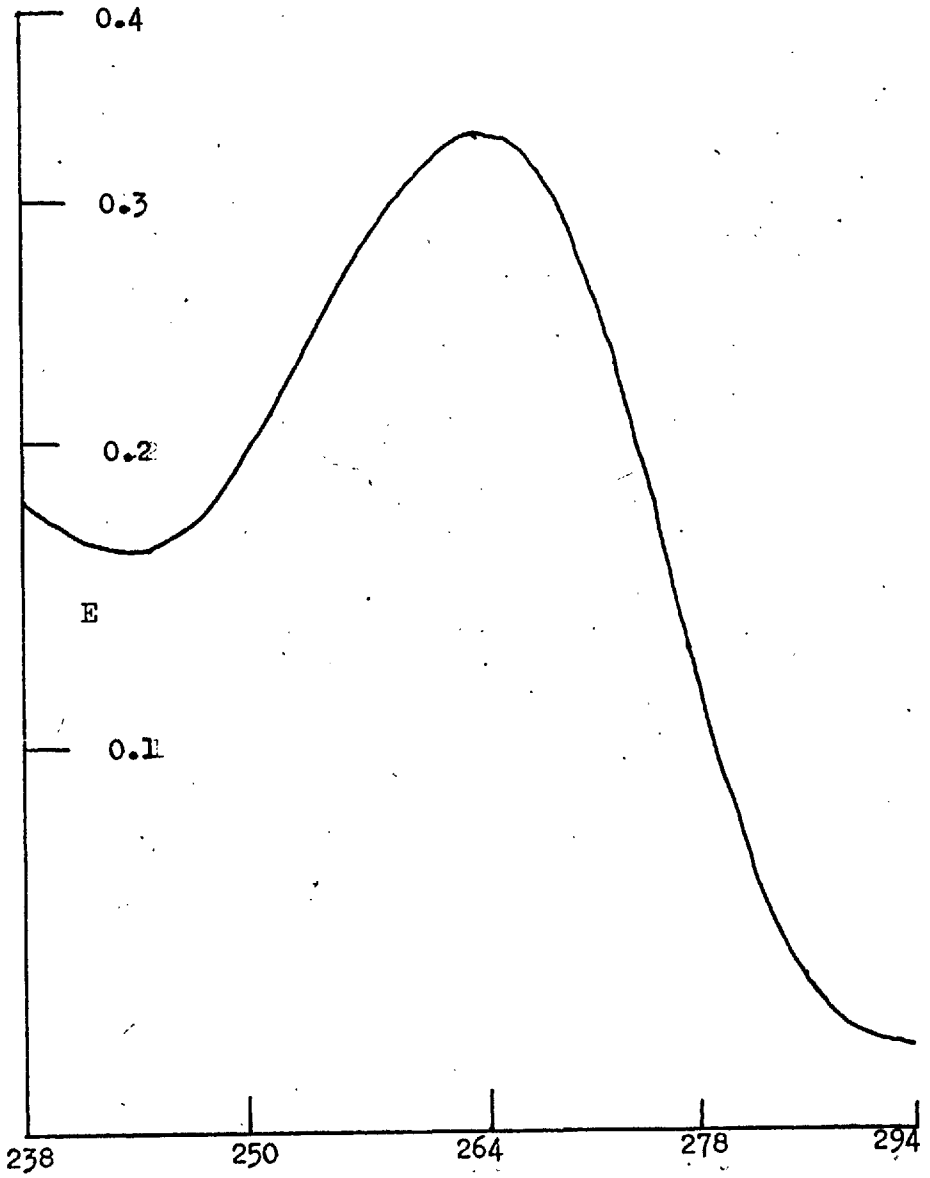
The method used was that of Konigsberg and Hill (1962).

N-ethylmorpholine buffer - N-ethylmorpholine 12.0 ml; acetic acid 0.3 ml; 95% ethanol 100 ml; water 77.7 ml.

Glycopeptide (18.2 mg) prepared by method I and decarbobenzoxylated by catalytic hydrogenation was dissolved in 2.0 ml of N-ethylmorpholine buffer. Phenylisothiocyanate (0.02 ml) was added and the solution incubated at 32° for 3 hr. The solution was then taken almost to dryness on a rotary evaporator (40-50°) and the yellowish oil that remained was extracted twice with benzene. Any remaining volatile material was removed under high vacuum at 60° for 5 min. The residue was dissolved in 2.0 ml of trifluoroacetic acid and left at 22° for 1 hr. The trifluoroacetic acid was removed by evaporation and the residue was left in a vacuum desiccator over calcium chloride and sodium hydroxide flakes.

A dry flaky substance was obtained which dissolved completely and easily in 0.01 ml of water and was precipitated by the addition of

Fig. 20



Spectrum of PTH-asparagine

ethanol to 80-90%. The precipitate was separated by centrifugation at 1500 rev./min for 5 min, the alcoholic supernatant removed and evaporated to dryness.

Paper chromatography of the products.

Descending chromatography was performed on Whatman's No 1 paper which for the first chromatogram was prepared by spraying with 0.5% starch solution and drying in air. For the second chromatogram the paper was not pretreated but the starch was incorporated in the detecting reagent. The PTH-glycopeptide (1.01 mg) was dissolved in 0.1 ml of water and 10 μl were applied to the paper.

The material left after evaporation of the alcoholic supernatant was dissolved in three drops of ethanol and 10 μl were applied to the paper.

PTH-asparagine, PTH-aspartic acid and PTH-leucine were used as marker substances.

The solvent system was mixture C of Fraenkel-Conrat et al (1955) composed of heptane-n-butanol - formic acid in the proportions 40:20:40. Papers were equilibrated overnight in a chromatographic tank containing the solvent and for two hours prior to the experimental run the solvent was allowed to run down a blank sheet of Whatman's No 1 paper.

The chromatogram took 12 hr to develop after which it was dried in an oven at 40°.

The PTH compounds were detected by spraying with an iodine-sodium azide reagent (Smith, 1960) made by mixing 1 volume of a solution of iodine (2.54 gm) and potassium iodide (8.0 gm) made up to 100 ml with water and 2 volumes of a solution of sodium azide (1.5 gm) made up to

100 ml with water. If the paper was not pretreated with starch the sodium azide was made up to 0.5% in starch.

Hydrolysis of the PTH-glycopeptide.

Approximately 5 mg of PTH-glycopeptide was dissolved in 1.0 ml of 2N HCl (made by dilution from constant boiling HCl) and heated in a boiling water bath for 2½ hr. The hydrochloric acid was removed by evaporation to leave a slightly charred residue.

This was dissolved in 0.25 ml of ethanol (leaving a small amount of insoluble material) and 50 µl was spotted on untreated paper.

PTH-asparagine, PTH-aspartic acid, PTH-leucine and unhydrolysed PTH-glycopeptide were run as markers. The PTH compounds were detected by the iodine-sodium reagent as described above.

The hydrosylate and markers were also run in the solvent system pyridine:N- heptane in the proportions 30:70 (Fraenkel-Conrat et al, 1955). The patterns obtained are shown in figs. 21 and 22.

Ultraviolet spectrum of the PTH-glycopeptide.

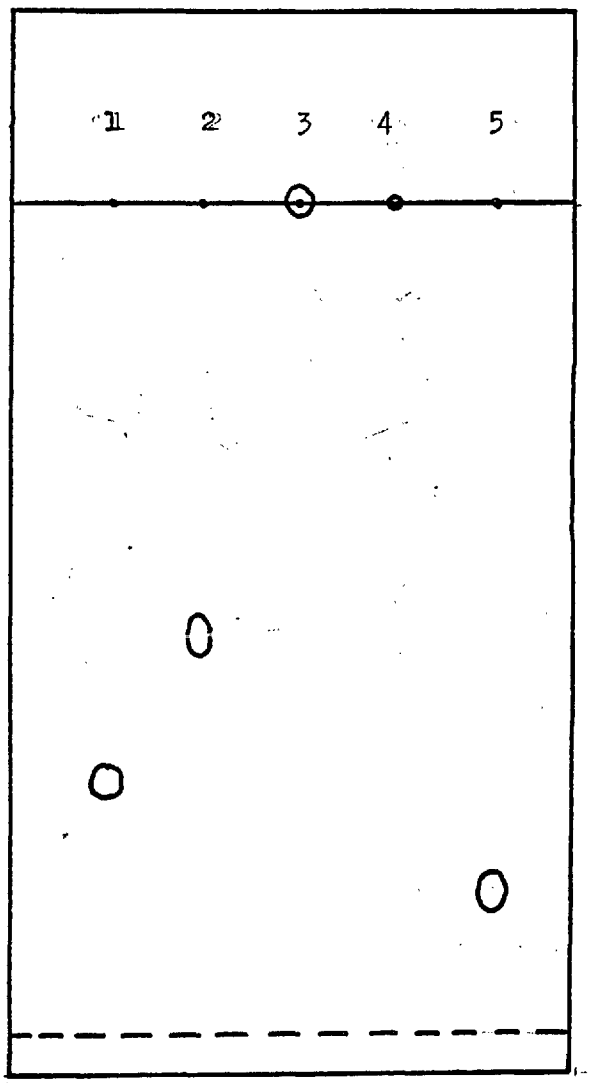
PTH-glycopeptide (2.31 mg) was dissolved in water and made up to 5.0 ml. Two portions, on of 1.0 ml and the other of 0.5 ml were estimated for mannose by the orcinol-sulphuric acid method as described previously. The results of the estimationsare shown in fig. 23 and table 19

0.5 ml of the PTH-glycopeptide solution was diluted to 2.5 ml and the ultraviolet spectrum recorded with the Unicam SP 700. Fig. 24

RESULTS AND DISCUSSION

The method of preparing PTH compounds was seen to be satisfactory

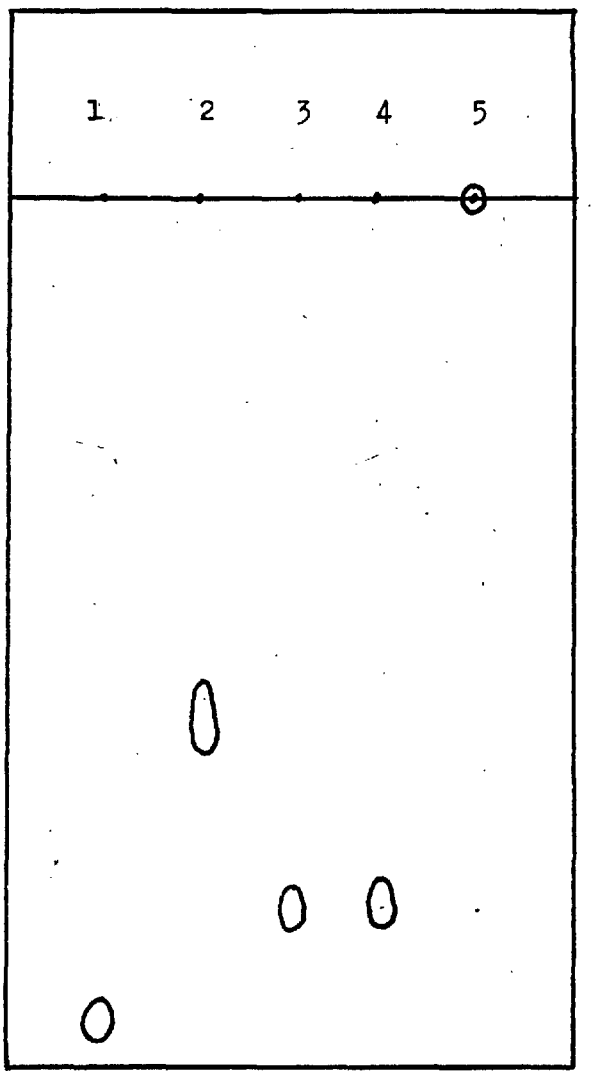
Fig. 21



Paper chromatography of PTH-glycopeptide in heptane-N-butanol-formic acid

1. PTH-aspartic acid 2. PTH-asparagine 3. PTH-glycopeptide 4. ethanol supernatant 5. PTH-leucine

Fig. 22



Paper chromatography of hydrolysed PTH-glycopeptide in heptane - N-butanol - formic acid

1. PTH-leucine 2. PTH-asparagine 3. hydrolysed PTH-glycopeptide 4. PTH-aspartic acid 5. PTH-glycopeptide

Table 19

Mannose estimation of PTH-glycopeptide

	$E_{505\ m\mu}$	Corr. for blank
Blank	0.023	
100 µgm mannose	0.253	0.230
200 µgm "	0.494	0.471
300 µgm "	0.780	0.757
400 µgm "	1.10	1.08
500 µgm "	1.46	1.44
0.5 ml glyco.	0.236	0.213
1.0 ml glyco.	0.474	0.451

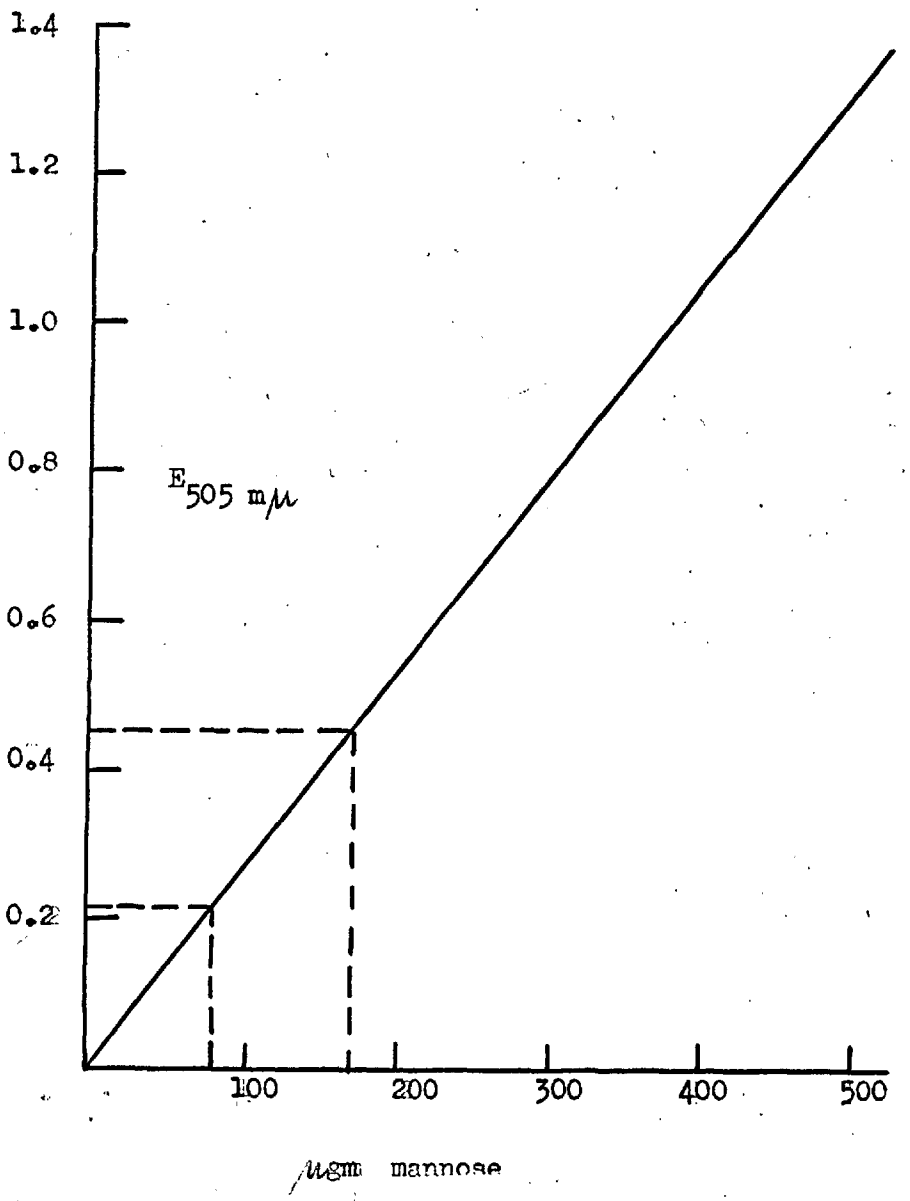
Concentration of glycopeptide = 0.46 mg/ml

Table 20

Reaction of PTH-amino acids with aqueous pyridine

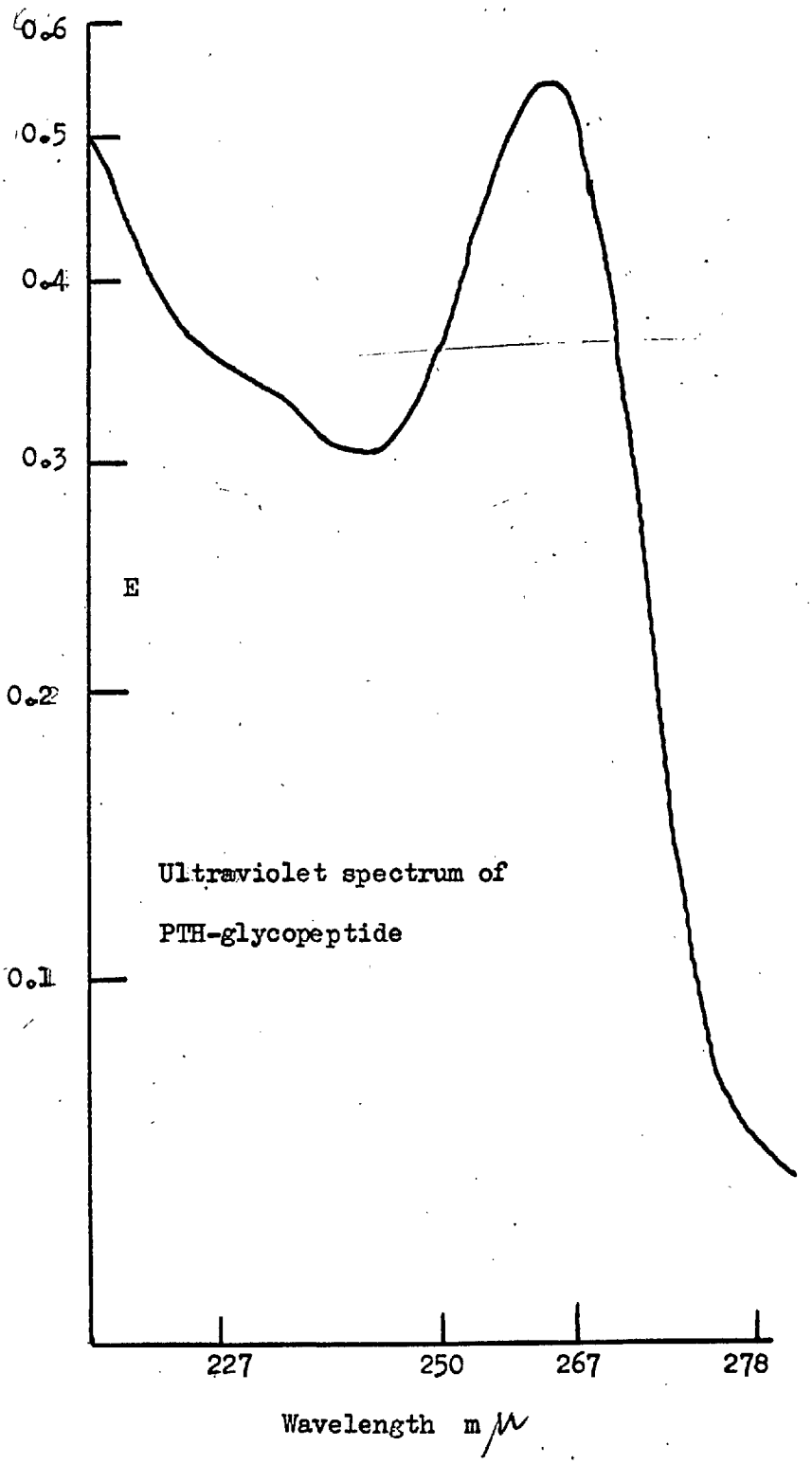
Amino acid	Reaction	Amino acid	Reaction
Alanine	-	Leucine	-
Valine	-	Threonine	-
Methionine	-	Arginine	-
Proline	-	Asp. acid	-
Cysteine	-	Lysine	v. weak
Glycine	-	Phenylalanine	v.v. weak
Glutamic acid	-	Histidine	+
Serine	-	Asparagine	+++
Isoleucine	-	Tyrosine	+

Fig. 23



Mannose estimation of PTH-glycopeptide

Fig. 24


0.6
0.5
0.4
0.3
E
0.2
0.1
Ultraviolet spectrum of
PTH-glycopeptide
227
250
267
278
Wavelength mμ

by the ultraviolet spectrum of PTH-asparagine which showed a maximum at 264 m with no indication of any PTC compound.

Since the products from the reaction of the glycopeptide with phenylisothiocyanate were readily and completely soluble in a very small volume of water it seemed unlikely that any cleavage of the carbohydrate-aspartic acid bond had occurred as the PTH-amino acids are rather sparingly soluble in water. The apparently complete precipitation at 80-90% ethanol strengthens this interpretation.

The results of paper chromatography were quite unequivocal since neither the aqueous solution of the precipitate nor the ethanolic supernatant contained any detectable free PTH-aspartic acid or indeed any PTH compound moving from the origin. However in the case of the aqueous solution a strongly reacting spot was present at the origin and in the case of the alcoholic supernatant only a weakly positive spot was observed in the same place.

The ultraviolet spectrum of the aqueous and alcoholic fractions gave further confirmation, showing a compound with a maximum at 267 mμ and a minimum at 242 mμ in the former fraction and virtually no absorbing material in the latter. Moreover there was no trace of any remaining PTC compound.

On the basis of mannose estimations, and assuming the presence of 5 residues of mannose per mole of glycopeptide, the PTH-glycopeptide had a molar extinction of 14600, a value which is within the expected range.

The above observations strongly suggested that hydantoin formation had not resulted in the release of any PTH-aspartic acid.

This was further confirmed by the results of acid hydrolysis of the PTH-glycopeptide. Chromatography in the two solvent systems

mentioned showed the presence of a PTH compound with the same mobility as PTH-aspartic acid. The pyridine-heptane system caused considerable trailing and was thus rather unsatisfactory but even so the results were consistent with the presence of PTH-aspartic acid.

This experiment gives conclusive evidence of a β-aspartyl linkage between the carbohydrate and protein in ovalbumin. The more precise identification of a substituted asparagine as suggested by Johansen et al, (1961) and Nuenke and Cunningham (1961) is indicated by a chance finding during a search for a solvent that might be useful for chromatography of the PTH-glycopeptide.

One of the solvent systems investigated was the mixture ethyl acetate - pyridine - water -acetone in the proportions 10:5:10:2 (Malpress and Hytten, 1958). Ethyl acetate was added until the mixture separated into two phases, the upper having sp. gr. 0.951-0.953. The lower layer was discarded and the upper layer equilibrated with an equal volume of water.

The layers were separated and PTH-glycopeptide (0.5 mg) added to 5.0 ml. of the lower phase. It was intended to investigate the distribution of the PTH-glycopeptide between upper and lower phases by examination of the ultraviolet spectrum of each layer but after a few minutes it was observed that the solution of PTH-glycopeptide began to develop a distinct yellow colour. Examination of the spectrum showed a curve quite different from that usually seen in PTH compounds.

The experiment was repeated using PTH-asparagine and a similar yellow colour was found to develop in a few minutes. Investigation of each of the components of the solvent system indicated that aqueous pyridine was responsible for the reaction. A solution of PTH-asparagine

and PTH-glycopeptide was spotted on filter paper, dried in air and dipped in aqueous pyridine. An intense yellow spot developed in a few minutes.

Seventeen other amino acids were spot tested in the same way (table 20) and only PTH-asparagine, PTH-tyrosine and PTH- histidine were found to give a positive reaction. PTH- aspartic acid was negative. The nature of the reaction is not known but the similarity between PTH-asparagine and PTH-glycopeptide is remarkable. An analogous pattern is shown by the reaction of ninhydrin (1% in acetone + trace of pyridine) with the glycopeptide and asparagine when spotted on paper (Fletcher et al, 1963 a). These two substances give a brown colour instead of the usual blue or purple given by most other amino acids and peptides.

This curious reaction of PTH-asparagine should also be borne in mind when using solvent systems containing pyridine for the separation of amino acids as their PTH derivatives.

INVESTIGATION OF THE CARBOHYDRATE-PROTEIN LINKAGE IN OVALBUMIN

INVESTIGATION OF THE CARBOHYDRATE-PROTEIN LINKAGE IN OVALBUMIN

INTRODUCTION

It would appear that the linkages between carbohydrate and protein in the glycoproteins are predominantly of two types, i) O-glycosidic linkages with serine and threonine and ii) N-glycosidic linkages with aspartic acid. There is also a possibility that in some glycoproteins there are glycosidic ester linkages with the ω-carboxyl groups of aspartic and glutamic acids. Recently Butler and Cunningham (1965), have isolated a glycopeptide from collagen that contains hydroxylysine, arginine, histidine, glycine, glutamic acid, lysine and methionine none of which are generally implicated in carbohydrate-protein linkages. One possibility would seem to be a linkage with the δ-hydroxy group of hydroxylysine but there is, as yet, no evidence to support this. Another possibility is an N-glycosidic link with the imidazole group of histidine, but again there is no experimental evidence.

It is with the N-glycosidic linkage to aspartic acid that the present study has been concerned but a brief survey of the state of knowledge of both types will put the problem into perspective.

Establishment of the O-glycosidic nature of the carbohydrate-protein bond in some glycoproteins comes mainly from work on ovine submaxillary gland (OSM) and bovine submaxillary gland (BSM) glycoproteins. However the first evidence was presented by Tsugita and Akabori (1959) who prepared a glycopeptide from an β-amylase of Aspergillus oryzae, which was non-reducing and contained serine as the only amino acid.

In the case of OSM, Gottschalk and Graham (1959) had shown that the carbohydrate was present as a large number of disaccharide units of the structure sialyl-(2→6)-N-acetylglucosamine. Pigman (1963) and Hashimoto, Tsuiki, Nisisawa and Pigman (1963) prepared a glycopeptide from this source containing serine and threonine in amounts of 0.56 and 0.58 moles respectively per mole of heterosaccharide unit and only traces of aspartic and glutamic acids.

By similar methods Blix (1963) has prepared glycopeptides from OSM in which at least some of the carbohydrate must be linked to serine and threonine although it is possible that some might be linked to glutamic acid. These conclusions were in contrast to the belief then held by Gottschalk and his colleagues (Graham, Murphy and Gottschalk, 1963) that in OSM the linkage was predominantly of the glycosidic ester type. In fact in later work (Bhavanandan, Buddecke, Carubelli and Gottschalk, 1964) Gottschalk has established beyond doubt that at least half of the linkages are O-glycosidic involving serine of threonine. The disagreement on the nature of this linkage arose because the alkaline lability of the bond was interpreted as an indication of its being an ester. However the β-elimination reaction characteristic of serine and threonine that have substituents abolishing the charge on the amino and carboxyl groups adequately explains the ease with which a substituent on the hydroxyl group is removed by mildly alkaline conditions. The result of this reaction on a serine or threonine residue in peptide linkage and involved in a glycosidic bond through its hydroxyl group would be the formation of the unsaturated α-amino-acrylic and α-aminocrotonic acids respectively.

Bhavanandan et al (1964) have followed this reaction by measuring the loss of serine and threonine residues and concomitant release of N-acetylglucosamine, under alkaline conditions.

Evidence for the ester type of linkage was also obtained from the products of reduction by lithium borohydride, a reagent which reduces esters to the corresponding alcohols but is claimed not to react significantly with amides except under severe conditions. However solubility problems necessitated conditions of greater severity than would have been desired. Identification of homoserine and δ-hydroxynorvaline was interpreted as evidence of ester linkages with the ω-carboxyl groups of aspartic and glutamic acids respectively.

The N-glycosidic or glycosylamine linkage was first proposed by Johansen, Marshall and Neuberger (1961), although Neuberger (1938) many years earlier suspected that the unidentified nitrogenous component he detected in ovalbumin was somehow involved. The basis of this belief was the demonstration of the release of one mole of ammonia per mole of glycopeptide on mild acid hydrolysis. Working independantly Nuenke and Cunningham (1961) and later Lee and Montgomery (1962) and Yamashina and Makino (1962) confirmed these findings but the latter group proposed an iminoether type of linkage in which the oxygen of the amide group of asparagine is directly linked to the reducing carbon of a sugar residue.

Using a fragment from the ovalbumin glycopeptide containing only N-acetylglucosamine, aspartic acid and "excessive nitrogen" which was isolated by partial acid hydrolysis (2 $\underline{N}$ H_2SO_4 at 100° for 20 min)

Bogdanov, Kaverzneva and Andrejeva (1962) also came to the conclusion that the most probable structure was a N-glycosidic linkage.

Proof of this structure was brought nearer when Marks, Marshall and Neuberger (1963) synthesised 2-acetamido-1-(L-β-aspartamido)-1, 2-dideoxy β-D-glucose, the model compound of the proposed linkage. Comparison of a partial acid hydrolysate of this compound with a similar partial acid hydrolysate of the glycopeptide (Fletcher, Marshall and Neuberger, 1963,b) by paper electrophoresis produce a "fingerprint" giving convincing evidence in favour of this type of bond. Furthermore, similar treatment of 2-(L-β-aspartamido)-2-deoxy-D-glucose, the model compound of the structure proposed by Rosevear and Smith (1958) gave an entirely difference "fingerprint" from the glycopeptide.

A linkage involving aspartic acid in glycopeptides from human γ-globulin was also indicated by the work of Rosevear and Smith (1958) who suggested that the bond was either that of an α-amide or an ester. Later Rothfus (1961) working in the same laboratory with the same protein suggested that the linkage was between the β-carboxyl group of aspartic acid and the amino group of glucosamine. Nolan and Smith (1962 a and b) proposed a similar linkage in rabbit γ-globulin and a γ-globulin from bovine colostrum.

Evidence for the linkage involving the β-carboxyl group of aspartic acid was based upon its resistance to papain and leucineaminopeptidase (Rosevear and Smith, 1961). A similar structure linking aspartic acid to the amino group of glucosamine has been proposed for the glycopeptides from a number of glycoproteins by Micheel, Ostman and Pielmeir (1963).

Winzler and Inoyue (1961) described the preparation of a glycopeptide from α_1-acid glycoprotein in which they proposed a linkage between the amino group of glucosamine and the γ-carboxyl group of glutamic acid.

Further studies by a number of groups working independently (Yamashina, Pan-I and Makino, (1963; Tsukamoto, Yamamoto and Miyashita, 1964 and Bogdanov, Kaverzneva and Andreyeva, 1964, and Marshall and Neuberger, 1964) have achieved the crystallisation of the N-acetyl-glucosamine-aspartic acid fragment enabling more precise comparisons to be made with the synthetic model compound (AADG). Both compounds have similar melting points, they have the same optical properties ($[\alpha]_D$ = + 18.6 - 24^0 No mutarotation), they were both non-reducing and both yielded one mole of ammonia on acid hydrolysis. Elementary analysis and infrared spectra were also in agreement although the latter investigation did not distinguish the proposed structure from 2-L-β-aspartamido-2-deoxy-D-glucose. The results of chromatography and electrophoresis under a variety of conditions showed the two materials to be indistinguishable. It seems therefore that the nature of the linkage in ovalbumin is well established. The only possibility that has not been investigated is the situation in which the acetamido and the aspartamido groups are transposed, however, by analogy with other naturally occurring products this does not seem to be a likely structure. The imino-ether and aspartyl-glucosamine structures are definitely excluded by these experiments.

Although the evidence in favour of the N-acylglycosylamine type

of linkage is overwhelming there has as yet been no direct chemical demonstration that unequivocally locates the nitrogen atom of the readily released ammonia. Thus, there has been no identification of a glycosylamine or asparagine in any of the degradation products however they were prepared.

A means of stabilising one or other of the bonds by which this molecule of ammonia is held in the compound is needed so that subsequent hydrolysis would yield a compound to which the nitrogen was still bound. If the proposed structure is assumed to be correct then the bond concerned is essentially a substituted amide and stabilization would be achieved if it were reduced to a secondary amine. Experiments described in the following pages were designed to obtain a modified glycopeptide of this form by reduction with lithium aluminium hydride.

REDUCTIONS WITH COMPLEX METAL HYDRIDES

The history of the use of this group of compounds for the reduction of organic substances begins with the paper of Finholt, Bond and Schlesinger in 1947 in which they described the preparation of lithium aluminium hydride (LAH) and gave details of a number of its chemical and physical properties. A considerable number of other complex metal hydrides have been synthesised of which those finding most common use in organic chemistry have been lithium borohydride and sodium borohydride (Schlesinger et al, 1953 and Schlesinger and Brown, 1940). The reducing power of these three agents differs considerably, LAH being quite clearly the most powerful and sodium borohydride the least powerful.

Table 21 shows some of the groups that are reduced by each of the reagents.

Table 21

Functional groups reduced by lithium aluminium hydride

Functional group	Product	Moles LAH required
Aldehyde	Primary alcohol	0.25
Ketone	Secondary alcohol	0.25
Ester	Primary alcohol	0.5
Carboxylic acid	Primary alcohol	0.75
Amide $-CONH_2$	Primary amine	1.0
Amide -CONHR	Secondary amine	0.75
Amide $-CONR_2$	Tertiary amine	0.5
	Aldehyde	0.25

from Brown (1962)

Since the primary aim of the present experiments is to reduce an amide bond it is clear that only LAH will suffice.

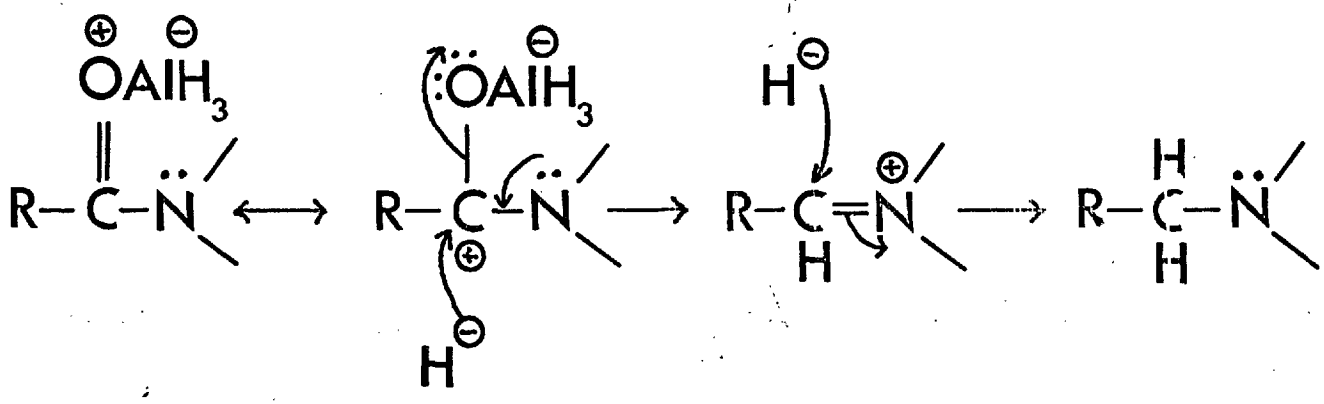
The lesser reducing power of lithium borohydride has been shown to be of limited use in the determination of the C-terminal amino acid in peptides and proteins by converting the free carboxyl group, after esterification, to an alcoholic group and then isolating the resulting amino alcohol, (Chibnall and Rees, 1950). However some workers have shown that lithium borohydride is not entirely specific and that some of the peptide bonds are cleaved and others are reduced to secondary amines. This observation has been confirmed in the present studies during the synthesis of 2,4-diaminobutane-1-ol.

Mechanism of the reduction of amides to amines by LAH.

It has been proposed (Micovic and Mihailovic, 1953; Mousseron, Jacquier, Mousseron-Canet and Zagdoun, 1952) that the reaction involves the attack of a positive AlH_2^+ ion on two molecules of the amide, yielding a complex aluminium ion on which hydrolysis forms the corresponding amine. However, in certain circumstances, as for instance when there is steric hindrance, the action of LAH upon amides gives aldehydes or alcohols and the amine component, and these reactions would seem to demand a different mechanism.

To overcome this difficulty Gaylord (1954 and 1956) has suggested that the attack is by the aluminohydride ion AlH_4^- in both cases, so explaining the formation of three different products by a single mechanism. The aluminohydride ion is believed to exist in ether solution in equilibrium with aluminium hydride and the hydride ion (Paddock, 1951), and it is postulated that the latter is involved in an attack upon the carbonyl carbon atom as shown in fig. 25

Fig. 25



Mechanism of reduction of amides by lithium aluminium hydride

from Gaylord (1956)

leading to cleavage of the carbon-oxygen bond and formation of the amine.

It is pertinent to consider the action of LAH on N-formylamines since it probably helps to explain the reduction products of N-benzyloxycarbonyl derivatives which are to be discussed later. N-formyl derivatives have been prepared by Blicke and Chi-Jung Lu (1953) by the action of chloral on a number of amines and they reduced them with LAH to give N-methyl compounds according to the reaction:-

$$R - NH - C{:}O.H \xrightarrow{LAH} R - NH.CH_3$$

The reductions were accomplished by refluxing for 6 hr in ether with equimolar quantities of LAH.

In the case of N-acetyl compounds it has been shown that an analogous reaction takes place, an example of which is the reduction of N-acetyl tryptamine (Eiter and Svierak, 1952) to N-ethyl tryptamine by the use of LAH in ether.

Expected products of the reduction of the glycopeptide with LAH.

A knowledge of the reducing properties of LAH as extensively discussed by Gaylord (1956) suggest that probable products of the reduction of the glycopeptide would be an N-ethylglucosamine residue from each of the N-acetylglucosamine residues and 2,4-diaminobutane-1-ol from the asparagine residue. The five mannose residues are all in glycosidic linkage and so would not be expected to undergo any alteration.

For the purpose of the present thesis only 2,4-diaminobutane-1-ol

considered since the identification of this substance would be sufficient to indicate the N-glycosidic nature of the linkage.

A thorough search of the literature has failed to uncover any reference to this compound. It was therefore necessary to devise a method for its synthesis.

The method chosen was the reduction by LAH of N-benzyloxycarbonyl asparagine. It was hoped that the N-benzyloxycarbonyl group would be cleaved from the asparagine, as it is by catalytic hydrogenation, the carboxyl group would be reduced to a primary alcohol and the amide group would be reduced to a primary amine. The product should be readily identifiable by electrophoresis at a pH at which both amino groups are charged. It was proposed to carry out the reduction in tetrahydrofuran, the benzyloxycarbonyl group making the asparagine sufficiently soluble to permit the reaction to proceed at a reasonable rate.

It has been shown by Nystrom and Brown (1947) that the LAH reduction of carboxylic acids proceeds without difficulty according to the reaction:-

$$4\ R\text{-}COOH + 5\ LiAlH_4 \longrightarrow (R\text{-}CH_2OH)_4LiAl + 2\ LiAlO_2 + 4\ H_2$$

The amino acids have also been shown to give the corresponding amino alcohols without difficulty but with the notable exception of glycine which fails to give ethanolamine as expected (Vogl and Pohm, 1952) when using 5% LAH in tetrahydrofuran.

An interesting reaction occurs in the case of succinamic acid and

some of its derivatives. The acid itself yields 4-amino-1-butanol (Barry and Twomey, 1951) but the reduction of 2-dodecylsuccinamic acid is accompanied by ring closure to yield 3-dodecylpyrrolidine, and a similar reaction occurs with 2-dodecyl-3-methylsuccinamic acid (Barry et al, 1950). Similarly Karrer and Portmann (1948) have shown that LAH reduction of diethylglutamate leads to the expected product 2-amino-1,5-pentanediol but also gives by a side reaction 2-hydroxymethylpyrrolidine. However acetylation of the amino group and reduction with lithium borohydride (Grassman, Hormann and Endres, 1955) gives the straight chain product in high yield on subsequent removal of the acetyl group.

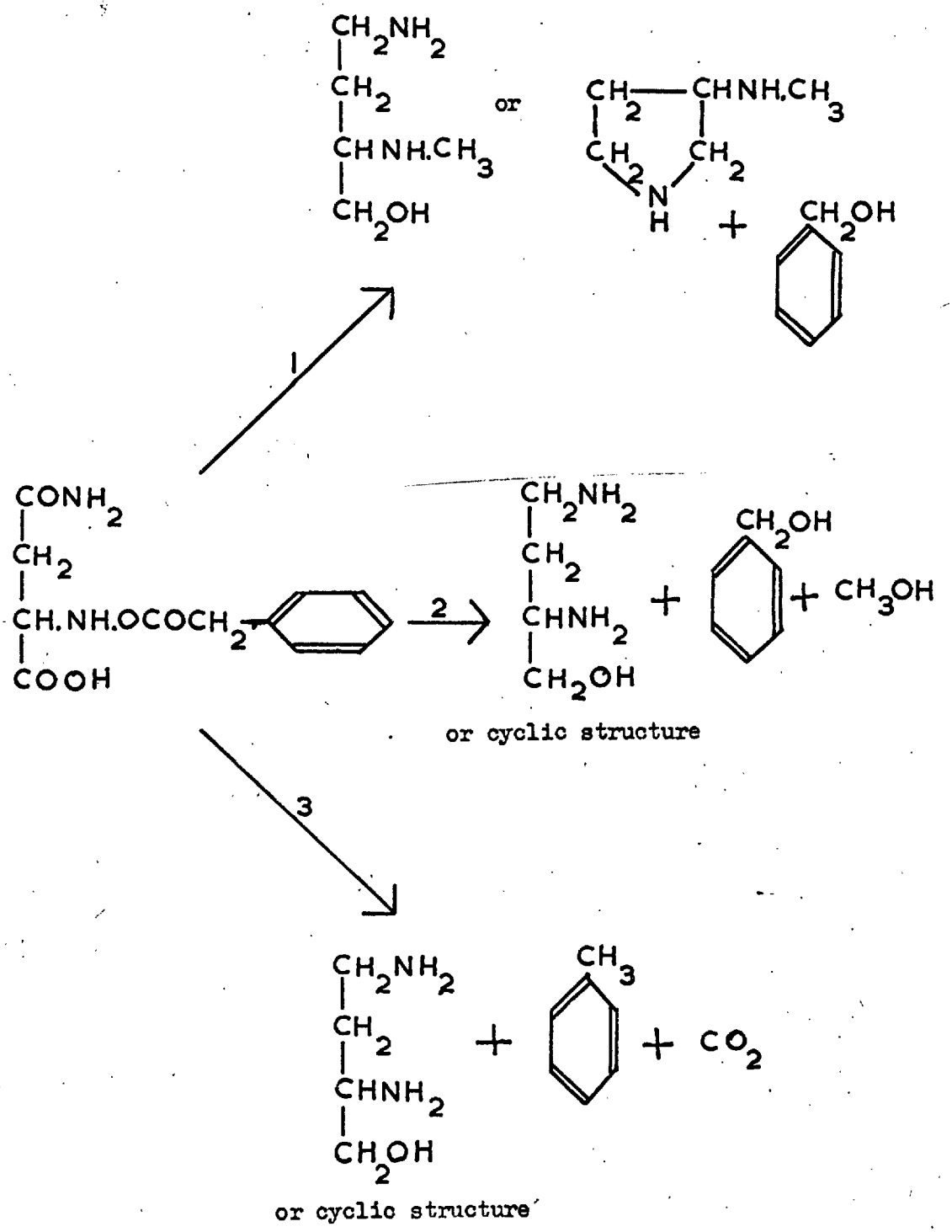
It was therefore necessary to consider the possibility that a similar cyclisation might occur in the preparation of 2,4-diamino-butane-1-ol.

Moreover the reaction of LAH on benzyloxycaronyl groups was not fully appreciated at the beginning of this investigation but it was soon apparent that the reduction products were different from those obtained by catalytic hydrogenation. Fig. 26 shows a number of theoretically possible pathways and their respective products.

Most commonly the products of reduction of carbamates by LAH are N-methylamines, thus the reduction of ethyl-N-phenyl carbamate gives N-methylaniline (Gaylord, 1955) and the reduction of N-carbomethoxysarcosine gives dimethylaminoethanol (Wessely and Swoboda, 1951). In a study of the reduction of the benzyloxycarbonyl group in various peptides and amino acids Karrer and Nicolaus (1952) have reported the preparation of N-methyl derivatives.

Fig. 26

The reduction of N-benzyloxycarbonyl asparagine by lithium aluminium hydride



Thus the reduction of N-benzyloxycarbonylglycine ethyl ester yields benzyl alcohol and N-methylethanolamine and the reduction of N-benzyloxycarbonylglycyl-L-lucine ethyl ester gave the corresponding fully reduced derivative.

However a different type of reaction has been reported by Jolles and Fromageot (1951) who have reduced the α-benzyl ester of ethyl carbobenzoxy-DL-aspartyl-β-glycinate and found the products to be the dipeptide diol, benzyl alcohol, ethanol, toluene and carbon dioxide.

It is proposed to discuss the experimental procedures and results involved in the reduction of N-benzyloxycarbonyl asparagine at this stage and to continue the discussion later.

EXPERIMENTAL

Preparation of N-benzyloxycarbonyl asparagine.

A modification of the method of Bergman and Zervas (1932) was used, the preparation being repeated several times with minor variations.

L-asparagine (6.65 gm; 0.05 moles) obtained from BDH was dissolved in distilled water (125 ml) with gentle warming. Whilst still warm sodium bicarbonate (10.5 gm; 0.125 moles) was added.

The mixture was shaken with a mechanical shaker and benzyloxycarbonyl chloride (9.5 ml; 0.055 moles) was added in four approximately equal portions over a period of 30 min. Shaking was continued for 2 hr. The reaction mixture was extracted twice with 10-15 ml of ether and the clear aqeous solution so obtained was acidified to pH 1.6 by the dropwise addition of concentrated HCl. A thick white precipitate formed which was collected by filtration and allowed to dry in air for 12 - 24 hr. The product had a melting point of 158°.

recrystallisation was achieved from hot methanol. M.P.162-165° (uncorr.); Bergman and Zervas give 165°(corr.) Yield 8.26 gm; 62%

Reduction of N-benzyloxycarbonyl asparagine by lithium aluminium hydride (LAH).

LAH (6.1 gm) was added slowly over 20-30 min to tetrahydrofuran (150 ml). The tetrahydrofuran (THF) was prepared by shaking 1 litre with stannous chloride (10.0 gm) and leaving to stand at room temperature for 2 hr. After this period the potassium iodide test for peroxides was negative. The THF was then distilled, the fraction boiling between 65 and 66° being collected. The distillate was left standing over sodium hydroxide flakes for 24-48 hr. The distillation was repeated and the distillate refluxed over sodium wire for 6 hr. The THF was redistilled and stored over sodium wire in a dark bottle.

During the addition of the LAH to the THF a considerable amount of heat was evolved so a reflux condenser was fitted to avoid excessive evaporation.

When the mixture had cooled N-benzyloxycarbonyl asparagine (7.2 gm) was added in small portions over 20-30 min. A vigorous reaction started with the evolution of much heat. The mixture was refluxed for approximately 18 hr.

Excess LAH was destroyed by the cautious addition of 50:50 THF-water until all reaction had ceased with the formation of a bulky white precipitate. The mixture was filtered and the precipitate was extracted by refluxing with 50 ml of THF for 30 min. The filtrates were combined and evaporated to dryness at 50° yielding 5-6 ml of a pale yellow oil.

Distillation under high vacuum gave a clear colourless oil that could be collected in two fractions, the first distilling at 65° and the second at a temperature above 76°. In most experiments the distillate was collected as a single fraction.

Preparation of 2-aminobutane-1,4-Diol.

This was achieved by the LAH reduction of the diethyl ester of aspartic acid and the product was characterised as the crystalline oxalate according to the method of Karrer, Portman and Suter (1948).

Preparation of the diethyl ester of L-aspartic acid.

The method used was a modification of that of Neuman and Smith (1951). L-aspartic acid (5.0 gm) was suspended in ethanol (60 ml) and dry hydrochloric acid passed for 4 hr. The reaction mixture was left standing at room temperature for 1 hr and was then evaporated to dryness. The residue was redissolved in ethanol and the solution again evaporated to dryness. This was repeated several times until no HCl remained. The white crystalline product was recrystallised from methanol-ether in the proportions 20:80 to give a substance with M.P. 109-110° (Neuman and Smith give 109-110°). Yield 96%. The preparation was repeated as above.

Reduction of diethyl ester of L-aspartic acid.

Aspartic acid diethyl ester (7.5g) was partially dissolved in 125 ml of dry ether and left a room temperature for 18 hr. Excess LAH was destroyed by the cautious dropwise addition of 12.0 ml of water and the insoluble residue was removed by filtration. The filtrate was evaporated to dryness and the oily residue dissolved in 20.0 ml of methanol. A weak solution of oxalic acid in methanol was added to precipitate lithium oxalate which was removed by filtration. The filtrate was evaporated to

dryness to give an oily residue which was distilled under high vacuum. A clear, pale yellow oil was collected between 120-126°.

The oil (382.4 mg) was dissolved in ethanol (2.0 ml) and oxalic acid (230 mg) was added. A gummy, insoluble whitish substance was produced which became crystalline on standing for 3-4 hr at 4°. Recrystallisation was achieved from hot methanol to give a substance with M.P. 105°

Purification and characterisation of the reduction products of asparagine.

Electrophoresis

Electrophoresis was performed in formic acetic buffer (formic acid 70 ml; acetic acid 15 ml made up to 2,500 ml with water; pH 1.9) on Whatman's 3MM paper for 0.75 hr at 1000 volts. L-asparagine and 2-amino butane-1,4-Diol were run as markers. Staining by the ninhydrin method (1% ninhydrin in acetone containing traces of pyridine) indicated three positive spots. The major one of these stained purple and had a mobility corresponding to two positive charges relative to 2-aminobutane-1,4-Diol. Of the minor components one had slightly greater mobility than the major and stained grey-brown and the other had a mobility corresponding with a single positive charge and stained purple. There was also a fourth very minor component, staining blue and running with slightly greater motility than asparagine. Fig. 27

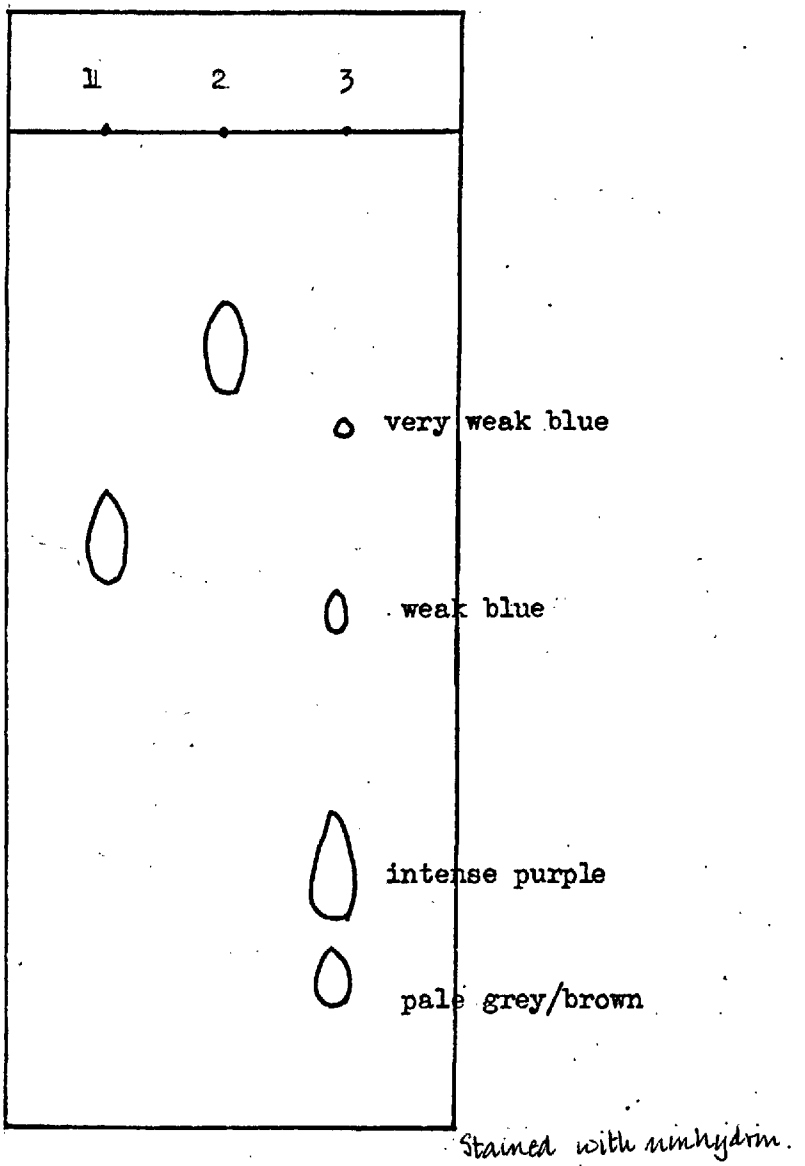
Ultraviolet spectrum.

The ultraviolet spectrum of each of the distillation fractions was recorded with the Unicam SP 700. Both fractions showed the presence of the benzene ring, the first containing relatively more than the second. Fig. 28

Chromatography on Dowex-50.

A column 2.5 x 55 cm of Dowex-50 x 8 200-400 in H^+ form was set up

Fig. 27



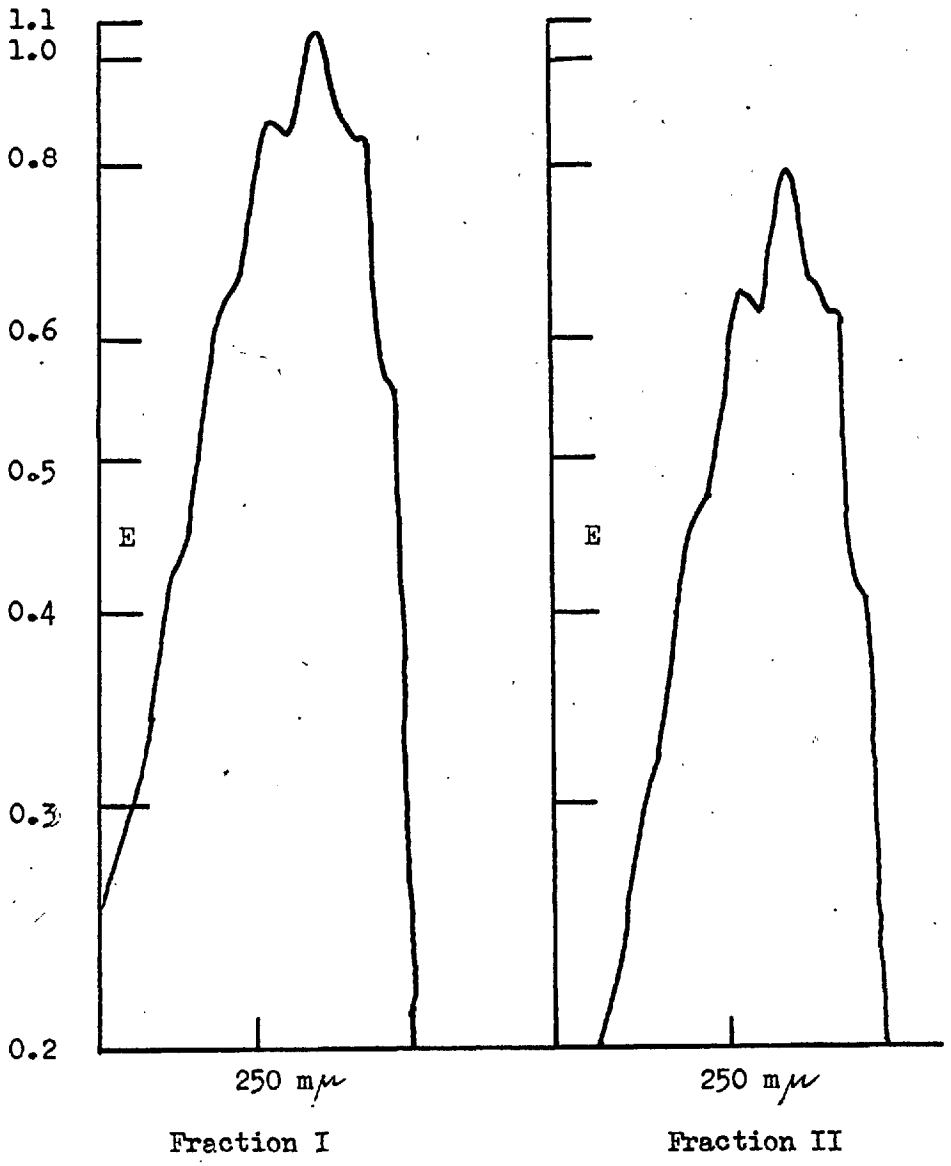
1. Aminobutanediol

2. Asparagine

3. Reduction products of cbzo-asparagine

Eleotrophoresis of reduction products of N-benzyloxycarbonyl asparagine in formic - acetic buffer pH 1.9

Fig. 28



Ultraviolet spectrum of distillation fractions of reduction products of N-benzyloxycarbonyl asparagine

and 5 ml of distillate in 5 ml of water allowed to run in. Elution was started with distilled water, 10 ml fractions being collected until 700 ml had run through. Elution was then changed to an HCl gradient being obtained by setting up two interconnecting bottles, one of which contained 1 litre of 1 N HCl and the other 1 litre of 3 N HCl, the bottle containing the weaker solution being connected to the column. The gradient was followed by elution with 3 N HCl.

Portions from alternate tubes 4-68 were assayed by taking readings at 262 mμ, the wave length at which the benzene ring would be expected to absorb. The effluent from the HCl gradient was assayed first by spot testing alternate tubes on filter paper with the ninhydrin reagent and secondly by taking portions from the relevant tubes and estimating them by the ninhydrin method (Moore and Stein, 1948) as described on page 25

The elution curve showed a small peak between tubes 130 and 146 and a large peak between tubes 190 and 216. Elution with 3 N HCl showed a third peak between tubes 246 and 251 giving a brown colour with ninhydrin. The three peaks were named FI, II and III respectively and electrophoresis showed that FI corresponded to the component possessing one positive charge, FII corresponded to the component reacting blue with ninhydrin and possessing two positive charges and FIII corresponded to the component possessing two positive charges and reacting grey-brown with ninhydrin. Fig. 29. Table 22

Characterisation of the fractions eluted from Dowex-50.

i) Oxidation with permanganate

The fractions eluted with water from Dowex-50 were pooled and extracted three times with 200 ml of ether. The ether extract was taken to

Table 22

Fractionation of reduction products of benzyl-oxycarbonyl asparagine on Dowex-50

Tube number	$E_{262\ m\mu}$	Tube number	$E_{262\ m\mu}$
4	0.208	38	7.12
6	0.170	40	26.2
8	0.230	42	25.68
10	0.148	44	23.68
12	0.134	46	18.96
14	0.132	48	11.52
16	0.128	50	12.20
18	0.112	52	10.30
20	0.234	54	7.00
22	0.244	56	6.60
24	0.240	58	5.46
26	0.234	60	4.60
28	0.226	62	3.60
30	0.212	64	3.0
32	0.208	66	2.4
34	0.194	68	2.2
36	0.226		
	$E_{570\ m\mu}$		$E_{570\ m\mu}$
130	0.101	198	5.16
132	0.114	200	3.84
134	0.149	202	4.34
136	0.179	204	2.39
138	0.117	206	2.04
140	0.054	208	1.52
142	0.045	210	0.90
144	0.039	212	0.54
190	0.11	214	0.54
192	0.11	216	0
194	3.46	218	0
196	4.88		

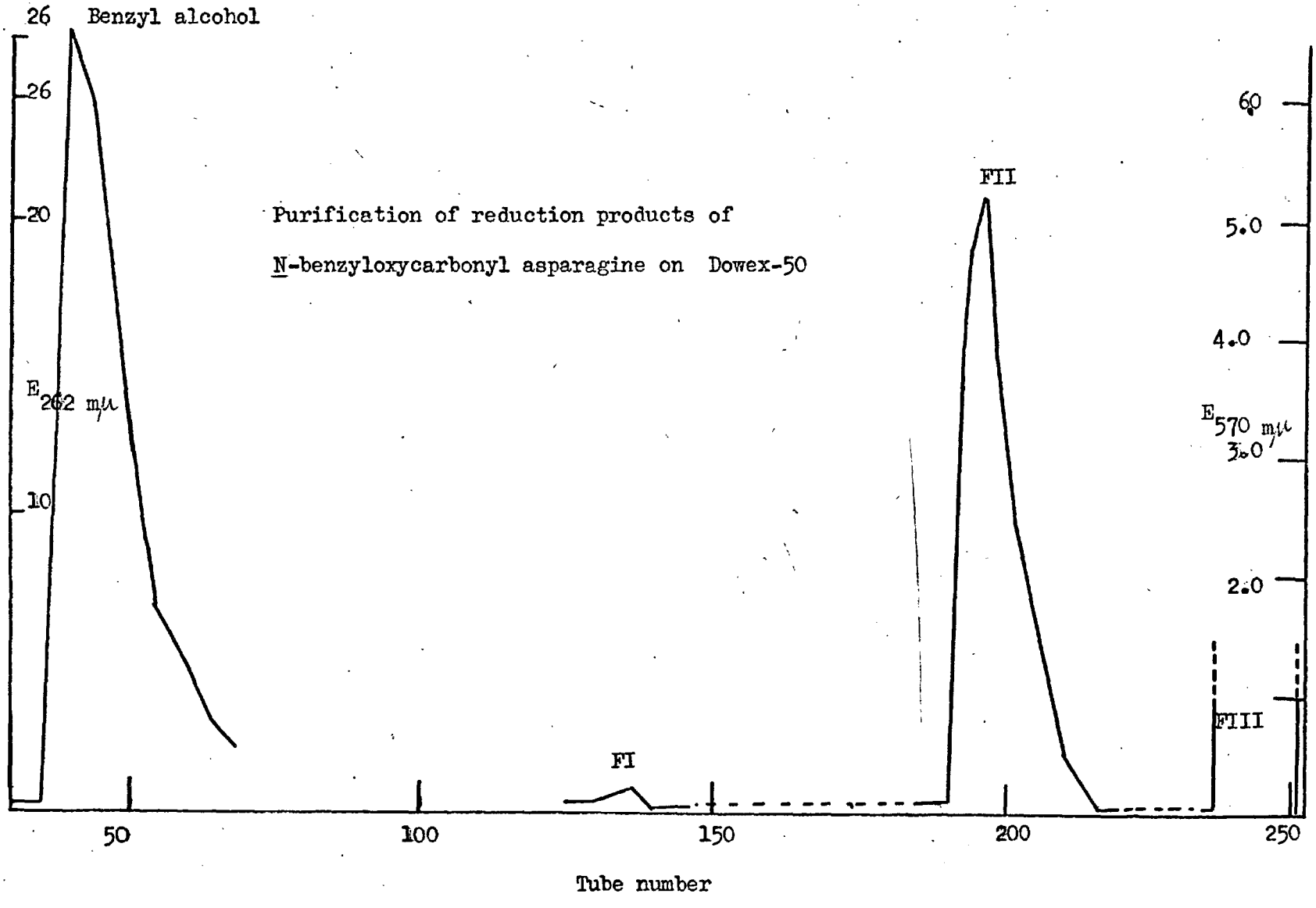

26
Benzyl alcohol
26
20
10
$E_{262\ m\mu}$
Purification of reduction products of
N-benzyloxycarbonyl asparagine on Dowex-50
FII
FI
FIII
60
5.0
4.0
3.0
2.0
$E_{570\ m\mu}$
50
100
150
200
250
Tube number

Fig. 29

dryness on a rotary evaporator to yield a colourless oil with a faintly aromatic odour. Oxidation of this oil was achieved by reaction with a hot solution of potassium permanganate. A white precipitate formed on acidification which was filtered and dried in air. This substance sublimed between 75° and 90° to form rectangular and rhomboidal crystals that melted sharply at 119° thus identifying it as benzoic acid.

ii) Reaction of fraction eluted with water with phenylisocyanate

The oily product (1 ml) obtained from the ether extract of the water eluant was mixed with phenylisocyanate (1.2 ml) and heated for 10 min in a boiling water bath after which it was extracted twice with 5 ml of 100/120 petroleum ether. Crystals formed on cooling which were recrystallised from 100/120 petroleum ether to form colourless needles with M.P. 77°; the benzyl derivative has M.P. 76° (Mc Elvain, 1953).

iii) Reaction with p-nitrobenzoyl chloride

The oily product (1.0 ml) was mixed with p-nitrobenzoyl chloride (0.5 gm) and pyridine (1.0 ml) and heated in a water bath at 100° for 30 min. The mixture was cooled and 10 ml of 5% sodium bicarbonate was added, stirring was continued whilst cooling in an ice bath until the product was crystalline. The substance was recrystallised twice from ethanol to give a product with M.P. 85° (McElvain gives 85° for benzyl derivative). Both the previous two methods were those of McElvain (1953).

It is concluded from the above results that one of the reduction products is benzyl alcohol.

Characterisation of the fractions eluted with HCl

The three components reacting with ninhydrin (FI, II and III) were isolated by pooling the relevant tubes and evaporating to dryness.

Attempts to crystallise as the hydrochloride were unsuccessful.

Formation of dinitrophenyl derivatives

The dinitrophenyl (DNP) derivatives were formed by reacting the relevant compound with 2,4-dinitrofluorobenzene in alkaline aqueous solution.

Only the derivatives of FII and FIII were formed. FII was prepared by distillation from aqueous alkaline solution as described on page

The distillate (approximately 50 ml) was shaken with dinitrofluorobenzene (0.4 ml) and sodium bicarbonate (0.5 gm) for 18 hr at room temperature. An intense yellow oil was formed. The aqueous supernatant was pipetted off and the residue shaken for 30 min with 25-30 ml of 1 $\underline{N}$ NaOH. A yellow solid material formed on the sides of the vessel, the supernatant was poured off and the residue extracted three times with dry ether. The substance was recrystallised from hot ethyl acetate to give a product decomposing at 160°.

FIII was obtained by evaporating to dryness the material in tubes 246-251 referred to above. The DNP derivative was formed by the same method that has been described for FII. The substance was recrystallised from hot ethanol to give a product with M.P. 184-185°.

The ultraviolet spectrum of FII in 50:50 ethanol-water showed two absorption maxima, one at 362 mμ and another at 404 mμ. The molar absorption of the first was 26, 900 and the second was 21,800, if the molecular weight was taken to be 435.

The ultraviolet spectrum of FIII in 50:50 ethanol-water showed a single

maximum at 386 mμ. The molar absorption was 25,800 if the molecular weight is taken to be 418. The spectra are shown in fig. 30.

Formation of the oxalates of FI, FII and FIII.

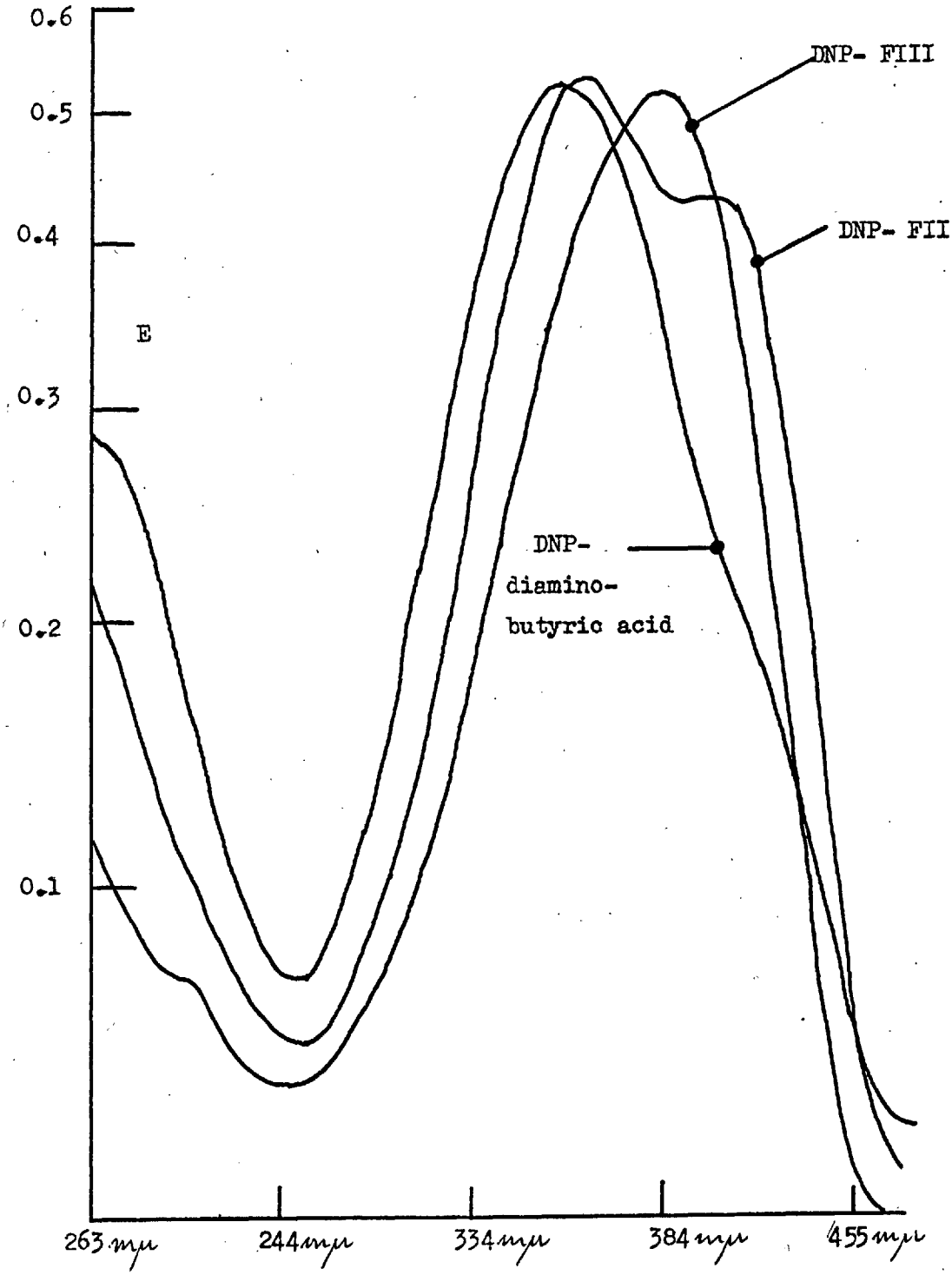
Dowex-1 was generated in the oxalate form by suspending the resin in saturated oxalic acid for 1 hr, decanting the supernatant and resuspending the resin in fresh saturated oxalic acid for a further hour. The resin was washed repeatedly with distilled water until excess oxalic acid had been removed and then poured as a slurry into a column 2.2x28 cm

FI was applied in aqueous solution and the column was eluted with water. Fractions of 2 ml were collected and a single drop from each tested with a drop of 10% calcium chloride solution. The tubes which produced a precipitate by this test were pooled and evaporated to dryness A gummy semi crystalline material was formed which was taken up in a small volume of ethanol and crystallisation achieved by the addition of dry ether and leaving at 4° overnight. The colourless feathery crystals melted at 148-150°. The infrared spectrum is shown in fig. 16)

A similar method was employed with FII but the final product proved difficult to crystallise. This was eventually achieved by dissolving the substance in a small volume of water, adding ethanol until a slight cloudiness appeared and leaving at 4°. Repetition of this process gave a pure white crystalline substance melting at 163° and having $[\alpha]_D$ = +7.9° (c 3.67 in water); elementary analysis, found C, 35.13; H, 7.87; N, 11.16; O, 45.39. The infrared spectrum is shown in fig. 2(b)

The oxalate of FIII was crystallised without difficulty from methanol-water to give a substance with M.P. 155-157° and having $[\alpha]_D$ = -8.35 (c 0.96 in water); elementary analysis, found C,37.91;H,5.95;

Fig. 30



Spectra of DNP- FII, DNP- FIII and DNP-diaminobutyric acid

N, 10.02; O, 46.12. The infrared spectrum is shown in fig 3(b)

Further observations on FI, II and III.

Distillation of an aqueous alkaline solution (after extraction with ether) of a mixture of FI, II and III yielded an alkaline distillate (pH 9.64) that gave a purple colour with ninhydrin when spot tested on filter paper. The substance responsible for the ninhydrin colour had the same electrophoretic mobility as FII, and its oxalate formed by the neutralisation of the distillate with oxalic acid and crystallised from methanol-water had M.P. 162°. The elementary analysis was closely similar to the oxalate of FII (found, C, 35.13; H, 7.87; N, 11.16; O, 45.39). It was concluded that the steam distillate substance was FII.

Oxidation of FII and FIII with potassium permanganate.

An aqueous solution of FII (as the hydrochloride) was titrated to pH 12.05 with N NaOH. Potassium permanganate (0.5 N) was added dropwise up to a total of about 6 ml. The potassium permanganate was decolourised and a dark brown precipitate of manganese dioxide was formed. The mixture was left standing overnight and then filtered.

FIII and a commercial sample of 2,4-diaminobutyric acid (L.Light) were treated similarly.

Electrophoresis in formic-acetic buffer (see page 103) of oxidised FII showed four spots reacting with ninhydrin, i) having the same mobility and colour as FIII, ii) having the same properties as FII, iii) having slightly less mobility that FII and staining a light blue colour and iv) a material with less mobility than iii) and having a purple colour with ninhydrin.

Oxidised 2,4-diaminobutyric acid gave spots corresponding with iii) and iv).

2,4-diaminobutyric acid before oxidation gave a single spot not corresponding with i), ii), iii) or iv).

Oxidised FIII gave a single spot staining purple and having slightly less mobility than the starting material but not corresponding with any of the spots mentioned above.

The patterns obtained are shown in fig. 31.

Titration of FII and FIII.

Electrometric titration of FII and FIII was performed using a lithium glass electrode and a Radiometer pH meter. Titration of both substances as the hydrochlorides or sulphate was done in water and in the case of FII also in formaldehyde.

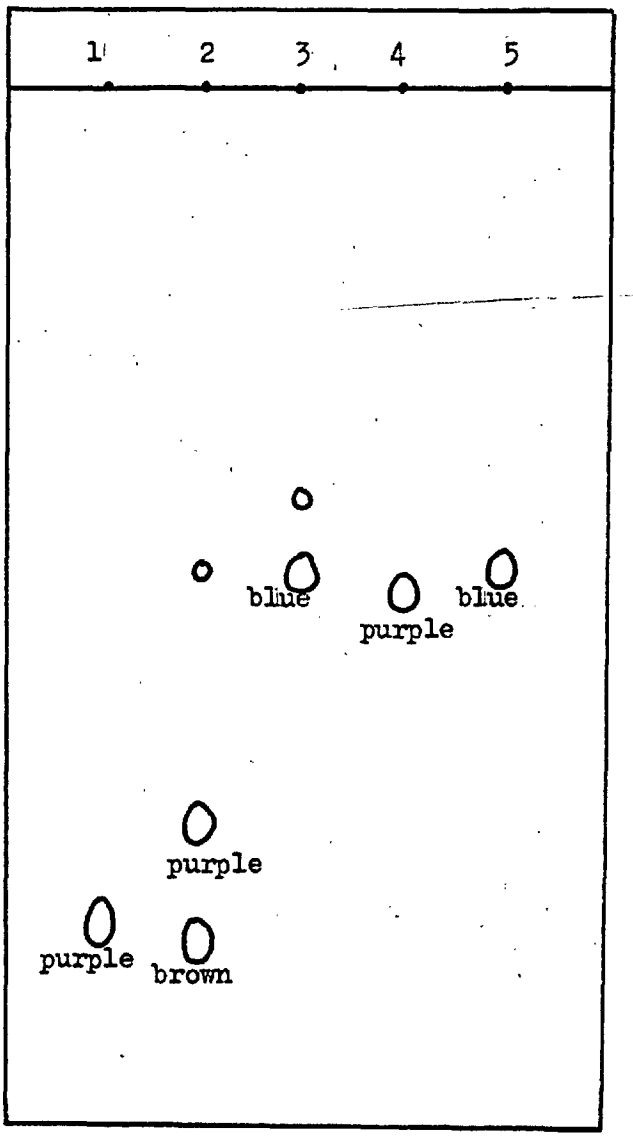
The titration of FII was repeated on several occasions, in one case an unpurified sample was used that was known to contain small amounts of FIII (as judged by the staining of electrophoretic strips). The material was prepared via the formation of the phosphotungstates of the crude products of reduction. The phosphotungstate was dissolved in 50% acetone in water and decomposed with barium hydroxide. The insoluble barium phosphotungstate was removed by filtration; the supernatant which contained the free diamine was neutralised with sulphuric acid and evaporated to dryness.

The diamine sulphate (7.12 mg) was dissolved in water and titrated with 0.1 N NaOH and HCl. If it is assumed that the substance is present as the *mono* sulphate then 7.12 mg is equal to about 34.4 μmoles.

Using the same material formaldehyde was added in slight excess

Fig. 31

Electrophoresis of oxidation products of FII and FIII in formic/acetic buffer pH 1.9



1. Oxidised FIII
2. FI, FII and FIII
3. Oxidised FII
4. Diaminobutyric acid
5. Oxidised diamino butyric acid

of two moles per mole of diamine, that is, one mole of formaldehyde per mole of amino group.

In another case a sample of pure FII of unknown quantity was obtained by distillation from alkaline aqueous solution and was titrated with $\underline{\underline{N}}$ HCl and $\underline{\underline{N}}$ NaOH.

The curve of the latter titration is shown in fig.32

FIII (approximately 50 mg) prepared by purification on a Dowex-50 column was titrated with 1 $\underline{\underline{N}}$ HCl and 2 $\underline{\underline{N}}$ NaOH.

The titration curve is shown in fig. 33

The reaction of FII and FIII with acid and alkali.

Four experiments were performed.

i) FII was refluxed for 2 hr in 2$\underline{\underline{N}}$ HCl.

ii) FII was refluxed for 2 hr in 2 $\underline{\underline{N}}$ NaOH.

iii) FIII was refluxed for 2 hr in 2 $\underline{\underline{N}}$ HCl.

iv) FIII was refluxed for 2 hr in 2 $\underline{\underline{N}}$ NaOH.

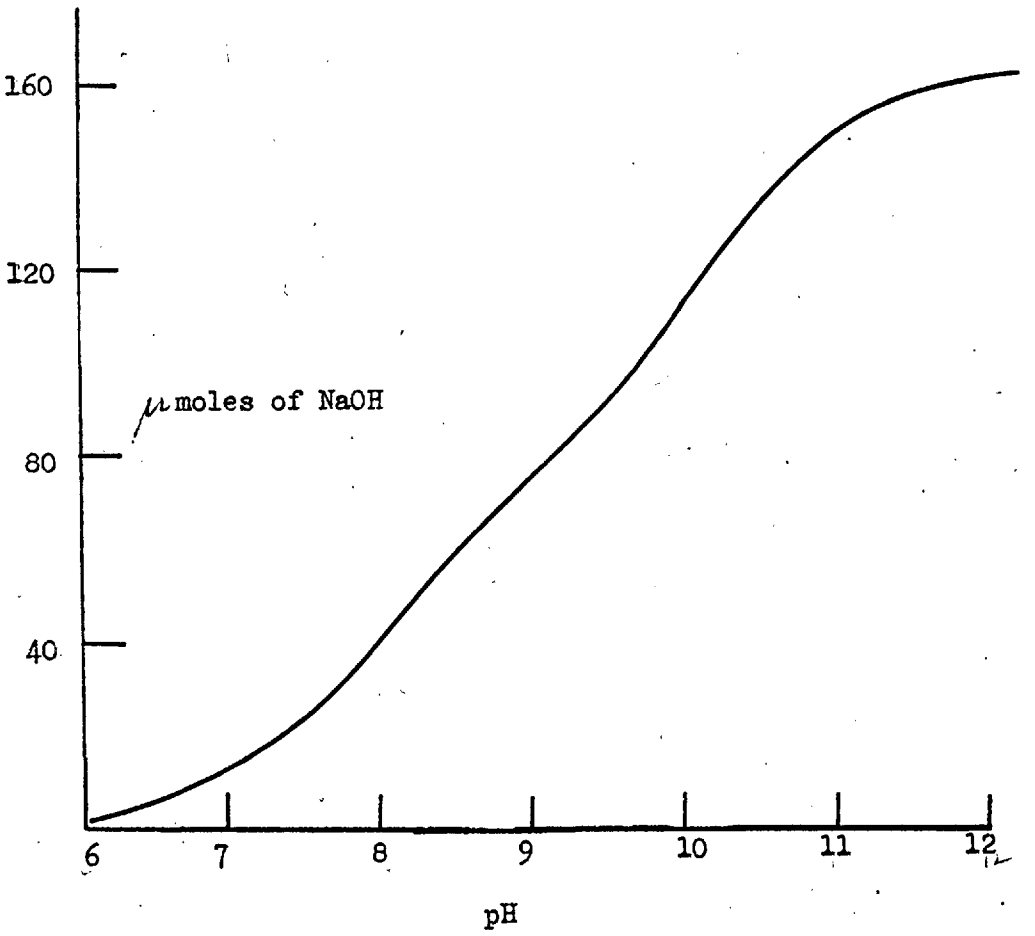
When refluxing was complete the reaction mixture was cooled, neutralised and examined by electrophoresis in the formic/acetic buffer described previously. The patterns obtained are shown in fig. 34

The nitrogen content of FII hydrochloride.

FII HCl (10.045 gm) obtained by purification on Dowex-50 was weighed on a microbalance and dried for 2 hr at 60° in vacuo. Reweighing showed a water content of 5.4%.

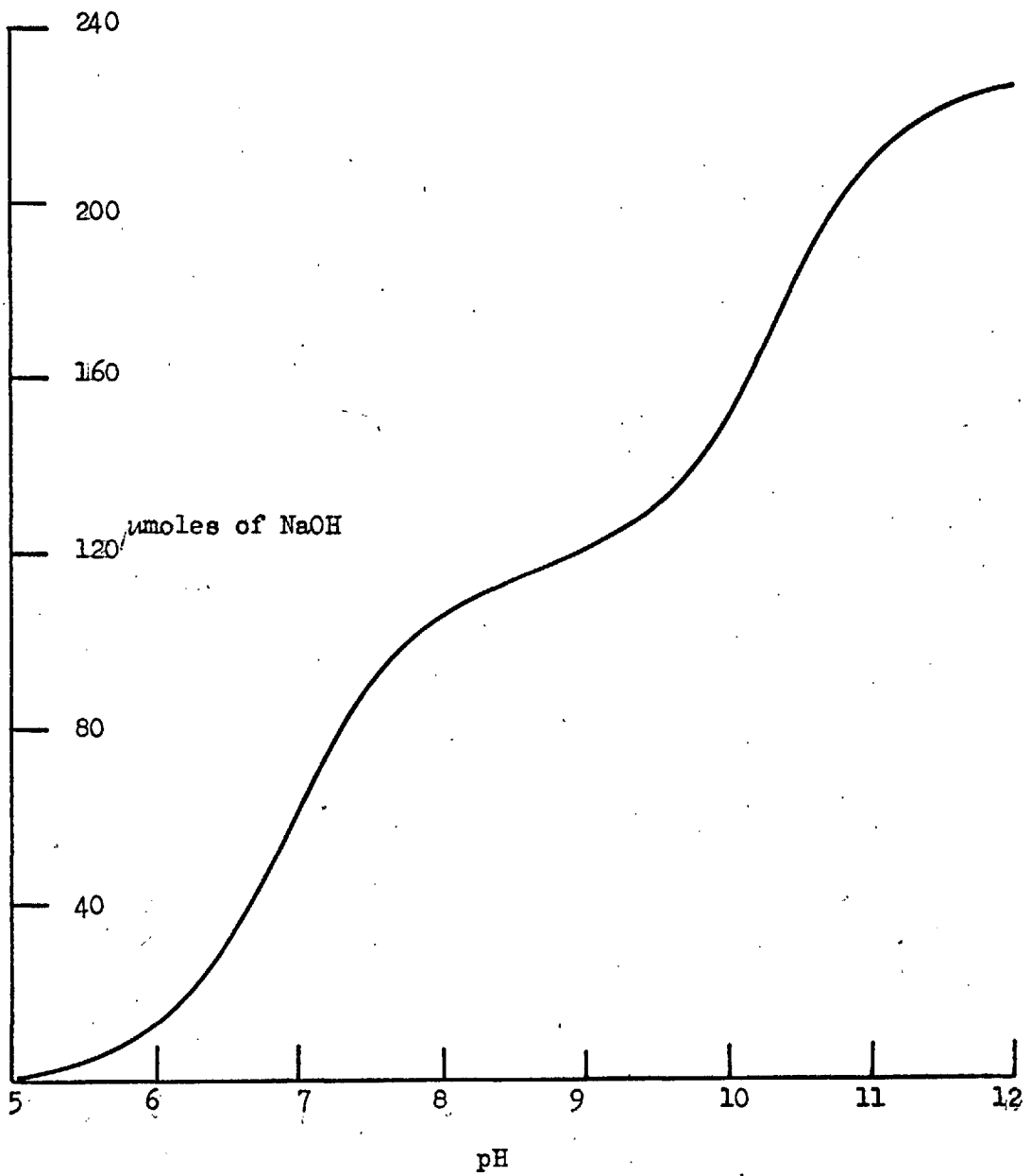
For nitrogen estimation FII HCl (6.42 mg) was dissolved in water and made up to 2.0 ml. A micro-Kjeldhal method was used, the sulphuric acid digest being distilled from sodium hydroxide into 4% boric acid containing bromocresol green and methyl red as indicators. The ammonia

Fig. 32



Titration curve of FII

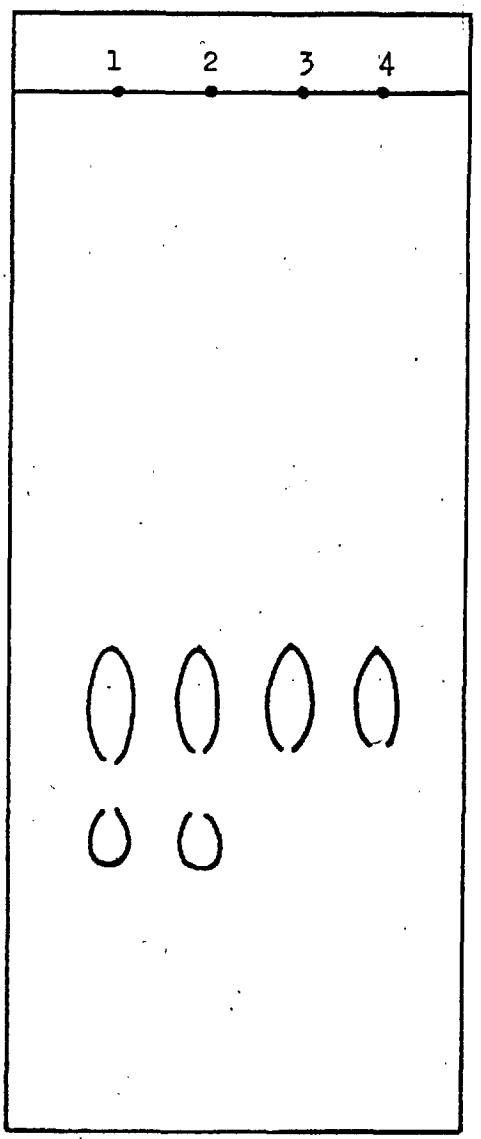
Fig. 33



Titration curve of FIII

Fig. 34

Behaviour of FII and FIII with acid and alkali. Electrophoresis in formic/acetic buffer pH 1.9



1. FIII after acid treatment
2. FIII after alkaline treatment
3. FII after acid treatment
4. FII after acid treatment

so released was titrated with N/70 sulphuric acid (1.0 ml = 0.2 mg N). 4-amino-2 methylaminobutane-1-ol dihydrochloride requires 14.7% N.

Ninhydrin estimation of FII HCl.

Ninhydrin estimations were performed as described on page using a dilution of the same solution that was used for the nitrogen estimations. The mean of four estimations was used and a colour value calculated from a glycine standard curve. The values obtained and the standard curve are shown in fig. 36

RESULTS AND DISCUSSION

Material eluted with water.

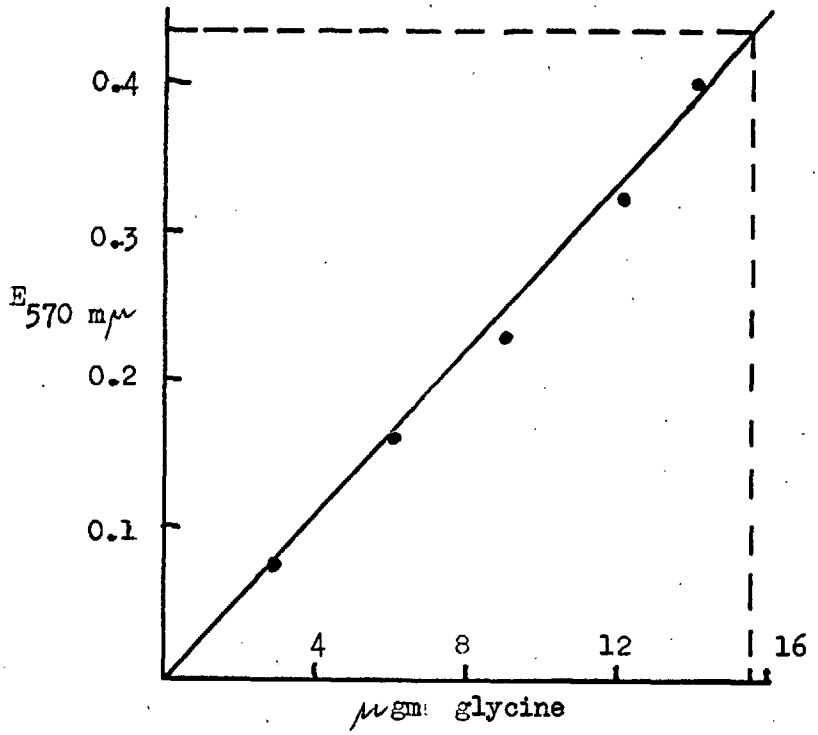
In the experimental section it has already been stated that one of the reduction products is benzyl alcohol, identified by the isolation of benzoic acid after oxidation by permanganate. This conclusion is further confirmed by the preparation of benzyl p-nitrobenzoate and benzyl-N-phenylcarbamate from the eluate.

From this it is clear that the course of the reaction proceeds largely by either pathway 1 or 2, (fig.26). In one experiment curve reduction products of N-benzylcarbonyl-L-asparagine (5.23 gm) were purified on a column of Dowex-50 as described previously and from the water elute was extracted 2.30 gm of benzyl alcohol. The theoretically expected amount is 2.50 gm so this corresponds to a yield of 96%. It is concluded from this high yield that pathway 3 makes no significant contribution.

Material eluted with hydrochloric acid.

Electrophoresis of the unpurified starting material showed three

Fig. 36



Ninhydrin value of FII

Table

	$E_{570\ m\mu}$	Corr. for blank
Blank	0.085	
3 μgm glycine	0.160	0.075
6 μgm glycine	0.245	0.160
9 μgm glycine	0.310	0.225
12 μgm glycine	0.410	0.325
15 μgm glycine	0.485	0.400
32.1 μgm FII	0.308	0.223
64.2 μgm FII	0.522	0.437
64.2 μgm FII	0.520	0.435
128.4 μgm FII	0.950	0.865

substances reacting with ninhydrin (FI, II and III). FI, the substance bearing a single positive charge was present only in very minor quantities and will not be considered further. It was assumed that FII and FIII, both of which bear a double positive charge were the products of pathways 1 or 2 (fig. 26).

The difference in ninhydrin colour of the two components was slightly suggestive that FII (purple) was the straight chain compound probably possessing one amino group and one methylamino group and that FIII (grey-brown) was the cyclic compound possessing one amino group and one methylamino group.

Elementary analysis of the oxalate of FII was in agreement with that expected for 4-amino-2-methylaminobutane-1-ol mono-oxalate dihydrate which requires C, 34.4; H, 8.2; N, 11.4; O, 46.0; found, C, 35.13; H, 7.07; N, 11.16; O, 45.39. The dry weight estimation of the FII dioxalate sent for analysis indicated the presence of 13% water. The dihydrate requires 15% of water.

Titration data showing pK values of 10.1 and 8.1 corresponding with the 4-amino and 2-methylamino groups respectively are in agreement with this structure. Thus the two pK's of 1,3-propanediamine are 10.62 and 8.64 (Bertsch, Fernelius and Block, 1958), a difference of 2 pH units. A hydroxyl group on the carbon atom adjacent to an amino group would be expected to lower the pK by about 1 pH unit, (ethylamine has a pK of 10.63; Evans and Hamann, 1951; and ethanolamine has a pK of 9.50; Bates and Pinching, 1951), but N-methyl substitution would be expected to raise it by about 0.4 of a pH unit, (glycine has a pK of 9.78 and sarcosine a pK of 10.20 Long, 1961).

This means that the 2-methylamino group might be expected to have its pK lowered by about 0.6 of a pH unit so that a value of 8.1 is reasonable. The value of 10.0 for 4-amino group is also within the expected range.

The products of permanganate oxidation of FII have similar electrophoretic and ninhydrin staining properties to the oxidation products of 2,4-diaminobutyric acid, which further supports the identification of FII as the straight chain compound, (it would not be expected that the difference between a methylamino and an amino group would be detected by these methods).

Nitrogen estimation of FII as the hydrochloride gave a value of 14.7% which is identical with the calculated requirement of 14.7%. Ninhydrin estimations based upon this value indicated a colour yield of 0.32 per amino group if the colour yield of glycine is taken as equal to one. This low colour yield might be taken as evidence in favour of one of the amino groups being N-methylated since the N-methylamino group has a colour yield of only 0.29 relative to a free amino group as calculated from a comparison of sarcosine with glycine (Moore and Stein, 1954).

A consideration of the spectrum of the DNP-derivative of FII (fig. 30) shows distinct similarities to the spectrum of di-DNP-diaminobutyric acid (preparation as described on page 110). Thus there is a maximum at 358 mμ (362 mμ FII) and a shoulder at 404 mμ in both compounds. The shoulder is absent in the DNP-derivative of FIII (fig. 30

Elementary analysis of FII as the free base, although desirable, was found to be impractical due firstly to its volatility in water vapour so making the preparation of a dry sample very difficult and secondly to its spontaneous decomposition over a few hours to a thick

sticky, black gum.

Treatment with 2 N HCl and 2 N NaOH at 100° for 2 hr shows FII to be unchanged when examined by electrophoresis thus indicating a fairly stable structure.

The infrared spectrum is shown in fig. 46)

From the observations described above it is concluded that FII is 4-amino-2-methylaminobutane-1-ol.

The characterisation of FIII has been slightly less successfully accomplished than in the case of FII. This material is not steam distillable and appears as a thick oil or gum in either the free state or as the hydrochloride. The elementary analysis of the oxalate indicates a carbon-nitrogen ratio of 9:2 suggesting that it is probably the dioxalate and that one of the amino groups is methylated. A structure compatible with this ratio is 3-aminomethyl pyrrolidine dioxalate and requires an elementary analysis of C, 38.6; H, 5.72; N, 10.0; O, 45.7. This compares closely with the found composition of C, 37.9; H, 5.9; N, 10.0; O, 46.1; and by themselves would have been strongly suggestive of this structure. Dry weight estimation indicated the presence of 3% water so it is probable that the compound is not hydrated. However the behaviour of this compound in acid and alkali, in which it is converted to a substance that is electrophoretically indistinguishable from FII, would seem to be against it, as a pyrrolidine would have been expected to be stable under these conditions. However since no information is available on the stability of amino pyrrolidines no firm conclusions should be drawn from this.

The pK values of 10.4 and 7.0 would seem to be consistent with that expected of a 3-amino pyrrolidine which has an imino and amino group on adjacent carbon atoms. A comparable situation is seen in the case of ethylene diamine which has pK's of 7.00 and 10.09 (Bertsch, Fernelius and Block, 1958). The slight discrepancy of the higher pKs is probably due to the 3-amino group of the pyrrolidine being N-methylated (see page 122)

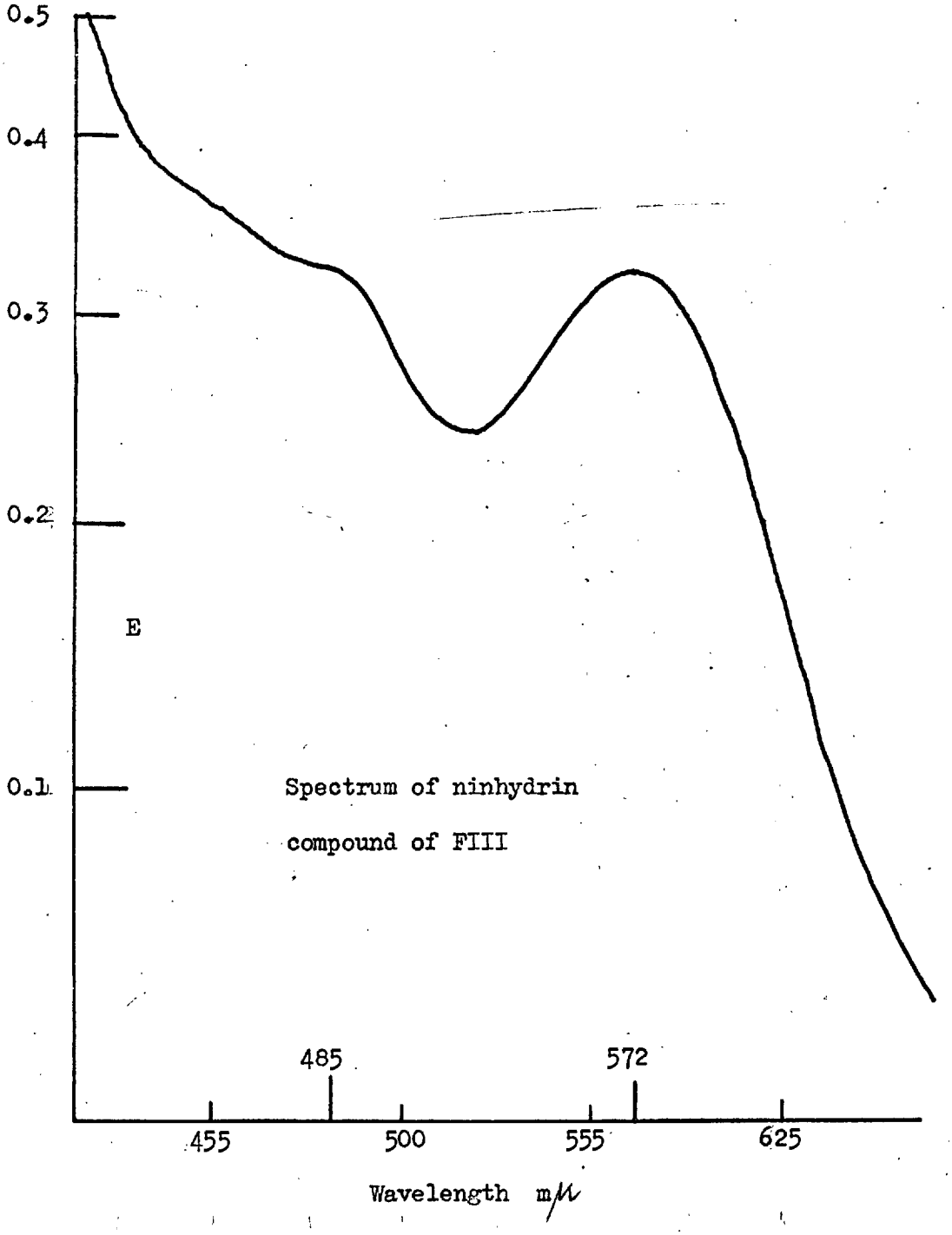
Fig.35 shows the spectrum of the products of the ninhydrin reaction with FIII. It is of interest to note that there are two maxima, one at 485 mμ and another at 572 mμ. The latter is the one usually observed in most amino acids except in the case of proline which absorbs at 440 mμ. This is in the same region as the first mentioned maximum of FIII and could be interpreted as indicating the presence of a similar chromophore to that in proline.

Oxidation by permanganate under the mild conditions described in the experimental section is also of interest since FIII is apparently resistant, the product failing to show any difference from the starting material when examined by electophoresis. This would be expected for the pyrrolidine structure since there is no primary alcoholic group present.

It was concluded that there was strong evidence in favour of FIII being 3-methylamino pyrrolidine dioxalate.

It seemed worthwhile however to investigate alternative methods of preparing 2,4-diaminobutene-1-ol free of the N-methyl-group, since the reduction of glycopeptides might not always involve the use of their N-benzyloxycarbonyl derivatives.

Fig. 35


0.5
0.4
0.3
0.2
E
0.1
Spectrum of ninhydrin
compound of FIII
485
572
455
500
555
625
Wavelength mμ

Alternative method for the preparation of 2,4-diaminobutane-1ol.

A careful consideration of the various compounds that might be expected to yield 2,4-diaminobutane-1-ol suggested that the problem of finding a suitable blocking group for the amino groups was almost impossible to solve if LAH was to be used as the reducing agent.

It seemed possible that the difficulty might be overcome if the milder reducing agent lithium borohydride were used.

As mentioned earlier (page 94) Chilmall and Rees (1958) have used this reagent in an attempt to devise a method for the identification of the C-terminal amino acid in proteins and peptides and they claim that its use does not cause any appreciable reduction of peptide bonds. However, Bailey (1952) has objected to the method on the ground that intermediate boron compounds are formed that are not easily cleaved.

The scheme proposed involved taking 2,4-diaminobutyric acid as the starting material and then converting it first to the N,N-diacetyl derivative and secondly forming the methyl ester. The lithium borohydride reduction of this substance should only affect the ester group, converting it to a primary alcohol and leaving the two N-acetyl groups intact. These could then be cleaved by mild acid hydrolysis to yield free 2,4-diaminobutane-1-ol.

EXPERIMENTAL

Preparation of 2,4-diaminobutyric acid.

This compound was prepared by one of the methods described by Adamson (1939).

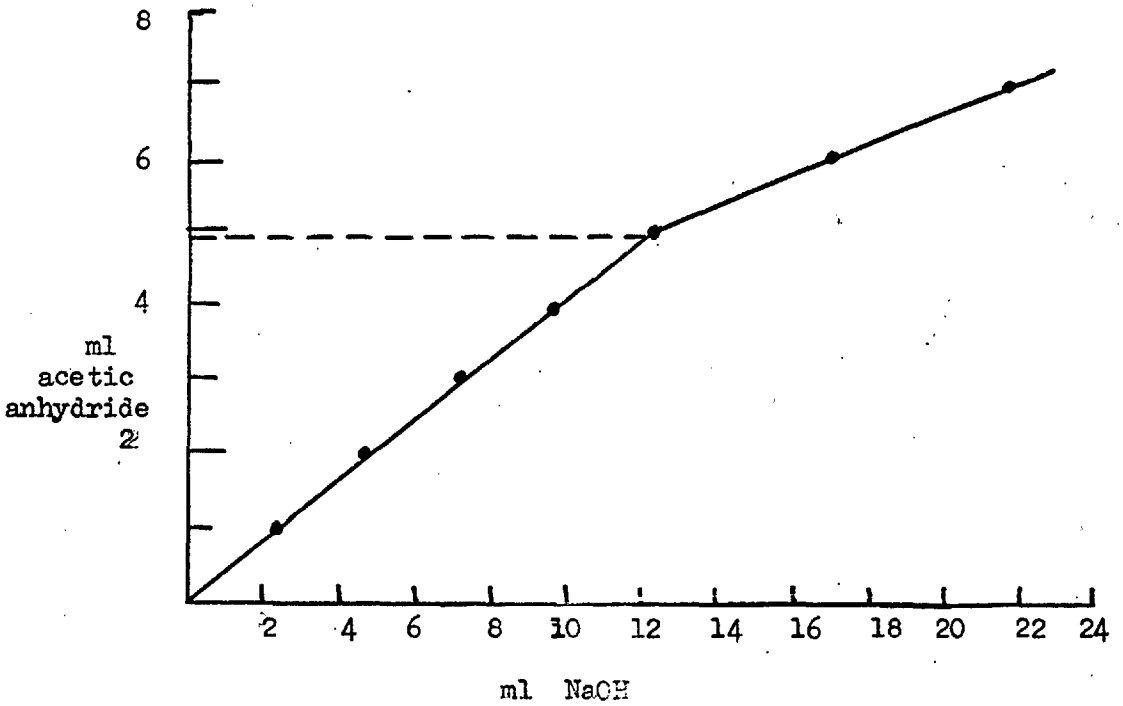
L-glutamic acid (30.0 gm; 0.204 moles) obtained from BDH was added to concentrated sulphuric acid (100 ml) and chloroform (60 ml)

and the mixture was stirred. Sodium azide (16.0 gm) was added in small portions keeping the temperature at 45-50°. When the evolution of nitrogen had ceased (4-5 hr) the mixture was poured on to ice and the aqueous extract so obtained was treated with hot saturated barium hydroxide until the reaction was weakly acid to Congo Red. Barium sulphate was removed by filtration and picric acid (60.0 gm) in 1400 ml of hot water was added. Large leafy crystals of the dipicrate had formed after 24 hr at 4°, the product at this stage having M.P. 180°. This product was dissolved in 500 ml of hot water and concentrated hydrochloric acid (200 ml) added. After cooling the picric acid was removed by filtration, the filtrate extracted three times with ether and then evaporated to dryness. The crystals of the dihydrochloride so obtained were ground with ethanol, collected by filtration and dried in air. Yield 16.4 gm (42%); M.P. 198°; $[\alpha]_D$ +13.9° (c 4.0 in water); the infrared spectrum is shown in fig. 5(i). Adamson gives +14.6°(C,3.67 in H_2O).

Preparation of the N,N -diacetyl derivative of 2,4-diaminobutyric-acid dihydrochloride.

Diaminobutyric acid dihydrochloride (4.77 gm) was dissolved in 30 ml of water and a solution of sodium hydroxide (4.2 gm) in 25 ml of water was added from a burette until the pH (measured by glass electrode) was 11.25. Acetic anhydride was then added in 1.0 ml portions, the mixture being stirred and the pH kept constant by the addition of sodium hydroxide from the burette. The reaction proceeded rapidly, its course being shown in fig. 37 and table 23 after the theoretically required amount of acetic anhydride had been added (4.9 ml) the uptake of sodium hydroxide abruptly doubled, indicating that acetic anhydride was no longer

Fig. 37



Reaction of diaminobutyric acid with acetic anhydride

Table 23

ml acetic anhydride	ml NaOH 4.42 M	pH
0	0	11.25
1.0	2.3	11.25
2.0	4.6	11.25
3.0	7.1	11.20
4.0	9.6	11.30
5.0	12.3	11.25
6.0	17.0	11.30
7.0	21.6	11.25

reacting with amino groups but was being hydrolysed to two moles of acetic acid.

The reaction mixture was acidified to pH 3 by the addition of hydrochloric acid and then evaporated to dryness. The residue consisted of sodium chloride in a nearly colourless oil which was extracted into a small volume of methanol. A portion of the extract was kept on one side from which the N,N'diacetyl compound was crystallised from ethanol-ethyl acetate. M.P. 155° $[\alpha]_D$ = -13.3° (c 3.0% in water.) Yield 95-100% on basis of acetic anhydride uptake.

Preparation of N,N'diacetyldiaminobutyric acid methyl ester.

Methylation was effected by passing diazomethane directly into a solution of the substance in methanol, the methanol extract described in the last section being used without further purification.

Diazomethane was prepared by the modified method of De Boer and Backer (1963). The distillation apparatus that was used had been carefully flamed at all joints and edges so as to minimise the risk of explosion during the preparation.

Potassium hydroxide (5 gm) was dissolved in 7.5 ml of water and placed in the reaction vessel with 25 ml of ethanol. This mixture was heated to 60-65° on a water bath and a solution of p-tolysulphonylmethyl-nitrosamide (N-methyl-nitrosotoluene-p-sulphonamide; 21.5 gm) in 100 ml of ether was added from a dropping funnel. The diazomethane so prepared was distilled directly into the methanol extract until the solution acquired a permanent yellow colour. The excess diazomethane was destroyed with a few drops of acetic acid and the product evaporated to dryness to yield a pale yellow oil which was extracted three times into dry ether. The extracts were pooled and after 48 hr approx-

imately 1 gm of pure white crystals were obtained having M.P. 100° and $[\alpha]_D = -33.7°$ (c 1.66 in water). Yield approximately 20%.

The infrared spectra of N,N'-diacetyldiaminobutyric acid and its methyl ester are shown in figs. 6+7 (See addendum page 196a).

The reduction of N,N'-diacetyldiaminobutyric acid methyl ester with lithium borohydride.

Preparation of solution of lithium borohydride

Lithium borohydride (approx. 5 gm) supplied by BDH was added to 50 ml of tetrahydrofuran (dried as described previously) and refluxed for 4-5 hr. The remaining insoluble material was filtered off through a glass sinter to give a clear colourless filtrate which was stored at 4°.

Reduction of the ester.

N,N'-diacetyldiaminobutyric acid methyl ester (250 mg) was dissolved in 30 ml of tetrahydrofuran and the solution of lithium borohydride (10 ml) was added added and the mixture refluxed for 6 hr, cooled and water added dropwise until all excess lithium borohydride had been destroyed. The insoluble decomposition products were removed by filtration, extracted by refluxing 4-5 min with ethanol and filtered. The filtrates were combined and evaporated to dryness to give a whitish gummy residue which was extracted into ethand leaving a small amount of insoluble white powder. The ethanol extract was evaporated to dryness to give a gummy white partially crystalline material. In a repeat of this experiment, which was performed in precisely the same way, the product at this stage was taken up in 5 ml of water, a drop of which was found to react strongly on paper, with the ninhydrin reagent.

Paper electrophoresis of the products in the formic-acetic buffer

and under the conditions described previously , showed the presence of four ninhydrin positive components, (fig. 38) one, present in small amount, having the same colour and mobility as diaminobutyric acid, a second rather more intense spot with slightly less mobility than FII, and a third, the major component, having the same mobility as as FII, but giving a slightly redder colour and a fourth minor component, grey in colour, and with about the same mobility as FIII.

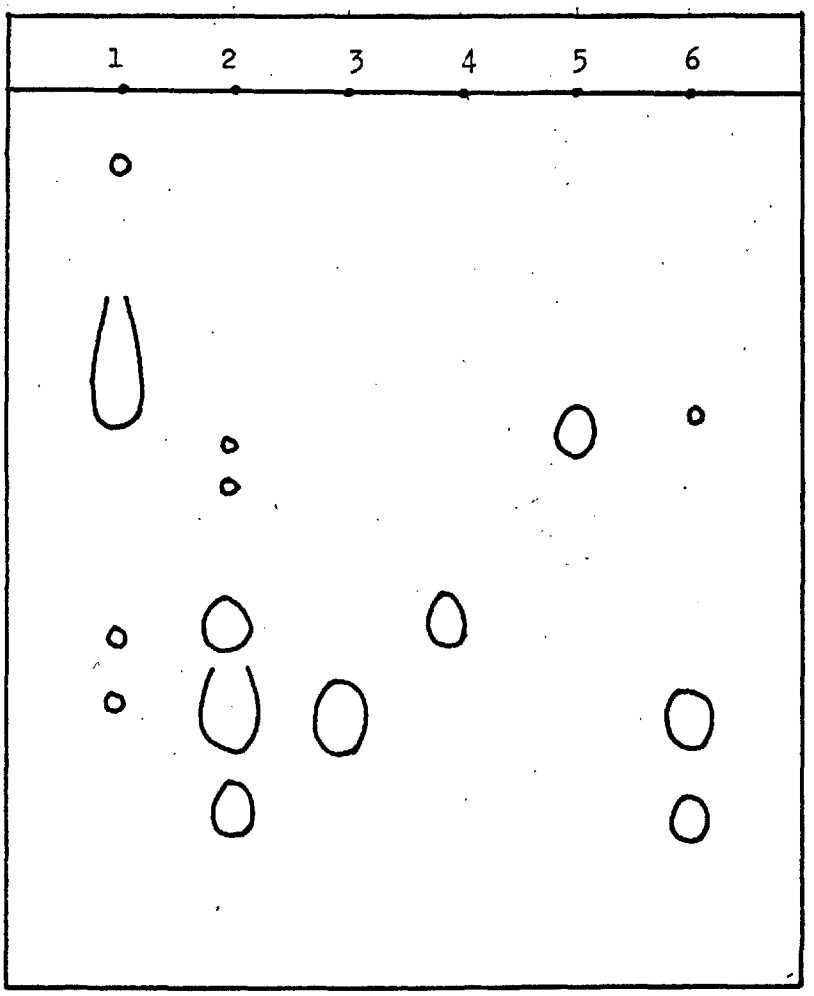
Fractionation of reduction products of the methyl ester on Dowex-50

Dowex-50 x 8; 200-400 mesh in the hydrogen form was made into a column 3 x 37 cm.

The reduction products were applied and the column was eluted with a hydrochloric acid gradient starting at 1 N and going to 3 N. Fractions of 10 ml were collected and on completion of the gradient elution was continued with 3 N hydrochloric acid. Alternate tubes were tested by spotting on filter paper and staining with the ninhydrin reagent. Two groups of tubes were observed to give a positive reaction, the first between tubes 156-168 and the second between tubes 188-198. Pairs of tubes from the relevant regions were pooled (each pair being numbered by a Roman numeral), evaporated to dryness, taken up in 5 ml of water and estimated by the ninhydrin method (fig. 39 and table 24). Three peaks were apparent and were named PI, PII and PIII as indicated.

Paper electrophoresis of each peak showed that PI corresponded with the spot having the same mobility as FIII and that PII corresponded with the spot having slightly less mobility. For some unexplained

Fig. 38



Electrophoresis of reduction products of diacetyl-diaminobutyric acid methyl ester

1. Before hydrolysis
2. After hydrolysis
3. PI
4. PII
5. Diaminobutyric acid
6. FI, FII and FIII

Table 24

Fractionation of reduction products of diacetyldiaminobutyric acid methyl ester on Dowex-50

Fraction number	$E_{570\ m\mu}$
I	0.249
II	0.607
III	1.60
IV	1.00
V	0.534
VI	0.439
VII	0.164
VIII	0.068
IX	0.048
X	0.027
XI	0.106
XII	0.308
XIII	0.646
XIV	0.642
XV	0.394
XVI	0.167
XVII	1.30
XVIII	0.620
IXX	0.345
XX	0.364

Fractionation of reduction products of diacetyl-diaminobutyric acid methyl ester on Dowex-50

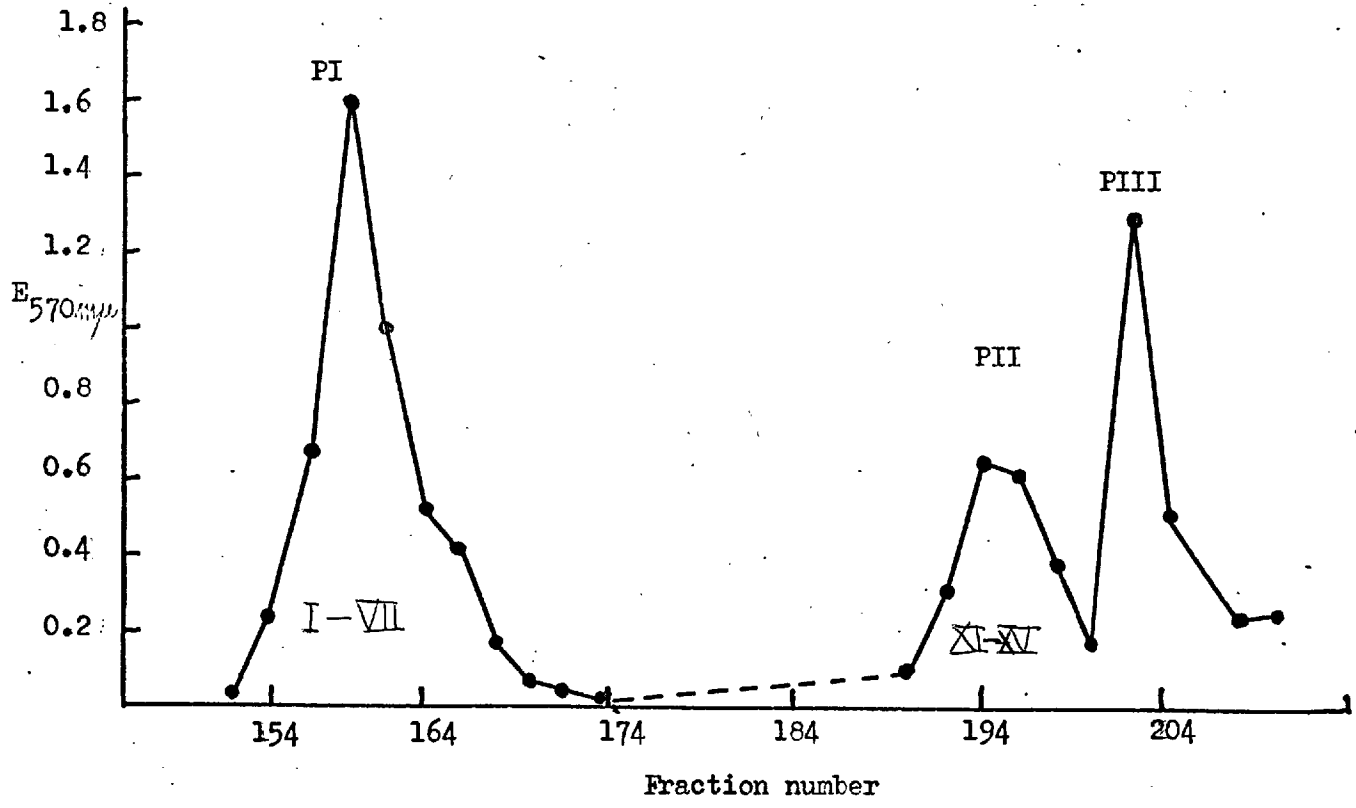


Fig. 39

reason the material in the third peak, PIII, was not detectable on the electrophoretic strip with the ninhydrin reagent.

Preparation of the oxalates of PI and PII

The relevant tubes (I-VII for PI and XI-XV for PII) were pooled, evaporated to dryness and the residues of each dissolved in 2 ml of water.

A column of Dowex-1 (1.0 x 24.0 cm) generated in the oxalate form as described on page 111 was set up and washed exhaustively with water. Fractions of 5 ml were collected and a drop from each was tested with a few drops of calcium chloride. Tubes 1 and 2 gave a precipitate of calcium oxalate so they were pooled and evaporated to dryness to give a pure white partially crystalline substance with M.P. 156-157°. The infrared spectrum is shown in fig. 8(i) . Elementary analysis showed C, 33.1; H, 5.7; N, 10.9; O, 50.3.

The column was regenerated, washed exhaustively, the solution of PII applied and treated as described above to yield approximately 15 mg of pure white crystals having M.P. 129-130°. The infrared spectrum is shown in fig. 9(i) . Elementary analysis showed C, 34.3; H, 6.2; N, 8.5; O, 51.0.

DISCUSSION

Since the object of this experiment was to prepare an unequivocal sample of 2,4-diaminobutane-1-ol only those two compounds that had electrophoretic mobilities closely similar to PII were considered. It is fairly clear from the way in which the reduction has yielded a multiplicity of products that the reaction has not achieved the

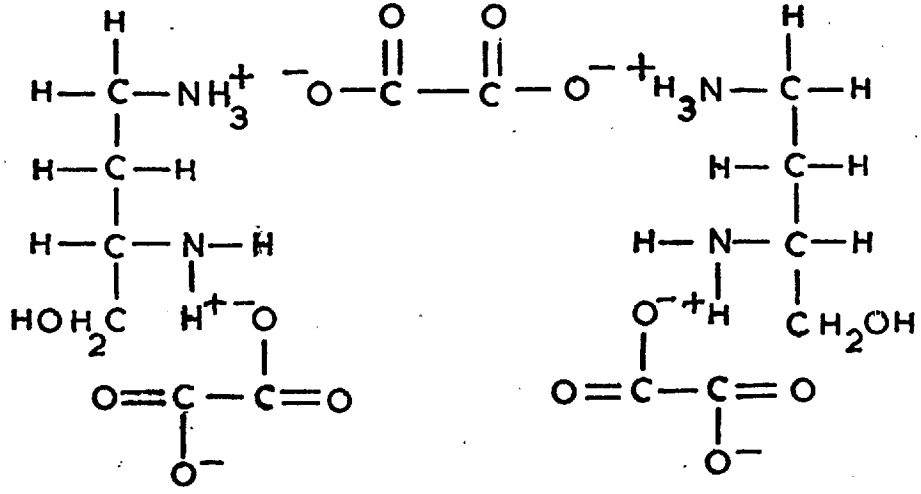
main object. The results are of interest however, in that they throw some light on the reactivity of lithium borohydride. The appearance of a ninhydrin positive substance in the reduction products prior to acid hydrolysis is probably indicative of some cleavage of acetamido groups although it is possible that this may be explained by the formation of an N-ethyl derivative. Electrophoresis of the reduction products before hydrolysis (fig. 38) shows a singly charged material and faint traces of doubly charged substances which is in agreement with the splitting or reduction of acetamido bonds. This is in contrast with the usually accepted view that amide bonds are not affected by lithium borohydride.

The elementary analysis of PI is in close agreement with the structure shown in fig. 40, which contains one and a half moles of oxalate per mole of diamine and a single molecule of water. This structure requires C, 35.3; H, 5.9; N, 10.9; O, 50.5.

It is therefore concluded that PI is probably 2,4-diaminobutane-1-ol.

The elementary analysis of PII indicates a carbon-nitrogen ratio of 9.5:1 which is difficult to reconcile with any likely structure, particularly as the infrared spectra of PI and PII are closely similar. It seems most likely that the difficulty is caused by the presence of oxalic acid as an impurity. The very small amount of material available did not allow further investigation to clear up this problem.

The experiment demonstrates that conclusions drawn from reductions with lithium borohydride should be made with caution, because of the possibility of unexpected side reactions.



Proposed structure of PI oxalate

Fig. 40

Following the preparation of these compounds it was decided to proceed with the reduction of the model compound, 2-acetamido-1-(L-β-aspartamido)-1,2-dideoxy-β-D-glucose.

Reduction of the N-benzyloxycarbonyl derivative of 3,4,6-tri-O-acetyl-2-acetamido-1-(L-β-aspartamido)-1,2-dideoxy-β-D-glucose with LAH

The material used in this reduction was synthesised and kindly given by Dr. R.D. Marshall. It represents the model compound of the linkage between asparagine and N-acetylglucosamine that is found in the ovalbumin glycopeptide. It was proposed to reduce the compound with LAH in tetrahydrofuran and to identify the products after acid hydrolysis. The substances involved in the reaction and its expected course are shown in fig. 41.

During the reduction it was expected that the O-acetyl groups would be cleaved to give ethanol and the original hydroxyl groups on the sugar, the acetamido group would give a secondary amine as would the group in the linkage and the N-benzyloxycarbonyl group would give an N-methyl group. Hydrolysis was then expected to cleave the bond between the C-1 of the sugar and the aspartamido nitrogen to yield equimolar amounts of N-ethylglucosamine and 4-amino-2-methylamino-butane-1-ol.

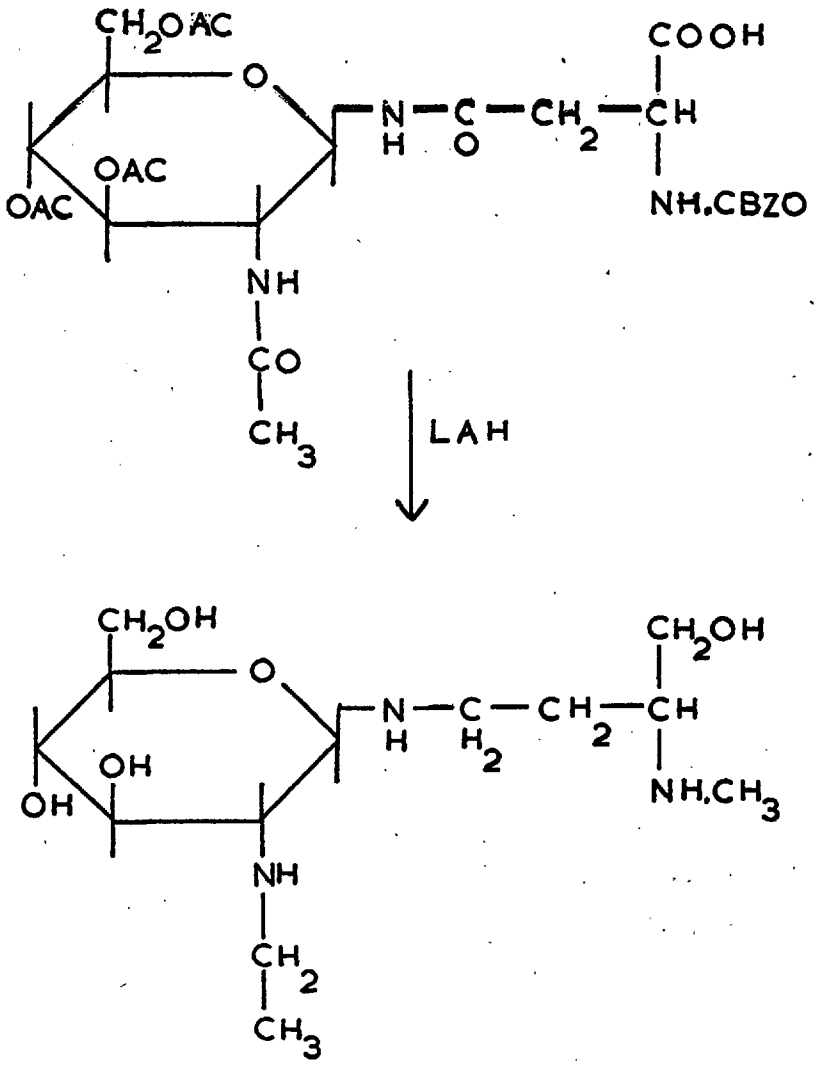
EXPERIMENTAL

Reduction of the N-benzyloxycarbonyl derivative of 3,4,6-tri-O-acetyl-2-acetamido-1-(L-β-aspartamido)-1,2-dideoxy-β-D-glucose.

The model compound (11.5 mg) was dissolved in 8-9 ml of hot tetra-

Fig. 41

The reduction of the N-benzyloxycarbonyl derivative of 3,4,6-tri-O-acetyl-2-acetamido-1-(L-β-aspartamido)-1,2-dideoxy-β-D-glucose with lithium aluminium hydride



hydrofuran, a small amount remaining out of solution. LAH (50.0 mg) was added and the mixture heated under reflux for 24 hr. Normal hydrochloric acid (1.3 ml) was added to destroy the excess LAH and the mixture evaporated to dryness. Ethanol (95%; 4-5 ml) was added and most of the material went into solution. The small insoluble residue was removed by centrifugation and the supernatant was found to be positive to the ninhydrin reagent on paper. The alcoholic supernatant was evaporated to dryness yielding sheaves of crystals in a yellowish oil. The whole residue was dissolved in 0.5 ml of water and the solution brought to pH 8 by the dropwise addition of normal sodium hydroxide causing the precipitation of a whitish substance which was removed by centrifugation. The supernatant gave a positive ninhydrin reaction.

Paper electrophoresis and paper chromatography of the products

2-acetamido-1-(L-β-aspartamido)-1,2-dideoxy-β-D-glucose (AADG) before reduction, after reduction and after reduction and acid hydrolysis was examined by descending paper chromatography (Whatman's 3MM) in phenol-ammonia (500 gm crystalline phenol in 125 ml of water; 1 ml of .880 ammonia was added to 200 ml of phenol solution).

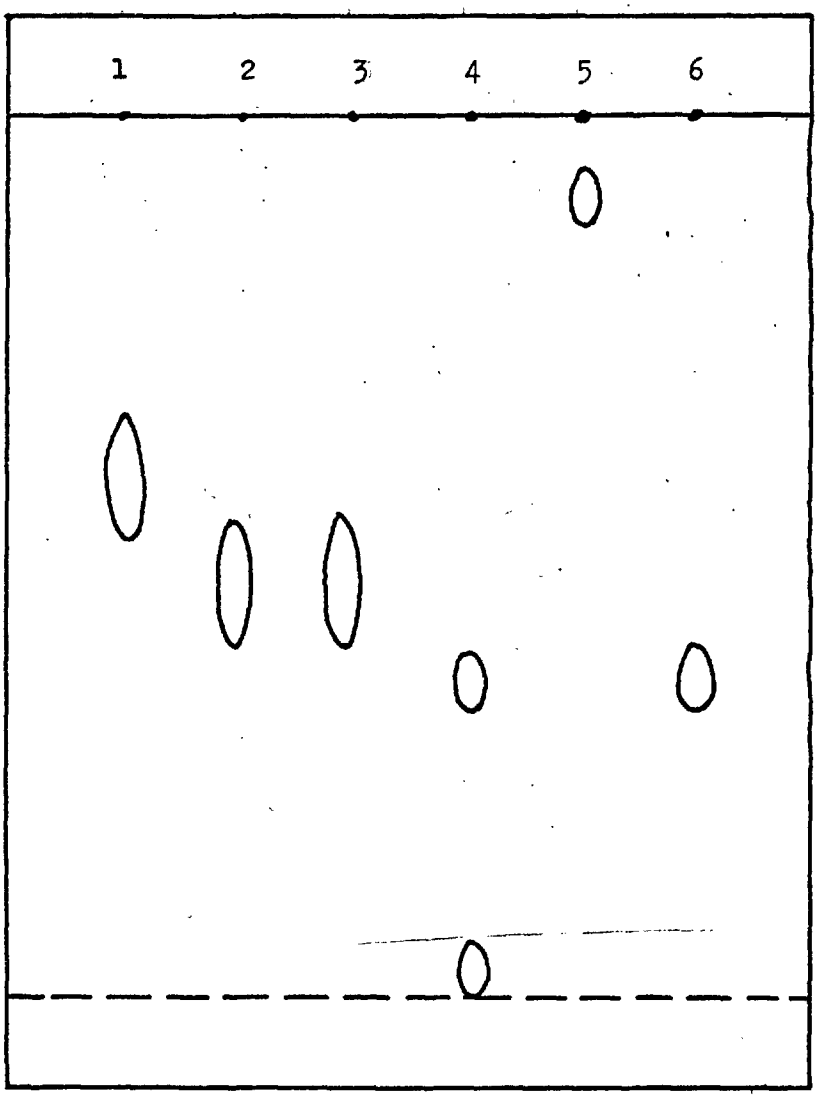
Paper electrophoresis was performed on Whatman's 3MM in the formic-acetic buffer of pH 1.9, for 45 min at 1000 v on the products of the hydrolysis of reduced AADG and the reduction products of N-acetylmethylglucosaminide after acid hydrolysis (see below). The patterns obtained are shown in figs. 42 and 43.

The reduction of a mixture of the α and β anomers of N-acetylmethylglucosaminide with LAH

A mixture of the α and β anomers of N-acetylmethylglucosaminide

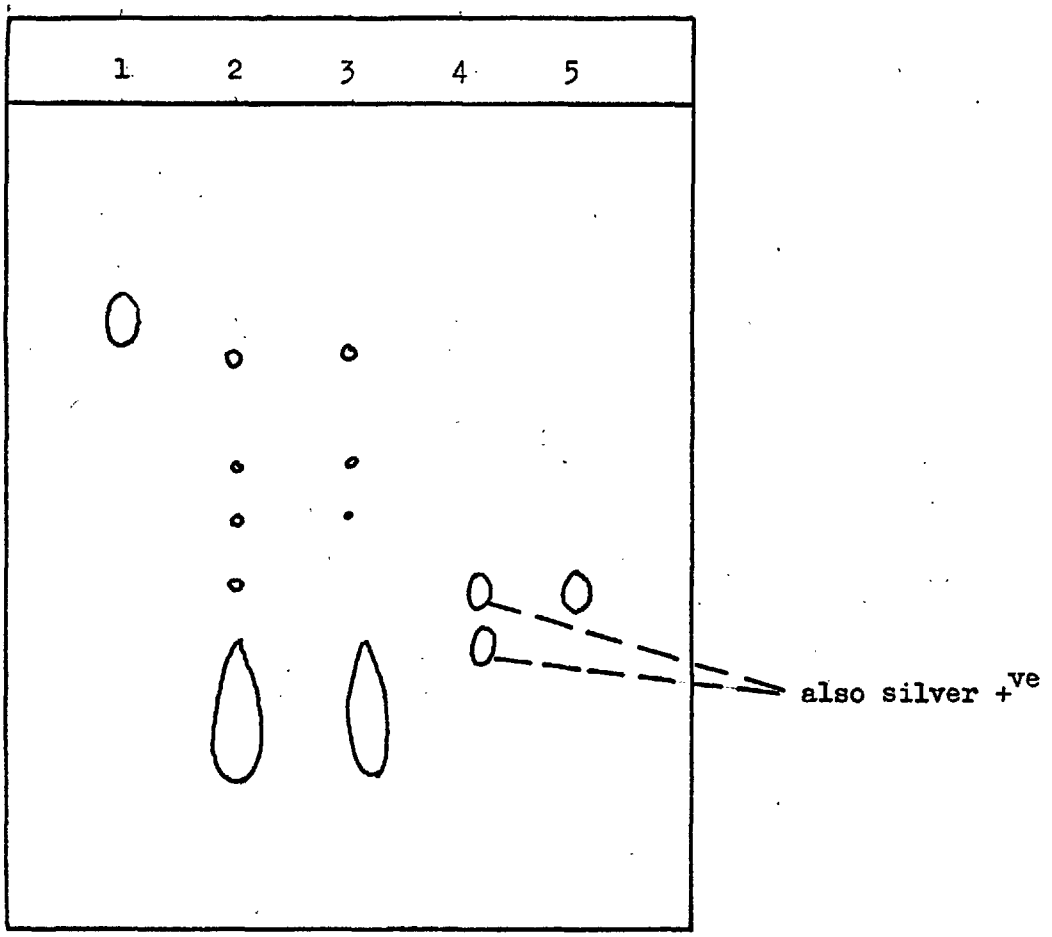
Fig. 42

Paper chromatography of reduced AADG in phenol/ammonia



1. AADG
2. Reduced AADG
3. Hydrolysed reduced AADG
4. FI, FII and FIII
5. Aspartic acid
6. Glucosamine

Fig. 43



Electrophoresis of reduced AADG in formic/acetic buffer pH 1.9

1. AADG
2. Reduced AADG
3. Hydrolysed reduced AADG
4. N-ethylglucosamine
5. Glucosamine

was kindly prepared by V. Bhoyroo according to the method of Zilliken, Rose, Braun and Gyorgy (1955) modified slightly by the use of Zeocarb 225 instead of Amberlite IR-120. The preparation had M.P. 179-180° and $[\alpha]_D$ = +44° (c 1.86 in water). (Zilliken et al give $[\alpha]_D$ = +105°).

The glucosaminide (206.2 mg) was added to 15 ml of tetrahydrofuran with LAH (200 mg) and refluxed for 24 hr. The tetrahydrofuran was removed by centrifugation and excess LAH was destroyed by the dropwise addition of dilute hydrochloric acid. A precipitate formed which was removed by centrifugation and the supernatant evaporated to dryness. The two residues were extracted with a small volume of water and the extract evaporated to dryness. The pale yellow product so obtained was dissolved in 2 ml of pyridine and treated by shaking with acetic anhydride, (1.5 ml) at 4° for 18 hr. Excess reagents were removed by repeated additions of 2-3 ml of water followed by evaporation to dryness. The oily residue was extracted into 3-4 ml of chloroform which on evaporation yielded a product that was negative to both the ninhydrin and silver nitrate tests.

The acetylated reduced methylglucosaminide so prepared was then hydrolysed under the same conditions as the reduced AADG (constant boiling HCl for 6 hr at 100°). The hydrolysate was evaporated to dryness, 3-4 ml of water added and the evaporation repeated to give a brown solid residue which was dissolved in the minimal quantity of methanol. Ether was added until a dark coloured substance came out of solution which was separated by centrifugation. The virtually colourless supernatant was evaporated on a watch glass giving sheaves

of colourless crystals which had M.P. (decomp.) 160-180° and in aqueous solution was ninhydrin and silver positive. (Glucosamine has M.P. 110°; decomp.).

Results of paper chromatography and electrophoresis

Fig. 42 shows the relative mobilities of the various substances in phenol-ammonia. AADG without any protecting groups ran with the least mobility and gave a brown colour with ninhydrin (Fletcher et al. 1963 a), after reduction but prior to hydrolysis it ran as a single spot with greater mobility and stained blue with ninhydrin, suggesting that the reduction had occurred as anticipated and had altered the asparagine type of structure. No change was apparent in the substance after acid hydrolysis. The reduction products of N-acetyl-methylglucosaminide ran with the same mobility as glucosamine.

Paper electrophoresis (fig. 43) at a pH (1.9) at which all amino groups would be expected to be fully ionised indicates that reduced AADG (after acid hydrolysis) runs as a single major component staining blue-purple with ninhydrin and having a mobility corresponding with approximately three positive charges as compared with unreduced AADG. There were also three or four minor spots with lesser mobility. It was concluded from these results that LAH reduction of AADG has effected the changes shown in fig. 41. The production of two new cationic groups is in agreement with its observed electrophoretic mobility. As is to be expected the presence of the charged groups in the linkage and on C-2 of the sugar render the N-glycosidic bond very resistant to acid hydrolysis. The minor

components presumably indicate a slight amount of breakdown.

Similarly electrophoresis of the reduction products of N-acetylmethylglucosaminide (fig. 43) showed the presence of two components, one having the same mobility as glucosamine and the other having slightly greater mobility. They both stained purple with ninhydrin. Two strips run under the same conditions showed that both components and glucosamine stained black with silver and that with an aniline-diphenylamine reagent (Smith, 1960) only the more mobile component gave a slightly positive reaction. It was concluded that the reduction of N-acetylmethylglucosaminide produces N-ethylglucosamine but in the present preparation there is contamination with glucosamine. The decomposition point is in fair agreement with the figure of 169-171° found by Mr. B. Wilson (personal communication) and that of Carson (1955) who found 180-181°.

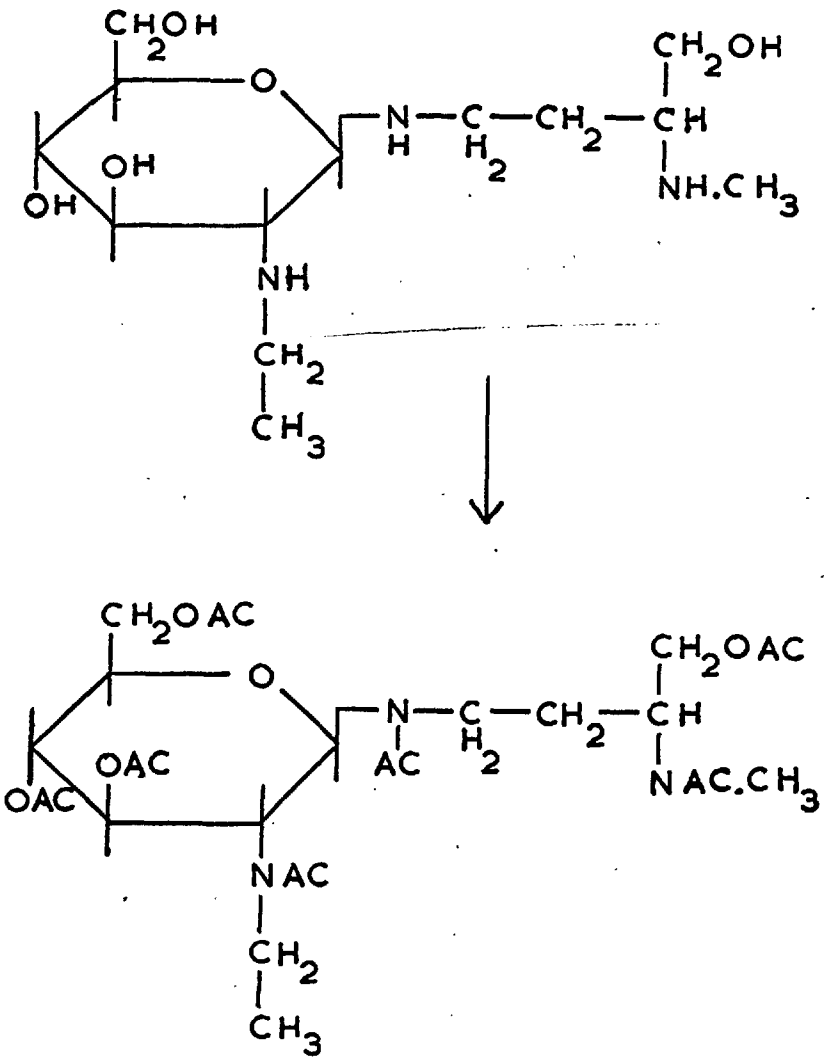
It is clear from these results of the reduction of AADG that no material corresponding with diaminobutanol, or its N-methyl derivative, has been produced. It was therefore necessary to modify the reduced material so that it was susceptible to acid hydrolysis. The method chosen was to fully acetylate the compound with acetic anhydride in pyridine to give the substance shown in fig.44.

Experimental

The acetylation of reduced AADG

The hydrolysate of the reduction products of AADG was dissolved in pyridine (3.0 ml) and acetic anhydride (1.5 ml) was added and the mixture left at 4° for 18 hr. Excess reagent and solvent were removed by repeated additions of small volumes of water and evapor-

Fig. 44



Acetylation of reduced AADG

ation to dryness.

Hydrolysis of acetylated reduced AADG

The product was dissolved in constant boiling HCl (3.0 ml) and gently refluxed for three hours. The hydrolysate was evaporated to dryness, 2-3 ml of water was added and the evaporation repeated. The residue was dissolved in 0.25 ml of water.

Paper electrophoresis and chromatography of the hydrolysate

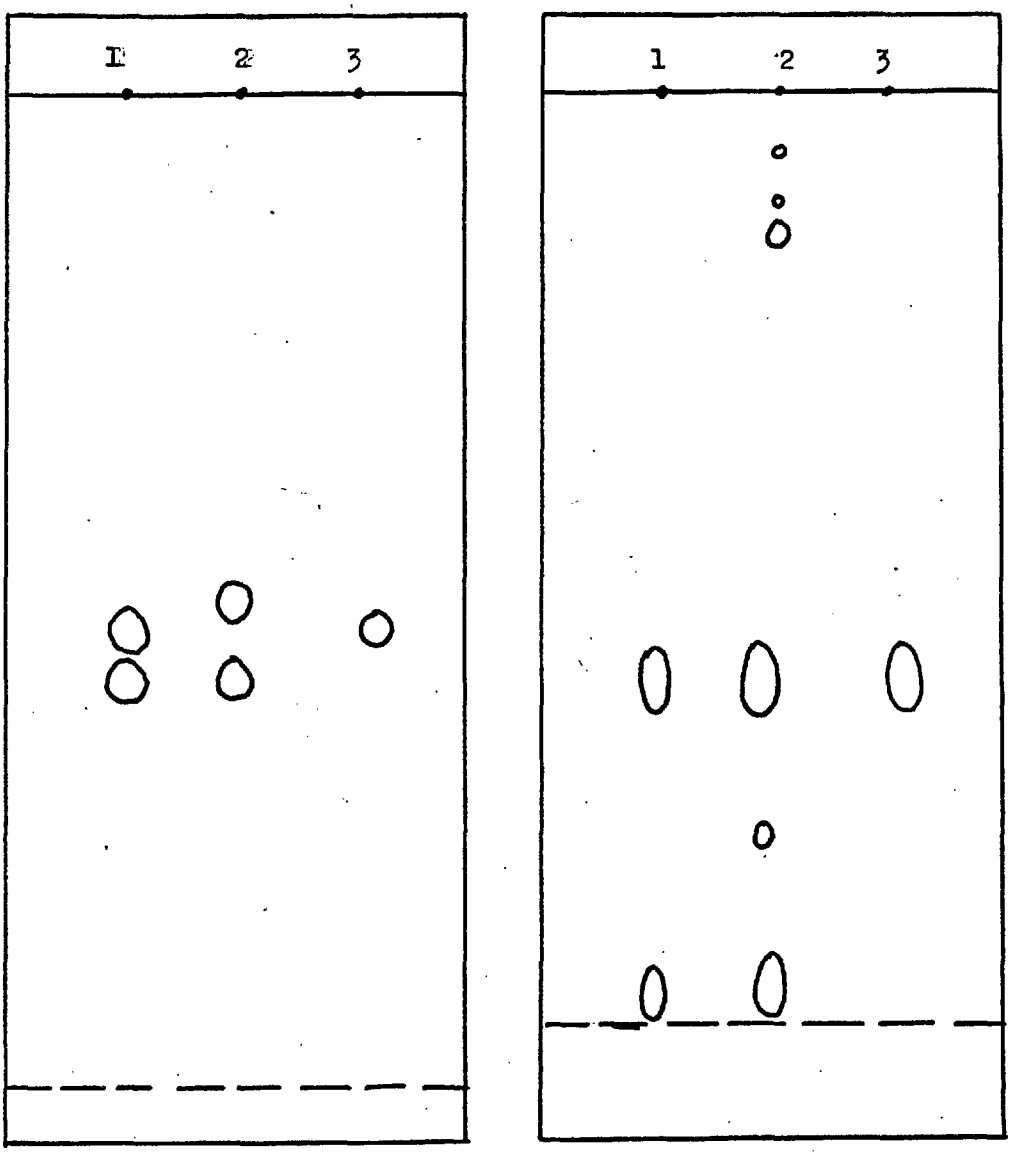
Descending paper chromatography was performed on Whatman's 3MM in the solvent systems i) phenol-ammonia (as described previously) and ii) ethyl acetate-pyridine-acetic acid-water in the proportions 5:5:1:3 (Fischer and Nebel, 1955).

4-amino-2-methylaminobutane-1-ol (referred to in future as AMAB and is synonymous with FII), N-ethylglucosamine and glucosamine were used as markers. The patterns obtained are shown in figs. 45 and 46.

Results and discussion

Figs. 45 and 46 indicate that in solvent i) AMAB runs as two components (evidently containing FIII as an impurity), the one with greater mobility being FII (if ninhydrin colour is to be taken as a reliable criterion). The hydrolysate of AADG runs as several components two of which correspond closely with the two components of AMAB (FII and FIII). Glucosamine and N-ethylglucosamine would appear to run with the same mobility as each other, so it is not possible to determine if AADG has yielded any N-ethylglucosamine, although there is a spot with the correct mobility.

Fig. 45

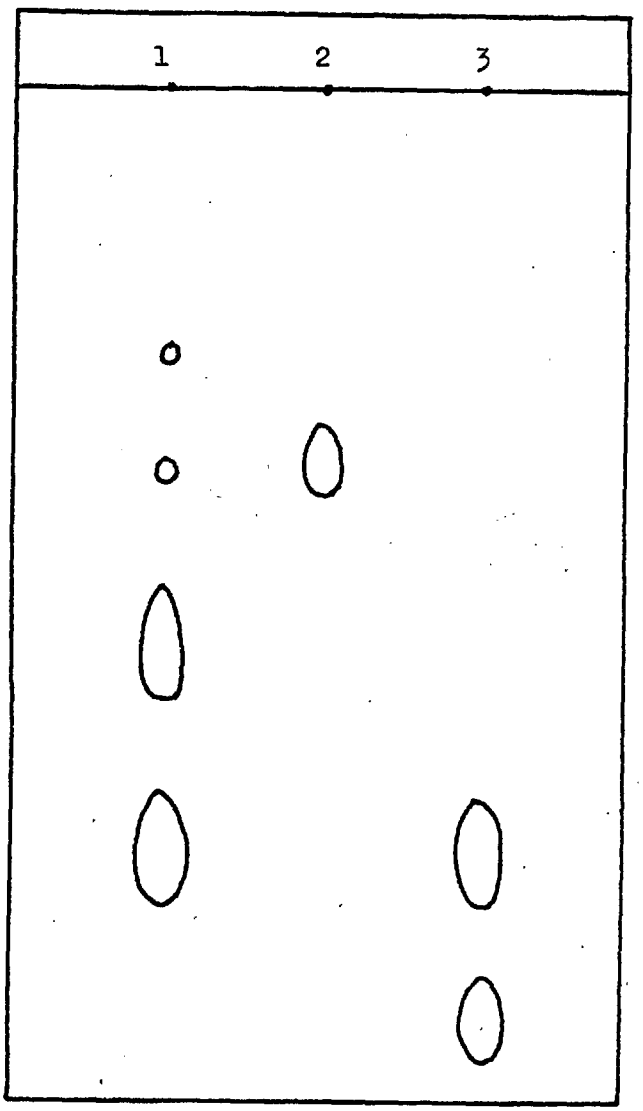


EtAc - Pyr - HAc - H_2O Phenol - ammonia

Paper chromatography of hydrolysed acetylated reduced AADG

1. FII and FIII
2. Hydrolysed acetylated reduced AADG
3. N-ethylglucosamine and glucosamine

Fig. 46



Electrophoresis in formic/acetic buffer pH 1.9 of hydrolysed acetylated reduced AADG

1. Hydrolysed acetylated reduced AADG
2. Glucosamine
3. FII and FIII

In solvent ii) AMAB again runs as two components but in this case the one attributed to FII is the slower. The hydrolysate of reduced AADG runs as two components, one of which corresponds closely with the spot attributed to FIII and the other having fair correspondence with FII. Glucosamine and N-ethylglucosamine again would appear to be unseparated by this solvent.

The results of paper electrophoresis are quite unequivocal, giving two major and two minor components. The faster of the major components runs as a discrete spot having identical mobility with FII and giving the same colour with ninhydrin. The other major component is probably unhydrolysed reduced AADG. Of the two minor spots, the faster corresponds with glucosamine, but the slower is not readily identifiable although it is possibly a small amount of aspartic acid from unreduced material.

It is concluded that the hydrolysis of acetylated reduced AADG results in the formation of AMAB (FII). It is possible that the failure to identify N-ethylglucosamine is due to its very low colour yield with ninhydrin (1.5% of the colour given by glycine; Mr. B. Wilson, personal communication). This may also explain the failure to separate it from glucosamine by chromatography.

Since this experiment gave the desired results it was decided to apply similar procedures to the glycopeptide.

Experimental

Acetylation of the N-benzyloxycarbonyl glycopeptide

N-benzyloxycarbonyl glycopeptide (88.6 mg; prepared by pronase-carboxypeptidase digestion) known to contain aspartic acid as the

only amino acid was dissolved in 4.0 ml of pyridine and 3.0 ml of acetic anhydride. The solution was shaken at 4° for 24 hr and excess reagent and solvent removed by repeated addition of a small volume of water and evaporation to dryness. The residue was extracted into dry ethyl acetate and evaporated to dryness.

Since the theoretical molecular weight of the benzyloxycarbonyl glycopeptide is 1680, 88.6 mg is equivalent to 53.0 μmoles. It was calculated from the data of Brown (1951) that each mole of fully acetylated glycopeptide would require 18 moles of LAH so that 53.0 moles would require approximately 1 millimole. A threefold excess (114 mg) was used. The glycopeptide was dissolved in 30 ml of tetrahydrofuran, the LAH added and the mixture refluxed for 6 hr after which N HCl (6.0 ml) was added to destroy the excess LAH. The mixture was evaporated to dryness leaving a gluey residue which was extracted with 0.1 ml of water. Insoluble material was removed by centrifugation and ethanol was added to 90-95%. After 2 hr at 4° a flocculent white precipitate had formed which was separated by centrifugation and dried in a vacuum dessicator over P_2O_5 and sodium hydroxide flakes.

Mannose estimation of reduced glycopeptide

The orcinol-sulphuric acid procedure described previously was used.

Reduced glycopeptide (10.215 mg) was dissolved in water and made up to 10.0 ml. Reading from a standard curve it was seen that 0.3 ml of the solution contained 100 μgm of mannose, which represents a mannose content of 32.7% for the glycopeptide. The lowness of

this figure (theoretical content on the basis of 5 moles of mannose per mole of glycopeptide is 53%) is probably caused by impurities in the preparation, notably salt. However the estimation does show that a glycopeptide containing fraction has been isolated from other reduction products. The mannose estimation is shown in fig. 47 and table 25.

Ultraviolet spectrum of the reduced glycopeptide

The glycopeptide solution (0.5 ml) referred to in the previous paragraph was diluted to 10.0 ml to give a concentration of 0.051 mg/ml. Examination with the recording spectrophotometer (Unicam SP 700) in Suprasil cells of light path 1.0 cm gave the spectrum shown in fig. 48.

The molar extinction at 205 mμ was 4850; the molar extinction of the unreduced glycopeptide at the same wavelength is 8400. The spectrum is shown in fig.48a.

Acid hydrolysis of the reduced glycopeptide

The reduced glycopeptide (6.4 mg) was dissolved in 1.0 ml of constant boiling HCl and heated at 100° for 6 hr. The HCl was removed by evaporation leaving a brownish residue which was dissolved in 0.5 ml of water.

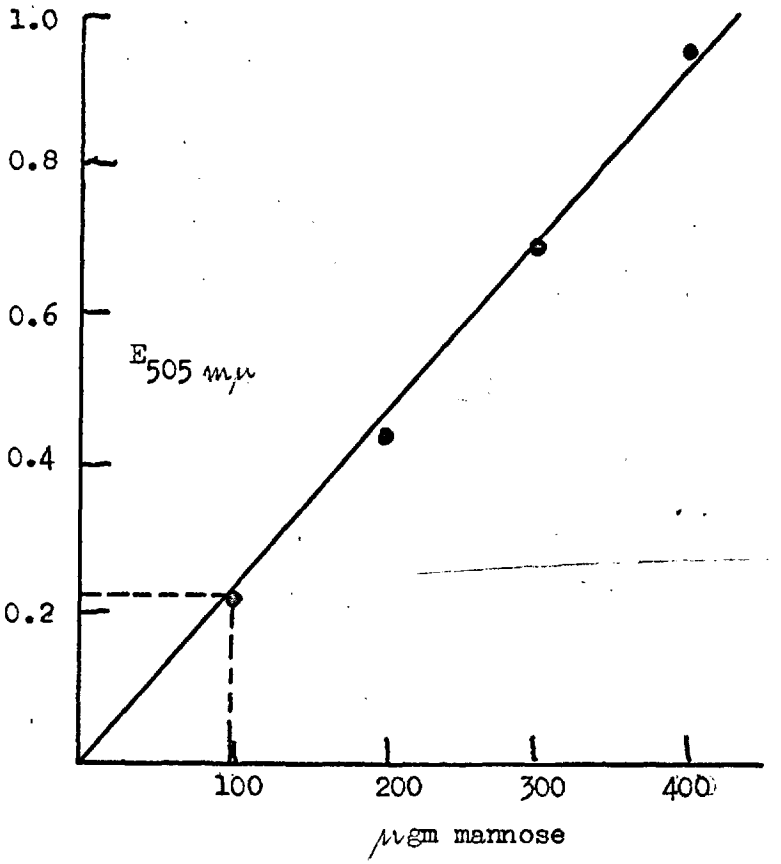
Chromatography and electrophoresis of the hydrolysate

Descending paper chromatography was performed in the solvent systems i) ethyl acetate-pyridine-acetic acid-water in the proportions 5:5:1:3, and ii) phenol-ammonia (as described previously).

Duplicate runs were made and aspartic acid, mannose, glucosamine and AMAB were used as markers. The results are shown in figs. 49 and 50.

Paper electrophoresis was performed under the conditions as

Fig. 47

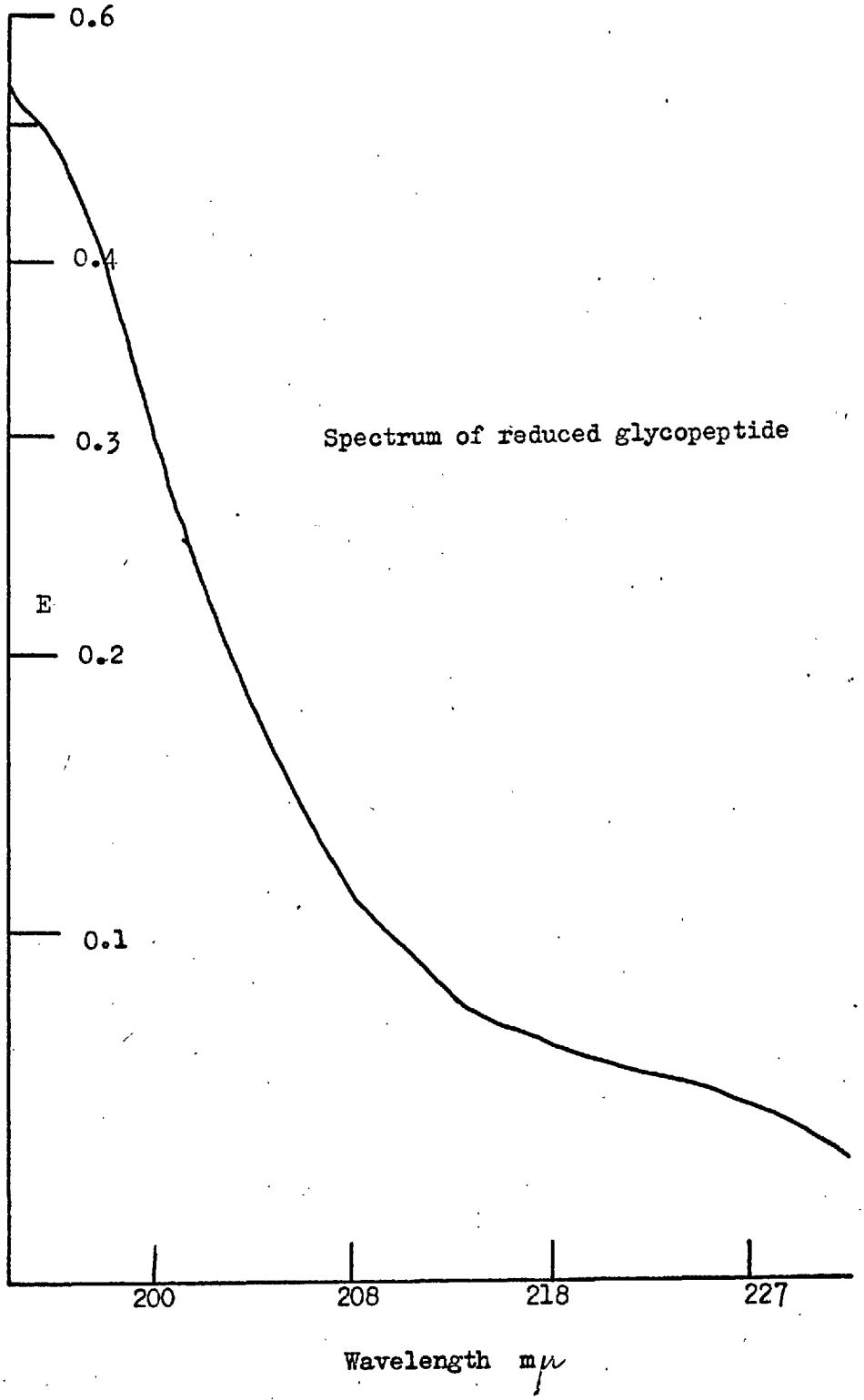


Mannose estimation of reduced glycopeptide

Table 24

	$E_{505\ m\mu}$	Corr. for blank
Blank	0.013	
100 μgm mannose	0.227	0.214
200 μgm mannose	0.448	0.435
300 μgm mannose	0.703	0.690
400 μgm mannose	0.965	0.952
0.34 mg glyco.	0.238	0.225
0.34 mg glyco.	0.235	0.222

Fig. 48



Spectrum of reduced glycopeptide

Fig. 48a

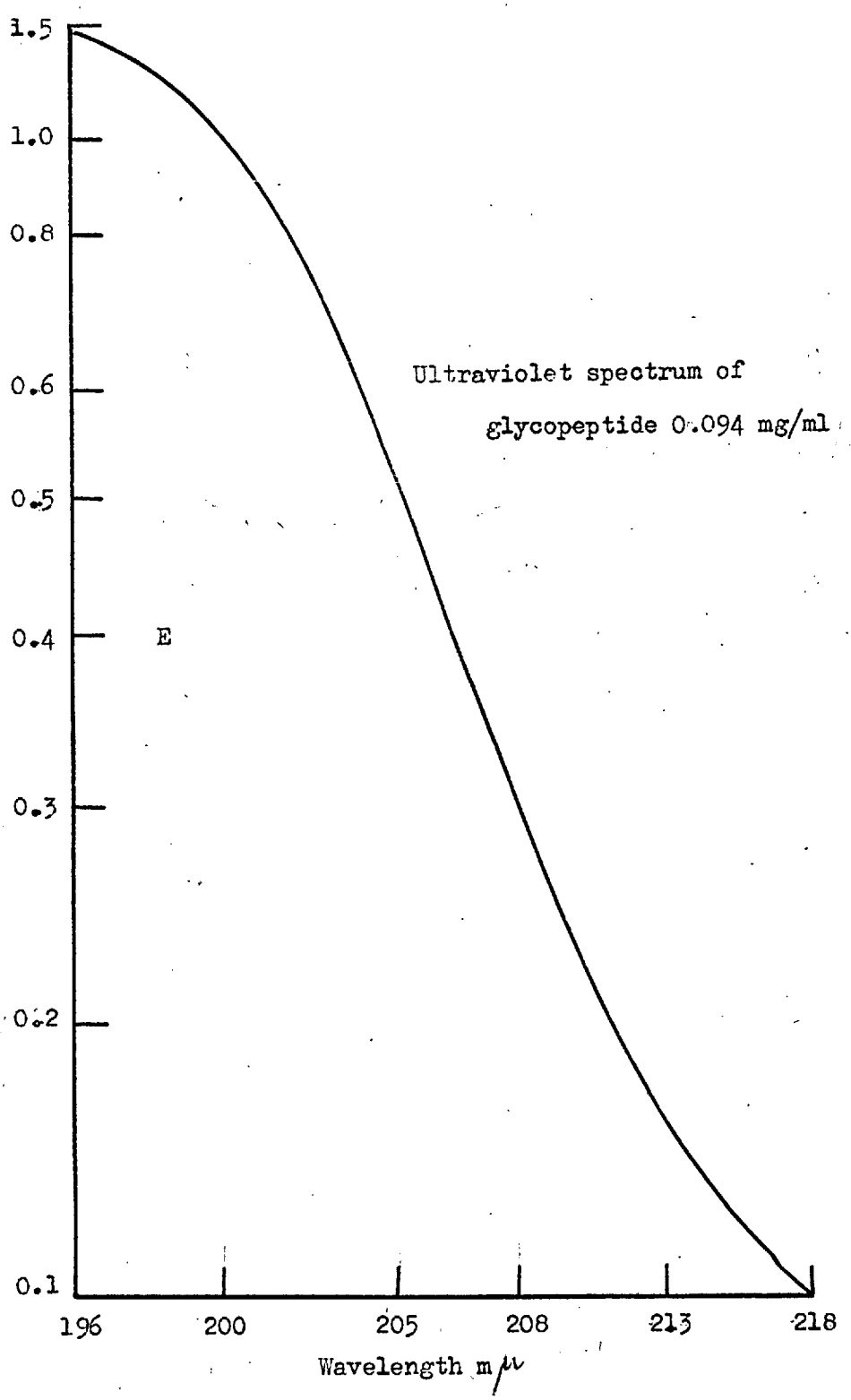
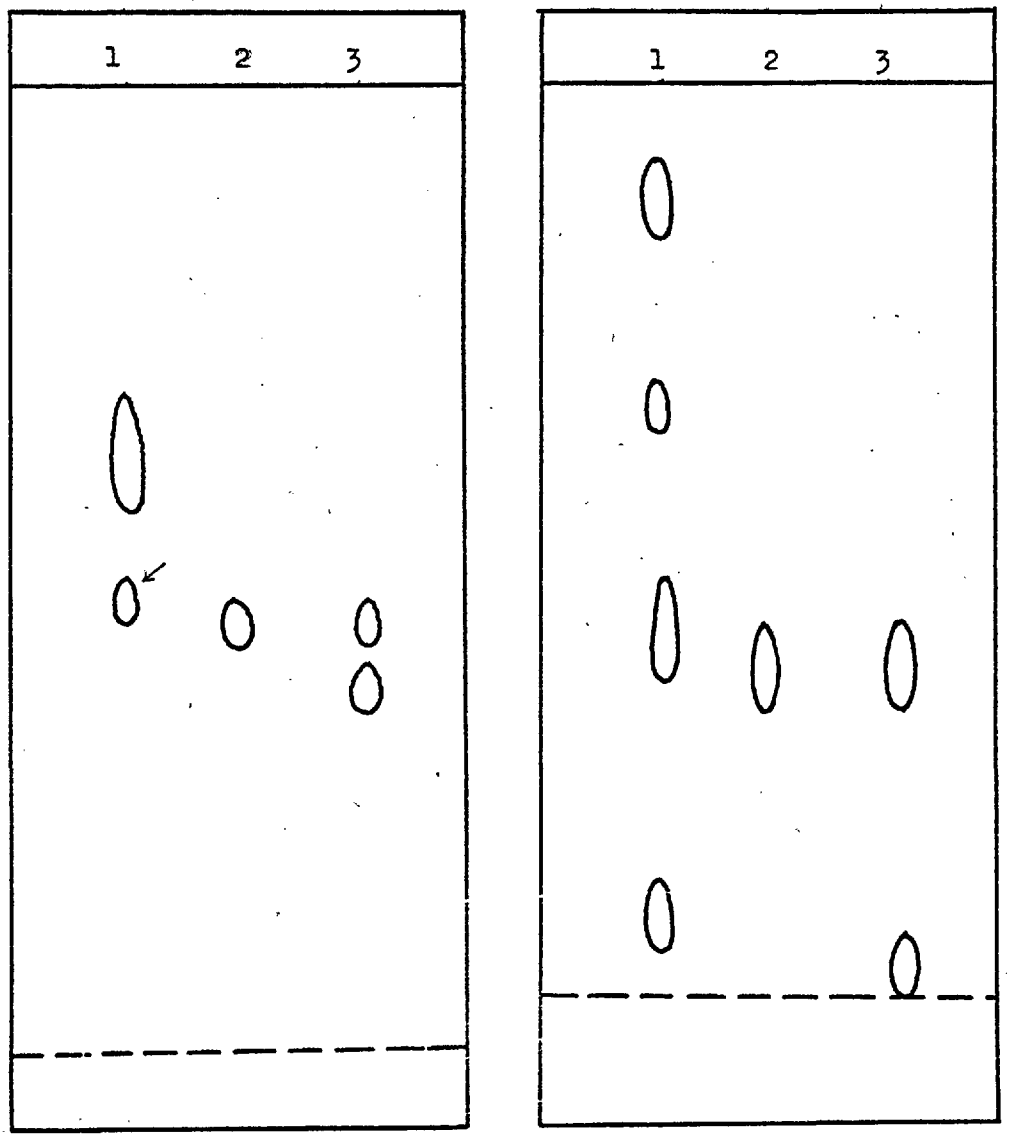

1.5
1.0
0.8
0.6
0.5
0.4
0.3
0.2
0.1
E
196
200
205
208
213
218
Wavelength mμ
Ultraviolet spectrum of
glycopeptide 0.094 mg/ml

Fig. 49



Et.Ac - Pyr - HAc - H_2O Phenol - ammonia

Paper chromatography of reduction products of glycopeptide

1. Hydrolysed reduced glycopeptide
2. N-ethylglucosamine and glucosamine
3. FII and FIII

described previously. The patterns obtained are shown in fig. 50

The substances were detected by the ninhydrin and the silver methods.

Results and discussion

Chromatography

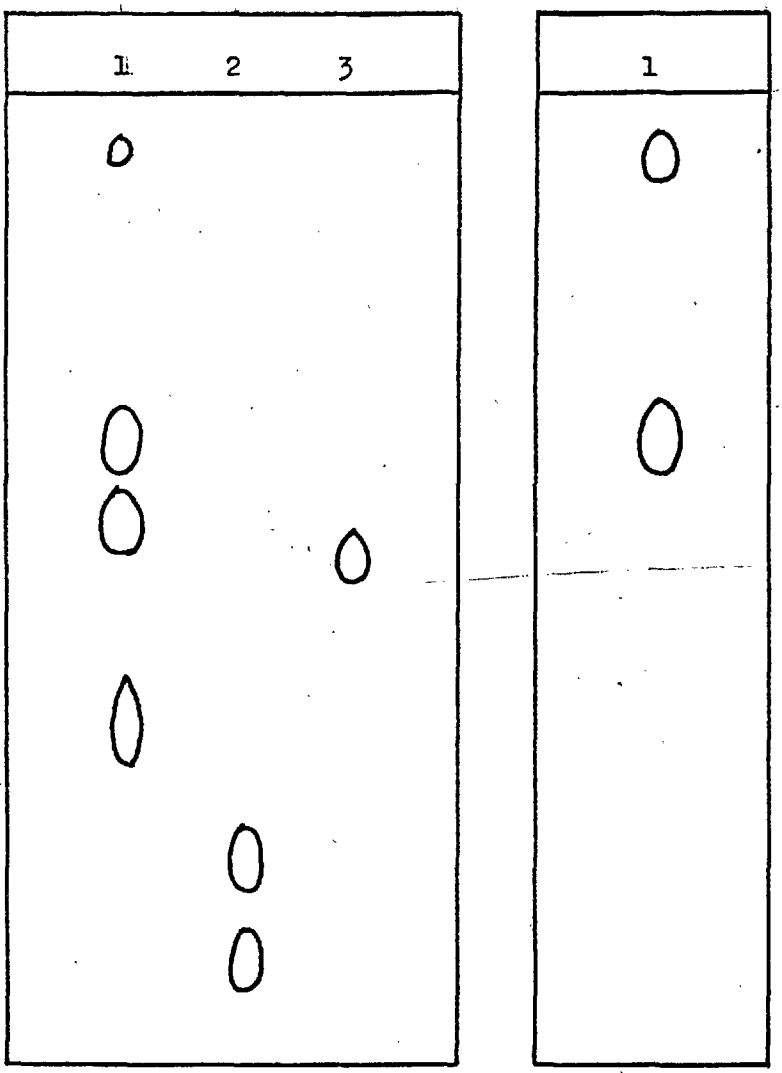
It is apparent that acid hydrolysis of the glycopeptide has yielded four or five different compounds. There is no satisfactory correspondence of any of these with AMAB as had been hoped. In the absence of synthetic model compounds as markers it is not possible to attribute the components obtained to any particular degradation product except that in one case one of the spots can be identified with reasonable certainty as glucosamine (indicated in fig. 49). This suggests that the reduction was incomplete and that some of the acetamido groups have remained unchanged. It was also noted that no aspartic acid could be detected, indicating complete reduction of all the carboxyl groups.

Electrophoresis

Again it is seen that the hydrolysis has failed to give any substance corresponding with either AMAB or aspartic acid, although several other degradation products have been separated.

It is concluded that although the reduction is incomplete, the failure to detect any aspartic acid (or aminobutanediol) indicates that in all molecules the amide bond of the linkage has been reduced. Furthermore the marked resistance to hydrolysis is explained if the acetamido group on the sugar residue to which the aspartic acid is attached has also been reduced to give an N-ethyl group.

Fig. 50



Electrophoresis of reduction products of glycopeptide in formic/acetic buffer pH 1.9

1. Hydrolysed reduced glycopeptide
2. FII and FIII
3. Aminobutanediol

There would then be two positively charged groups in close proximity to the bond that is to be split which would effectively prevent the approach of the proton necessary for hydrolysis.

These findings were in agreement with the results obtained with AADG. It was therefore decided to acetylate the reduced glycopeptide and repeat the hydrolysis.

Acetylation of the reduced glycopeptide

A solution of the glycopeptide (2.0 ml; containing 2.4 mg glycopeptide) was evaporated to dryness and dissolved in 3.0 ml of pyridine and 2.0 ml of acetic anhydride. The solution was shaken at 4° for 18 hr. Excess reagent and solvent were removed by evaporation and the residue was dissolved in a small volume of water.

Electrophoresis of the products

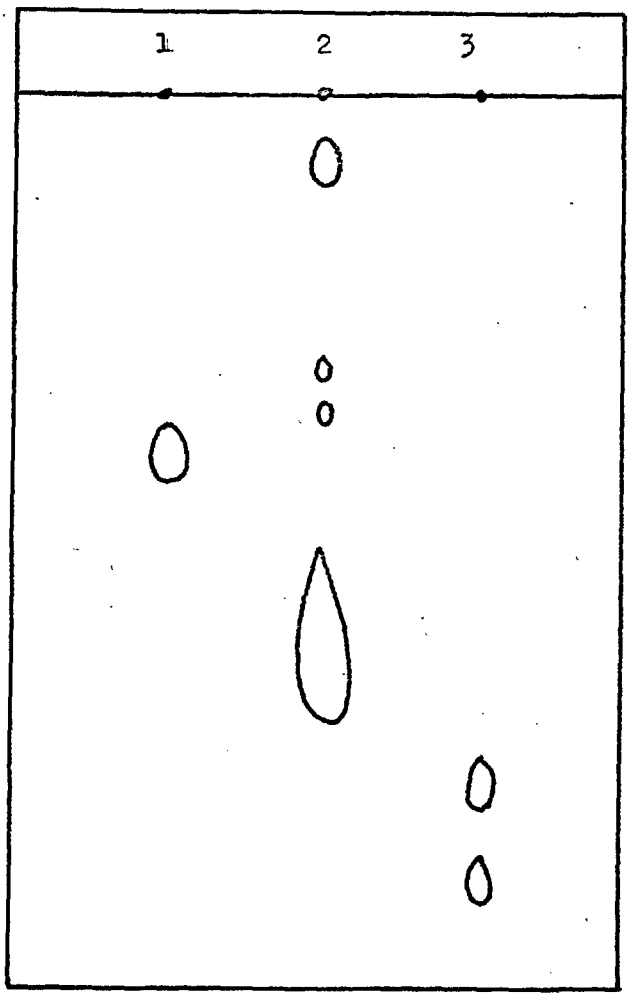
The conditions employed were as described before. The pattern obtained is shown in fig. 51.

Results and discussion

The electrophoretic pattern obtained by staining with ninhydrin indicates that the hydrolysis has yielded two main components, one with very little mobility (probably only that due to electroendosmosis) and the other of greater intensity with a mobility equivalent to 1.5 positive charges as compared with glucosamine. This latter compound gave a brownish colour with ninhydrin. There were also two minor components, one of which was probably attributable to glucosamine. Most notable however, was the absence of any spot corresponding with ANAB.

The compound with very little mobility (which also gave a brown-

Fig.. 51



Ninhydrin

Electrophoresis of acetylated hydrolysed reduced glycopeptide in formic/acetic buffer pH 1.9

1. Glucosamine
2. Hydrolysed acetylated reduced glycopeptide
3. FII and FIII

ish colour with ninhydrin) was possibly unhydrolysed partially reduced glycopeptide.

The identification of the major component will be discussed later.

Since no AMAB has been obtained by this method an alternative hydrolysis scheme was used. It was considered that the failure to detect AMAB was possibly due to the interference of degradation products from the sugar residues that are not present in AADG. It seemed possible therefore that if a preliminary mild acid hydrolysis were performed to cleave any susceptible glycosidic bonds (those not involving the reducing carbon atom of an amino or N-ethylamino sugar) and the fragment containing the aspartic acid-carbohydrate linkage separated, acetylated and hydrolysed, then a situation more closely resembling the case of AADG might be achieved.

Further experiments on the reduced glycopeptide

Reduced glycopeptide (5.5 mg) was dissolved in 3.0 ml of 2 N HCl and heated under reflux for 3 hr.

The hydrolysate, without further treatment, was applied to a column of Dowex-2 generated in the acetate form and was eluted with water.

Fractions of 5.0 ml were collected by hand and spot tested on paper by the ninhydrin method. A positive reaction was observed in tubes 2, 3, 4 and 5 (the pH was neutral). The tubes were pooled and evaporated to a small volume and applied to a column of Dowex-50 generated in the hydrogen form. Elution was commenced with water and run until 120 ml had been collected when the elution was changed

to 2 N HCl. Fractions of 10.0 ml were collected and the pH observed to become acid in tube 5. Spot testing on filter paper withninhydrin gave a positive reaction in tubes 5 and 6 which were pooled and evaporated to dryness.

The residue was dissolved in 3.0 ml of pyridine and 2.0 ml of acetic anhydride and shaken at 4° for 18 hr. The reagent and solvent were removed by evaporation and the residue hydrolysed in 2 N HCl by heating under reflux. The hydrochloric acid was removed by evaporation and the residue was dissolved in a small volume of water.

Electrophoresis of the products of hydrolysis

The conditions were as described before. The pattern obtained is shown in fig. 52.

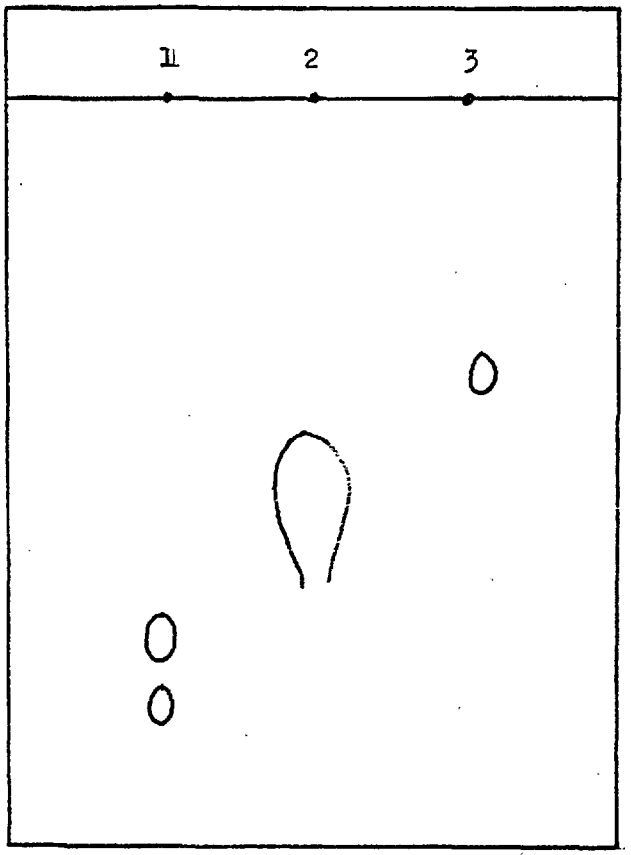
Results and discussion

Electrophoresis shows a single component staining brown with ninhydrin and having 1.5 positive charges relative to glucosamine and AMAB. None of the latter compound could be detected for certain although the single component tended to extend forwards into the region characteristic of AMAB.

It seems possible that the single brown staining component observed in each of the hydrolysates may be explained in the following way.

From the studies of several groups of workers (Fletcher et al, 1963; Clamp and Hough, 1965; Montgomery et al, 1965) it is likely that the heterosaccharide unit is a branched structure possibly of the form shown in fig.1. It is reasonably certain that the N-acetyl-

Fig. 52



Ninhydrin

Hydrolysate of acetylated reduced glycopeptide after preliminary purification on Dowex-2. Electrophoresis in formic/acetic buffer pH 1.9

1. FII and FIII
2. Hydrolysate of glycopeptide
3. Glucosamine

glucosamine that is involved in the linkage is also linked at either the 3, 4 or 6 position to a second N-acetylglucosamine residue. This structure might help to explain the major component possessing 1.5 positive charges. The molecule that would be expected to arise from the LAH reduction of an asparagine-N-acetylglucosamine-N-acetylglucosamine complex would be AMAB-N-ethylglucosamine-N-ethylglucosamine which would possess four positive charges and would be expected to be very resistant to acid hydrolysis. The discrepancy in the mobility between the observed and that expected with four positive charges is possible due to the large size of the molecule and firm adsorption to the paper.

The most probable explanation of the findings, therefore, is that the reduction yields a fragment that is sufficiently stable to acid hydrolysis to prevent the liberation of any AMAB. The failure of acetylation to overcome the problem is possibly due i) to a failure to acetylate the N-ethyl groups and the secondary amine in the linkage or ii) to the rate of deacetylation under the conditions of hydrolysis greatly exceeding the rate of cleavage of the bonds holding the individual residues together. The first possibility would appear to be a little unlikely since the procedure has been a success in the case of AADG.

The failure of these experiments necessitated the development of an alternative method to overcome the difficulties of hydrolysis.

It was proposed to prepare a fully methylated, N-benzyloxycarbonyl glycopeptide which after reduction with LAH would be made to react with 2,4-dinitrofluorobenzene to form the DNP derivative, and would

then be hydrolysed to give di-DNP-AMAB and the DNP derivatives of variously methylated N-ethylglucosamines. Paper chromatography would then be used to compare the products of reduction with a genuine sample of di-DNP-AMAB (the preparation of this compound was described earlier).

Experimental

Methylation of N-benzyloxycarbonyl glycopeptide

The glycopeptide was carbobenzoxylated as described previously. A solution (5.0 ml) of this material containing approximately 75 mg of glycopeptide was evaporated to dryness and the residue dissolved in 1.0 ml of dimethyl sulphoxide (obtained from B.D.H.). Barium oxide (1.2 gm) and iodomethane (1.0 ml; obtained from B.D.H.) were added and the mixture left at 36° for 18 hr after which a further addition of similar quantities of dimethyl sulphoxide, barium oxide and iodomethane was made. This was again repeated after 8 hr and the mixture left for a further 18 hr at 36° after which it was extracted with 10 ml of chloroform. An emulsion formed which was broken by centrifugation for 15 min at 2500 rev/min. The chloroform layer was pipetted off and the residue extracted three times into chloroform. The chloroform extracts were pooled, washed once with water and then dried over anhydrous sodium sulphate. The extract was then evaporated to give a brownish oil which was dissolved in 1-2 ml of chloroform and dropped into cold 80/100 petroleum ether. A viscous brownish oil separated out and long needle shaped crystals of dimethyl sulphoxide formed on standing in the cold for 1 hr. The petroleum ether and dimethyl sulphoxide were decanted off and the

oily residue dissolved in 1.0 ml of chloroform. The treatment with 60/100 petroleum ether was repeated twice, a pale brown viscous oil being obtained which was dried in a vacuum dessicator over P_2O_5. An infrared spectrum performed in tetrahydrofuran was very poorly defined and no definite estimate of the degree of methylation could be made.

Reduction of methylated benzyloxycarbonyl glycopeptide with LAH

The methylated glycopeptide was dissolved in 5.0 ml of tetrahydrofuran and 150 mg of LAH was added. The mixture was heated under reflux for 4 hr. Excess LAH was destroyed by the addition of tetrahydrofuran-water in the proportions 50:50 and the insoluble material was removed by filtration. The residue was extracted three times by refluxing for 20 min in tetrahydrofuran. The filtrates were pooled and evaporated to dryness. Spot testing of the filtrate on filter paper gave a brown colour with ninhydrin.

An attempt to acetylate and hydrolyse the material was made at this stage but electrophoresis failed to demonstrate any AMAB. Silver staining gave a pattern that was very similar to that found in the previous experiment.

Formation of the DNP derivative of the reduced methylated glycopeptide

The reduced methylated glycopeptide solution was made alkaline by the addition of 20-30 mg of sodium carbonate. 2,4-dinitrofluorobenzene (0.01 ml) in ethanol was added giving a clear solution which was stood at room temperature for 18 hr. The intense yellow solution so obtained was pipetted off the excess sodium carbonate and evaporated to dryness. The residue was hydrolysed in 5.0 ml of 6 N HCl by

heating under reflux for 1 hr. The HCl was removed by evaporation and the residue dissolved in 0.5 ml of ethanol.

Paper chromatography

Descending paper chromatography on Whatman's 3MM was performed in the following solvent systems.

i) Butanol-acetic acid-water in the proportions 120:30:50.

ii) Distilled water.

iii) 70% N-propanol-water

iv) 50% N-propanol-water with 1% ammonia.

v) 30% N-propanol-water

vi) N-hexane-pyridine in the proportions 70:30.

vii) N-hexane-pyridine in the proportions 80:20.

viii) N-heptane-pyridine in the proportions 70:30.

Di-DNP-AMAB (FII), DNP-FIII, di-DNP-diaminobutyric acid and dinitrophenol were used as marker substances. The first two were prepared as described previously.

Results and discussion

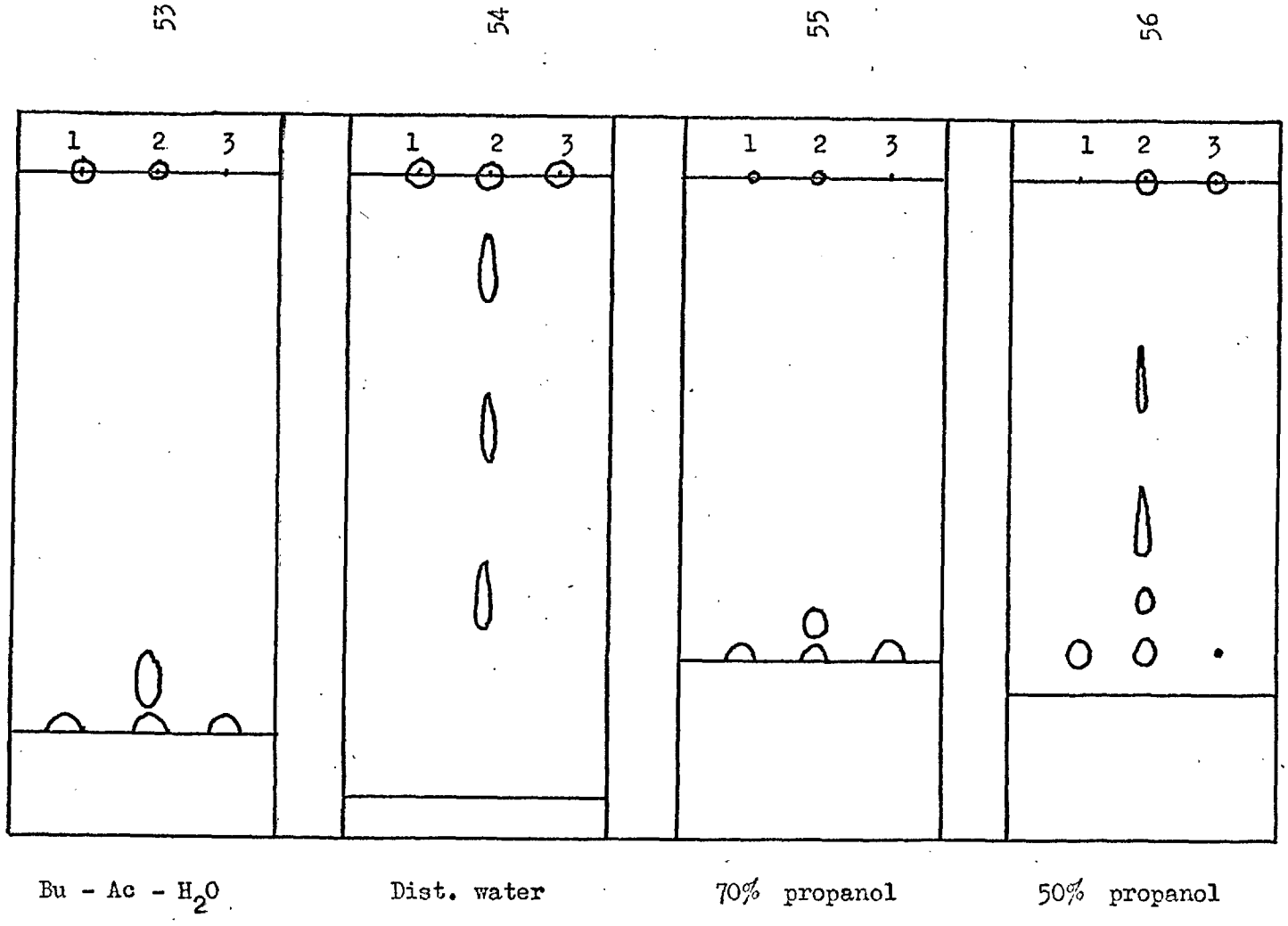
The chromatograms obtained by the use of the solvent systems listed above are shown in figs. 53-60. In each case the hydrolysate of the DNP-reduced methylated glycopeptide yields spots corresponding with di-DNP-AMAB and DNP-FIII. In some of the solvents the relevant substances have run either withthe solvent front or have remained at the origin, but in the cases of solvents iv), vi) and vii) quite satisfactory intermediate mobilities have been obtained. Moreover in solvent iv), di-DNP-AMAB has been separated from DNP-FIII and spots corresponding with both of these substances are apparent

in the hydrolysate.

It is concluded from this experiment that substitution of the reduced glycopeptide by a group that is resistant to acid hydrolysis (DNP-) allows the liberation of the substituted AMAB. The reactions involved also liberate other DNP substituted materials that have not been identified.

It is clear from this experiment that the proposed N-acyl glycosylamine linkage to the β-carboxyl group of aspartic acid is correct for in no other way could di-DNP-4-amino-2-methylaminobutane-1-ol arise. The single molecule of ammonia released by mild hydrolysis is therefore correctly assigned to a position in the linkage. The utilisation of this procedure as a general method for determining the nature of the carbohydrate-protein linkage in glycoproteins is probably limited by the need to prepare a glycopeptide containing only the amino acid to which the carbohydrate is attached. If other amino acids are present then reduction with LAH would give a polyamine that would be totally resistant to hydrolysis so preventing the liberation of AMAB or its DNP derivative.

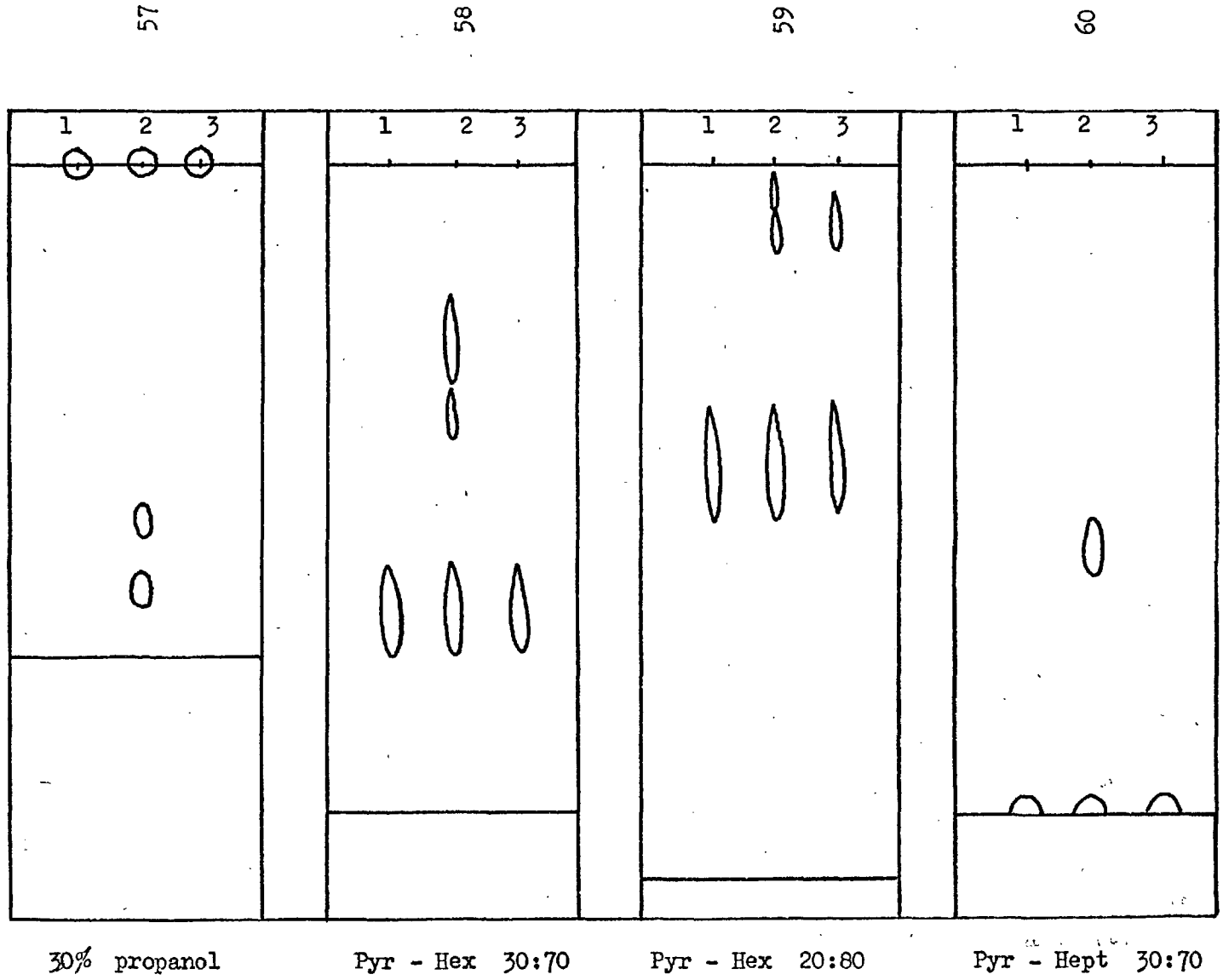
Figs. 53 - 56



Paper chromatography of hydrolysate of DNP reduced methylated glycopeptide

1. DNP- FII 2. Hydrolysate of DNP-glycopeptide 3. DNP- FIII

Figs. 57 - 60



Paper chromatography of hydrolysate of DNP reduced methylated glycopeptide

1. DNP- FII 2. Hydrolysed DNP-glycopeptide 3. DNP- FIII

THE INVESTIGATION OF THE GROUP IONISING AT HIGH pH IN THE ASPARTIC ACID-CARBOHYDRATE COMPLEX FROM OVALBUMIN AND STUDIES ON RELATED COMPOUNDS

THE INVESTIGATION OF THE GROUP IONISING AT HIGH pH IN THE ASPARTIC ACID-CARBOHYDRATE COMPLEX FROM OVALBUMIN AND STUDIES ON RELATED COMPOUNDS

INTRODUCTION

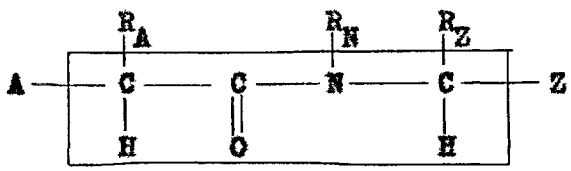
The titration of the ovalbumin glycopeptide having aspartic acid as the only amino acid would be expected to show two ionisable groups, one corresponding with the α-carboxyl group and the other corresponding with the amino group of aspartic acid. The carbohydrate component would not be expected to make any contribution since all three glucosamine residues are acetylated.

An earlier titration of a glycopeptide containing several other amino acids besides aspartic acid (Johansen et al, 1963) showed the two expected ionisations but also gave some indication that a third group ionising at a high pH might be present. In the experiments described in this thesis it is this third group that has been studied and accordingly titrations have been continued up into the pH range 12.0-13.0. In a recent paper Montgomery, Lee and Wu (1965) have also noticed an ionisation in this range but have attributed it to degradation products.

The localisation of this group presented something of a problem but it was suspected that one of the amide bonds in the molecule might be involved. On this assumption it was considered worthwhile to examine the ultraviolet spectrum of the compound in the region in which the amide bond is known to absorb.

Ley and Arends (1932) demonstrated that acetamide absorbs in

the 200 mμ region and five years later Magill et al (1937) noticed absorption below 240 mμ in peptides. This absorption amounted to more than the absorption of the component amino acids and they attributed the excess to the peptide bonds. More recently Ham and Platt (1952) have shown this absorption to be a broad band with a maximum at 185 mμ which they considered corresponded to a transition. In extending these studies Saidel and coworkers in a number of papers (Saidel, 1955; Saidel and Lieberman, 1958) have investigated the effect of various substitutions on the basic peptide chromophore which they represent thus:-



They have shown that absorption of this chromophore is markedly affected by the presence of ionisable groups on the various possible substitutents. Thus, an ionised carboxyl at Z results in a considerable increase in absorption and if molar extinctions are plotted against pH an S shaped curve is produced with an inflexion at the pK of the carboxyl group. This indicates that the spectral change is not a direct effect of acid upon the peptide link but is an indirect effect operating through the carboxyl group.

It seems possible that a similar change might be observable in the glycopeptide in the region in which the ionisation is detected by electrometric titration.

In a preliminary experiment it was shown that N-acetylglucosamine

in water has an amide absorption spectrum with a molar extinction of 2060 at 205 mμ. Before further discussing the high pK of the glycopeptide it is proposed to consider the behaviour of N-acetyl-glucosamine in alkali.

Experimental

Free N-acetylglucosamine was examined by potentiometric titration and also by measuring the ultraviolet absorption at different pH's in the alkaline range.

An estimate of the molar extinction was obtained by taking three weighed amounts of N-acetylglucosamine (21.635, 21.930 and 22.42 mg) dissolving each in water and making up to 1 litre. Three spectra of each solution were recorded and the extinction of each measured at 205 mμ. The values obtained were 2370; 2320; 1760; 1910; 1910; 1980; 2080;2120 (one result differed so widely from the rest that it was not included) giving a mean of 2060.

Free N-acetyl glucosamine (30.2 mg) was dissolved in approximately 1 ml of 0.1 M KCl, made up to 1.5 ml and titrated with a glass electrode (Radiometer) using a micro-cell of 2-3 ml capacity through which a slow current of nitrogen was passed. Measurements were made at 20° and additions of acid or alkali were made with an Agla micrometer syringe. The same apparatus was used in all other potentiometric titrations that are described. The curve obtained is shown in fig. 61 and the calculations are shown in table 26.

Spectrophotometric titration was performed at 26° by making up

a stock solution of N-acetylglucosamine (233.4 mg in 100 ml of water). Portions (2.0 ml) of this stock solution were taken and diluted with 3.0 ml amounts of sodium hydroxide solutions of different concentration. The pH of the resulting mixture was measured with a glass electrode and a solution of sodium hydroxide of matching pH was made up for use in the reference cell. Suprasil cells of light path 0.1 cm were used. A recording spectrophotometer (Unicam SP 700) was used and spectra were recorded from about 200 mμ to 240 mμ. Readings were taken at 218 mμ and the values were plotted against pH. The curve obtained is shown in fig. 62 and the calculations are shown in table 27.

Results and discussion

An examination of the titration curve of N-acetyl glucosamine obtained by the electrometric method indicates a pK value of about 11.8. The calculated value obtained after correction for the concentration of hydroxyl ions and taking the mean of five readings was 11.76 at 20°.

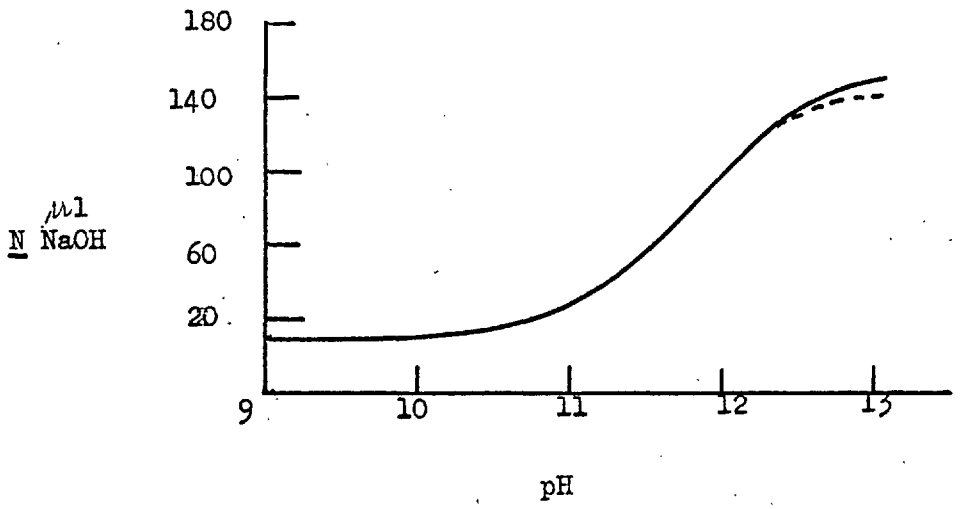
The value calculated from the curve obtained by spectrophotometric titration, taking the mean of six readings was 11.65 at 26°.

The acidic properties of the sugars was first noted by Kullgren (1898) who formulated the reaction thus,

$$R - OH \longrightarrow R - O^{-} + H^{+}$$

Michaelis and Rona (1913) observed that alcohols such as glycerol and mannitol were also weakly acidic but to a considerably lesser degree than the sugars. Some years later Urban and Shaffer

Fig. 61



Potentiometric titration of N-acetylglucosamine 30.2 mg

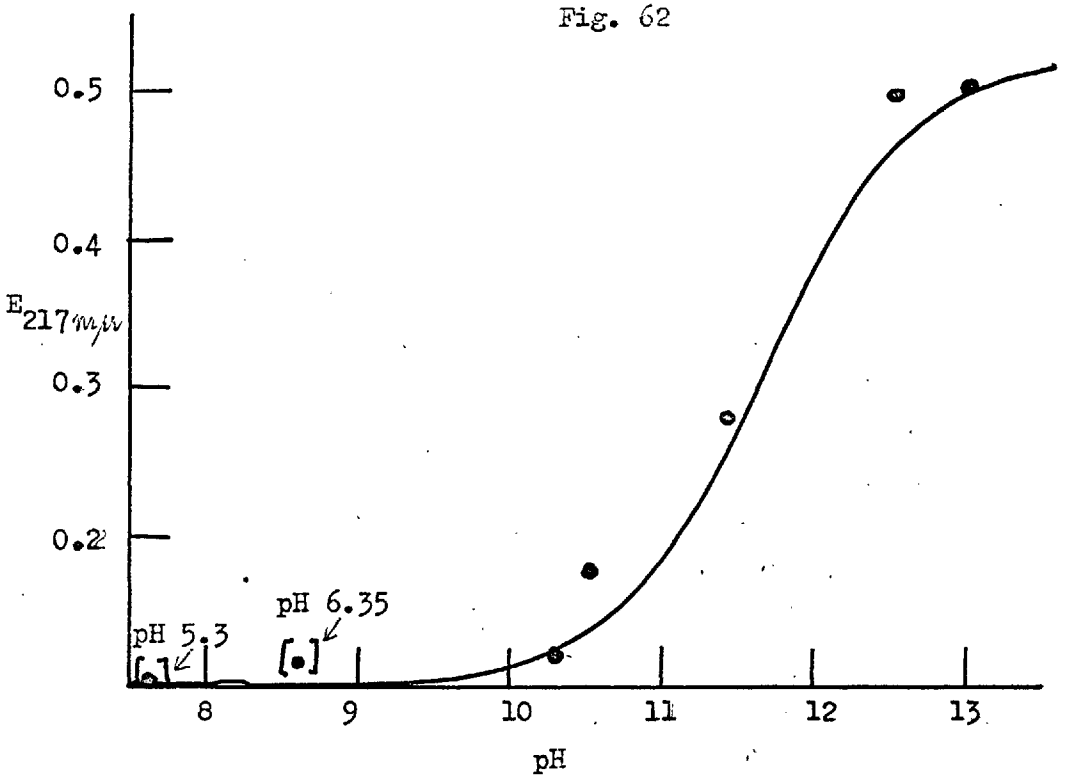
Table 26

Calculations of pK

pH	[HA]	$[A^-]$	$\{OH^-\}$	$\frac{[HA]+\{OH^-\}}{[A^-]-\{OH^-\}}$	log	pK_a
10.8	0.078	0.013	0.00043	6.21	0.794	11.59
11.2	0.065	0.026	0.00107	2.66	0.392	11.59
11.6	0.052	0.039	0.0027	1.51	0.179	11.78
11.9	0.039	0.052	0.00675	1.01	0.004	11.90
12.0	0.026	0.065	0.0085	0.610	-0.214	11.79
12.3	0.013	0.078	0.0135	0.342	-0.342	11.88

Mean value of pK_a = 11.76 at 20°

Fig. 62



Spectrophotometric titration of N-acetylglucosamine

Table 27

Calculations of pK

d_M = optical density of the unionised molecule = 0.105

d_I = optical density of the ion = 0.520

pH	d	$d_I - d$	$d - d_M$	$\log \frac{d_I - d}{d - d_M}$	pK
10.3	120	400	15	+ 1.43	11.73
11.0	175	345	70	+ 0.69	11.69
11.5	275	245	170	+ 0.16	11.66
12.0	395	125	290	- 0.365	11.63
12.5	470	50	365	- 0.863	11.64
13.0	505	15	400	- 1.43	11.57

Mean value of pK_a = 11.65 at 26°

(1931) postulated that the sugars were in fact polybasic acids and they determined the values of two dissociation constants for glucose, fructose and sucrose. In the case of the latter compound they suspected the presence of a third acidic group having a pK value in the region of 14.2. Table 28 summarises some of the sugars and other relevant substances and their pK values.

It seems reasonable to attribute the pK_1 of the sugars to an ionisation on the hydroxyl group at C-1 although there is little comment to be found in the literature on this point. The values observed (in the region of pH 12) are in quite good agreement with figures obtained from a consideration of analogous structures. Thus the pK of ethanol (16.2; Ballinger and Long, 1960) is lowered by about two pH units if a second oxygen atom is linked to the carbon atom of the primary alcoholic group, as in the case of the hydrated form of acetaldehyde which has a pK of 14.2 at 0° (Euler, 1906). A similar situation is seen if the pK of trichloroethanol (12.24 at 25°, Ballinger and Long, 1960) is compared with the pK of chloral hydrate (9.77 at 25°, Gustaffson and Johansen, 1948). In this case the second oxygen atom causes a lowering of the pK by about 2.5 pH units. However the difference is accounted for by the fact that the pK of acetaldehyde was measured at 0° whereas the pK of ethanol is an extrapolated value based on pK's of other substances measured at 25°.

Furthermore the pK's of ethylene glycol (14.24 at 19°, Michaelis, 1913; 15.1 at 25°, Ballinger and Long, 1960), glycerol (14.15 at

Table 28

The pK values of some weak acids

Substance	pK_1	pK_2	T^o	Method	Reference
Ethanol	16.2				Ballinger & Long (1960)
Methanol	15.5		25		" "
Acetamide	15.1			C	Branch & Clayton (1928)
Acetaldehyde	14.2		0		Euler (1906)
Ethylene glycol	14.24		19	H	Michaelis (1913)
Glycerol	14.15		17.5	H	Michaelis & Rona (1913)
Erythritol	13.9		18	H	" "
α-methyl glucoside	13.71		16.5-19	H	" "
β-methyl glucoside	13.58		15.5-19	H	" "
Sorbitol	13.6		17.5	H	" "
"	14.14		0	G	Thamsen (1952)
"	13.57		18	G	"
Dulcitol	13.46		19.0	H	Michaelis & Rona (1913)
Mannitol	13.47		17.5	H	" "
"	14.09		0	G	Thamsen (1952)
"	13.50		18	G	"
Glucose	12.18		17.5-18	H	Michaelis & Rona (1913)
"	12.92		0	G	Thamsen (1952)

Substance	pK_1	pK_2	T^o	Method	Reference
Glucose	12.43		18	G	Thamsen (1952)
"	12.09	13.85	25	H	Urban & Shaffer (1931)
"	12.11	13.81	25	H & C	Hirsch & Schlagg (1929)
"	12.34	14.10	17-18.5	H	Urban and Shaffer (1931)
Galactose	12.28		17-18.5	H	Michaelis & Rona (1913)
Mannose	11.97		17-18.5	H	" "
Fructose	12.05		18.5	H	" "
"	11.68	13.24	25	H	Urban & Shaffer (1931)
"	11.7	13.81	25	H & C	Hirsch & Schlagg (1929)
"	12.5	14.18	4	H	Urban & Shaffer (1931)
Sorbose	11.56		17.5	H	Michaelis (1913)
Rhamnose	12.2		15.5	H	"
Xylose	12.14		18.0	H	"
Arabinose	12.34		17.0	H	Michaelis & Rona (1913)
Sucrose	12.62		18.5-19	H	" "
"	12.60	13.52	25	H	Urban & Shaffer (1931)
"	12.51	13.52	17.5	H	Hirsch & Schlagg (1929)
Maltose	12.05		17.5	H	Michaelis (1913)
Lactose	12.22		16.5	H	Michaelis & Rona (1913)
Raffinose	12.74		18.5	H	" "

H = hydrogen electrode C = conductivity G = glass electrode

17.5°, Michaelis and Rona, 1913) indicate that neighbouring hydroxyls, through a combination of inductive and statistical effects, also lower the pK of an isolated alcoholic group by about 2 pH units. Thus the combined effects of these two factors would give an expected pK of approximately 12.2 in the case of the reducing sugars. It is not possible to decide if this ionisation occurs in the straight chain aldehydic form or in the ring form since in both cases the carbon atom at position 1 is linked to two oxygen atoms, that is, the hydrated aldehydic group in the former and the oxygen of the ring in the latter.

In agreement with the above assignment are the observed pK's of the α- and β-methylglucosides which are about 1.5 pH units higher than in the free sugar, and thus correspond more closely with the values observed in the sugar alcohols and in the pK_2 values of the reducing sugars.

In the case of N-acetylglucosamine the pK_1 is distinctly lower than in the other sugars (by about 0.4-0.5 of a pH unit), an effect that is presumably due to the presence of the acetamido group at C-2, although the difference in the pK's of N-acetylglycine (3.67 at 20°; King and King, 1956) and glycollic acid (3.83 at 25°; Nimms, 1936) is only 0.16 of a pH unit. There is no obvious reason for the discrepancy in the effect of the acetamido group but it is possible that steric factors may play a part. It is however fairly certain that the acetamido group has a distinctly greater acidifying effect than a hydroxyl group on a neighbouring ionisation.

The titration of AADG

The same experimental techniques were used as in the previous titrations.

For the potentiometric titration AADG (10.33 mg) was dissolved in water and titrated against N HCl and N NaOH. The curve obtained is shown in fig. 63.

For the spectrophotometric titration AADG (1.4 mg) was dissolved in 5.0 ml of water and treated with alkali as previously described. The absorption was read at 213 mμ, using 1.0 cm Suprasil cells. The curve obtained is shown in fig. 64.

The titration of the glycopeptide

The same experimental techniques were used as before.

Glycopeptide (16.34 mg) prepared by pronase-carboxypeptidase digestion and known to contain aspartic acid as the only amino acid was dissolved in approximately 1 ml of 0.1 M KCl and potentiometrically titrated with 0.1 N HCl and 0.1 N NaOH. The curve obtained is shown in fig. 65. The spectrophotometric titration is shown in fig. 66

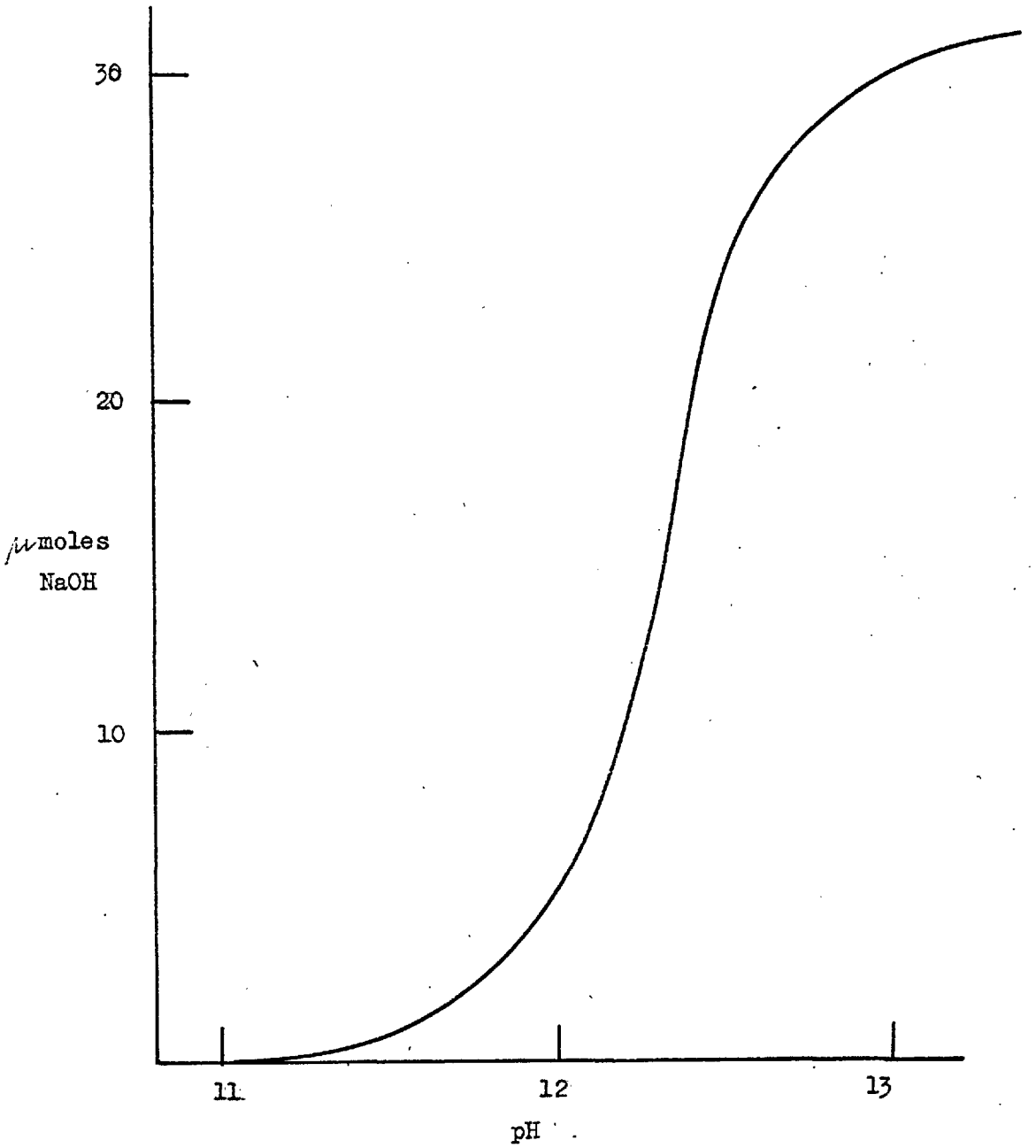
The titration of the N-benzyloxycarbonyl derivative of N-(L-β-aspartyl)-β-glucosylamine.

The material used in this experiment was prepared and kindly given by Dr. G. S. Marks, (Marks and Neuberger, 1961).

The substance (73.98 mg) was dissolved in approximately 1 ml of water and titrated with 1.0 N HCl and 0.5 N NaOH.

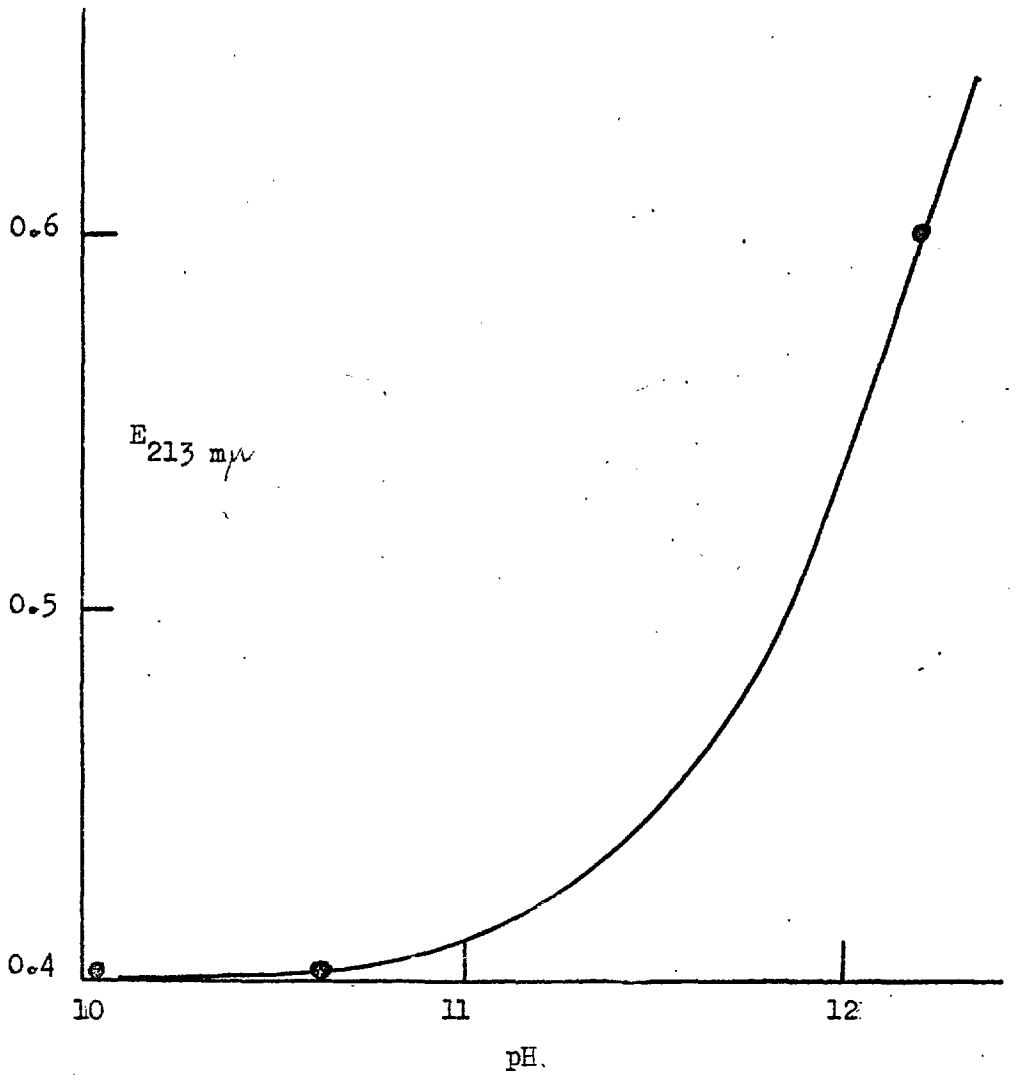
Spectrophotometric titration was not performed.

Fig. 63



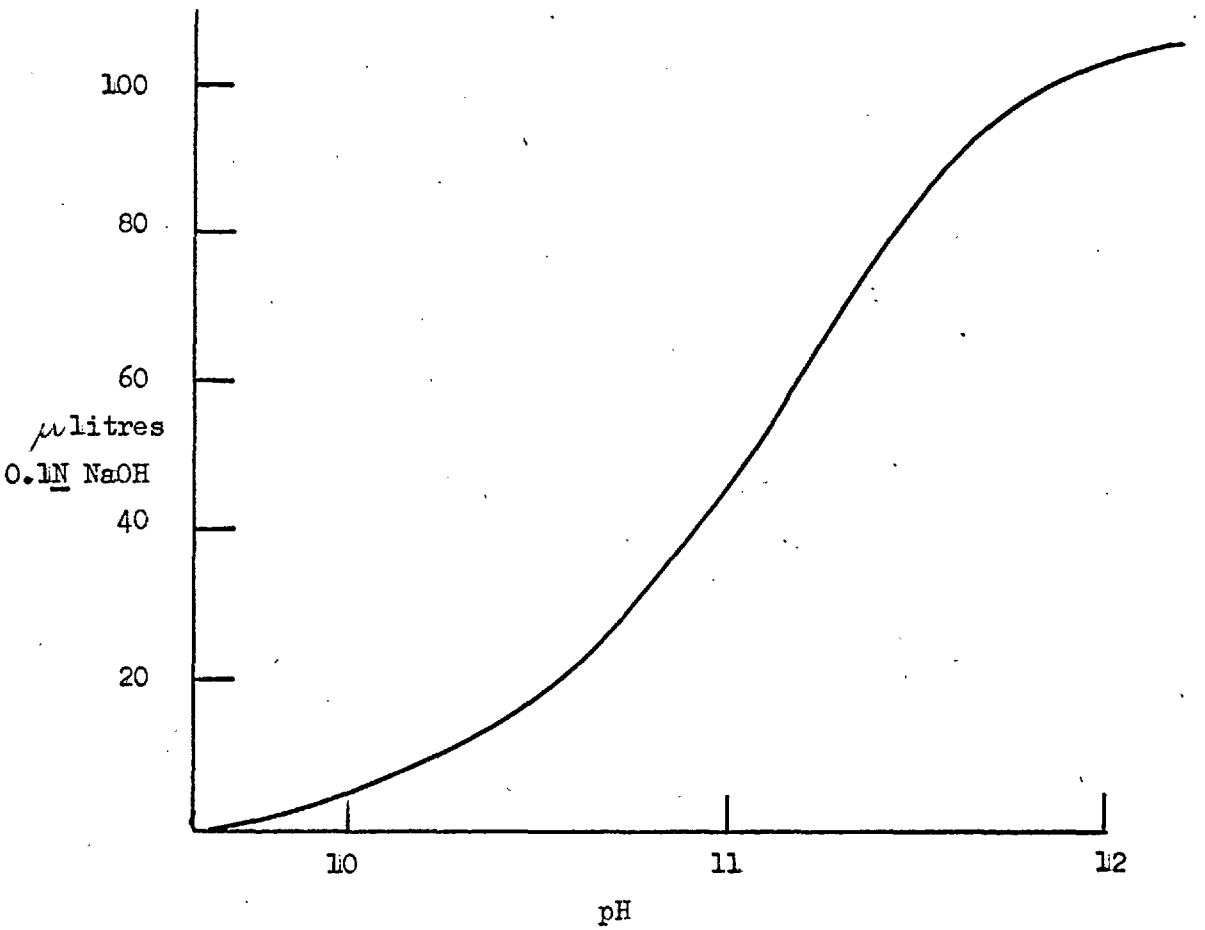
Potentiometric titration of AADG

Fig. 64



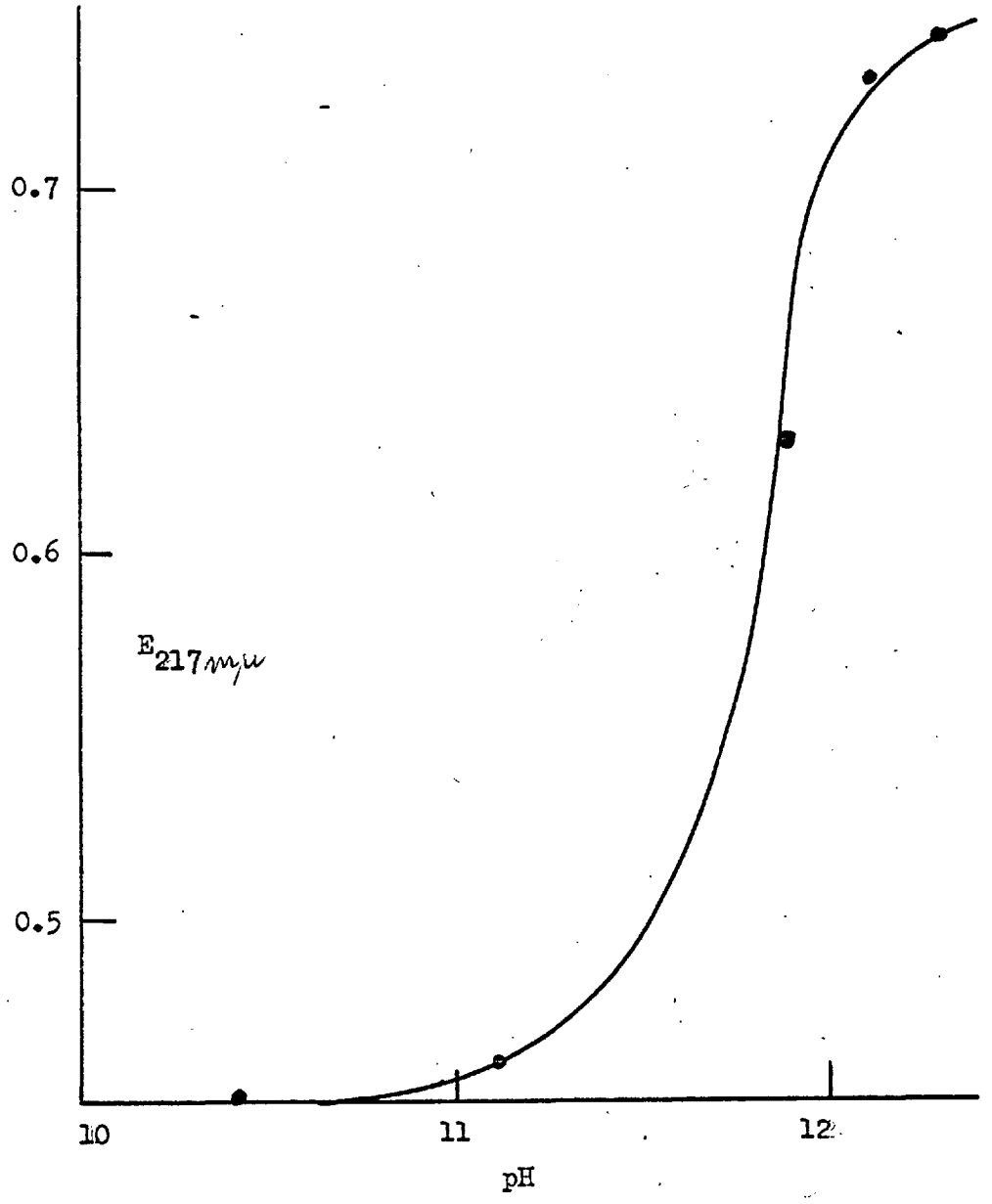
Spectrophotometric titration of AADG

Fig. 65



Potentiometric titration of the glycopeptide

Fig. 66



Spectrophotometric titration of the glycopeptide

The titration of diacetylethylenediamine

Potentiometric titration of this substance on several occasions failed to give convincing evidence of any pK in the range accessible to this method.

For the spectrophotometric titration diacetylethylenediamine (20.095 mg) was dissolved in water and made up to 500 ml and treated as described previously. The curve so ontained is shown in fig. 67.

Preparation of diacetylethylenediamine

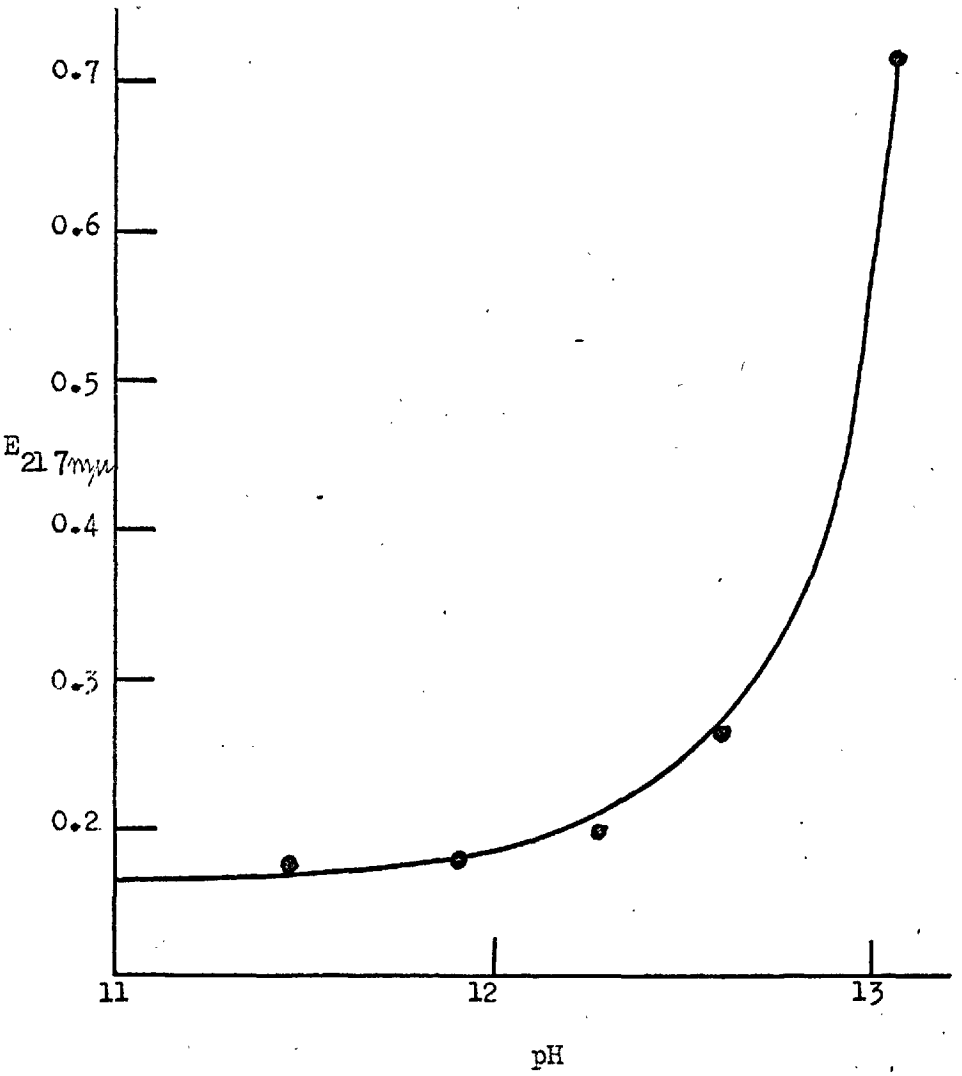
Ethylenediamine (40.0 ml) was mixed with 60 ml of water and acetic anhydride (110 ml) was slowly added with cooling under running water. The reaction mixture was left at room temperature for 20 min and then evaporated to dryness. The product was twice crystallised from ethanol to give a substance with M.P. 172-173°.

Results and discussion

It is seen from the curves that are obtained from the titration of AADG by both the potentiometric and the spectrophotometric methods that this substance possesses a group with a pK of 12.3. In the case of the glycopeptide the curve obtained by the spectrophotometric method indicates a pK of 11.9 but that obtained by the potentiometric method gives the lower value of 11.25. The reasons for this discrepancy are not clear. It is probably explicable in terms of a difference in experimental technique. It has not been possible to repeat the titrations because sufficient glycopeptide of the necessary composition has not been available.

This type of ionisation and its associated spectrophotometric

Fig. 67



Spectrophotometric titration of N,N'-diacetylethylenediamine

changes have not previously been described in proteins or glycoproteins. However the ionisation of amide groups has been described in a wide variety of compounds and in some cases the change in ultraviolet absorption has also been investigated. The ionisation constants of these substances vary over a wide range according to the structure of the molecule in which the amide group is present. In particular neighbouring carbonyl groups as in the grouping .CO.NH.CO.NH.CO. are responsible for greatly increasing the acidity of the substance.

Three questions concerning the third pK of the glycopeptide arise, i) is the pK value that is observed in reasonable agreement with theoretical considerations derived from analogous structures, ii) what is the basis of the spectral change and, iii) what is the most likely form of the ionising structure?

In an attempt to answer question i) it is seen from table 28 that the pK of acetamide is 15.1 and from the discussion on the pK of N-acetylglucosamine it has been shown that the effect of the ring oxygen in a sugar is to reduce the pK of a group on C-1 by about 2 pH units. Furthermore it seems that the effect of an acetamido group as compared with a hydroxyl group on C-2 is to lower the pK of a group on C-1 by about 0.5 of a pH unit. If a further lowering by an uncertain amount, due to hydroxyl groups in the molecule, is taken into account then the observed value of 11.8 (spectrophotometric titration) is probably in accordance with expectations.

With regard to question ii), Stuckey, (1947) has investigated

the absorption spectrum of hydantoin and has drawn attention to the possibility of keto-enol tautomerism in the .CO.NH. group having shown that the second carbonyl group in the .CO.NH.CO. group facilitates enolisation in alkaline solution. Using the open chain compounds acetylurea and hydantoic acid Stuckey has shown that the former exhibits keto-enol tautomerism whereas the latter does not, which would appear to confirm the effect of the second carbonyl group.

Schauenstein and Perko (1954) also working with the hydantoins have come to essentially the same conclusions, attributing the absorption changes to the -C=N- group. With regard to question iii) it appears that the anion is likely to be of the form C=N-

In the case of AADG and the glycopeptide a very similar grouping is found in the linkage between sugar and amino acid, except that the second carbonyl group is replaced by the ring oxygen of N-acetylglucosamine. It also seems probable that the acetamido group on C-2 facilitates tautomerisation and ionisation of the amide bond in the linkage since no ionisation is observed in aspartylglucosylamine (the model compound without the 2-acetamido group).

In the cases that have just been discussed it is clear that the keto-enol tautomerism and the ionisation are closely interrelated events or indeed different aspects of the same event. In the case of free N-acetylglucosamine however, the spectral changes possibly have a different origin. That is, they are not an expression of an ionisation occurring within the amide group but are a response to

an ionisation occurring on a neighbouring carbon atom (the hydroxyl on C-1). Thus there is a spectral change analogous to that investigated by Saidel and coworkers where the changes are caused by an ionisation at a distance from the peptide bond.

However it is also possible that the ionisation occurring in the linkage in the glycopeptide and AADG has an effect on the absorption of the acetamido group of the N-acetylglucosamine in the same way as the ionisation at C-1 does in free N-acetylglucosamine.

It is suggested that in glycopeptides of suitable composition this ionisation at high pH and its associated spectral changes might be of use in detecting the N-glycosidic type of linkage between N-acetylglucosamine and aspartic acid.

SUMMARY

The primary object of these studies has been to elucidate the precise nature of the chemical bond linking carbohydrate to protein in ovalbumin.

As a preliminary to any such studies it was necessary to prepare a glycopeptide containing only the amino acid (aspartic acid) to which the carbohydrate is attached. Two enzymic procedures were used. The first involved the use of a fairly non-specific protease (which is almost certainly a mixture of enzymes) known as Pronase-P which is derived from the micro-organism Streptomyces griseus. Digestion by this enzyme was followed by substitution of the free amino group of the glycopeptide by the benzyloxycarbonyl group and further digestion with carboxypeptidase. This was found to yield a glycopeptide of the required structure. The second enzymic procedure that was investigated involved digestion with papain followed by digestion with pronase. However this procedure yielded a glycopeptide containing fractional molar quantities of amino acids other than aspartic acid. It would appear that papain digestion gives a fragment that does not possess the specificity requirements for pronase to cleave the remaining amino acids.

The molecular weight of the glycopeptide prepared by the first method was estimated by measuring the molar extinction of a weighed amount of the N-benzyloxycarbonyl substituted glycopeptide. The

molar extinction of the benzyloxycarbonyl group was measured for a number of known compounds and a mean value calculated. A value of 1570 was obtained for the unsubstituted glycopeptide which is in agreement with the known composition of five residues of mannose, three residues of N-acetylglucosamine and one residue of aspartic acid.

The linkage was shown to involve the β-carboxyl group of aspartic acid by forming the phenylthiohydantoin derivative of the glycopeptide. It could be demonstrated that this procedure did not lead to any cleavage of the carbohydrate-protein bond as would have happened if the linkage had been through the α-carboxyl group.

Although it has been fairly certain for a number of years that the linkage was that of an N-glycoside there has never been any unequivocal chemical demonstration of the nitrogen in the bond since this is released as a mole of ammonia on hydrolysis. The possibility of reducing the bond from an amide to an amine by the use of lithium aluminium hydride (LAH), and isolating the diamino compound formed from aspartic acid (asparagine as it is in the glycopeptide) was studied.

It was necessary to synthesise this diamine (2,4-diaminobutane-1-ol) for use as a model compound and some methods of achieving this have been investigated. The reduction of N-benzyloxycarbonyl-asparagine with LAH and the characterisation of the products has been described. The main product was identified as 4-amino-2-methyl-

aminobutane-1-ol (AMAB). An alternative scheme involving the reduction of N,N'-diacetyldiaminobutyric acid methyl ester with lithium borohydride has also been investigated. A brief study of the LAH reduction of N-acetylmethylglucosaminide suggests that the acetamido group is reduced to an N-ethyl group.

Similar reductions of the model compound of the linkage (2-acetamido-1-(L-β-aspartamido)-1,2-dideoxy-β-D-glucose; AADG) by LAH have been made to yield AMAB. For some inadequately explained reason the reduction of the glycopeptide cannot be made to give this compound by the method that was successful with AADG. However the formation of the DNP derivative of the reduced glycopeptide yields di-DNP-AMAB on acid hydrolysis so demonstrating the N-glycosidic nature of the linkage. The synthesis of the DNP derivatives of the model compounds has also been described.

Potentiometric titration of the glycopeptide some years ago suggested the presence of an ionising group in the high alkaline range. This has been more carefully investigated and both potentiometric and spectrophotometric (using the absorption of the amide bond) titration curves have been plotted indicating an acid dissociation in the pH range 11-12. Similar titrations of AADG gave comparable results. It was suggested that the site of the ionisation was in the carbohydrate-protein bond and a consideration of compounds possessing similar groupings lent support to this idea. Thus spectrophotometric titration of diacetylethylenediamine indicated a pK

of 13+. It is concluded that the ionisation involves the release of a proton from the imino group in the carbohydrate-protein linkage.

Free N-acetylglucosamine was also titrated by both methods and pK values of 11.76 at 20° and 11.65 at 26° were obtained. This ionisation has been assigned to the hydroxyl at C-1 and its low value in comparison with other sugars is in agreement with the known acidifying effect of a neighbouring acetamido group.

ADDENDUM

Elementary analysis of di-*N*-acetyldiaminobutyric acid

Requires, C, 47.5; H, 6.93; N, 13.9; O, 31.7.

Found, C, 47.1; H, 6.71; N, 14.2; O, 32.0.

Elementary analysis of di-*N*-acetyldiaminobutyric acid methyl ester

Requires, C, 50.0; H, 7.36; N, 13.0; O, 29.6.

Found, C, 49.6; H, 6.97; N, 13.3; O, 30.1.

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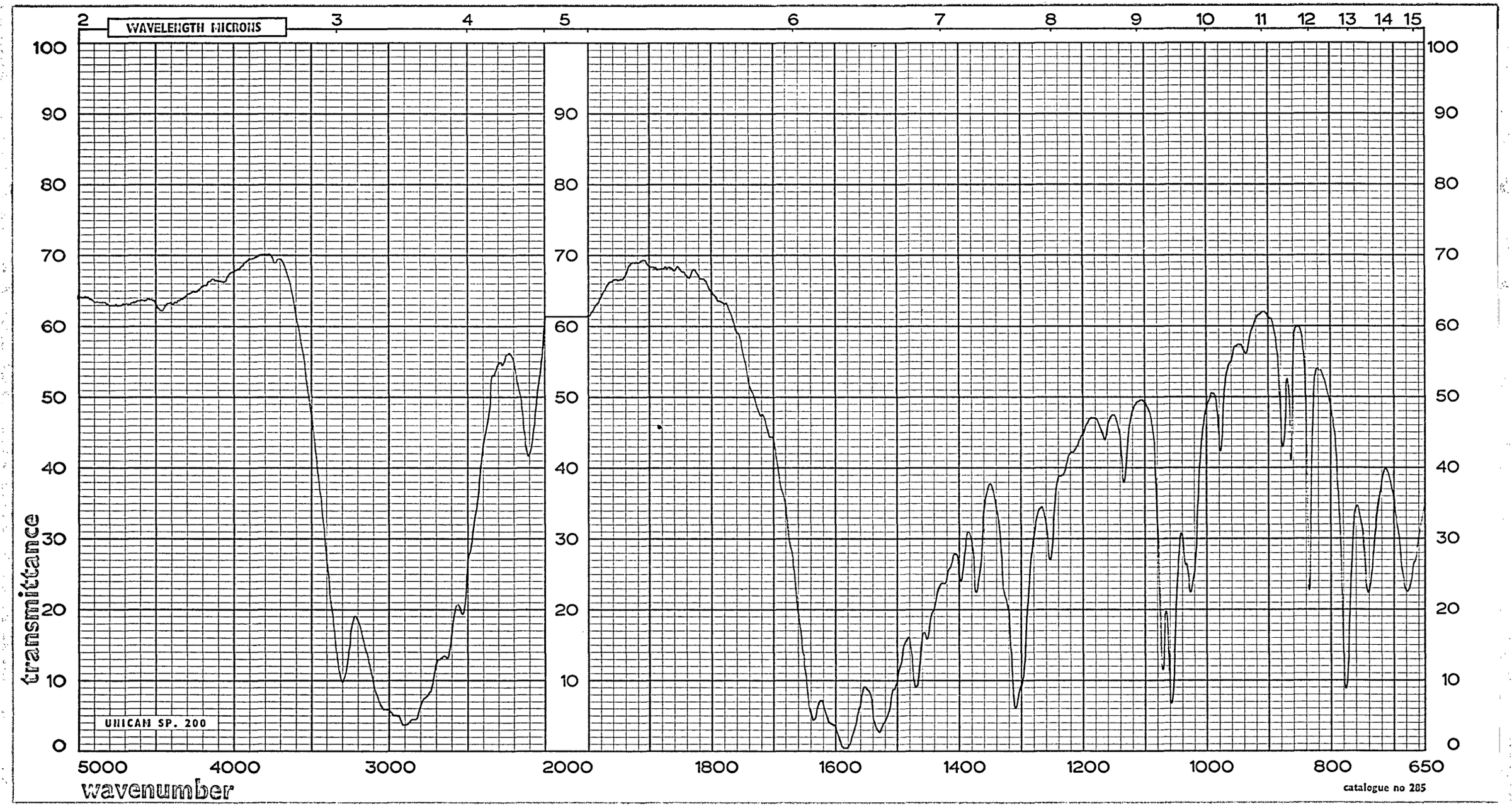
List of infrared spectra

F I oxalate	1 (1)
F II oxalate	2 (1)
F III oxalate	3 (1)
F II (free)	4 (1)
2,4-diaminobutyric acid HCl	5 (1)
Diacetyldiaminobutyric acid	6 (1)
Diacetyldiaminobutyric acid methyl ester	7 (1)
P I oxalate	8 (1)
P II oxalate	9 (1)

INFRARED SPECTROPHOTOMETER

UNICAM SP.200

UNICAM INSTRUMENTS LIMITED, CAMBRIDGE, ENGLAND



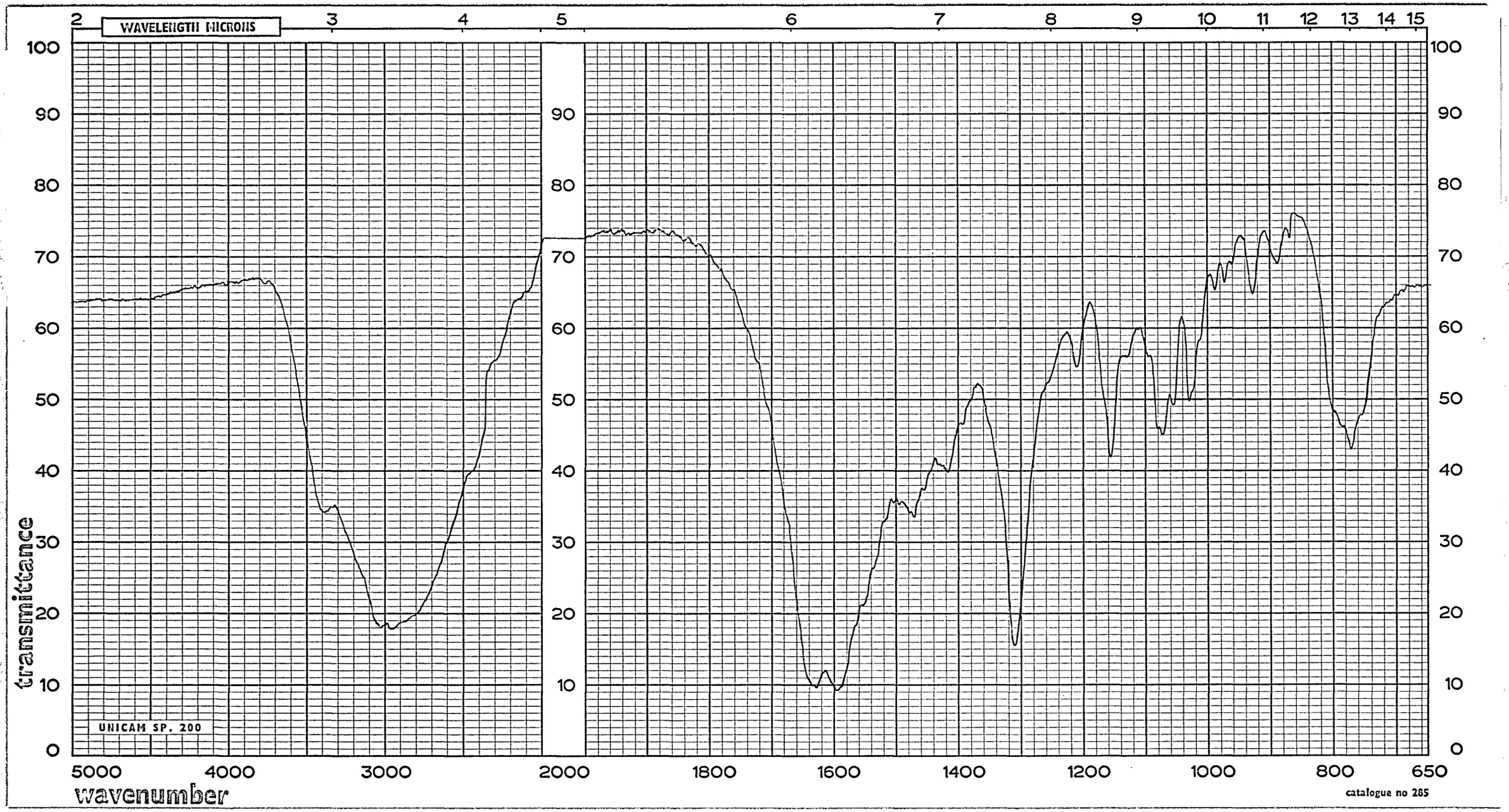
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ALIGN WITH INDEX ON THE RECORDER ▽	SAMPLE FI REFERENCE	FORMULA	PHASE Kel disc THICKNESS REMARKS 2.5%	NUMBER 1 (i) DATE OPERATOR	REFERENCE

INFRARED SPECTROPHOTOMETER

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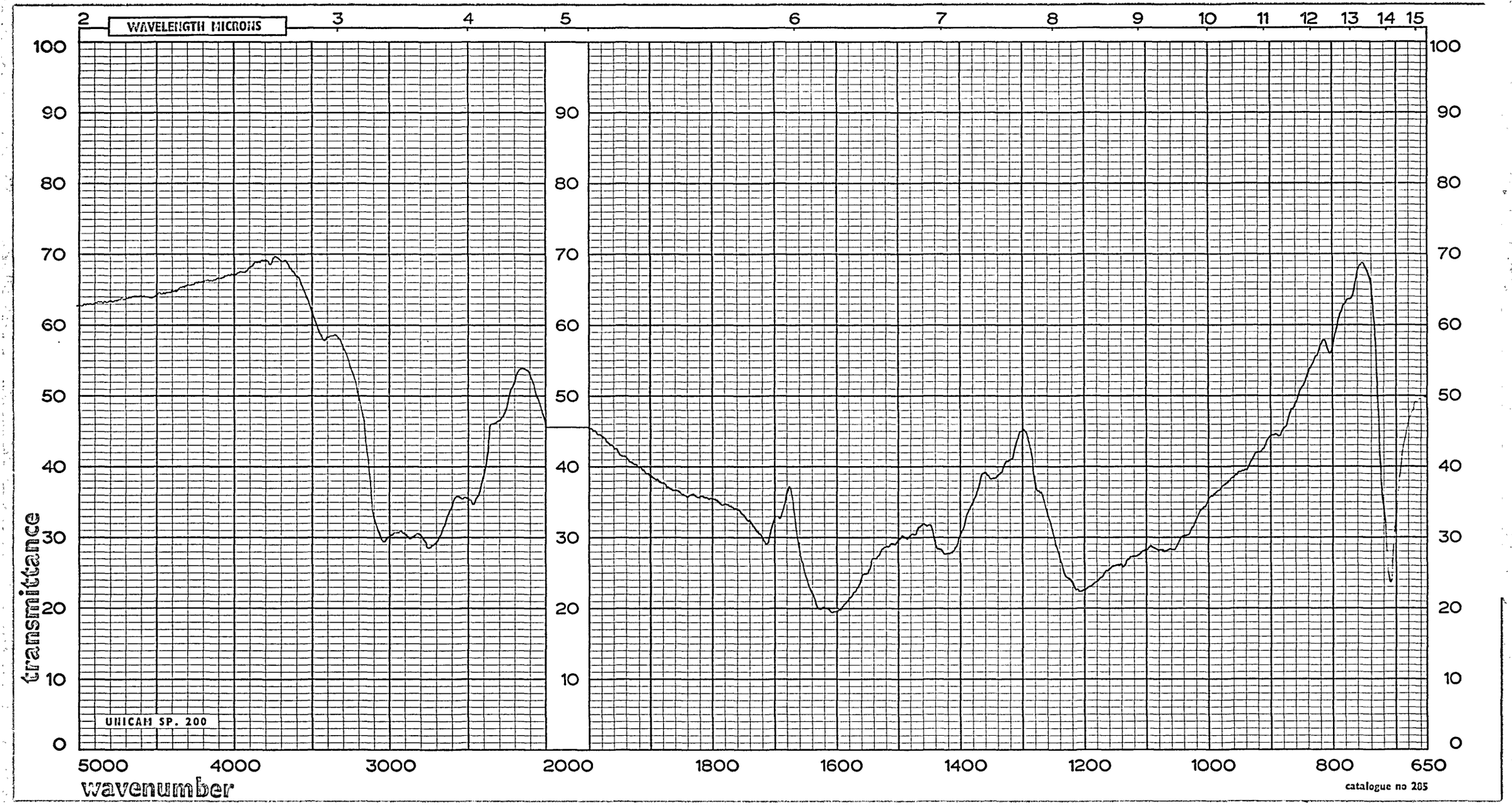


ALIGN WITH INDEX ON THE RECORDER ▽	SAMPLE F II oxalate REFERENCE	FORMULA	PHASE KCl disc THICKNESS REMARKS 1 %	NUMBER 2 (i) DATE OPERATOR	REFERENCE

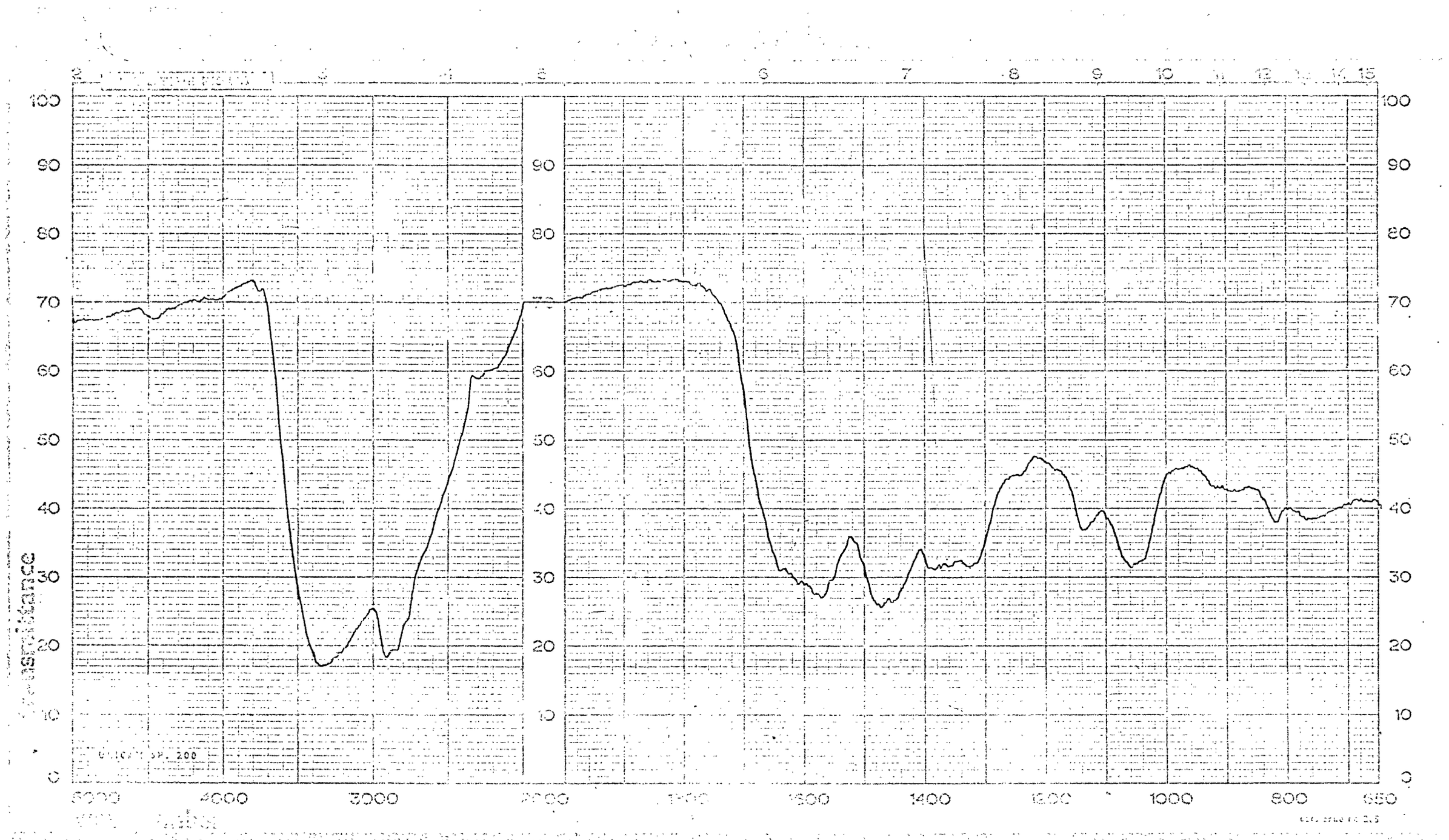
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ALIGN WITH INDEX ON THE RECORDER ▽	SAMPLE FIII oxalate REFERENCE	FORMULA	PHASE KBr disc THICKNESS REMARKS 1%	NUMBER 3 (i) DATE OPERATOR	REFERENCE



SAMPLE Free FII
Dessication dried

REFERENCE

FORMULA

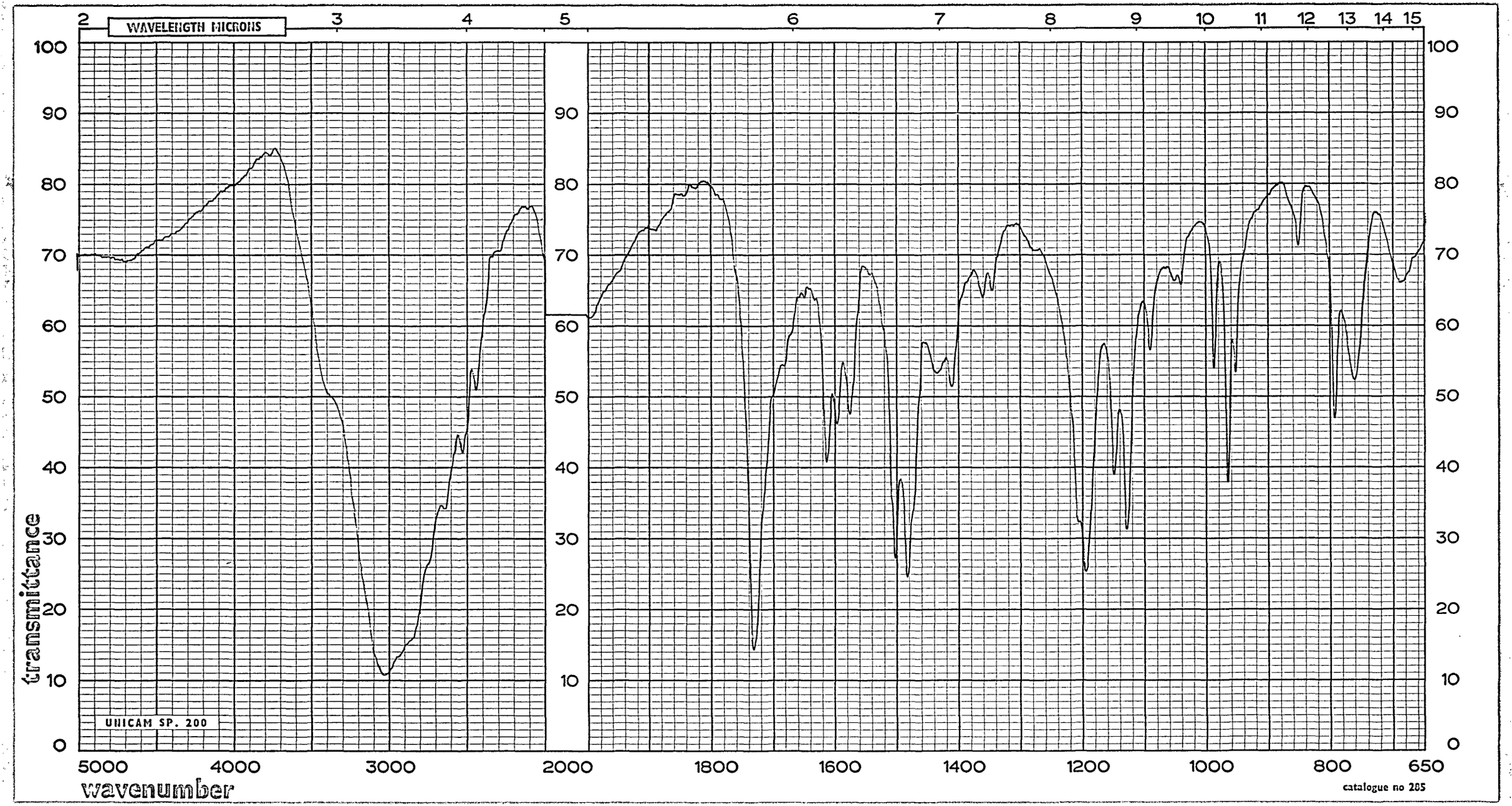
PHASE Oil smeared between
THICKNESS two KBr discs.
REMARKS

NUMBER 44 [4(i)]
DATE 9.3.65
OPERATOR A.P.F.

INFRARED SPECTROPHOTOMETER

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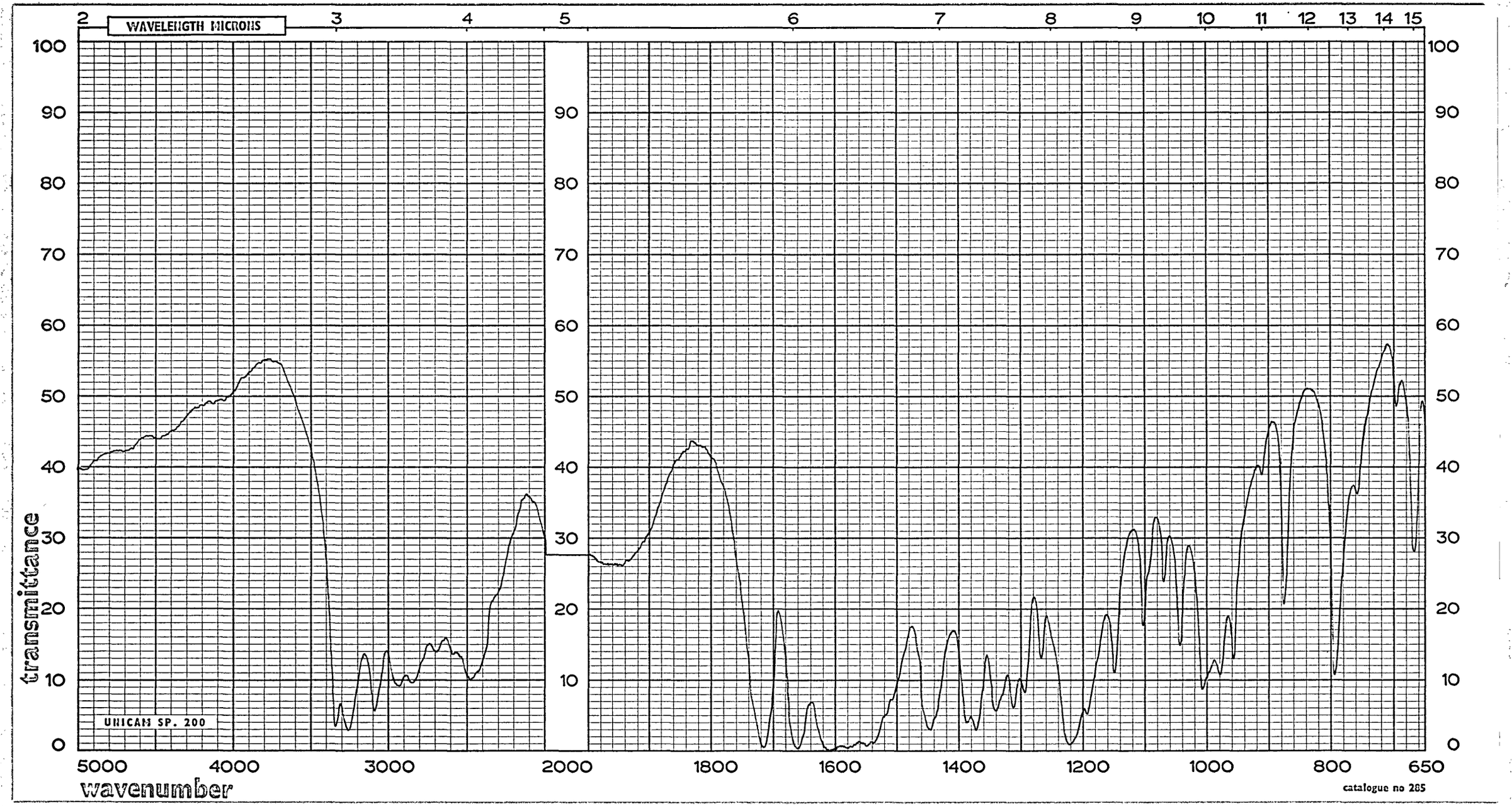


ALIGN WITH INDEX ON THE RECORDER ▽	SAMPLE 2,4-Diamino butyric acid HCl REFERENCE	FORMULA	PHASE KBr Disc THICKNESS REMARKS 2·5%	NUMBER 5 (i) DATE OPERATOR	REFERENCE

INFRARED SPECTROPHOTOMETER

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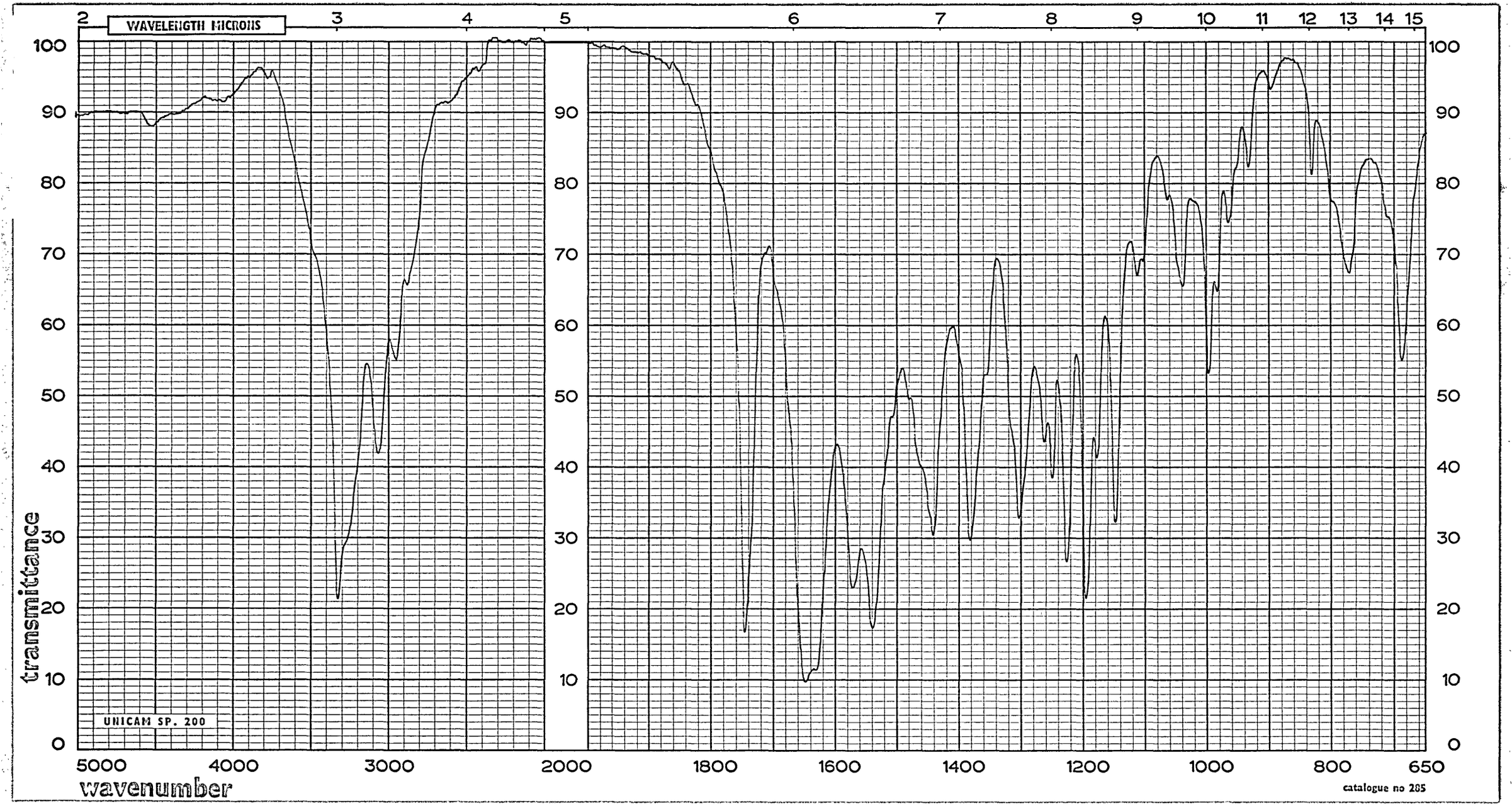
catalogue no 285

ALIGN WITH INDEX ON THE RECORDER ▽	SAMPLE Diethyl diaminobutyric acid REFERENCE	FORMULA	PHASE KCl disc THICKNESS REMARKS 2·5%	NUMBER 6 (i) DATE OPERATOR	REFERENCE

INFRARED SPECTROPHOTOMETER

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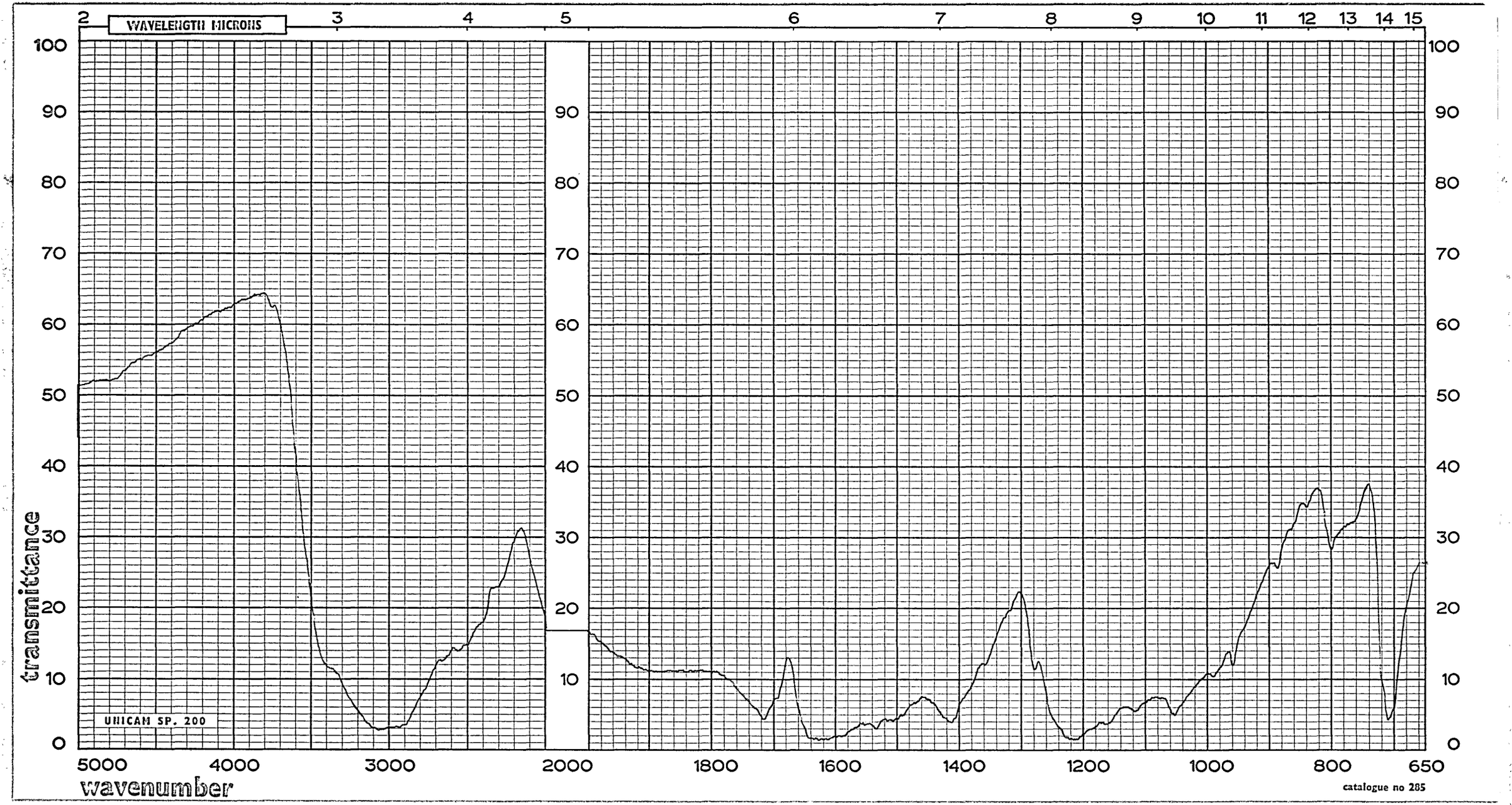


ALIGN WITH INDEX ON THE RECORDER ▽	SAMPLE Dimethyl thiomalic acid methyl ester. REFERENCE	FORMULA	PHASE Nuj mull THICKNESS REMARKS 2·5%	NUMBER 7 (i) DATE OPERATOR	REFERENCE

INFRARED SPECTROPHOTOMETER

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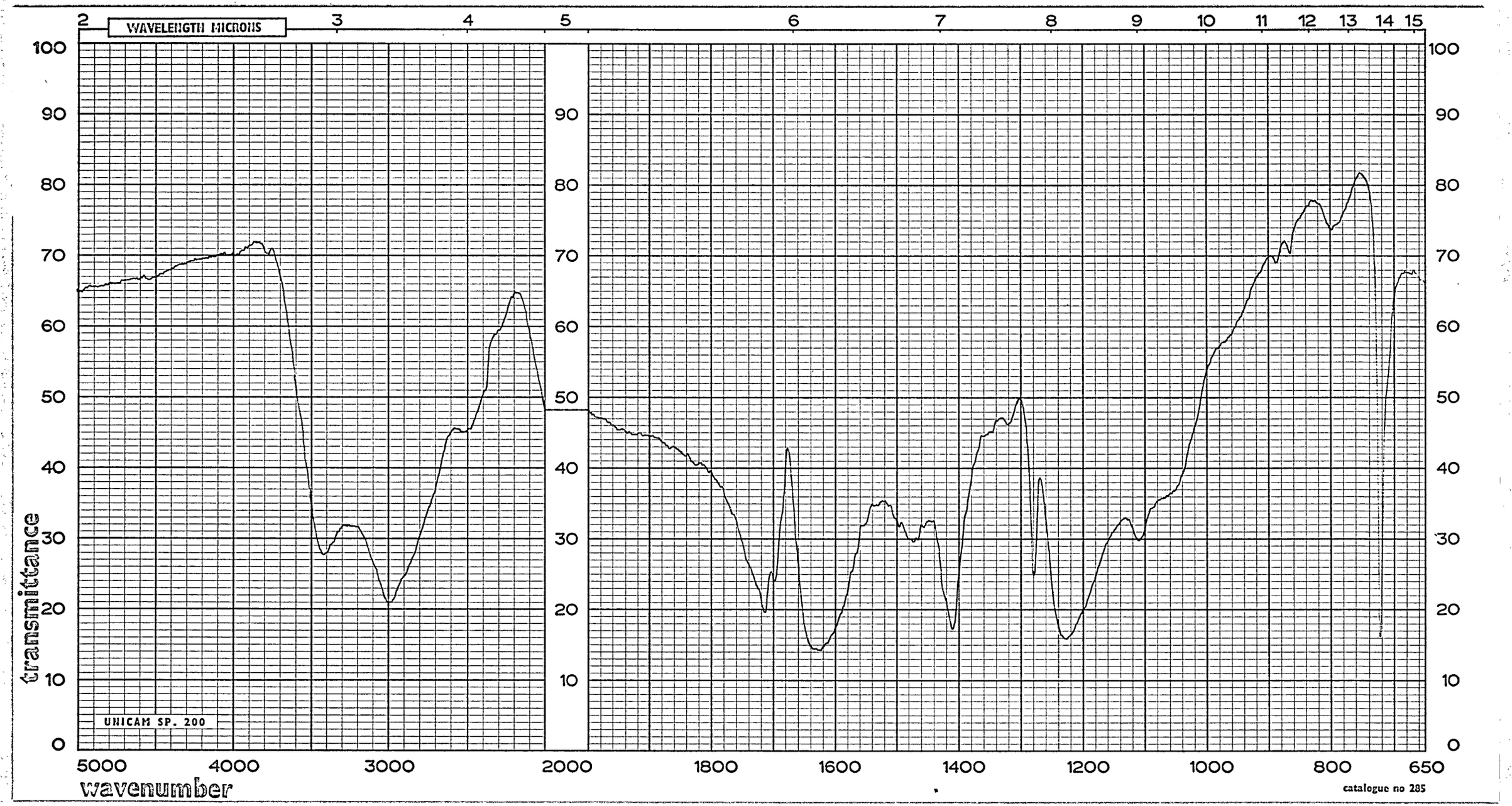
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ALIGN WITH INDEX ON THE RECORDER ▽	SAMPLE P I oxalate REFERENCE	FORMULA	PHASE KCl Disc THICKNESS REMARKS 2.5%	NUMBER 8 (i) DATE OPERATOR	REFERENCE

INFRARED SPECTROPHOTOMETER

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catalogue no 285

ALIGN WITH INDEX ON THE RECORDER ▽	SAMPLE P II Decarate REFERENCE	FORMULA	PHASE Kel Kisi THICKNESS REMARKS 2·5%	NUMBER 9(i) DATE OPERATOR	REFERENCE