ISOLATION OF DESMOSINE AND ISODESMOSINE AND INVESTIGATION OF THEIR PROPERTIES

being a thesis presented by

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to the University of St. Andrews,

in application for the degree of

Master of Science.

D E C L A R A T I O N

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition and that no part of it has been presented previously for a higher degree.

The research was carried out in the Department of Biochemistry and Microbiology in the United College of St. Salvator and St. Leonard, the University of St. Andrews, under the supervision of Professor G. R. Tristram.

MAGNUS BABALOLA AKIN-JOHNSON.

C E R T I F I C A T E

I hereby certify that Magnus Babalola Akin-Johnson has spent seven terms as a matriculated post-graduate student under my direction, and that he has fulfilled the conditions of 1974, No. 2 Resolution of the University Court (St. Andrews), and that he is qualified to submit the accompanying thesis for the degree of Master of Science.

A C K N O W L E D G E M E N T

I hereby express my gratitude to the Chief of Naval Staff, Nigerian Navy for granting me study leave with pay during the duration of the course.

I also express my gratitute to Professor G. R. Tristram for his understanding, interest and personal guidance during the course of this work.

I should also remember to thank Messers Jim Crawford, Jim Hunter, Angus Grieve, Bill Blyth, Colin Armit and Ellis Jaffray for their technical assistance.

Finally, I should express my thanks to my wife, Mrs. Fadeke Akin-Johnson and my children for their moral support during our stay in St. Andrews.

A B S T R A C T

Two different methods were selected for the isolation of elastin, and ultimate isolation of cross linked amino acids - Desmosine and Isodesmosine. The first method for isolating the elastin was by Serafini-Fracassini and Tristram (1965), and the second was by Starcher and Galione (1975). The elastin isolated by the first method was hydrolysed in 6M HCl for 24 hours, and the hydrolysate was subjected to electrodialysis, the catholyte collected being redialysed three more times.

Two different sets of membranes (BDH ionic exchange membranes, and a combination of gelatinised formalin linen/paper parchment) were separately employed for separating the basic amino acids in the catholytes. JEOL amino acid analyser was used to estimate the concentrations of the basic amino acids.

The elastin isolated by the second method was hydrolysed under reflux in 6M HCl for 36 hours, and the cross linking amino acids were adsorbed unto cellulose, eluted with water to Dowex resin, and finally with 4N Ammonium hydroxide.

The concentrated material was then applied to Dowex 50 in an hydrogen form, and eluted with Sodium Citrate buffer both at room temperature and 55°C.

Gradient elution with HCl was used to separate the cross linking amino acids with either Dowex 50 or Sephadex ion exchange as the resin.

The effect on cross links on the amino acids were also investigated.

CONTENTS OF THE THESIS

1. INTRODUCTION

1.1 INTRODUCTION

Elastic tissue which is widely distributed in mammalian organisms, contains elastin as the main protein component, in association with other macromolecules such as collagen and mucopolysaccharides (Serafini-Fracassini and Tristram) (1965). Hoeve and Florey (1974) said that elastin confers rubber-like mechanical properties on elastic tissue. In the native state, as seen under electron microscope, elastin is surrounded by an envelope of microfibrils about 100Å in diameter; Karrer (1960), Greenlee (1966), Ross et al (1969, 1970).

Partridge and Davis (1955) said that the rubber like elasticity of the hydrated elastin fibril leads one to suggest that it is a disordered structure composed of bundles of randomly contorted peptide chains lying generally parallel to the fibre axis. Elasticity is used here in the sense in which rubber is called elastic, and a small portion will produce a fairly large elongation, which vanishes on removal of the tension.

For the greater part of length, the peptide chains must be free to take up independent thermal motion, and to account for the insolubility and the swelling properties of the fibre, the presence of cross links at rather wide intervals is assumed. These cross links might possibly be established by the presence of crystalline regions containing polar groups in close apposition, but according to Partridge et al (1955) there appears to be little evidence for such a structure from established X-Ray diffraction studies.

Extensive electron microscopy and X-ray diffraction studies, carried out on elastic tissue and on isolated elastin have given no clear cut answer, and it is still uncertain, whether elastin molecules form part of a filamentous structure, or are arranged in an unorientated manner, Gross (1949), Franchi and De Robertis (1952), Lansing et al (1951), Linden et al (1955), Rhodin and Dalhamn (1955), Ramachandran and Santhanam (1957), Kawase (1959), Keech (1960 a, 1966 b), Pease and Molinari (1960), Charles (1961), Cox and Little (1961), Low (1961), Gotte and Serafini-Fracassini (1963), Gotte et al (1965).

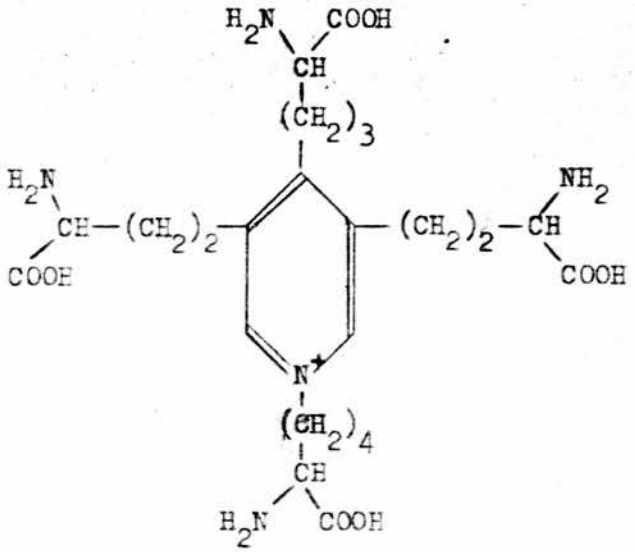
Serafini-Fracassini and Tristram (1965) viewed elastic fibre under electron microscope, and found that it occurs in a form which does not exhibit any characteristic structure, such as is found in tissues which are predominantly collagenous in nature.

This finding presented a problem to the workers as they were not sure whether the lack of structural features in the tissue reflects absence of molecular orientation in the main protein component (elastin) or not.

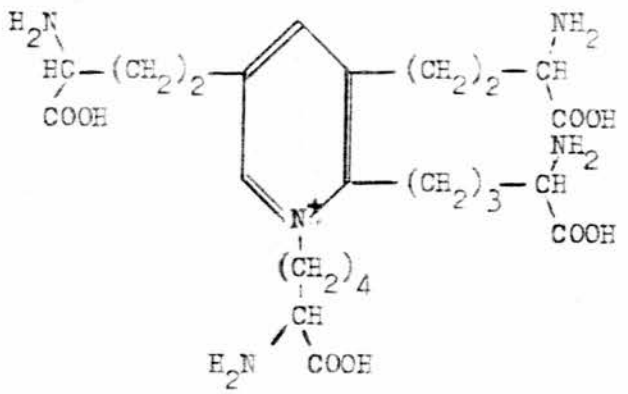
Elastin is resistant to the action of prolonged autoclaving, and shows no tendency to dissolve in either hydrogen bond breaking solvents such as neutral urea solution or in organic solvents such as phenol or cresol. Partridge et al (1955) therefore inferred that the probable type of bond involved in the small number of cross links present would be primarily covalent.

In order to ascertain if cystine bridges are important in stablishing the protein, Partridge et al (1955) treated elastin powder with performic acid under conditions which ensured the conversion of the whole of cystine half residues into cysteic acid, thus breaking all the disulphide bonds. The oxidised protein remained, insoluble in neutral 40% (w/v) aqueous urea, and 90% (w/v) aqueous phenol, both at room temperature and 100°C .

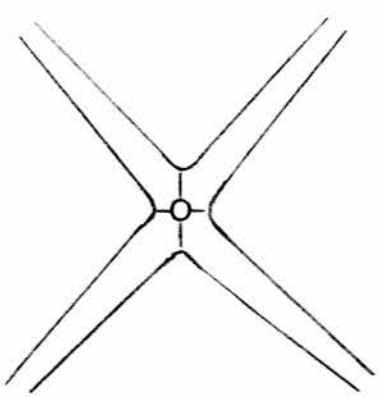
Figure 1.



DESMOSINE



ISODESMOSINE



Cross-linking of four pertide chains.

This confirmed that disulphide bridges have little effect on the solubility properties of the protein and other stable cross links were known to be present. This work was carried out before the amino acid composition of elastin (ref. TABLE 1), and the type of the bonds that make up the elastin filament were known.

Volpin et al (1976) have provided conclusive evidence by their work on tropoelastin (the non-crosslinked precursor protein of fibrous protein) and α-elastin, that hydrophobic association is the predominant driving force for formation of filamentous elastin in vivo.

The cow aorta contains 40% to 60% of elastin per cent dry weight; Davril and Han (1974). Elastin contains a variety of cross linking compounds that are responsible in large measure for some of the mechanical properties of elastic fibre, Paz et al (1974). The first of cross linking compounds discovered were called DESMOSINE and ISODESMOSINE, Thomas et al (1963), Partridge et al (1966). Both are pyridinium compounds derived from lysine residues in immature elastin. The structure is shown in Figure I.

Studies by Partridge et al (1963) have shown that the two isomeric amino acids (Desmosine and Isodesmosine) each possess four α-amino acid groups and that these

complex amino acids have a polyfunctional nature which make them ideal for covalent cross links since they may potentially unite up to four different peptide chains. The covalent cross links provide the elasticity and insolubility of elastin.

The discovery of the new amino acids of elastin came as a result of an attempt to isolate regions of a peptide network containing the cross linking by the use of a sucession of proteases followed by amino acid carboxy peptidases. The fraction which was suspected to contain the cross link was bright yellow in colour, and had a white flourescence. It seemed as if the cross linking agent might be associated with the agent responsible for the yellow colour of elastin. After the final hydrolysis of this fraction with acid yielding the cross linking molecules (Desmosine and Isodesmosine), they turned out to be colourless. Acid hydrolysis degradates the yellow colour and flourescence of elastin. Such molecules would be capable of acting as cross linking agents uniting four different protein chains of the pyridinium ring. Anwar (1966) believes that they represent an example of the poly functional molecules that can convert simple linear polymers into net work gels, and serve not only to cross link protein chains, but are responsible for the rubber like elasticity and connective tissue properties.

Serafini-Fracassini and Tristram (I965) expressed the view that the requirements for elastic recoil of elastin are most adequately provided by a system of polypeptide chains with an even spacing of pyridinium residues. These residues with potential peptide units, are looked upon as junction points (probably Y-Shaped) which connect the chains in planar conformation, rather than in a three dimensional cross linked system. From this study, Serafini-Fracassini and Tristram concluded that desmosines are concerned only in planar interlocking of polypeptide chains, and that other forces must be responsible for the maintenance of the lateral stability of elastin. Since as much as 80% of the side chains are hydrophobic, they inferred that the hydrophobic bonds, must play a dominant role in the stability of the elastin, because of the small amount of polar amino acids and desmosines.

The aggregation or co-acervation theory by Partridge et al (I955) which says that the α-elastin (a fragmentation product of fibrous elastin), co-acervates when its temperature is raised, favours occurence of hydrophobic bonds which become stronger on raising its temperature to about 50°C, Scheraye (I963). Volpin el al (I976) provided evidence that as a result of increase in temperature of tropoelastin, filaments are formed, which align in paralled arrays with about 50Å centre to centre distance, which are same as those of α-elastin and of mature cross linked elastin.

1.2 ISOLATION AND PURIFICATION

Methods for isolating and purifying elastin depend upon the insolubility and relative inertness of this fibrous protein, and all preparations of elastin represent insoluble residue, after attempts to remove contaminants by various mild extraction procedures, Anwar (1966).

The methods most commonly used include:

(a) The use of 89% formic acid at 45°C for 72 hours and extraction with boiling alkali, Lansing et al (1952).

(b) Extraction with 0.25M oxalic acid at 100°C, Partridge et al (1955).

(c) Extraction in chloroform/methanol (3:1), homogenisation in 1% Sodium Chloride, dehydration in Acetone and repeated autoclaving, Serafini-Fracassini and Tristram (1965)

(d) Boiling in water repeatedly and dehydration in Acetone, Starcher and Galione (1975)

All these methods involve high temperature and this can lead to cleavage of the peptide bonds, without solubilising the protein, Franzblau (1971).

Enzymic procedures have also been attempted and the advantage of these, is that the risk of cleaving the peptide bond is reduced.

Hospelhorn and Fitzpatrick (1961) used trysin (E.C.3.4.4.4) followed by collagenase to remove contaminating proteins. Miller and Fullmer (1966) used repeated guanidine treatment followed by collagenase. Davril and Han (1974) used pancreatic elastase (E.C.3.4.4.7) thermolysin and pancreas proteases.

The enzymic methods yielded amino acid composition slightly different from those elastins prepared by hot alkali and auto claving, Oluoha (1980).

TABLE I

Amino Acid composition of elastin isolated by various methods from the ligamentum nuchae and visceral pleura of adult cattle. Results are expressed as amino acid residues/1000 residual

	Ligamentum Nuchae				Visceral Pleura				
Isolation Method	Collagenase	Auto Claved	Formic Acid	Alkali	Collagenase	Collagenase +Trypsin	Auto Claved	Formic Acid	Alkali
Hyp	10.5	11.0	9.0	9.6	9.0	9.8	9.7	10.1	9.5
Asp	7.1	7.3	5.4	5.4	15.6	6.5	10.3	6.4	6.2
Thr	8.0	8.1	7.5	6.3	11.7	7.6	9.4	7.9	6.5
Ser	6.7	6.9	5.6	5.3	9.9	7.1	8.0	7.4	6.5
Glu	14.4	15.7	13.9	15.5	27.4	15.2	20.7	17.1	16.6
Pro	125.0	120.0	122.5	116.0	108.5	121.6	128.0	124.0	117.0
Gly	338.0	334.0	343.0	334.0	314.0	334.6	332.0	335.0	340.0
Ala	220.0	224.0	224.0	223.0	198.5	205.4	212.0	216.0	221.0
Val	140.0	142.5	144.0	145.8	131.5	141.5	139.0	141.0	145.0
Ileu	26.0	25.4	24.5	27.6	27.0	28.1	25.3	26.0	25.9
Leu	58.4	60.8	57.7	64.0	70.0	65.8	62.0	61.0	62.4
Tyr	7.2	6.5	6.0	7.5	9.5	9.6	5.5	7.2	6.6
Phe	28.8	27.5	27.6	32.4	29.2	32.4	24.0	29.2	28.9
Lys	3.9	3.8	3.5	3.5	15.5	7.4	6.9	4.8	4.5
Arg	6.8	6.9	6.4	4.3	23.4	7.6	7.7	7.4	4.2
Total	1000.8	1000.4	1000.6	1000.2	1000.7	1000.2	1000.5	1000.5	1000.8

Francis et al (1973)

The amino acid composition of elastin though unique varies with the species, the tissue under investigation and the age of the animal, Field et al (1978). The amino acid composition of human aortic elastin was reported by Lansing et al (1951); Fitzpatrick (1965) and Gotte et al (1965). Serafini-Fracassini and Tristram (1965) agreed with the findings of these workers, but believed that despite the fact that some modification did occur with aging, figures presented by former workers showed some inconsistencies. Gotte et al (1965) inferred from Ultra Violet studies that the content of desmosine increases with aging. Serafini-Fracassini and Tristram (1965) on the other hand showed that the amount of desmosine (0.71 - 0.79) percent, and Isodesmosine (0.5 - 0.59) percent remained constant in foetal, normal and old adult elastin, and that the supposed changes in composition are limited to minor constituents e.g. Aspartic acid, glutamic acid, methionine and histidine, the last two being absent from foetal elastin. This is definately at variance with the finding of Gotte et al (1963).

As no satisfactory method of elastin purification has been achieved, many investigatiors tried isolating elastin in precursor which they hoped would help in the elucidation of elastin structure.

Weissman et al (1963) were able to prove that pigs deprived of copper had a lowered elastin content. The deprivation of copper causes rupture of aorta in the animal.

Smith et al (1972) extracted salt soluble elastin (pro elastin) and found that its amino acid composition is similar to that of insoluble elastin with the exception of higher lysine content and absence of crosslinking amino acids in the former. Sandberg et al (1969) characterised the soluble elastin (pro elastin) and called it TROPOELASTIN. 86% of its amino acid residues are non-polar and it lacks desmosine and isodemosine (ref TABLE II).

T A B L E II

Amino Acid Composition of Mature Elastin and Tropoelastin

	COW (I)		PIG (I)	COW (2)		TROPOELASTIN
Source	Ligamantum nuchae	Aortic	Aortic	Ligamantum nuchae	Aortic	From Copper Deficient pig aorta
Hydroxypro	I3.I	I5.5	II.0	8.I	I0.6	9.9
Asp Acid	6.5	7.6	6.2	5.8	6.5	3.7
Threonine	9.4	9.6	I3.6	9.3	9.6	I4.4
Serine	9.I	I0.0	II.4	8.7	9.2	II.6
Glutamic Acid	I5.7	I6.2	I9.I	I5.5	I6.0	I6.9
Proline	II7.0	I22.0	II7.0	II6.I	II2.7	I04.0
Glycine	332.0	3II.0	330.0	329.7	332.4	326.0
Alanine	228.0	223.0	234.0	228.0	223.9	230.0
Valine	I38.0	I37.0	I20.0	I32.2	I3I.3	I32.0
Half Cystine	0.0	0.0	0.0	0.0	0.0	0.0
Methionine	0.0	0.0	0.0	0.0	0.0	0.0
Isoleucine	25.0	26.7	I7.8	24.0	23.5	I6.0
Leucine	59.9	64.8	54.2	59.7	58.2	45.2
Tyrosine	6.2	8.I	I5.9	5.9	7.5	I6.3
Pheryl Alanine	29.I	33.0	33.0	29.4	29.7	26.5
Histidine	0.5	0.5	0.5	0.5	0.5	0.0
Lysine	2.7	5.I	6.2	3.3	4.6	43.3
Arginine	4.6	6.2	6.I	5.8	5.9	4.3
Isodesmosine	0.9	I.2	I.2	I.6	I.9	0.0
Desmosine	I.4	2.0	I.8	2.2	2.4	0.0
Lysinonorleucine	I.2	0.9	0.9	I.I	I.0	0.0
Merodesmosine	0.0	0.0	0.0	0.2	0.2	0.0

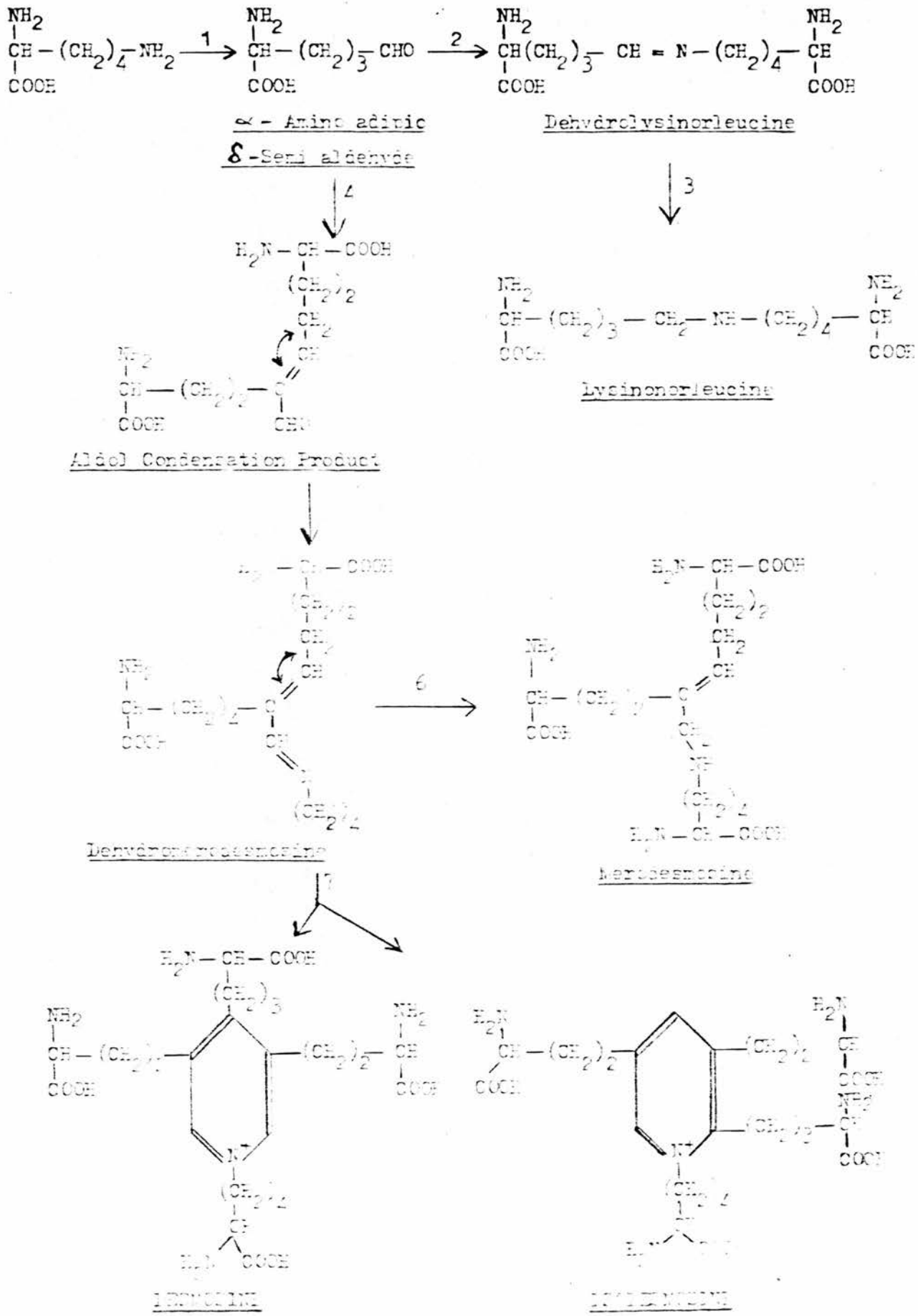
I. Rasmussen et al (I975) Formic Acid Cyanogen bromide method.

2. Serafini-Fracassini et al (I975).

3. Smith et al (I972).

BIOSYNTHETIC PATHWAY SCHEME (1)

Francis et al (1973)



The peptide bonds are formed by the α-amino and α carboxyl groups.

Reaction 1 is oxidative deamination of the ε-amino groups of selected lysine residues - Miller et al (1967).

Reaction 2 is a Schiff base reaction of the aldehydic residues with ε-amino groups of other lysine residues, or in aldol condensations with other similar aldehydic residues.

Reaction 3 - Interaction of a residue of the semi aldehyde with a lysine residue.

Reaction 4 - Aldol condensation of two semi aldehydic residues.

Reaction 5 - From interaction of the aldol condensation product of Reaction 4.

Reaction 6 - Reaction between Dehydromerodesmosine and the ε-amino group of a third lysine residue (Schiff base formation)

Reaction 7 - Interaction of dehydromerodesmosine with a fourth lysine residue, Starcher et al (1967).

Lent et al (1969) presented evidence for the presence of α-amino adipic acid and δ semi aldehyde and its aldol condensation product. Starcher et al (1967) and Paz et al (1971) reported the presence of both merodesmosine and dehydromerodesmosine in elastin isolated without exposure to alkali reduction.

The work of Francis et al (1973) on pathway of biosynthesis of desmosine (Scheme 1 refers) shows that the levels of lysine and aldol condensation product decrease with maturity, while the levels of desmosine and Isodesmosine increase. The concentration of dehydromero-desmosine and dehydrolysinonorleucine decrease with ageing, while no major changes occur in levels of merodesmosine and lysinonorleucine.

I.4 PROPERTIES OF DESMOSINE AND ISODESMOSINE

Desmosine and Isodesmosine are unique amino acids in Elastin. They contain lysine derived cross links, and contribute in a large measure to the protein's elastic mechanical properties. Elastin crosslinks are found in alanine enriched areas of the molecule and are formed from lysine molecules separated by two or three alanine residues, Sandberg et al (I97I, I972), Foster et al (I974). Grey et al (I973) proposed that the clustering of alanine and lysine residues favours an α-helix conformation with the lysine side chains appositively positioned on one side of the helix, allowing for easy condensation of the juxtaposed cross link precursors after enzymic oxidation. Foster et al (I976) proposed from circular dichroism studies that desmosine enriched peptides, though rich in alanine are in an extended helix, rather than α-helix.

The work of Baurein et al (I976) on photolysis of Desmosine and Isodesmosine by Ultra violet light, shows desmosine can be maximally degraded by irradiation at 274nm and Isodesmosine at 285nm. These wave lengths do not correspond to the absorption maxima of the cross links but to shoulders of the main absorption peaks. When they were irridated at their optimum wave lenghts, but at various pH, both desmosine and isodesmosine seem quite stable at pH greater than 8.5. Between pH 8.0 and 5.0, the photolytic rate is maximum and decreases slightly at more acidic pH. Below pH 4.0, one of the products of photolysis is free lysine.

I.5 STRUCTURAL MODEL

The insolubility of elastin in non hydrolytic and organic solvents made researchers look for its precursor, - TROPOELASTIN.

Sandberg et al (I97I, I972) digested tropoelastin with trypsin, and obtained a number of small peptides. Their amino acid sequence was determined by the dansyl-Edman method and by mass spectrometry. Two of the peptide groups, - Ala - Ala - Ala - Lys - and - Ala - Ala - Lys -, are repeated six times in the polypeptide chain. Due to the repeating structure, they proposed that these peptides represent the areas of the chain involved in the formation of desmosine and isodesmosine links of insoluble elastin.

Gerber et al (I974) were able to release single chain peptides from the carboxyl groups of desmosine cross links also by the use of Edman degradation. They identified twelve possible lysine sequences which are likely to be involved in the formation of the cross links. Six pairs of lysine in the sequence -Lys - Ala - Ala - Lys -, and six additional pairs of lysine in the sequence - Lys - Ala - Ala - Ala - Lys -, two of such pairs meeting to form desmosine in isodesmosine cross links.

The sequences of amino acids found by Gerber et al (I974) are similar to those found by Sandberg et al (I969, I97I). The work of Gerber et al (I975) shows that the sequence of C-terminal peptides fall into two distinct classes, one starting with the hydrophobic residue, the other with Alanine. Three of the four lysine residues, which are preceded and followed by Alanine or similar small amino acid are oxidised by the enzyme Lysyl oxidase, while the fourth lysine residue,

which has a hydrophobic residue, e.g. Phenyl alanine or leucine on its carboxyl and is protected from action of lysyl oxidase, and thus retains its ε amino group and donates the nitrogen to the pyridine ring; Gerber and Anwar (1975), Baig et al (1980). The function of the enzyme, lysyl oxidase is to catalyse the formation of the aldehyde.

--Ala-Ala-Lys-Ala-Ala-Ala-Lys (Peptide starting with alanine e.g. Ala-Gly-Ala)

+

--Ala-Ala-Lys-Ala-Ala-Lys (Peptide starting with hydrophobic amino acid e.g. Phe Ala-Gly-Ala.

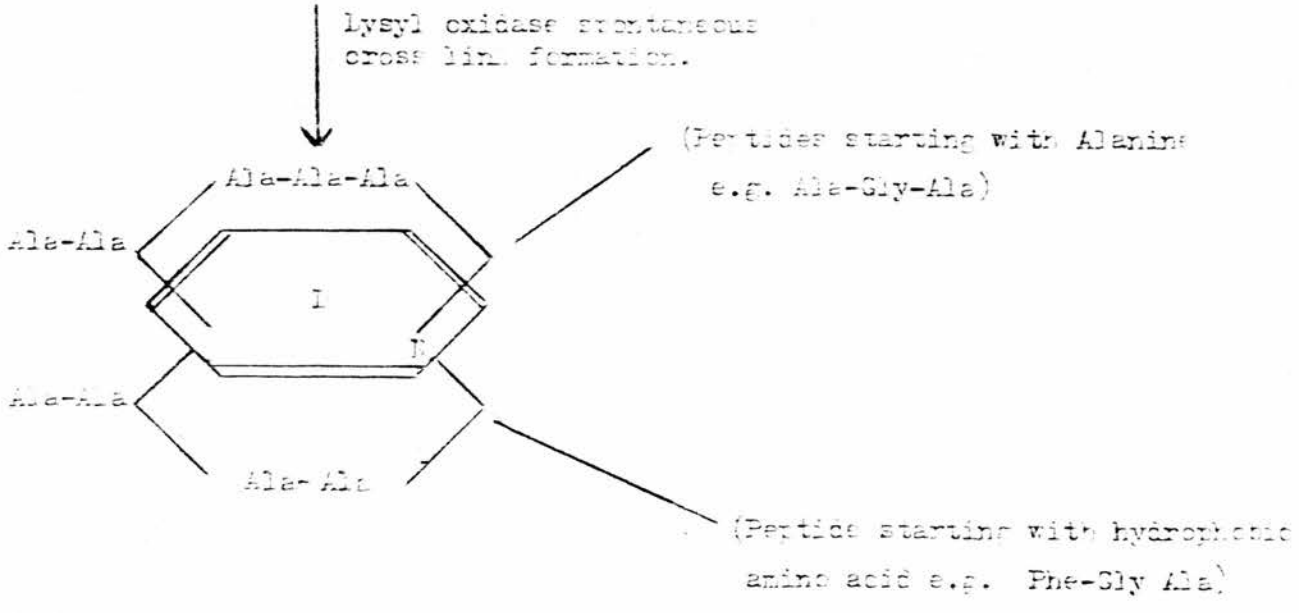


Figure 2 (a)

Peptide isolated from Porcine aorta. The sequence of the -COOH of the peptide differ from species to species.

Foster et al (1974) showed that each peptide in tropoelastin contains on a molar basis, one residue of Serine, one of Glutamic acid, one of Tyrosine, and two or three of Phenyl alanine, and that these amino acids tend to cluster near the desmosine cross link regions. The sequencing data from the work of Sandberg et al (1971), Foster et al (1973) show that the seven tyrosine residues located, five occupy a position adjacent to the carboxyl group of lysine, and also the sequence - Ala-Ala-Glu-Phe- follows a lysine residue at a minimum two to three times within the molecule.

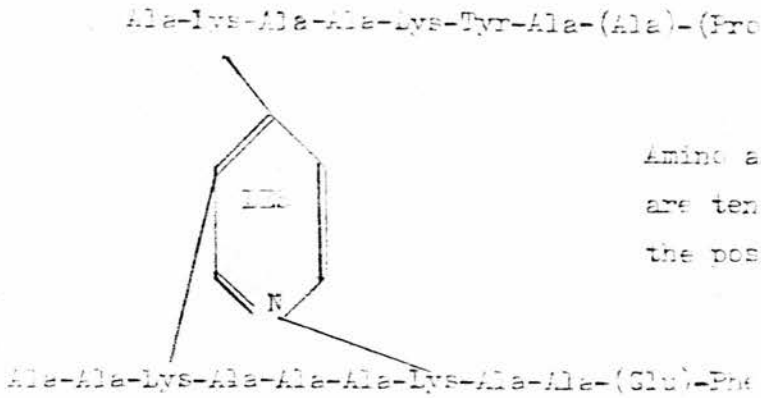


Figure 2 (b)

Cross link isolated form ligamentum nuchae of bovine.

The unique role which Foster et al (1973) gave to Tyrosine, that it inhabits the deamination of neighbouring lysine, is not in accord with the work of Gerber et al (1975), Baig et al (1980) discussed earlier.

Foster et al (1974) showed that both desmosine and isodesmosine can occur in the same sequence position. Davril and Han (1974) however suggest that they occur at separate sites close together on the peptide chain.

Trypsin has been used to cleave tropoelastin, and tyrosine was shown to be the N-terminal amino acid.

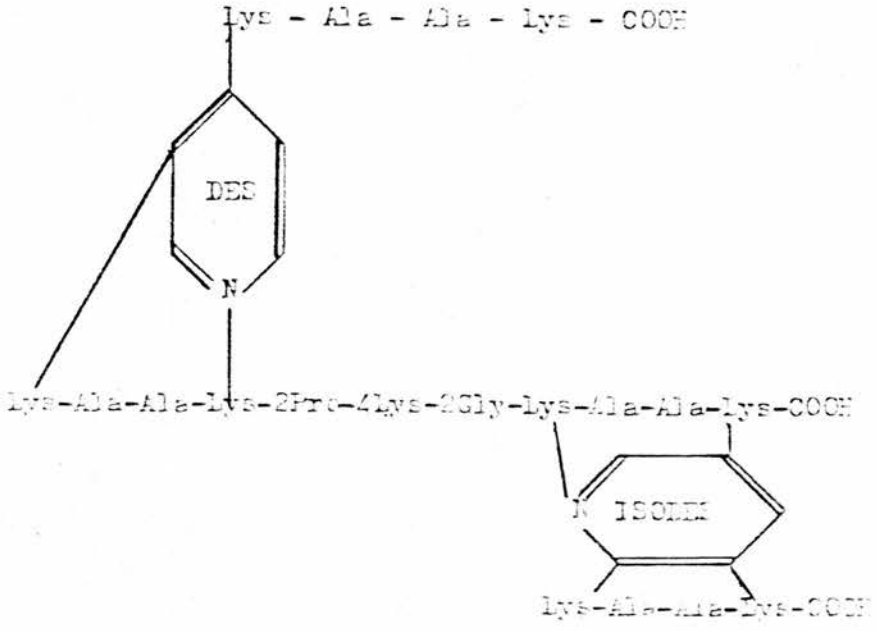


Figure 2 (c)

As trypsin cleaves at the α-carboxyl of lysine, Sandberg (1976) suggested that tyrosine follows the -Lys-Ala-Ala-Lys sequence. The worker therefore postulated that oxidation of adjacent lysine by lysyl oxidase can be prevented by this arrangement.

This residue could therefore form a dehydro desmosine ring by condensing with three oxidised lysinyl residues (allysine). It was also suggested that the tyrosine lying adjacent to such a residue would allow interactions between its π electron systems and the pyridinium nucleus. This would provide a conducting path for electrons in the final oxidation step leading to formation of desmosine and isodesmosine. This view was supported by Francis et al (1973), Scheme (I) refers, and Foster et al (1974), while Gerber and Anwar (1975) advocated a hydrophobic interaction for the final oxidation step.

I.6 METHODS OF SEPARATION

The concentrations of desmosine and isodesmosine in elastin are small, compared to the concentrations of hydrophobic amino acids (ref. TABLE II). This is why the separation of both amino acids first created some problems.

Previous methods for isolation of the desmosines involve separation by ion exchange chromatography using non-volatile buffers, gel filteration and a high voltage electrophoresis.

Partridge et al (1964) used a column packed with sulphonated polystyrene resin, and measured extinctions of the fractions (1.3ml) at 275nm.

Corbin (1969) modified the standard amino acid analysis procedures, by adding a third column (60cm resin height) packed with Beckman Custom Research type PA-28 to resolve the desmosine and isodesmosine.

Ledvina and Bartos (1967) used a method based on the chromatography combination of ion-exchange chromatography and molecular exclusion and measured the fractions collected at 274nm.

Anwar (1965) used a 50cm column with 0.2N citrate buffer pH 4.45.

Thornhill (1971) used gel filteration on Bio-gel p-2 and recovered the desmosine by evaporation of the appropriate fractions.

Moczar et al (1971) instead used high voltage electrophoresis.

Green et al (1972) believe that gel filtration and high voltage electrophoresis have a draw back, in that they fail to separate desmosine for isodesmosine, while the ion exchange methods necessitate the use of desalting columns. The workers therefore recommended the use of volatile buffers and fractionation was done on Aminex A5 column, maintained at 50°C. Qualitative identification was achieved by high voltage paper electrophoresis and standards were included in the runs.

Starcher and Galione (1976) used the hydrophilic nature of desmosine which preferentially adsorbs to cellulose fibres in mixtures of organic solvents. Resolution of the isomers was achieved on a polystyrene resin column.

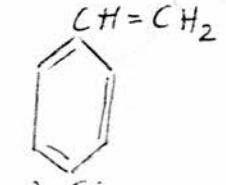
1.7 ION EXCHANGE CHROMATOGRAPHY - HISTORICAL AND GENERAL

Many of the chemical manipulations routinely encountered in the laboratory can be quickly and efficiently carried out using ion exchange chromatography. The technique is used in protein separation. It depends largely on interaction between the fixed charged groups on the ion exchange resin, and the charged groups of the substance to be separated i.e. amino acids.

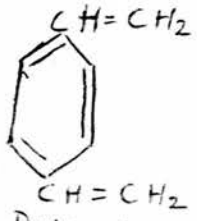
Modern ion exchange technology began in 1935 by Adams and Holmes, who discovered that resins are capable of exchanging ions. These synthetic resins are solids that may be pictured structurally as being composed of two parts. The fundamental framework of this ion exchange substance is an elastic, three dimensional hydrocarbon network or matrix, the second part of their structure is hydrophilic in nature and consists of ionisable groups (either acidic or basic) chemically bonded to the hydrocarbon framework. The organic network is fixed, is insoluble in most common solvents, and can be termed as chemically inert. The ionisable or functional groups attached to the matrix have active (mobile) ions that can react with or be replaced by other ions.

The chemical behaviour of an ion exchange resin is determined by the nature of the functional groups that are attached to the hydrocarbon skeleton. There are two classes of ion exchange polymers: Cation exchangers whose functional groups can react with cations of surrounding solution, and Anion exchangers whose funtional groups can react with the anions of the surrounding solution.

A cation exchange resin is generally prepared by copolymerisation of styrene and divinyl benzene



a) Styrene



b) Divinyl benzene

Fig. 3

During the polymerisation process, linear chains of polystyrene are formed which later become covalently linked to each other at intermittent points, by divinyl benzene cross links, and these result in a three dimensional insoluble hydrocarborn net work. Sulphonic acid groups ($SO_3^-H^+$) are then introduced in to most of the benzene rings of the styrene-divinyl benzene polymer, on addition of Sulphuric acid. The end product is a cation-exchange resin.

An anion exchange resin can also be prepared by first chloromethylating the benzene rings of the three dimensional styrene-divinyl benzene copolymer to attach - CH_2Cl groups and then causing these to react with a tertiary amine e.g. trimethylamine. This gives the chloride salt of a strong base exchanger. The cross linked vinyl benzene resins are insoluble in concentrated acids, bases and salts and are resistant to oxidation, reduction and radiation.

1.8 PHYSICAL PROPERTIES OF ION EXCHANGE RESINS

(a) Particles size and form:

Most ion exchange resins are sold in the form of spherical beads. In a typical preparation, the particles may range from Imm to less than 0.04mm. The coarser particles (50 - 100 mesh) are usually used in batch operations (ref. 1.10a). The finer resin particles (200 - 400 mesh or smaller) are utilised in column operations (ref. 1.9b)

(b) Swelling and Porosity

As ion-exchange resins are elastic three dimensional polymers, they can have no definite pore size, only a steadily increasing resistance of the polymer network limits the uptake of ions or molecules of increasing size. The beads when immersed in water imbibe a limited amount of the liquid to form a homogenous gel like structure. The resins have no appreciable porosity until swollen in a suitable solvent, such as water. The amount of swelling is directly proportional to the number of hydrophilic functional groups attached to the polymer matrix, and is inversely proportional to the degree of divinyl benzene cross linking present in the resin.

(c) Crosslinkage

The degree of crosslinking is the more important factor in determining to what extent a resin is free to swell or shrink. The degree of cross linking is expressed as the fraction of divinyl benzene that is contained in the styrene divinyl benzene resin beads. The content of divinyl benzene varies from 1% to 16% in commercially available resins, with 8% cross linking being considered ideal for a general purpose ion exchange resin.

1.9 CHEMICAL PROPERTIES OF ION EXCHANGE RESINS

(a) Equivalency of Ion Exchange Reactions

Ion exchange reactions progress stoichiometrically; i.e. for every ion equivalent removed from a solution by an exchanger, one ion equivalent of like charge must be released.

(b) Capacity of Exchangers

The number of electrical charges i.e. the number of exchange sites on the resin matrix per unit weight of material, expresses quantitatively the capacity of an ion exchanger for counter ions. Capacity ratings are usually assumed to relate to the H-form of cation exchangers and the Cl-form of anion exchangers.

(c) Selectivity of the Resins for the Counter Ion

When an exchanger is added to an electrolyte solution containing a counter-ion different from that initially bound to the resin, an equilibrium is established.

The position of the equilibrum depends upon the relative concentration of the competing counter ions, Na^+ and K^+, both in solution and on the exchanger, assuming equal affinities of the counter-ions for the exchanger.

$$RSO_3^- Na^+ + K^+ + Cl^- \rightleftharpoons RSO_3^- K^+ + Na^+ + Cl^-$$

Affinities displayed by ion exchange resins are not equal for some common monovalent cations, selectivity sequences are in following increasing order.

$$Li^+ < H^+ < Na^+ < NH_4^+ < K^+ < Rb^+ < Cs^+ < Ag^+$$

and for some divalent cations the order is

$$Cu^{++} < Cd^{++} < Ni^{++} < Ca^{++} < Sr^{++} < Pb^{++} < Ba^{++}$$

For anions the sequence in increasing order is

$$F^- < \text{acetate} < \text{formate} < Cl^- < Br^- < I^- < NO_3^- < SO_4^{--} < \text{Citrate}.$$

Selectivity sequences depend upon the nature of the functional group (the fixed ionic group) of the ion exchanger. Since ion exchange involves electrostatic forces, selectivity at first glance should depend mainly on the relative charge and the ionic radius of the (hydrated) ions competing for an exchange site. Divalent ions should be attracted to an exchanger more that monovalent ions, because affinities are inversely proportional to their hydrated radii.

1.10 BASIC OPERATION

There are two ways of bringing a solution into contact with an ion-exchange resin.

(a) Batch Method

Where contact of the exchanger and solution is made by stiring or shaking, i.e. forming a slurry. After equilibration, the exchanger is separared from the solution phase by filtration or centrifugation.

(b) Column Method

Consists of allowing the solution to move by gravity, or forcing it to flow through a column of resin contained in a tube or column. The eluates are then collected in fractions and analysed. The column method is more widely used than the batch method, because the batch method is time consuming, laborious and allows experimental errors to accumulate. This becomes very apparent if the batch method is repeated continously (Cascade or multistage operation) to improve the separation. Also in the column method, neither the resin, nor the liquid in the column is homogeneously mixed. For these reasons, column method is used where complete exchange is necessary, or when required to separate with maximum efficiency a group of ions having similar exchange potentials.

There are three types of column chromatography - Frontal, Displacement and Elution analysis, the latter being the most widely used.

(i) Frontal Development

This is the simple form of chromatography and consists of continuously passing a sample through the column of resin. The rate at which the various components move down the column, depends upon their relative selectivity co-efficients. The component of the mixture with the lowest co-efficient will move down fastest.

(ii) Displacement Development

The essential requirement for this process is that ions of the sample have a higher affinity than the ion initially bound to the exchanger.

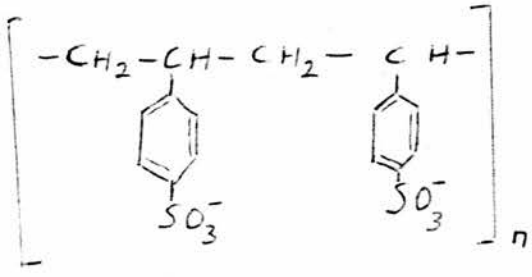
(iii) Elution Analysis

In this method, all the components of the sample can be completely resolved and quantitatively recovered. The chief draw back is that only small quantities of solute can be analysed. Sample is applied to the top of the column, and a solution of higher ionic strength is run through the column, which moves the solute bands down through the column while those with higher relative selectivity co-efficients are retarded.

1.11 ION EXCHANGE AMINO ACID ANALYSERS

S. M. Partridge and collaborators of Cambridge developed ion exchange chromatography with sulfonated polystyrene resins as a method for separating mixtures of amino acids in protein hydrolysates. Tristram and Smith (1963) reported that S. Moore and W. H. Stein later perfected the technique, that is now widely used for amino acid analysis of proteins. The method passed from non-automated to semi automated method, Hamilton and Anderson (1959) Moore and Stein (1951, 1954), Moore et al (1958) and finally to fully automated procedure, Spackman et al (1958) performed amino acid analysis by ion exchange chromatography.

The amino acid mixture is applied to a column of the resin



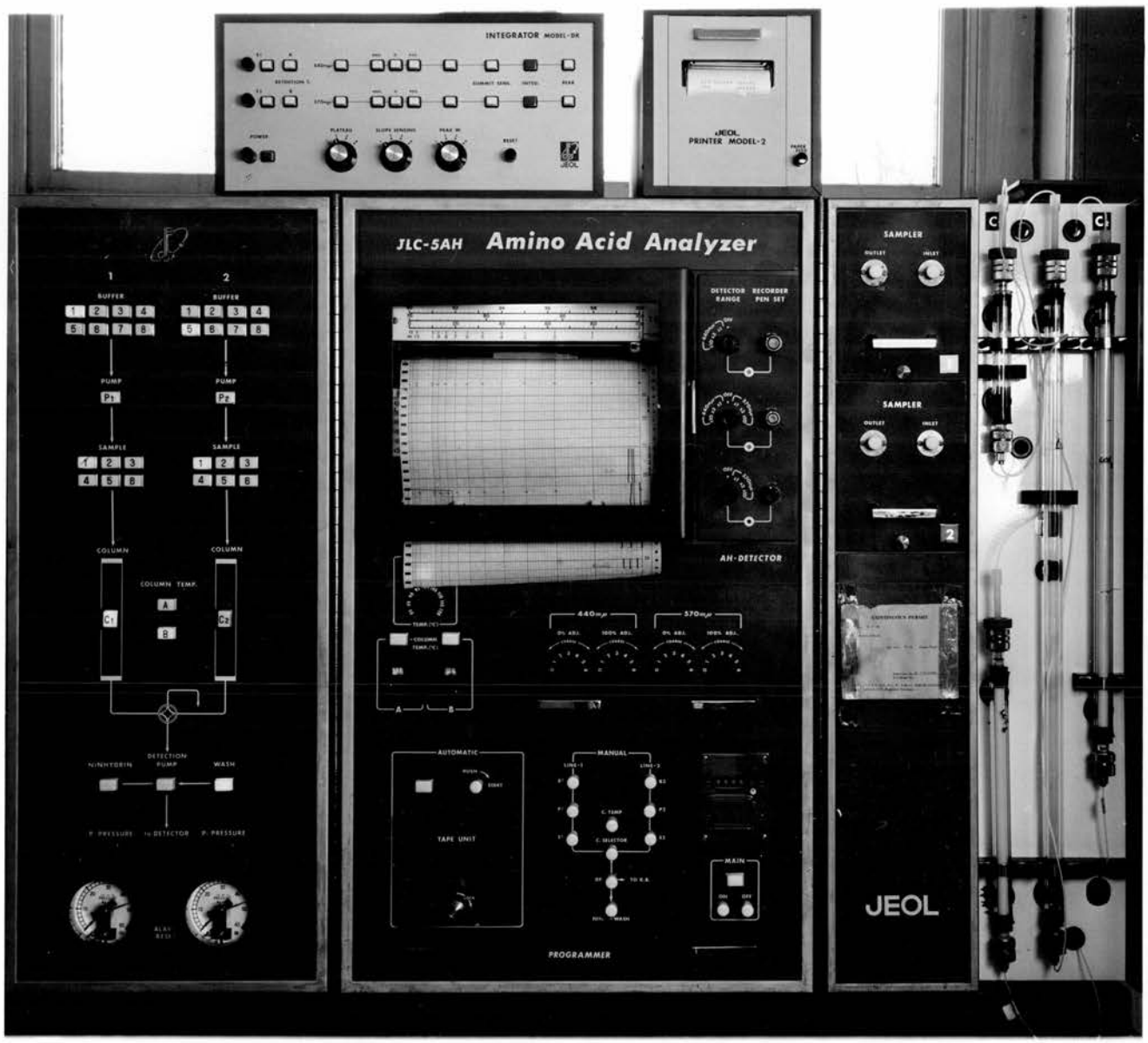
Sulphonic acid cation exchanger of polystyrene.

Figure 4.

and then eluted by passing a buffer a specified pH and ionic strengh through the column. The positive charges of an amino acid are attracted to the resin by electrostatic forces, and the hydrophobic regions of amino acids interact with the non-polar benzene ring.

At any pH, a certain fraction of any amino acid exists in positively charged forms. An amino acid with a higher $[AA^+] / [AA^\circ]$ ratio will move through the column slower than one of equal non-polar charcter with a lower $[AA^+]/ [AA^\circ]$ ratio. Generally, the amino acid, with the lower $[AA^+]/ [AA^\circ]$ ratio will elute before the one with a higher ratio, provided they have equal non-polar attractions for the resin. This means that a protein will stick to a cation exchange resin below its iso-electric point and be released when the pH approaches its isoelectric pH. The protein can also be released by competition from other ions if its ionic strength is raised.

The sulphonated polystyrene resin, is equlibrated with 0.2M Sodium hydroxide solution to charge its sulphonic acid groups with Na^+. This is referred to as sodium form of the resin. The resin may also be prepared in the protenated or H^+ form by washing it with acid. Amino acid mixture is added to the Na^+ form of the resin.



JLC-5AH FULL-AUTOMATIC AMINO ACID ANALYZER.

DIAGRAM 1

At an acid pH, amino acids are largely cations with net positive charge. The cationic amino acids tend to displace some of the bound Na^+ ions from the resin particles, the amount of displacement varies slightly among different amino acids, because of small differences in degree of ionization. As the pH and the ionic strength of the eluting medium are increased, the amino acids move down the column, at different rates, and the eluate is collected in small fractions and analysed by ninhydrin reaction. So the most anionic e.g. Glutamic acid appear first, and the most cationic e.g. lysine appear last.

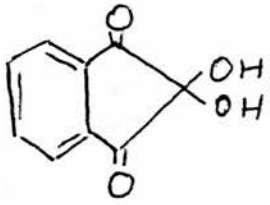
Modern amino acid analysers have incorporated auto samplers of different capacities, sophisticated control and data handling systems. The factors that influence the present day columns, and the materials used in their design as enumerated by Akiode (1980) are the following:

1. The structure of the cation-exchange resin in the column.
2. The dimensions of the column.
3. The composition and flow rate of the eluting buffers, which resolve the amino acids on their passage through the column.
4. The composition and reaction conditions of the chemical detection systems to give high sensitivity, greater resolution and decreased analysis time.

1.12 JLC - 5AH

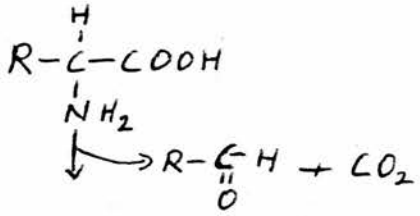
The automatic amino acid analyser used for the project is called Full Automatic Amino Acid Analyser JLC-5AH (see Pages 33 and 36). It consists of two columns. The hydrolyzates of proteins are used and the eluting buffers pH 5.28, pH 4.25, pH 3.25 are forced through the column under pressure and analysed quantitatively after reaction with ninhydrin (ref. Page 37) the attached chart.

NINHYDRIN REACTION

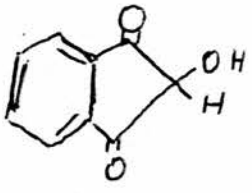


Ninhydrin

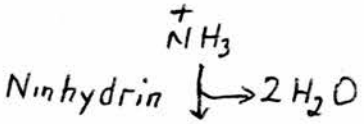
+

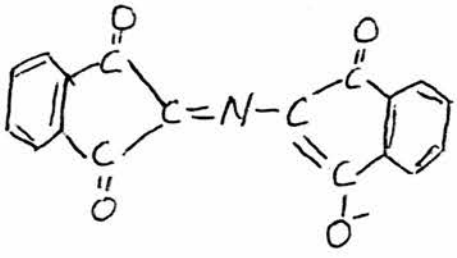


Amino acid



Hydrindantin





Purple pigment.

FLOW DIAGRAM

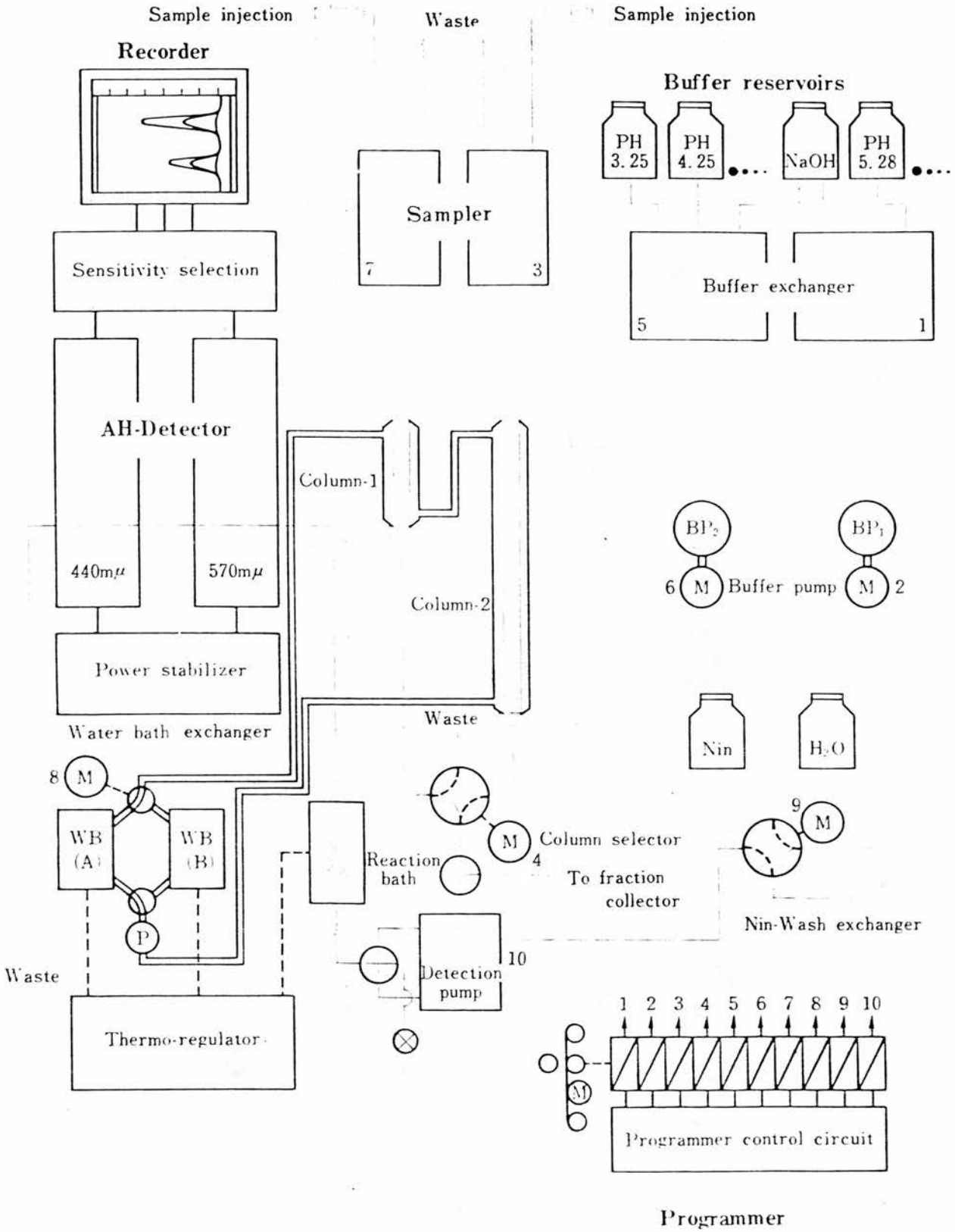
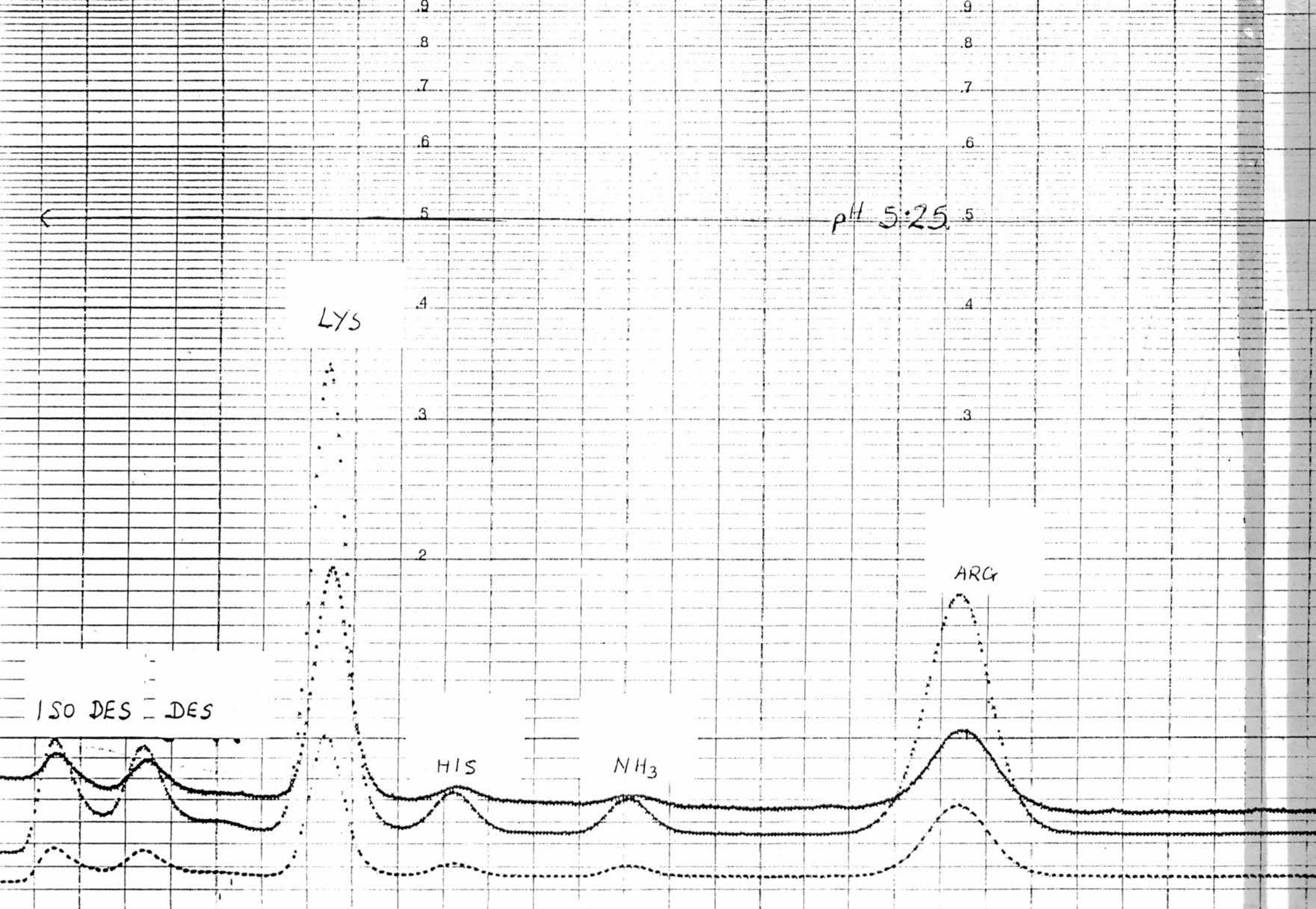
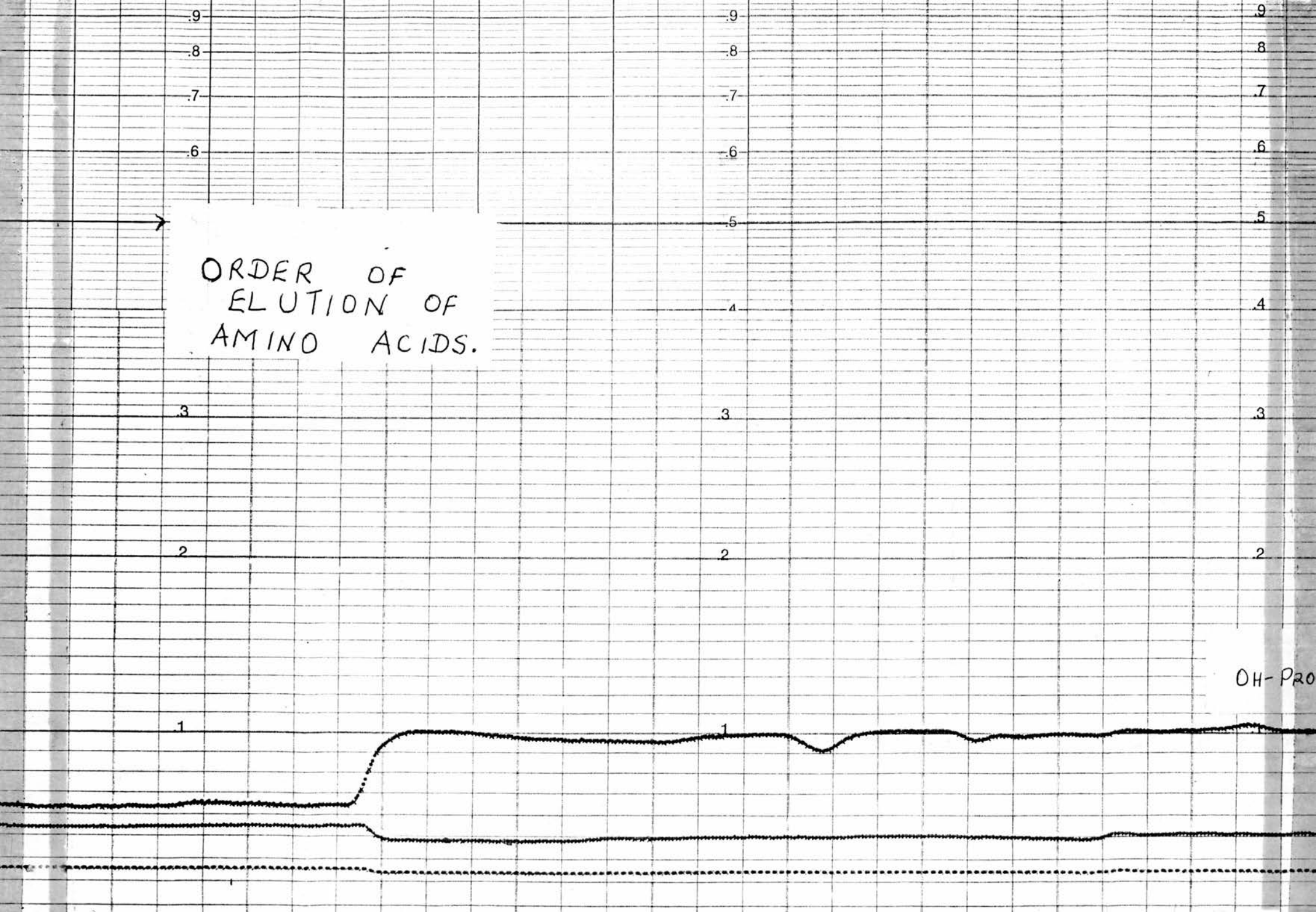
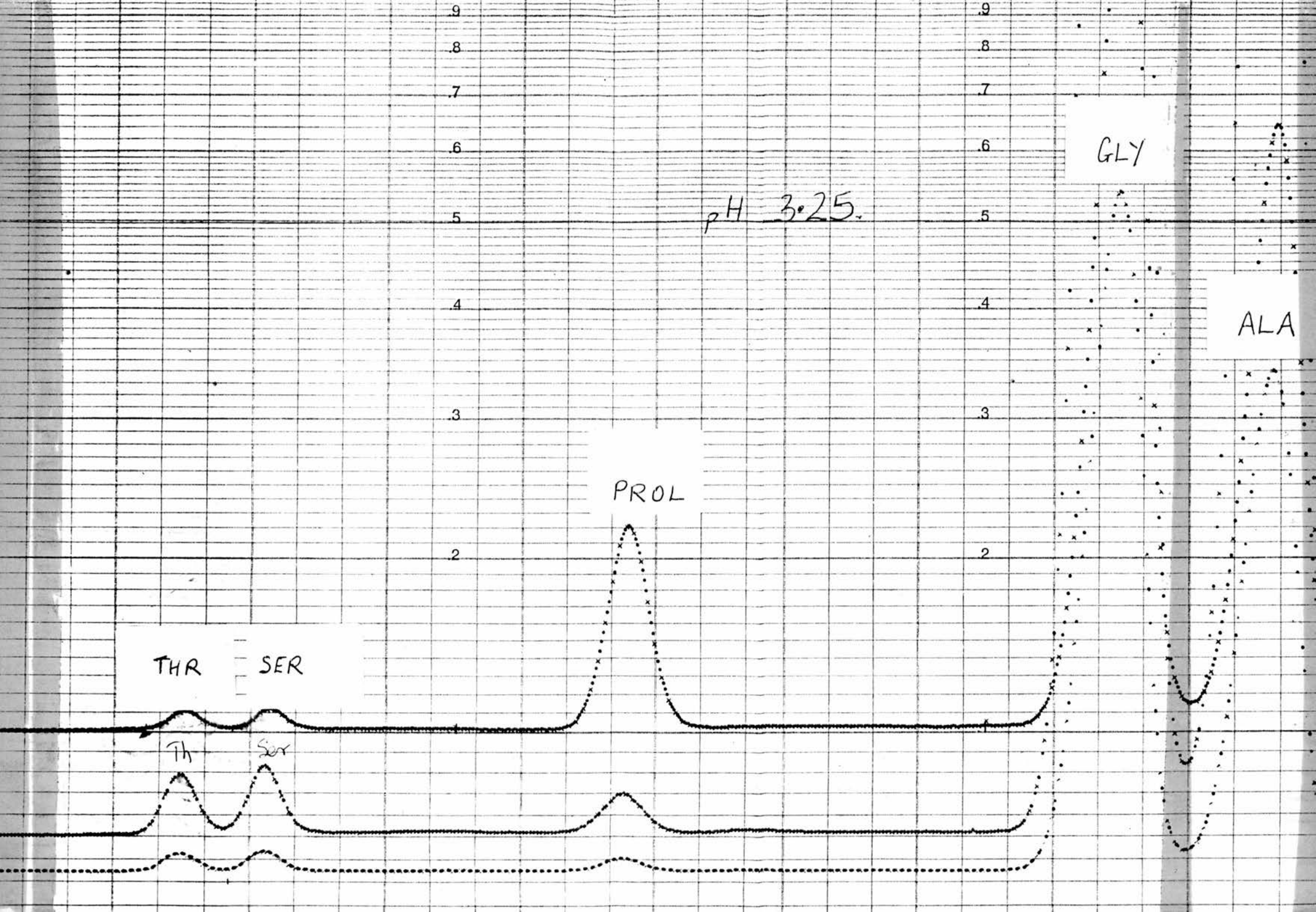
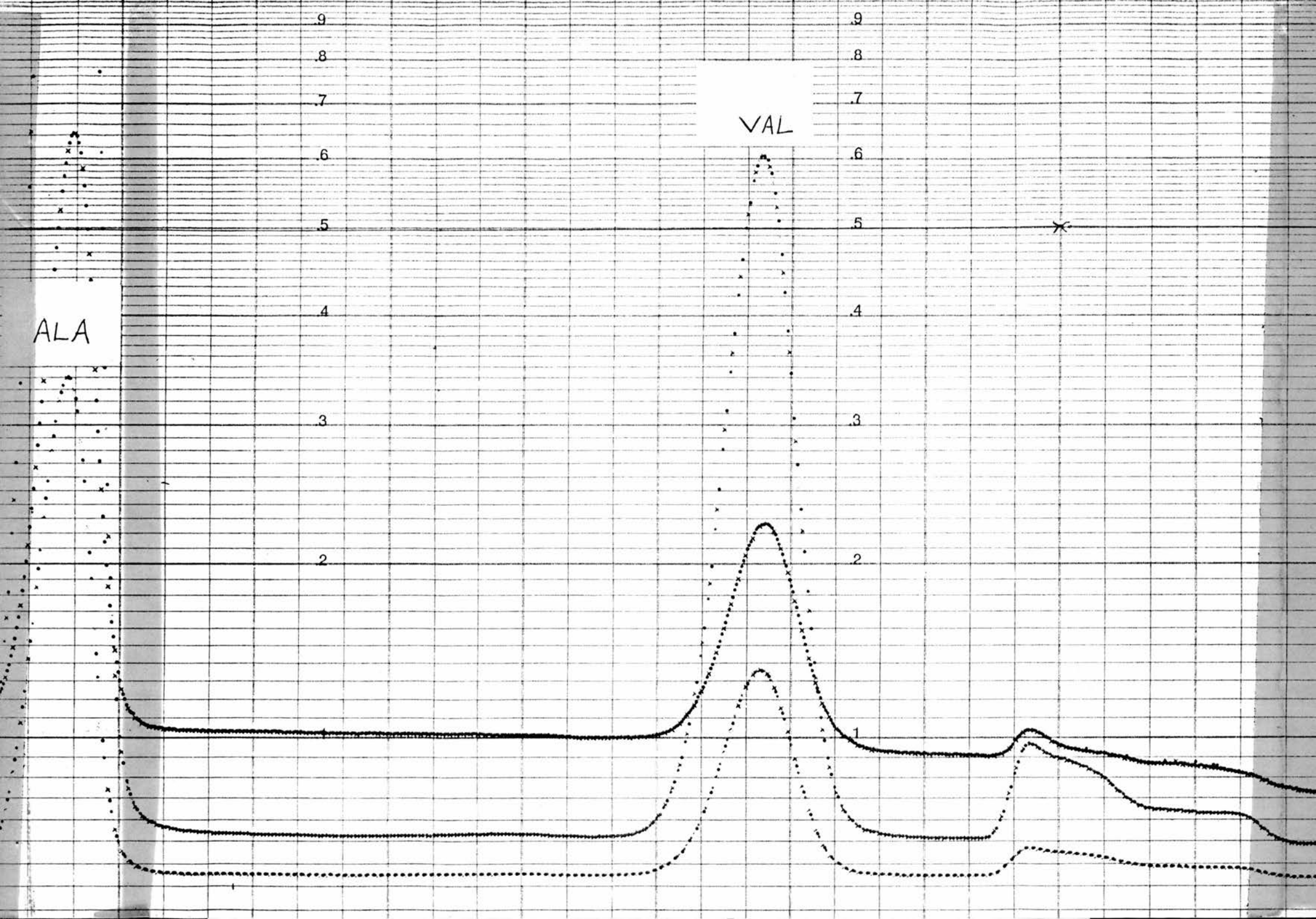


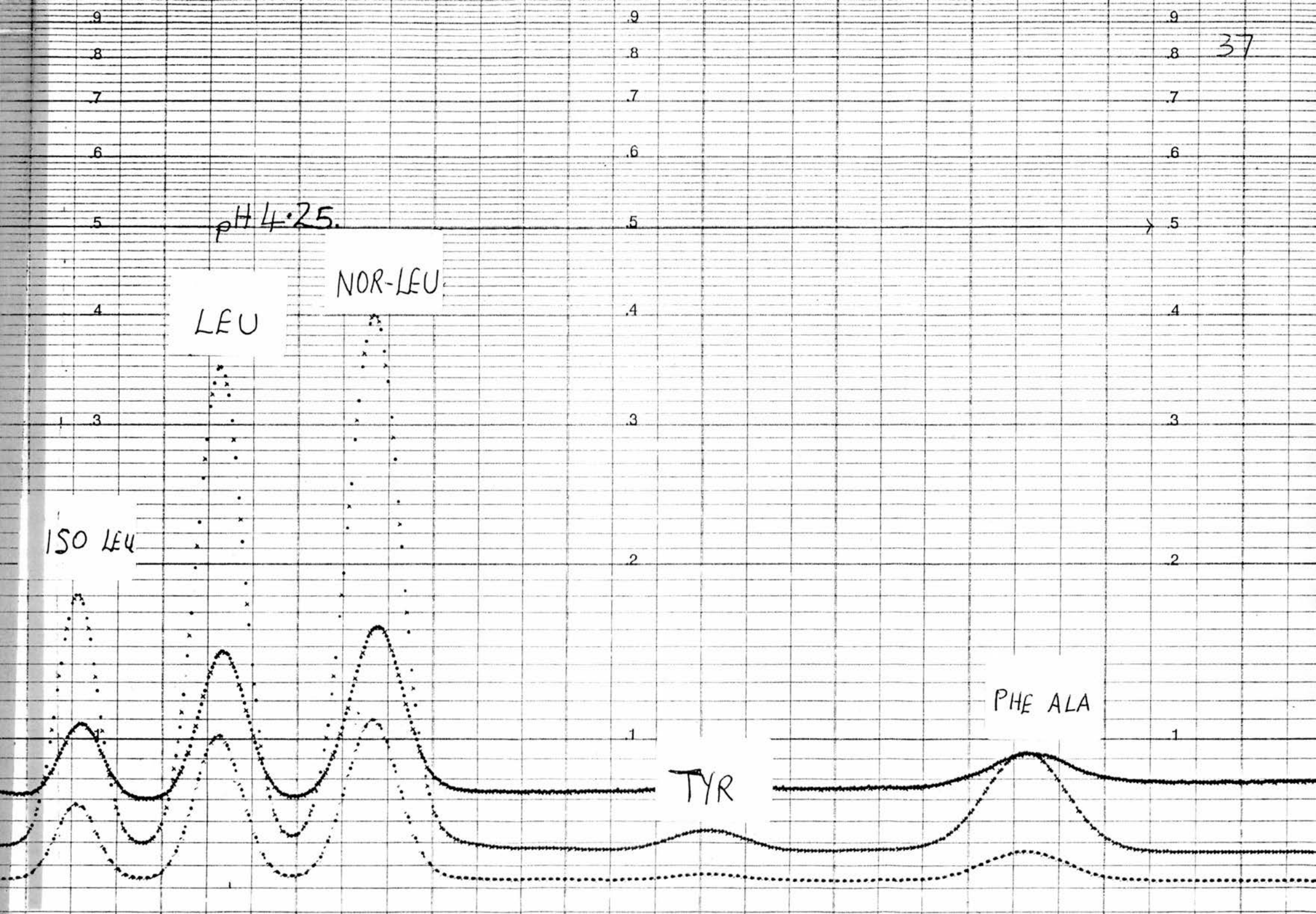
DIAGRAM 2


pH 5.25
LYS
ISO DES
DES
HIS
NH3
ARG


ORDER OF ELUTION OF AMINO ACIDS.
OH-PRO


pH 3.25
GLY
ALA
PROL
THR
SER
Th
Ser


VAL
ALA
.9
.8
.7
.6
.5
.4
.3
.2
1


pH 4·25.
NOR-LEU
LEU
ISO LEU
TYR
PHE ALA

The order of elution is as in the attached chart. Page 37.

(a) Short Column

15cm x 0.9cm. Buffer pH 5.25 $\pm$ 0.10, Na^+ 0.35N Resin LCRI, Isodesmosine, Desmosine, Lysine, Histidine Ammonia, Arginine.

(b) Long Column

70cm x 0.9cm. Resin LCRI

(i) pH 3.25 $\pm$ 0.08, Na^+ 0.2N, Aspartic Acid, Threonine, Serine, Glutamic acid, Proline, Glycine, Alanine, Valine.

(ii) pH 4.25 $\pm$ 0.10, Na^+ 0.2N. Isoleucine, Leucine, Nor-Leucine (internal standard) Tyrosine and Phenyl alanine.

The operating parameters for optimal chromatographic performance are:

(1) Column temperature - Generally increase in temperature causes increase in movement of amino acids.

(2) Buffer pH. At acidic pH, some amino acids e.g. Aspartic acid are most sensitive.

(3) Salt concentration.

(4) Resin - The resin with the highest degree of crosslinking compartible with the desired separation must be used. The height of resin in the column also affects the separation of amino acids.

(5) Length of column - As discussed in Section I.I2, column length is essential in separation of amino acids. This is why there are two columns Short and Long.

(6) Detection Reagent - The most widely employed detection reagent is based on the reaction of amino acids in column effluent with a heavily buffered solution (pH 5.5) of ninhydrin in the presence of reducing agents such as Stannous Chloride. When the hydrindantin (reduced ninhydrin) is added directly to the reagent mixture, care should be taken to avoid air oxidation taking place during the transfer to the analyzer reservoir. Also on the formation of hydrindantin by the addition of Stannous Chloride, precipitation of tin salts occurs in the reagent reservoir and flow lines of the analyzer. The precipitate can be removed by periodic flushing with water.

James (I97I) suggested that the use of Titanous Chloride is more convinient than Stannous Chloride for preparation of ninhydrin reagent.

The absorbance of resulting complex (Ruthmann's purple) is measured at a wavelength of 570nm for most amino acids, and 440nm for proline which gives a yellow colour.

(7) Purity of Samples - Chemical companies such as Sigma and British Drug House provide suitable tested chemicals, solutions and calibration standards for use in Amino acid analysers.

1.13 IDENTITIFICATION OF PEAKS - QUALITATIVE ANALYSIS

The position (i.e. retention time) of a peak is used to identify the amino acids separated, though a standard has to be put up. The peaks of unknown components are compared with the peaks of the standard chromatogam, run under same conditions.

1.14 QUANTITATIVE ANALYSIS

The quantity of each amino acid is proportional to the peak areas of the chromatogram.

The measurement of the peak areas, can be carried out in one of two ways -

(a) Absorbance Method

This accumulates absorbance dots at either 570nm for most amino acids and 440nm for proline

or

(b) HW Method

This is calculating the product (HW) of the peak height (H) and its half width (W). The product (HW) is proportional to the peak area.

The objective of this project was to isolate two rare amino acids (desmosine and isodesmosine) by more than one method, vary the techniques in some ways and see the effect of these modifications on the results. The properties of the amino acids were also investigated to see the effect of the crosslinks on their structures.

2. APPARATUS

2.1 APPARATUS

JLC-5AH Amino acid analyser and Intergrator Model DK and JEOL Printer Model 2 from Jeolco House, Colindale London, Tecam Dri Block DB-3H Heating block drilled to hold test tubes sizes 10 x 75mm and 16 x 159mm and connected to Tecam Laboratory Temperature Controller, Carl Zeiss Spectrophotometer PMQ 11 supplied by Degenhardt and Co. Ltd. of London, LKB 3400B Radi Fraction Collector from United Kingdom LKB Instruments Ltd. LKB House South Croydon, Surrey. pH Meter 26 from Radiometer Copenhagen, Rotary Evaporator (Buchi, Switzerland) with a water bath kept at 45°C, Pressure Steam Steriliser (Portable Universal Electric Model) connected to an Autoclave Timer Control Box from Industrial Control Services Ltd Maldon Essex, Type SSB3 Water bath from Grants Instruments (Cambridge) Ltd. Barrington Cambridge, Serial 2450 Magnetic Stirrer from Grants Instruments Cambridge, Type LB4X Water bath from Grants Instruments Cambridge, Shandon Southern Power Pack (Vokam 500 - 150) Serial No. A09D 013 from Shandon Southern Products Ltd. Astmoor, Runcorn Cheshire, Nitrogen Cylinder from British Oxgen Corporation, Electrodialser made of perspex, Unicam SP 800 Spectrophotometer from Unicam Instruments Ltd. Cambridge England, Buchi (Switzerland) Rotary Evaporator Model R.10, Chromatography column (16.5" x 2.5") from Pharmacia, Water pump serial 500091 Type P2 from Grants Instruments, Cambridge, Edwards Vacuum Freeze Dryer by Edwards High Vacuum Ltd. Crawley Sussex, Edmund Buhler Tubingen peristaltic pump Type MPL, Aminco-Bowman Spectrophotofluorometer by Aminco.

2.2a JEOL JLC-5AH

See diagrams 1 and 2, pages 33 and 36 for the block diagram and flow diagram of JLC - 5AH.

As shown in the flow diagram, the JLC - 5AH is equipped with 8-step buffer exchangers, two buffer pumps and two samplers. The buffer exchangers, buffer pumps, samplers and columns, connected in this order, comprise two independent flow systems. One of the flow systems is selectively connected to the detection system by the column selector. The other is connected to waste port by the column selector. Part of the effluent in the former is sampled by the sampling pump and mixed with a ninhydrin solution from the ninhydrin pump. The mixture is colour developed in the reaction bath and delivered in to flow cell installed in the detector, where its absorbance is measured. The sampling pump and the ninhydrin pump are incorporated in the same drive unit and deliver the liquid synchronously at a flow rate of 2:1. The residual effluent can be fractionated by a Fraction Collector.

The optical detector, consisting of two sections, detects the absorbances of two wavelenghts of 440mn and 570nm. This optical detector employs high sensitivity photo tubes as detection elements. An independent amplifier which maintains great stability for sustained operating periods even at high sensitivity, is provided for each wavelength. The sensitivity of the amplifier can be varied in 3 ranges: x1, x3 and x10.

A chromatogram of the wavelenghts of 440nm and 570nm is recorded with any of three ranges of sentivity. In fact it is recorded in three curves, since two ranges of sentivity can be simultaneously selected in either of the two wavelenghts.

2.3b Conditions used for analysis were:

		Column 1 S.C.	Column 2 L.C.
	Stationery Phase	JEOL Resin LC-R-1	JEOL Resin LC-R-1
	Mobile Phase	Sodium Citrate	Sodium Citrate
(i)	Flow Rate buffer pump	1.22ml/min	0.93ml/min
(ii)	Flow Rate of Detection Pump	(a) Sample pump	0.42ml/min
		(b) Ninhydrin pump	0.21ml/min
		Total	0.63ml/min
(iii)	Column temperature	55°C	60°C
(iv)	Reaction Bath Temperature	95°C	
(v)	Chart speed: 12cm/hr		
(vi)	Pump Pressures	4 - 6kg/cm^2	8 - 10kg/cm^2
(vii)	Range	570mn Blue 70~100% fs (x3)	440nm Red 70~100%
		Green 0~100% fs (x1)	
(viii)	Light path length	2.0mm	

The column was packed with JEOL Resin LC-R-1. This is a special spherical ion exchange resin for 15cm and 70cm columns.

2.2c Loading of Sample into the Analyser

The inlet and outlet of the system were rinsed with distilled water injected from 10ml or 5ml Sabre disposable syringe and excess water and air were removed with Sabre disposable syringe. The sample load which was 0.8ml was slowly injected with 2ml Sabre sterile disposable syringe.

2.2d Integrator

JLC-DK digital Integrator attacned to the Analyser was used and the results compared with calculations by HW method.

2.3a The Carl Zeiss Spectrophotometer PMQ 11

As shown in figure 5, the spectrophotometer consists of the following parts:

Illuminator	Detector Housing
Monochromator	Indicator Instrument
Sample Changer	Power Supply Unit for the Lamps

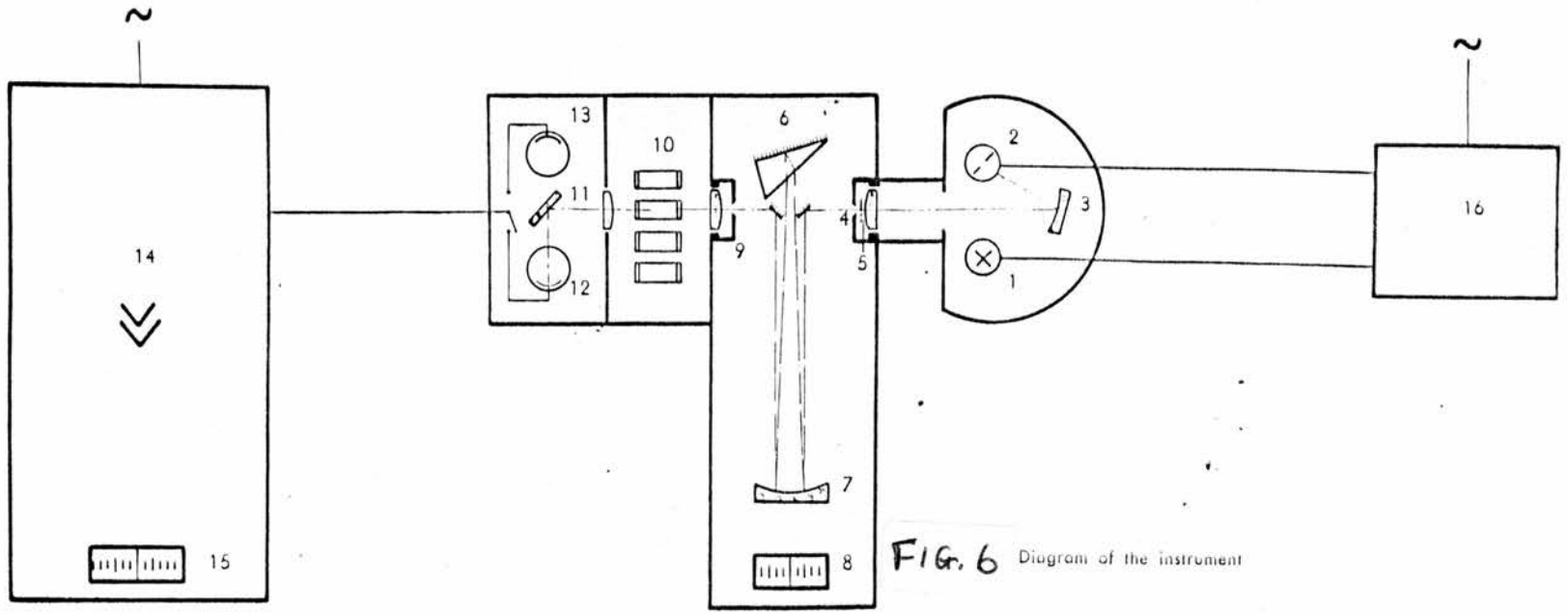


FIG. 6 Diagram of the instrument

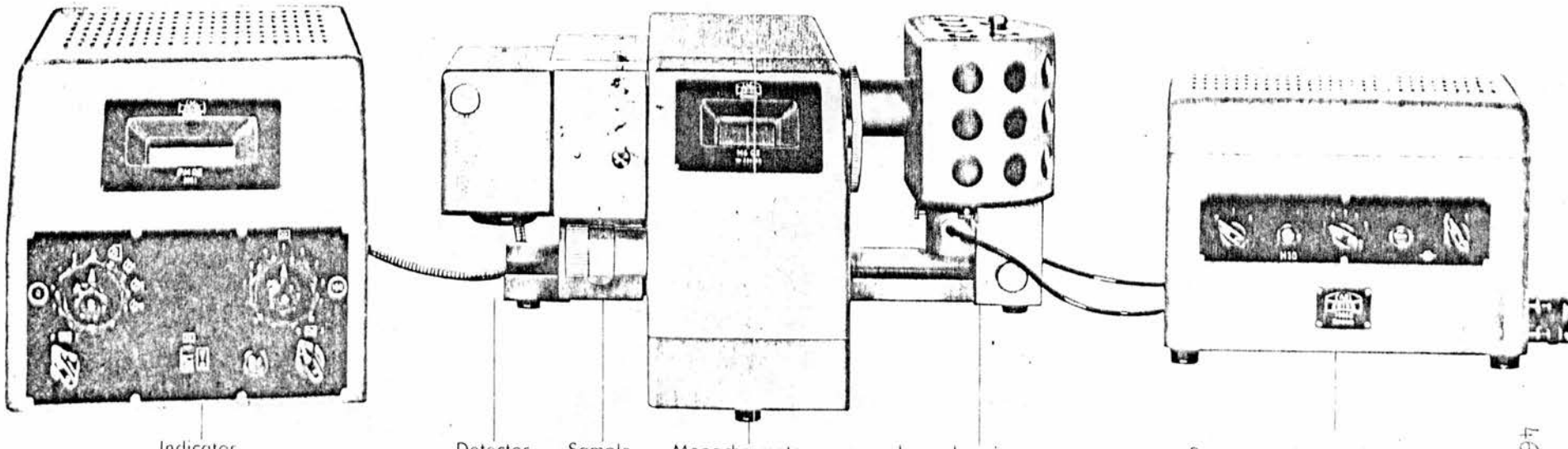


FIG. 5. View of the instrument

C O N S T R U C T I O N

Figure 5 shows a view of the instrument. The construction by a combination of easily separable individual parts offers manifold possibilities of application. The monochromator as the central part of the arrangement is provided with an optical bench on which the lamp housing with the light sources is mounted at the right and the sample changer with the detector housing at the left. The part for supply of current, amplification and indication of the measuring value are united in the indicator device.

The power supply unit for the lamps contains regulating devices for constant feeding of the lamps, independent of fluctuations in the main voltage.

Figure 6 clearly shows the mode of operation of the instrument. Two light sources 1 (incandescent bulb) and 2 (hydrogen lamp) can be imaged, by means of a swivel mirror 3, on the entrance slit 4 of the monochromator. A chopper diaphragm 5 in front of the entrance slit modulates the light. The approximately monochromatic radiation leaving the exit slit 9 of the monochromator traverses one of the four shiftable cells 10 in which the test solutions are contained and falls via a swivel mirror 11 on one of the two radiation detectors 12 or 13.

For measurement in the visible and ultraviolet ranges a multiplier 12 (photocell with secondary electron amplification) is provided, for measurement in the near infrared a photocell or a photoconductive cell 13. The current coming from the detector is amplified in the indicator device 14 and indicated on instrument 15. A separate power supply unit 16 feeds the incandescent bulb 1 and the hydrogen lamp 2 used for measurement in the UV.

3. MATERIALS AND METHOD

MATERIALS AND METHOD

3.1a Materials - Reagents

All the reagents were of analar grade and were obtained from British Drug House Chemical Limited, London unless otherwise stated.

3.1b M & B: Pronalys Grade

Concentrated Hydrochloric Acid, Methanol, Ammonia solution specific gravity 0.88, n-Butanol

3.1c Other Materials

Bovine Ligamentum nuchae was supplied by courtesy of Robert Alexander Butcher of Market Street in St. Andrews. CM Sephadex C25 Cation exchanger was bought from Pharmacia Upssala Sweden Fine Chemicals Company, London. Cation exchange membrane (Na^+ form) Product No. 55164 and Anion Exchange membrane (Cl^- form) Product No. 55165 were bought from BDH Chemicals, Vegetable Parchment membrane was supplied by courtesy of Dr. Russell, Linen cloth was supplied by courtesy of Mrs. J. Tristram, Dowex 50W (50 x 12 - 400) Hydrogen form was supplied by Sigma Chemical Company St. Louis, Missouri, U. S. A.

3.2 MEMBRANES

(a) Preparation of Anodic Membrane - (Albanese I940)

This was based on the method of Albanese (I940). The concentration of Gelatin used was 5% and not I%. The linen was soaked in 5% gelatin. This crosslinked the methane bridges of the gelatin and it became insoluble. It was hardened by soaking the linen in 4% formaldehyde.

(b) Cathodic Membrane

The vegetable parchment was prepared as specified in British Standard I820 : I96I. The vegetable parchment was made from pure bleached vegetable fibre by treatment with Sulphuric acid. It would be capable of withstanding boiling water without dis-intergration, and invariably white in colour. The parchment should not contain:

(i) added starch, gelatine, casein or formaldehyde.

(ii) loading materials in the form of mineral matter, as shown by an excessive ash content, nor more than the limiting quantities of arsenic, copper, iron and lead, and also not show the presence of benzoic acid, boric acid or borates and sulphur dioxide or sulphites. The maximum content of acidity is 0.02 per cent of acid calculated as Sulphuric acid, not more than 9 per cent of moisture, 0.6 per cent of ash, 2 parts per million of arsenic, 30 parts per million of copper, I0 parts per million of water soluble copper, 70 parts per million of iron, I5 parts per million of water

soluble iron, 20 parts per million of lead, 1.5 percent of water soluble matter, 0.2% of soluble non volatile reducing matter, and free from glycerine.

3.3 COMMERCIAL MEMBRANES

The second type of membranes used were bought from British Drug House Chemicals.

They were:

(a) The Cation Exchange Membrane (Na^+ form) Product No. 55164 and

(b) Anion Exchange Membrane (Cl^- form), Product No. 55165.

3.4 REAGENTS

Preparations Of Solutions For JLC-5AH Analyser

(a) BUFFERS

The three buffer solutions were prepared from analytical reagents as shown in the table below:

	pH 5.28 ± 0.10 at 20°C ± 5°C	pH 3.25 ± 0.08 (0.2N Na^+) at 20°C ± 5°C	pH 4.25 ± 0.10 (0.2N Na^+) at 20°C ± 5°C
Sodium Citrate dihydrate	171.5g	98.5g	98.5g
Benzyl alcohol (1% v/v) cm^3	50.0	-	-
Concentrated HCl cm^3	32.5	61.5	42.0
n^- Octoic Acid cm^3	0.5	0.5	0.5
Thiodiglycol	-	25.0	25.0
Methanol (7% v/v) cm^3	-	350.0	-
Final Volume Litres	5	5	5

(b) Sodium Acetate Ninhydrin Reagent

For 1 litre Ninhydrin reagent

(i) Sodium Acetate Solution for Ninhydrin

Reagents:	Sodium Acetate trihydrate	136.1g (A.R.)
	Glacial Acetic Acid	25.0cm^3 Pronalysis
	Final volume with deionised water	250.0cm^3

Preparation:

136.1g Sodium Acetate trihydrate ($CH_3COONa.3H_2O$) were dissolved in about 150cm^3 deionised water and gently heated for about 15 minutes to expel Carbon dioxide. The solution was then filtered when warm in to 250cm^3 volumetric flask and allowed to cool. 25cm^3 glacial acetic acid was added and the mixture after cooling to room temperature was made up to 250cm^3 mark with deionised water.

(ii) Re-distilled 2 - methoxy ethanol for Ninhydrin Reagent

2 Methoxy-ethanol is to keep hydrindatin - reduced ninhydrin in solution. The solvent for ninhydrin should be free from peroxides, hence 2 methoxy ethanol purchased was re-distilled to obtain 2-methoxy ethanol free from peroxides.

Solution A

This was prepared by adding 3cm^3 Conc.Sulphuric acid (Pronalysis) to 30g Ferrous Sulphate ($FeSO_4.7H_2O$) and then 55cm^3 deionised water.

1% V/v of this solution A was added to 6 litres of 2-methoxy ethanol and distilled. The first 5% V/v of the distillate at 125°C (768mn pressure) was discarded as well as the final 10% V/v left undistilled. The distillate was tested to be free from peroxides, according to the method of Eastoe and Courts (1972) or by adding 2ml of the solvent to 1ml of freshly prepared 4% aqueous Potassium Iodide. A colourless to light straw-yellow colour is satisfactory.

(iii) Preparation of the Ninhydrin Reagent for the Analyser

	Ninhydrin Reagent
Sodium acetate buffer solution	$250cm^3$
Redistilled 2 methoxy ethanol	$750cm^3$
Ninhydrin	20g
Stannous Chloride	0.6g
Final Volume	1 litre

$250cm^3$ of Sodium Acetate buffer solution (b(i) was placed in Ninhydrin reservoir pressure bottle covered with dish cloth and $750cm^3$ redistilled 2-methoxy-ethanol (b(ii) were added, and continously stirred with the magnetic stirrer. Under pressure, nitrogen gas (free from oxygen) was passed into the mixture for about 20 minutes. 20g of 2,2-Dihydroxyindandion-(1,3), (Ninhydrin A.R. from Merck) was added to the mixture, with stirring. The nitrogen gas was bubbled into the mixture until all the ninhydrin had dissolved.

The reducing reagent, 0.6g Stannous Chloride ($SnCl_2.2H_2O$) was added to the solution and nitrogen gas passed in to the ninhydrin reagent with stirring till all dissolved to form a red wine colour.

(iv) Age and Stability of Ninhydrin Reagent

Due to aging of ninhydrin reagent with time, standard measurements were made before and after running samples. The solution was stored in the ninhydrin reservoir pressure under nitrogen, covered with dark cloth. It is advisable to run a standard sample regularly, during the life of the reagent to check the area peak of each amino acid with the standard peaks.

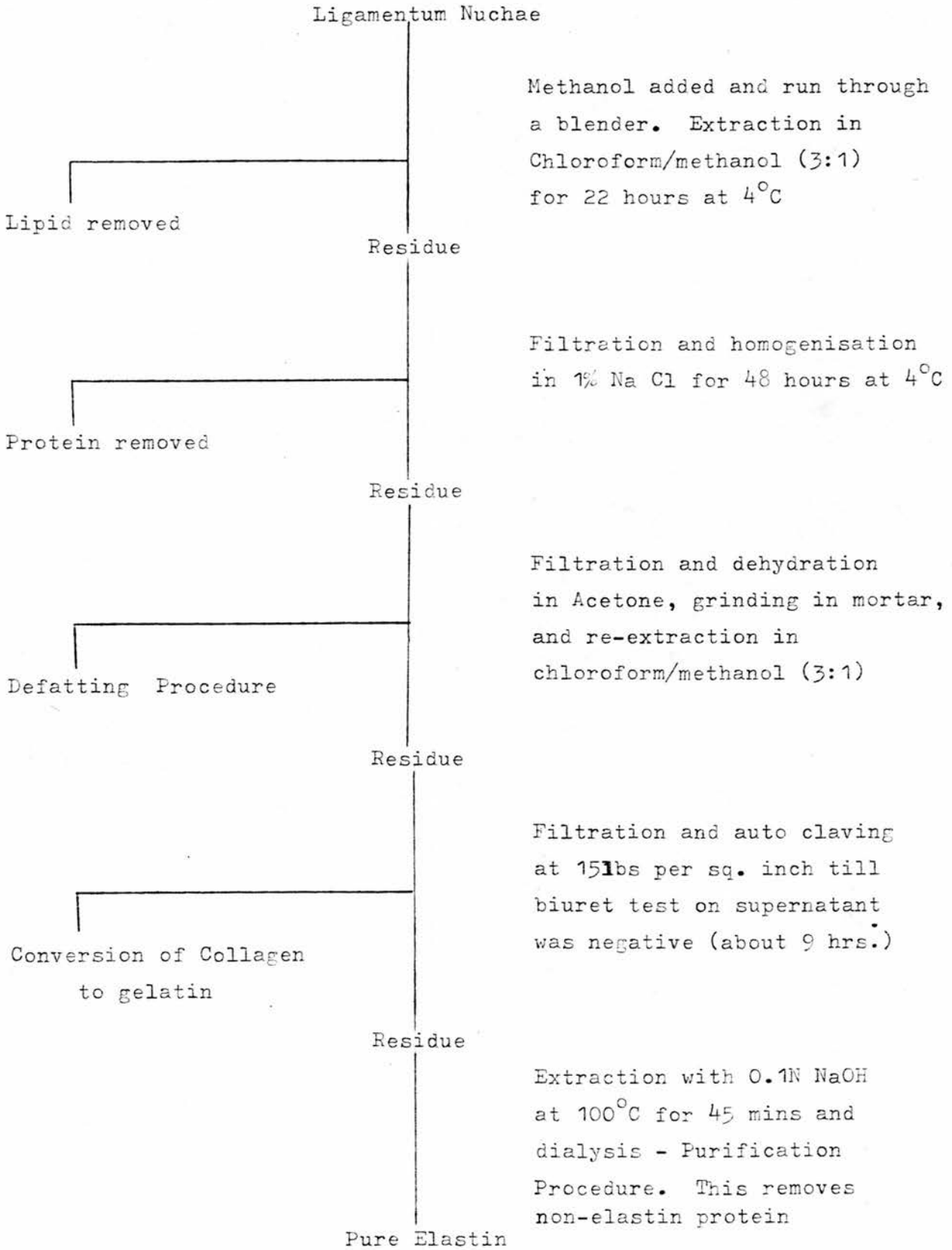
3.5 PREPARATION OF ELASTIN

Two different methods were used for isolating Bovine aortic elastin. The first method modified in some aspects was according to Serafini-Fracassini and Tristram (1965) and the second method was according to Starcher and Galione (1975).

(a) Method of Serafini-Fracassini and Tristram 1965

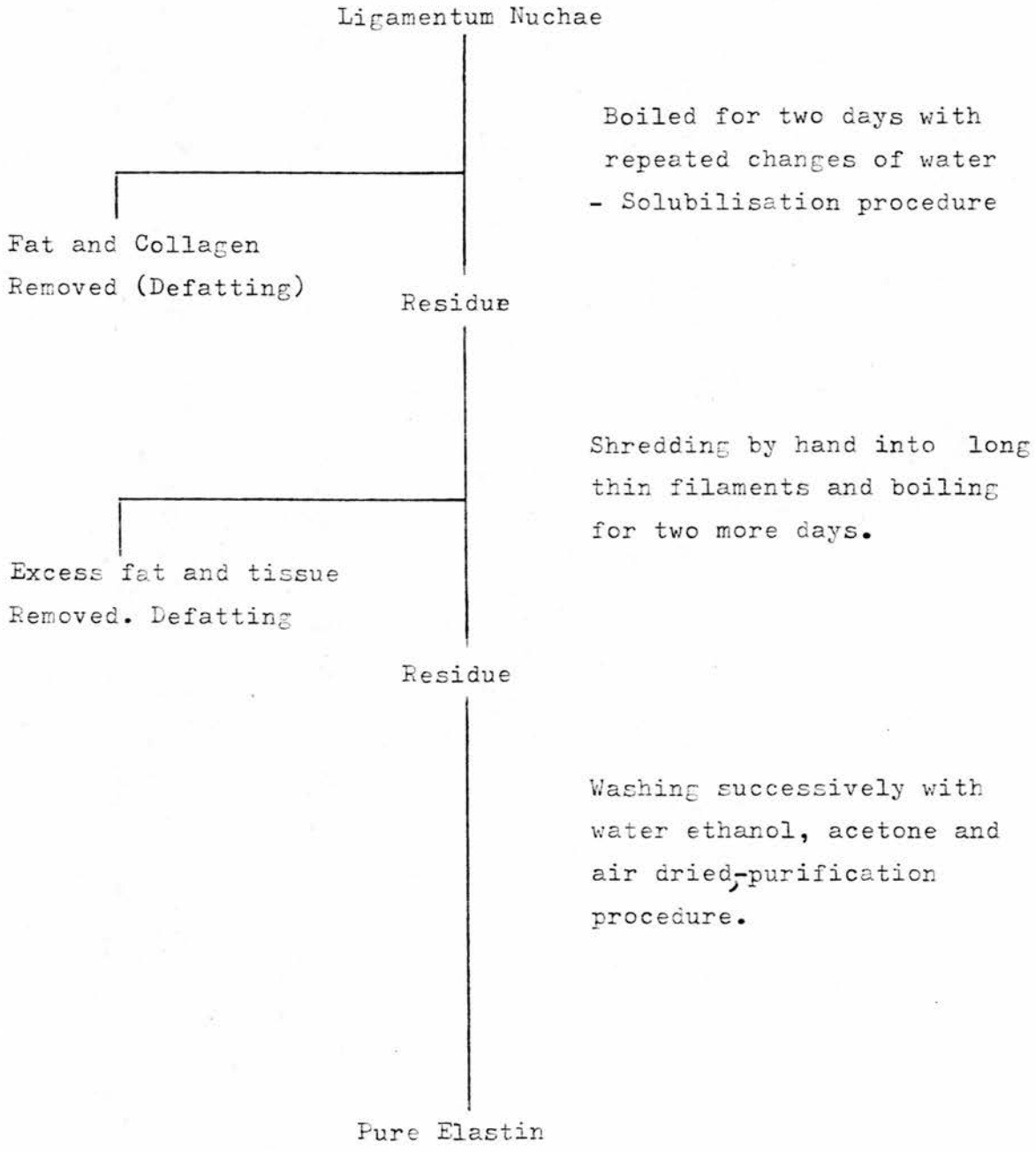
SCHEME FOR ISOLATION OF BOVINE AORTIC ELASTIN

SCHEME 3



(b) Method of Starcher and Galione (1975)

Scheme 4



3.6 GENERAL METHODS FOR HYDROLYSING PROTEIN

3.6a I N T R O D U C T I O N

The limiting factor in obtaining the highest degree of accuracy of the amino acid composition of a protein as a true reflection of the composition of the parent protein was, and is still the problem of hydrolysis as determined from composition of the hydrolysate. The problems involved are:

1. Destruction or decomposition of certain labile amino acids during hydrolysis.
2. Hydrolysis time, for example Tristram and Smith (1963) suggested 20, 40, 70 and 140 hours for hydrolysis, and
3. Conditions for perfect hydrolytic procedures as designed by Kimmel et al (1959) and Mahowald et al (1962).

The general methods for hydrolysis of proteins are:

1. Acid Hydrolysis
2. Alkaline Hydrolysis
3. Enzymatic Hydrolysis

3.6b Acid Hydrolysis

Chibnall et al (Macpherson 1946) hydrolysed protein by heating it in a boiling water bath with 10 volumes of 11N. HCl until all the protein had dissolved. This was diluted with water, the solution was then brought to 6N. with respect to Hydrochloric acid and hydrolysis continued under reflux for 24 hours. Linderstrom-Lang (1952) found that the losses of amino acids by the method of Chibnall et al could be reduced by hydrolysing the protein in excess 6M HCl prepared by distillation in an all-glass apparatus. This view was also confirmed by the work of Dustin et al (1953). Smith and Stockell (1954) hydrolysed the protein by heating it in sealed pyrex tubes at 105°C, with 500 volumes of 6M HCl, which has been redistilled three times in glass. Residual air was got rid off, from the pyrex tubes before they were sealed in later experiments, as black humid was being formed by the air, and this led to a contamination of the colour of the hydrolysate. Excess HCl was removed by evaporation in vacuo at 40°- 50°C. The usual method now is to heat the protein with excess 6M HCl at 100 to 120°C for 10 to 24 hours usually in evacuvated sealed tube (under nitrogen blanket). Little or no racemisation of the amino acids takes place under these conditions, though not all amino acids are quantitatively recovered.

3.6c Alkaline Hydrolysis

Tryptophan is degraded by hydrolysing protein in 6M HCl, but it is stable to alkaline hydrolysis, Tristram and Smith (1963). This method was not used in this project.

3.6d Enzymatic Hydrolysis

Hill and Schmidt (1962) carried out complete enzymatic hydrolysis of some proteins, and recommended that despite its many advantages, it should not replace acid hydrolysis. In this project more attention was paid to basic amino acids. The usual method of heating the protein in 6M HCl was adopted, though the time of the hydrolysis was different in the two chosen methods.

3.7 Elastin Hydrolysis - Serafini-Fracassini and Tristram (1965) Method

Some of the purified elastin was dried for three days in a small plastic beaker in an Edward Vacuum Freezer dryer.

(i) 0.5g of the dried elastin was weighed accurately on a Sartorius Chemical balance and put in 150 x 20 mm hydrolysis tube. The screw cap of the hydrolysis test tube had on its inside, an inert material made of Poly Tetra Fluoro Ethylene (P.T.F.E.). Some amount of Pronalysis 6M Hydrochloric acid was then added.

Nitrogen gas was bubbled through for about 10 minutes, and the hydrolysis tube quickly closed with the screw cap. It was then placed in an home made oil bath kept at $108^{\circ} \pm 1^{\circ}C$ for 24hours.

(ii) 0.2g of dried elastin was weighed accurately on a Sartorius Chemical balance into a 125 x 16mn hydrolysis tube, with a screw cap, also having P.T.F.E. material on the inside of the tube. Pronalysis Hydrochloric acid 6M was added, and nitrogen gas was bubbled through for about 10 minutes. The hydrolysis tube was then put in a heating block maintained at a temperature of $100^{\circ}C$ for 24 hours.

(iii) 5g of dried elastin was weighed accurately into a 500ml Quickfit B34/35 round bottom flask. Excess 6M pronalysis Hydrochloric acid was added, and nitrogen gas was bubbled through. A Quickfit reflux condenser was attached to the flask and the flask was suspended by a retort stand, and made to sit in 12.5mm heated Isomantle. The tap was turned on, and bumping granules were added to the flask to decrease rate of bumping. The hydrolysis was continued for 24 hours at a temperature of $104^{\circ} \pm 2^{\circ}C$ in a fume cupboard.

3.8 Elastin Hydrolysis - Starcher and Galione (1975)

Method

The hydrolysis was done in a 5 litre Quickfit round bottom flask connected to a reflux condenser. Owing to the fact that the weight of the elastin was 1.4kg the dried elastin was added in bits into the flask, and a large excess of 6M HCl was added. The flask was supported by a retort stand, and also made to sit on a 250m heated Isomantle, kept at 105 ± 2°C. Some amount of pronalysis HCl was added too, to increase the strength of the 6M HCl. All the elastin dissolved in the acid, and a reflux condenser was attached to the flask and the tap turned on. The hydrolysis was done for 36 hours in a fume cupboard.

3.9 Methodology

The method of isolating desmosine and isodesmosine are different in the two chosen methods after the hydrolysis of the elastin. In the first method (see section 3.9a) electrodialysis was carried out, and the catholytes collected were run on an amino acid analyser. In the second method (see section 3.10a) chromatography was done, and Sodium citrate buffer was used for elution. The eluates were collected on a fraction collector, and run through an amino acid analyser.

3.9a ELECTRODIALYSIS

Principle

The method of electrodialysis has been in use for a long time. The basic amino acids, placed in the centre compartment of a three compartment cell was subjected to the influence of a direct current at a suitable pH. The basic amino acids would carry a net positive charge and migrate to the cathode. By selecting a pH at which both the basic and mono amino mono carboxylic amino acids are completely or substantially dissociated (i.e. when the net charge is maximal for the former, and zero for the latter) an almost complete separation can be got. Dicarboxylic acids will migrate to the anode. Complete separation can not be affected in a single run. Albanese (1940) applied this method to the analysis of protein hydrolysate so also were Theorell and Akesson (1942), and they all believed that complete separation could be effected with a run. Macpherson (1946) observed that because of diffusion, some mono amino acids entered the cathode compartment. He suggested redialysis, at a selected pH of 5.8 and this would reduce the contamination. To obtain complete separation the dialysis might be done four times.

3.9b Description of the Apparatus

The dialyser was based on the type built by Williams, and reported by Macpherson (1946). It consisted of Perspex, the end compartments of about 100cm^3 capacity, were fitted with draining tubes. The centre compartment was constructed to contain about 150cm^3 so that catholyte and washings may be transfered for continuous electro dialysis, without concentration, as a peristaltic pump was connected to drive the hydrolysate through the system. The centre compartment was made to drain into a tube which served as a reservoir, and another tube led from the reservoir, through the system, and was made to end up at the centre compartment. The electrodes were made of carbon, and had a diameter of 10cm each. A current of 50 mA was applied using Shandon Power Pack (Vokam 500 - 150).

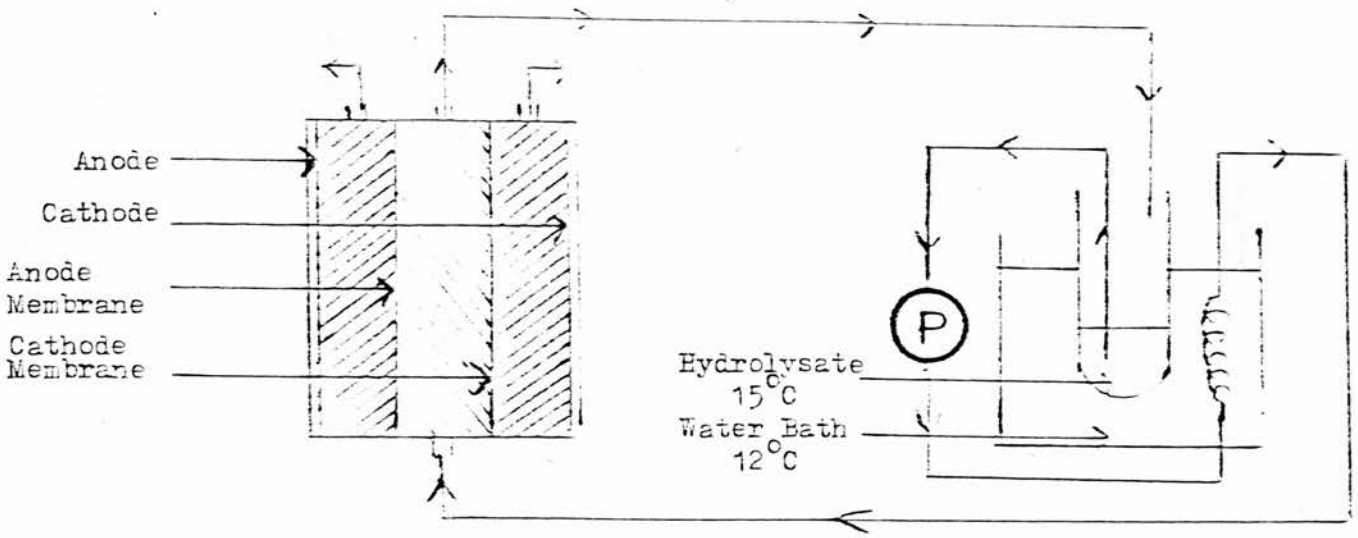
3.9c Procedure

The three compartments were filled with distilled water. The hydrolysate was made up to about 120cm^3, and washed into a tube suspended in a water bath kept at $15^\circ \pm 1^\circ$C. This tube served as the reservoir. The peristaltic pump and the power pack were switched on, and the dialysis was allowed to continue for several days depending on the concentration of the hydrolysed elastin in the hydrolysate and the membrane in use.

(i) For micro estimation ($\leq$ 0.5g), the membranes recommended by Albanese (1940) were used. As described on page 50 the anodic membrane consisted of a gelatinised linen soaked in 4% formaldehyde and the cathodic membrane consisted of vegetable parchment. This combination was used to minimise endosmotic effects.

(ii) For Macro estimation ($>$ 0.5g), the BDH commercial membranes were used, (see text page 51). The time spent for the dialysis, corresponded to the weight of hydrolysed elastin in the hydrolysate.

E L E C T R O D I A L Y S E R



P = Peristaltic Pump

Figure 7.

(iii) The duration of the first run (using any of the membranes combination) would depend on the quantity and nature of the amino acids present and also on the amount of H Cl remaining, as Chlorine gas would be liberated into the anode compartment. The current eventually dropped below 50mA, as the catholyte was being withdrawn by washing the cathode compartment through with distilled water. During the run the contents of the anode compartment should be run off hourly and replaced by distilled water. The main purpose of the washing through, was to remove most of the colour, and all H Cl free and bound. In addition to the basic amino acids, the catholyte would contain a large proportion of the other amino acids since the pH of the centre compartment would be very low (acidic) during most of the run.

A pH indicator - Bromocresol purple (few drops) was now added to the catholyte which was then adjusted with a pH meter to a pH of 5.8, by addition of ammonia solution.

The dialyser was thoroughly washed, and set up for the second run, the catholyte being placed in the centre compartment. The second run would take less time than the first run, and the current would therefore fall quicker from 50mA. The catholyte was again collected during the second run by washing through the cathode compartment with distilled water. The anode compartment was also washed through, and chlorine gas would no more be liberated into it.

The pH of the catholyte was made to 5.8, the pH indicator used was the earlier one (Bromo cresol purple) and a pH meter was used to measure the pH. The pH of the second catholyte would still be on the acid side, and ammonia solution was used to bring the pH to 5.8. The procedure was repeated as described above. The third run would take less time than the second run. The pH of the second catholyte might not be acidic as reported above, if the quantity of the amino acids in the hydrolysate was small, (0.2g to 0.5g).

The third catholyte would be collected as described above, and the pH made up to 5.8. The pH would invariably be on the basic side (> pH 7.0). Dilute sulphuric acid was added using bromocresol purple as indicator to bring the pH to 5.8. Each Catholyte was taken to dryness on a Buchi Rotary Evaporator at a temperature of 45°C, before redialysis.

(iv) Life of Membrane:

From experience, if the BDH membranes were being used, a set of membrances should be used for the 1st run, as this takes several days, and another set for the 2nd, 3rd and 4th runs. If the linen and vegetable purchment were being used, a set of the membranes , should be used for the 1st run, another for the 2nd run, and another for both 3rd and 4th runs.

(v) Estimation of the Amino Acids on an Amino Acid Analyser

Each catholyte was concentrated in vacuo on a water bath at a temperature of 45°C to remove ammonia present, before it was made up to 100cm^3 mark with distilled water. 0.5cm^3 of the catholyte was transferred to a Quick fit 25cm^3 round bottom flask and evaporated on a Buchi Rotary evaporator kept at 45°C. This was used for the estimation of the amino acids, while the 99.5cm^3 left was put in the centre compartment for re-dialysis.

The dried sample was dissolved in 10cm^3 of distilled water, and 0.8cm^3 out of it was applied to the Amino acid analyser for the estimation of the amino acids.

(vi) Calculation of the Amino Acids

The method used in this project is the HW method (Ref. Page 40 in text). The Jeol ruler specifically calibrated for the purpose was used for the measurement of the peaks. The ruler estimates the absorbance characteristics of amino acid - ninhydrin colouration at 570nm and 440nm.

The HWs of the amino acids and nor-leucine (internal standard) for both short and long columns, or Homo-arginine (for short column runs only), using 0.1uM/ml standard, and the concentration of the unknown are calculated thus

FIG. 8.

FLUORESCENCE SPECTRA
HYDROLYSED ELASTIN
EXCITATION/FLUORESCENCE SPECTRA
EXCITATION EMISSION
340/415
350/425
370/435

X.....mV/cm

Y.....mV/cm

Datum:

Name:

Nr.:

Blatt Nr.:

$$\text{Ratio} \quad \frac{\text{HW of each amino acid component}}{\text{HW nor-leucine}}$$

The nanomoles of each amino acid is calculated thus

$$\frac{\text{Ratio of unknown amino acid}}{\text{Ratio of standard amino acid}} \times 80\text{n. moles}$$

Sampler - capacity is $0.8cm^3$, and 0.1 uM/ml of internal standard (nor-leucine) is equivalent to 80 nano Moles, the number of moles in $1cm^3$ is calculated from colour yield.

3.10 ION EXCHANGE CHROMATOGRAPHY

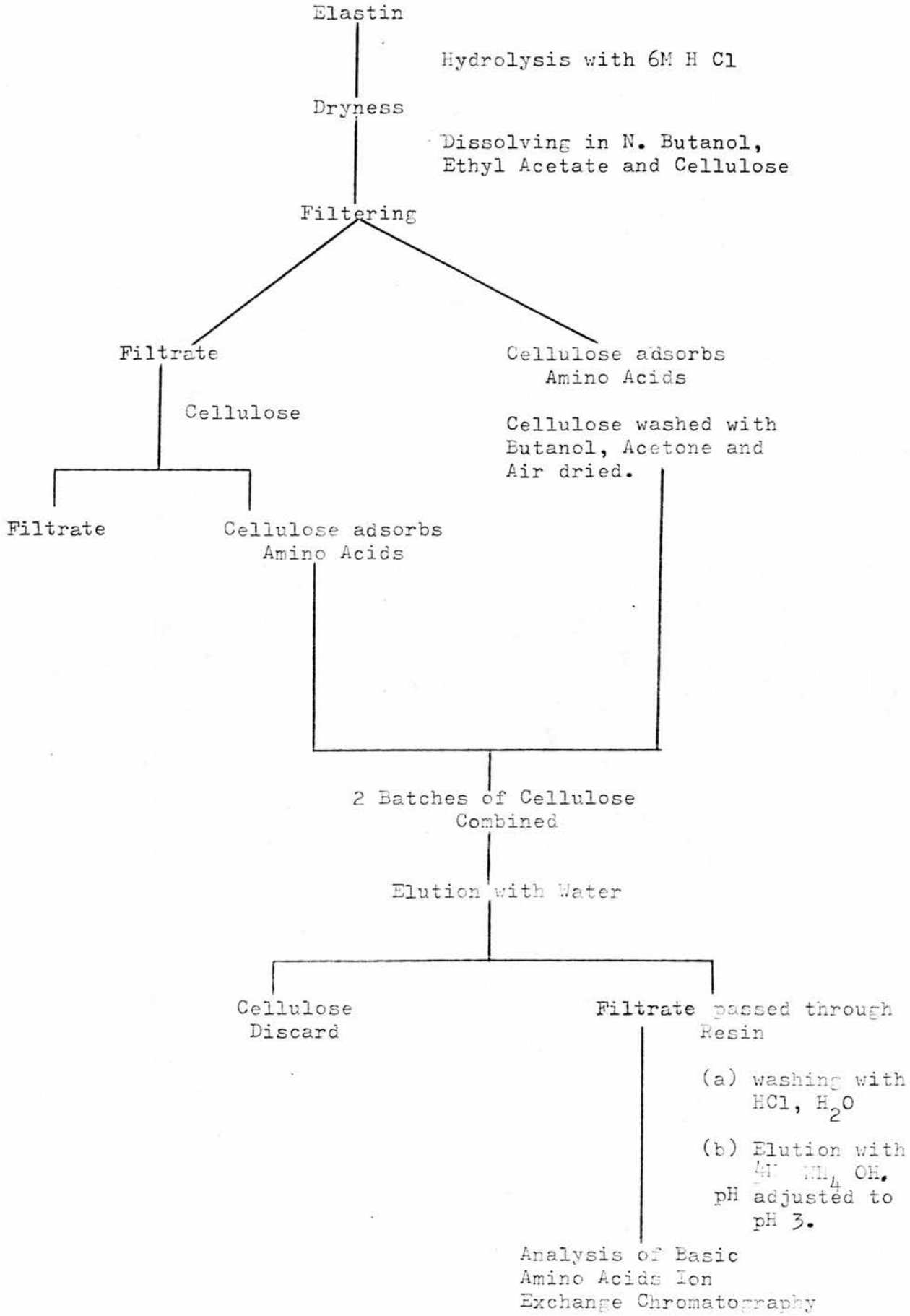
Column chromatography was used to purify the Desmosine and Isodesmosine in the second chosen method. This method was the one used by Starcher and Galione (1975).

SOME NOTES ON THE TECHNIQUE (Ref. Scheme 5)

(a) Starcher and Galione (1975) believed that by extracting twice with paper, it was possible to extract virtually all the desmosine, leaving the other amino acids in solution. The other cross linking amino acids, lysinonorleucine and merodesmosine as well as considerable lysine were also concentrated on the cellulose paper. Washing the cellulose with acetone was necessary in order to remove the butanol which has a high boiling point, and is difficult to remove by flash evaporation.

The desmosine which elute from the paper with H_2O, adsorb readily to the Dowex 50 and remain adsorbed when the resin was washed with 0.5N HCl. This removed most of the neutral and acidic amino acids with the exception of small amounts of leucine, isoleucine, tyrosine and phenyl alanine. These amino acids eluted early from Dowex 50 column, and were separated from the desmosines.

S C H E M E 5


Elastin
Hydrolysis with 6M H Cl
Dryness
Dissolving in N. Butanol, Ethyl Acetate and Cellulose
Filtering
Filtrate
Cellulose adsorbs Amino Acids
Cellulose
Cellulose washed with Butanol, Acetone and Air dried.
Filtrate
Cellulose adsorbs Amino Acids
2 Batches of Cellulose Combined
Elution with Water
Cellulose Discard
Filtrate passed through Resin
(a) washing with HCl, H_2O
(b) Elution with 4N NH_4OH. pH adjusted to pH 3.
Analysis of Basic Amino Acids Ion Exchange Chromatography

3.10b PREPARATION OF SODIUM CITRATE BUFFERS

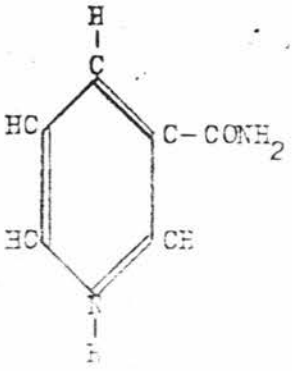
pH	Na^+ conc	Citric Acid	NaOH	Conc HCl	TDG	Brij	Phenol	Total Volume
3.5	0.2N	210g	82.5g	106.5ml	50ml	27ml	10g	10L
4.55	0.2N	210g	82.5g	47ml	-	27ml	10g	10L

The pHs checked with a pH Meter 26 (Radiometer Copehangen) and necessary adjustments were made. TDG stands for Thiodiglycol.

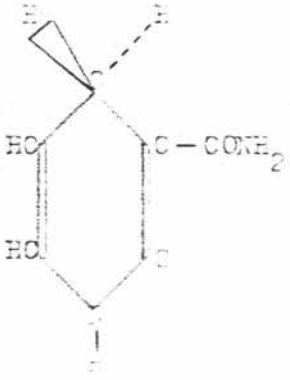
3.10c TRIALS OF SOME TECHNIQUES AFTER ELUTING THE AMINO ACIDS WITH 4N AMMONIUM HYDROXIDE

In the original method of Starcher and Galione (1975) thin layer chromatography using butanol/acetic acid/water (4:1:1) was the technique used in indentifying the desmosine and isodesmosine. In this project some other methods were tried to see if they would produce ideal results. Since desmosine and isodesmosine are shown to possess a pyridinium ring (Franzblau 1971) it was felt that they would behave like pyridine nucleotides and give a large absorbance peak at 340nm, and this was why in these methods the wavelength of 340nm was used. (See Figure 10 and Page 72A)

(i) A continous flow through Carl Zeiss Spectrophotometer was used in monitoring the Optical density of the eluates. The experiment was carried out at room temperature, and the wavelength of the spectrophotometer was set at 340nm. This is the wavelength of maximal absorption for reduced Nicotonamide Adenine Dinucleotide. (NADH).



NAD^+ oxidised form.

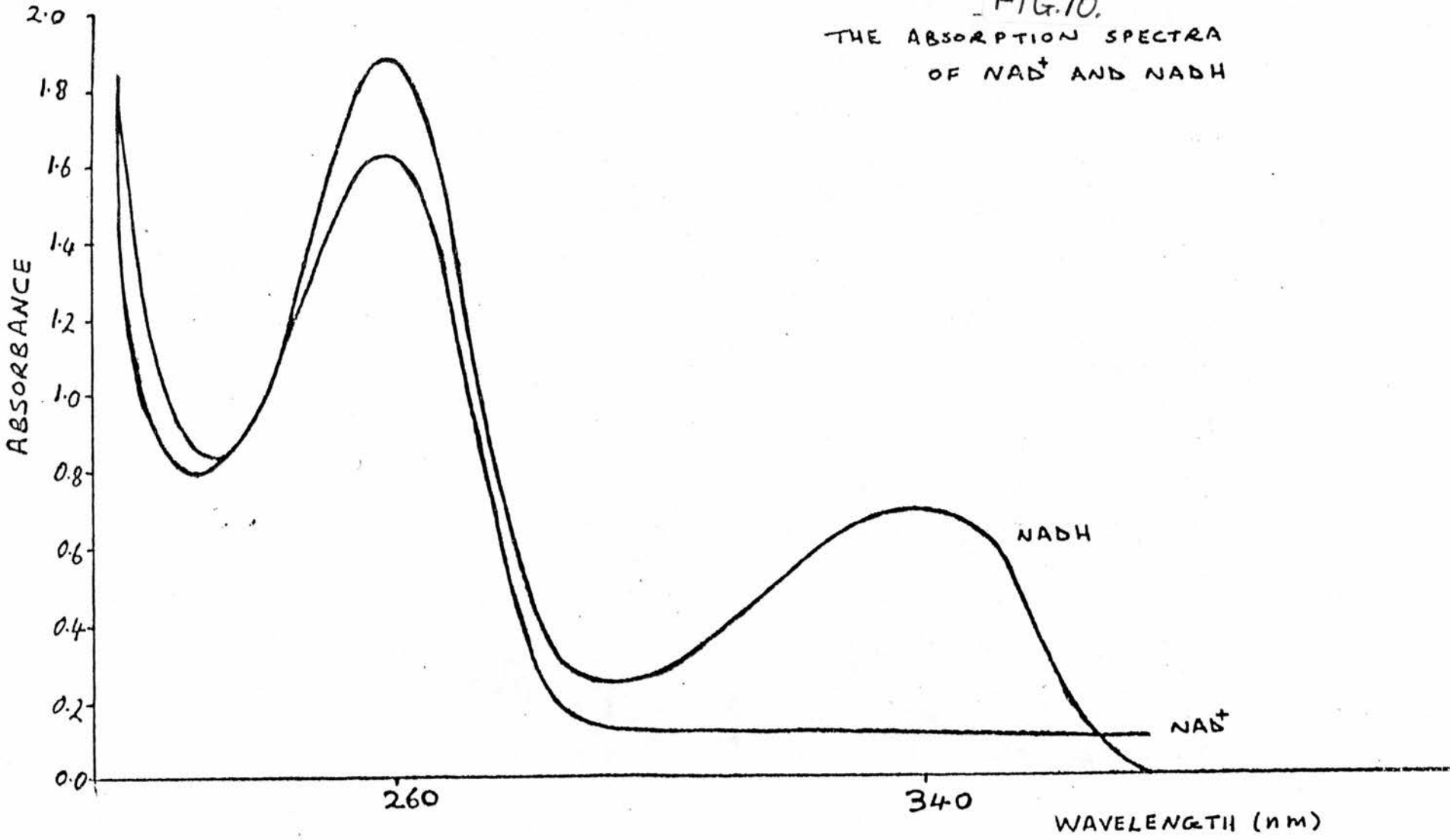


NADH reduced form.

Figure 9.

FIG. 10.

THE ABSORPTION SPECTRA OF NAD^+ AND NADH



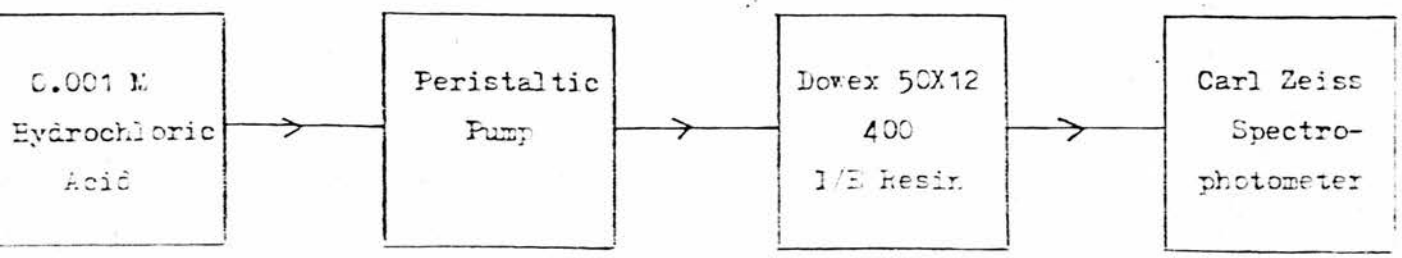


Figure 11.

5cm^3 of sample containing 29.79g of hydrolysed elastin was applied to the column.

(ii) The apparatus was set up as in 3.10c (i). The difference in the two was that a gradient was used. This was achieved by drawing 4M HCl into 0.01M HCl, to increase its molarity. The acid with increasing strength was then used to elute the applied amino acids out of the Dowex resin.

CONDITIONS FOR THE TEST

(a) The two flasks containing the two acids of different strength must be on the same level.

(b) The volume of acids in the two flasks must be same.

(c) A magnetic stirrer must be employed, to keep stirring the acid of a lower strength.

(d) Similar Volumetric flasks (i.e. same capacity) must be used.

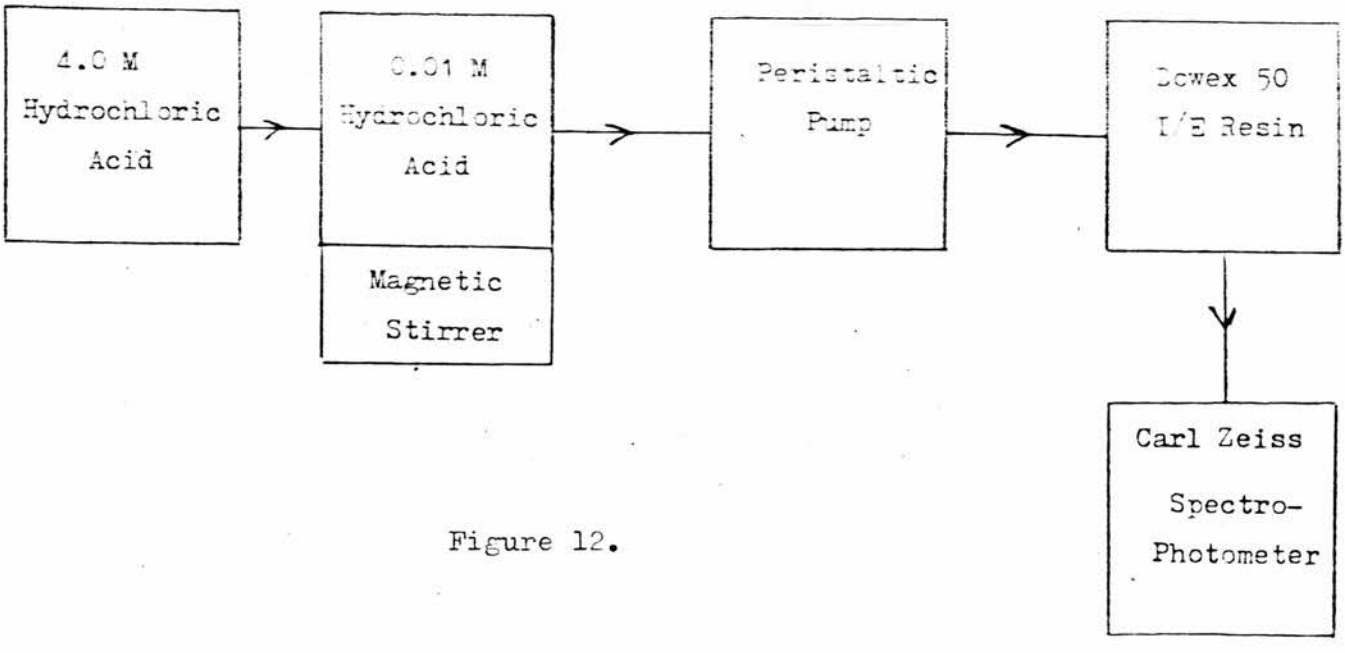


Figure 12.

The experiment was carried out at room temperature and the optical density of the eluate was monitored with a Carl Zeiss Spectrophotometer at 340nm. $5cm^3$ of sample containing 29.79g of hydrolysed elastin was applied to the column.

(iii) The apparatus was set up as in 3.10c (i), but the column contained Sephadex C25. C denotes that it is cation exchanger, while 25 is used to denote the degree of porosity.

Principle of Sephadex Ion Exchanger

Sephadex ion exchangers are produced by introducing functional groups on to Sephadex, a cross linked dextran. These groups are attached to glucose units in the matrix by stable ether linkages. Sephadex is hydrophilic; and swells readily in aqueous solutions.

The Sephadex C-25 was first soaked in distilled water, and carefully applied to the column, care being taken to avoid air bubbles, not to be trapped in the Sephadex. $5cm^3$ of sample containing 29.79g of hydrolysed elastin was then applied to the column, and 0.001N HCl was used for elution.

(iv) The apparatus was set up as in Section 3.10c(ii). The strength of the two acids used were 4N HCl and 0.01N HCl. All the conditions set out in section 3.10c(ii) were obeyed, and a peristaltic pump was used to pump the acid of increasing concentration into the Sephadex.

$5cm^3$ of sample containing 29.79g of hydrolysed sample was also applied to the column.

HCl was used for elution in these four methods because Citrate buffer or Acetic Acid absorbs in the Ultra Violet.

3.10d Ion Exchange Chromatography and Collection of Samples with a Fraction Collector

(i) 60ml of the sample containing 357.45g of hydrolysed elastin was applied to the column containing Dowex 50 x 12 - 400. The size of the column was 16.5" x 2.5". The resin was cycled first with 2N. HCl, H_2O, 2N.NaOH,H_2O and equilibrated with 0.2M Sodium Citrate buffer pH 3.25. The experiment was carried out at ambient temperature. After application of the sample to the column, the desmosineswere eluted with 0.23M Sodium Citrate buffer pH 4.55, and collected in ten milliliter fractions, on a Radi Rac Fraction Collector. The buffers were prepared as shown in Section 3.10b.

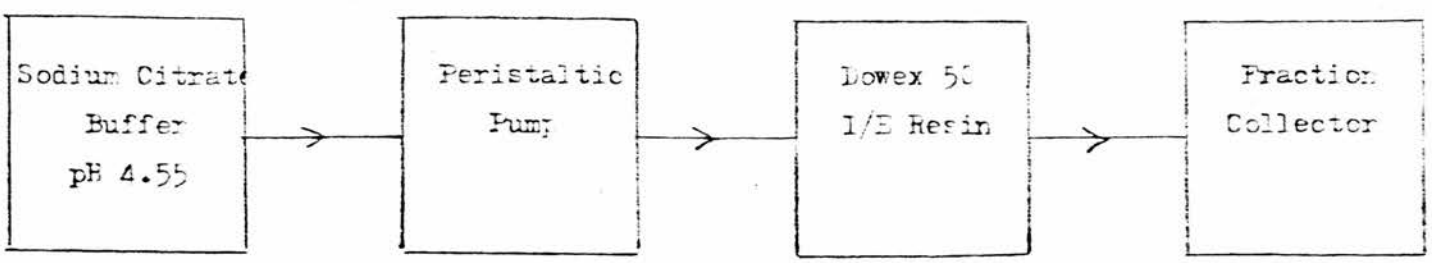


Figure 13.

3.10d(ii) The apparatus was set up as in section 3.10d(i). The major difference in the technique was that a water jacket maintained at 55°C was circulating round the column. This technique is similar to that of Starcher and Galione (1975), and 10 millilitre fractions were collected in 250 test tubes.

The samples were analysed on an amino acid analyser, in portions of five test tubes. A standard chromatogram of the applied sample was done, and the peaks compared with those of the eluates. The run was done till no more basic amino acids were detected by the amino acid analyser. The samples that had basic amino acids, were then pooled together, and taken almost to dryness on a Buchi Rotary Evaporator at 45°C. The concentrated sample was dissolved in a buffer of pH 3.25, and the elution process repeated. The basic amino acids were again detected by pooling five consecutive eluates together, and running them through an amino acid analyser.

The fractions that contained pure Isodesmosine and Desmosine were pooled together, and put in a Buchi Evaporator set at 45°C to concentrate it.

The concentrated material was then desalted by application to Dowex 50 resin in the Hydrogen form. The resin was washed with 0.5N. HCl, followed by water, eluted with 4N. NH_4OH, and evaporated to an oil. The oil was triturated with acetone until desmosine and isodesmosine precipitated as a fine powder. The precipitate was filtered dry through sintered glass and rapidly transferred to storage vials and desicated.

3.11 CRYSTAL FORMATION

One of the pooled samples (See UV Chart - Figure 49 that contained large amount of desmosine and isodesmosine, was taken almost to dryness on a Buchi Rotary Evaporator set at 45°C. The concentrated sample was transferred to a 100ml flask, a drop of toluene was added with a glass rod, and the glass rod was made to stand in the flask, and covered with parafilm. The flask was then transferred to a fridge to see if crystals of desmosine and isodesmosine would form.

Formation of Desmosine Picrate and Isodesmosine Picrate

This was done according to the method of Tristram (1939). A sample of a 4th Catholytes that contained mainly desmosine and isodesmosine was concentrated to about $1cm^3$, the flask was washed down with $6cm^3$ of 93% C_2H_5OH. The resulting liquid was adjusted with absolute alcohol or water so that a faint turbidity remained, and it was then kept on ice. An alcoholic solution of picric acid, kept on ice was then added, and the picrate allowed to crystallise in the fridge.

4. RESULTS AND DISCUSSION

4. RESULTS

4.1. ELECTRODIALYSIS

(a) Micro Method

In the micro methods (where 0.5g of elastin was hydrolysed) there was a good separation of the amino acids. Very small amounts of non-polar amino acids passed through the cathode membrane by the end of the third redialysis (that is 4th Catholyte). The two non polar amino acids detected by the amino acid analyser were Glycine and Alanine on the long column. These two non-polar amino acids are the most abundant in elastin, as shown by the figures published in Tables 1 and 11 in the text.

On the short column, all the basic amino acids in elastin were detected. The charts are shown in figures
The charts prove conclusively that the set of membranes (paper parchment and gelatinised linen) will isolate basic amino acids if the dialysis is rerun three more times, but that desmosine and isodesmosine cannot be completely separated. The calculations of the amounts of different amino acids that were obtained from 0.5g elastin are tabulated on page 82.

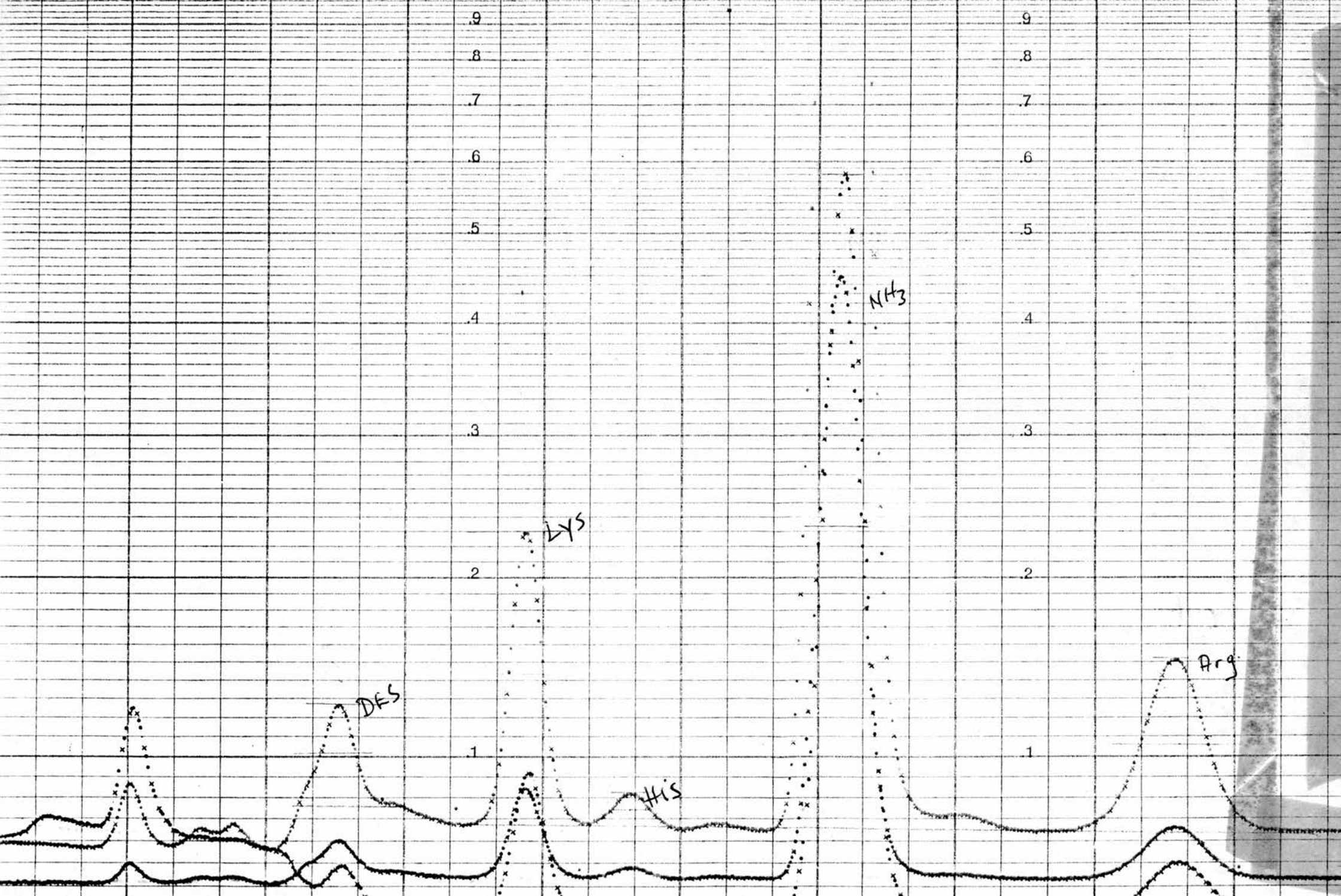
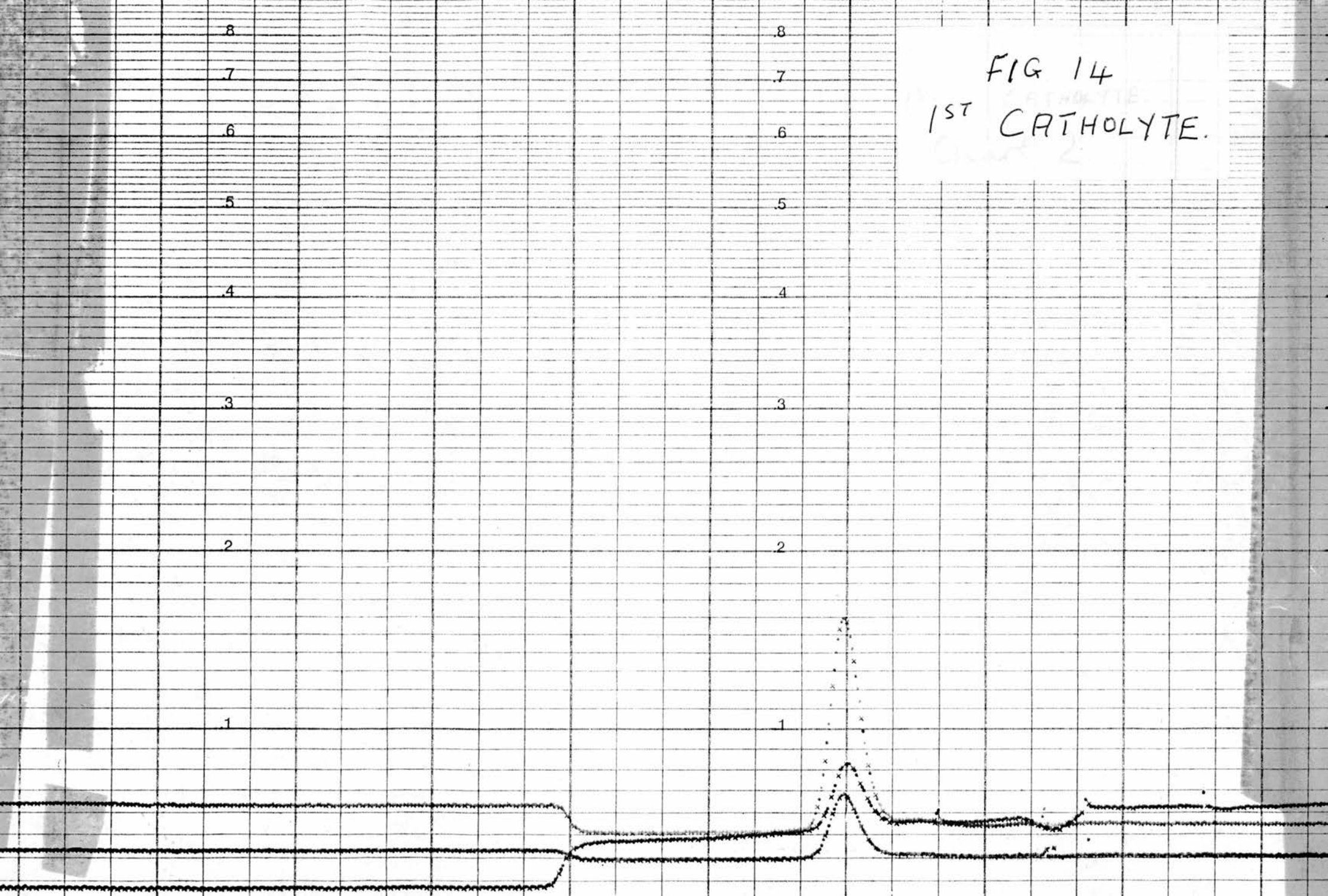
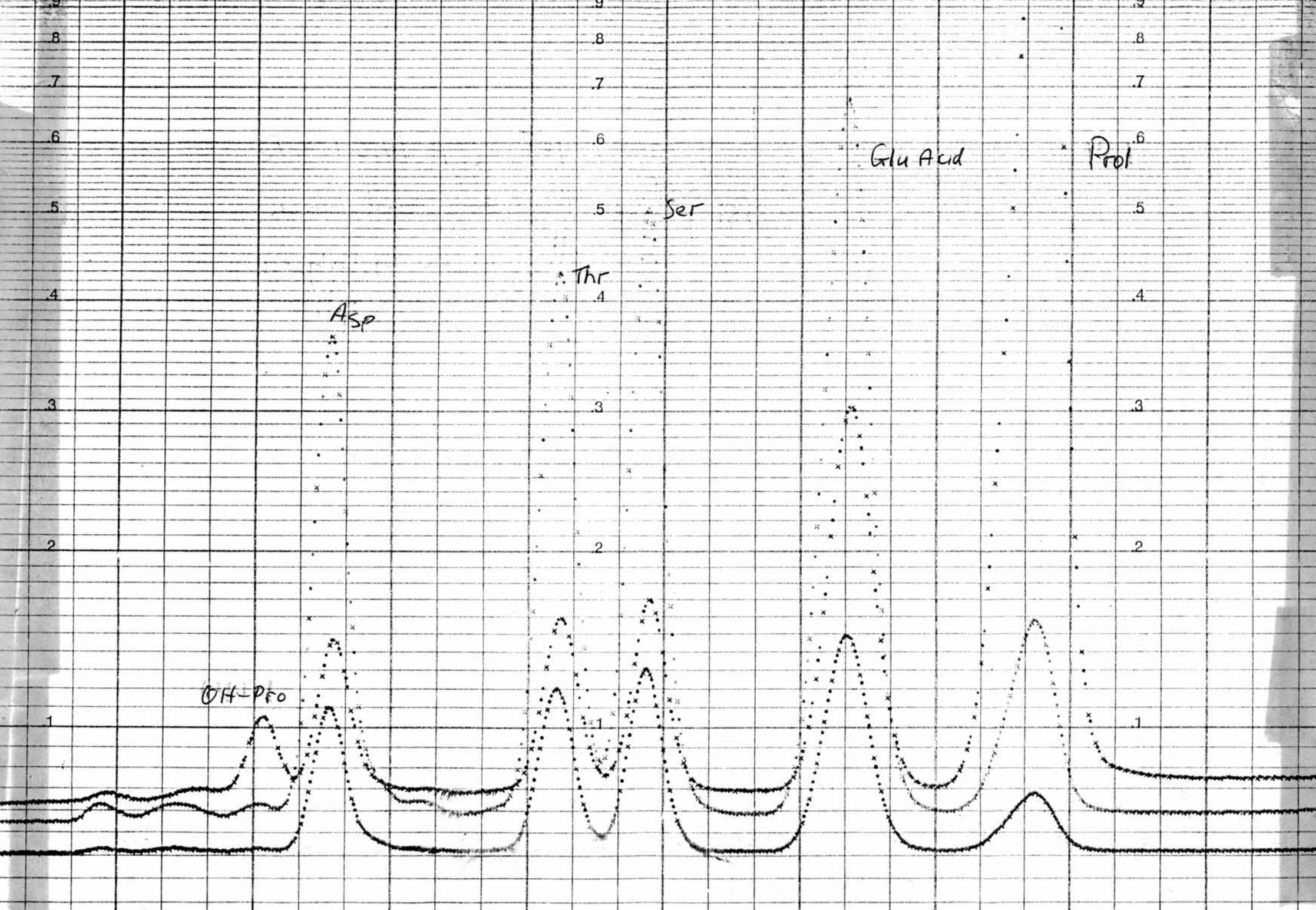
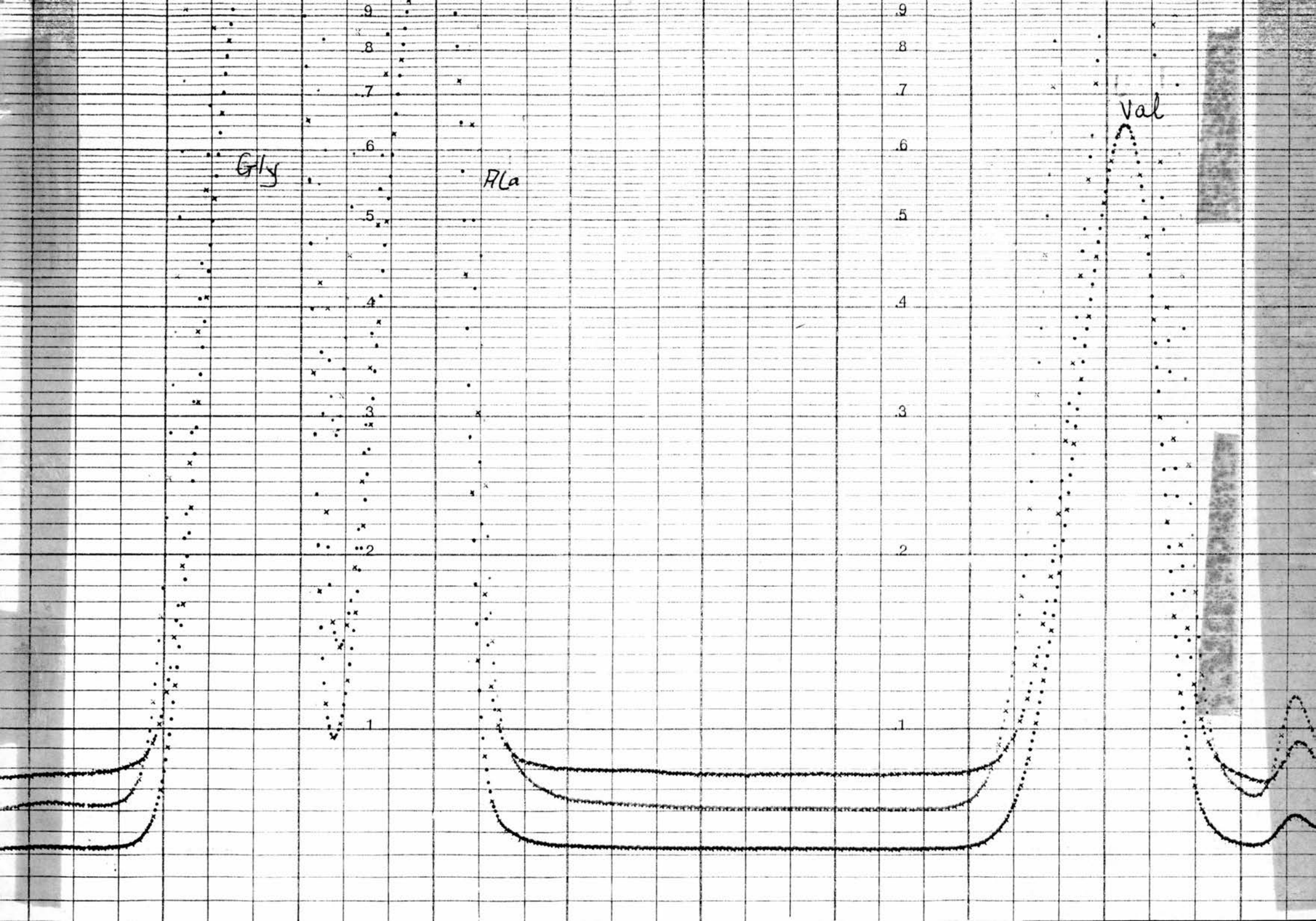
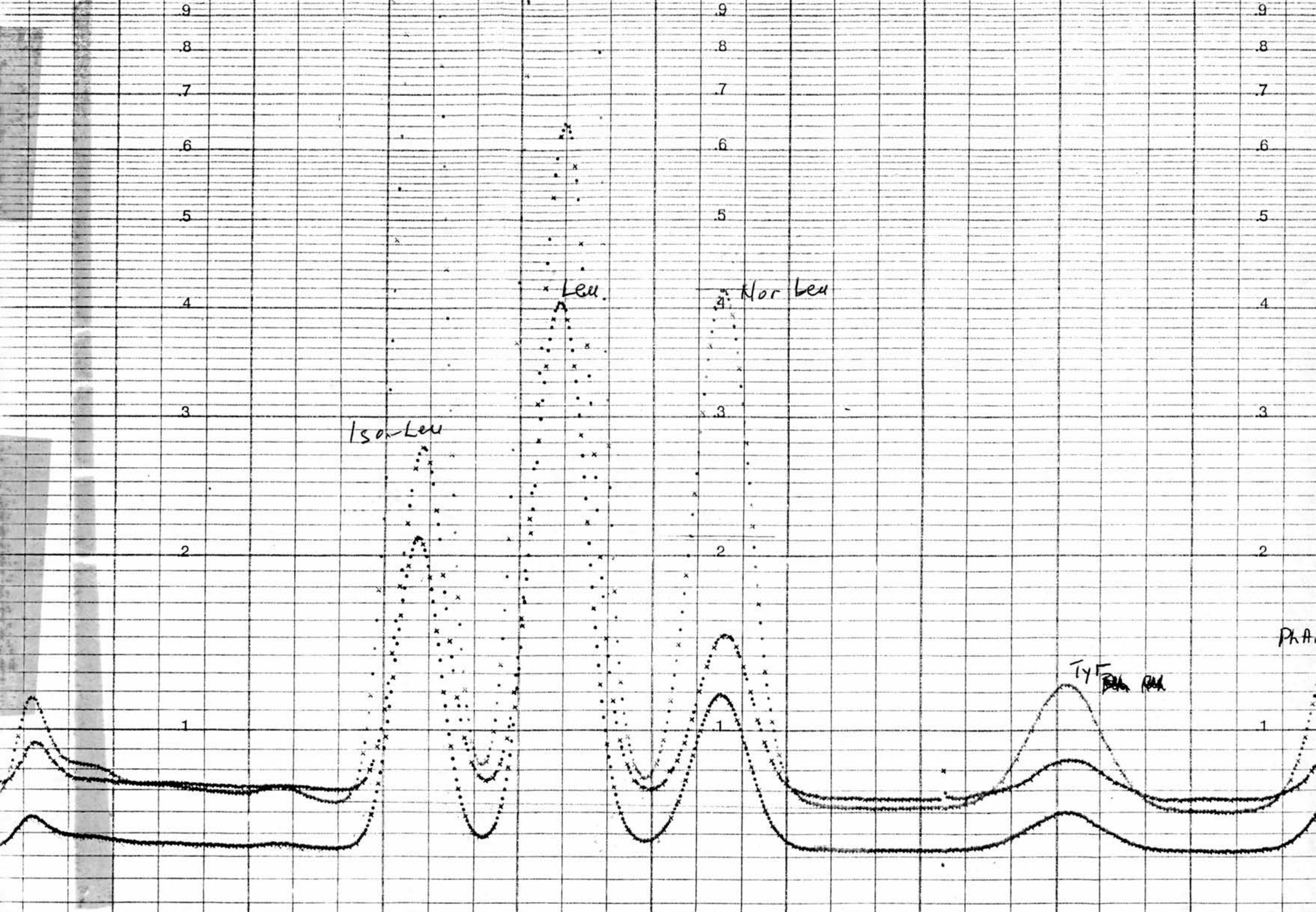

.9
.8
.7
.6
.5
.4
.3
.2
.1
NH3
LYS
DES
HIS
Arg

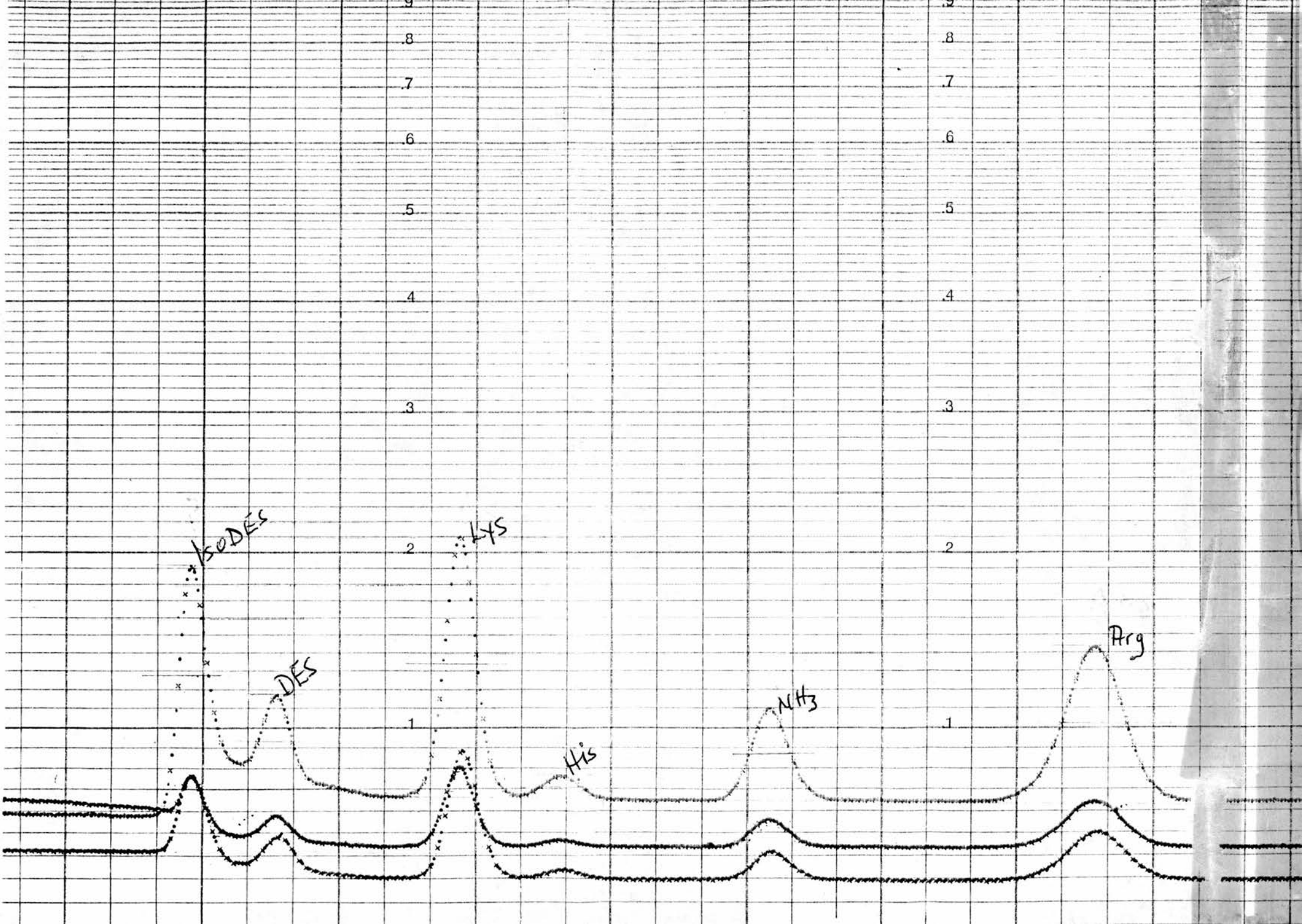
FIG 14
1ST CATHOLYTE.




Asp
Thr
Ser
Glu Acid
Prol
OH-Pro


Gly
Ala
Val
9
8
7
6
5
4
3
2
1


Iso-Leu
Leu
Nor Leu
Tyr
Pha


.9
.8
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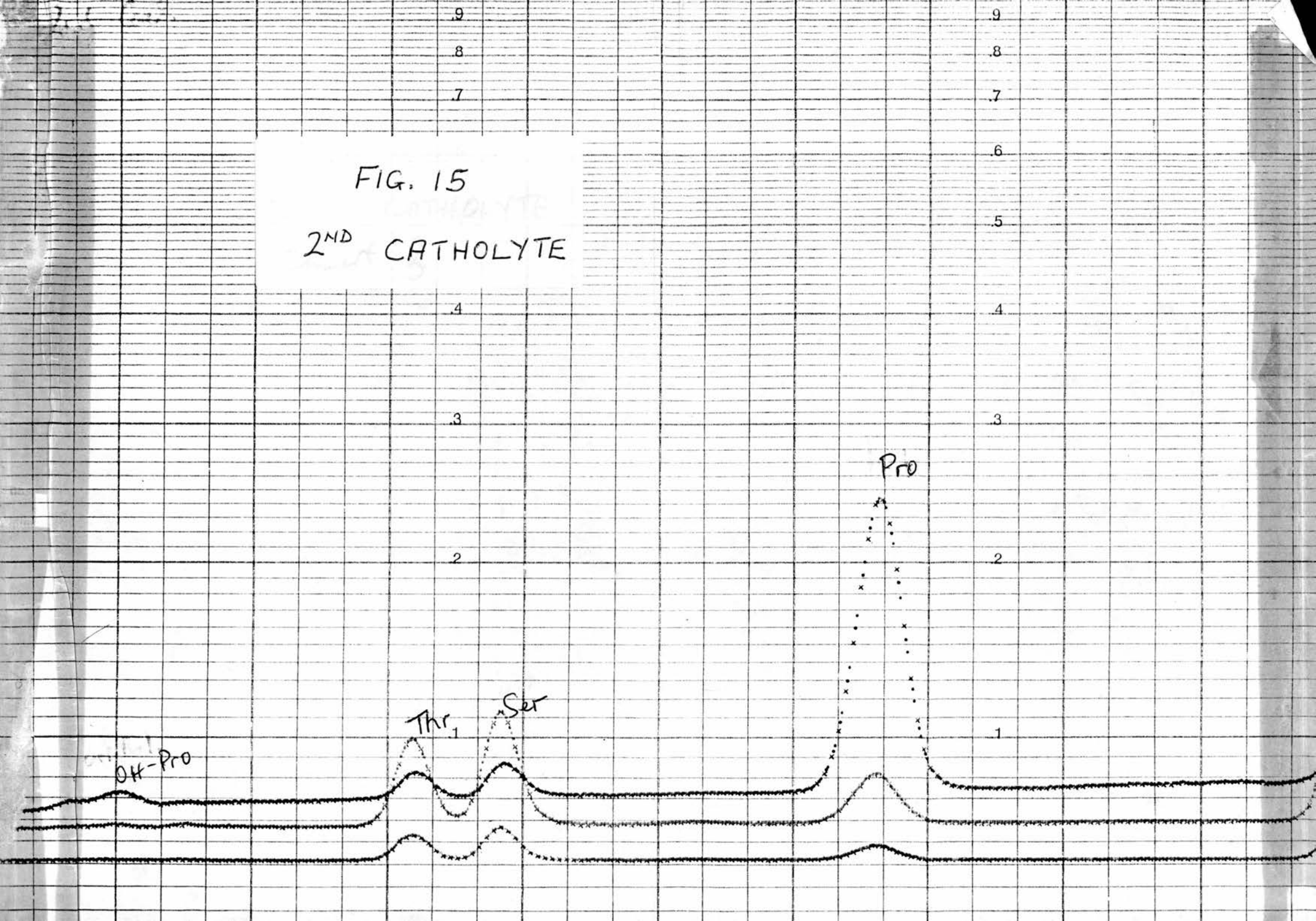
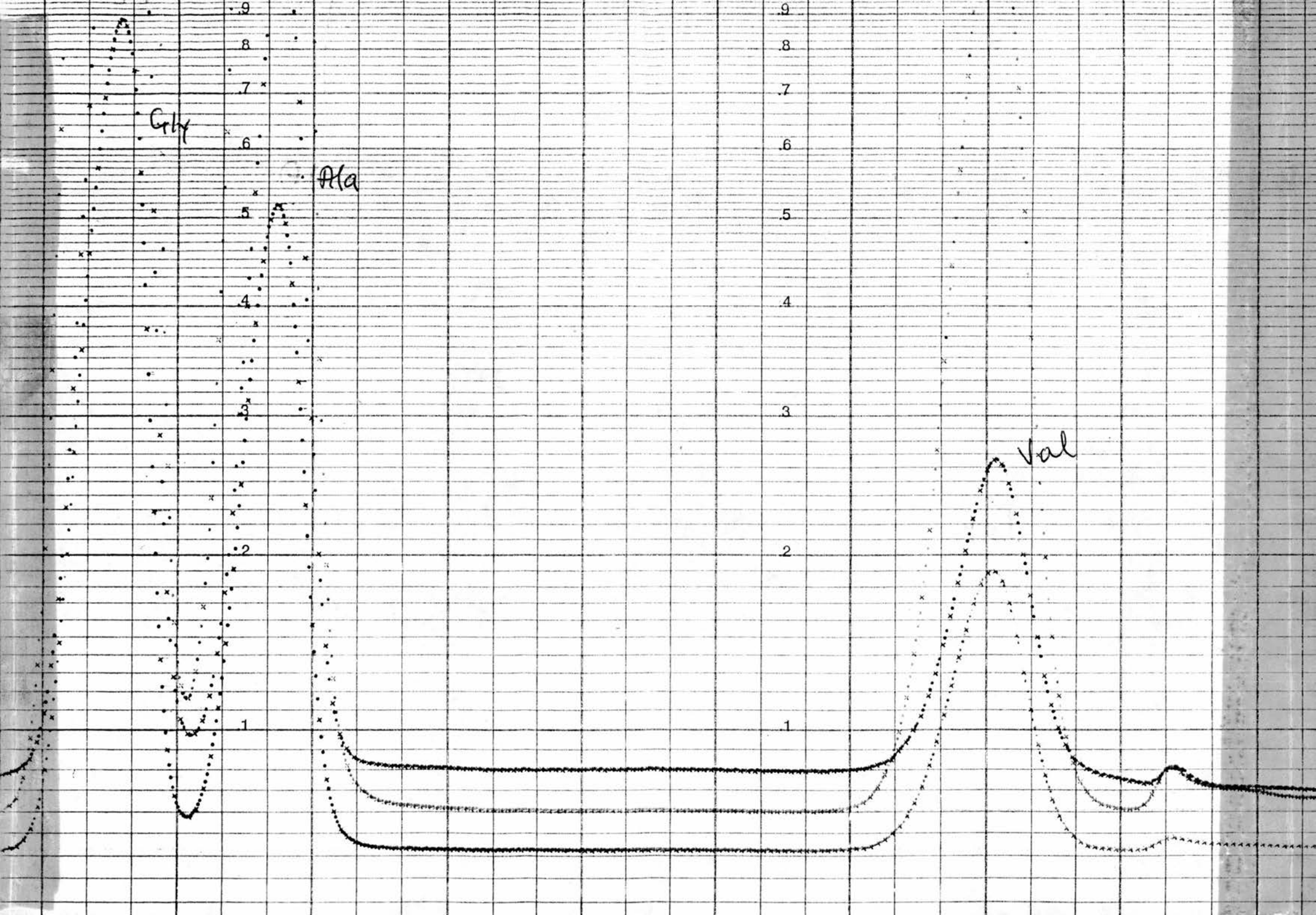
ISODES
DES
LYS
HIS
NH3
Arg

FIG. 15

2ND CATHOLYTE




Gly
Ala
Val
.9
.8
.7
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.3
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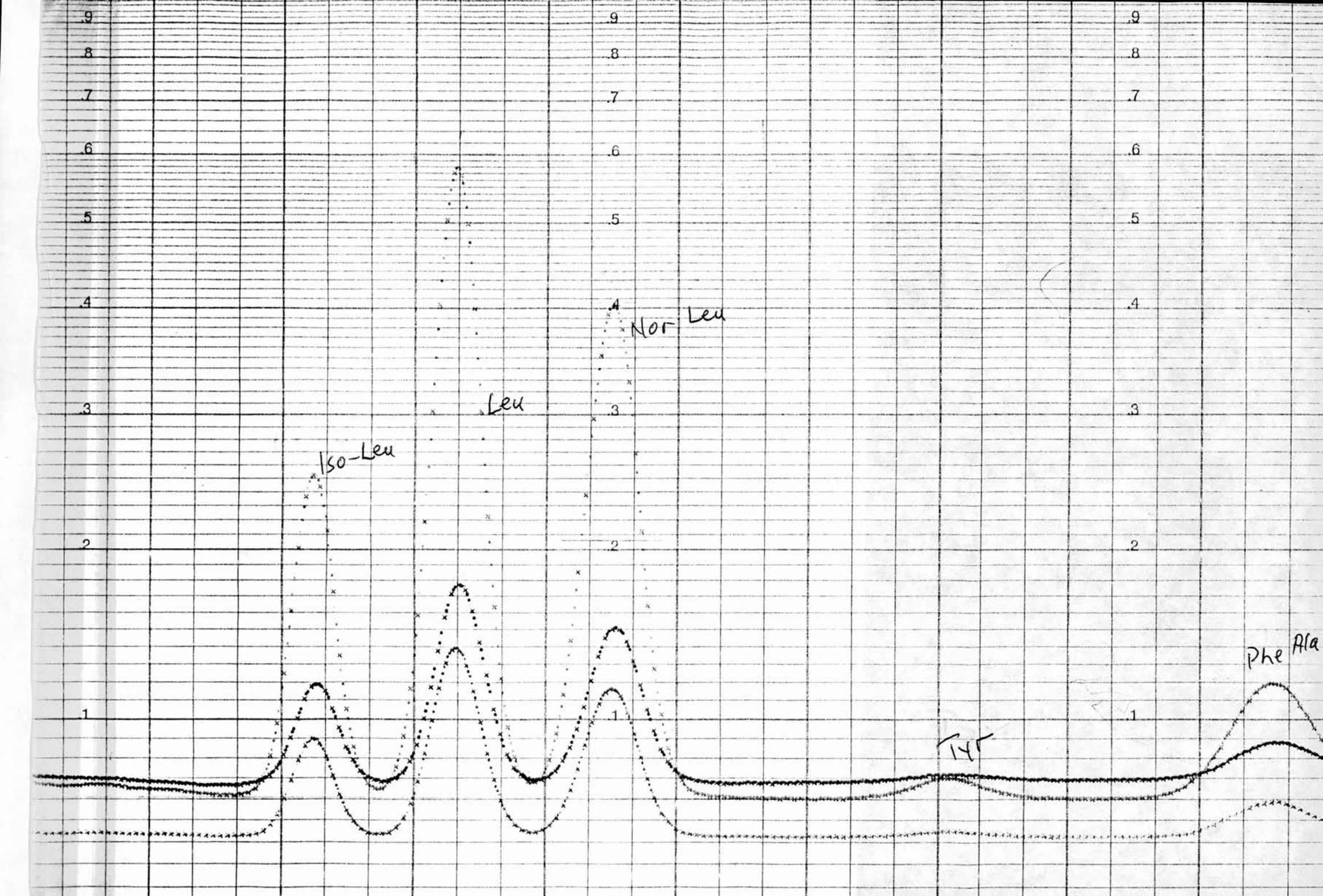
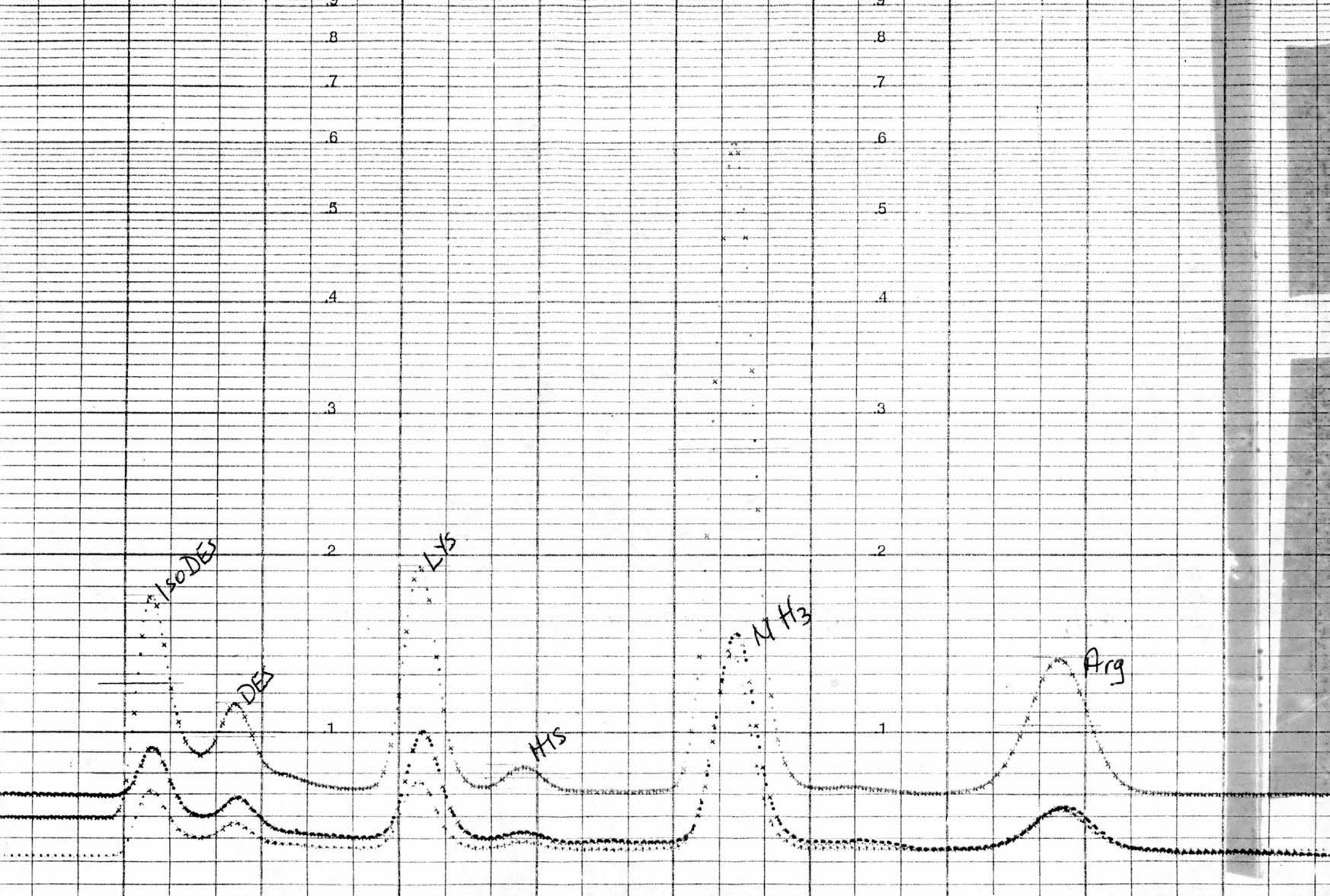
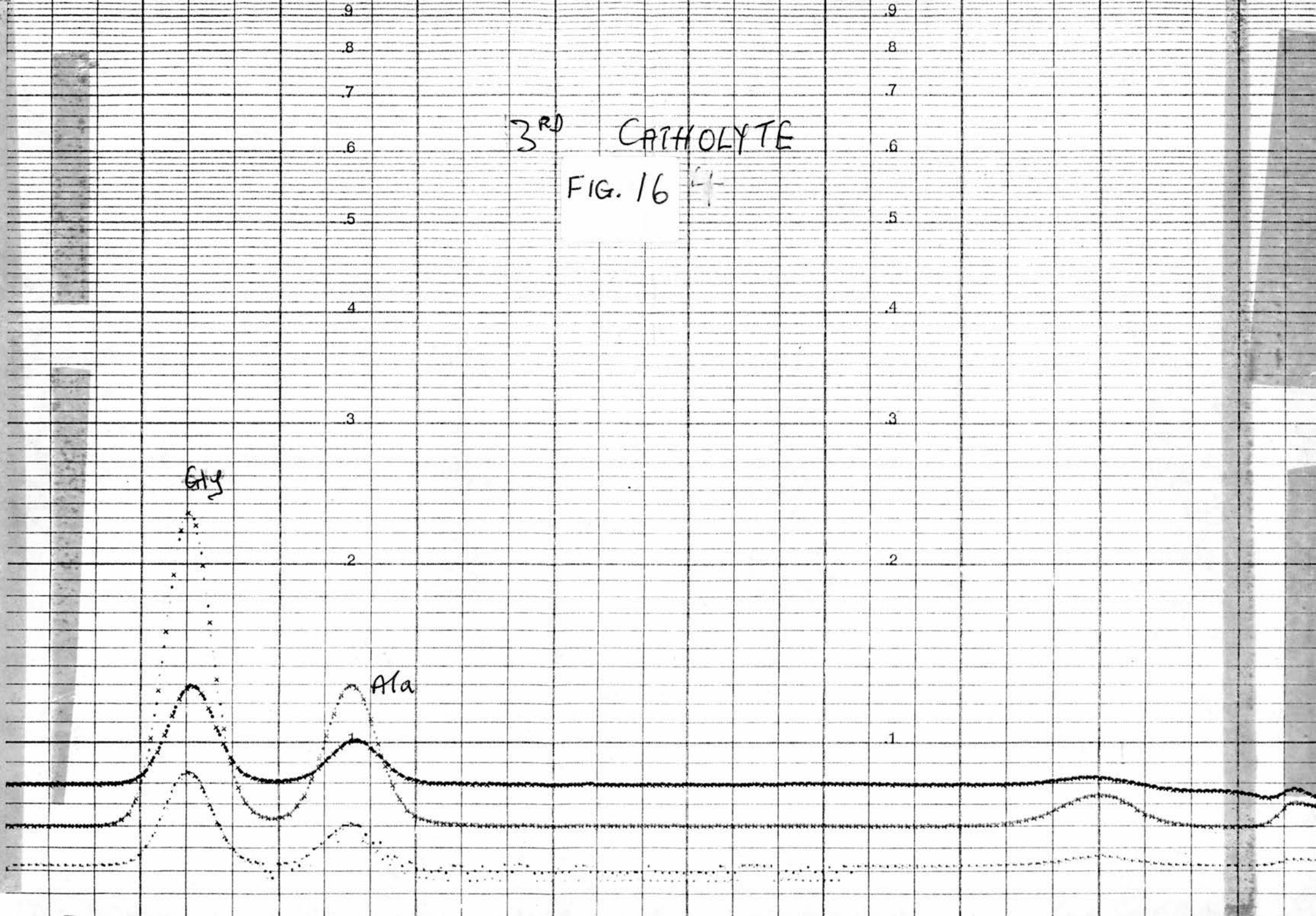
.1

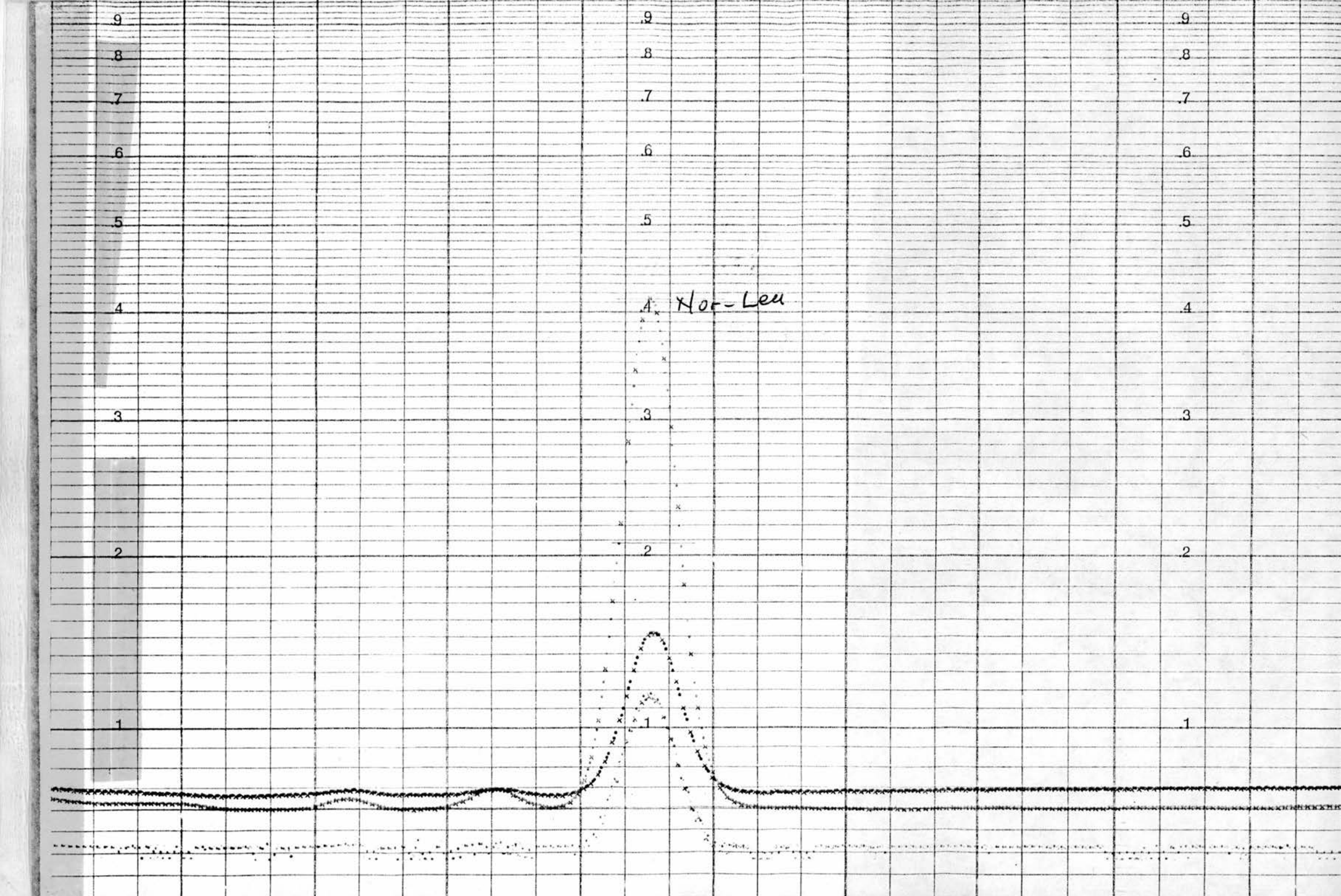

.9
.8
.7
.6
.5
.4
.3
.2
.1
Iso-Leu
Leu
Nor Leu
Tyr
Phe
Ala


.9
.8
.7
.6
.5
.4
.3
.2
.1
IsoDES
DES
LYS
HIS
MH3
Arg

3RD CATHOLYTE

FIG. 16




.9
.8
.7
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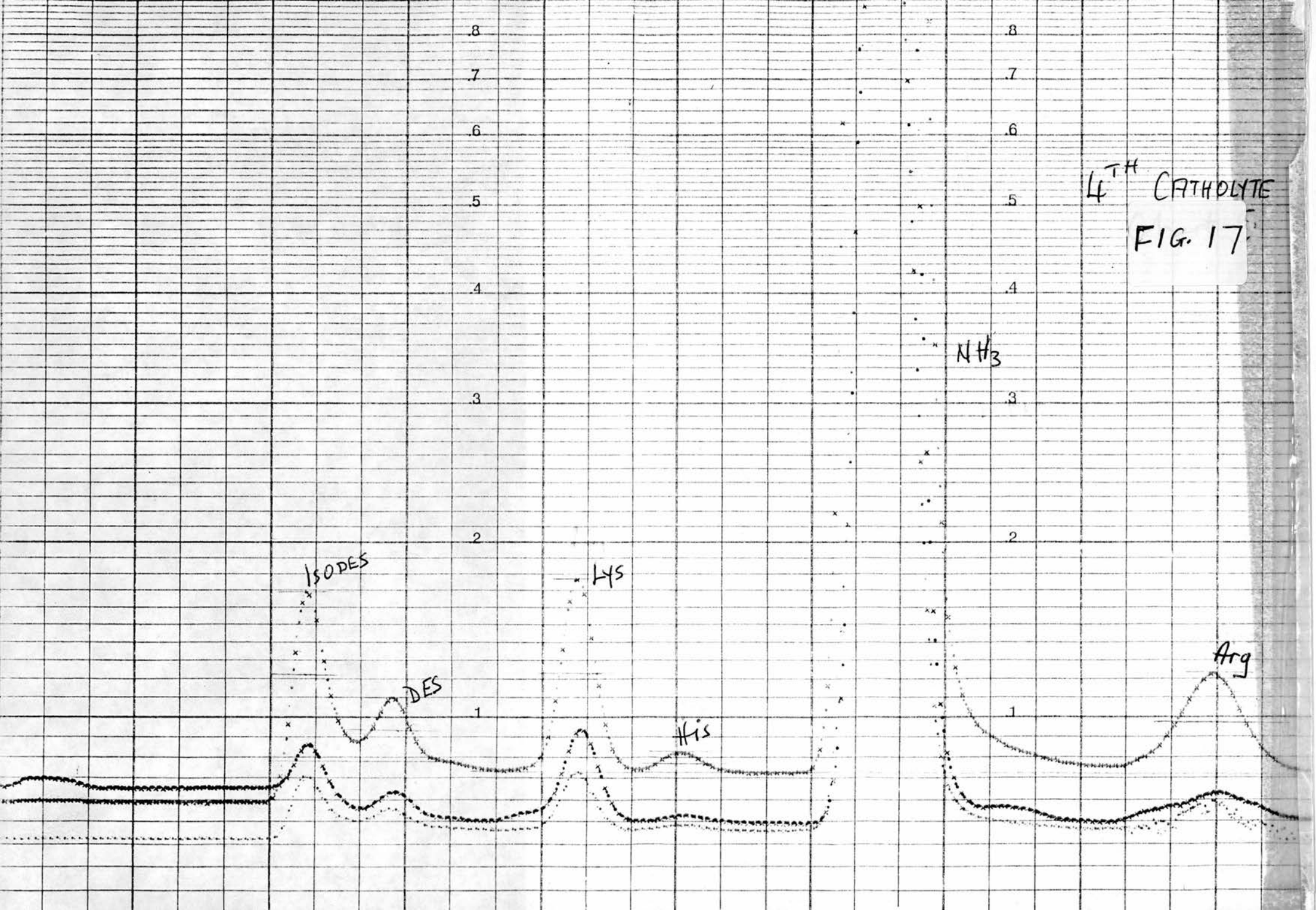
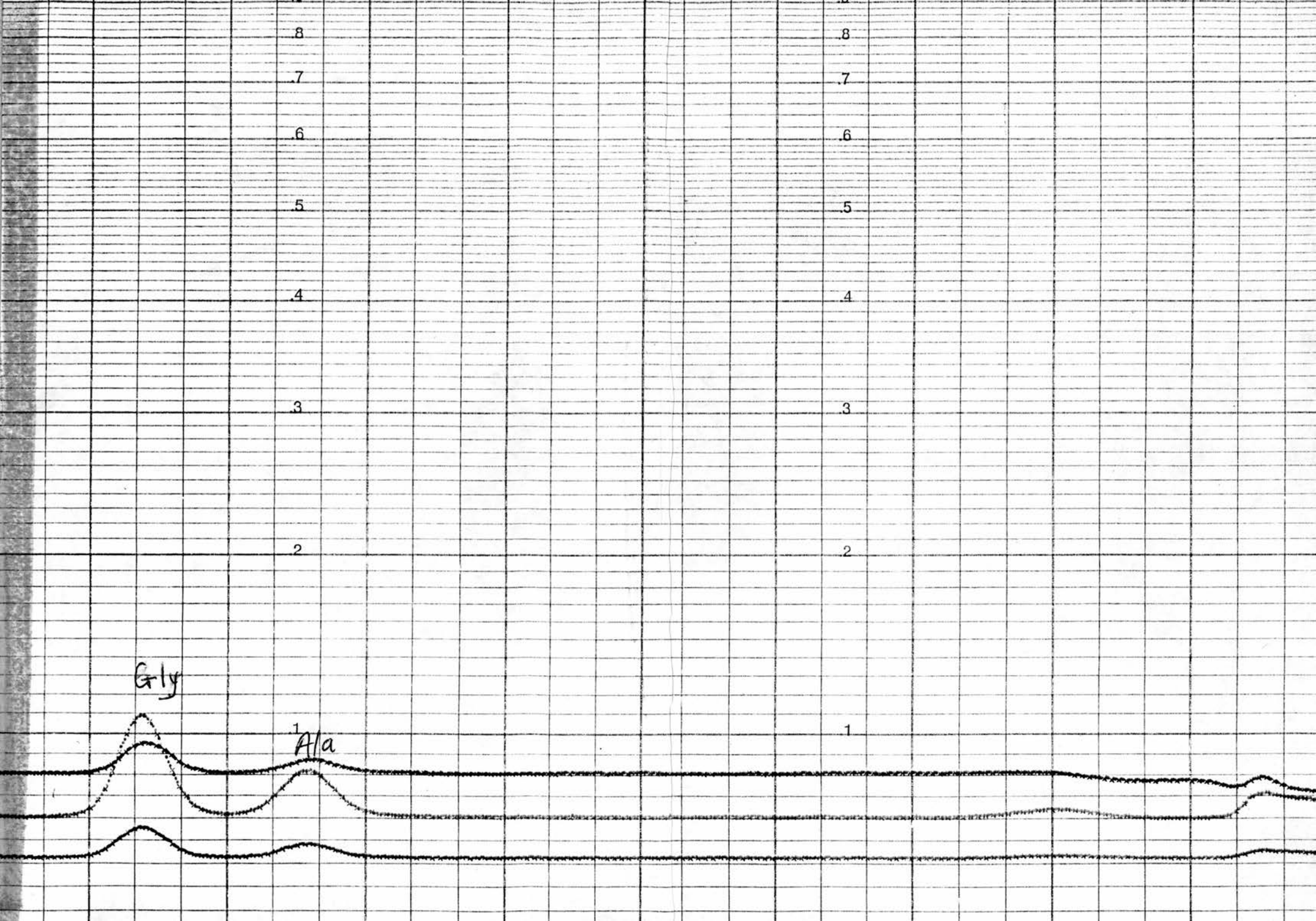
.5
A Hor-Leu
.4
.3
.2
.1



4TH CATHOLYTE

FIG. 17


.8
.7
.6
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.4
.3
2
1
Gly
Ala

ULTRAVIOLET SPECTROPHOTOMETER

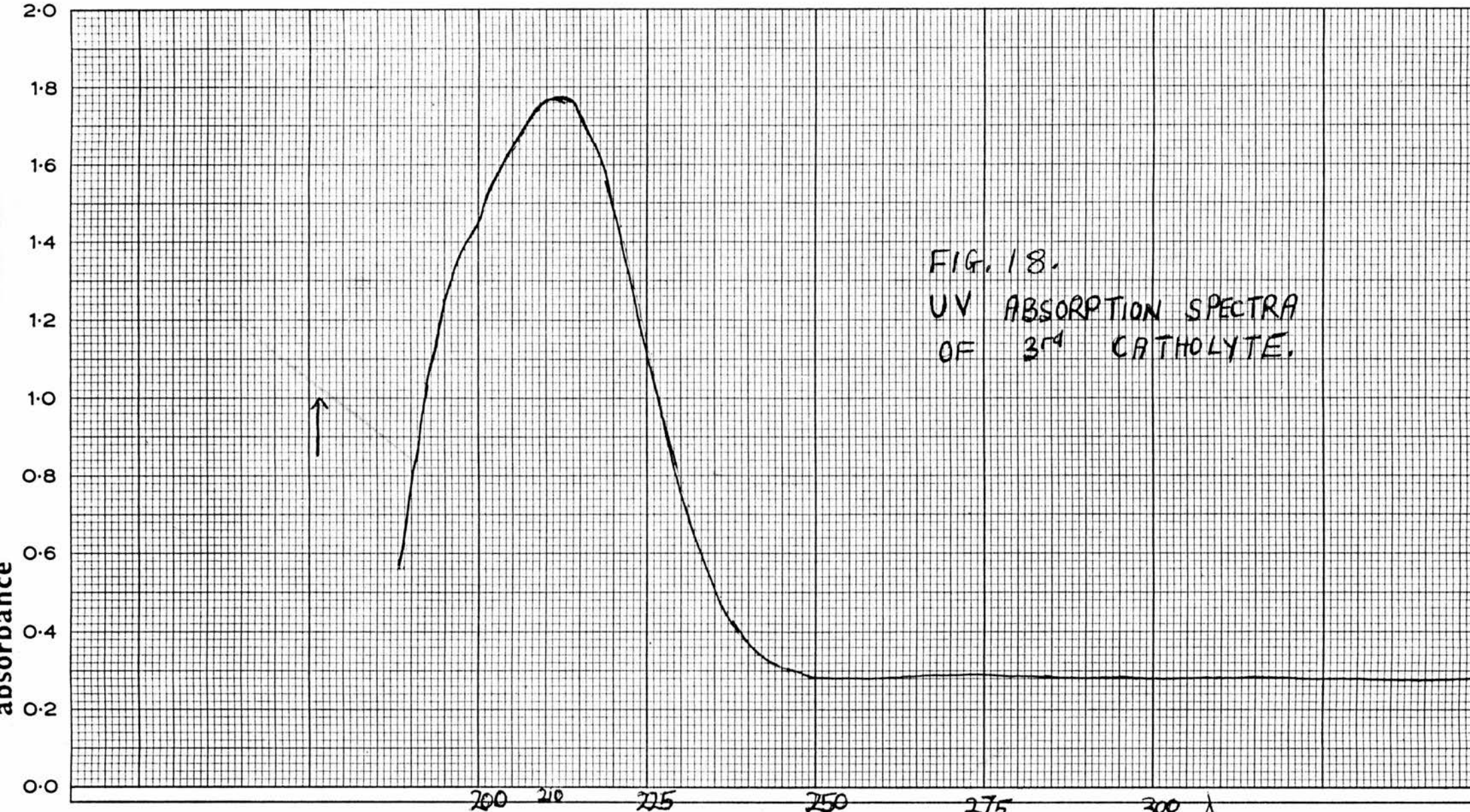


FIG. 18.
UV ABSORPTION SPECTRA
OF 3rd CATHOLYTE.

Intek DUN/102/1007 6008

ALIGN WITH INDEX ON THE RECORDER

SAMPLE AND FORMULA

CONCENTRATION

REFERENCE _

PATH LENGTH MM.

SCAN SPEED FAST ☐ SLOW ☐

DATE

OPERATOR

ULTRAVIOLET SPECTROPHOTOMETER

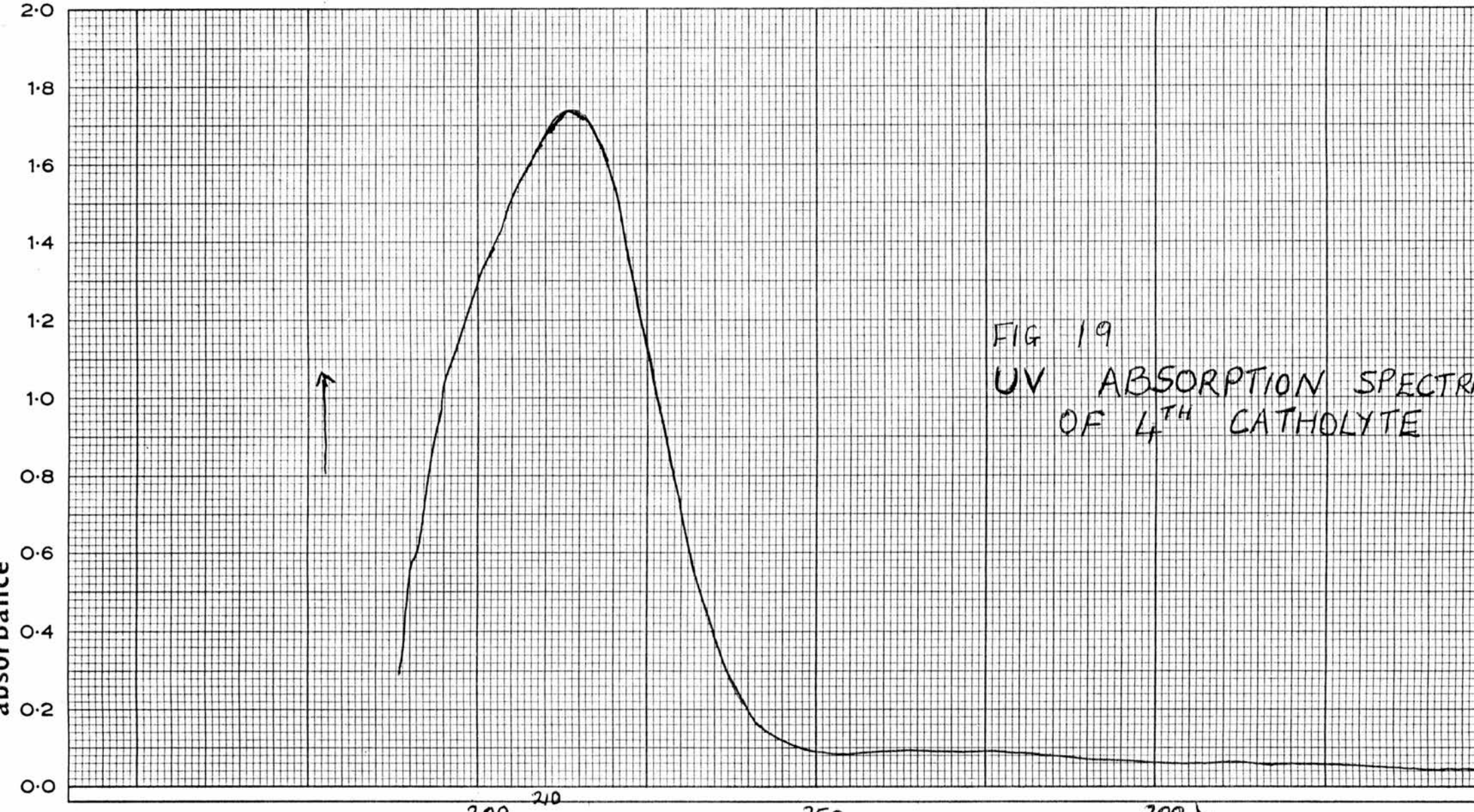


FIG 19
UV ABSORPTION SPECTR
OF 4TH CATHOLYTE

ALIGN WITH INDEX
ON THE RECORDER

SAMPLE AND FORMULA

CONCENTRATION
REFERENCE _
PATH LENGTH MM.

SCAN SPEED FAST ☐ SLOW ☐
DATE
OPERATOR

4.1

(b) Ultra Violet Absorption Spectra Results

The absorption spectra of the 3rd and 4th catholytes were done on SP 800, and are shown in figures 18 and 19. The wavelength of maximal absorption of the two are very close (212nm and 213nm), despite the fact that the 3rd catholyte contained more neutral and acidic amino acids as contamimanants.

4.2 MACRO METHOD - ELECTRODIALYSIS

For the macro method, the ion exchange membranes supplied by BDH were used. The results obtained from hydrolysates of 5g and 9g of elastin are tabulated on pages 83 and 84. The experiment was set up as in the micro method, the only differences are the weight of hydrolysed elastin and the membranes.

4.3 TABULATION OF RESULTS

(a) Electrodialyser Results

Table 3

(i) Weight of Elastin 0.5g

100cm^3 of hydrolysate contained 0.5g hydrolysed elastin

0.5cm^3 of " " 2.5mg " "

Solution made up to 2.5cm^3

2.5cm^3 solution contained 2.5mg hydrolysed elastin

0.8cm^3 " " 0.8mg " "

0.8mg of hydrolysate contained 28.14n.moles lysine

0.5g of hydrolysate contained 17587.5n moles lysine

1 Mole of lysine weighs 146.19g

17587.5n moles lysine weighs 2.57mg

	1st Catholyte	2nd Catholyte	3rd Catholyte	4th Catholyte
Isodesmosine)	Single peak	28.63mg	24.34mg	24.34mg
Desmosine)		14.31mg	10.02mg	10.02mg
Lysine	2.57mg	2.67mg	2.37mg	2.08mg
Histidine	0.36mg	0.36mg	0.36mg	0.36mg
Arginine	4.49mg	4.09mg	3.54mg	2.73mg
% of Isodesmosine		5.73	4.87	4.87
% of Desmosine		2.86	2.00	2.00

Table 4

(ii) Weight of Elastin 5g.

100cm^3 of hydrolysate contained 5g hydrolysed elastin

0.5cm^3 of hydrolysate contained 25mg hydrolysed elastin

Solution made up to 10cm^3

10cm^3 of solution conatined 25mg

0.8cm^3 of solution contained 2mg

	1st Catholyte	2nd Catholyte	3rd Catholyte	4th Catholyte
Isodesmosine		115.67mg	83.03mg	55.54mg
Desmosine		112.23mg	52.68mg	73.29mg
Lysine	35.56mg	33.43mg	25.32mg	30.82mg
Histidine	5.7mg	1.28mg	3.25mg	3.96mg
Arginine	57.79mg	48.53mg	22.90mg	37.73mg
% Isodesmosine		2.31	1.66	1.11
% Desmosine		2.24	1.05	1.46

(iii) Weight of Elastin 5g

Table 5

	1st Catholyte	2nd Catholyte	3rd Catholyte	4th Catholyte
Isodesmosine	234.78mg	62.98mg	22.90mg	85.89mg
Desmosine	157.48mg	103.08mg	57.26mg	97.34mg
Lysine	41.78mg	34.42mg	36.79mg	40.75mg
Histidine	5.76mg	2.88mg	3.84mg	5.28mg
Arginine	58.78mg	39.80mg	42.53mg	48.53mg
% Isodesmosine	4.69	1.38	0.46	1.72
% Desmosine	3.15	2.06	1.14	1.95

Table 6

(iv) Weight of Elastin 9g

100cm^3 of hydrolysate contained 9g hydrolysed elastin

0.5cm^3 of hydrolysate contained 45mg hydrolysed elastin

Solution made up to 20cm^3

20cm^3 solution contained 45mg hydrolysed elastin

0.8cm^3 solution contained 1.8mg hydrolysed elastin

	1st Catholyte	2nd Catholyte	3rd Catholyte	4th Catholyte
Isodesmosine	-	286.31mg	206.14mg	389.38mg
Desmosine	-	171.78mg	125.97mg	80.16mg
Lysine	42.73mg	37.98mg	30.86mg	34.03mg
Histidine	11.53mg	4.80mg	3.84mg	3.84mg
Arginine	70.88mg	59.98mg	41.44mg	39.26mg
% Isodesmosine		3.18	2.29	4.33
% Desmosine		1.91	1.40	0.89

4.4.

(a) DISCUSSION OF RESULTS

From the data in Section 4.3, it is evident that better results were obtained with the micro method, compared with the macro method. The percentage recovery of desmosine and isodesmosine obtained, were much higher than what previous workers obtained. This makes this technique unacceptable.

The wavelengths of maximal absorption of the 3rd and 4th catholytes were 212nm and 213nm respectively. These values are much lower than what any previous worker quoted. This could therefore be used to support the inference that this technique is not ideal.

(b) Comments on the method

It was found that the 1st electrodialysis took about 48 hours. The anodic membrane (linen) had gelatinised formalin coated on it, and because of the long time the electrodialysis lasted, the linen had lost part of its gelatinised formalin. The cathodic membrane (paper parchment) was strengthened by making it double. If this was not done, one would discover that after about 24 hours, the paper parchment would burst, and the accuracy of the results will be adversely affected. Also if the linen has lost its gelatinised formalin, there will be free movement across the membrane. There is a likelihood that the composition of the linen material used in this experiment was different from the linen Macpherson (1946) used.

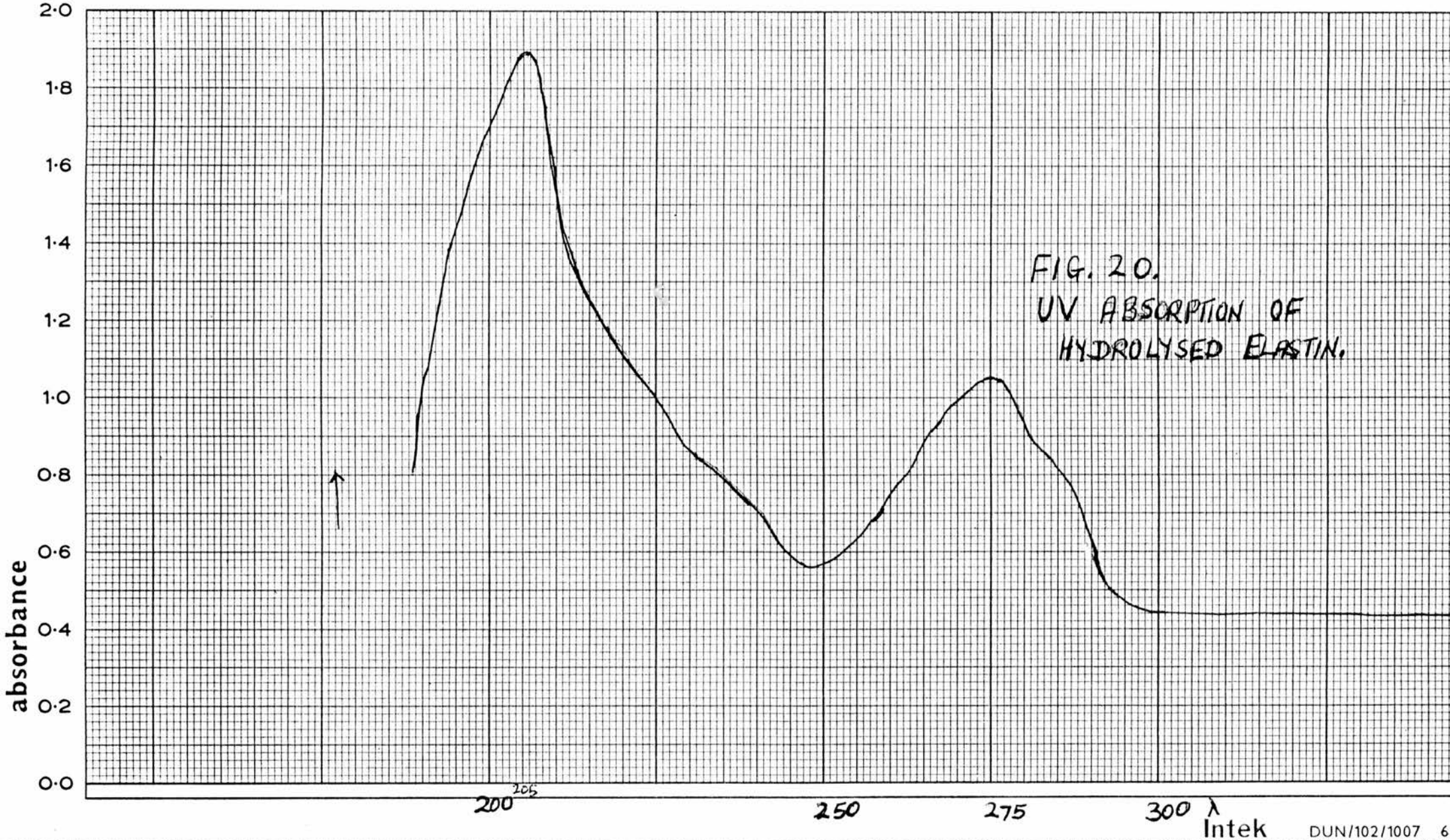


FIG. 20.
UV ABSORPTION OF
HYDROLYSED ELASTIN.

Intek DUN/102/1007 6

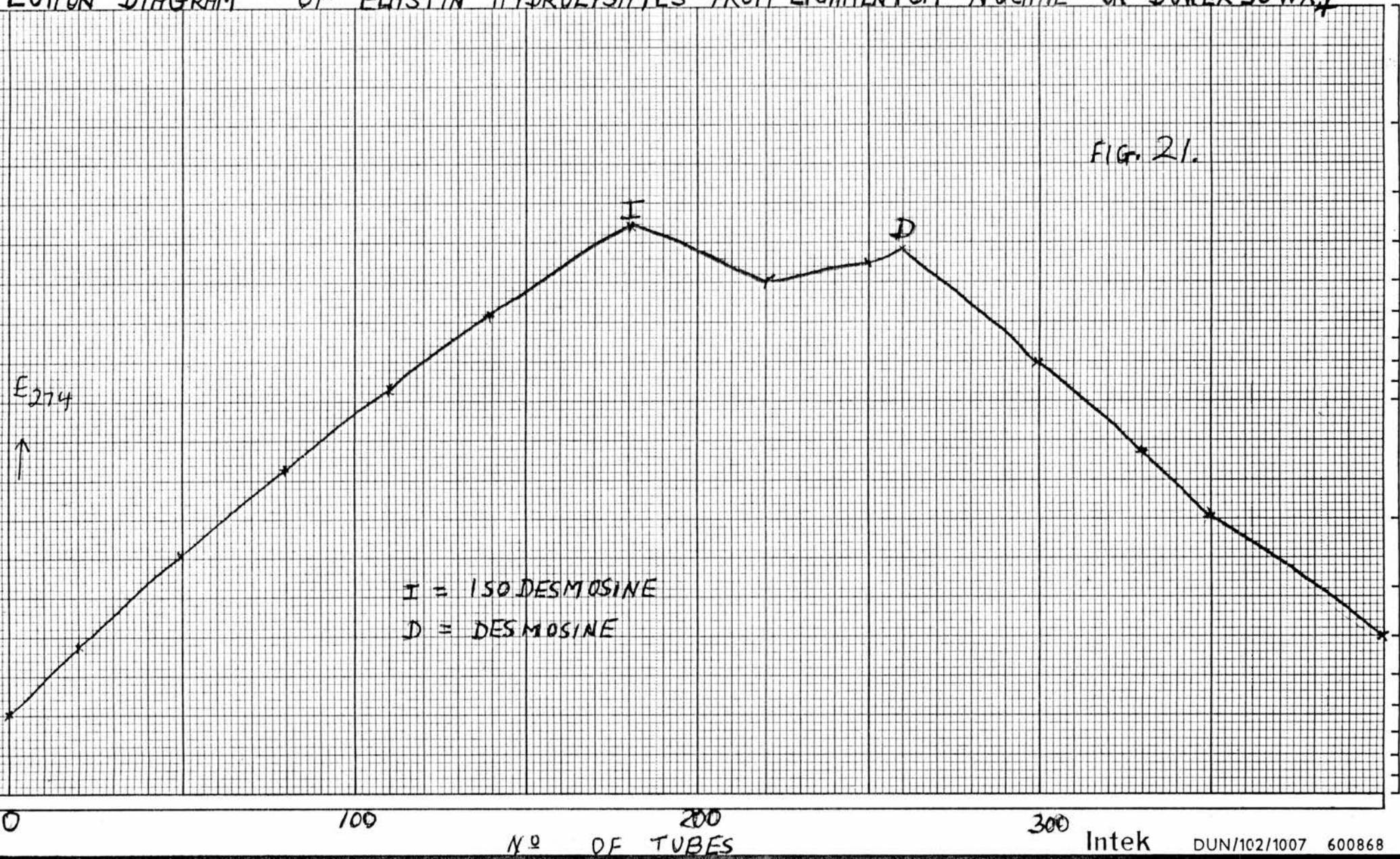
ALIGN WITH INDEX
ON THE RECORDER

SAMPLE AND FORMULA

CONCENTRATION
REFERENCE _
PATH LENGTH MM.

SCAN SPEED FAST ☐ SLOW ☐
DATE
OPERATOR

LUTION DIAGRAM OF ELASTIN HYDROLYSATES FROM LIGAMENTUM NUCHAE ON DOWEX 50WX4



Intek DUN/102/1007 600868

SAMPLE AND FORMULA

CONCENTRATION
REFERENCE
PATH LENGTH MM.

SCAN SPEED FAST ☐ SLOW ☐
DATE
OPERATOR

REF. NO.

The BDH membranes withstood the heat from the power pack better. These membranes were not utilised to separate micro amounts. It is possible that if they are used to separate micro amounts they will give better results than the linen paper parchment membranes.

4.5 Column Chromatography using Dowex 50

Sodium citrate buffer contained 'Brij 35' - See page 72

(a) Elution done at ambient temperature

The second method chosen for the estimation of desmosine and isodesmosine, after isolating the elastin was by the method of Starcher and Galione (1975). The hydrolysed elastin was run through a chromatography column containing Dowex 50. Eluates were collected, and these were analysed on an amino acid analyzer. As discussed in Section 3.10d (i), the first experiment was done at ambient temperature. It was found that from about tube 115, isodesmosine started to elute. There was no chart that showed where the desmosine started to elute, and only one chart showed a shoulder on isodesmosine peak, showing that there was contamination with another basic amino acid. It is surprising that there were no charts showing definite desmosine peaks where as many of the charts showed large peaks of isodesmosine. The only reason one could give to this might be because of the temperature at which the experiment was carried out. The elution of isodesmosine stopped at tube 210. Arginine was present in all the fractions estimated, and its elution ceased on about the same fraction. So only isodesmosine and arginine were detected on the amino acid analyzer, and it can be assumed that desmosine eluted together with the isodesmosine. See figures 22 - 26.

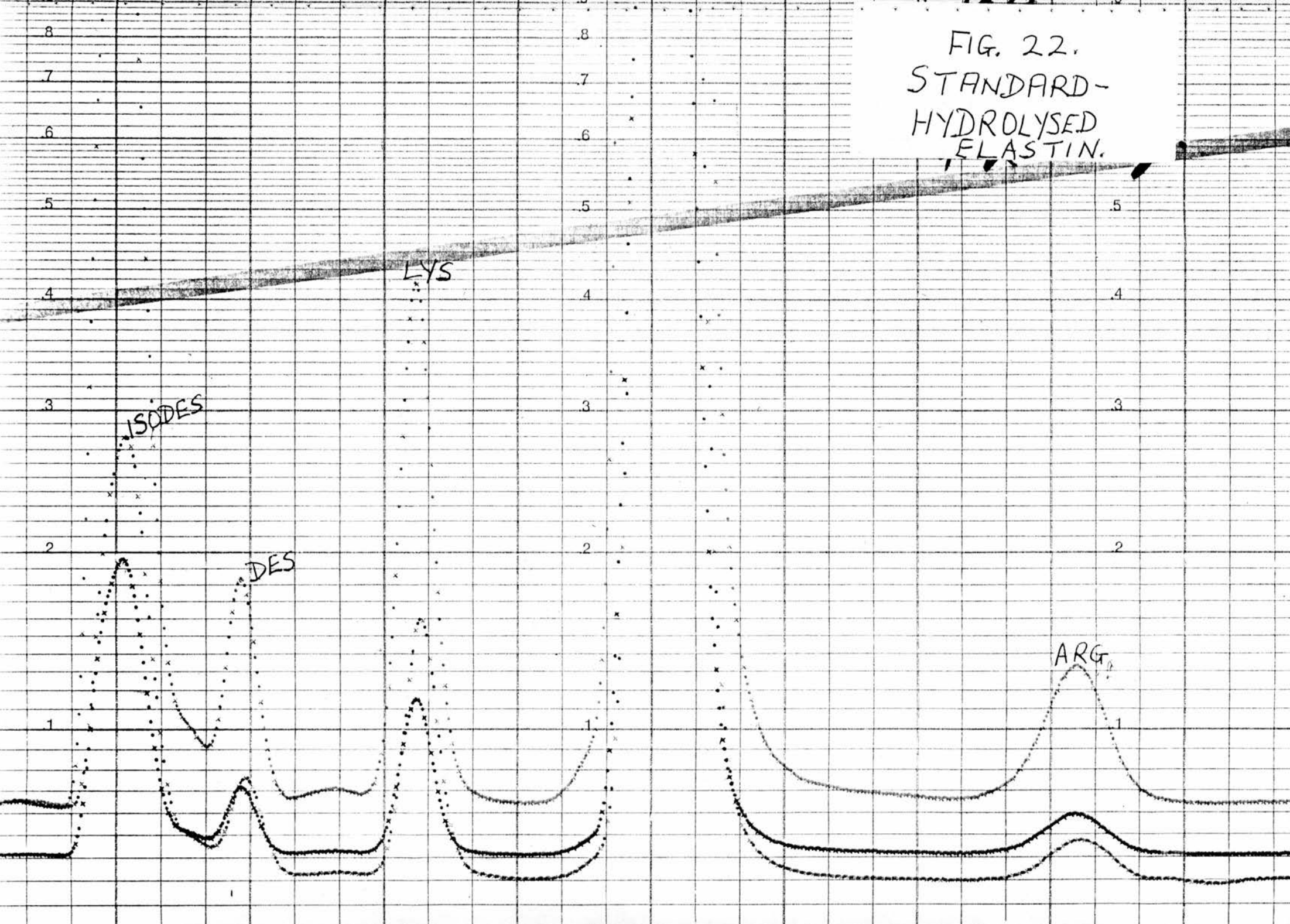


FIG. 22.
STANDARD-
HYDROLYSED
ELASTIN.

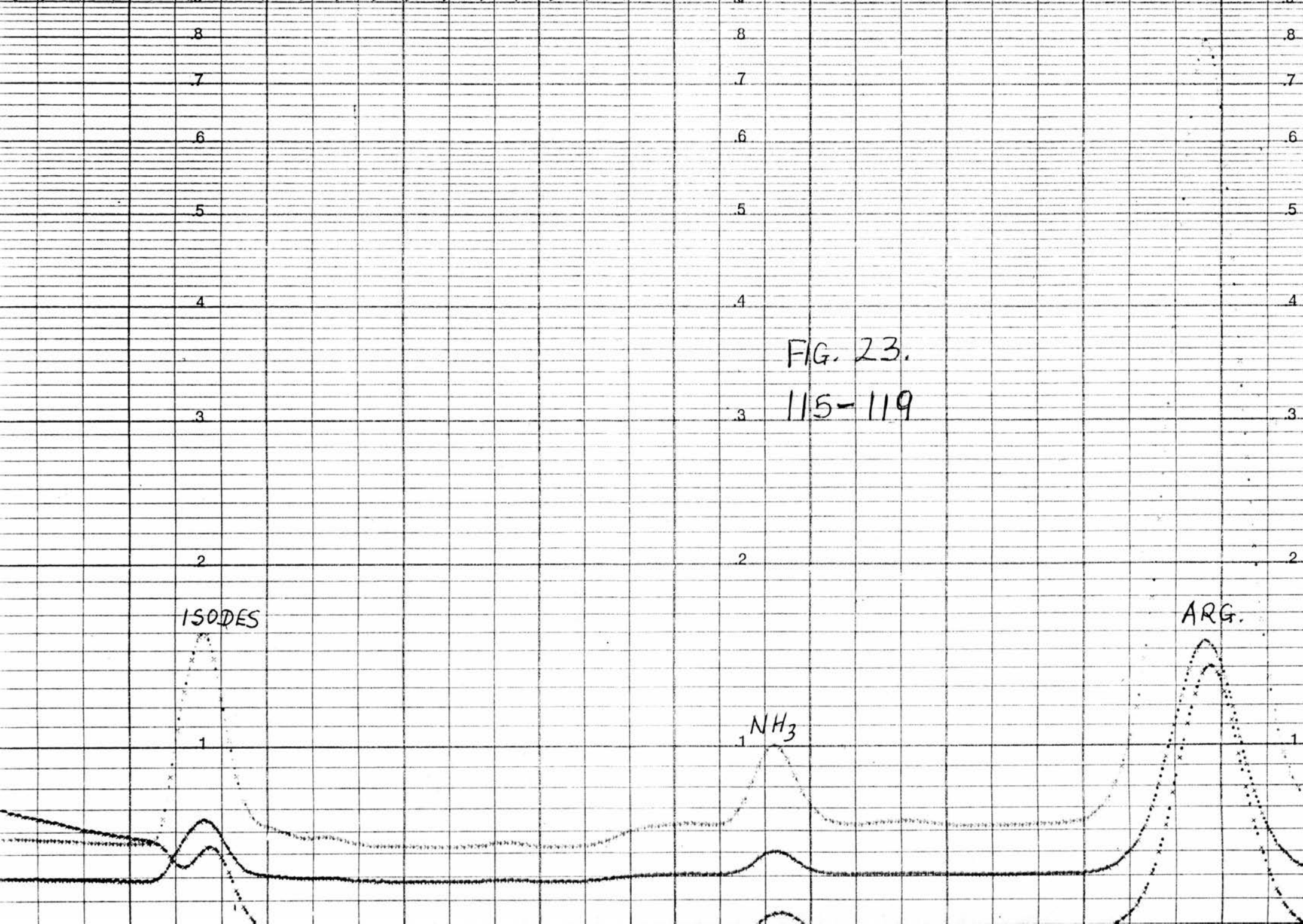
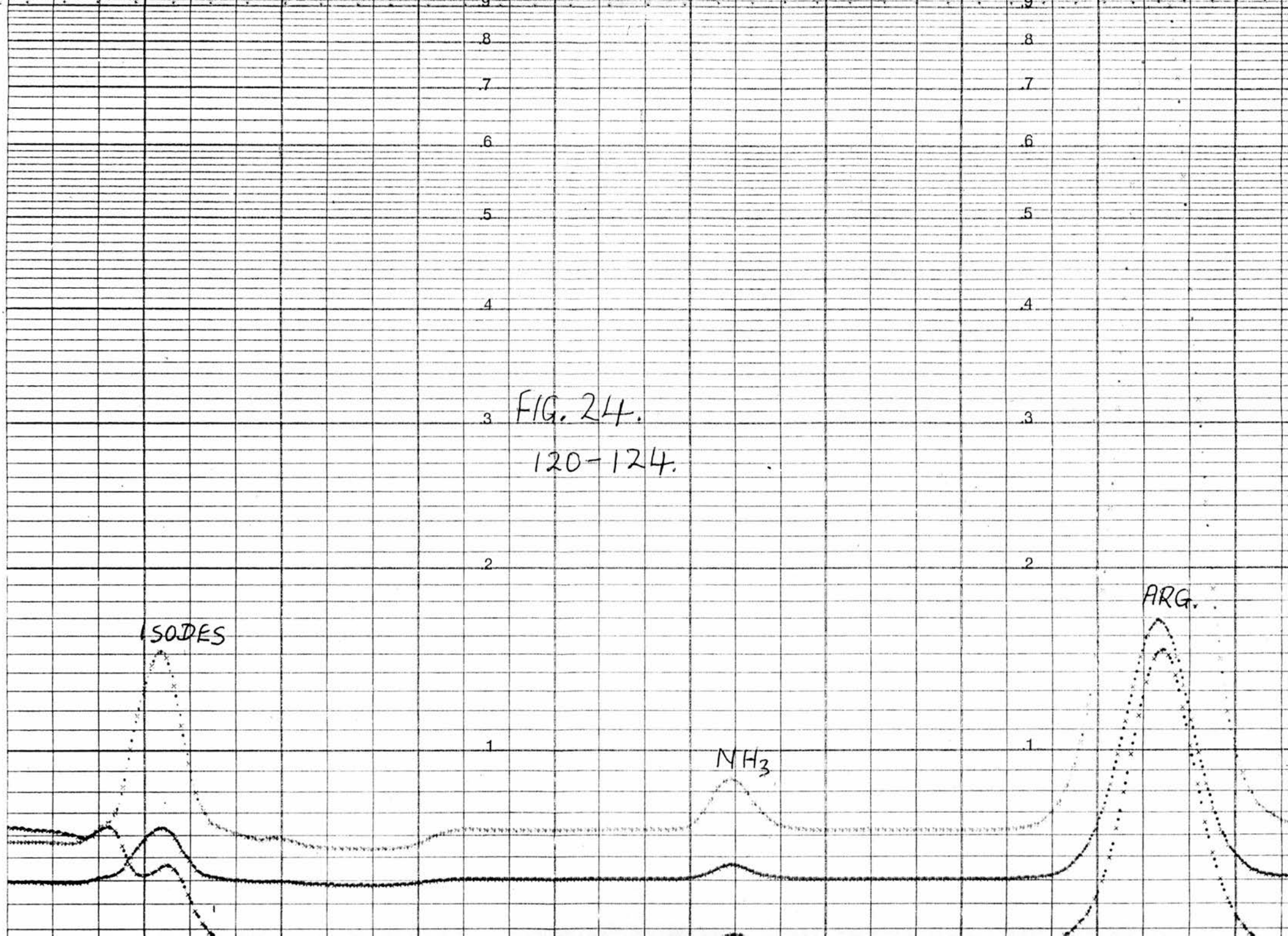


FIG. 23.
115-119

FIG. 24.

120-124.



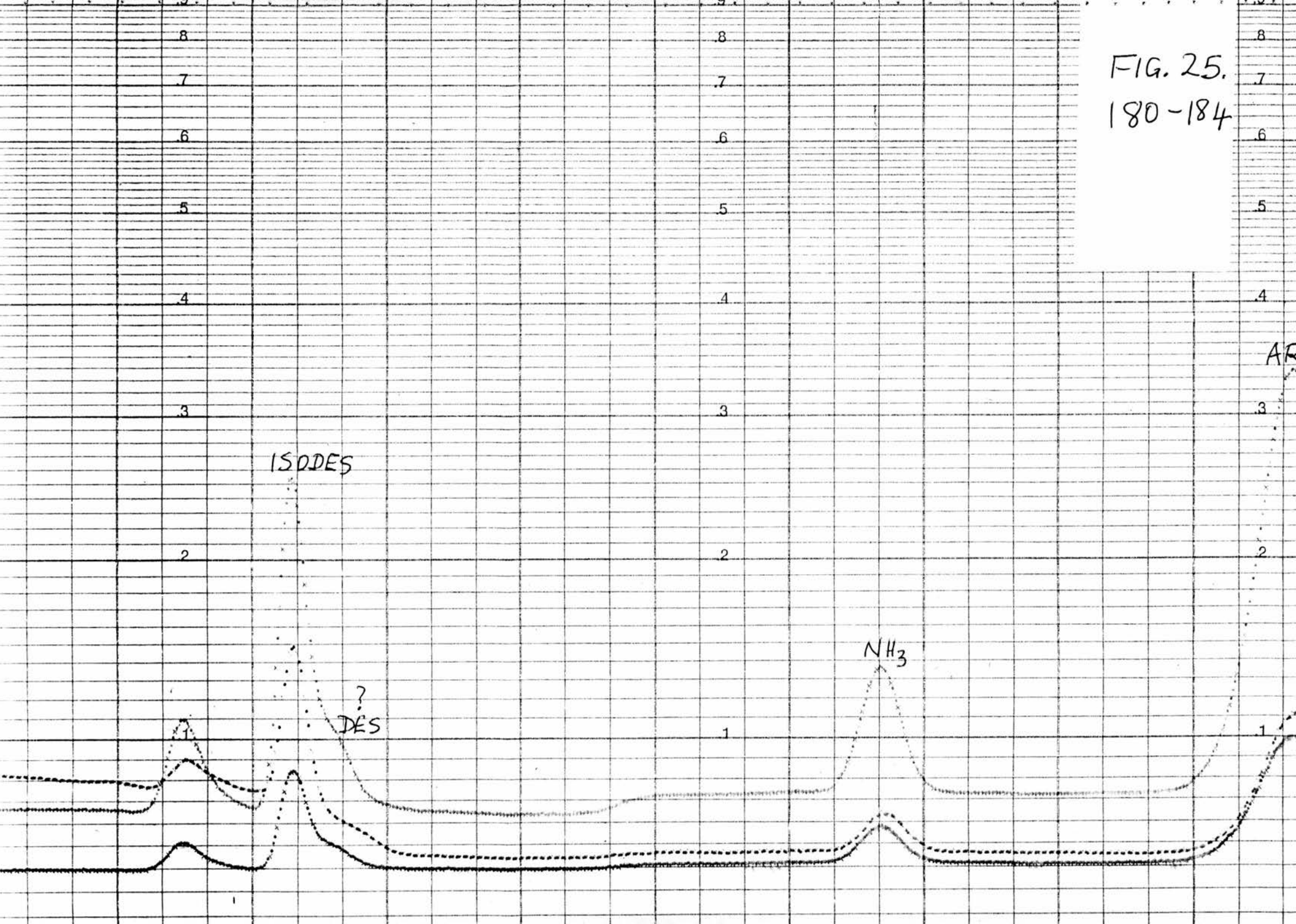


FIG. 25.
180-184

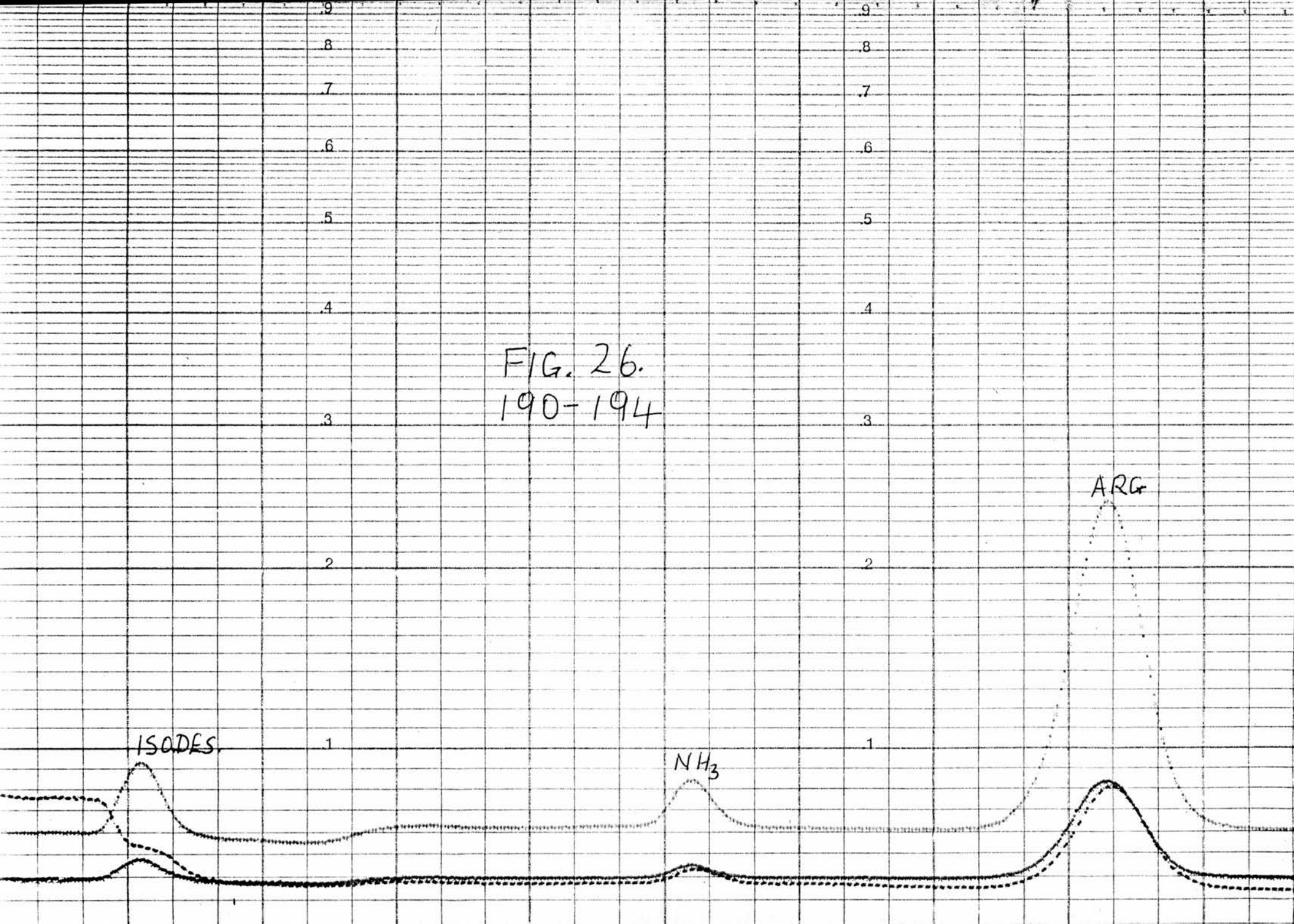


FIG. 26.
190-194

ULTRAVIOLET SPECTROPHOTOMETER

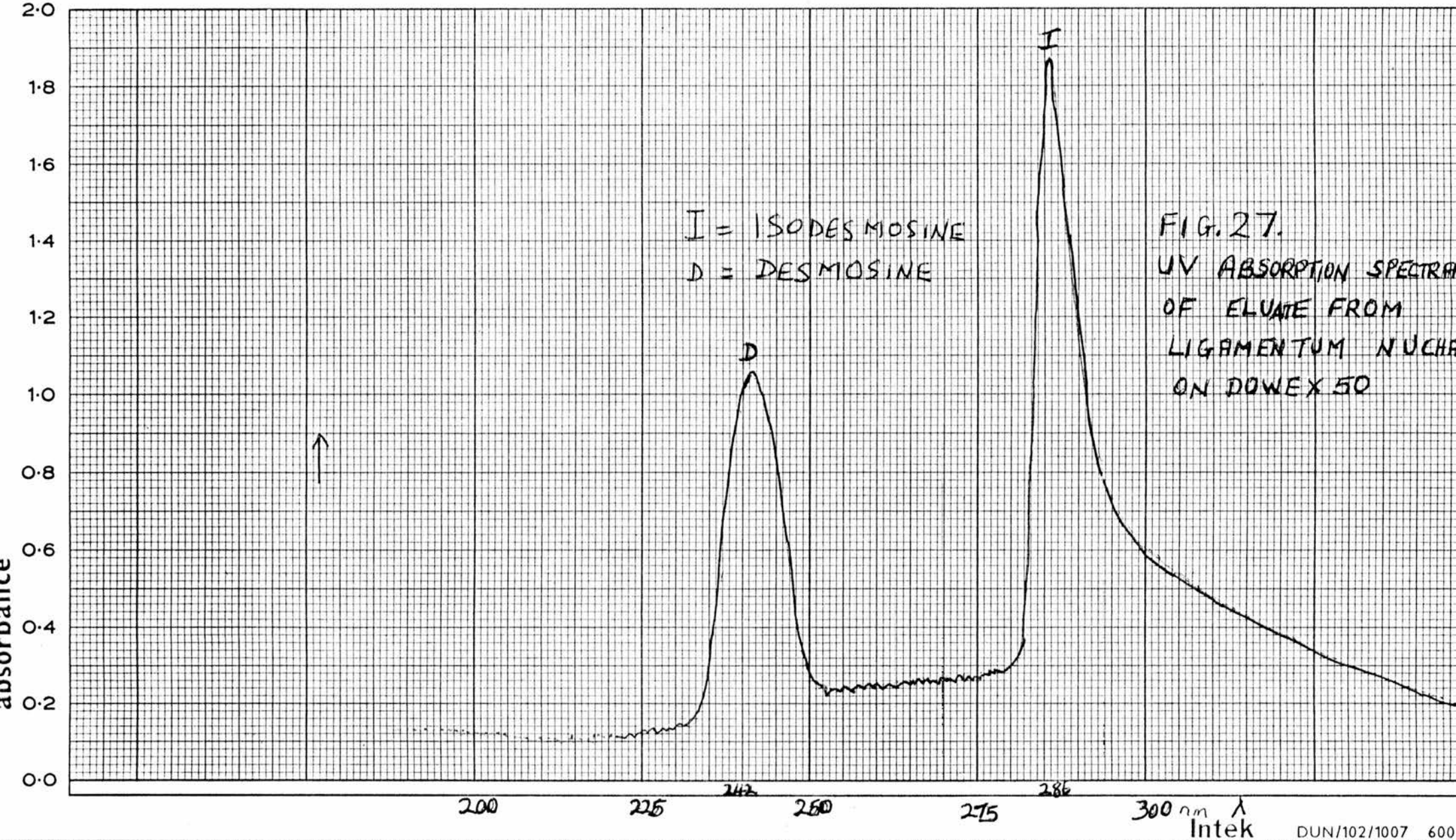


FIG. 27.
UV ABSORPTION SPECTRA
OF ELUATE FROM
LIGAMENTUM NUCHE
ON DOWEX 50

Intek DUN/102/1007 600

ALIGN WITH INDEX
ON THE RECORDER

SAMPLE AND FORMULA

CONCENTRATION

REFERENCE

SCAN SPEED FAST ☐ SLOW ☐

DATE

(b) Elution at 55°C See Figures 28 - 34

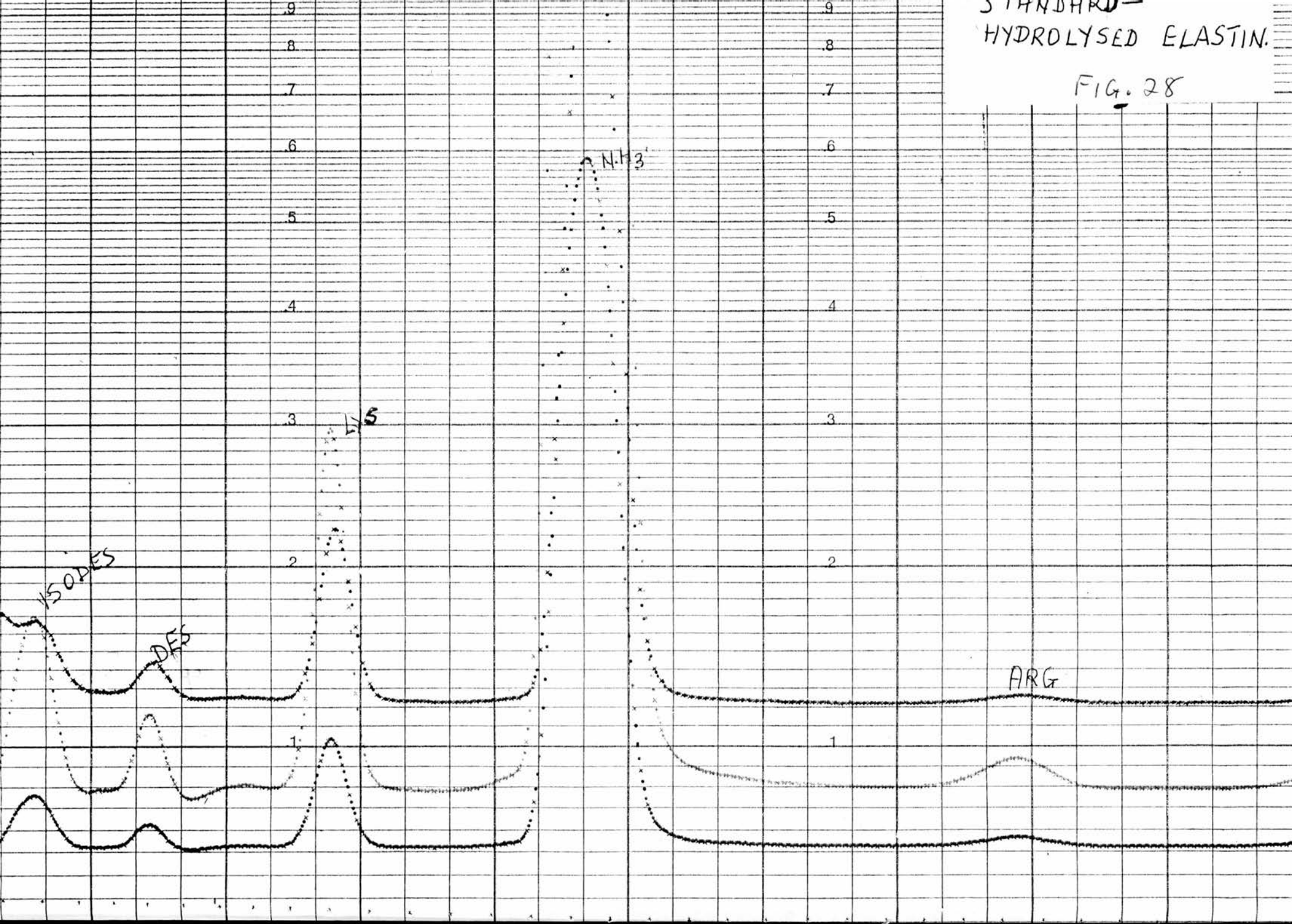
The buffer used was the same as the one used for the elution at ambient temperature. A water jacket maintained at 55°C, was made to circulate round the chromatography column that contained Dowex 50 (See Section 3.10d (ii). The elution of isodesmosine started from around tube 61. The amounts increased progressively to about tube 145, and started to decrease to about tube 230. Lysine and histidine started to appear from fraction 71. Histidine being a minor constituent stopped eluting after fraction 75. The amount of lysine too decreased from fraction 81, though trace amounts were detected to about fraction 165. Desmosine started to elute from fraction 71, reached its peak at 210, and started to decrease till only trace amounts were shown in fraction 230. Arginine did not appear in any of these fractions. The results of this experiment confirm the findings of Starcher and Galione (1975), that application of large amounts of desmosines to the column, resulted in overlap of the isodesmosine and desmosine peaks.

(c) Elution done at 55°C

Buffer free of 'Brij 35'. See page 52 for preparation of sodium citrate buffer. The experiment was set up as in Section 3.10d (ii), but the buffer was free of 'Brij 35'. The elution of isodesmosine started from tube 16 and desmosine was not detected until fraction 151. Lysine and trace amount of histidine showed up along with desmosine. The elution of isodesmosine and desmosine continued to about fraction 375. See figures 35 - 39.

STANDARD
HYDROLYSED ELASTIN.

FIG. 28



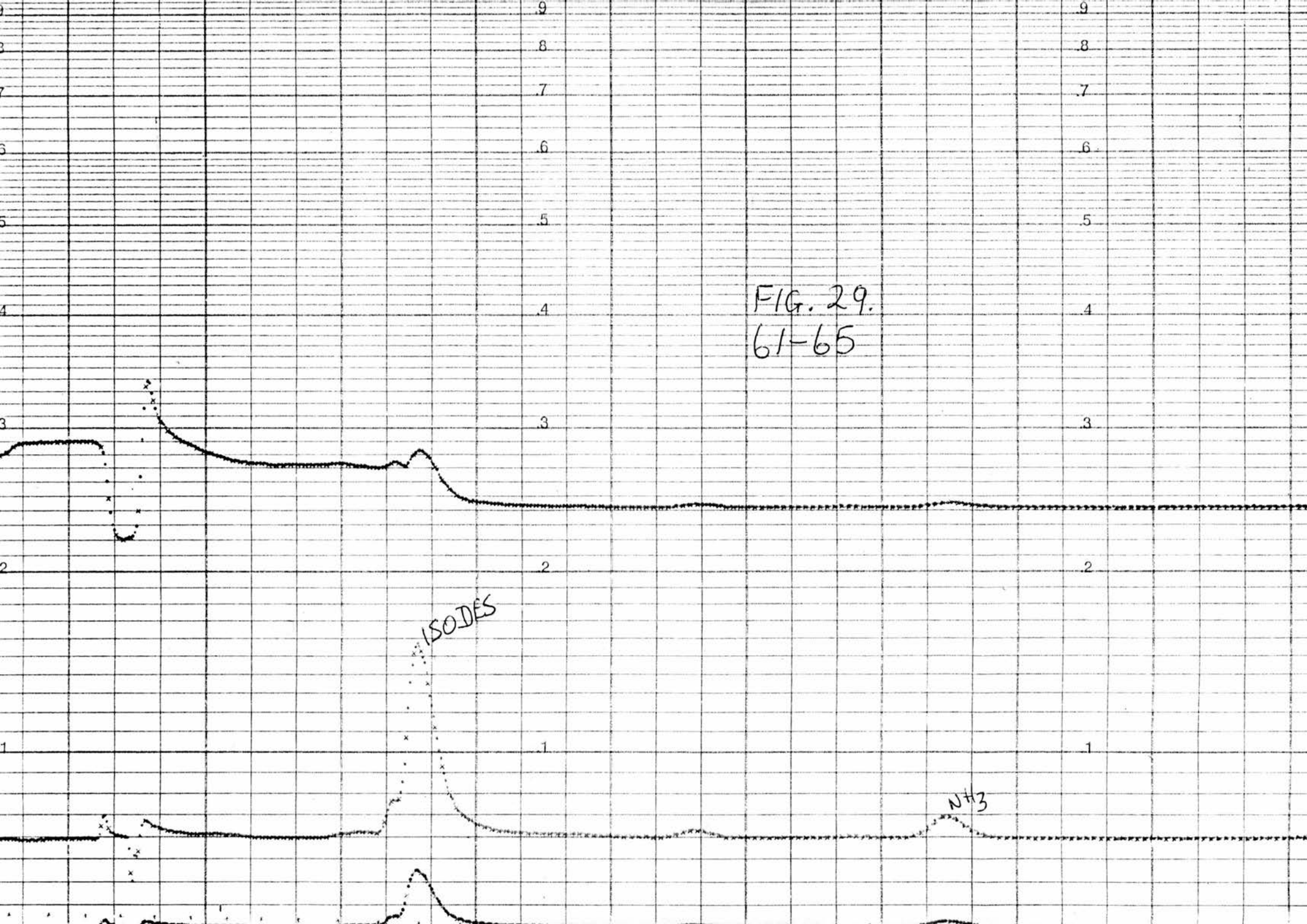
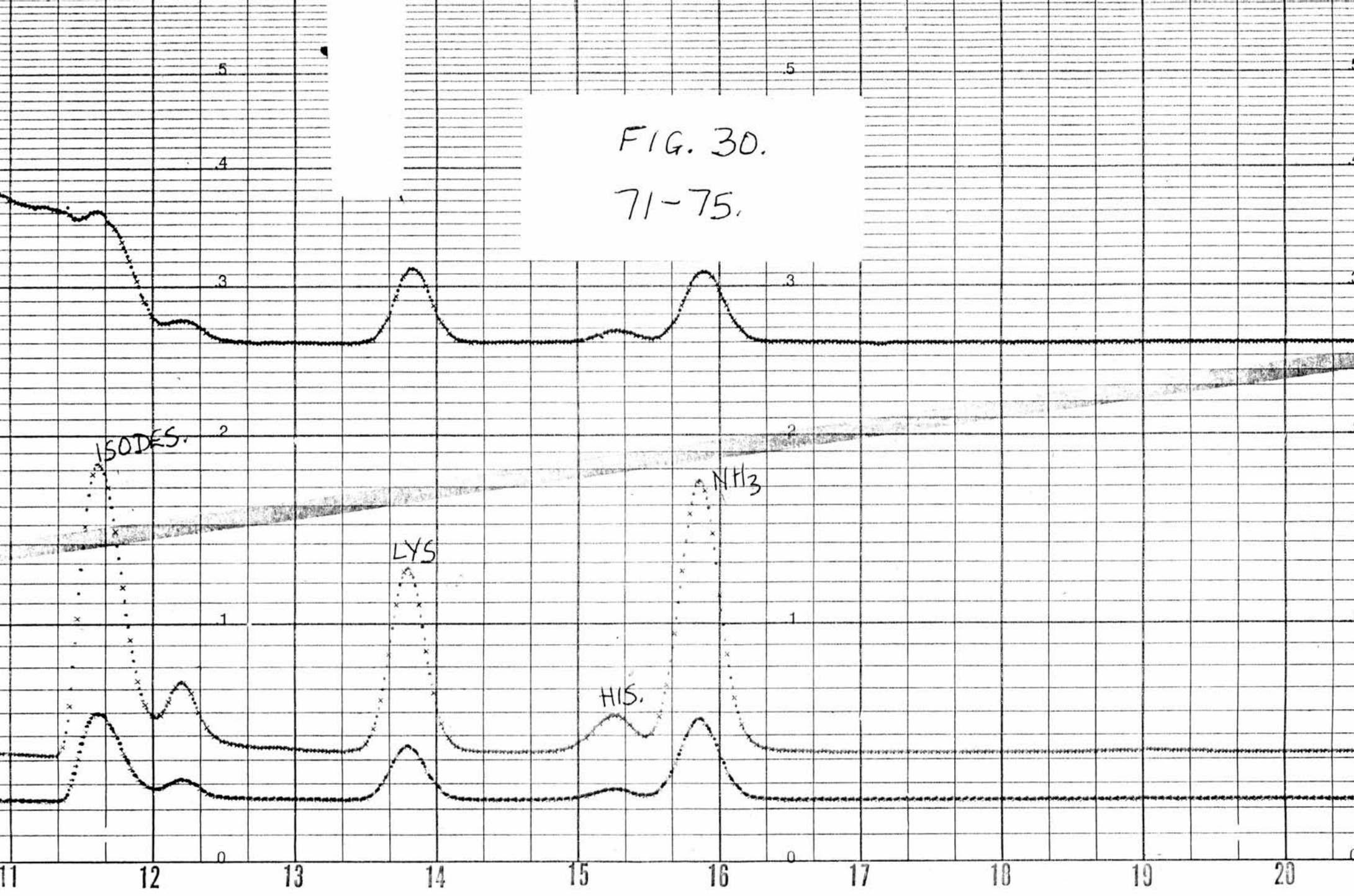
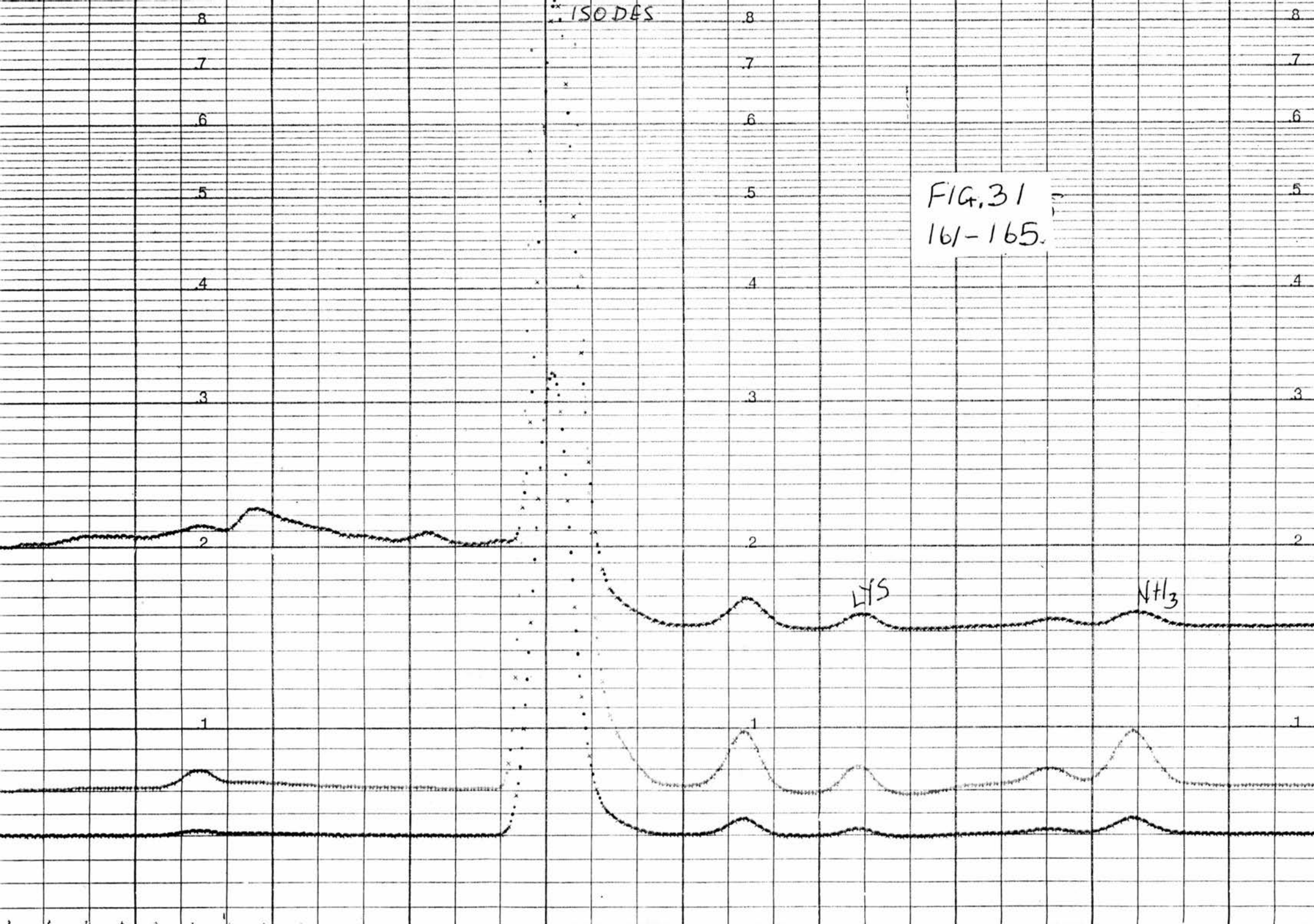


FIG. 29.
61-65




ISODES
FIG. 31
161-165.
LHS
NH3

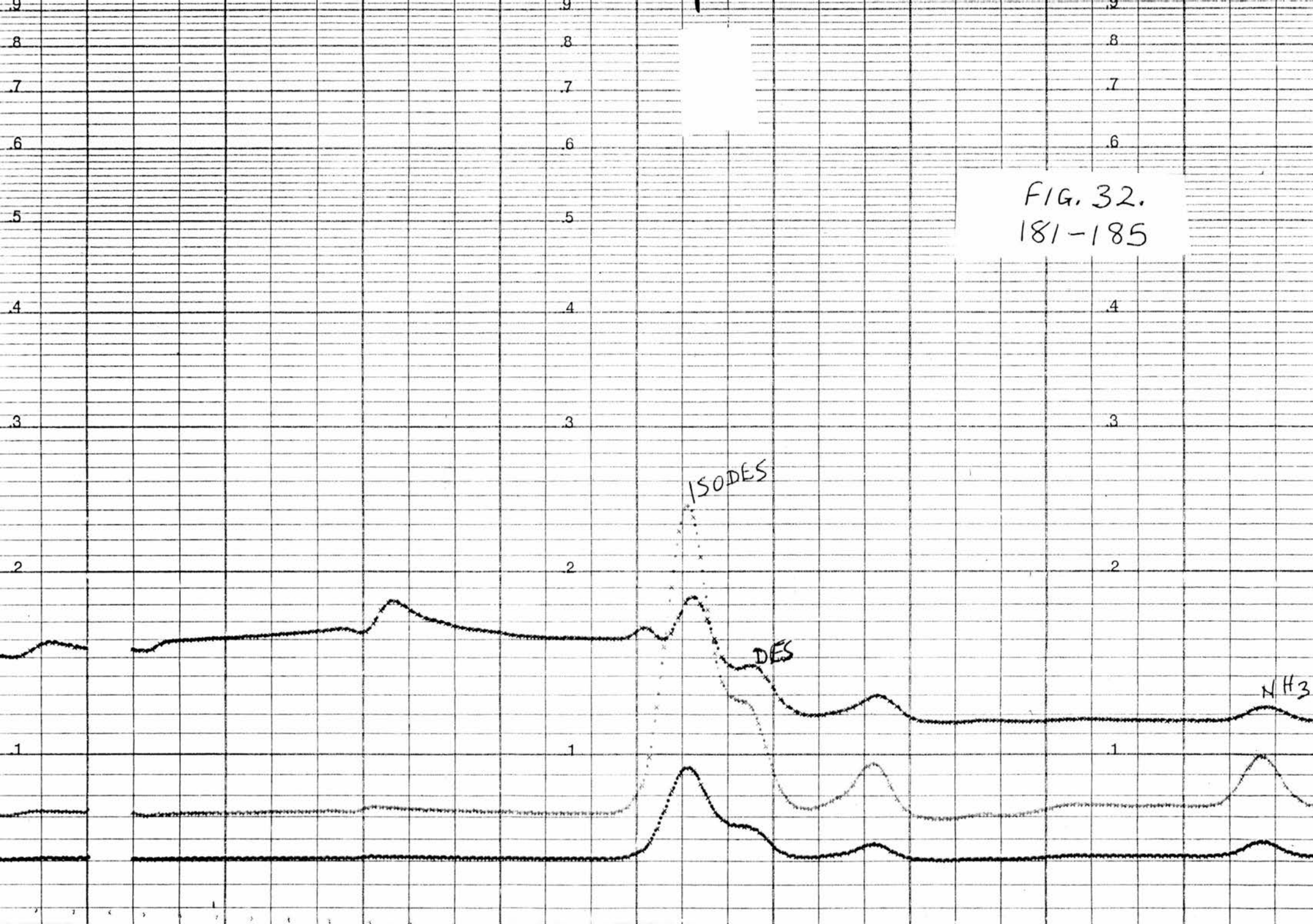


FIG. 32.
181-185

FIG 33
186-190

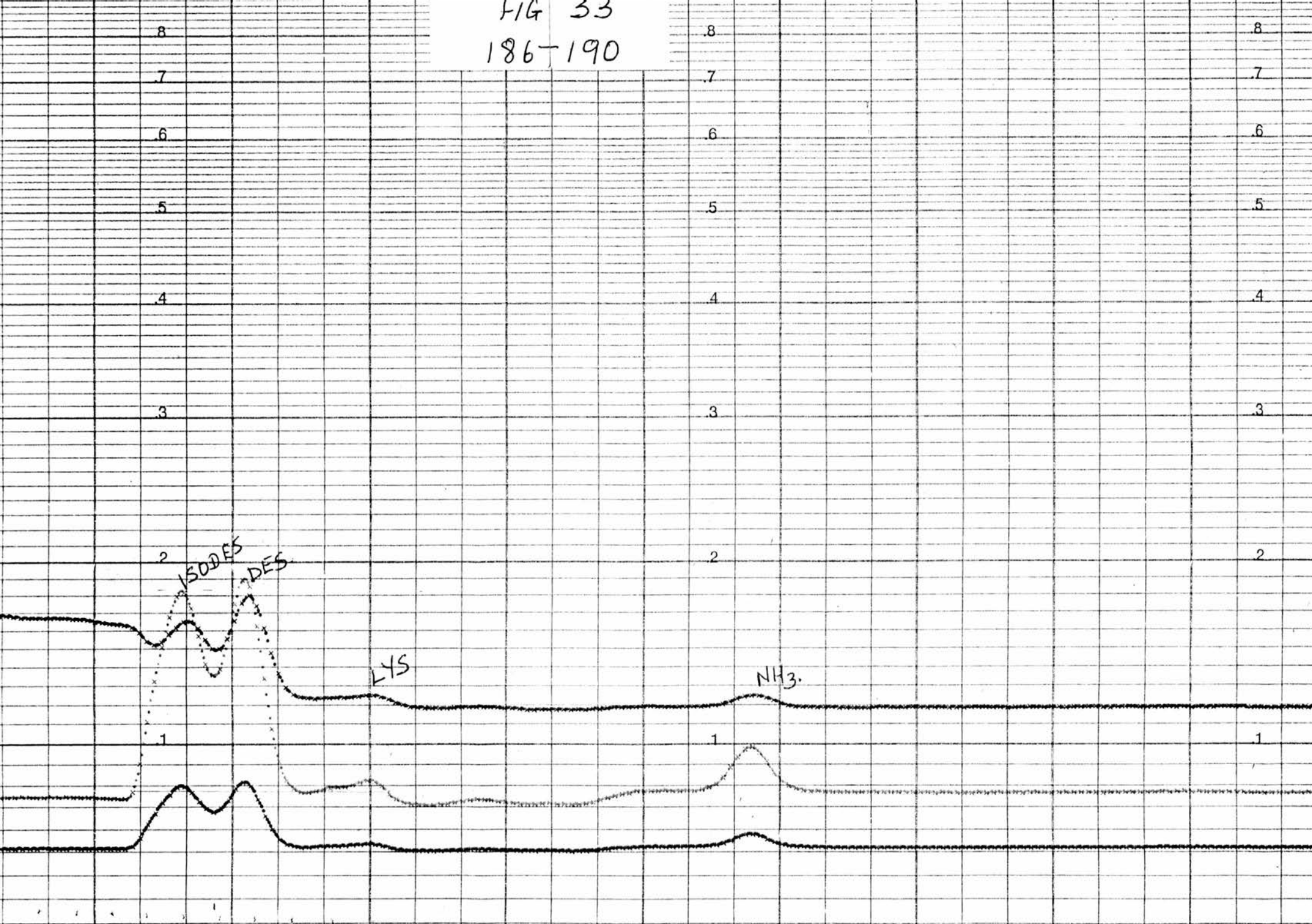
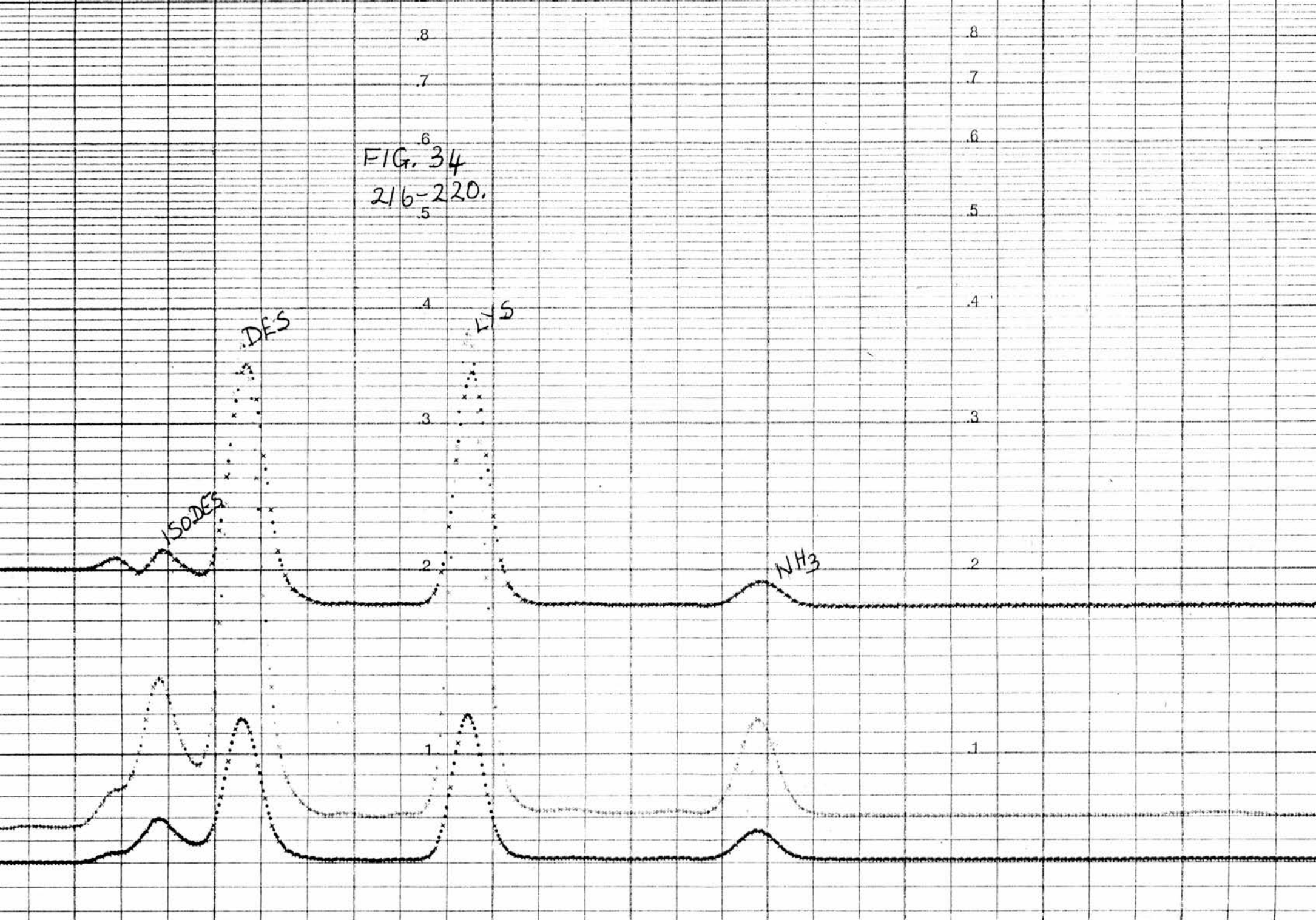


FIG. 34
216-220.



4.6. Comments on the Method

(a) The pattern of elution obtained with the Sodium Citrate buffer at 55°C appeared better than that at room temperature. The only reason for this would be because of the difference in the temperature of both experiments.

The Sodium Citrate with 'Brij 35', also produced a better elution pattern compared to the buffer free of 'Brij 35'. This can also be attributed to the presence of 'Brij 35'.

(b) Short Coming of Method

(i) In all three elution methods discussed in Section 4.5, the fractions containing the desmosine and isodesmosine were many. Over one hundred tubes of ten ml. fraction in each tube, contained the two amino acids, and this is a large volume to carry to the next stage of the experiment.

(ii) It was because of this large volume containing the amino acids, that necessitated that the fractions should be pooled together and concentrated in vacuo on a water bath set at 45°C. This is a further purification step to free the two amino acids of contaminants like lysine.

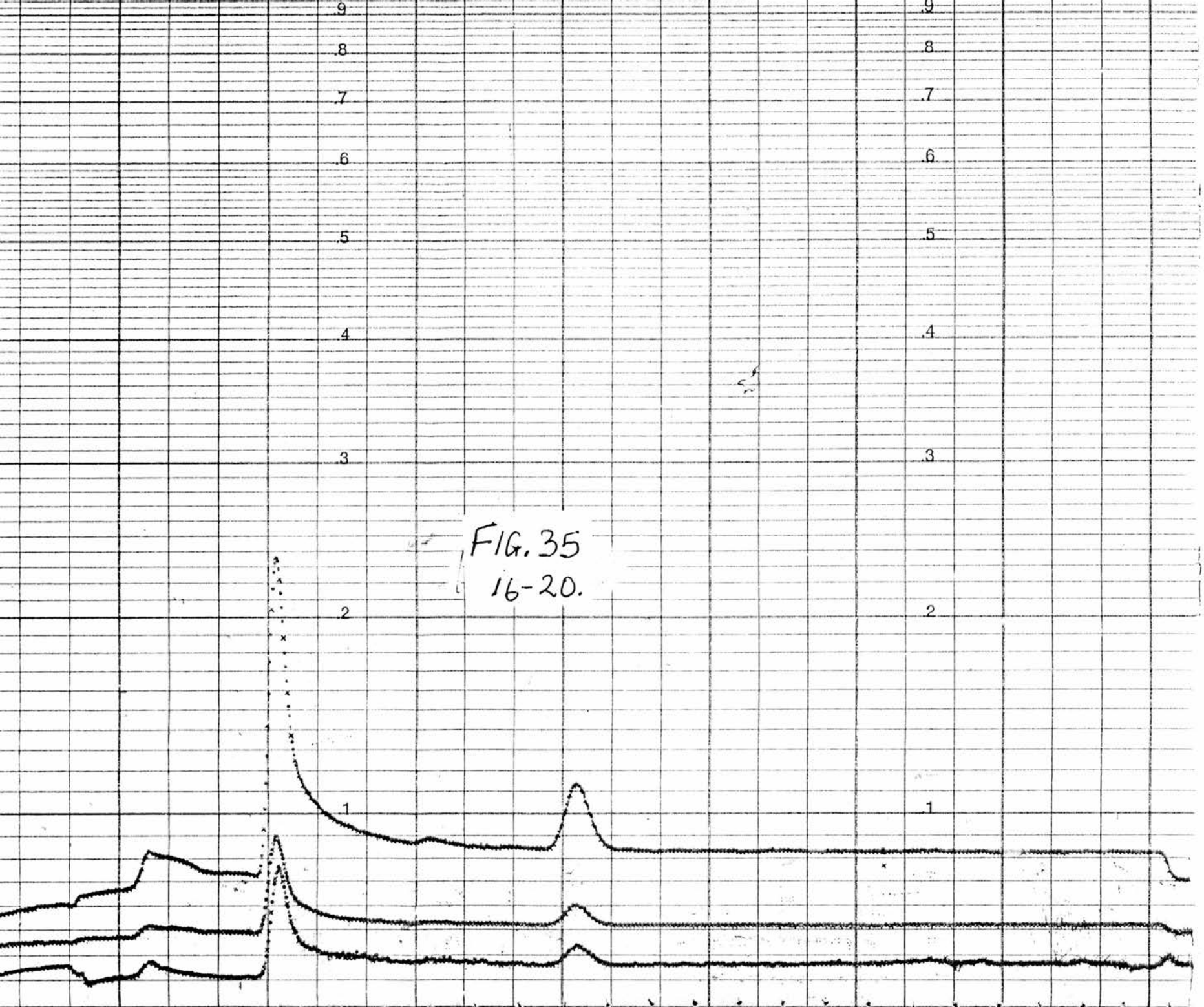
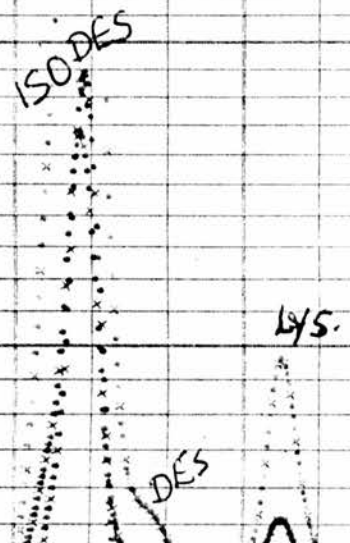


FIG. 35
16-20.

FIG. 36.

151-155



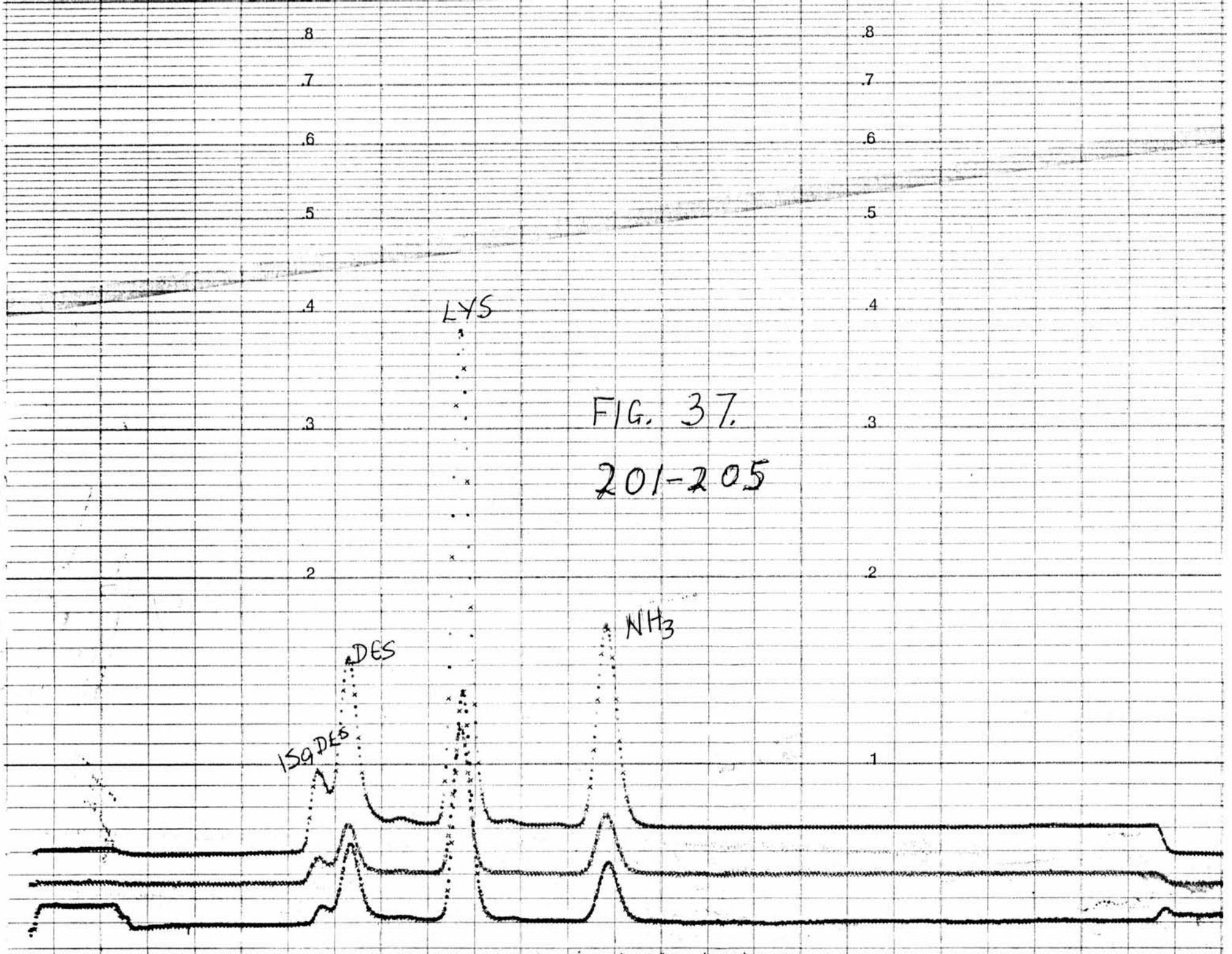


FIG. 37.

201-205

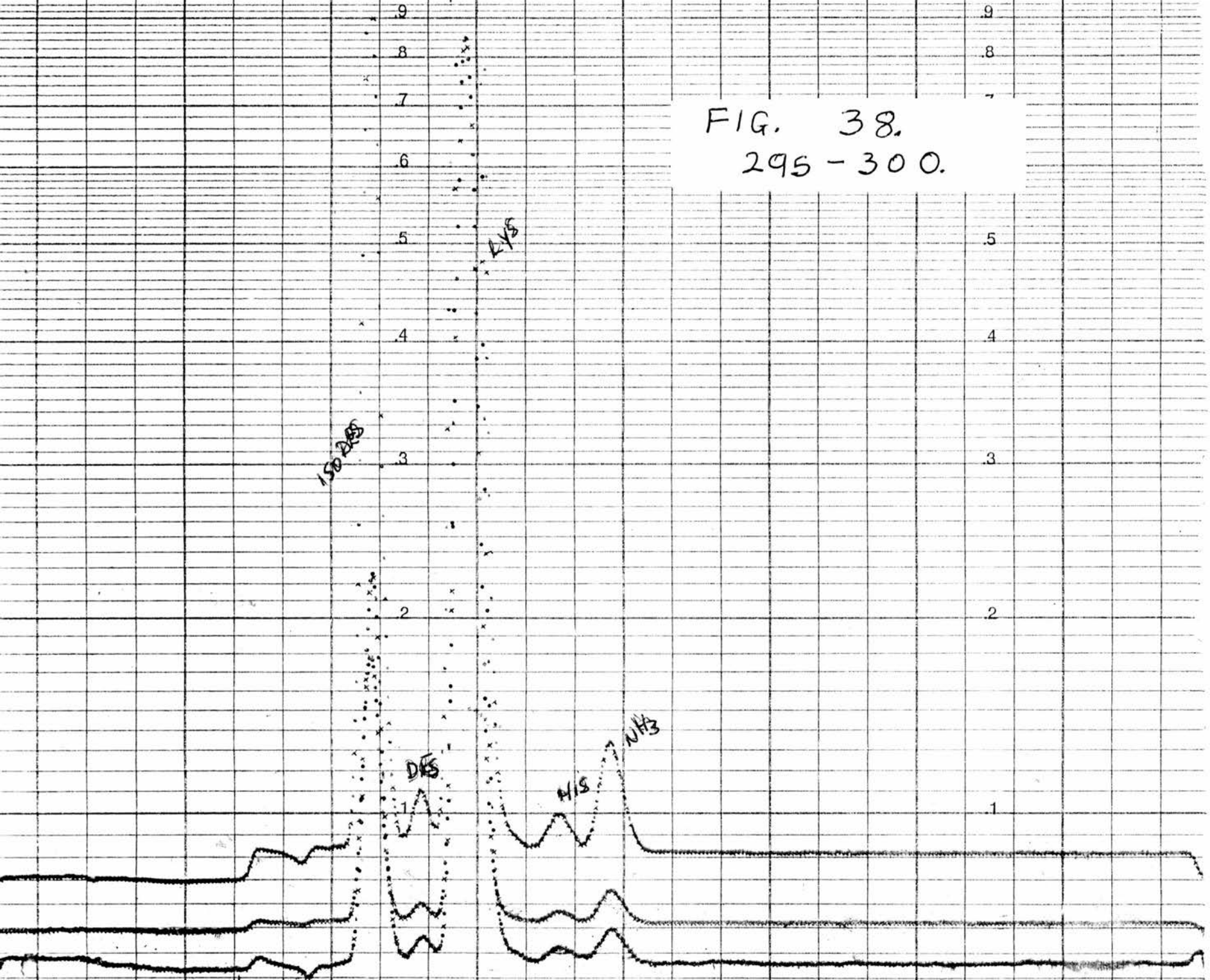
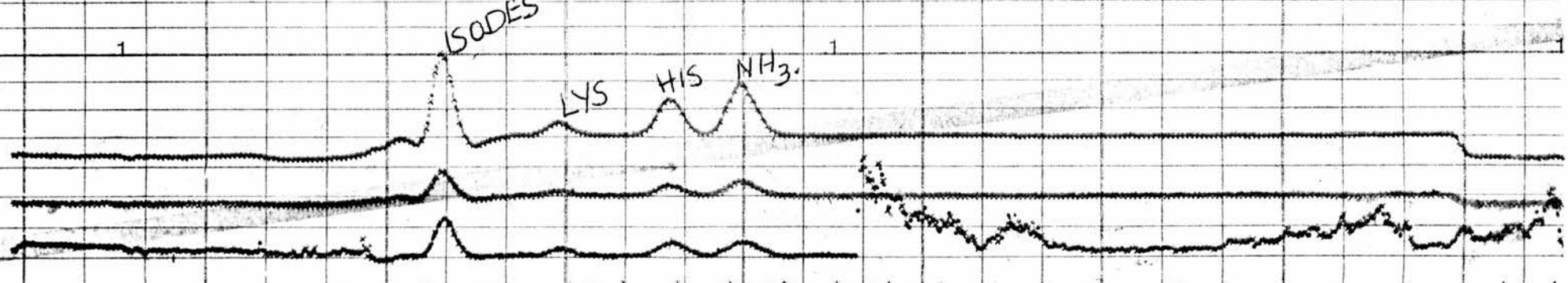


FIG. 38.
295 - 300.

FIG. 39.
396 – 400.



(iii) A lot of care has to be taken when concentrating the fraction in vacuo because of the presence of 'Brij 35' as it causes unnecessary foaming. 'Brij 35' is Polyoxyethylene lauryl ether. It can be removed by soaking the fractions in Acetone. The amount of ammonia in the fractions appeared to increase because of this, and it could be removed by treatment of the fractions with an ion exchange cellulose (Cellex CM).

(iv) The fractions have to be analysed in groups of five serially. The amino acid analyzer can only analyse a maximum of three batches of five fractions per day.

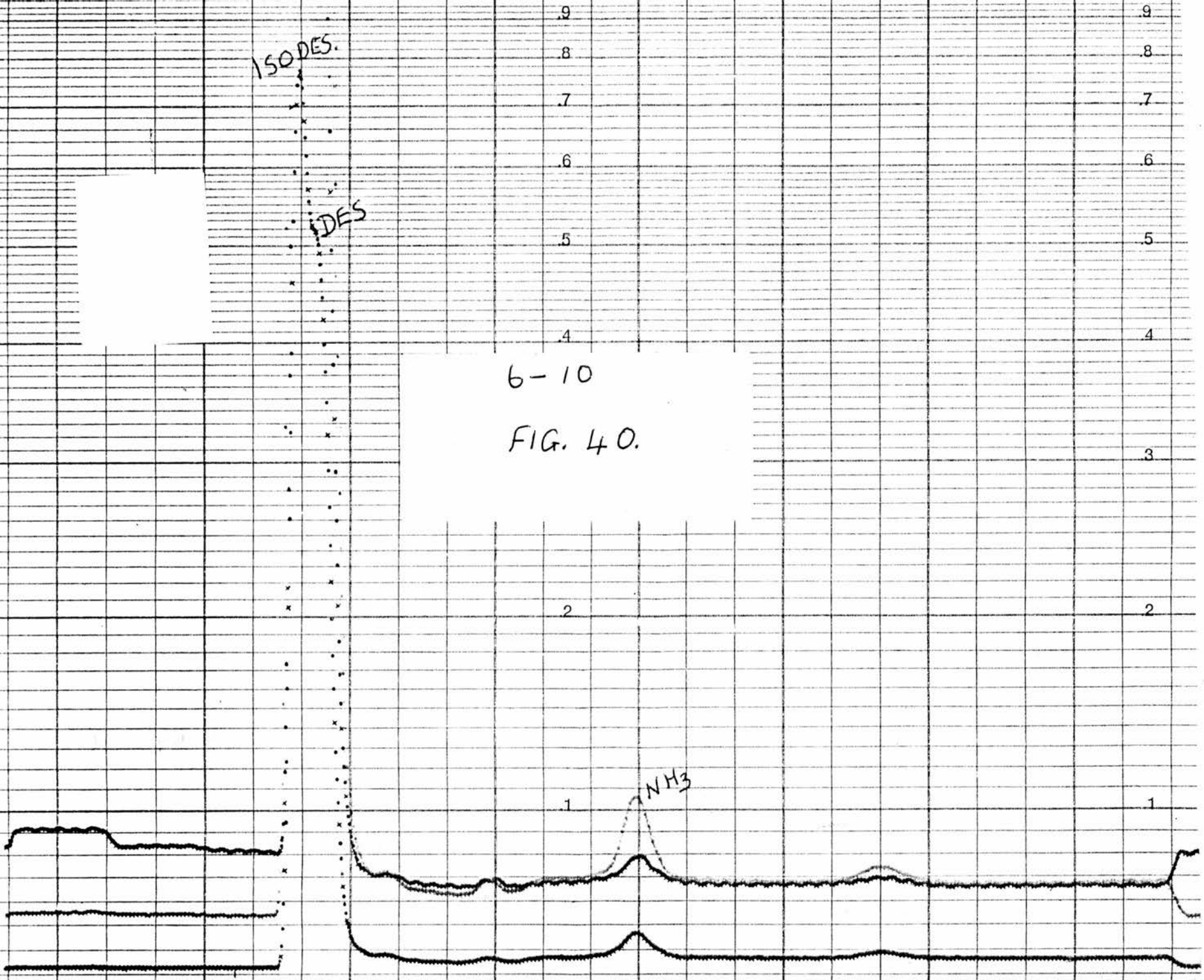
4.7 Effect of Reapplication of Basic Amino Acid Fractions unto Dowex 50 Resin

(a) Buffer with 'Brij 35' - Elution at 55°C

The pattern of results obtained after the concentrated solution had been reapplied to the column, were very different from the result in Section 4.5b. The isodesmosine started to elute from about fraction 5. Desmosine started to elute from about fraction 26. Only Desmosine and Isodesmosine eluted in this experiment. Elution ceased around fraction 130. See Figures 40 - 44.

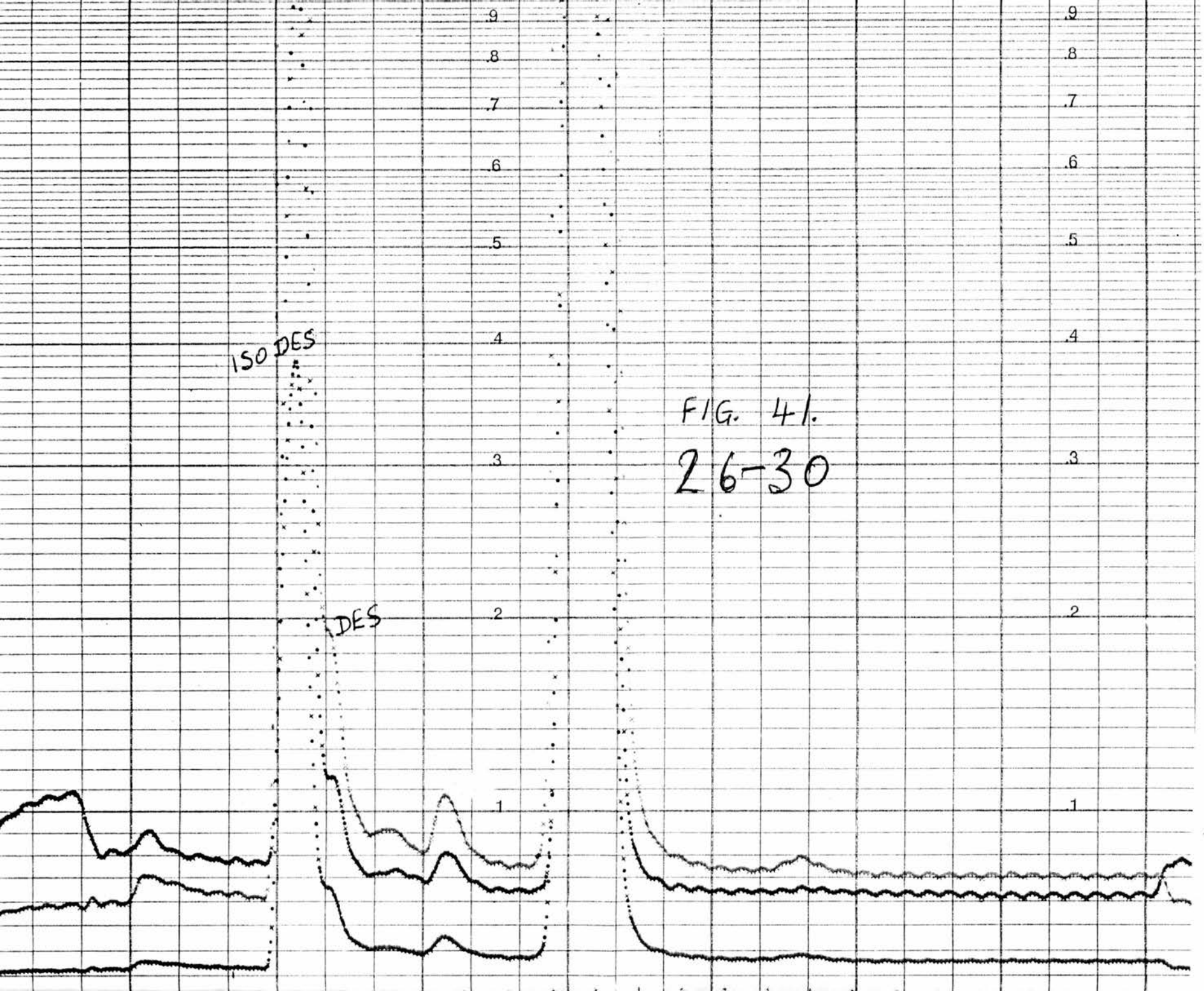
(b) Buffer free of 'Brij 35' - Elution at 55°C

Elution of desmosine and isodesmosine started from tube 11 and by tube 40, there was no more left in the column. Some lysine still passed through the column along with the desmosine and isodesmosine. See Figures 45 - 48.



6-10

FIG. 40.


ISO DES
DES
FIG. 41.
26-30

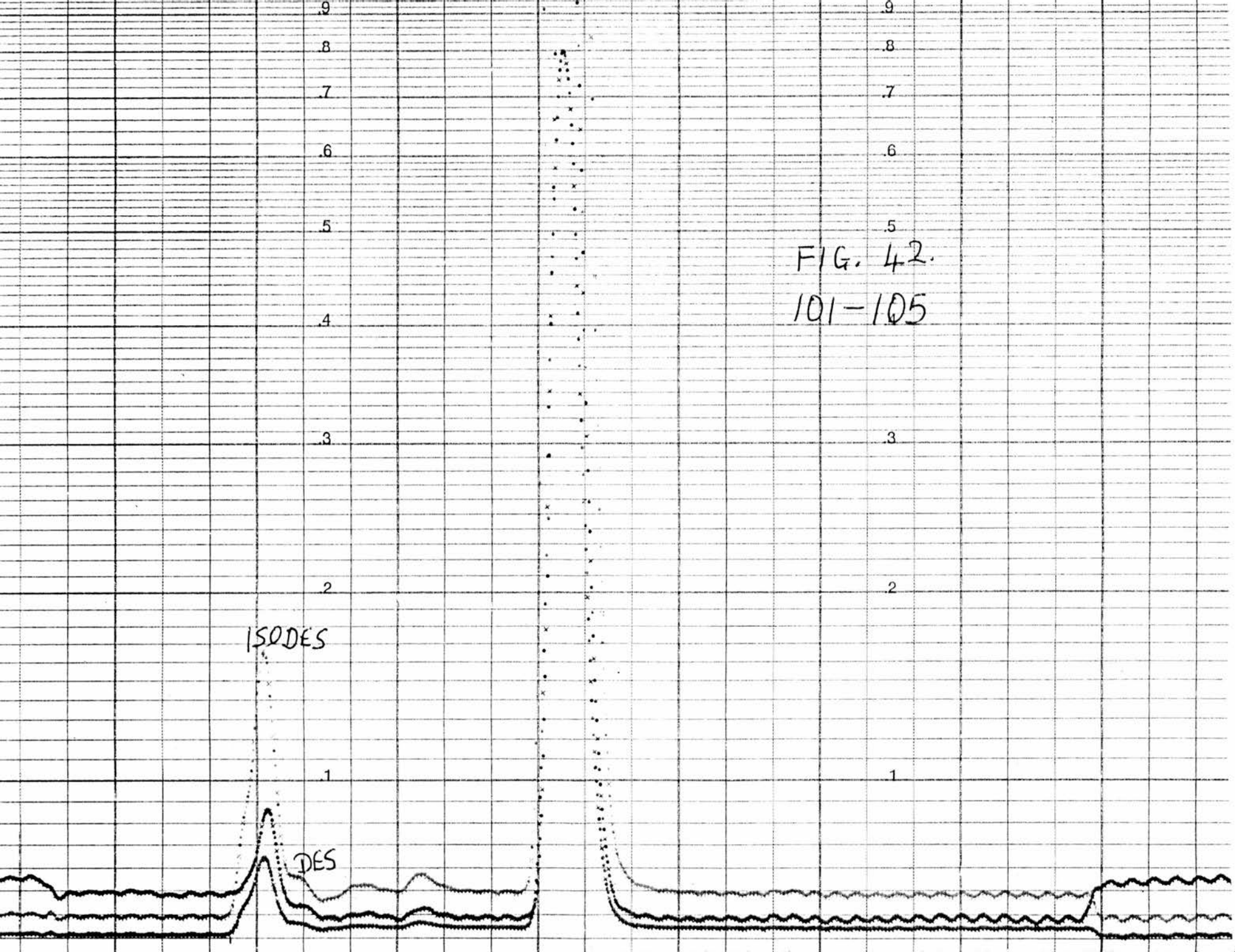


FIG. 42.
101-105

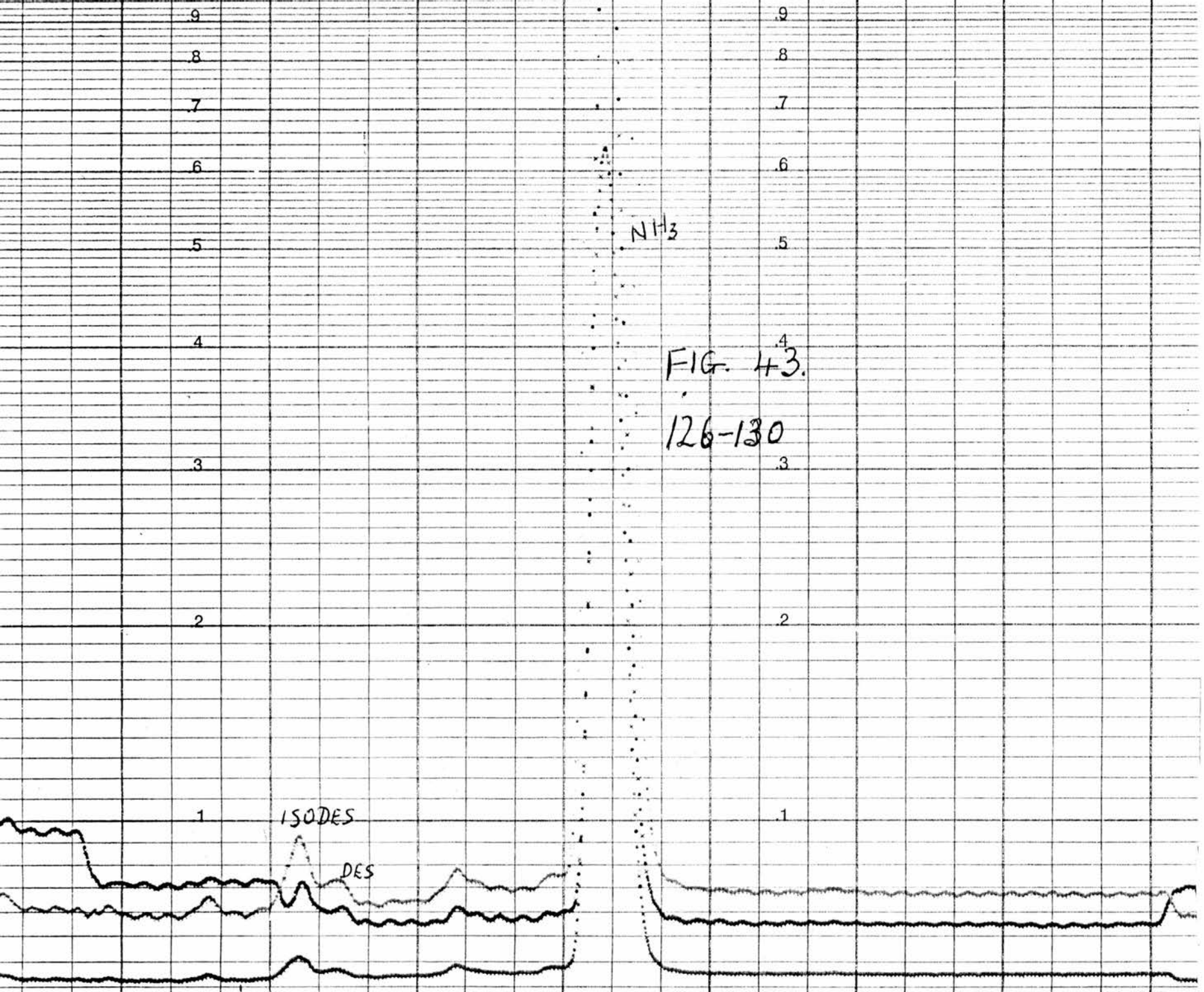
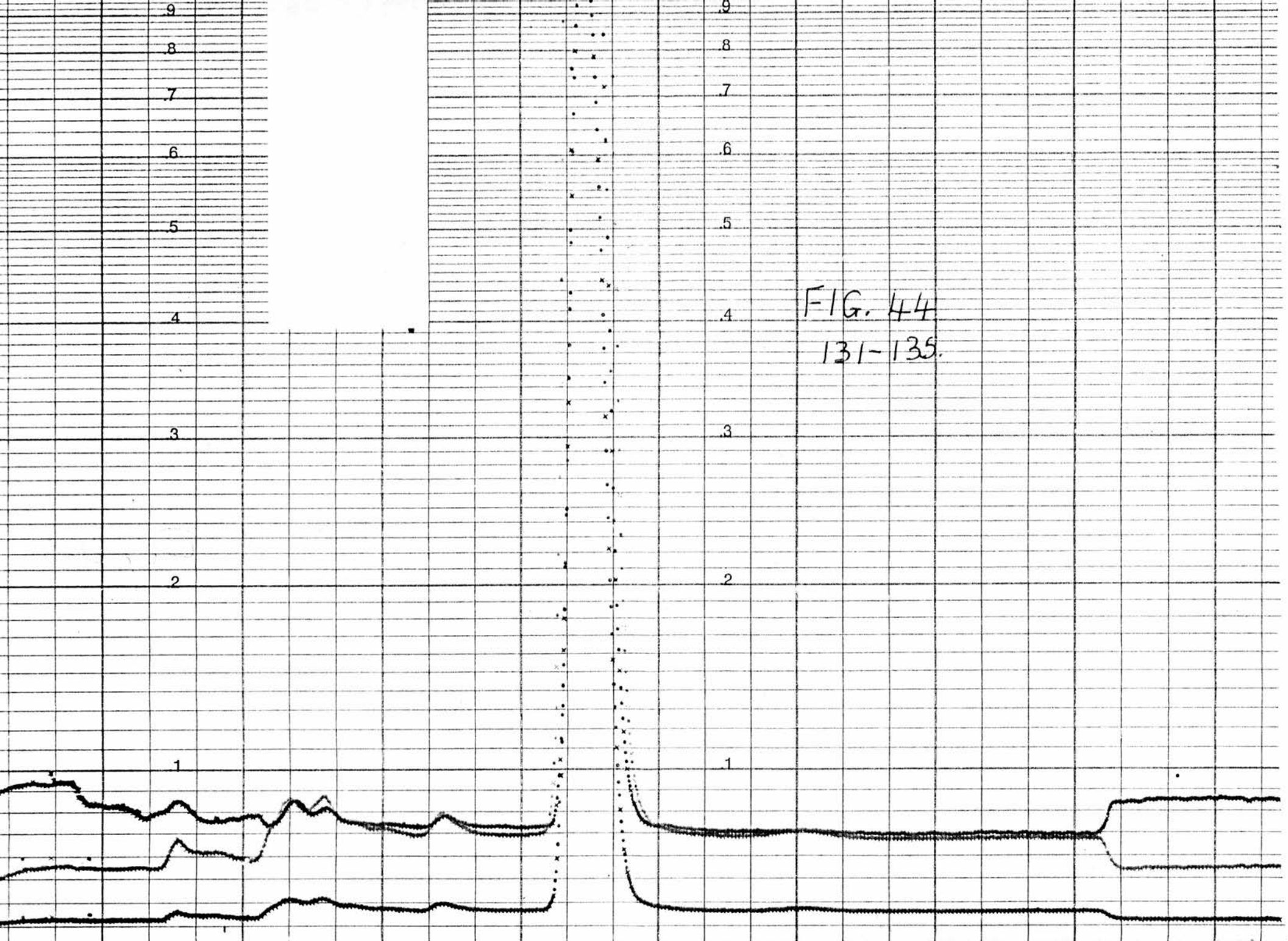


FIG. 43.

126-130


.9
.8
.7
.6
.5
.4
.3
.2
.1
FIG. 44
131-135.

(c) Comments on the Results

The results obtained on application of the concentrated fractions to the Dowex resin seemed better in the buffer that had 'Brij 35'. This goes to confirm that the addition of 'Brij 35' to the buffer produces a better elution.

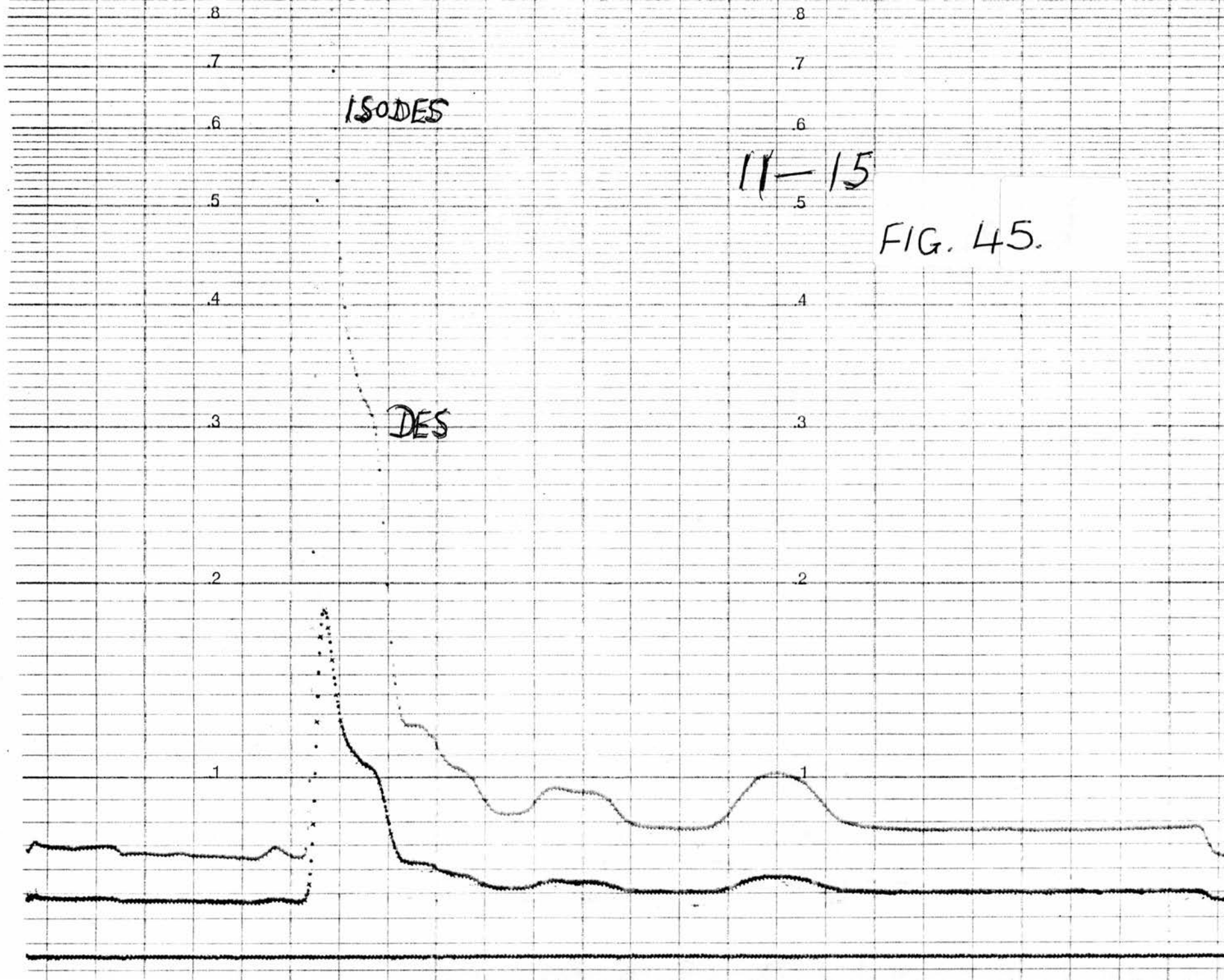
4.8 Results got with either Dowex or Sephadex Ion Exchange Resin

(a) The first experiment with Dowex (See Section 3.10c (i) did not produce any separation. This could be due to the low strength of the eluting HCl (0.001M).

(b) The second experiment (See Section 3.10c (ii) a gradient was applied. The optical density of the solution passing through the flow cell varied wildly. This could be due to channelling, and the experiment was abandoned.

(c) The third experiment (Ref. Section 3.10c (iii) produced a poor resolution. Experiment was abandoned because of this.

(d) The fourth experiment (Ref. Section 3.10c (iv) did not produce a good result either, and was abandoned. There was shrinkage of the Sephadex, because of the increasing strength of the acid used for elution.



11-15

FIG. 45.

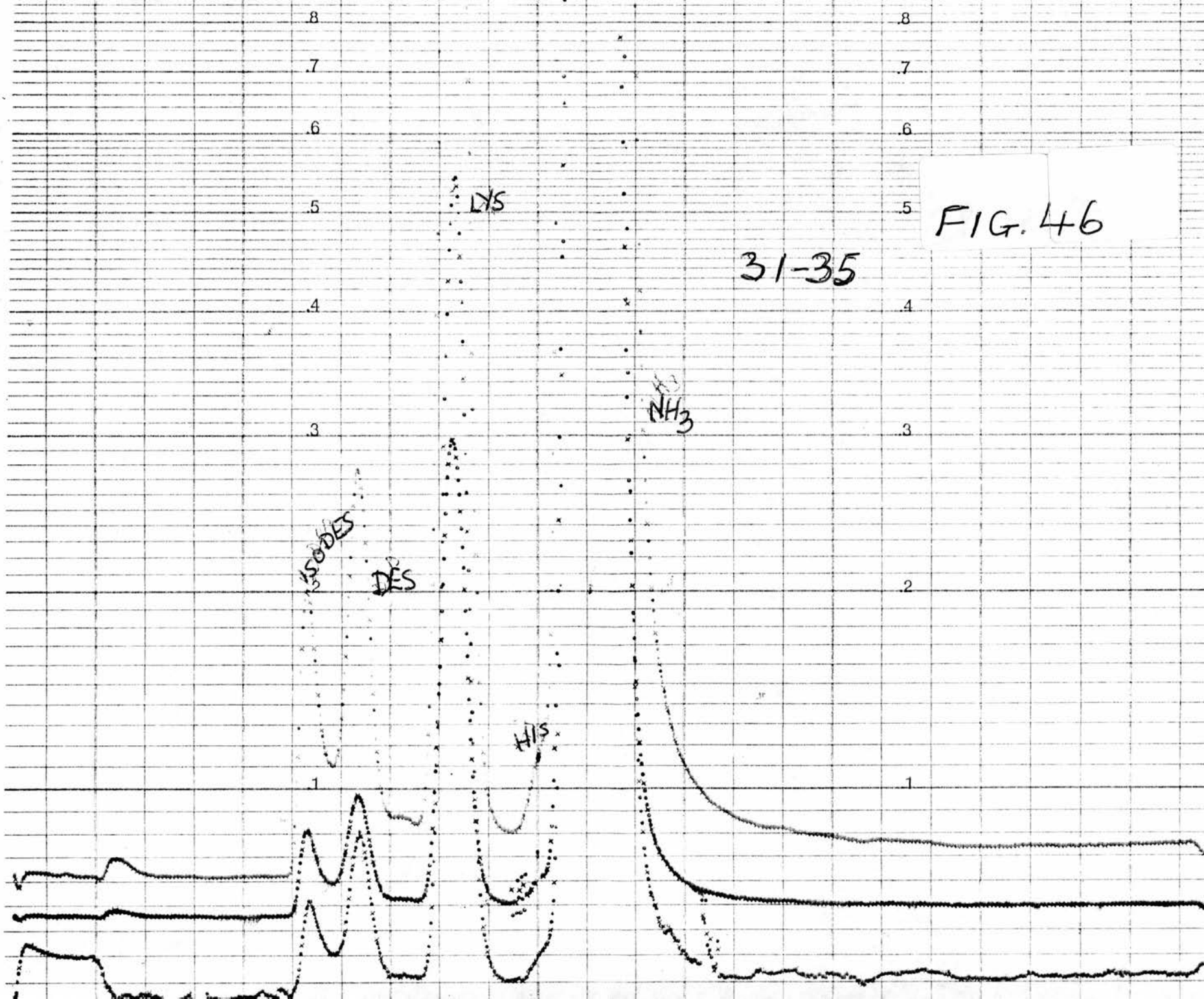


FIG. 46

31-35

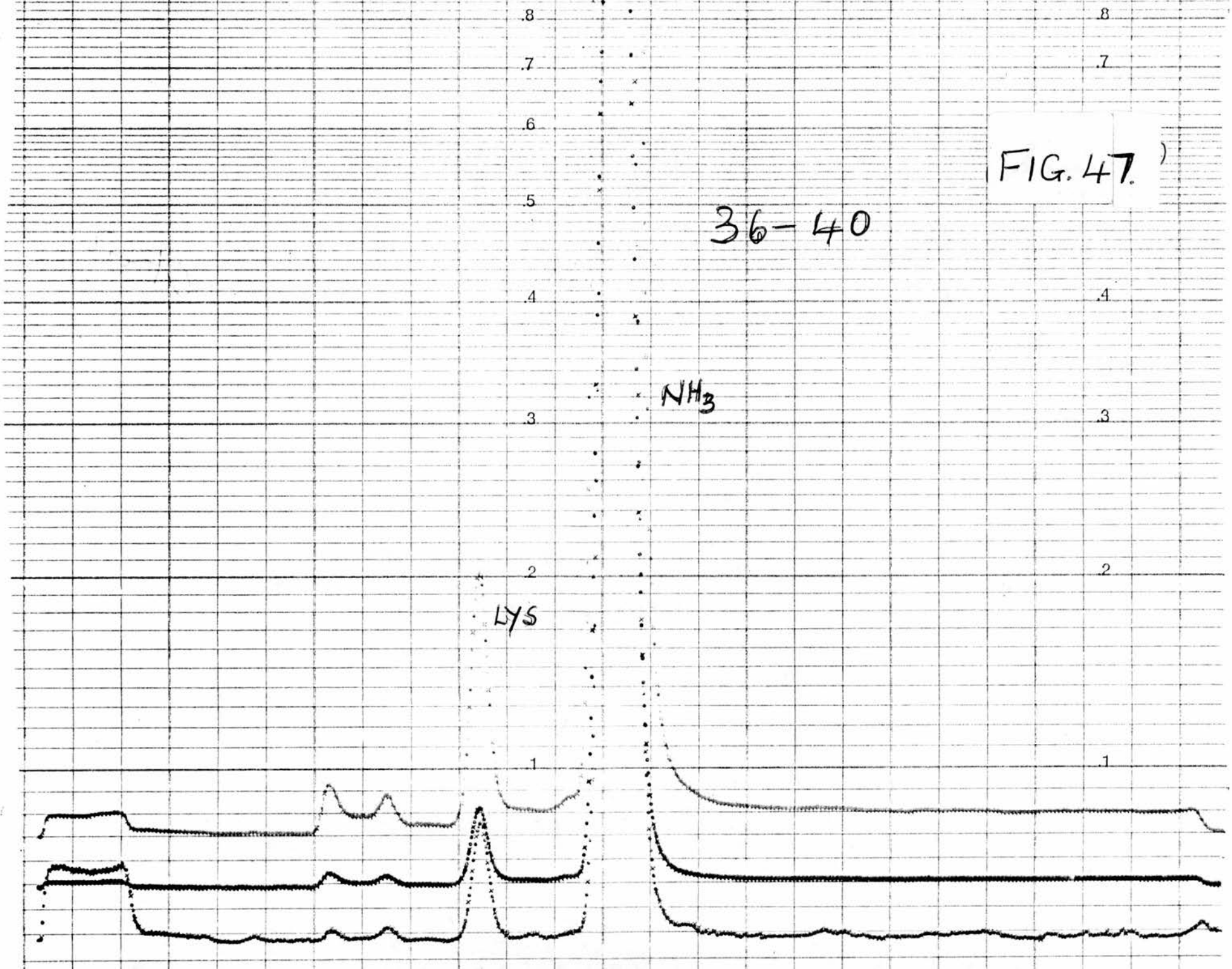
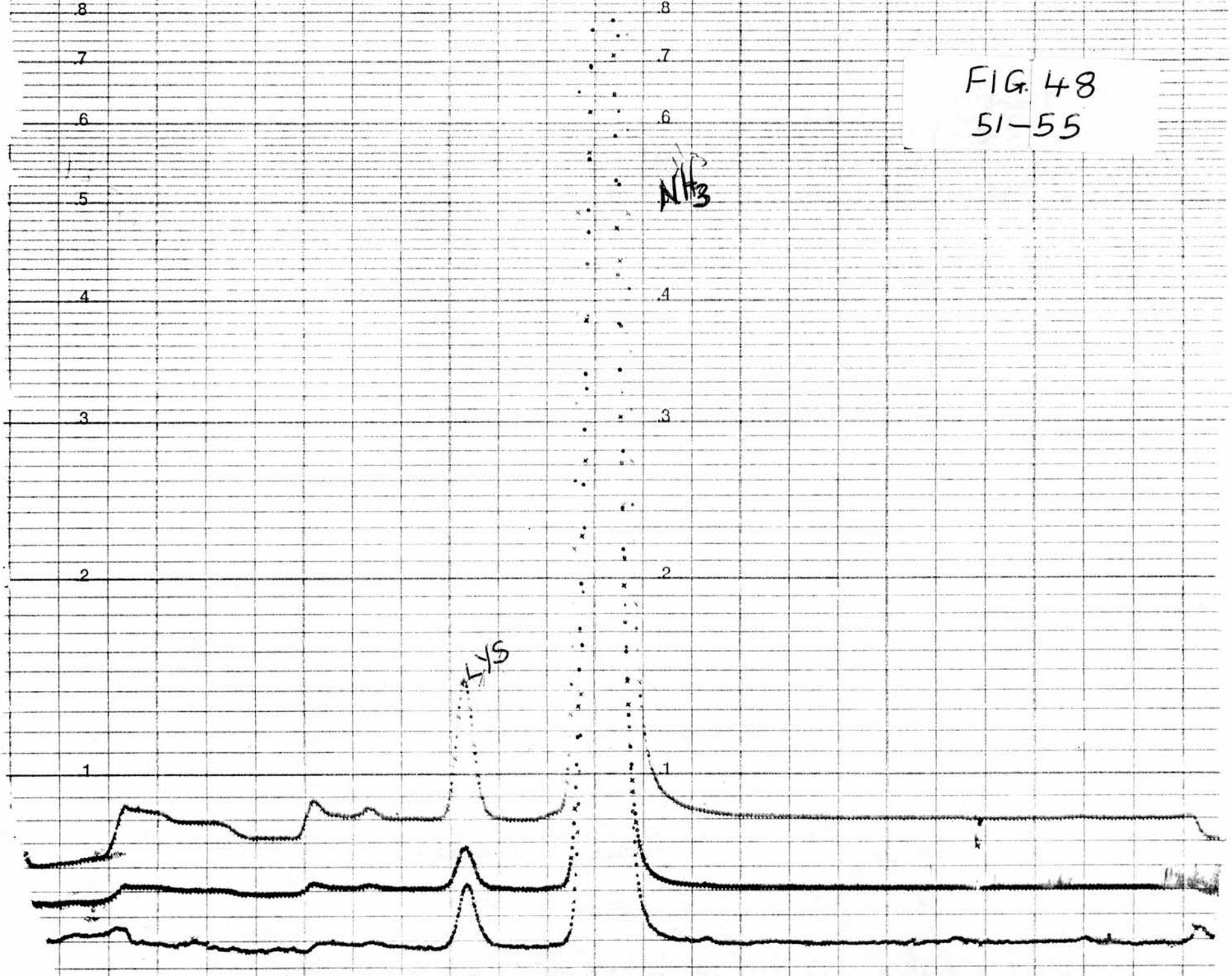


FIG. 47.
36-40
NH3
LYS
.8
.7
.6
.5
.4
.3
.2
.1

FIG. 48
51–55



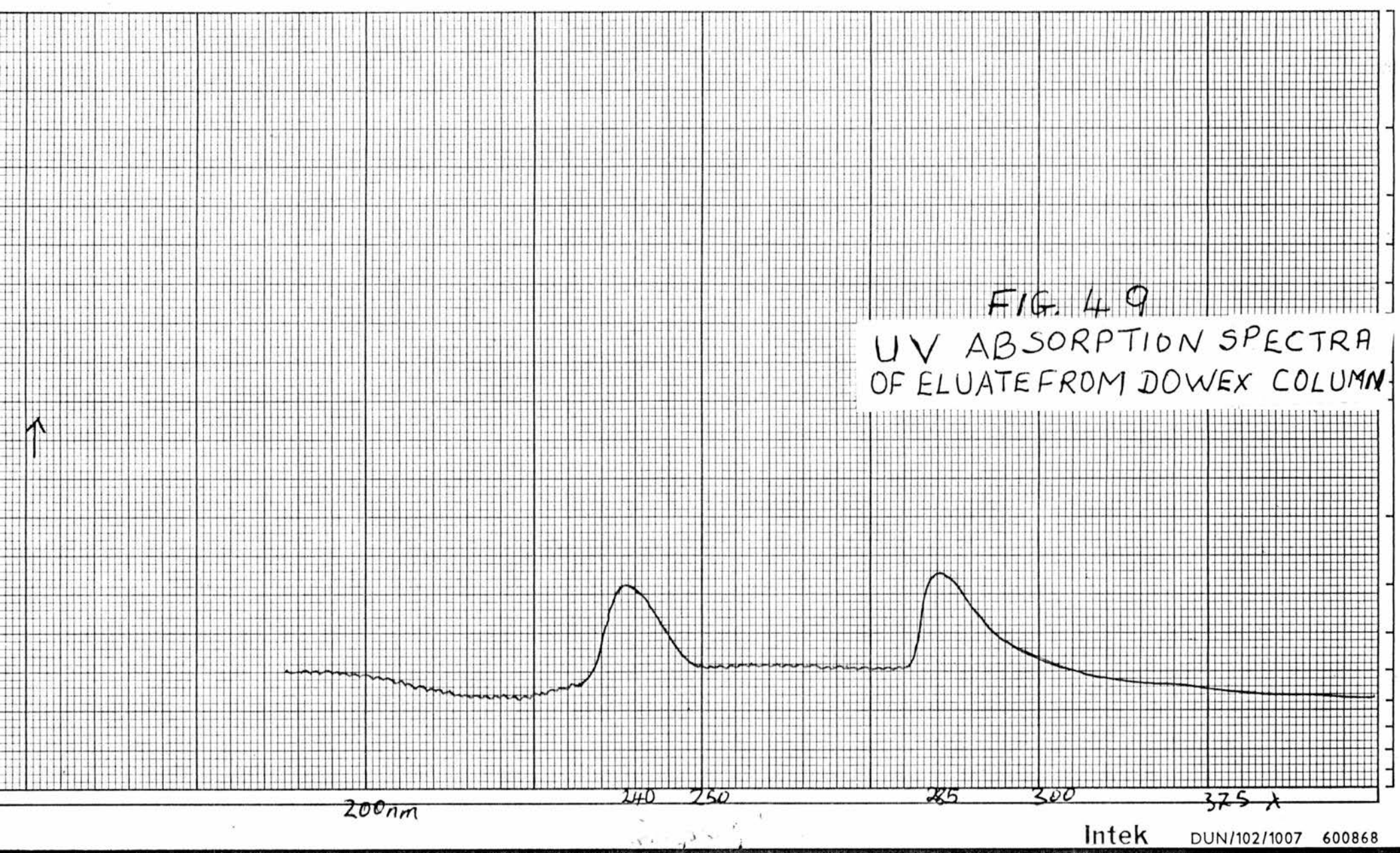


FIG. 49

UV ABSORPTION SPECTRA OF ELUATE FROM DOWEX COLUMN

Intek DUN/102/1007 600868

DEX
DER

SAMPLE AND FORMULA

CONCENTRATION
REFERENCE _
PATH LENGTH MM.

SCAN SPEED FAST ☐ SLOW ☐
DATE
OPERATOR

4.9 Ultra Violet Absorption of Eluates Containing Desmosine and Isodesmosine

The scanning on SP 800 of the eluates that contained large amounts of desmosine and isodesmosine is shown in figure 49. The wavelength of maximal absorption for desmosine is 240nm; and that of isodesmosine is 284nm. These are very close to values quoted by Anwar (1966). He quoted 235nm for desmosine, and 280nm for isodesmosine. The difference in his experiment and this can be traced to the pH of the Sodium Citrate used in the two experiments. Anwar (1966) used a slightly more acidic buffer (0.38N citrate buffer, pH 4.26) while 0.23M Sodium Citrate pH 4.55 was used in this experiment.

4.10 Investigation of the Properties of Desmosine and Isodesmosine

(i) Formation of Crystals (Ref. Section 3.11)

Crystals were formed after the sample had been in the fridge for about three weeks. It was assumed that the crystals formed were mixtures of desmosine and isodesmosine.

(ii) Formation of Desmosine and Isodesmosine Picrates

No crystals were formed. Tristram (1939) was able to form lysine picrate from leaf proteins. The inability to form picrate might be due to internal interaction of the charged groups of the desmosine molecule.

4.11 Fluorescence Values

In all experiments the excitation wavelength of the beam is written as the prefix, while the emission wavelength is given at the suffix.

(i) Elastin

The fluorescence spectra of the elastin used for the experiment is shown on page 68A. The values obtained were 340/415, 350/425 and 370/435 at pH 3.4.

This can be compared with the values quoted by Anwar (1980) in her work on hydrolyzate of egg shell membrane. She quoted the value of 340/415 for the fluorescence spectra of the egg shell membrane which she said would be the same with that of elastin.

(ii) Catholyte of Desmosine and Isodesmosine

The fluorescence value obtained when the 4th catholyte of desmosine and isodesmosine was excited at 340nm was somewhat higher than that obtained when the hydrolysed elastin was excited.

The work of John and Thomas (1971) shows that pH of medium affects the fluorescence values.

Elastin	340/415	pH 3.4
Catholyte	340/430	pH 9.5

(iii) Crystals of Desmosine and Isodesmosine

The fluorescence values obtained when the crystals were dissolved in 0.1M Tris buffer are as follows:

274/345

340/393

The fluorescence spectra were very similar to those obtained by Anwar (1980). The fact that the value for the emission fluorescence was 393 and not 415 could be due to the difference in pH of the samples.

Fluorescence were obtained under two different conditions:

(a) The hydrolysate of elastin at pH 3.4 (Figure 8).

and

(b) A solution of desmosines in Tris buffer pH 11.5 (Figure 50)

The effect of the pyridinium nucleus which is a charged molecule in the hydrolysate of elastin of sample (a) and the effect of pyridine nucleus which has no charge (sample b) explains the difference in the fluorescence spectra.

4.12 Table 7

Elementary Analysis of Crystals

Sample Isolated	Manufactured Sample
Molecular Weight	
(By Mass Spectrogram)	(By Calculation)
	526
Percent Composition	
Carbon	54.75
Oxygen	24.34
Nitrogen	13.31
Hydrogen	7.60

5. C O N C L U S I O N

5. CONCLUSION

From the results obtained on the percentage concentration of the desmosines in the catholytes, it is evident that the results were much higher than those obtained by previous workers. The yields of isodesmosine and desmosine obtained by Starcher and Galione (1975) in 25lbs (11.36kg) bovine ligament was 15g each, equivalent to a percentage of 0.26 for both amino acids.

Serafini-Fracassini and Tristram (1965) worked on human aortic elastin and found the yield of desmosine to be (0.71 - 0.77%) and isodesmosine to be (0.51 - 0.59%). Every worker agrees that the levels of isodesmosine and desmosine in bovine ligament or human aorta are very low and variable, the yields obtained from electrodialysis were higher than those of other workers. This is best exemplified by comparing duplicate preparations from 5g elastin.

In the first experiment, the yield of isodesmosine was equivalent to 1.11 - 2.31%, while the yield of desmosine was equivalent to 1.05 - 2.24%. In the second experiment the yields were equivalent to 0.46 - 4.99% for isodesmosine, and 1.14 - 3.14% for desmosine. These results show the inconsistencies of the method, and it is therefore not recommended.

When 9g elastin was used, the yields were equivalent to 2.29 - 4.30% of Isodesmosine and 0.89 - 1.91% of Desmosine.

The yield of isodesmosine in samples of 3rd and 4th catholytes was the same (4.87%), while the yield of desmosine was the same (2.00%). All the yields no matter what set of membranes were utilised were much higher than what other workers obtained.

Using the method of Starcher and Galione (1975), the yields of the desmosines was 0.23% which is in agreement with those of Starcher and Galione (1975).

X.....mV/cm Datum: Nr.:

Y.....mV/cm Name: Blatt Nr.:

FIG. 51

FLUORESCENCE SPECTRA
4TH CATHOLYTE
EXCITATION / FLUORESCE
274 / 344
340 / 430

X.....mV/cm

Y.....mV/cm

Datum:

Name:

6. BIBLIOGRAPHY

6. BIBLIOGRAPHY

ADAMS, B.A. and HOLMES, E.L. (1935) J.Soc. Chem. Ind. 54, (1935); British Patents 450308-9 (1936).

AKIODE, C.A. (1980) M.Sc. Thesis University of St. Andrews.

ALBANESE, A.A. (1940) J. Biol. Chem. 134, 467.

ANWAR, K. (1980) M.Sc. Thesis University of St. Andrews.

ANWAR, R.A. (1966) Can. J. Biochem. 44, 725 - 734.

BAIG, K.M.; VLAOVIC, M. and ANWAR, R.A. (1980) Biochem. J. 185, 611 - 616.

BAURAIN, R. LAROCHELLE, J-F. and LAMY, F. (1976). Eur. J. Biochem. 67, 155.

CHARLES, A. (1961) Brit. J. Derm. 73, 57

CHIBNALL A.C., REES M.W. and WILLIAMS, E.F. (1943) Biochem. J. 37, 373 - 374.

CORBIN, K.W. (1969) Anal. Biochem. 32, 118

COX, R.C. and LITTLE, K. (1961) Proc. Roy. Soc. London B 155, 231

DAVAIL, M. and HAN, K. (1974) FEBS Letters Vol. 43 No. 3, 331 - 335.

DUSTIN, J.P., SCHRAM, E.; MOORE, S. and BIGWOOD, E.J. (1953) Bull. Soc. Chem. Biol. 35, 1137

EASTOE, J.E. (1961) Biochem. J. 79, 652

FIELD, J. M. , RODGER, G. W., HUNTER J. C., SERAFINI-FRACASSINI, A. and SPINA, M. (1978). Archives of Biochemistry and Biophysics 191, 705

FITZPATRICK, M.; HOSPELHORN, V.D. and LAKE, S. (1965) Amer. Heart J. 69, 211

FOSTER, J. A.; GRAY W. R. and FRANZBLAU C. (1973a) Biochemica et Biophysica Acta 303, 363.

FOSTER, J. A.; BRUENGER E.; GRAY, W. R. and SANDBERG L.B. (1973b) J. Biol. Chem. 248, No. 8, 2876

FOSTER, J. A.; RUBIN L.; KAGA H. M.; FRANZBLAU, C.; BRUENGER E. and SANDBERG, L. B. (1974) J. Biol. Chem 249, 6191

FRANCHI, C. M. and DE ROBERTIS, E. (1951), Proc. Soc. Exp. Biol. N.Y. 76, 515

FRANCIS G.; JOHN, R. and THOMAS, J. (1973) Biochem. J. 136, 45.

FRANZBLAU, C. (1971). In Comprehensive Biochemistry, 26C, 659, Ed. Florkin, M. & Stotz, E. H. Amsterdam: Elsevier.

GERBER, G. E. and ANWAR R. A. (1974) J. Biol. Chem. 249, 5200

GERBER, G. E. and ANWAR, R. A. (1975) Biochem J. 149, 685

GOTTE, L. and SERAFINI-FRACASSINI, A. (1963), . J. Atheroscler Res; 3 247

GOTTE, L. MENEGHELLI, V. and CASTELLANI, A. (1965) In "Structure and Function of Connective and Skeletal Tissue" (Butterworth: London).

GRAY, W. R.; SANDBERG L. B. and FOSTER J. A. (1973)
Nature 246, 461

GREEN, R. A.; FOSTER, J. A. and SANDBERG, L. B. (1973)
Anal. Biochem. 52, 538

GREENLEE T. K. Jr.; ROSS, R. and HARTMAN J. L. (1966)
J. of Cell Biology. 30, 59

GROSS, J. (1949). J. Exp. Med. 89, 699

HAMILTON B. and ANDERSON R. A. (1959), Anal. Chem 31, 1504

HILL, R. L. and SCHMIDT W. R. (1962)
J. Biol. Chem 237, 389

HILL, R. L. and SCHMIDT, W. R. (1962)
J. Biol. Chem. 237, 941

HOEVE, C. A. J. and FLORY P. J. (1974)
Biopolymers 13, 677

HOSPELHORN, V. D. and FITZPATRICK, M. J. (1961).
Biochemical Biophysical Research Communications
6, 191

JAMES, L. (1971) J. Chromatography 59, 178

KARRER, H. E. (1960). J. of Ultra Structure Res. 4, 420.

KAWASE, O. (1959) Bull. Res. Inst. Diabetic Med. Kumamoto
University 9, (Suppl.) 8.

KEECH, M. K. (1960a). J. Biophys Biochem Cyt; 7, 533

KEECH, M. K. (1960b). J. Biophys. Biochem Cyt 7, 539

KHYM, J. X. Analytical Ion Exchange Procedures in Chem.
and Biol. Theory Equipment Techniques
Prentice-Hall, Inc. Englewood Cliffs. N.J.

KIMMEL, J. R. Markowitz H. and Brown, D. M. (1959)
J. Biol. Chem. 234, 46

LANSING, A. I.; ROSENTHAL, T. B.; ALEX, M. and DEMPSEY, E.W. (1952). Anat. Rec. 114 555

LEDVINA M. and BARTOS, F. (1967). J. of Chromatography 31, 56

LENT, R. W.; SMITH, B.; SALCEDO, L. L.; FARIS, B. and FRANZBLAU, C. (1969). Biochem. 8, 2837 - 2845.

LINDEN, I. H.; LADEN, E.; ERICKSON, J. O. and ARMEN D. (1955) J. Invest. Derm 24, 83

LINDERSTROM-LANG, K. (1952) - Personal Comm. Re: Tristram and Smith (1963).

MACPHERSON, H. T. (1946). Biochem. J. 40, 470 - 480

MAHOWALD, T. A.; NOLTMANN, E. R. and KUBY, S. A. (1962) J. Biol. Chem., 237, 1138.

MECHAM, R. P. and FOSTER, J. A. (1978). Biochem. J. 173, 617 - 625

MILLER, J. E. and FULLMER H. M. (1966). J. Exp. Med. 123, 1097

MILLER, E. J.; PINNELL S. R.; MARTIN, G. R. and SCHIFF-MANN E. (1967) Biochem. Biophys. Res. Commun. 26, 132 - 137

MOCZAR, E.; ROBERT, B. and ROBERT, L. (1972). Anal. Bioch. 45, 422.

MOORE S. and STEIN W. H. (1949). J. Biol. Chem. 178, 53

MOORE S. and STEIN W. H. (1949). J. Biol. Chem. 178, 79

MOORE, S. and STEIN, W.H. (1951).

J. Biol. Chem. 192, 663

MOORE, S. and STEIN, W.H. (1954a).

J. Biol. Chem. 211, 893

MOORE, S. and STEIN, W.H. (1954b).

J. Biol. Chem 211, 907

MOORE, S. SPACKMAN, D.H. and STEIN, W.H. (1958)

Anal. Chem. 30, 1185

MOORE S. and STEIN W.H. (1960).

Methods in Enzymology, Vol. VI.

Acedemic Press N.Y. 819.

OLUOHA, U.C. (1980) M.Sc. Thesis University of St. Andrews.

PARTRIDGE, S.M. and DAVIS, H.F. (1955)

Biochemical J. 61, 21

PARTRIDGE, S.M.; DAVIS, H.F. and ALDAIR, G.S. (1955)

Biochemical J. 61, 11

PARTRIDGE, S.M., ELSDEN, D.F. and THOMAS, J. (1963)

Nature London 197, 1297

PARTRIDGE, S.M.; ELSDEN, D.F.; THOMAS, J.; DORMAN, A.;

TELSER, A. and HO, P-L. (1964)

Biochem. J. 93, 30C

PARTRIDGE, S.M.; ELSDEN, D.F. and THOMAS, J. (1964)

Biochem. J. 93, 30C.

PAZ, M.A.; HENSON, E.; BLUMENFIELD, O.O.; SEIFTER, S. and

GALLOP, P.M. (1977).

Biochem. Biophys. Res. Communication 44, 1518 - 1523

PAZ, M. A.; PEREYRA, B.; GALLOP, P. and SEIFTER, S. (1974)
J. of Mechanochem Cell Motility 2, 231

PAZ, M. A.; GALLOP, P. M.; BLUMENFIELD, O. O.; HENSON, E. and SEIFTER, S. (1971).
Biochem. Biophys. Res. Communication 43, 289

PEASE, D. C. and MOLINARI, S. (1960).
J. Ultrastruct. Res. 3, 447

RAMACHANDRAN, G. N. and SANTHANAN, M. D. (1957).
Proc. Indian Acad. Sci. 45A, 124

RASMUSSEN, B. L.; BRUENGER, E. and SANDBERG, L. B. (1975)
Anal. Biochem. 64, 255 - 259

RHODIN, J. and DALHANN, T. (1955)
Exp. Cell. Res. 9, 371

ROSS, R. and BORNSTEIN P. (1969).
J. of Cell Biology 40, 366

ROSS, R. and BORNSTEIN P. (1970) Chemistry and Molecular
Biology of Intercellular Matrix 1, 641,
(Ed.) Balazs E. A. Academic Press London.

SANDBERG, L. B.; WEISSMAN, N. and GRAY, W. R. (1971)
Biochem. 10, 52

SANDBERG, L. B.; GRAY, W. R. and BRUENGER, E. (1972).
Biochemica et Biophysica Acta 285, 453

SANDBERG, L. B.; WEISSMAN, N. and SMITH, D. W. (1969).
Biochem. 8, 2940.

SANDBERG, L. B. (1976). Inter. Review of Connect. Tissue
Res. 7, 159

SCHERAGA, H. A. (1963). In The Proteins; Ed. H. Neurath
Vol. 1, 527 (New York: Academic Press).

SCHUBERT, M. and HAMMER, D. A Primer on Connective Tissue
Biochem. Printed in U.S. by Lea and
Febiger of Philadephia.

SERAFINI-FRACASSINI, A. and TRISTRAM, G. R. (1965).
Proceedings of the Royal Society of
Edinburgh, Section B (Biology).
Vol. LXIX Part IV (No. 8) 334 - 342.

SERAFINI-FRACASSINI, A.; FIELD, J.M.; RODGER, G.W. and SPINA, M.
(1975) Biochemica et Biophysica Acta 386, 80

SMITH, D. W.; BROWN, D. and CARNES W. H. (1972).
J. Biol. Chem. 247, 2427

SMITH, E. L. and STOCKELL, A. (1954).
J. Biol. Chem. 207, 501

SPACKMAN, D. H.; STEIN W. H. and MOORE, S. (1958).
Anal. Chem. 30, 1190

STARCHER, B. C.; PARTRIDGE S. M. and ELSDEN D. F. (1967)
Biochem. 6, 2425.

STARCHER, B. C. and GALIONE M. J. (1975).
Preparative Biochemistry 1976. Vol. 5,
Part (5 - 6) 455 - 460.

THEORELL, H. and AKESSON, A. (1942). Biochem. J. 40, 471.

THOMAS, J.: ELSDEN, D. F. and PARTRIDGE, S. M. (1963).
Nature, London 200, 651.

THORNHILL, D. P. (1972). Anal. Biochem. 46, 119.

TRISTRAM, G. R. (1939). Biochem. J. 33 (2), 1274.

TRISTRAM, G. R. (1949). Adv. in Pro. Chem. 5, 84.

TRISTRAM, G. R. and SMITH, R. H. (1963). Advances in Protein Chem. 18, 227.

VOLPIN, D.; URRY, D. W.; COX, B. A. and GOTTE, L. (1976) Biochemica et Biophysica Acta 439, 253 - 258.

WEISSMAN N.; SHIELDS, G. S. and CARNESS, W. H. (1963). J. of Biological Chemistry 238, 3115.