STUDIES

on

THE CHEMICAL STRUCTURE of CYTROCHROME c

by

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C O N T E N T S.

GENERAL INTRODUCTION.

The proteins are the chemical foundations of all living matter, without which maintenance of life would not be possible.

Protein substances perform many varied functions in the body. Some form structural units such as hair, nails, muscle fibres, tendons and skin tissue. Others undertake specific physiological roles, examples of which are the enzymes, and a number of the hormones.

The antibodies, which help to protect the organism against infection by foreign substances, are also proteins, as are the viruses, a unique group of apparently non-living matter, which, nevertheless, are able to reproduce in a suitable living host.

The diverse actions which the proteins display must be due to differences in their structures. All, on hydrolysis, give rise to the same simple products, the amino acids, which must be linked in different proportions and sequences, in the intact protein molecule, to give each its characteristic function.

Furthermore, proteins with the same function in different species are known, in some instances, to differ from each other in chemical and physical properties. This means that there are probably small differences between the structures of these proteins.

The elucidation of the fine structure of the proteins is of fundamental importance to-day, because the behaviour of different proteins cannot be properly understood until the chemical structures which govern their properties are known.

In the present work a study has been undertaken of some aspects of the structure of the haem-protein cytochrome c, isolated from horse heart. This protein belongs to a class of substances which have the same iron-porphyrin, haem, as their prosthetic group. This class includes haemoglobin, the oxygen carrying pigment in the blood, catalase, an enzyme which catalyses the decomposition of hydrogen peroxide, and peroxidase, another enzyme which utilises hydrogen peroxide for the oxidation of a number of substances. Cytochrome c, on the other hand, is responsible for the transfer of electrons during the oxidation of substrates in the cell. As these substances have the same prosthetic group, the differences in their biological activities must be a reflexion of differences in the structures of the protein parts of their molecules. It therefore seemed of importance to learn something of the structure of cytochrome c in this connection.

Cytochrome c is associated with a number of similar compounds, the cytochromes, which were first described by MacMunn in 1886. He found, in various organs and tissues of all types of animals, a class of pigments having four characteristic/

characteristic absorption bands in the reduced state. The bands disappeared on oxidation, and MacMunn rightly concluded that these compounds were concerned in respiration in tissues. This introduced a new concept, as previously it had been believed that respiration took place in the blood. He named these substances myo- and histohaematins and demonstrated that they were haemochromogen in nature. Although he made an oxalic acid extract of washed skeletal muscle, in which he observed absorption bands at 554.5 - 548.5 mμ, he was unable to proceed further with the isolation of the substances responsible for this. He believed, unfortunately, that these compounds replaced haemoglobin in muscle, in which they carried on a similar role to the latter in blood. This involved him in serious argument with Hoppe-Seyler, who maintained that myo- and histohaematins were merely modified forms of haemoglobin. Although MacMunn tried to defend his views, he was no match for Hoppe-Seyler, and his work was disregarded.

Many years later, in 1925, Keilin repeated and confirmed MacMunn's experiments. He shewed that these compounds are even more widely distributed than MacMunn realised, being present in plants and unicellular organisms, as well as in animal tissues. Because of their wide distribution Keilin called them by the general name of cytochromes.

Keilen found that the four-banded spectrum of these compounds varied little from tissue to tissue, and careful

microspectroscopic/

microspectroscopic studies of baker's yeast showed that the bands had maxima as follows:- a 604 mμ; b 566 mμ; c 550 mμ; and d, which appeared to display three maxima at 532, 528 and 521 mμ. This type of spectrum was not immediately identifiable with that of a reduced haemochromogen, but Keilin (1925) proposed that the cytochrome spectrum was due to the combined spectra of three distinct substances, each consisting of the absorption bands as shown below.

Cytochrome	α (mμ)	β (mμ)
a	604	532
b	566	528
c	550	521

This gives the effect of four main absorption bands as the d band of cytochrome is made up of the three bands of cytochromes a, b and c. The reduced spectrum of cytochrome c has been confirmed, but there is some doubt as to the true spectra of cytochromes a and b.

Apart from the cytochromes mentioned above, a number of others have been detected, but, on account of the difficulties involved in their isolation, are so far only distinguished by differences in their absorption spectra.

The Isolation of Cytochrome C.

Cytochrome c, unlike the other cytochromes, has been extracted from the cell, and obtained in a soluble state. This is facilitated by its remarkable stability to heat

and/

and strong acids.

The substance was first prepared by Keilen in 1930, who extracted it from lysed yeast cells with sodium bisulphite and dithionite, and precipitated it with sulphur dioxide in the presence of calcium chloride. The preparation was extensively contaminated with cellular proteins, but some purification was effected by precipitating a portion at least of these with sulphur dioxide, while the cytochrome c was kept in solution in the reduced state. Although this preparation was successfully used by Hill and Keilin (1930) for their experiments, it could not be regarded as a method suitable for isolating the protein.

Zeile and Reuter (1933) attempted to purify cytochrome c by adsorbing it on a column of kaolin in the oxidised state. On reduction the cytochrome c was eluted from the column, while the impurities remained adsorbed on the kaolin. The method would not appear to have been very satisfactory, because the molecular weight of the material collected was 18,000, which is much higher than the figure accepted to-day.

Theorell (1935, 1936) described an improved method for the preparation of cytochrome c from ox and horse hearts. In principle the method was to extract dried, defatted minced heart with 0.1N - H_2SO_4, after which the impubities/

impurities were precipitated by adjusting the pH to neutrality. Cytochrome c, with an iron content of 0.17%, was obtained by fractionating the concentrated supernatant fluid with ammonium sulphate. This rather crude preparation was further purified by adsorption on barium sulphate, followed by elution with 0.1N - HCl, or by adsorption on cellophane and eluting with dilute ammonia. The final material contained 0.34% iron, with a calculated molecular weight of 16,000, which Theorell claimed represented pure cytochrome c.

This method was the basis for subsequent preparations, and was considerably improved by Keilin and Hartree (1937), who extracted the minced heart directly with dilute trichloroacetic acid. This eliminated the labour of defatting and drying the muscle and also precipitated contaminating proteins more efficiently.

In 1939 Theorell and Åkeson discovered that the iron content of cytochrome c preparations could be increased to 0.43% by prolonged electrophoresis, at the isoelectric point of the protein, in the apparatus designed by Tiselius (1938). By this means cytochrome c migrated towards the cathode which separated it from impurities, while these tended to move towards the anode. The purified material had a molecular weight of 13,000 calculated on sulphur analysis.

Preparation of pure cytochrome c could be performed on

a large scale by this method, but it is not suitable for most laboratories on account of the expensive equipment required. However, a simpler method for preparing cytochrome c, with an iron content of 0.43%, was devised by Keilin and Hartree (1945), who modified their previous technique. A solution of cytochrome c containing 0.34% iron, and saturated with ammonium sulphate, was adjusted to pH 10 with 0.88 s.g. ammonia, and allowed to stand overnight. A precipitate settled out leaving a solution of cytochrome c, with the higher iron content, which was found to be electrophoretically pure. This modification was found, however, to be unsatisfactory when employed in this laboratory (Blain, 1950).

Tint and Reiss (1950 a, b) examined, by electrophoresis, samples of cytochrome c from the hearts of different species prepared according to the method of Keilin and Hartree (1937, 1945). They found, in the case of cytochrome c from horse heart, that pure material could be obtained without resort to electrophoresis or the modification introduced by Keilin and Hartree (1945). They also found that the iron content of both horse and beef heart cytochrome c was higher than 0.43%, and that the molecular weights of beef, horse, chicken and pig cytochrome c were 12,350, 12,270, 13,270, and 13,000 respectively. These are close to the values found by Theorell and Åkeson (1941 a) for horse heart cytochrome c, but nevertheless show significant variations

between some of the species.

The Structure of Cytochrome c.

Cytochrome c appears to have a remarkably stable structure, because it is not denatured by heat and is stable to dilute acids and alkalis. Apart from its biological function, it is relatively unreactive, and does not combine with substances which react, under normal conditions, with cytochrome oxidase and haemoglobin.

Within the pH range of 4-11 it is not autoxidisable and does not react with carbon monoxide, hydrogen sulphide or hydroxylamine. Below pH 4 and above pH 11 reduced cytochrome c is modified to form an autoxidisable compound which does react with carbon monoxide (Keilin and Hartree, 1939; Theorell and Åkeson, 1941b; Tsou, 1951a). Native cytochrome c, however, does react with cyanide (Potter, 1941; Horecker and Kornberg, 1946) and with azide (Horecker and Stannard, 1948).

It is, therefore, probable that the structure of cytochrome c differs markedly from that of haemoglobin, for example, which has the same prosthetic group (Zeile and Reuter, 1933). The differences are likely to be evident both in the structures of their proteins and in the mode of linkage of the proteins and their prosthetic groups.

Previous work has been mainly centred on the nature

of the haem-protein linkage in cytochrome c. Hill and Keilin (1930) isolated the porphyrin from cytochrome c by the action of sulphur dioxide, because it could not be done by the more general method with acid acetone, which was known to split haem from haemoglobin. This indicated that a more stable linkage between the protein and the prosthetic group occurs in cytochrome c than in haemoglobin.

Theorell (1937) found that porphyrin c, prepared after the hydrolysis of cytochrome c with HCl, contained 1-2 atoms of sulphur per molecule. L-cystine could be split from this porphyrin by the action of hydrogen bromide in glacial acetic acid. Theorell (1938) concluded that the prosthetic group was linked, through its vinyl side chains, to two cysteine residues in the protein, by means of thioether linkages.

Support has been lent to this view by the work of Paul (1950), who split the porphyrin from the protein by the action of silver salts, which are known to cleave thioether linkages (Peters and Wakelin, 1947).

Complementary evidence has also been provided by Davenport (1952), who identified mesoporphyrin after reductive cleavage of cytochrome c with sodium amalgam. On the other hand, protohaematin, horse liver catalase and ox haemoglobin, under the same conditions, gave rise to the unsaturated protoporphyrin. This indicates that unmodified vinyl groups are not reduced by sodium amalgam. If it is

assumed that sodium amalgam reduces vinyl side chains involved in the linkage of the prosthetic group to the protein, with simultaneous removal of iron, in cytochrome c, then these findings support Theorell's conclusions.

The prosthetic group is also linked to the protein by bonds between its iron atom and nitrogenous groups in the protein moiety, as evidenced by the characteristic haemochromogen type of spectrum of the whole molecule. Theorell and Åkeson (1941b,c,d), by titration, spectrophotometric and magnetometric measurements, showed that the iron atom was probably attached by bonds to the nitrogen atoms of two histidine residues in the protein molecule. They further concluded, from this work, that in cytochrome c both imidazole groups are in a favourable position for co-ordination with the iron atom. In haemoglobin, according to Conant (1932), there is supposed to exist either no binding, or only a weak binding, of one of the two imidazole groups, on account of the unfavourable position for electrostatic co-ordination of this imidazole group.

Paul (1951) attempted to confirm these conclusions by treating cytochrome c with fluorodinitrobenzene (Sanger, 1945), and estimating the extent of labelling in the imidazole groups of histidine, after hydrolysis. His results were not absolutely conclusive, but he concluded that at least one histidine molecule was linked, through its imidazole group, to iron.

If these conclusions are correct, it can be understood why the protein-prosthetic group complex in cytochrome c is much more stable than in haemoglobin. The two types of linkages between the protein and the prosthetic group in cytochrome c will confer remarkable stability to the whole molecule. This is reflected in its ability to withstand heat, acids and alkalis, and its non-reactivity with substances which combine with some of the other haem proteins.

Little work has been carried out so far on the nature of the protein moiety of cytochrome c. Keilin and Hartree (1937) estimated the amounts of the basic amino acids and cystine in an acid hydrolysate of cytochrome c containing 0.34% iron. These amino acids were separated from the monoamino acids by precipitation with phosphotungstic acid. Determinations were carried out using specific colour reactions for cystine, histidine and arginine, while lysine was estimated as the difference between the values for the above amino acids and the value for the total base fraction. The amount of tryptophan was determined on a separate sample, which was digested with trypsin. These methods were not very accurate, and therefore little reliance can be placed on the results.

Theorell and Åkeson (1941 a) carried out a more detailed analysis of the protein of cytochrome c, from which the prosthetic group was removed after hydrolysis. They still used similar methods to the previous workers, but were able, in addition, to isolate samples of glutamic and aspartic

acids and leucine. The yields of these amino acids were low, as would be expected in such a complex procedure.

The introduction of modern methods for amino acid analysis has led to great advances being made in this type of work. We have therefore applied the techniques of chromatography to a quantitative study of the amino acids in purified horse heart cytochrome c. In addition, an attempt has been made to elucidate some of the sequences in which the amino acids occur in the intact protein. The results of these studies are recorded in the following pages.

S E C T I O N I.

THE USE OF ION EXCHANGE CHROMATOGRAPHY

FOR PURIFYING CYTOCHROME c.

INTRODUCTION.

Paleus and Neilands (1950) were among the first workers to employ an ion exchange resin for the purification of a protein. They obtained promising results when they used the polycarboxylic cation exchange resin, Amberlite IRC - 50 for purifying cytochrome c. Advantage was taken of its high isoelectric point, 10.05, which allowed the adsorption of concentrates of the protein on the resin at pH 9. A golden-coloured protein impurity, with a low iron content, which was not adsorbed, was eluted from the resin by washing with pH 9 0.1M ammonium acetate/ammonium hydroxide buffer. The cytochrome c could then be eluted by raising the pH to 10.8 with ammonium hydroxide.

Later these authors prepared highly purified cytochrome c from ox heart by continued elution of the column, after removal of the impurities, with the pH 9 buffer. This resulted in the fractionation of the cytochrome c into three bands, the first reduced and the others oxidised. The second band appeared to be the purest as it had an iron content of 0.466%. The physical constants of this fraction gave very similar results to those calculated for a theoretically pure sample by Tint and Reiss (1950).

This method proved satisfactory for the preparation of relatively pure cytochrome c, but as the material was collected in dilute solution it had to be concentrated by precipitation with trichloroacetic acid. This procedure modifies

the cytochrome c into an enzymically inactive product spectroscopically identical with native cytochrome c (Margoliash, 1952).

Pirrie, working in this laboratory, found that the cytochrome c could be eluted from the column, in small volume, by raising the concentration of the pH 9 ammonium acetate/ammonium hydroxyde buffer from 0.1M to 0.5M, instead of increasing the pH.

Neilands (1952) also improved the technique by applying the charge to the column, at a lower pH, in pH 7 0.1M sodium phosphate buffer. The impurities were washed off in this buffer, and the cytochrome c eluted, in one band, with saturated ammonium acetate .

Following on the work of Pirrie and Neilands, Leaf (1953) applied the cytochrome c to the column, in 0.1M sodium phosphate buffer at pH 7. After removal of impurities the cytochrome c was eluted in two fractions, one reduced and the other oxidised, by increasing the molarity of the buffer to 0.25M.

In the present experiments on the purification of cytochrome c the last mentioned procedure has been mainly used.

EXPERIMENTAL.

Preparation of cytochrome c.

Cytochrome c was prepared from minced horse heart muscle according to the method described by Keilin and

Hartree (1945). In order to reduce denaturation of the protein as far as possible, solutions were added to the muscle extract through a capillary tube, the rate of flow being controlled by a screw clip. The mixture was always mechanically stirred to prevent frothing, which causes denaturation.

The ammonium sulphate-trichloroacetic acid precipitation was generally repeated before examining the preparation.

Dialysis was carried out in running tap water for 48 hours, followed by three changes of 0.3% ammonium hydroxide or distilled water, which was sufficient to remove all traces of ammonium sulphate.

It was found, in our experience, that cytochrome c did not keep well in solution in chloroform. After about two weeks a small amount of a white precipitate was observed in many instances. Denaturation, as indicated by testing the solution with carbon monoxide (Tsou, 1951) was shown to have taken place. Therefore, after the final dialysis, the preparations were lyophylised, and stored, at room temperature, in a desiccator over calcium chloride.

The yield from 6 kg. of minced muscle was usually 6-700 mg. dried cytochrome c.

Preparation of Amberlite IRC - 50.

The resin was finely powdered in a coffee grinder and passed through a 100 mesh sieve. It was washed several

times in water, by decantation, to remove the small particles of resin, which tended to slow the rate of flow and resulted in clogging of the column. The resin was then washed with 5% NaOH followed by 5% HCl, the process being repeated twice, before the pH was raised to 7, measured with a glass electrode, by titration with 2N - NaOH. About 20 g. of this resin was poured in a slurry with pH 7 0.1M sodium phosphate buffer into a glass tube (20 cm. x 3 cm.) fitted with a sintered glass disc at the bottom. Washing of the resin was continued with phosphate buffer until the influent and effluent had the same optical density at 280 mμ, and the same pH, after which it was ready for use. After each experiment the resin was washed twice alternately with 100 ml. of 5% NaOH and 100 ml. of 5% HCl before being adjusted to pH 7 again.

Measurement of Denaturation of Cytochrome c.

There was the possibility that denaturation of cytochrome c was taking place while it was in contact with the resin, and it was decided to study this in two ways.

In the first the biological activity was tested according to the method of Potter and Dubois (1942). To the sample of cytochrome c, in 2.5 ml. of water, was added 0.3 ml. of 0.25 M pH 7.4 sodium phosphate buffer and 0.2 ml. of rat kidney enzyme preparation containing cytochrome oxidase and succinic dehydrogenase. With no added substrates the cytochrome oxidase oxidised the cytochrome c, and the

absorption at 550 mμ was measured in the Unicam SP 500 spectrophotometer. 0.01 ml. of 0.5 M sodium succinate was then added to the system along with 0.03 ml. of 0.1 M neutralised cyanide which stopped the action of the cytochrome oxidase. The cytochrome c was rapidly reduced, and the absorption at 550 mμ again measured. From these results the concentration of biologically active cytochrome c was calculated.

In the second method advantage was taken of the fact that denatured cytochrome c forms a complex with carbon monoxide (Tsou, 1951a) which reduces the intensity of the absorption of reduced cytochrome c at 550 mμ. The absorption at this wave length was measured before and after carbon monoxide was bubbled through a solution of reduced cytochrome c.

Estimation of Iron.

The iron content of the cytochrome c preparations was measured according to the method of Drabkin (1941).

Spectrophotometry.

The absorption of the cytochrome c, in suitable concentration in pH 7 0.1 M sodium phosphate buffer, was measured at 280 mμ in the oxidised state, while absorption at 535 mμ and 550 mμ was estimated after reduction with 1-2 mg. sodium dithionite.

Estimation of Nitrogen.

Nitrogen was estimated by the micro-Kjeldahl method

as modified by Ma and Zuasaga (1942). Samples were digested with 1.5 ml. of concentrated nitrogen-free H_2SO_4, with the addition of a bead of mercury as catalyst. Digestion was generally continued for a period of four hours after clearing (cf. Hiller, Plazin and Van Slyke, 1948), after which the flask was allowed to cool and the sides washed down with 2 ml. of distilled water. Distillation was carried out in the apparatus described by Markham (1942). Before the addition of 10 ml. of 40% sodium hydroxide to liberate the ammonia, 1 ml. of saturated sodium thiosulphate was added to precipitate the mercury which would otherwise interfere with the subsequent distillation. The ammonia was received into a conical flask containing 6 ml. of 2% boric acid and four drops of the mixed indicator (5 parts of 0.1% bromo cresol green and 1 part of 0.1% methyl red in 95% ethanol). Collection was started only when the distillate had reached the bottom of the condenser, which eliminated the possibility of any sulphur dioxide, coming from the thiosulphate, collecting in the receiver. The trapped ammonia was titrated against 0.01N sulphuric acid.

RESULTS.

In Table 1 are shown the results obtained on three preparations of cytochrome c examined after two precipitations, but not separated on Amberlite IRC - 50. The preparation, R.P., was examined by Pirrie after a single Precipitation.

TABLE 1.

Analytical values for Cytochrome c not purified on Amberlite IRC-50.

Prepn.	% Fe.	$\varepsilon_{280\ m\mu}$.	$\varepsilon_{535\ m\mu}$.	$\varepsilon_{550\ m\mu}$.	$\frac{\varepsilon_{550\ m\mu}}{\varepsilon_{280\ m\mu}}$.	$\frac{\varepsilon_{550\ m\mu}}{\varepsilon_{535\ m\mu}}$.	$\frac{\varepsilon_{550}}{N}$.	$\frac{N}{Fe}$.
1	-	23,967	8,158	25,810	1·077	3·16	12·44	37·0
2	-	24,084	7,935	26,776	1·11	3·38	11·52	41·5
3	-	23,558	7,787	28,058	1·19	3·60	13·15	37·5
R.P.	0·34	27,100	7,364	27,100	1·00	3·68	9·9	49·02
"Theoretical"	0·45	-	-	-	-	3·83	15·15	33·45

The molecular extinction coefficients, at the three wave-lengths, were calculated from the iron analysis. Thus

$$\varepsilon_{mol.} = \frac{E.}{\text{Fe content (g/l.)}} \times 56$$

The concentration of cytochrome c was based on the nitrogen content of each sample instead of on its dry weight, as it is more accurate. Consequently the percentage of iron and nitrogen in the cytochrome c preparations were not calculated.

In the last line of Table I are shown the ratios determined on pure samples of horse heart cytochrome c by other workers. The ratio of ε 550 mμ/ε 280 mμ is taken from the results of Tint and Reiss (1950b). The ratio ε 550 mμ/N was calculated assuming that $\varepsilon_{mol.}$ at 550 mμ equals 28,000 and that the molecular weight is 12,270 (Tint and Reiss, 1950b). The nitrogen content is taken as 15.08%, using the value determined by Theorell and Åkeson (1941a). The N/Fe ratio was calculated using the same figure for nitrogen and a value of 0.45% for the iron content (Tint and Reiss, 1950b).

Comparison of the results obtained on the crude preparations of cytochrome c, and the values calculated for a pure specimen show, as would be expected, considerable differences. The largest variations are found in the ε550 mμ/N and the N/Fe ratios for Pirrie's preparation,

which had only been precipitated once.

The results indicate that the preparations before purification do not all have the same composition. This is not in accordance with the findings of Keilin and Hartree (1945), who believed that their method provided constant production of cytochrome c containing 0.34% iron.

Pirrie found that after a single precipitation the iron content was usually 0.34%, but in the other preparations, which had been precipitated twice, the iron content was higher.

The Behaviour of Cytochrome c on Amberlite IRC - 50.

In early experiments the Amberlite IRC - 50 was used at pH 9 in which the cytochrome c was placed on the column in pH 9 0.1 M ammonium acetate buffer. The cytochrome c remained adsorbed at the top, while a golden-coloured fraction separated from the main band and was eluted with the 0.1 M buffer. On increasing the molarity of the buffer to 0.5 M the cytochrome c started to move down the column and was collected in a small volume in the eluate. There was very little reduced cytochrome c present, or at least it did not separate from the main cytochrome c band.

In the main, separation was carried out at pH 7. In a typical experiment about 200 mg. of cytochrome c was dissolved in 2 ml. of pH 7 0.1 M sodium phosphate buffer and placed on the column, which was then washed

with this buffer. A light brown band soon started to separate from the front of the red-coloured material and was eluted from the column. By this time a small amount of pink material had also moved from the main red band and was found, after elution, to display the absorption bands of reduced cytochrome c, when examined in the Hartridge reversion spectroscope. After the column had been washed with a further 20 ml. of 0.1 M buffer, the molarity was raised to 0.25 M which eluted the main cytochrome c fraction in another 20 ml. of buffer. This material was found to consist of oxidised cytochrome c. In the initial experiments the molarity of the buffer was increased from 0.1 M to 0.25 M in 0.05 M stages, but the cytochrome c could not be eluted in a buffer concentration less than 0.25 M.

A number of other narrow bands of red-coloured material remained on the column, which were presumably small amounts of cytochrome c modified during preparation. No attempt was made to examine these further.

The main oxidised cytochrome c fractions were pooled and dialysed in running tap water overnight and then in distilled water until the dialysate was free from phosphate as indicated by the method of Allen (1940). Samples were then taken for iron, nitrogen and spectrophotometric analysis.

The results for one preparation (4) are shown in Table 2 before and after passage through an Amberlite IRC - 50

TABLE 2.

Analytical values for Cytochrome c before and after purification on Amberlite IRC-50.

Prepn.	% Fe.	$\varepsilon_{280\ m\mu}$.	$\varepsilon_{535\ m\mu}$.	$\varepsilon_{550\ m\mu}$.	$\frac{\varepsilon_{550\ m\mu}}{\varepsilon_{280\ m\mu}}$.	$\frac{\varepsilon_{550\ m\mu}}{\varepsilon_{535\ m\mu}}$.	$\frac{\varepsilon_{550}}{N}$	$\frac{N}{Fe}$.
4 (before)	-	24,637	7,906	27,200	1·104	3·44	13·1	37·0.
4 (after)	-	24,230	7,600	27,125	1·12	3·59	15·81	30·6.
R.P.(before)	0·34	27,100	7,364	27,100	1·00	3·68	9·9	49·02.
R.P.(after)	0·46	23,000	7,500	27,600	1·20	3·68	14·26	34·72.
"Theoretical"	0·45	-	-	-	-	3·83	15·15	33·42.

TABLE 3.

Extinction ratios of Cytochrome c, prepared from horse heart, before and after passage through an Amberlite IRC-50 column.

BEFORE		AFTER	
$\varepsilon_{550\ m\mu}/\varepsilon_{535\ m\mu}$	$\varepsilon_{550\ m\mu}/\varepsilon_{280\ m\mu}$	$\varepsilon_{550\ m\mu}/\varepsilon_{535\ m\mu}$	$\varepsilon_{550\ m\mu}/\varepsilon_{280\ m\mu}$
3.48	1.05	3.67	1.21
3.21	1.11	3.78	1.16
3.44	1.09	3.59	1.19
---	---	3.69	1.22
3.44	1.06	3.81	1.29

column. Figures are also given for Pirrie's once precipitated preparation before and after purification. In our experiment there is an increase in the absorption ratios as well as reduction in nitrogen, as instanced by the increase in the ε 550 mμ/N ratio. The N/Fe ratio has considerably decreased, which indicates that nitrogenous material containing little or no iron has been removed from the cytochrome c. Pirrie's results show the same features, with a very striking increase in the iron content, to a value close to that for pure cytochrome c.

Spectrophotometric analysis of the fast running brown material showed that it had a very high absorption at 280 mμ, characteristic of protein, but low absorption, after reduction, at 550 mμ.

The extinction ratios of a number of other preparations of cytochrome c, measured before and after passage through the Amberlite IRC - 50 column, are recorded in Table 3. It can be seen that in most cases the ε 550mμ/ ε 535 mμ ratio has increased, after purification, to a value close to that measured by Tint and Reiss (1950b) for electrophoretically pure horse heart cytochrome c. The ε 550 mμ/ ε 280 mμ ratio in all cases is near the values of 1.17 (Paleus and Neilands, 1950) and 1.19 (Margoliash, 1954b) obtained for pure beef heart cytochrome c and horse heart cytochrome c respectively.

When testing whether denaturation of the cytochrome c on the column had occurred it was found that the results obtained by the two methods described paralleled each other very closely. It was therefore decided to examine the preparations for denaturation by the carbon monoxide method only, as this procedure was simpler to carry out. In most cases there was no indication of denaturation on the column, although in one preparation denaturation had occurred to the extent of 6%. This, however, was not a typical finding.

DISCUSSION.

Our experiments show that cytochrome c prepared according to the method of Keilin and Hartree (1945) is contaminated with a non-cytochrome protein, which has an isoelectric point lower than cytochrome c itself. This is in agreement with the results obtained by Paleus and Neilands (1950) on beef heart cytochrome c. We have not examined the nature of this impurity further, but Margoliash (1954a) is of the opinion that it consists of globin from myoglobin, in horse heart preparations. In one experiment, in this laboratory, the pigment was examined spectroscopically and was found to display a weak metmyoglobin spectrum which accords with this view.

Paleus and Neilands (1950) obtained three cytochrome c fractions, after prolonged elution with pH 9 buffer, the first of which was reduced, while the other two were oxidised. In the present work only small amounts of the reduced com-

pound were obtained. The main reduced band probably represents the faster reduced band described by Paleus and Neilands, while the narrow bands, usually three in number, may correspond to the second reduced band obtained by these authors. Neilands (1952), on the other hand, eluted all the fractions in one band by means of saturated ammonium acetate.

In our experience more effective separation is obtained by eluting at a constant pH, and increasing the molarity of the buffer as required. This method has also been adopted by Boardman and Partridge (1953) and Margoliash (1954a,b). The latter author used an initial buffer concentration of 0.25 M at pH 7, while we have found that 0.1 M buffer was sufficient to separate the non-cytochrome c fraction from the main band. With buffer concentrations greater than 0.1 M, in our experiments, all the material passed rapidly through the column with less efficient separation. However the main cytochrome c band obtained by Margoliash (1954b) from the column at pH 7 appeared to consist of a mixture of both oxidised and reduced cytochrome c, which he separated from each other on a second column at pH 9.6. We obtained the same separation using a single column at pH 7.

Margoliash (1954b) observed that reduction of cytochrome c, during chromatography, only takes place when the protein is adsorbed on and eluted from the resin, and not in solution. This is not a unique property of Amber-

lite IRC - 50, but reduction was also shown to occur on other adsorbents. The rate of reduction of cytochrome c increases as the pH of the column is increased, until pH 11, when it rapidly becomes autoxidisable. Thus Margoliash found that rechromatographing , at pH 7, a sample of cytochrome c, previously run through a column at pH 9.6, resulted in the increased formation of reduced cytochrome c. At a relatively low pH, as used in our work, only small amounts of the reduced compound would be expected to be formed.

We have also found, as has Margoliash, a third fraction of three narrow bands which were only slowly eluted with 0.25 M ammonia. We have not examined this fraction, but Margoliash eluted it in 0.5 M buffer, and examined it in certain enzyme systems. In the succinic-oxidase and cytochrome-oxidase systems it displayed very low activity, but ascorbic acid was rapidly oxidised by this fraction in the same enzyme system without the addition of the cytochrome-oxidase preparation. Cytochrome oxidase was not required because this cytochrome c fraction is autoxidisable. This ability to oxidise ascorbic acid rapidly appears to be a property of cytochrome c modified by trichloroacetic acid. This fraction is entirely indistinguishable from normal cytochrome c, when examined spectroscopically, but can be easily separated from the latter on Amberlite IRC - 50. The fact that we observed

observed several narrow bands, in this fraction, may indicate that different degrees of modification have taken place.

The molecular extinction coefficient at 550 mμ for purified horse heart cytochrome c , as shown in Table 2, was found to be 27,125. This is a little lower than the figure obtained by other workers. Pirrie found a value of 27,600, while other estimations were 27,700 (Margoliash, 1954b), and 27,840 ± 600 (Tint and Reiss, 1950b) for an electrophoretically pure preparation. The last result shows that the determination is liable to considerable variation, but at its lower limit agrees well with our figure.

The ε mol, at 550 mμ for beef heart cytochrome c was calculated to be 26,400 ± 560 by Tint and Reiss (1950b), which is lower than the value for horse heart cytochrome c. However the value calculated from the molar absorption (β) at 550 mμ, measured by Theorell and Åkeson (1941b), was found to be 28,300. The same data, determined by Paul (1951b), gave a figure of 27,600. From these results it would appear therefore that there is probably no significant difference between the values for beef and horse heart cytochrome c.

We have calculated that the ε mol. at 535 mμ is 7,600 for horse heart cytochrome c. This is in close agreement with the values of 7,500, found by Pirrie, and 7,203 ± 170

determined by Tint and Reiss (1950b). Margoliash (1954b), on the other hand, obtained a much higher figure of 9,200.

The value for beef heart cytochrome c is similar to that for horse heart cytochrome c. Tint and Reiss (1950b) estimated it to be 7,230 ± 170, and the figure of 7,630 was calculated from the data of Paul (1951b).

Comparison of Tables 1, 2 and 3 shows that the absorption ratios increase after purification, and that they approach very nearly the values found by other workers for pure cytochrome c. Tint and Reiss (1950b) found that the $\varepsilon_{550m\mu}/\varepsilon_{535m\mu}$ ratio for an electrophoretically pure preparation of horse heart cytochrome c was 3.83 ± 0.13, while Margoliash (1954b) calculated that the $\varepsilon_{550\ m\mu}/\varepsilon_{280\ m\mu}$ ratio was 1.19.

The corresponding values, 3.82 and 1.17, for beef heart cytochrome c (Paleus and Neilands, 1950) show that these values are identical for both beef and horse heart cytochrome c.

On purification the ratio of $\varepsilon_{550\ m\mu}/N$ increases as would be expected but the value of 15.81 for preparation 4 is higher than the figure calculated for pure cytochrome c. This may have been due to under-estimation of nitrogen in the sample. This ratio for impure preparations is low, but not excessively so, in the case of preparation 3 (Table 1). This indicates that cytochrome c can be prepared in a fairly pure state by means of the Keilin and Hartree

(1945) method. Tint and Reiss (1950a,b) were able to prepare electrophoretically pure cytochrome c from horse heart, using only this method.

The N/Fe ratio falls as the purification of the cytochrome c proceeds. The value for the purified preparation 4 (Table 2) is lower than that calculated for pure cytochrome c, but, as mentioned before, this may have been due to a low estimate for nitrogen in the sample. However, it does indicate that non-iron containing nitrogenous material has been removed. The value of 34.72, calculated by Pirrie, is near to the value for the pure material.

Comparison of the results obtained from our purified preparations of horse heart cytochrome c, and those obtained by other workers, are in reasonably good agreement, which indicates that the material we have finally obtained probably represents pure cytochrome c.

S E C T I O N II.

THE AMINO ACID COMPOSITION

OF CYTOCHROME c.

INTRODUCTION.

The first step in studying the chemical structure of a protein is to make a quantitative examination of its amino acid composition. Until recently this was a very dificult task to perform as the amino acids have similar chemical structures, and hence similar physical and chemical properties. These render their separation from each other before estimation by the classical methods of organic chemistry a lenghy, if not impossible, operation.

The history of the measurement of the amounts of amino acids in a protein is essentially the history of the development of techniques designed to make use of small differences between the physical and chemical properties of the amino acids, for their separation from each other.

The methods used by the early workers have now been discarded because of the technical difficulties involved and because only about ten amino acids could be measured accurately. However, they were useful in that they provided methods for separating the amino acids into groups according to their properties and could often be used for preparing samples of amino acids where quantitative recovery was not so important.

Some of these methods are worthy of mention, if only on account of the skill and ingenuity which were used to

devise them.

Emil Fischer (1901) introduced a method for the fractional distillation of the ethyl esters of the mono-amino acids under reduced pressure. The technique was very laborious, and was not quantitative, but considerable information was obtained concerning the overall features of the amino acid compositions of proteins, while a good method was provided for the isolation of pure amino acids.

The nitrogen distribution method first introduced by Van Slyke in 1910 was a big advance at that time. The amino acids, or groups of amino acids, could be estimated by measuring the amounts of nitrogen in different fractions, without isolating the amino acids individually. This saved time and labour, and also reduced the amount of protein required for the estimations. While the results obtained were not, in general, very satisfactory, fairly accurate figures could be obtained for the amounts of arginine, cystine, histidine and lysine.

Dakin (1918) utilised the different solubilities of amino acids in water, ethanol and butanol, to separate them into five groups. The technique used prevented the racemisation of amino acids, and could be used for the preparation of the natural isomers in surprisingly high yields. In the case of zein 100% recoveries were quoted, but again this method has been abandoned in favour of the

modern techniques.

The methods described so far were intended for the separation and estimation of all the amino acids, but they were mainly unsatisfactory because there was incomplete separation of some of these. At best, accurate estimation was limited to about ten amino acids.

Between 1920 and 1940 emphasis was placed on methods for the estimation of individual amino acids. It was possible to isolate the basic amino acids by precipitation with phosphotungstic acid. They were then estimated separately by specific colour reactions. MacPherson (1946) improved the separation of the basic amino acids by electrodialysis, and this method has been used with success quite recently. However, no satisfactory methods had been devised for the estimation of each of the mono-amino acids.

The nearest approach to a complete method was the precipitation technique introduced by Chibnall, Rees and Williams (1943), which gave good results in these workers' hands. It had the disadvantage of requiring large amounts of protein, besides being a laborious and exacting procedure.

Another method, with limited use, was employed by Gale (1945), who isolated specific decarboxylases for tryosine, lysine, arginine, ornithine, histidine and glutamic acid, which could be used for the estimation of

these amino acids, by measuring the CO_2 produced after reaction with the appropriate enzyme. Krebs (1950) extended the method to include aspartic acid by converting it into glutamic acid using the enzyme transaminase. The technique has the advamtage of being simple and, unlike microbiological assay, is not dependent on the variations which occur in an intact growing organism, but can be accurately controlled.

In the last ten to fifteen years, however, a number of rapid and accurate general techniques have been described, which have the added advantage, in most cases, of being applicable to separations and estimations on a microscale.

It is worth considering some of these methods, although we have relied entirely on chromatographic techniques for our analyses on cytochrome c.

Microbiological Assay.

Snell and his co-workers were largely responsible for the adaptation of this method for the quantitative estimation of amino acids. This procedure had previously been used for work on vitamins. Snell (1945, 1946) reviewed this technique, which depends on the fact that when the growth of an organism is governed by the presence of a limited amount of an essential metabolite, the growth will increase to a maximum, as the concentration of this metabolite is increased.

The method has been extensively used for the estimation

of amino acids and, until chromatographin techniques were fully developed, provided many of the results on protein analysis. The technique has numerous advantages. It is highly specific, and can be used for estimating very small amounts of amino acids. There is no need to carry out laborious separations before estimation. With the relatively inexpensive equipment used, the method can be adapted to become a rapid routine operation, as the same procedure is used for each amino acid. However, the handling of bacteria requires rather a specialised knowledge, and to obtain the best results the services of a good bacteriological laboratory would almost certainly have to be available.

There are several disadvantages attached to the method, the most important one being due to lack of information on the complete metabolic processes which take place in bacterial cells. Until fuller knowledge is gained concerning these, doubts will arise as to the validity of some of the results obtained.

It is doubtful whether any organisms are available which utilise D-amino acids, and, as a certain amount of racemisation may occur during hydrolysis of the protein, this will lead to low recoveries of amino acids on assay. In addition, if any amino acid is originally present in the protein, as a D-isomer, it will not be estimated by this procedure. Similarly there will be an inability to

detect any amino acid not yet discovered, which could presumably affect the assay of other amino acids (Tristram, 1949).

While there are organisms available for the estimation of most of the amino acids, so far none has been found which requires hydroxyproline, and although this amino acid does not occur frequently in proteins, there may be other more important instances.

Finally, as in all other methods of amino acid analysis, rigorous control experiments must be carried out to ensure the highest possible accuracy.

Isotope Dilution.

This technique was devised by Shemin (1945) and Foster (1945), and is the one method of amino acid analysis which is capable of giving almost theoretical results. The method consists of the addition of an isotopically labelled amino acid to a protein hydrolysate, and the isolation of the total labelled and unlabelled amino acid. The extent of dilution gives a measure of the amino acid in the original mixture.

One advantage is that the recovery of the amino acid does not require to be quantitative, but it must be isolated in a pure state. Tristram (1953) suggests that utilisation of the new methods of separation, such as chromatography, would be helpful in obtaining pure products, thus eliminating the less satisfactory precipitation procedures.

A considerable amount of labour is involved in the preparation of the labelled amino acids, which, of course, must be pure. Appreciable error may result if D-isomer is present in a DL mixture of the added compound, but Shemin and Foster (1946) claim that the D- and L-isomers can be separated.

Although the method is very attractive, it has not been extensively used, because of the expensive equipment required. Where only small amounts of protein are available, the isolation of each amino acid in sufficient quantity might not be possible, and more information may be obtained by using a method which requires less material.

Another isotopic method, isotope sampling, has been used by Keston and his associates (1949, 1950). This involves the quantitative reaction of an amino acid with a group carrying radioactive atoms. To facilitate isolation a large amount of the non radioactive derivative of the amino acid is added, and after purification the amount of amino acid originally present in the protein can be assessed. The amino acid derivatives used by these workers are prepared by reaction with p-iodophenyl sulphonyl chloride, labelled either with I^{131} or S^{35}. The method has given good results in so far as it has been used. There is a danger, however, that low recoveries will be obtained because of incomplete conversion of the amino acid to the radioactive derivative. This technique is also suitable

for the estimation of N-terminal amino acids, but its use is limited by the short half lives of the iodine and sulphur isotopes.

Other Methods of Estimation.

A number of other methods for the separation of amino acids have been described, which have not been used widely so far.

Paper ionophoresis has been adapted by Durrum (1950) for the separation of amino acids, but its application appears to be limited at present, as the amino acids can be separated more efficiently by chromatographic methods.

Battersby and Craig (1952) have used their counter current distribution technique (Craig et al., 1950) for the separation of the amino acids of tyrocidine A. This method is suitable for the isolation of pure amino acids, in relatively large amounts, and will probably receive wider attention in the future. It is important to check on the physical and chemical properties of an amino acid, whenever possible, as further proof of its presence in a protein. This is only possible when each amino acid has been isolated in the free state.

Chromatographic Methods.

The introduction of chromatographic methods for the separation of amino acids has led to rapid advances being made in this field. No other technique has had such spectacular success in the history of protein chemistry.

Chromatography has been defined as "the technical procedure of analysis by percolation of fluid through a body of comminuted or porous rigid material irrespective of the physico-chemical processes that may lead to the separation of substances in the apparatus " (Gordon, Martin and Synge, 1944). There are three main types of chromatography, adsorption, partition and ion exchange, each of which will be discussed briefly.

Adsorption Chromatography.

In this type of chromatography true adsorption of the amino acids takes place on the material comprising the column. The degree of adsorption on any particular medium is dependent on the structure of each amino acid. Thus the aromatic amino acids are very strongly adsorbed on charcoal, and this has been used as a method for their separation from a mixture of amino acids (e.g. Schramm and Primosigh, 1943). After the other amino acids have passed through the charcoal the aromatic amino acids can be eluted with an organic solvent such as phenol or ethyl acetate.

Schemes for the separation of the neutral amino acids have not been developed so successfully, but Turba, Richter and Kuchar (1943) effected the separation of valine and leucine, and valine and methionine, on columns of active carbon.

Utilisation of a series of columns of different ad-

sorbents for the separation of the amino acids into groups has been suggested by Schramm and Primosigh (1944), but this has not been used much.

Adsorption chromatography has been extensively developed, however, by Tiselius, as described in his review (Tiselius, 1947). He introduced two new types of analysis, frontal and displacement analysis, as well as using the usual elution technique. In all these methods he examined the effluent fluid, from the columns of activated charcoal, with a micro interferometric arrangement which determines the concentration of solutes by means of refractive index measurements.

In frontal analysis the mixture being examined is continuously supplied to the top of the column, so that the fastest component emerges from the column closely followed by a mixture of the first and second components, and so on. The resolving power of this method is not high, as no true separation takes place, and has not been used to any extent for work on amino acids. Its potential application is in assessing the number of components in a mixture of peptides (e.g. Synge and Tiselius, 1947).

Displacement development has been found to give better results. In this technique, instead of the usual eluting solvent, a displacing agent is added to the top of the column, which is more strongly adsorbed than any of the other components present. This prevents tailing on the column, but here again there is no interruption between

the bands. This difficulty has been overcomeby the carrier displacement technique (Tiselius and Hagdahl, 1950), in which zones of other substances are interposed between the amino acid components according to their degrees of adsorption.

The major deterrent to the use of these techniques is the delicate and expensive apparatus required, which is hardly justified when cheaper and more efficient methods are available.

In general, adsorption chromatography has not been found to have such wide application as either partition or ion exchange chromatography for the separation of amino acids.

Partition Chromatography.

This excellent method we owe to Martin and Synge (1941), who introduced it after studying the partition of acetylated amino acids in chloroform-water systems. Instead of using a large series of separating funnels they decided to use a column of inert material, such as silica gel, to support the aqueous phase of a two component solvent system, while the second organic phase was allowed to run down the column. This had the effect of separating a mixture of acetylated amino acids, placed on the column, into single components, according to their partition coefficients in the solvent system.

In the first experiments a mixture of acetyl mono-

amino acids was separated on silica gel using a water-chloroform mixture. The amino acids were detected on the column with methyl orange, incorporated in the aqueous phase, and collected in the effluent when they were estimated by titration with barium hydroxide.

The method was further developed by Gordon, Martin and Synge (1943a,b,c,d) and by Tristram (1946) for the estimation of the monoamino acids in a number of proteins. It was also used to examine the amino acids in peptides from partial hydrolysates of proteins. Fairly satisfactory results were obtained.

At about the same time Consden, Gordon and Martin (1944), who had been experimenting with methods for separating amino acids on cellulose columns, introduced an elegant technique for the separation of amino acids and small peptides on sheets of filter paper. The paper holds a certain amount of water in its fibres, which acts as the stationary phase, while the organic phase is allowed to move over the paper. By virtue of the mechanical adaptability of paper two dimensional chromatograms can be run, which increases the resolving power of the method considerably. The chief advantage of the method is that only small amounts of material need to be used, e.g. about 300 µg. are required for the identification of all the amino acids in a protein hydrolysate. The amino acids can be detected on the paper by means of a

suitable spraying agent such as ninhydrin.

This method has been used in the present work for studying the qualitative amino acid composition of cytochrome c and of some peptides from it. Although we have never attempted to use this method quantitatively, many workers have done so (e.g. Polson, Mosley and Wyckoff, 1947; Woiwood, 1948a,b; Heynes and Anders, 1951). It is doubtful whether the results obtained are very accurate, and where sufficient material is available column chromatographic methods give more reliable results.

Chromatography of acetylated amino acids on columns of silica gel suffered from two main disadvantages. Variations occurred from batch to batch of silica gel, which affected the reproducibility of the separations obtainable by specific solvent systems. Difficulty was also experienced in carrying out the quantitative acetylation of amino acids. Synge (1944), however, obtained reproducible results using columns of potato starch for the separation of free amino acids, instead of their acetylated derivatives.

There is considerable discussion as to whether the separations on starch are due to partition or to adsorption. Martin (1950) has put forward a very good argument for the former view, but Tristram (1953) is of the opinion that the starch acts as a solid polar adsorbent. Moore and Stein (1948a) also consider that some adsorption takes place, especially in the case of the aromatic amino

acids.

Starch chromatography has been adapted by Stein and Moore (1948) and Moore and Stein (1949) for the determination of amino acids. By the use of three solvent systems all the commonly occurring amino acids can be separated and then estimated by the modified quantitative ninhydrin reaction (Moore and Stein, 1948). This method gave very good results for β-lacto-globulin and bovine serum albumin (Stein and Moore, 1949) when compared with results from other techniques. The authors claim that the degree of accuracy is 100 ± 3% for all except the dicarboxylic amino acids, which, because of a small amount of esterification in the solvents used on the column, give recoveries of 100 ± 6%. The method has the advantage of requiring only small amounts of protein, as little as 15 mg. being required for a single estimation on each amino acid. It was widely used by us for our work on cytochrome c until the introduction of the more powerful ion exchange technique by the same authors (Moore and Stein, 1951).

Ion Exchange Chromatography.

This method is likely to give the most satisfactory results on account of its high resolving power. Separation depends on a number of factors, the most important being due to differences of the net charges on the molecules of the amino acids. Adsorption probably also plays a part.

A number of exchange materials have been used, among them being alumina (Wieland, 1942), or silica gel (Schramm and Primosigh, 1944) for the retention of the basic amino acids, which could then be eluted with alkali. The acidic amino acids could be retained on acid alumina (Schramm and Primosigh, 1944) and later eluted with acid.

With the introduction of synthetic ion exchange resins, these have been used for the general separation of amino acids. Partridge and his co-workers have applied the principles of displacement chromatography to the separation of the amino acids into groups on the resin Zeo-Karb 215 (cf. Partridge and Westall, 1949; Partridge, 1949). These workers have extended the method to the preparation of crystalline amino acids on a series of columns (Partridge and Brimley, 1952).

Moore and Stein (1951) have devised a method for the quantitative estimation of amino acids on the cation exchange resin Dowex 50, which is similar to their technique described for separations on starch. The resin has the advantage, over the starch, of not being so easily overloaded, which means that more material can be placed on the column. This leads to more accurate determination of the amino acids present in small amounts. All the amino acids can be separated by two solvent systems and therefore results can be obtained more quickly than on starch. Single values for each amino acid can be obtained from about 15 mg. of protein with an accuracy, the authors claim of 100 $\pm$ 3%. We used this method for the estimation of the amino acids, especially the basic ones, in cytochrome c.

EXPERIMENTAL and RESULTS.

The Qualitative Amino Acid Composition of Cytochrome c.

The qualitative amino acid composition of cytochrome c was studied on two dimensional paper chromatograms.

A sample (1 mg.) of purified cytochrome c was dissolved in 1 ml. of 6 N-HCl, and the mixture hydrolysed, in a sealed tube, in an air oven at 106°for 18 hours. The hydrolysate was evaporated to dryness, taken up in a little water, and about 300 µg. of the material placed, as a small spot, on a sheet of Whatman No. 1 filter paper. Any acid remaining in the spot was neutralised by adding 1 drop of 0.88 sg. ammonia to it.

Two solvents were used throughout our work with paper chromatography. The first was the n-butanol-acetic acid-water system described by Partridge (1948), and the second was phenol saturated with water. When the paper was placed in the latter solvent several drops of 0.88 s.g. ammonia were run down the inside of the tank, which increases the R_F values of the basic amino acids in phenol. A few crystals of KCN were added to a separate dish, in the tank, containing water saturated with phenol, which kept the paper in an atmosphere of HCN. This helped to prevent the formation of oxidation products of phenol on the paper, as well as eliminating "pink front" near the faster amino acid spots (Consden, Gordon and Martin, 1944).

The paper was always run first in the long direction, as a descending chromatogram in the n-butanol-acetic acid solvent,

and then dried in a cabinet, in a stream of air, at 60° for one hour. The paper was then run in a direction at right angles to the first, as an ascending chromatogram in the form of a cylinder, in phenol. After development the paper was replaced in the cabinet for the minimum amount of time for drying, usually one and a half hours, because amino acids tend to be destroyed in the presence of phenol at elevated temperatures.

The amino acid spots were detected by spraying the paper with a solution of 0.2% ninhydrin, made up by dissolving 200 mg. ninhydrin in 50% ethanol containing 1 ml. of glacial acetic acid. A few drops of collidine were added prior to using the reagent. The acetic acid neutralises any ammonia on the paper, while the collidine is useful in differentiating some of the amino acids with which it gives characteristic colours. After the paper was thoroughly sprayed, it was held in front of an electric fire for approximately 30 seconds, when the amino acid spots could be identified.

Table 4 shows the results obtained from a typical chromatogram. Most of the common amino acids are contained in cytochrome c, although serine does not appear to be present. It did occur occasionally but there was no indication of it in our purest preparations either on paper or on a column, and it was concluded that, if present, it belongs to contaminating impurities. The absence of serine provides a useful check on the purity of cytochrome c

TABLE 4.

The Qualitative amino acid composition of Cytochrome c.

Amino acid.	Relative Intensity of Spot. *
Aspartic acid	++
Glutamic acid	+++
Cystine	++
Glycine	+++
Threonine	++
Alanine	++
Tyrosine	+
Valine Methionine	+
Phenylalanine	±
Proline	+
Leucines	+
Histidine	+
Lysine	+++
Arginine	±

* The + and - signs are purely arbitrary and are used to designate the relative size and intensity of each spot on the chromatogram.

preparations.

Tryptophan is known to be present in cytochrome c, but it is rapidly destroyed by acid hydrolysis, and was therefore not detected on paper chromatograms.

The Quantitative Amino Acid Composition of Cytochrome c.

Preparation of hydrolysates.

Each hydrolysate of cytochrome c was prepared by heating approximately 20 mg. of the purified cytochrome c with 2 ml. of 6 N-HCl in a sealed tube for 18 hours at 105°, after which the mixture was evaporated to dryness in a vacuum desiccator over H_2SO_4 and Na OH. Water was added and the mixture re-evaporated several times to remove HCl completely, before the hydrosylate was taken up in the appropriate solvent, and diluted to 2 ml. in a graduated stoppered tube. An aliquot, containing 3 mg. for the starch and 5 mg. for the Dowex 50 columns, was used for analysis. Another sample was taken for the estimation of total nitrogen, from which the amount of cytochrome c placed on the column was calculated.

Analysis on Starch Columns.

These experiments were carried out on columns of Gordon Slater starch, using the technique described by Stein and Moore (1948) and Moore and Stein (1949).

After the charge was placed on the column, the latter was mounted on a fraction collector and the rate of flow maintained at 1.5 ml. per hour under air pressure. The

collection of 0.5 ml. fractions was controlled by a timing device, which, on account of the relatively slow rate of flow, gave fractions of constant size.

Analysis of fractions. The contents of the tubes were analysed by the ninhydrin method of Moore and Stein (1948). In preparing the ninhydrin reagent, 350 mg. of $SnCl_2 . 2H_2O$, instead of 200 mg., was added per gram of recrystallised ninhydrin, because the larger amount appeared to keep the reagent, without deterioration, for a longer period. It was found, however, that after being stored for about a week, under nitrogen, the reagent developed a pale blue colour. This did not appear to affect the results but, in general, only sufficient reagent for three days was prepared at one time.

1 ml. of ninhydrin reagent was added to each tube, from an automatic pipette, and the tubes placed in a boiling water bath for 30 minutes.

Standard curves were prepared using 0.1 to 0.5 ml. samples of 0.001 M glycine, in 0.1 ml. intervals, dissolved in the solvent being used on the column.

Satisfactory straight line curves were at first obtained with the Unicam D.G. spectrophotometer, but after some time the curves did not obey Beer's law, the deterioration being traced to the instrument. Good results were, however, obtained with the Unicam SP 600 spectrophotometer. Test tubes could not be used with this instrument and each solution had to be transferred to 1 ml. cuvettes. This

operation was time consuming as four or five dozen tubes were being handled in each batch.

The colour yield occasionally varied, unaccountably, from the normal, and it was therefore necessary to include a set of standards with each batch of tubes. Previous workers, in this laboratory, carried out recovery experiments on amino acid mixtures from starch columns, and obtained, except for glutamic and aspartic acids, 100% recoveries. Corrections have been made for losses on the column, due to esterification with the solvent, for these two amino acids.

Results. On column S 45, the amino acids were separated using the solvent 2:1 n-propanol - 0.5 N HCl. The type of separation is shown in Fig. 1, and the results are recorded in Table 5. In this table the number of micromoles of each amino acid or group of amino acids recovered from the column is listed. From these values the percentage nitrogen of total nitrogen was calculated from

$$\frac{\text{micromoles recovered} \times \text{N atoms per mole of amino acid}}{\text{micromoles N added to column.}}$$

The moles of amino acid per mole of cytochrome c were calculated from

$$\frac{\text{micromoles recovered} \times \text{micromoles N per micromole of cytochrome c}}{\text{Total micromoles N added to column.}}$$

The micromoles of N per micromole of cytochrome c, calculated to be 134, were determined assuming that the molecular weight of cytochrome c is 12,270 (Tint and Reiss, 1950) and

Fig. 1.

Separation of the amino acids from a hydrolysate of cytochrome c on starch column S45. Solvent, 2:1 n-propanol-0.5N HCl.

The abreviations used for the amino acids are those suggested by Brand and Edsall (1947).

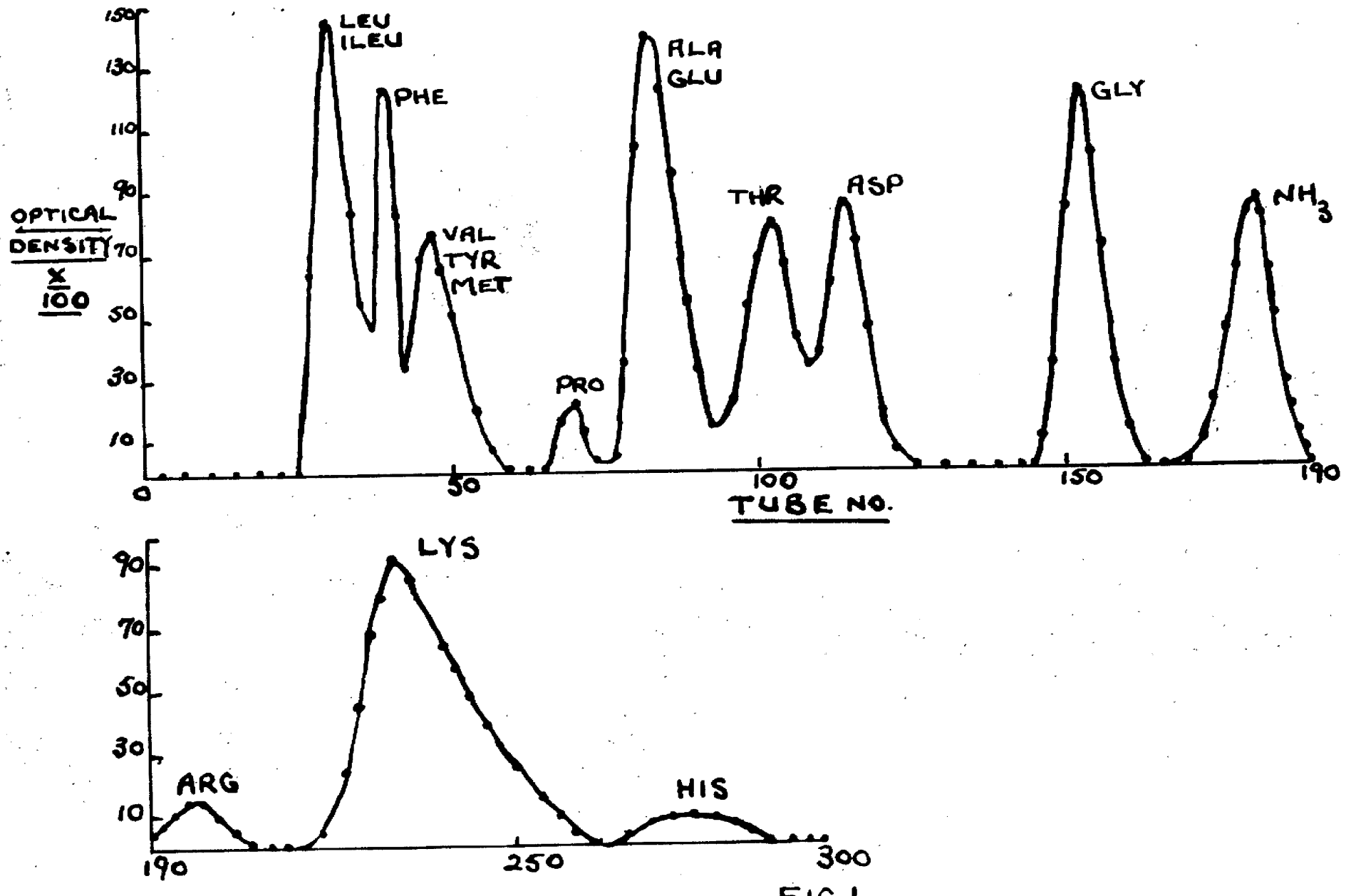

OPTICAL DENSITY X 100
150
130
110
90
70
50
30
10
LEU
ILEU
PHE
VAL
TYR
MET
PRO
ALA
GLU
THR
ASP
GLY
NH3
0
50
100
150
190
TUBE NO.
90
70
50
30
10
LYS
ARG
HIS
190
250
300

FIG I.

TABLE 5.

Results of separation of amino acids from Cytochrome c hydrolysate on column S45.

Amino acid.	Micromoles recovered.	Nitrogen % of Total Nitrogen.	Moles of amino acid per mole of Cytochrome c.
Glycine	2·31	7·61	10·14.
Leucine, Isoleucine, Phenylalanine, Valine, Tyrosine, Methionine	5·07	16·7	22·02.
Proline	0·875	2·87	3·78.
Serine	-	-	-
Threonine	2·07	6·83	9·03.
Cystine	-	-	-
Aspartic acid	1·68	5·54	7·32.
Alanine, Glutamic acid	3·15	10·4	13·74.
Arginine	1·35	4·45	1·47.
Lysine	9·12	30·0	19·8.
Histidine	1·62	5·35	2·35.
Ammonia	1.67	5.5	7.21.
Nitrogen recovered		95.5%.	

that the nitrogen content is 15.08% (Theorell and Åkeson, 1941a).

It can be seen from Fig. 1 that leucine, isoleucine, phenylalanine, valine, methionine, and tyrosine are incompletely separated from each other, and these were treated as a single group. There is also incomplete separation of threonine and aspartic acid, but these were partitioned according to the formula derived by Moore and Stein (1948). With this solvent there is no separation of glutamic acid and alanine, while cystine appears to have been completely destroyed.

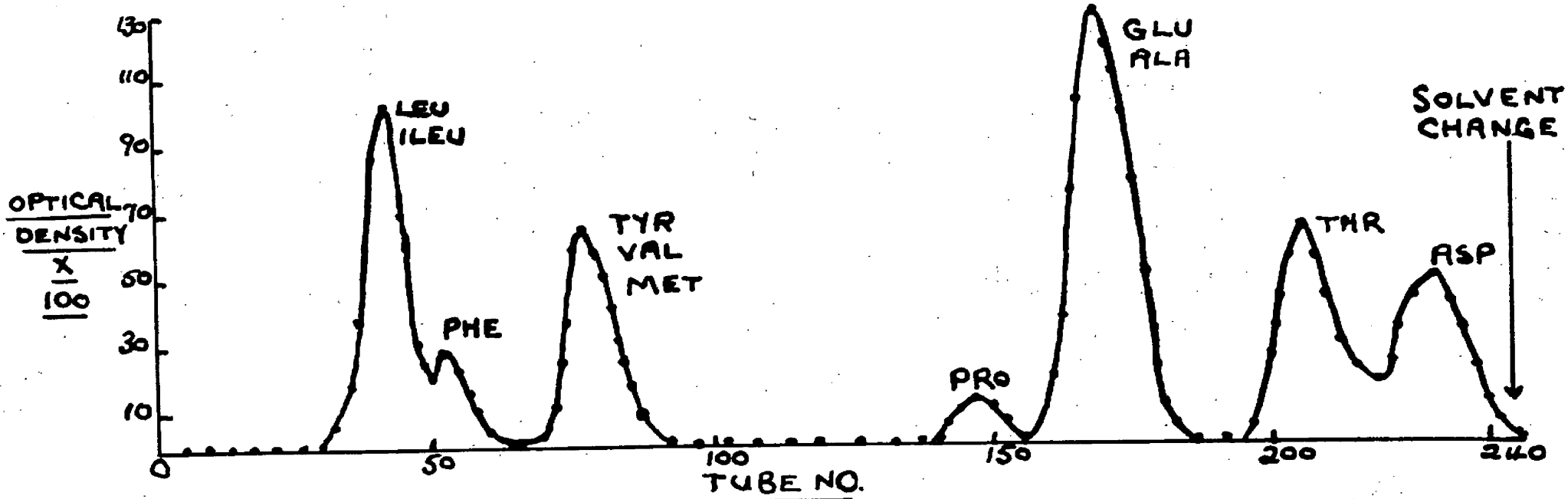
By means of the solvent system 1:2:1 n-butanol-n-propanol-0.1 N HCl leucine and isoleucine are partially separated from phenylalanine, while tyrosine, valine and methionine are obtained in another separate band on column S 49, shown in Fig. 2. After aspartic acid was eluted from the column the solvent was changed to 2:1 n-propanol-0.5 N HCl and a separation similar to that on column S 45 was obtained. The results obtained from this separation are recorded in Table 6.

The amino acids leucine, isoleucine, phenylalanine, tyrosine, methionine and valine were completely separated from each other on column S 48 using 1:1:0.288 n-butanol-benzyl alcohol-water as solvent. The type of separation is shown in Fig. 3 and the results recorded in Table 7.

It is possible to separate glutamic acid and alanine using a 2:1:1 mixture of tert.-butanol-sec.-butanol - 0.1 N

Fig. 2.

Separation of the amino acids from a hydrolysate of cytochrome c on starch column S49. Solvents, 1:2:1 n-butanol-n-propanol-0.1N-HCl, followed by 2:1 n-propanol-0.5N-HCl.


OPTICAL DENSITY X 100
130
110
90
70
50
30
10
LEU
ILEU
PHE
TYR
VAL
MET
PRO
GLU
ALA
THR
ASP
SOLVENT CHANGE
0
50
100
150
200
240
TUBE NO.

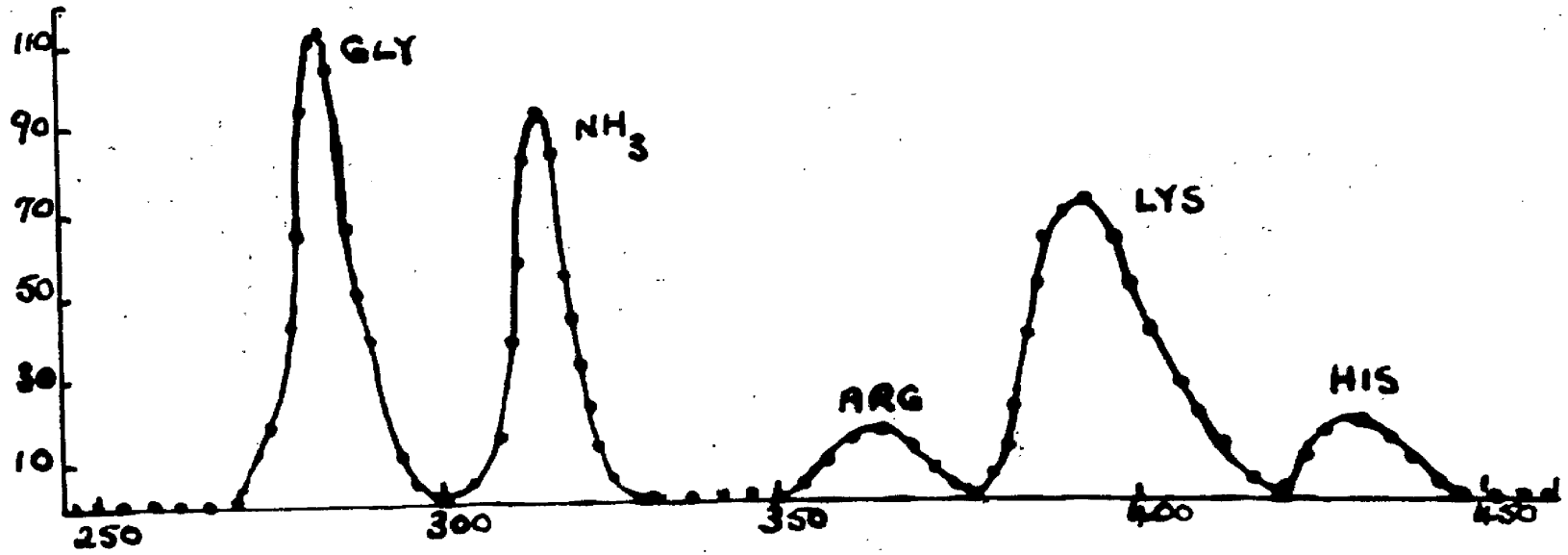

110
90
70
50
30
10
GLY
NH3
ARG
LYS
HIS
250
300
350
400
450

FIG. 2.

TABLE 6.

Results of separation of amino acids from Cytochrome c hydrolysate on column S49.

Amino acid.	Micromoles recovered.	Nitrogen % of Total Nitrogen.	Moles of amino acid per mole of Cytochrome c
Glycine	2·22	6·62	8·75.
Leucine	2·74	8·18	10·81.
Isoleucine	0·85	2·54	3·35.
Phenylalanine			
Proline	0·89	2·66	3·51.
Tyrosine	1·715	5·13	6·76.
Methionine			
Valine			
Serine	-	-	-
Threonine	1·92	6·03	7·97.
Cystine	-	-	-
Aspartic acid	1·7	5·4	7·14.
Alanine	3·9	12·05	15·91.
Glutamic acid			
Arginine	0·54	6·45	2·13.
Lysine	3·73	22·3	14·74.
Histidine	0·57	5·16	2·28.
Ammonia	1·46	4·39	5·8.

Nitrogen recovered 86·8%.

Fig. 3.

Separation of phenylalanine, leucine, isoleucine, methionine, tyrosine and valine, from a hydrolysate of cytochrome c, on starch column S48. Solvent, 1:1:0.288 n-butanol-benzyl alcohol-water.

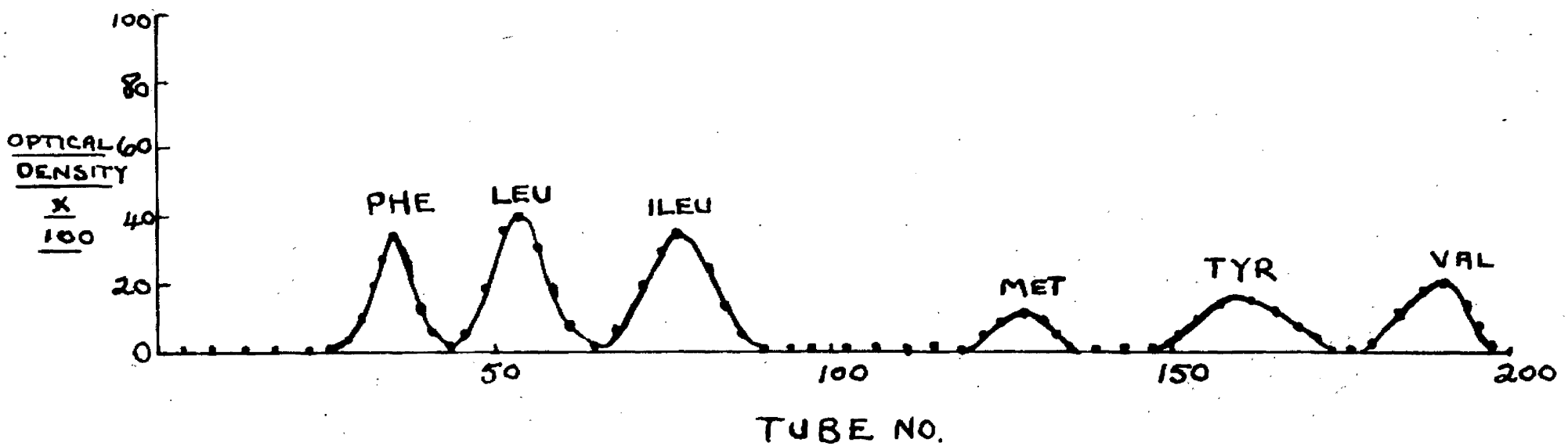

100
80
60
40
20
0
OPTICAL DENSITY
X
100
PHE
LEU
ILEU
MET
TYR
VAL
50
100
150
200
TUBE NO.

FIG. 3.

TABLE 7.

Results of separation of amino acids from Cytochrome c hydrolysate on column S48.

Amino acid.	Micromoles recovered.	Nitrogen % of Total Nitrogen.	Moles of amino acid per mole of Cytochrome c.
Valine	0·457	1·99	2·63.
Leucine	0·93	4·06	5·36.
Isoleucine	0·938	4·09	5·41.
Phenylalanine	0·585	2·55	3·36.
Tyrosine	0·74	3·23	4·27.
Methionine	0.281	1.22	1·61.

HCl but the results obtained are not very satisfactory, and it was decided to separate these on a 100 cm. Dowex 50 column.

The recovery of nitrogen from the columns can only be calculated for S 45 and S 49, because only in these cases were there sufficient amino acids estimated to enable this to be done. In S 45 95.5% nitrogen was actually recovered from the column. If to this is added the nitrogen contained in the prosthetic group plus that for 2 molecules of cystine and 1 of tryptophan, determined in subsequent experiments, the total nitrogen recovery is found to be 102.96%. 86.8% nitrogen was recovered from column S 49, and if the above corrections are again made, this brings the total recovery to 94.26%.

Analysis on Dowex 50 Columns.

The analysis of the basic amino acids on starch columns was found to be very variable, and it was hoped to obtain better results on the resin columns.

As there is also more efficient separation of the other amino acids, results from the resin columns should give better results than the starch columns.

250 mesh Dowex 50 resin was prepared for use in a 100 cm. column which was used for the separation of the neutral and acidic amino acids. Zeo-Karb 225, with a particle size of 50 μ, was employed in the 15 cm. column for estimating the basic amino acids. The technique devised by Moore and Stein (1951) was used throughout.

The columns were mounted on the fraction collector, fitted with a photoelectric drop counter, which ensured that the size of the fractions was constant. This was very important as the fractions had to be neutralised by the addition of acid or alkali, according to the solvent used, to pH 5, and then buffered with two drops of 0.1 M pH 5 sodium citrate.

Care had to be taken that the buffers, used as eluting solvents, were prepared as nitrogen-free as possible. Glass-distilled water which had been passed through a column of Zeo-Karb 225, to remove ammonia, was always used. Each reagent was examined by the ninhydrin method, to ensure that only those containing the lowest amounts of ninhydrin-positive material were used for preparing the solvents.

With the 15 cm. column it was found that the blank of the eluate containing the pH 6.5 citrate buffer was very high, and this was liable to interfere with the arginine band. This buffer did not contain any nitrogenous impurities, but it washed interfering material off the column, which had been retained from the pH 6.7 phosphate buffer. The contamination was traced to the disodium phosphate used to prepare the buffer, but although the salt was recrystallised several times little improvement was obtained.

Recoveries from the columns. A mixture of amino acids was made in the proportions previously found in hydrolysates of cytochrome c. Suitable aliquots were placed on the 100 cm.

and 15 cm. columns to test the separatory powers of the resin, and also the recovery of the amino acids from the columns.

The elementary analysis of each amino acid was checked by Mr. D. Cameron of the Chemistry Department, and all were found to be pure except proline. This amino acid was purified according to the method of Hamilton and Ortiz (1950), by conversion to the picrate, and decomposition of the salt with aniline. It was found to be pure after this treatment.

Similar separations to those described by Moore and Stein (1951) were obtained, but there was little or no separation of phenylalanine and tyrosine on the 100 c.m. column. Table 8 records the recoveries obtained, which were in general satisfactory. The recoveries of cystine and methionine were very low, probably due to decomposition on the column, while the recovery of isoleucine was also low for no apparent reason. Corrections were made, according to the recoveries, when calculating the results of analysis of the cytochrome c hydrolysates.

Results. The type of separation obtained on a cytochrome c hydrolysate on the 100 cm. column is shown in Fig. 4. The results obtained from this column, IR 9, are shown in Table 9. There was no separation of phenylalanine and tyrosine, while the methionine appears to have been completely destroyed, probably partly during hydrolysis and

TABLE 8.

Recoveries of Amino acids from Dowex 50 columns.

Amino acid.	Average recovery %.	Standard error.	Number of Chromatograms.
Glycine	99	± 0°94	3
Alanine	96.6	± 1.4	5
Valine	95	± 2.27	5
Leucine	97.1	± 4.6	3
Isoleucine	88.3	± 3.54	3
Proline	108	± 0.87	4
Phenylalanine	103.7	± 1.2	6
Tyrosine	92.3	± 3.0	2
Serine	96	-	1
Threonine	99.5	± 4.64	2
Cystine	62	± 4.91	3
Methionine	40.3	± 3.41	3
Aspartic acid	98	± 1.08	3
Glutamic acid	93.7	± 1.09	3
Arginine	91.3	± 1.45	3
Lysine	94.5	± 1.78	2
Histidine	98	± 7.14	2
Ammonia	99	± 2.45	3

Fig. 4.

Separation of the neutral and acidic amino acids from a hydrolysate of cytochrome c on the 100 cm. Dowex 50 column IR9. Solvents, 0.1M pH 3.42 sodium citrate buffer followed by 0.1M pH 4.25 sodium citrate buffer.

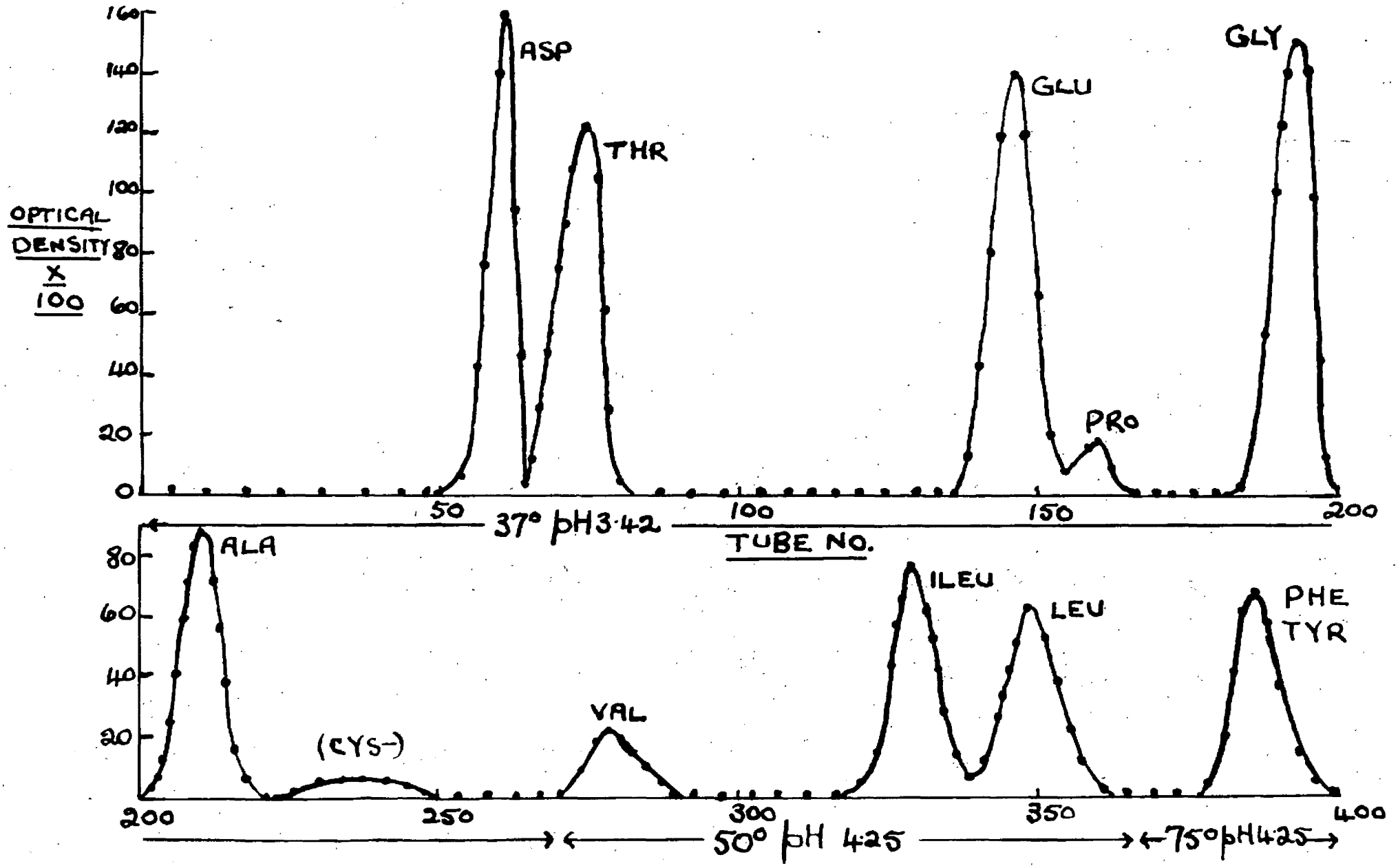

OPTICAL DENSITY X 100
160
140
120
100
80
60
40
20
0
ASP
THR
GLU
PRO
GLY
50
100
150
200
37° pH3·42
TUBE NO.
80
60
40
20
ALA
(CYS-)
VAL
ILEU
LEU
PHE
TYR
200
250
300
350
400
50° pH 4·25
75° pH 4·25

FIG 4.

TABLE 9.

Results of separation of amino acids from Cytochrome c hydrolysate on column IR9.

Amino acid.	Micromoles recovered. (corrected).	Nitrogen % of Total Nitrogen.	Moles of amino acid per mole of Cytochrome c.
Glycine	3•84	7.98	10•6.
Alanine	1.87	3•79	5•04.
Valine	0 .70	1•41	1•89.
Leucine	1•76	3.56	4•76.
Isoleucine	1•93	3.91	5•23.
Proline	1•04	2.10	2 .7.
Phenylalanine / Tyrosine	2•4	4•9	6•47.
Serine	-	-	-
Threonine	3.21	6•51	8.7.
Cystine/2	0•41	0.83	2•24.
Methionine	-	-	-
Aspartic acid	2•7	5•47	7•31.
Glutamic acid	3.69	7.8	10•25.

Fig. 5.

Separation of the basic amino acids, from a hydrolysate of cytochrome c, on the 15 cm. Zeo-Karb 225 column IR10. "A" comprises all the amino acids emerging before tyrosine. Solvents, 0.1M pH 5 sodium citrate buffer, 0.1M pH 6.8 sodium phosphate buffer and 0.2M pH 6.5 sodium citrate buffer.

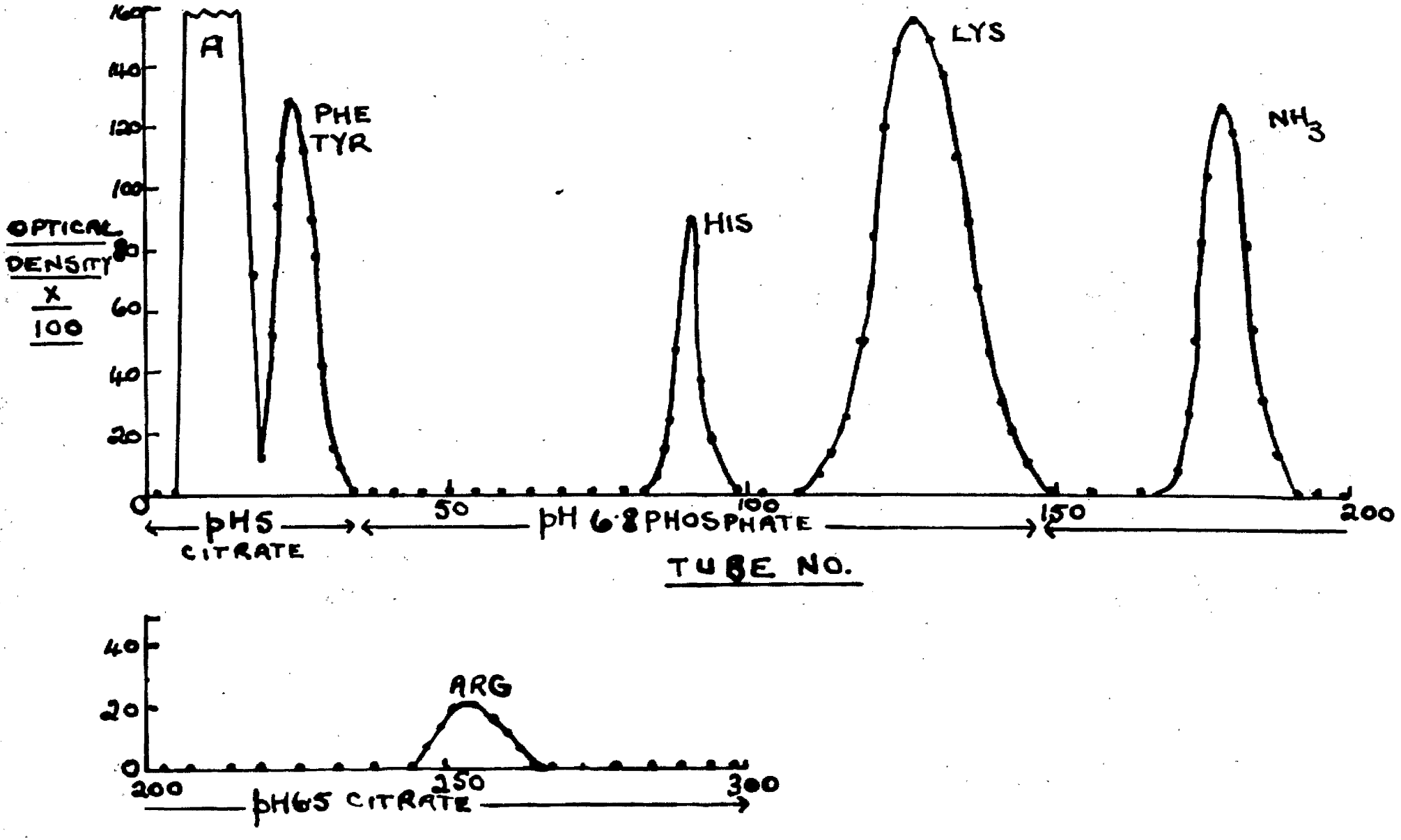

OPTICAL DENSITY X 100
160
140
120
100
80
60
40
20
0
A
PHE
TYR
HIS
LYS
NH3
50
100
150
200
pH 5 CITRATE
pH 6·8 PHOSPHATE
TUBE NO.
40
20
0
ARG
200
250
300
pH 6·5 CITRATE

FIG. 5.

TABLE 10.

Results of separation of amino acids from Cytochrome c hydrolysate on column IR10.

Amino acid.	Micromoles recovered. (corrected).	Nitrogen % of Total Nitrogen.	Moles of amino acid per mole of Cytochrome c
Phenylalanine Tyrosine	3·00	4·87	6·43.
Arginine	0·718	4·66	1·58.
Lysine	8·75	28·4	18·7.
Histidine	1·125	5·47	2·44.
Ammonia	4·57	7·4	9·74.

partly on the column.

No attempts were made to collect the basic amino acids from the 100 cm.column, as Moore and Stein (1951) found the recoveries were low. These were separated on the 15 cm. column as shown for column IR 10 in Fig. 5. The results for this column are given in detail in Table 10, while a summary of the results obtained from all the resin columns are listed in Table 12.

Estimation of Cystine.

The estimation of cystine, on both starch and resin columns, was very unsatisfactory, because it was largely destroyed during manipulation, and this resulted in a small band being obtained which could not be measured with much accuracy. It was therefore decided to oxidise cystine to cysteic acid, as suggested by Moore and Stein. Cysteic acid is eluted from the 100 cm. Dowex 50 column, in a sharp band, after 25 ml. of the pH 3.42 citrate buffer have passed through the column. It was hoped that this would increase the efficiency of the estimation of cystine.

Oxidation was carried out either by performic acid or bromine water. The oxidation by performic acid was carried out on a sample of known cystine, according to the method of Sanger (1949). 2 mg. of cystine were dissolved in 1 ml. 1 N-HCl and treated with 1 ml. of a mixture of 9:1 formic acid - hydrogen peroxide (30%). The reaction was allowed to proceed for 20 minutes, after which it was stopped by adding

10 ml. of water, and the mixture evaporated to dryness. No free cystine remained, as indicated by the nitroprusside test in the presence of cyanide. However, the recovery of cysteic acid was only about 60%, as estimated by the ninhydrin method, using a standard curve prepared with known cysteic acid.

Milder oxidation was then carried out with bromine water. Saturated bromine water was added to 10 mg. of cystine in 5 ml. 1 N-HCl until the mixture had a permanent faint yellow colour. The solution was allowed to stand for 30 minutes before being evaporated to dryness in a vacuum desiccator. No free cystine remained and the yield of cysteic acid was found to be 99%. A second experiment, in which the cystine was oxidised in the presence of a mixture of amino acids, in the proportions found in cytochrome c , gave a recovery of 100% cysteic acid. This indicates that the oxidation proceeds satisfactorily in the presence of other amino acids.

The recoveries of cysteic acid from the 100 cm. Dowex 50 column were 99% and 100%.

It would appear that the oxidation of cystine is more satisfactorily performed by bromine water than by performic acid, but this did not turn out to be the case when oxidation was carried out on cytochrome c itself. In experiment IR 17 oxidation was carried out with performic acid, when the yield of cysteic acid was calculated to be 1.38

moles per molecule of cytochrome c. The same yield was obtained when oxidation was performed with bromine water. These values are much lower than the figure of 2.24 moles of cysteine per molecule of cytochrome c obtained from column IR 9. It must be assumed, therefore, that the recovery of cysteic acid is low, and probably corresponds to two molecules of cysteine. The cysteine residues attached to the prosthetic group are not split from the porphyrin by oxidation, and therefore are not estimated.

It was found that tyrosine and methionine, in previous recovery experiments, are largely destroyed when a mixture of amino acids is oxidised either by performic acid or bromine water. The aspartic acid band is much larger than expected, presumably due to contamination with unknown products of oxidation. It was therefore decided that no reliance could be placed on the results for the amino acids, other than for cysteic acid, after oxidation.

Estimation of Proline.

Proline forms a very small band and it was decided to attempt to increase the sensitivity of its measurement by estimating it with ninhydrin at pH 1, according to the method described by Chinard (1952).

This technique was used to estimate half of the proline fractions recovered from one 100 cm. resin column. From the results it was calculated that there are 2.72 moles of proline per molecule of cytochrome c. 2.89

moles per molecule were estimated by the method of Moore and Stein (1948). It would appear, therefore, that there is very little difference between the sensitivities of the two procedures.

Estimation of Tryptophan.

Tryptophan is completely destroyed during acid hydrolysis, and Spies and Chambers (1949) have shown that 20% - 40% destruction of tryptophan takes place during hydrolysis of protein in 5N-NaOH in the presence of air at 100°. Until a method for hydrolysing cytochrome c without loss of tryptophan has been developed, this amino acid cannot be measured accurately. Leaf (1954) carried out a single estimation of tryptophan in cytochrome c using p-dimethylaminobenzaldehyde, as described by Graham, Smith, Hier and Klein (1947). He found that the content of tryptophan was 0.86%, which corresponds to 0.52 molecules per molecule of cytochrome c. As can be seen, the recovery appears to be very low.

DISCUSSION.

A general study of the results, summarised in Tables 11 and 12, shows that while agreement between the values for some of the amino acids is good, others display considerable variations. Part of these variations may be due to the limitations of the ninhydrin method. We have found that the results for standard solutions sometimes differed from the normal quite appreciably, for no apparent reason.

TABLE 11.

Summary of results from Starch columns (calculated in moles of amino acid per mole of Cytochrome c).

Amino acid.	S45.	S49.	S48.
Leucine	22.02	10.81	5·36.
Isoleucine			5·41.
Phenylalanine		3·35	3·36.
Tyrosine		6·76	4·27.
Valine			2·63.
Methionine			1·61.
Alanine	13·74	15·91	-
Glutamic acid			
Glycine	10·14	8·75	-
Cystine /2	-	-	-
Proline	3·78	3·51	-
Threonine	9·03	7·97	-
Aspartic acid	7·32	7·14	-
Arginine	1·47	2·13	-
Lysine	19·8	14·74	-
Histidine	2·35	2·28	-
Ammonia	7·21	5·8	-

TABLE 12.

Summary of results from Dowex 50 columns (calculated in moles of amino acid per mole of Cytochrome c).

Amino acid.	100 cm. IR9	15 cm. IR 10	15 cm. IR 11	15 cm. IR 21	15 cm. IR 22.
Leucine	4·76	-	-	-	-
Isoleucine	5·23	-	-	-	-
Phenylalanine	6·47	6·43	6·7	6·96	7·09.
Tyrosine					
Valine	1·89	-	-	-	-
Methionine	-	-	-	-	-
Alanine	5·04	-	-	-	-
Glutamic acid	10·25	-	-	-	-
Glycine	10·6	-	-	-	-
Cystine/2	2·24	-	-	-	-
Proline	2·7	-	-	-	-
Threonine	8·7	-	-	-	-
Aspartic acid	7·31	-	-	-	-
Arginine	-	1·55	-	1·59	1·64.
Lysine	-	18·7	17·8	18·02	16·49.
Histidine	-	2·44	2·58	2·4	2.71.
Ammonia	-	9·74	8·01	8·82	8·05.

It is possible that impurities may be present which are eluted in the same band as a known amino acid, and these will not be differentiated by the ninhydrin method. This would cause variations, and to eliminate this it would be necessary to estimate each amino acid by a specific method.

Variations are liable to occur in estimating the amino acids which occur in the protein in small amounts, because the peaks are inclined to be shallow and difficult to define, especially if the blank values in the vicinity are variable.

The values obtained for leucine and isoleucine are reasonably constant. There appear to be 5 residues of each present, as indicated in S 48 and IR 9. The total of 10.8 molecules of isoleucine and leucine in S 49 is therefore a little high, perhaps due to contamination with part of the phenylalanine band.

From S 48 and S 49 it is concluded that there are 3 residues of phenylalanine, and from an examination of the values obtained on the resin columns there is probably a total of 7 molecules of phenylalanine and tyrosine. This indicates that there are probably 4 molecules of tyrosine, which is supported by the value obtained in S 48.

From columns S 48 and IR 9 there appear to be 2 molecules of valine.

Only one value for methionine has been obtained, 1.61 molecules in S 48. This value is probably rather low because the small bands could not be very accurately

measured. These could not be increased, however, because the high content of the hydrophilic amino acids in cytochrome c, e.g.lysine, glycine and aspartic acid, is liable to upset the equilibrium between the starch and the solvent 1:1:0.288 n - butanol-benzyl alcohol-water. The amount of hydrolysate which can be placed on this column is therefore limited. Åkeson (1942), however, estimated that there are 2 residues of methionine in cytochrome c, and our result appears to be in agreement with his value.

The total of 6.76 molecules for tyrosine, methionine and valine obtained from S 49 is rather low compared with the 8 molecules probably present in cytochrome c. The total for leucine, isoleucine, phenylalanine, tyrosine, valine and methionine in S 45 is 22 molecules. This is in reasonable agreement with the total of 21 molecules obtained from individual estimations on other columns.

From IR 9 there appear to be 5 molecules of alanine and 10 of glutamic acid. The total of 15.91 for these amino acids, in S 49, is a little high, while this value is low in S 45.

Although glycine gives the low value of 8.75 molecules in S 49, it is probable from the other experiments, S 45 and IR 9, that there are 10 residues of this amino acid.

From column IR 9 it was calculated that 2 molecules of cysteine are present per molecule of cytochrome c. However, only 1.38 moles of cysteic acid were recovered after oxid-

ation of cytochrome c . Theorell and Åkeson (1941) estimated that there are 6 atoms of sulphur per molecule of cytochrome c, 2 of which are present as cysteine residues attached to the prosthetic group. This leaves 4 atoms of sulphur in the protein moiety, 2 of which belong to methionine (Åkeson, 1942). However, Åkeson was only able to measure the equivalent of 1 other atom of sulphur, which appeared to be present in a further molecule of cysteine.

In the present work it is probable that the 1.38 molecules of cysteic acid are derived from 1 molecule of cystine. The cysteine residues attached to the prosthetic group are probably not oxidised as no instance has yet been reported of the oxidation of thioether linkages. These residues will therefore not be estimated.

The values for proline are consistently lower on the resin than on the starch columns, which is interesting, but we have no explanation to offer for this at the moment. From the results there appear to be 3 residues of this amino acid.

The results for threonine are rather variable, but if it is assumed that the value of 7.97 is low in S 49, there are probably 9 molecules of threonine in cytochrome c.

Aspartic acid gives very constant results, which indicate that there are 7 residues of this amino acid.

The results for arginine on the resin columns were rather low, probably because of the very shallow peak ob-

tained, and also because of interference caused by the variability of the blank in this region. The band on the starch columns was much sharper, and probably the results from these are more reliable. Bearing this in mind, there appear to be 2 molecules of arginine in cytochrome c.

On the other hand, the histidine band on the starch columns was not well defined, while a sharp band was obtained on the Dowex 50 columns. It is probable that the results from the latter are more reliable, but all are found to be very constant. It is difficult to decide whether there are 2 or 3 molecules of histidine, as the results lie midway between these values.

The results for lysine on starch columns were not very satisfactory because the peak was not always smooth and the blank values were variable in this region. The values should therefore be treated with reserve. The figure of 19.8 residues from S 45 is very high, but there appears to have been some interfering material in the tail of this band, which may have led to the high result. In S 49 there were fluctuations in the colour yield with ninhydrin while lysine was being estimated, which has resulted in a low recovery. The values for the resin columns were much more constant and, apart from IR 22, all give approximately the same value of 18 molecules for this amino acid.

Little significance can be placed on the results for ammonia, because it is difficult to decide its origin.

Losses are liable to occur due to evaporation during the estimations, while it is possible that contamination is caused by ammonia from the atmosphere. From a study of the various results there appear to be 8 molecules of ammonia present in cytochrome c, as amide groups. This means that only a portion of the free carboxyl groups occurs as amides, as there is a total of 17 molecules of glutamic and aspartic acids.

The value of 0.52 molecules for tryptophan would appear to be low, but this may be due to the limitations of the method used for estimating it. Obviously further work must be done on this amino acid. Acid hydrolysis completely destroys tryptophan, and Spies and Chambers (1949) found that there are considerable losses of tryptophan when proteins are submitted to alkaline hydrolysis. There would appear, therefore, to be no really satisfactory method for estimating this amino acid, although Spies and Chambers (1949) obtained good recoveries using p-dimethylaminobenzaldehyde on intact proteins. From the present results there is probably only one residue of this amino acid in cytochrome c.

Comparison of the results from the present work with those of other workers.

Few analyses are reported in the literature concerning the amino acid composition of cytochrome c. In 1937 Keilin and Hartree made an examination of the basic amino

TABLE 13.

Comparison of the amino acid composition of Cytochrome c as determined by different workers. The values are given in moles of amino acid per mole of Cytochrome c.

Amino acid.	1.	2.	3.	4.	5.
Histidine	3	1	2	3	2-3.
Arginine	2	3	2	2	2
Lysine	22	13	18	16	18
Cystine	1	1	-	-	1
Tyrosine	4	2	3	2	4
Tryptophan	1	1	-	-	1
Glutamic acid	19	6	11	13	10
Aspartic acid		26* 32	7 18	6 19	7 17
Amide N	8	-	7	10	8
Leucine	9	9	5	5	5
Isoleucine		2 13	5 13	5 14	5 13
Phenylalanine		2	3	4	3
Alanine	33	3	5	4	5
Glycine		1	10	12	10
Valine		5	2	2	2
Hydroxyvaline		- 22	- 30	- 31	- 31
Threonine	-	2	9	9	9
Methionine	-	5	1	-	2
Proline	-	3	3	3	3
Serine	-	3	-	1	-

1. Theorell and Akeson (1941a).
2. Barbieri and Zamboni (1951).
3. Leaf and Pirrie (1951).
4. Buchanan (1952).
5. Present work.

* Asparagine.

acids of ox heart cytochrome c containing 0.34% iron. The methods for isolating and estimating the amino acids were not well developed at that time, with the result that the figures quoted by these authors are not very reliable. They recovered 9.1% lysine, which is very much lower than the more recent values of over 20%, while their results for histidine were much higher than the present figures.

The results obtained by different workers compared with those from the present work are shown in Table 13.

Theorell and Åkeson (1941a) estimated the amino acid composition of cytochrome c, from which the prosthetic group had been removed after hydrolysis. It is not clear whether these authors obtained their results from beef or horse heart cytochrome c as they appear to have regarded both as having the same composition. The material they used was of a high degree of purity, having been separated from contaminating material by electrophoresis. They used the classical methods of amino acid analysis, which were only suitable for estimating the basic amino acids and tyrosine, tryptophan and cystine. Comparing our results for these amino acids with those of Theorell and Åkeson, the biggest difference is found in the figures for lysine, 18 as against 22. We are inclined to place more reliance on our result as it was directly determined. Theorell and Åkeson calculated the amount of lysine from the difference between the nitrogen recovery for the other

basic amino acids and the total nitrogen of the basic fraction.

These workers estimated that there is one molecule of cystine present in the protein part of cytochrome c. This is apart from the two residues of cysteine which are involved in the linkage between the protein and the prosthetic group. Åkeson (1942) made a closer examination of the sulphur-containing amino acids in cytochrome c. He found that as well as the cysteine residues involved in thioether linkages between the protein and the prosthetic group there are probably the equivalent of two other cysteine residues in the molecule. These findings are in agreement with the present results.

We have determined that there are 4 molecules of tyrosine, which is the same as the value obtained by Theorell and Åkeson. In their results this figure is given as 5, which must be a misprint as their figure for the percentage nitrogen corresponds to 4 molecules of the amino acid.

The above authors isolated glutamic and aspartic acids and estimated them gravimetrically. Despite the difficulties involved in this procedure, their results are very close to the values obtained by us. They stated that they believed there was still part of this fraction not accounted for. The present results indicate, however, that their values were actually high, although their

material may have contained substances other than glutamic and aspartic acids.

The fraction which they isolated as leucine, isoleucine and phenylalanine gave a value of 9 molecules as against 13 found by us. This discrepancy is not surprising because, as stated by Theorell and Åkeson, only part of their leucine material was isolated.

The remaining amino acids, which these workers designated as alanine, glycine, valine and hydroxyvaline, were estimated to total 33 residues. This value was determined by subtracting the nitrogen of the fractions already determined from the total nitrogen, and therefore no direct measurements were made. Apart from the amino acids they mentioned, this fraction probably contained threonine, methionine, and proline. We estimate that these amino acids total 31 residues. Part of the "leucine" fraction was possibly contained in the 33 molecules found by Theorell and Åkeson. The total of 42 residues found by these workers for all the monoamino acids is in good agreement with the value of 44 found in the present work.

Åkeson (1942) determined the content of methionine per molecule of cytochrome c is 2 molecules, and our result, although low, is in fair agreement with this.

Theorell and Åkeson found 1 molecule of tryptophan, which our sole determination appears to support.

These authors estimated that there is a total of 143

atoms of nitrogen per molecule of cytochrome c, while the present work indicates a total of 133 - 136. These two values are probably in near agreement, because the former workers used a value of 13,000 for the molecular weight, while we used the value of 12,270 as calculated by Tint and Reiss (1950,b).

The results obtained by Barbieri and Zamboni (1951) bear little comparison to any of the other figures given. They claim that their estimations were carried out on purified cytochrome c, which contained 0.456% iron. The authors gave no experimental details apart from indicating that either chemical or microbiological methods were used in the determinations. The most striking difference is shown in their value for aspartic acid, which is very much higher than our estimate. These workers found 3 molecules of serine per molecule of cytochrome c, while in the present work we find that serine is absent from the pure material. There are also large discrepancies between many of the other determinations, and it is difficult to explain why this should be. However, we feel confident that the methods of Moore and Stein, which we have used, give results of a reasonable degree of accuracy, as has been proved by other workers using this technique for analysing various proteins.

We also show, for comparison with our results, the values obtained by Leaf and Pirrie (1951) for horse heart

cytochrome c on starch columns. These are very similar to our figures, and the few differences which occur are probably due to experimental error. We also quote the figures obtained by Buchanan (1952), who estimated the amino acid composition of beef heart cytochrome c on starch columns. There are some differences between his results and those described in the present work, the main ones being between glutamic acid, glycine and tyrosine. There also appears to be 1 molecule of serine in beef heart cytochrome c, and if these differences are significant it must be accepted that the proteins of beef and horse heart cytochrome c differ slightly from one another.

A study of the quantitative amino acid composition of cytochrome c reveals two characteristic features. The more striking is the high percentage of lysine in the molecule. There are no reports in the literature, to our knowledge, of a protein with such a high content of lysine. This explains the highly basic nature of the protein.

Most proteins contain serine in fairly large amounts, but this amino acid does not appear to be present in horse heart cytochrome c. The distribution of the other amino acids, however, is similar to that found in a number of other proteins.

S E C T I O N III.

THE INVESTIGATION OF SOME AMINO ACID

SEQUENCES IN CYTOCHROME c.

INTRODUCTION.

Knowledge of the overall amino acid composition of a protein does not provide information concerning the arrangement of the residues in the intact protein molecule, and in order to build up a picture of the fine structure of a protein, this information must be obtained.

At the present time the assumption is made that the amino acids are linked together in peptide chains, in the intact protein, by peptide bonds as postulated by Hofmeister (1902) and Fischer (1902). This means that in an open chain structure there will be an amino acid residue with a free amino group at one end of the chain, and one with a free carboxyl group at the other end.

The logical places to start the investigation of the amino acid sequences in a protein are at these positions.

Theorell and Åkeson (1941,a) found that there are 9 - 10 free amino groups, apart from the ε-amino groups of lysine in cytochrome c, while Blain (1950) found this value to be 6 - 7 amino groups. In the present work, therefore, an attempt has been made to investigate the nature of these amino groups.

After investigation of the free terminal groups in an amino acid chain, the next stage is to study the sequences of the amino acids in the chain. This is a formidable task to carry out on a large protein molecule, and this has not been attempted on cytochrome c. Tsou (1951,a), however, digested cytochrome c with pepsin and isolated from the

products formed an autoxidisable peptide PMc , which contains the haem prosthetic group. A study has been made of the free amino groups of this peptide, and preliminary experiments have been carried out to investigate the sequence of amino acids in it.

The identification of an N-terminal residue in an amino acid chain is carried out by altering the nature of the amino group of this residue in such a way that, on hydrolysis, it will be easily distinguished from all the other residues. This is generally achieved by treating the free amino group with a suitable reagent, which reacts quantitatively without altering the structure of the amino acid chain, and which is resistant to the conditions employed for hydrolysing peptide bonds. A number of reagents have been described for this purpose, which were used by the early workers. Fischer and Abderhalden (from Fox, 1945) frequently employed naphthalene-β-sulphonyl chloride, while Abderhalden and Stix (1923) investigated the use of 1;2:4 chlorodinitrobenzene, and prepared a number of dinitrophenyl (DNP) amino acid derivatives. The fact that elevated temperatures had to be used for the preparation of such derivatives, and the difficulties involved in separating the products, limited their use.

The most widely used labelling agent, and the one employed in the present investigations, is 1:2:4 fluorodinitrobenzene (FDNB), which was introduced by Sanger in 1945.

Because of the high reactivity of the fluorine atom, reaction proceeds at room temperature in alkaline solution, to give quantitative substitution, according to the author, but we have not always found this to be the case.

The DNP amino acid derivatives are easily detected, on account of their bright yellow colour, and a very comprehensive literature has grown up dealing with their separation by both paper and column chromatography. One disadvantage is that partial destruction of some of the DNP amino acids takes place during hydrolysis, but corrections can be applied for this. Two principal artefacts, dinitroaniline and dinitrophenol, are formed, but these can generally be easily detected and do not interfere with the results.

This technique has been applied to a number of proteins (e.g. Sanger, 1945; Porter and Sanger, 1948). It has been used, with outstanding success, for studying the products of the partial hydrolysis of insulin by Sanger and Tuppy (1951a, b) and Sanger and Thompson (1953 a,b).

New reagents for labelling N-terminal residues have been described, but so far none of these has been so extensively employed as FDNB.

Other methods for detecting free amino groups have been used, such as deamination of the terminal amino acid with nitrosyl chloride (Consden, Gordon and Martin, 1947), but Sanger and Thompson (1953a) found that dinitrophenylation

gives more clear cut results.

An ideal technique for studying the sequence of a chain of amino acid residues would be one by which the labelled N-terminal residue could be split off and identified, leaving the rest of the chain intact. The procedure could then be repeated until each amino acid had been identified. A method, along these lines, has been devised by Edman (1949,1950), in which the free amino group reacts with phenylisothiocyanate to form a phenylthiocarbamyl (PTC) derivative. On cleavage the phenylthiohydrantoin is formed which can be identified by paper chromatography. Additional proof of identity of the amino acid can be obtained by hydrolysing the phenylthiohydrantoin and identifying the free amino acid formed.

The method has been used by Fraenkel-Conrat and Fraenkel-Conrat (1951) for determining the N-terminal residues of insulin, while Ottesen and Wallenberger (1952) have applied it successfully to a number of synthetic peptides.

This technique has not been employed in the present work. Leaf (1954) was able to identify the N-terminal residue of PMc, using this method. He could not proceed further with the identification of the amino acid sequence on account of the insolubility of the PTC derivative in the reagents used for fission. Thompson (1954) has reported similar difficulties in his work on serum albumins.

All the present experiments have involved the identification of the N-terminal amino acid residues, but methods have been developed for examining the C-terminal residues. Chibnall and Rees (1951) and Fromageot and his co-workers (1950) independently worked out a method for identifying C-terminal residues by reducing the free carboxyl groups, after esterification, to the corresponding alcohols with metal hydrides. After hydrolysis the amino alcohols were identified on paper chromatograms.

Carboxypeptidase can be used for the splitting of the C-terminal amino acid from a peptide chain, by which means Lens (1949) identified the C-terminal residues of insulin.

After identification of the terminal residues in a protein or peptide, the sequence of amino acids is best studied by partially hydrolysing the molecule, under suitable conditions, and examining the products formed.

It has long been known that different peptide bonds in proteins and peptides have different degrees of stability to hydrolysing agents, and provided sufficiently mild conditions are used, the substrate will be broken into smaller units without being completely degraded to amino acids. Ideally, a number of selective hydrolytic agents should be used, which will preferentially attack different bonds, thus giving rise to "overlapping" sub-units.

Partial hydrolysis for studying amino acid sequences, has been employed frequently in early work, as shown in the

review by Synge (1943). The results obtained prior to the last 10 - 15 years were rather scanty on account of the inadequacy of the techniques for fractionating and investigating the products of hydrolysis. The introduction of chromatographic and other methods has, however, led to the rapid advancement of our knowledge of the sequences of amino acids in proteins and peptides.

Consden, Gordon and Martin (1947) devised a method for the separation of small peptides into groups by ionophoresis in silica gel. The groups were further fractionated on two dimensional paper chromatograms. The amino acid sequences of the peptides were studied by examining the products of hydrolysis, with and without deamination of the N-terminal residues. In this way the structure of the cyclopentapeptide gramicidin S was elucidated (Consden, Gordon, Martin and Synge, 1947). Using the same techniques Consden, Gordon and Martin (1949) identified a number of acidic peptides from partial hydrolysates of wool, a much more formidable task than working with a small peptide.

The most successful investigation of amino acid sequences, by separating the products of partial hydrolysates and examining their structures, has been achieved by Sanger and his co-workers. Their brilliant work led to a complete knowledge of the amino acid sequences in the molecule of beef insulin. Their success has stimulated work in this field, and the structures of at least two of the naturally

occurring peptides have also been worked out, e.g. the elucidation of the structure of beef vasopressin by Acher and Chauvet (1953,1954) and du Vigneaud et al. (1953), working independently.

In the present work we have employed essentially the same techniques as Sanger and his associates.

EXPERIMENTAL, RESULTS and DISCUSSION.

The N-Terminal Amino Acid Residues of Cytochrome c.

Preparation of DNP - Cytochrome c.

The DNP derivative of a purified sample of horse heart cytochrome c was prepared by the method used by Sanger (1945) for preparing DNP-insulin.

At the same time the DNP derivative of the "apo-enzyme" of cytochrome c was prepared, after removal of the prosthetic group by the action of silver nitrate, as described by Paul (1950).

To test the completeness of the reaction, a portion of the aqueous fraction of the DNP-cytochrome c hydrolysate, after removal of the ether-soluble products, was run on a two dimensional paper chromatogram. n-Butanol-acetic acid-water and phenol, saturated with water, were used as solvents. Free lysine was found to be absent from the hydrolysate, suggesting that the reaction with FDNB was complete.

Hydrolysis of DNP-Cytochrome c.

50 mg. of DNP-cytochrome c were hydrolysed with 5N-HCl

under reflux for 4 hours. A fairly large amount of insoluble material was still present at the end of this period. If hydrolysis was carried out for 24 hours, a clear solution was obtained but there was considerable loss in the yields of the ether-soluble DNP amino acids.

Examination of the DNP hydrolysate of cytochrome c.

The aqueous DNP hydrolysate of cytochrome c was extracted several times with 0.5 ml. quantities of ether. A small fraction of the ether extract, corresponding to 800 µg. of DNP-cytochrome c, was run on a one dimensional buffered paper chromatogram using tert.-amyl alcohol, saturated with 0.2 M pH 6 potassium phthalate (Blackburn and Lowther, 1951), as solvent. Three spots were obtained, apart from dinitrophenol and dinitroanaline. These appeared to correspond to DNP-alanine, DNP-serine and DNP-glutamic acid. When known DNP derivatives of these amino acids were run, together with a sample of the ether-solube fraction from the DNP cytochrome c hydrolysate, no new spots were obtained. This showed that the DNP amino acids had probably been correctly identified.

The separation of the ether-soluble DNP amino acids was carried out on columns of silica gel. The gel was prepared according to the method described by Tristram (1946). Ether saturated with 0.5 M pH 6.5 sodium phosphate buffer was used as solvent.

4g. of silica gel, mixed with 2 ml. of 0.5 M pH 6.5

phosphate buffer, which had been saturated with ether, were thoroughly ground in a mortar. The silica gel was then made into a slurry with the ether solvent and poured into a glass tube. After being well packed the column was ready for use.

The DNP ether soluble amino acids were evaporated to dryness, dissolved in 0.5 ml. of the buffered ether solvent, and placed on the column, which was then developed with the buffered ether. Four bands separated out, which were eluted from the column, and a small portion of each was taken for identification on paper chromatograms. The fastest band (R = 2.25) consisted of a mixture of dinitroaniline and dinitrophenol, the second (R = 0.26) was DNP-alanine, the third (R = 0.15) was DNP-serine, and the slowest, (R = 0.025) which was forced through by the addition of 1% n-butanol to the ether, was identified as DNP-glutamic acid.

Each fraction was extracted into 10 ml. of 1% sodium bicarbonate, and the concentration of the DNP amino acid in the solution measured in the Spekker absorptiometer. A standard curve was prepared at the same time using 20 µM to 100 µM solutions of DNP-valine, in 20 µM intervals.

Each DNP amino acid has approximately the same absorption properties at a given concentration, and therefore a curve, using DNP-valine only, gave satisfactory results.

The results of the quantitative measurements on the

TABLE 14.

DNP amino acids obtained from the hydrolysate of DNP-Cytochrome c.

DNP amino acid.	Recovery. (Moles of DNP amino acid per mole of DNP-Cytochrome c.)
DNP-alanine	0.098
DNP-serine	0.09
DNP-glutamic acid	0.015

TABLE 15.

DNP amino acids obtained from the hydrolysate of DNP-"apo-Cytochrome c."

DNP amino acid.	Recovery. (Moles of DNP amino acid per mole of DNP-Cytochrome c.)
DNP-alanine	0.082
DNP-serine	0.08
DNP-glutamic acid	0.019.

ether soluble DNP amino acids of whole DNP-cytochrome c are shown in Table 14. The results for the DNP-"apo-cytochrome c", which were obtained in the same manner, are shown in Table 15. It can be seen that the two sets of values parallel each other very closely.

The most striking feature of the results is the very low yield of the DNP amino acids. This cannot be due to destruction of the DNP derivatives during hydrolysis, because corrections were made for this when calculating the values. It was thought that there was perhaps a high rate of destruction of the DNP amino acids caused by the presence of DNP-cytochrome c in the hydrolysis mixture.

A recovery experiment was carried out in which 1.5 micromoles of DNP-alanine were refluxed with 3 ml. of 6 N - HCl for four hours. The acid mixture was extracted with ether and the extract run through a column of celite 535, using ether saturated with 0.2 M pH 6.5 phosphate buffer as solvent. The recovery of DNP-alanine was 90%. A second experiment was performed in which a mixture of 1.5 micromoles of DNP-alanine and 28 mg. of DNP-cytochrome c was refluxed and the acid mixture subsequently treated in the same manner as before: the recovery of DNP-alanine was found to be 90.6%. This not only indicates that the DNP protein did not cause destruction of the DNP-alanine, but that negligible amounts of this DNP amino acid are present in DNP-cytochrome c.

The cytochrome c which had been used to prepare the DNP-

cytochrome c had not been purified on Amberlite IRC - 50. It appears to be significant that DNP-serine was one of the ether-soluble DNP amino acids identified. This amino acid does not appear to be present in pure cytochrome c, and therefore it is concluded that probably all the DNP amino acids isolated are associated with small amounts of impurities attached to the cytochrome c. This would account for the low yields obtained.

An examination of the aqueous fraction of the DNP-cytochrome c hydrolysate on two dimensional paper chromatograms did not reveal the presence of any DNP amino acids apart from ε-DNP-lysine. From these results cytochrome c does not appear to possess any free amino groups, or at least any which react with FDNB, apart from the ε-amino groups of lysine.

Discussion.

It is very likely that the DNP amino acids recovered from the DNP-cytochrome c preparation were derived from impurities and not from DNP-cytochrome c itself.

The conclusions to be reached from this work are that either cytochrome c is a cyclic protein, which has no free N-terminal amino groups, or if it has N-terminal amino groups they are not able to react with FDNB.

The first conclusion is not in agreement with the results obtained by Theorell and Åkeson (1941a). They found, by the nitrous acid method, 9 - 10 free amino groups apart from the

ε-amino groups of lysine, in beef heart cytochrome c. Blain (1950), using the same method, found 6 - 7 free amino groups, other than the ε -amino groups of lysine, in horse heart cytochrome c. Buchanan (1952), on the other hand, reacted the free amino groups of beef heart cytochrome c with the acidic dye Orange G, as described by Fraenkel-Conrat and Cooper (1944). He could find no evidence for the presence of free basic groups other than those of lysine, arginine and histidine.

Desnuelle, Rovery and Fabre (1951) treated beef serum albumin with FDNB and were only able to isolate one molecule of DNP-aspartic acid per molecule of the protein. Previously Brand, Kassel and Saidel (1944) had found 10 free amino groups, other than the ε -amino groups of lysine, as determined by the Van Slyke method. It would appear from these results that the nitrous acid method gives values far in excess of the number of free amino groups present.

A similar situation has arisen in the present work. It may be concluded that the Van Slyke method gives excessively high results with cytochrome c and that there are probably no free amino groups, apart from the ε-amino groups of lysine, in the molecule.

It is unlikely that the FDNB was unable to react quantitatively with the free amino groups in cytochrome c, because it was able to combine with all the ε-amino groups of lysine.

It is interesting that the removal of the prosthetic group from the protein moiety of cytochrome c has not led to the appearance of any new amino groups. If the conclusions of Theorell and Åkeson (1941d) are correct, no free amino groups would be released on removal of the prosthetic group. Two imidazole groups of histidine should be available for reaction with FDNB, but there was no evidence of these in the present work.

It appears from the above results that cytochrome c is a cyclic protein, although it may have a number of terminal carboxyl groups, but these were not investigated. There is evidence that a number of other proteins exist which have no terminal amino groups. Among these are ovalbumin and chymotrypsinogen (Desnuelle and Casal, 1948) and myosin and tropomyosin (Bailey, 1951)

Estimation of the Extent of Hydrolysis of Cytochrome c with Pepsin.

Before preparing PMc it was decided to study the extent and rate of hydrolysis of cytochrome c with pepsin. A number of methods are available to measure these, but none were entirely satisfactory for use in this work. The Van Slyke nitrous acid method appears to give values in excess of the free amino groups present. Direct titration using an indicator is also not suitable because the end point is difficult to determine on account of the red colour of cytochrome c. The ninhydrin method of Moore and Stein (1948)

is probably the best for studying the action of pepsin in cytochrome c, and was the one mainly used in these studies.

A digestion mixture was set up as follows:

1.5 ml. of cytochrome c solution (24 mg./ml.)
2.3 ml. of water
1.2 ml. of 0.3 N-HCl (to bring the pH to 1.6 as measured with a glass electrode)
1.0 ml. of pepsin solution (1 mg./ml.)

The pepsin was found to have an activity of 0.37 haemoglobin units per mg. of pepsin nitrogen, as determined by the method of Anson (1938).

Blank estimations were carried out for both cytochrome c and pepsin. It was found that a final concentration of 140 µg. of cytochrome c in each tube gave a sufficiently low blank. The amount of pepsin in each tube did not increase the colour yield to an appreciable extent, and therefore no corrections were required, to allow for its presence.

After the addition of the pepsin to the digestion mixture, aliquots were withdrawn at various intervals and neutralised with the calculated amount of 0.1 N-NaOH. A small fraction of the neutralised solution was diluted to 4 ml. with 0.1 M pH 7 sodium phosphate buffer. The absorption of this solution, reduced with dithionite, was measured at 550 mµ in the Unicam S.P. 500 spectrophotometer.

The main bulk of the neutralised solution was treated with ninhydrin, as described in Section II.

Results.

It was found that hydrolysis was very rapid for the first 10 minutes (Fig. 6a). The rate of hydrolysis slowed down after this and appeared to be complete by the end of 90 minutes. No appreciable increase was detected after a further 24 hours. A small increase in the rate of hydrolysis was noted if a further 1 mg. of pepsin was added after the first 40 minutes, but this did not appear to increase the final extent of hydrolysis.

From the results it was estimated that 9 -10 peptide linkages are broken per molecule of cytochrome c. Differences of the order of one amino group liberated by hydrolysis cannot be detected by this method. We therefore had to be satisfied with estimating when hydrolysis was complete, as it is not possible to measure the absolute extent of hydrolysis using this technique.

In calculating the extent of hydrolysis the assumption is made that the amino groups of both peptides and amino acids react to the same extent with ninhydrin. Moore and Stein (1948) showed that certain dipeptides give the same colour yield as the amino acid with ninhydrin, but it is possible that larger peptides give lower values.

It would appear from the results that approximately 10% of the peptide bonds are split by the hydrolysis of cytochrome c with pepsin.

Tsou (1951a) observed that on the addition of pepsin to cytochrome c the absorption at 550 mμ, measured at pH 7, fell

Fig. 6a.

Graph showing the rate of hydrolysis of cytochrome c with pepsin.

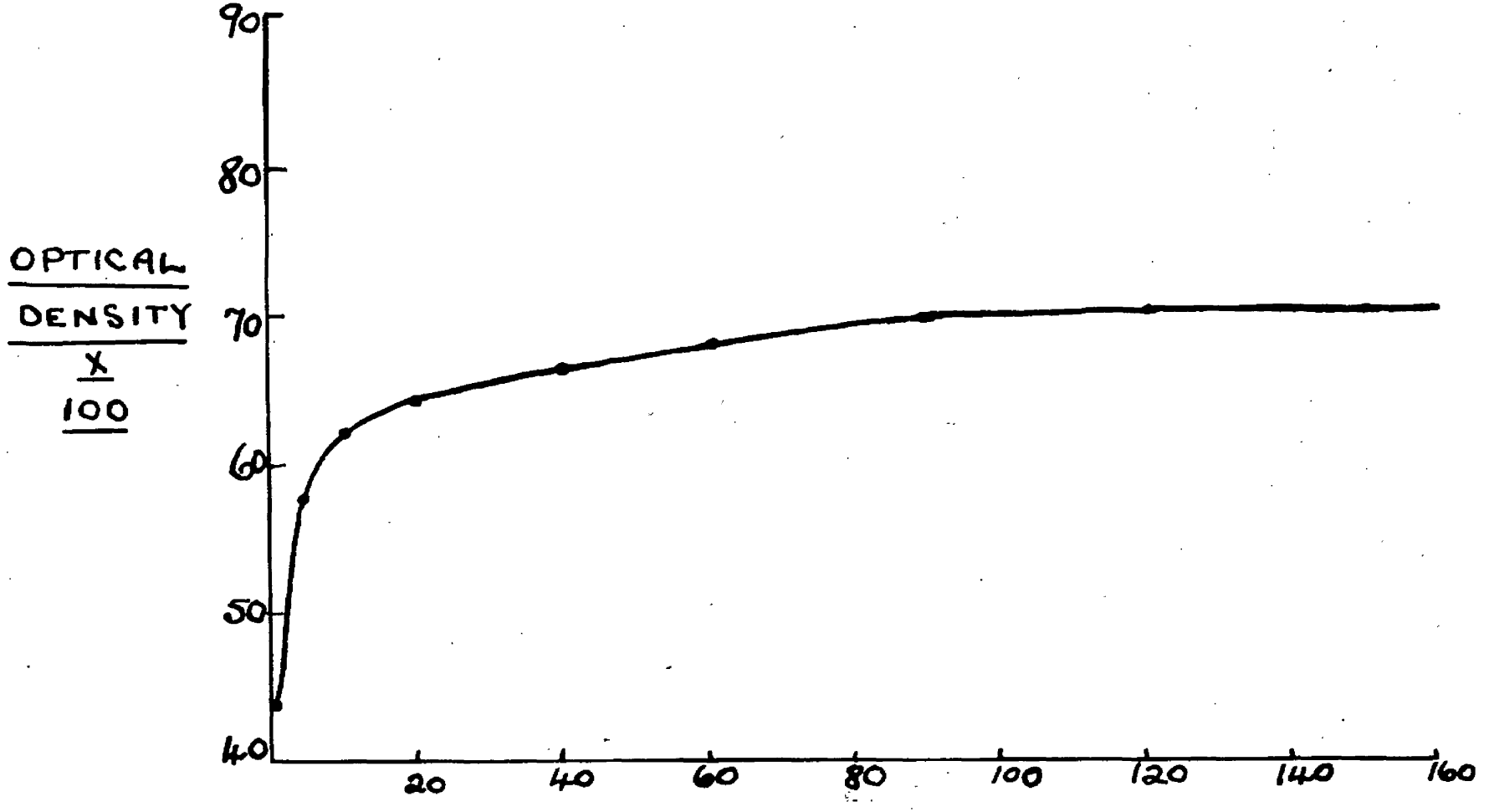

OPTICAL DENSITY X 100
90
80
70
60
50
40
20
40
60
80
100
120
140
160
TIME (MIN.)

FIG. 6(a)

Fig. 6b.

Graph showing the fall in absorption at 550 mμ when pepsin acts on cytochrome c. Measurements were made on samples of the hydrolysis mixture after reduction with dithionite.

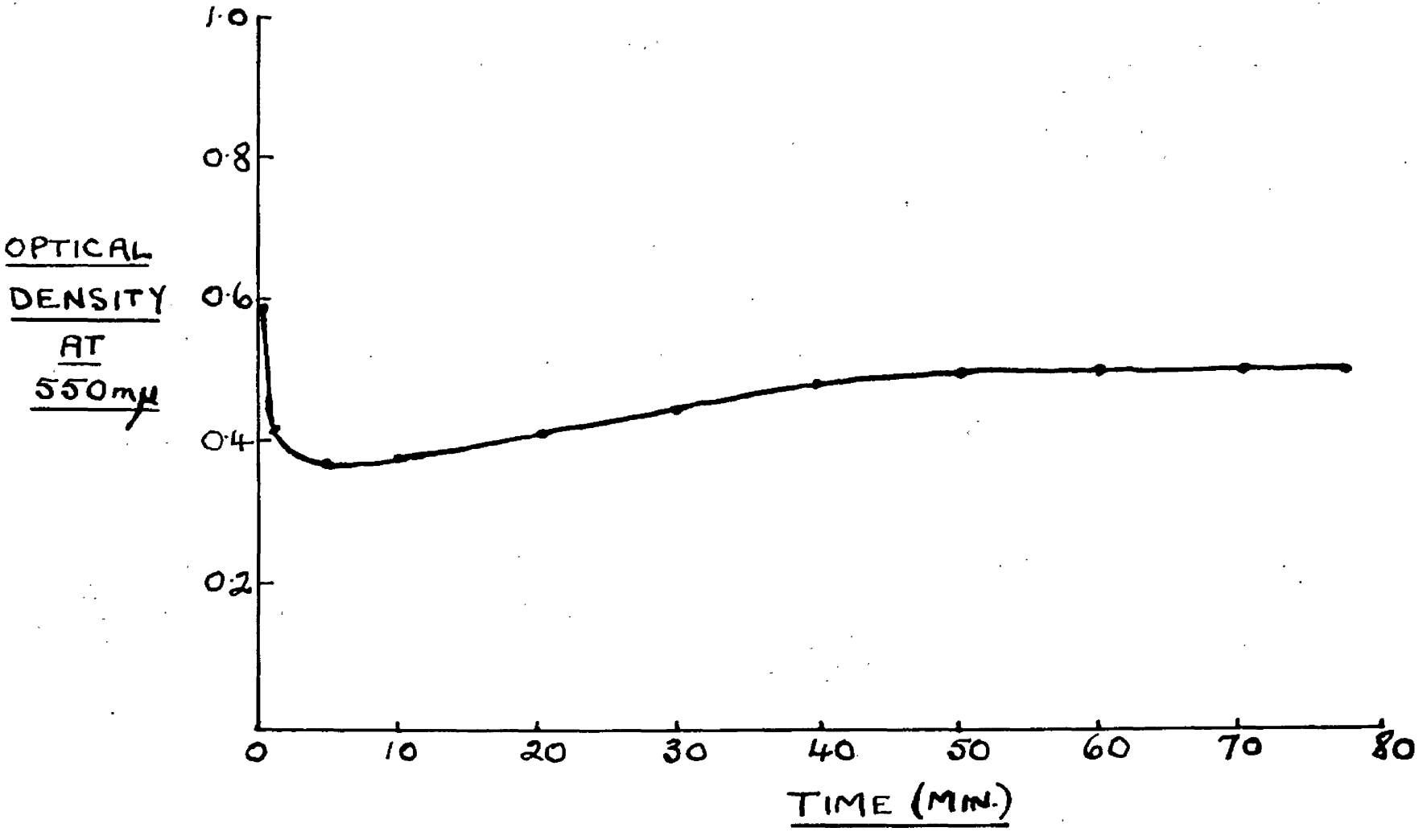

1·0
0·8
0·6
0·4
0·2
OPTICAL DENSITY AT 550mμ
0
10
20
30
40
50
60
70
80
TIME (MIN.)

FIG. 6(b).

rapidly, within a few minutes to a value only 60% of that given by the original solution. This absorption rose again to about 90% of the original within an hour. We have observed the same phenomenon (Fig. 6b) but, in general, the absorption did not rise again to a value as high as 90% of the original. This may have been partly due to the fact that the digestion was carried out at room temperature, but it also may mean that the end product of digestion is different from that obtained by Tsou. There is no explanation for these observations at the present time.

Preparation of Pepsin Modified Cytochrome c (PMc).

Having determined the extent and rate of hydrolysis of cytochrome c by pepsin, PMc was prepared according to the following scheme.

400 mg. of purified cytochrome c were dissolved in water to give a 1% solution, and 9 ml. of 0.3 N - HCl added to bring the pH to 1.6 as measured with a glass electrode. 5 mg. (185 haemoglobin units) of pepsin were then added in 1% solution, the mixture shaken, and then allowed to stand for 6 hours at room temperature. A second addition of 5 mg. of pepsin was made, and the mixture left for a further 18 hours. At the end of this time the solution was neutralised with the calculated amount of 1 N - NaOH, and the PMc isolated as described by Tsou (1951a). After the peptide was precipitated a second time, it was washed with 0.01 M pH 5 phthalate buffer, ethanol and ether, and allowed

to dry at room temperature.

Only about 8 mg. of PMc were obtained from each preparation, which represents a yield of 2%. A large amount of red-coloured material was left in solution after the first dialysis against 0.01 M pH 5 phthalate buffer, and therefore major losses probably occur at this stage. There may only be slight differences between this soluble material and the substance which we have isolated as PMc. However, in order to restrict the present studies to a product obtained by as standard a method as possible, and therefore of reasonably constant composition, all the soluble material was discarded.

In later experiments it was noted that preparations of PMc were not completely soluble in 0.03 N - HCl. All the red-coloured material appeared to dissolve, but a dark grey insoluble fraction could be separated off by centrifugation.

The Homogeneity of Preparations of PMc.

In preparing a peptide, such as PMc, it is possible that the material isolated will not be completely homogeneous. The PMc preparation was submitted to ionophoresis on paper in order to study the homogeneity of the material.

Sufficient PMc was applied, in aqueous solution, along a line across the centre of a strip of Whatman No. 1 filter paper (50 cm. x 5 cm.), to give a strongly coloured band. The paper was then placed in an ionophoresis apparatus, similar to that described by Durrum (1950), in 0.05 M pH 9.4 ammonium acetate/ammonia buffer and current passed at 260 volts with a voltage gradient of 5.2 volts/cm.for 18 hours.

Ionophoresis was carried out in the cold room at 4° to cut down evaporation of water from the paper. On completion of ionophoresis the paper was removed, dried in a cabinet in a stream of air, and then placed in a vacuum desiccator over H_2SO_4 overnight to remove all traces of ammonia.

The PMc was found to have separated into three red-coloured bands, 1A, 2A and 3A, which had all migrated towards the anode, the distances moved being 1.5 cm., 4 cm. and 8 cm. respectively. 2A was by far the most concentrated band. The paper was then treated with starch-iodide reagent, after exposure to chlorine gas (Rydon and Smith, 1952) but no other peptides could be detected.

From these experiments it would appear that PMc is not homogeneous. Attempts were made to separate PMc preparations on an Amberlite IRC - 50 column at different pH's but these were not successful. However, the important point is that no new colourless peptides appear to be present in the preparations. Fraction 2A forms the bulk of the coloured material, and it is likely that the fractions 1A and 3A differ very slightly from it. It was therefore decided to use the PMc samples as prepared, for further work, without attempting to fractionate them.

Properties of PMc.

On account of the small amount of material available, it was not possible to carry out a complete analysis of PMc. The iron content was found to be 2.77%, which gives a mole-

cular weight of 2,112, as compared with the value of 2,500 estimated by Tsou (1951b). The absorption spectrum of the reduced compound was found to be essentially the same as that of reduced cytochrome c, with the ε mol. at 550 mμ calculated to be 21,000, which is lower than that for cytochrome c.

A qualitative examination of PMc was made by running acid hydrolysates on two dimensional paper chromatograms using n-butanol-acetic acid-water and phenol, saturated with water, as solvents. Serine was found in a few of the preparations, but only those which did not appear to contain serine were used for studying the amino acid sequences in PMc. The amino acids present in a typical preparation, free from serine, are shown in Table 16. There was no evidence of the presence of proline, tyrosine and arginine. Methionine would be situated in the same position as valine, but later work showed that this amino acid was absent from PMc. Phenylalanine was not generally detected, but it did occur in a few of the preparations. Histidine could not be detected by the Pauli reaction (Dent, 1948) on two dimensional paper chromatograms, possibly because of the difficulty of completely removing phenol from the paper, which interferes with the reaction. However, histidine was detected by the Pauli reaction on one dimensional paper chromatograms of PMc hydrolysates, using n-butanol-acetic acid-water as solvent. Tryptophan would be destroyed by

TABLE 16.

Qualitative amino acid composition of PMc.

Amino acid.	Intensity of Spot.
Leucines	±
Valine	++
Alanine	+++
Threonine	+
Glycine	+
Glutamic Acid	+++
Aspartic Acid	±
Cystine	+
Lysine	+++

TABLE 17.

Amino acid composition of acid-soluble and acid-insoluble PMc fractions as determined by paper chromatography.

Amino acid.	Intensity of Spot. Acid-soluble.	Acid-insoluble.
Leucines	+	+++
Phenylalanine	-	±
Valine	++	++
Tyrosine	-	±
Proline	-	+
Alanine	++	+
Threonine	+	+
Glycine	+	+
Serine	-	++
Glutamic acid	+++	++
Aspartic acid	Trace	±
Arginine	-	±
Lysine	+++	+

acid hydrolysis, but Tsou (1951b) showed that it is probably not present in PMc.

When it was found that part of the PMc preparation was insoluble in dilute HCl, samples of the acid-soluble and acid-insoluble fractions were hydrolysed and examined on two dimensional paper chromatograms. The results, shown in Table 17, indicate that there are appreciable differences between the two fractions. The acid-insoluble material contains a number of amino acids not found in PMc, while the acid-soluble fraction has a composition very similar to that of the serine-free samples of PMc already examined. It is noteworthy that serine is only present in the acid-insoluble fraction, and it would appear, therefore, that the serine found in other PMc samples is derived from a non-PMc impurity, which can be removed by fractionation with dilute HCl. It is possible that this impurity is derived from the pepsin used for the preparation of PMc. Pepsin is known to contain a fairly high percentage of serine.

The Quantitative Amino Acid Composition of PMc.

Only one complete estimation of the quantitative amino acid composition of PMc was carried out. This was performed by Leaf (1952). The hydrolysate of PMc was prepared in the usual manner, and the amino acids separated on a starch column using the solvent system 1:2:1 n-butanol-n-propanol-0.1 N - HCl followed by 2:1 n-propanol-0.5 N HCl. The fractions were estimated by the ninhydrin method of Moore

TABLE 18.

Quantitative amino acid composition of PMc.

amino acid.	Moles of amino acid per mole of PMc.	
Leucine		2
Isoleucine		
Phenylalanine		
Valine	2	
Alanine	2	5
Glutamic acid	3	
Threonine	1-2	
Glycine	1	
Ammonia	3	
Lysine	3	
Histidine	1	
Cystine	0.17	

and Stein (1948). The results of the analysis are shown in Table 18. The leucine, isoleucine and phenylalanine fraction was contained in one band. It is therefore not certain that phenylalanine was actually present in this preparation of PMc. Glutamic acid and alanine were also contained in one band, but it was possible to partition them, after examining the results of another separation of the amino acids of PMc on the 100 cm. Dowex 50 column, on which these two amino acids are obtained as separate bands.

The N-Terminal Residues of PMc.

DNP-PMc was prepared by dissolving 4.7 mg. of the acid-soluble PMc in 1 ml. of 0.1% trimethylamine and treating the solution with FDNB according to the method described by Sanger and Thompson (1953a). The mixture was shaken for 8 hours. The DNP-PMc remained in solution but was precipitated out by the addition of a few drops of glacial acetic acid. The precipitate was washed with water, ethanol and ether, and allowed to dry at room temperature.

The DNP material was suspended in 1.5 ml. of 6 N - HCl and hydrolysed in a sealed tube in an air oven at 105° for 18 hours. The acid solution was then extracted three times with 0.5 ml. amounts of ether. A fraction of the ether extract was run on a one dimensional buffered paper chromatogram with tert.-amyl alcohol as solvent (Blackburn and Lowther, 1951). Three yellow spots were obtained, one of which was in the position of DNP-valine. The other two

were attributed to dinitrophenol, because both faded on exposure to HCl vapour (Sanger and Thompson, 1953a).

The remainder of the DNP ether-soluble material was run through a small column of celite 535 using ether, saturated with 0.5 M pH 5.3 sodium phosphate buffer, as solvent. There appeared to be no separation on the column and the DNP material was eluted in one fraction. This material was run, with suitable markers, on a buffered one dimensional paper chromatogram when three spots were obtained. The fastest was due to dinitroaniline, the second was in the position of DNP-valine, while the third was found to be dinitrophenol.

The spot in the position of DNP-valine was cut from the paper and eluted with water. The dinitrophenyl group was removed by means of baryta, as described by Mills (1950). Attempts were made to identify the free amino acid on a one dimensional paper chromatogram with n-butanol-acetic acid-water as solvent. These were unsuccessful because it was difficult to remove all the barium carbonate, formed after precipitation of the barium with CO_2, and this interfered with the separation of the spots on the paper. Some peptide material was derived from the paper during elution of the DNP spot, and this made positive identification of valine impossible. Aknown sample of DNP- valine, treated in the same way, gave no better results, and identification of the free amino acid had to be abandoned.

Examination of the aqueous fraction of the DNP-PMc hydrolysate on the usual two dimensional paper chromatograms did not reveal the presence of any DNP amino acids apart from ε-DNP-lysine. It was noted that some free lysine was also present, which showed that dinitrophenylation was not complete.

Similar results were obtained with several samples of the DNP derivative prepared from different batches of PMc.

From the present work it would appear that valine is the sole N-terminal residue in PMc. Leaf(1954), using the method of Edman (1950), has identified valine as the only N-terminal residue in PMc, which is in agreement with the present results.

The Partial Hydrolysis of PMc.

Preparation of Partial Hydrolysates of PMc.

Acid Hydrolysates.

Approximately 4 mg. of PMc were used for each hydrolysis, 1 ml. of 12 N - HCl was added, and the mixture placed in an air oven at 37° for the desired length of time. On completion of hydrolysis, the HCl was evaporated off in a vacuum desiccator over NaOH, the residue suspended in water and evaporated to dryness six times to remove completely all HCl. Sanger and Thompson (1953a) used silver oxide to precipitate bound HCl. Although we did not do this, the subsequent separations by ionophoresis appeared to be satisfactory.

Tryptic Hydrolysate.

To 3.2 mg. of PMc dissolved in 1.3 ml. of water was

added 0.4 mg. of trypsin dissolved in 0.5 ml. of 0.05 M pH 8.4 amonium acetate buffer. The amount of trypsin added was equivalent to 1.88×10^{-4} haemoglobin units, as determined by the method of Anson (1938). The mixture was allowed to stand at room temperature for 21 hours, after which the reaction was stopped by placing the hydrolysate in a boiling water bath for 10 minutes. The solution was placed directly on a paper strip on which ionophoresis was carried out.

Fractionation and Identification of the Peptides Produced after the Partial Hydrolysis of PMc.

The initial separations of the products of the partial hydrolysis of PMc by paper ionophoresis were carried out in the apparatus similar to that described by Durrum (1950). The separations were performed at 4° in the cold room.

Samples of approximately 4 mg. of hydrolysate were placed along a line across the middle of a strip of 3 MM Whatman paper (50 cm. x 7 cm.). The separation was carried out in 0.05 M pH 7 ammonium acetate buffer at an average of 200 volts with a voltage gradient of 4 volts/cm. for 16 hours. The strip was then placed in a stream of air until dry and left overnight in a vacuum desiccator over H_2SO_4 and NaOH to remove all traces of the buffer.

In the first experiment two narrow strips were cut from the paper, one being sprayed with ninhydrin, while the other was saturated with chlorine gas and sprayed with starch-iodine reagent, according to the method of Rydon and Smith

(1952) for detecting peptides. The latter method did not give very satisfactory results, only a few peptides being detected by it, and in later experiments the peptides were located by the ninhydrin method only.

The ninhydrin-positive areas on the narrow strip were noted and the corresponding regions cut from the main strip. The cuts were pointed at one end and the peptide material eluted with water into a capillary tube drawn out at both ends. An apparatus similar to that described by Sanger and Tuppy (1951a) was used for this purpose. The eluate from each capillary tube was placed on a small polythene disc and evaporated to dryness in a vacuum desiccator.

Further fractionation of the peptides was carried out on two dimensional chromatograms. One drop of 75% ethanol was used to extract the material from each polythene disc. The extract was drawn into a Carlsberg micropipette and approximately one-fifth placed on one sheet of Whatman No. 1 filter paper (31 cm. x 19 cm.), which was used as a marker chromatogram, while the remaining four-fifths were placed on another sheet of the same size. This process was repeated twice to ensure that all the material was extracted from the disc.

The chromatograms were run as described in Section II, developing them in the long direction with n-butanol-acetic acid-water and at right angles to this with phenol saturated with water. The marker sheets were sprayed with ninhydrin.

After spraying, peptides and amino acid spots in low concentration were frequently difficult to detect on account of the bluish background produced when traces of phenol were present on the paper. Phenol was therefore removed as completely as possible by washing the paper thoroughly with ether, before spraying, when much improved results were obtained.

The positions of the spots were noted on the marker sheets and the corresponding areas cut from the main sheet. These were eluted with water. One-half of the eluate was evaporated to dryness on a polythene disc and the residue dissolved in 0.1 ml. of 6 N-HCl. The acid solution was sucked into a capillary tube, which was then sealed off and hydrolysis allowed to proceed in an air oven at 105° for 18 hours. The hydrolysate was evaporated to dryness on a polythene disc. The residue was transferred to a sheet of chromatography paper after extracting it from the disc in three 0.1 ml. portions of 75% ethanol. The chromatogram was developed as before and the amino acids identified after spraying with ninhydrin.

The remaining half of the eluate was placed in a small test tube (7.5 cm. x 1 cm.), evaporated to dryness, and the DNP derivative of the peptide prepared according to the method of Sanger and Thompson (1953a), except that the reaction was allowed to proceed for 18 hours, instead of 2 hours as used by these authors. After the extraction of

excess FDNB with ether, the DNP material was evaporated to dryness, dissolved in 0.1 ml. of 6 N-HCl and hydrolysed as previously described. The hydrolysate was evaporated down on a polythene disc and then examined on a paper chromatogram.

In early experiments it was decided to attempt to identify the N-terminal residue of each peptide by noting its disappearance on chromatograms developed with n-butanol-acetic acid-water and phenol saturated with water. However, this proved unsatisfactory because the free amino group of the N-terminal residue did not react quantitatively with FDNB, and it was sometimes extremely difficult to detect reduction in intensity of any of the amino acid spots. Attempts were then made to determine the N-terminal residue by positive identification of the DNP amino acid as well as examining the amino acid spots present. The DNP hydrolysate was run on a descending, buffered chromatogram using buffered tert.-amyl alcohol as solvent (Blackburn and Lowther, 1951). Suitable DNP amino acid markers were also applied to the paper, which were chosen according to the amino acids identified in the unlabelled hydrolysate. After identifying the DNP derivatives present, the paper was developed in a direction at right angles to the first in phenol-ammonia. The paper was dried, washed with ether and sprayed with ninhydrin. The amino acid spots were fairly difficult to identify because they did not separate very well in tert.-amyl alcohol. After spraying, a deep blue background developed when the

paper was heated, presumably due to the presence of the phthalate buffer. In many cases, however, examination of the DNP amino acid and the free amino acid spots led to identification of the N-terminal residue of the peptide.

During this work it was noted that only in a few cases did dinitrophenylation appear to be complete, using the method of Sanger (1945), Schroeder and LeGette (1953) experienced the same difficulty. These authors modified the method so that the reaction proceeded to the extent of 90%. In the present work experiments were carried out on very small amounts of material. Sodium bicarbonate therefore could not be used as it would be difficult to remove it from the small amounts of the DNP derivatives formed. The later method of Sanger and Thompson (1953a), as stated above, did not give quantitative reaction. Lysine should always be converted into bis-DNP-lysine or ε-DNP-lysine according to its position in the peptide, but free lysine was found in a number of instances after reaction with FDNB. It is possible that the DNP group was split from some of the DNP derivatives during hydrolysis. Experiments with free amino acids, however, showed that the reaction with FDNB was incomplete.

Results.

Experiment P.

In this experiment a four-day partial acid hydrolysis was carried out on a purified sample of PMc.

Fig. 7 (a) shows the position of the ninhydrin-positive

areas found after the ionophoretic separation of the products of hydrolysis. The regions in which peptide material was detected by the method of Rydon and Smith (1952) are shown in Fig .7 (b). It can be seen that the reacting areas on the two strips did not correspond exactly. This is probably due to the fact that ninhydrin reacts more sensitively with small peptides and amino acids. Larger peptides are preferentially detected by the chlorine starch-iodide method. The red material, which had been applied at the origin, tended to migrate towards the anode, but the humin did not move at all.

Eight regions containing peptide material were identified as shown, and the main paper strip was cut up according to their positions. The red material which was present in P4 could not be eluted from the paper with water. It was found that fractions P1 and P4 did not give rise to ninhydrin-positive spots after fractionation on paper chromatograms. Unfortunately there was insufficient time to study these further. The remaining six fractions were separated on duplicate chromatograms. The marker chromatograms were sprayed with ninhydrin and the positions of the spots obtained are shown in Figs. 11 - 16.

Some of the spots were very faint and it was decided to examine the hydrolysates, corresponding to these, on one dimensional chromatograms using n-butanol-acetic acid-water as solvent. However, the results obtained were very unsatis-

factory. This was partly due to the inadequate separation of the amino acid spots and partly due to the presence of traces of a peptide which was eluted from Whatman No. 1 filter paper. This peptide was found to consist of glycine, threonine and glutamic acid. No reliance could therefore be placed on the results from these chromatograms. In later experiments two dimensional chromatograms were always used for studying the structures of fractions giving weak spots. There were no indications of the interfering peptide on these, presumably due to the small amount of the peptide present.

In this experiment attempts were made to identify the N-terminal residues by observing the reduction in intensity of any of the amino acid spots. This did not prove to be absolutely satisfactory, as mentioned previously.

The results obtained are presented in Tables 19 - 24, which require no further explanation. The abbreviations used for the amino acids are those suggested by Brand and Edsall (1947). The conventions used for describing the structures of peptides are the same as those employed by Sanger and Tuppy (1951a).

Experiment Q.

A second four-day acid hydrolysate was carried out on another sample of purified PMc.

The ninhydrin-positive fractions obtained after ionophoresis are shown in Fig. 8. Only two fractions were indicated by the chlorine starch-iodide reagent, and it was decided to abandon its use.

The main paper strip was cut into seven fractions, which were eluted with water. The fractions were further separated on two dimensional chromatograms into the spots shown in Figs. 17 - 23.

In this experiment attempts were made to identify the N-terminal residues of some of the fractions on buffered papers using tert.-amyl alcohol and phenol ammonia as solvents. The results were more satisfactory than those previously obtained. Where the intensities of the amino acid spots in the unlabelled hydrolysate were very low, no attempts were made to identify the N-terminal residue.

The results are presented in Tables 25 - 31.

Experiment M.

From the two previous experiments it was observed that hydrolysis was fairly extensive, as a number of free amino acids and dipeptides were obtained. In order to obtain a number of larger peptides it was decided to decrease the duration of hydrolysis to 24 hours.

No clear cut separation of the products of hydrolysis was observed after spraying a strip from the paper on which ionophoresis had been carried out. The main paper strip was therefore cut into nine arbitrary regions as shown in Fig. 9.

After elution and separation of the eluates on two dimensional chromatograms, fractions M1, M2, M7, M8 and M9 were found to contain no ninhydrin-positive material. The

spots obtained from the other fractions, of which M5 and M6 were combined, are shown in Figs. 24 - 26. The compositions of these fractions are recorded in Tables 32 - 34.

It can be seen from the results that hydrolysis was still considerable after 24 hours. The amount of free amino acids was reduced, however, and a number of larger peptides were obtained.

Experiment T.

Bergmann and Fruton (1941), working with synthetic peptide substrates, showed that trypsin splits the bonds linking the carboxyl group of lysine with the amino group of another amino acid. As PMc contains three residues of lysine, it seemed of interest to hydrolyse it by digestion with trypsin.

The peptides obtained from such a digestion were separated by paper ionophoresis into the fractions shown in Fig. 10. Fraction T1 contained all the red material originally applied to the paper but it did not contain any ninhydrin-positive material. Fractions T2, T3 and T4 were separated on two dimensional chromatograms into the spots shown in Figs. 27 - 29. The amino acid compositions of these spots are listed in Tables 35 - 37.

A larger number of peptides were obtained than were expected. Cleavage should only have occurred at three points, but the results show that six peptides were formed by the action of trypsin on PMc. It would appear, therefore that the trypsin used was contaminated with another enzyme, possibly chymotrypsin.

Fig. 7a.

Diagram of the ninhydrin-positive fractions obtained after separating the products of hydrolysis in experiment P by paper ionophoresis.

Fig. 7b.

Diagram of the fractions which were detected by the method of Rydon and Smith (1952) after separating the products of hydrolysis in experiment P by paper ionophoresis.

Fig. 8.

Diagram of the ninhydrin-positive fractions obtained after separating the products of hydrolysis in experiment Q by paper ionophoresis.

Fig. 9.

Diagram showing the arbitrary fractions into which the paper strip was divided after separating the products of hydrolysis in experiment M by paper ionophoresis.

Fig. 10.

Diagram of the fractions obtained after separating the products of hydrolysis in experiment T by paper ionophoresis. T1 was red-coloured, while T2, T3 and T4 were the ninhydrin-positive fractions.

In all cases the material was applied to the paper in the region indicated by the dotted line.

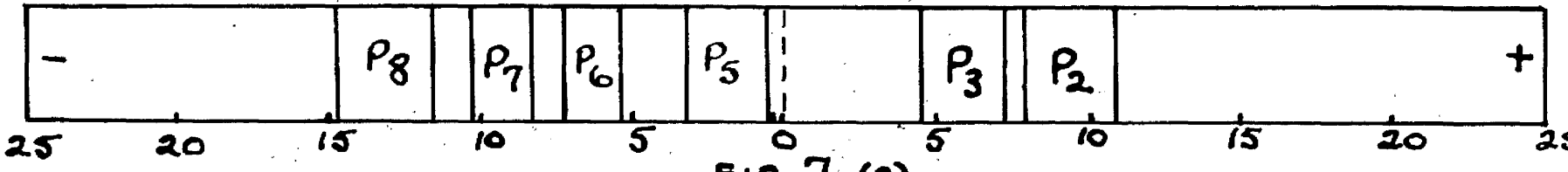


FIG. 7 (a)

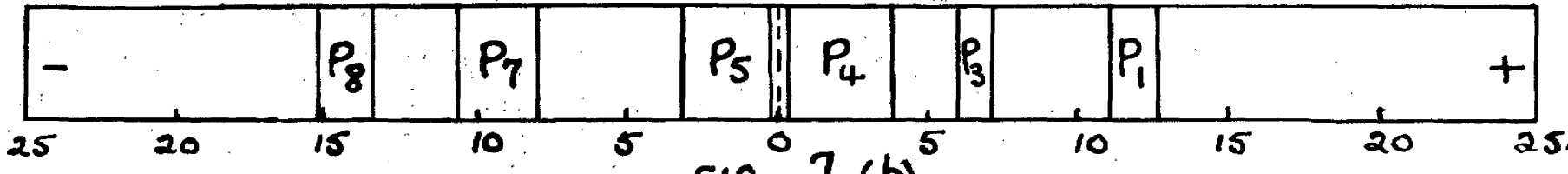


FIG. 7 (b)

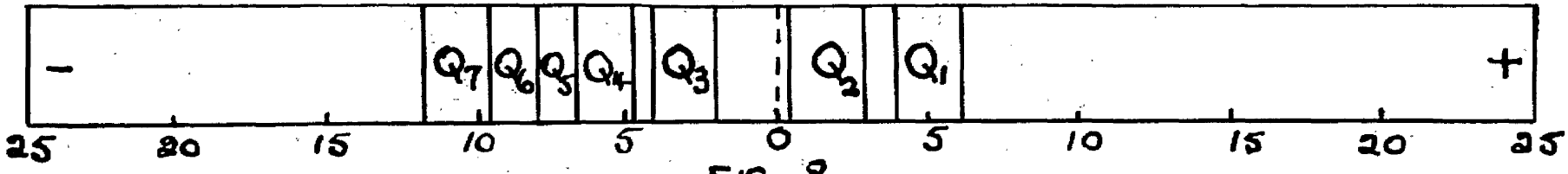


FIG. 8

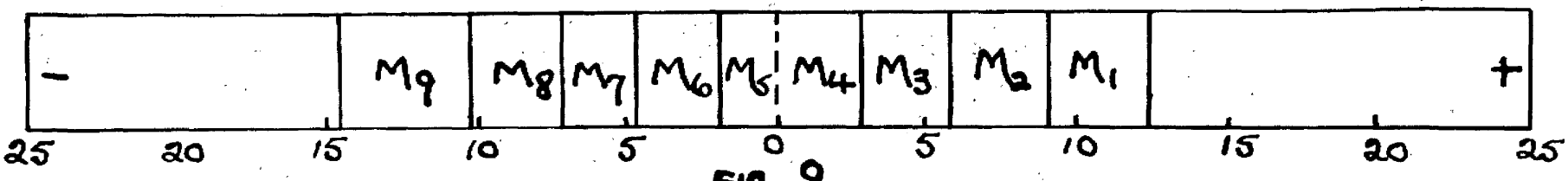


FIG. 9

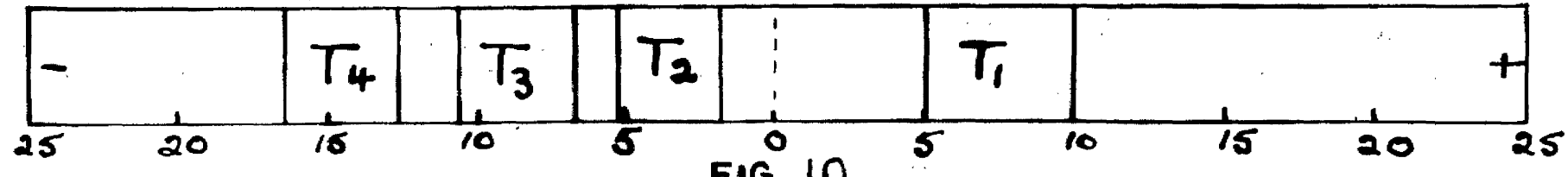


FIG. 10

Fig. 11.

Chromatogram of fraction P2.

In all the chromatograms, the abscissae represent the R_F values in n-butanol-acetic acid-water and the ordinates, the R_F values in phenol-ammonia.

The fractions were applied in the position marked by the small dotted circle.

Fig. 12.

Chromatogram of fraction P3.

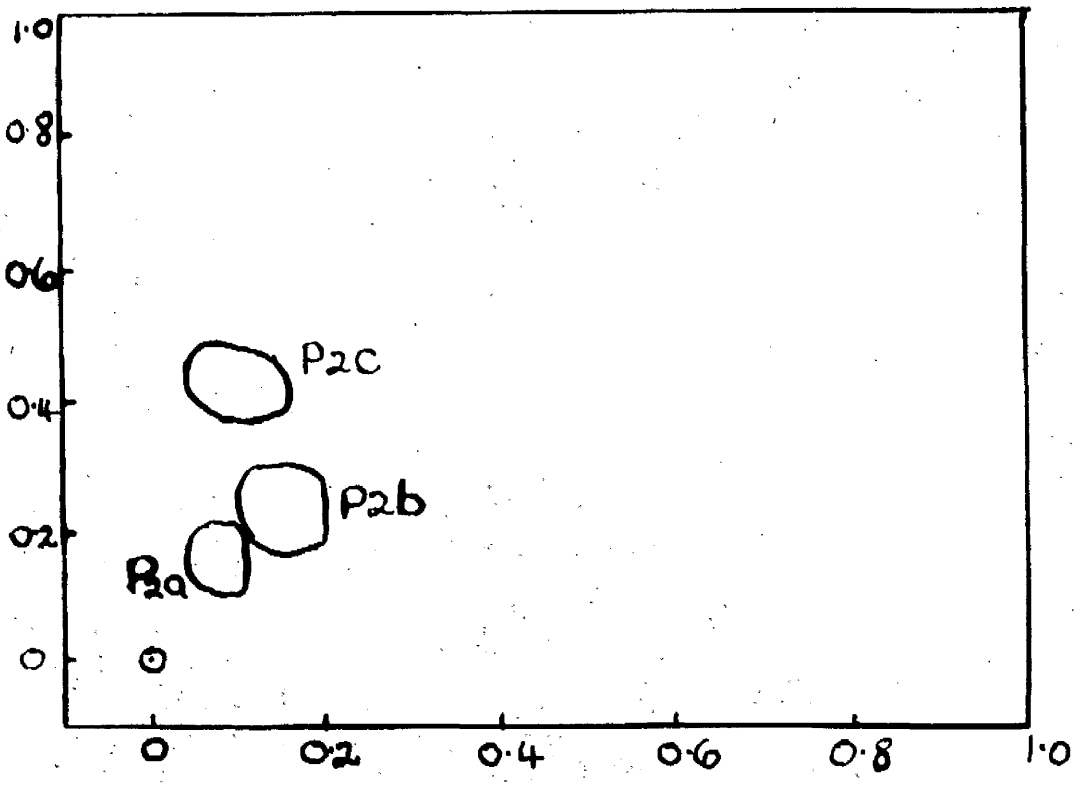

1·0
0·8
0·6
0·4
0·2
0
P2c
P2b
P2a
0
0·2
0·4
0·6
0·8
1·0

FIG. 11.

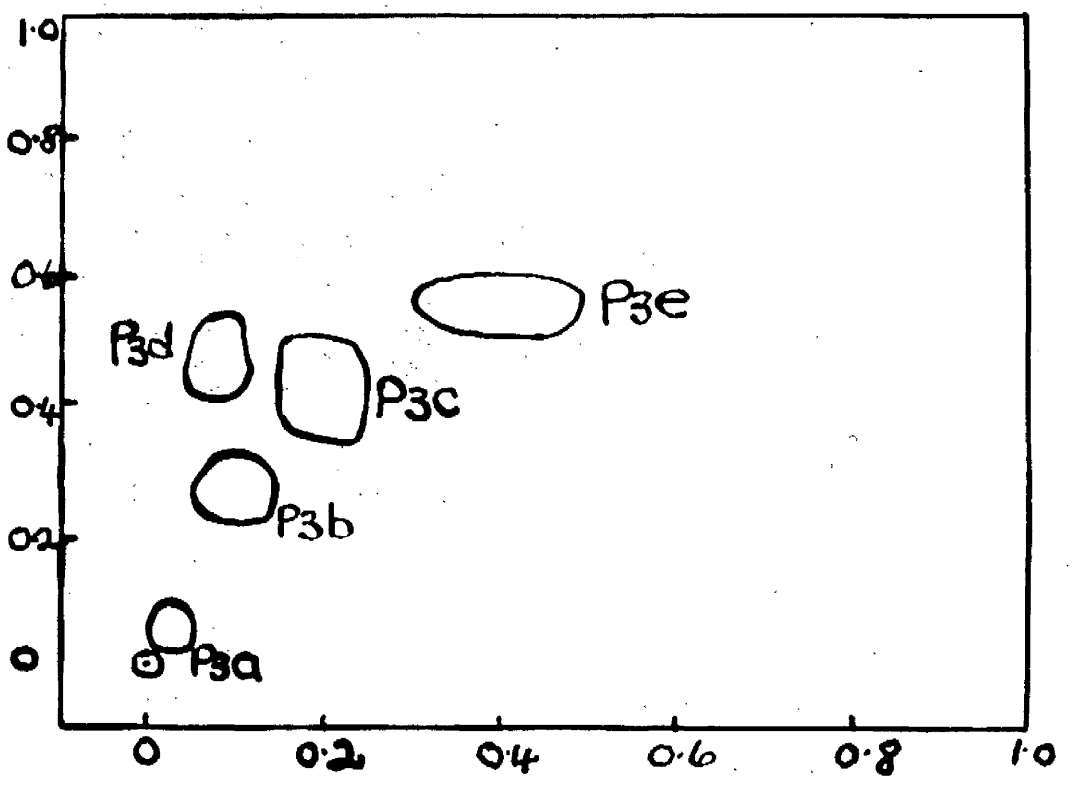

1·0
0·8
0·6
0·4
0·2
0
P3e
P3d
P3c
P3b
P3a
0
0·2
0·4
0·6
0·8
1·0

FIG. 12.

Fig. 13.

Chromatogram of fraction P5.

Fig. 14.

Chromatogram of fraction P6.

Fig. 15.

Chromatogram of fraction P7.

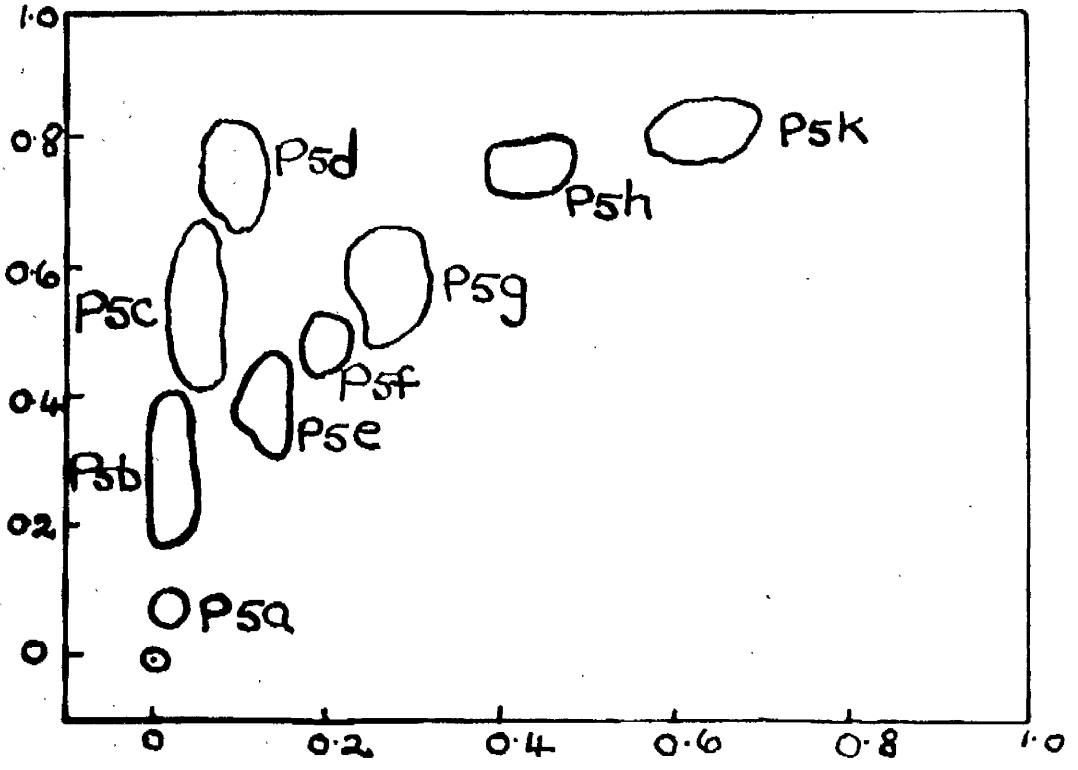


FIG. 13.

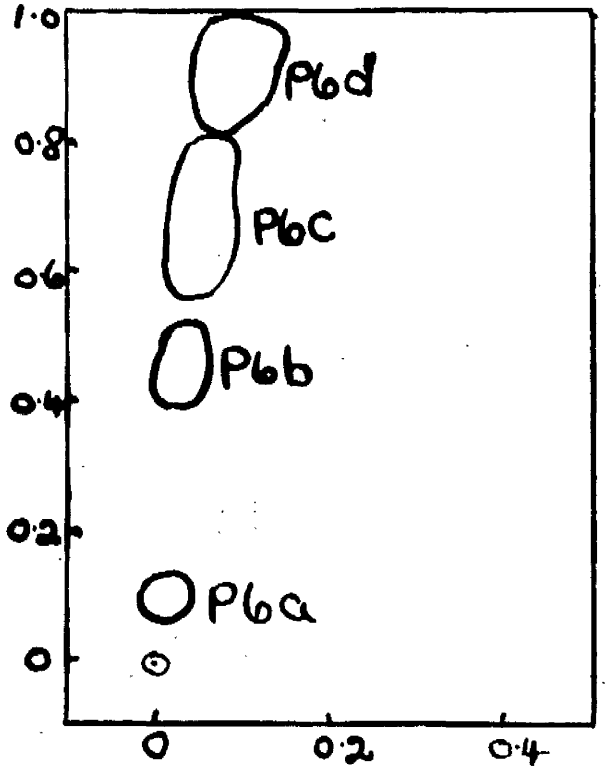


FIG. 14

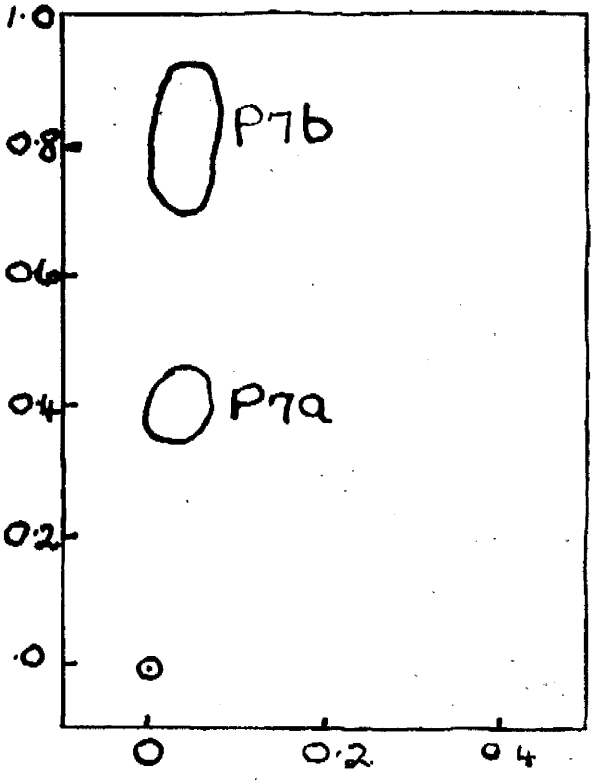


FIG. 15

Fig. 16.

Chromatogram of fraction P8.

Fig. 17.

Chromatogram of fraction Q1.

Fig. 18.

Chromatogram of fraction Q2.

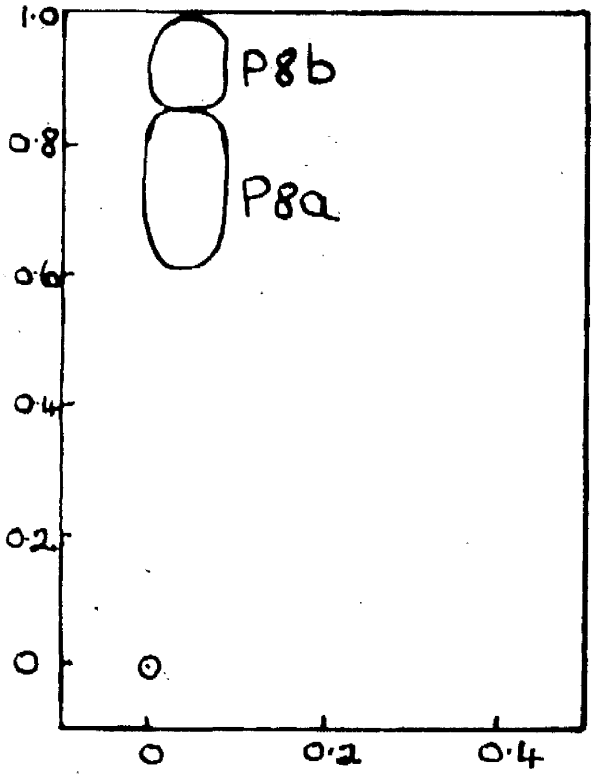


FIG. 16.

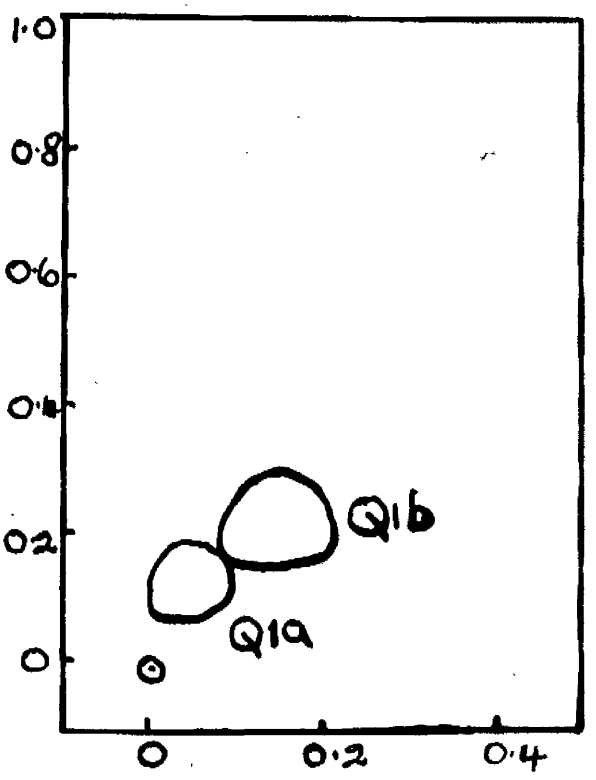


FIG. 17.

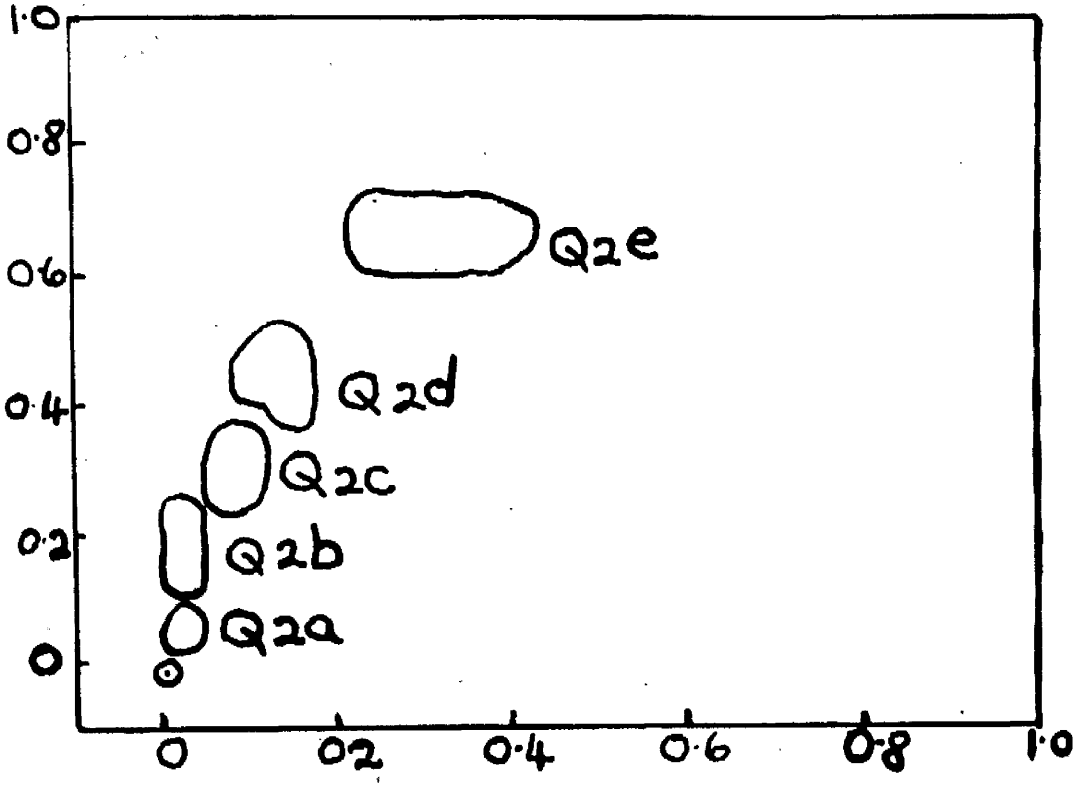


FIG. 18.

Fig. 19.

Chromatogram of fraction Q3.

Fig. 20.

Chromatogram of fraction Q4.

Fig. 21.

Chromatogram of fraction Q5.

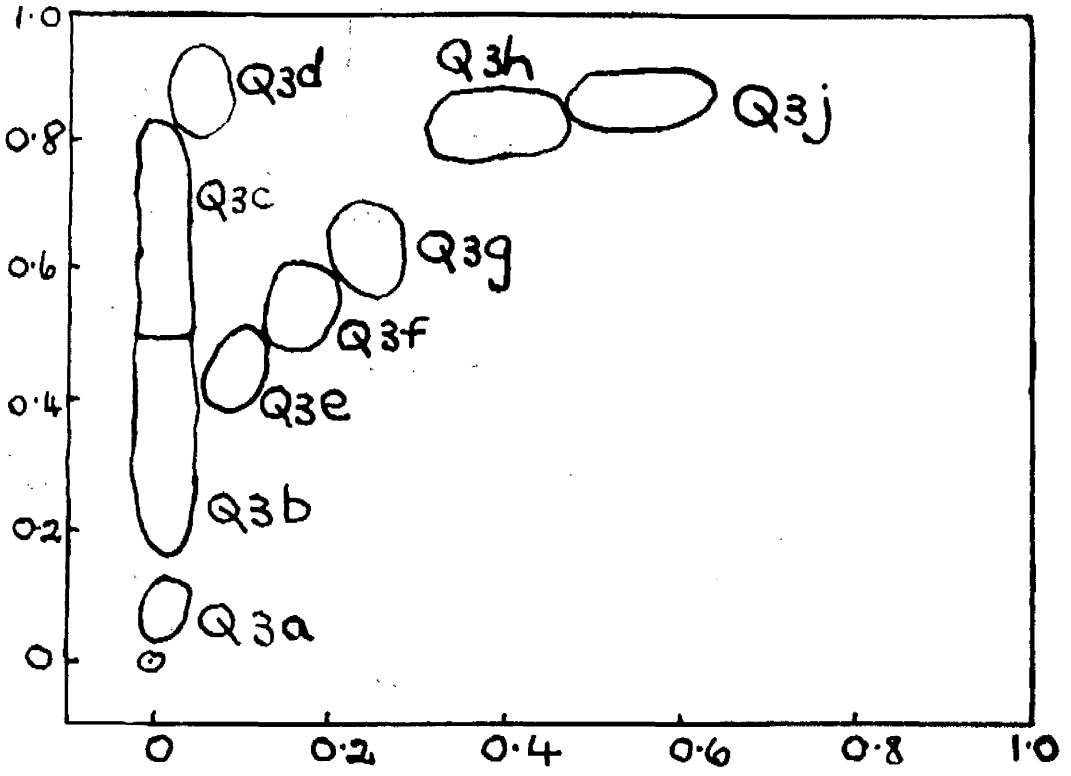


FIG. 19.

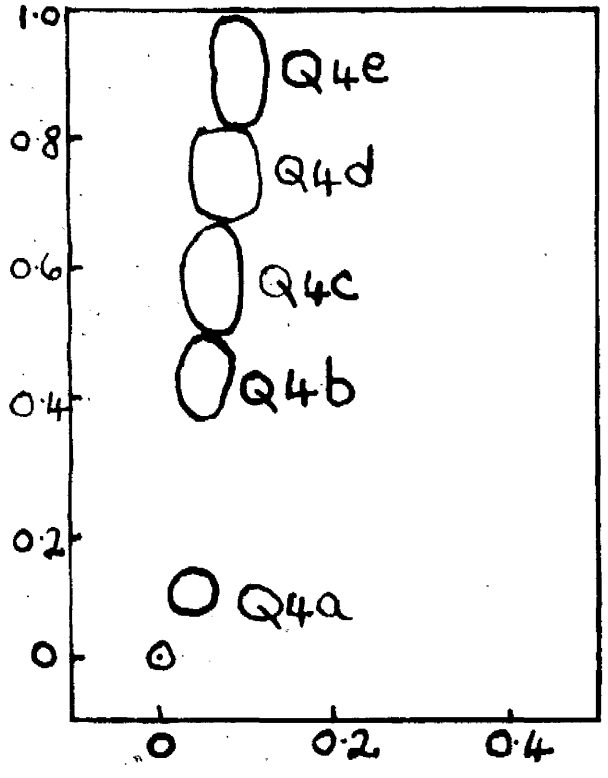


FIG. 20.

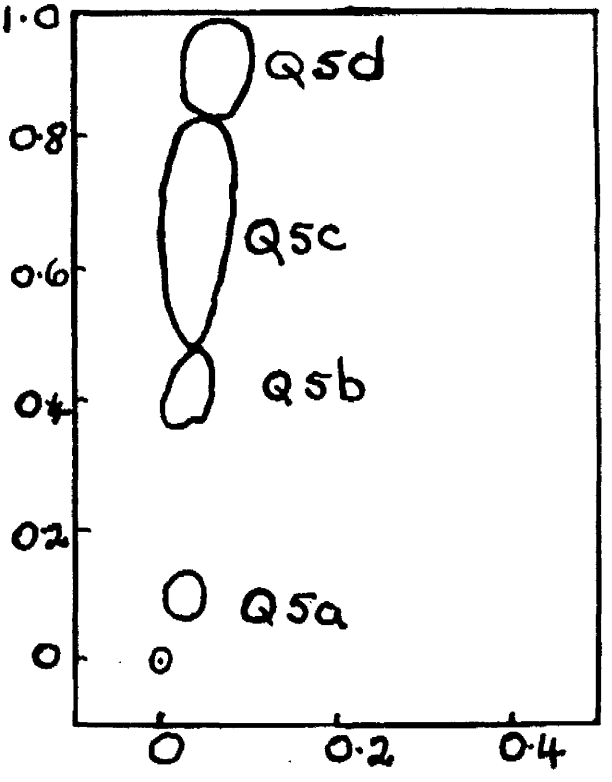


FIG. 21.

Fig. 22.

Chromatogram of fraction Q6.

Fig. 23.

Chromatogram of fraction Q7.

Fig. 24.

Chromatogram of fraction M3.

Fig. 25.

Chromatogram of fraction M4.

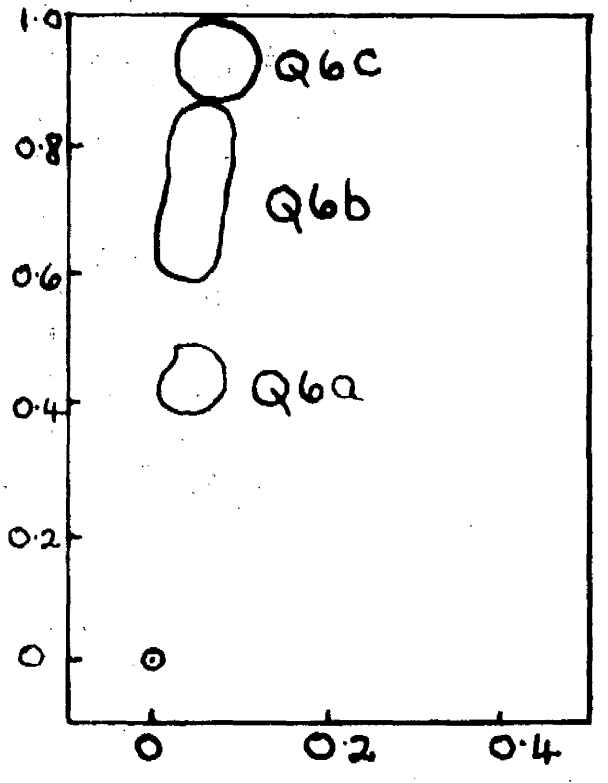


FIG. 22.

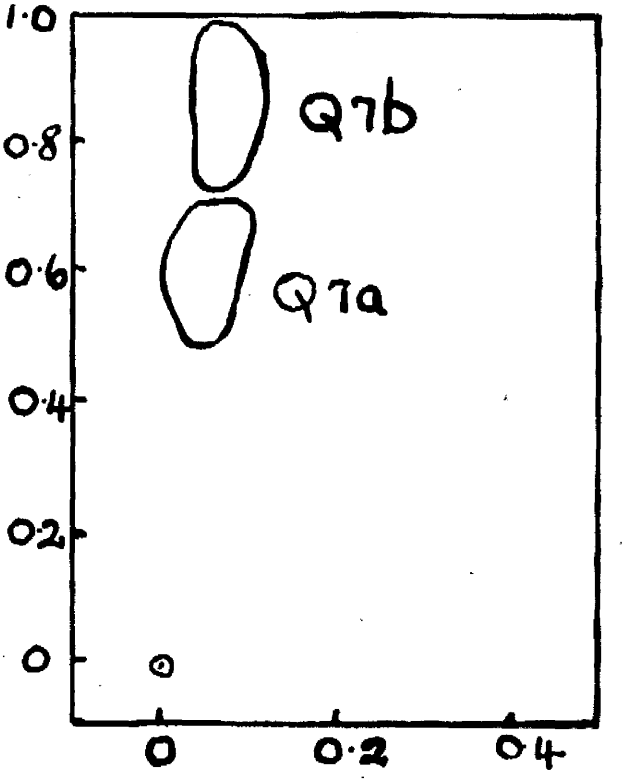


FIG. 23.

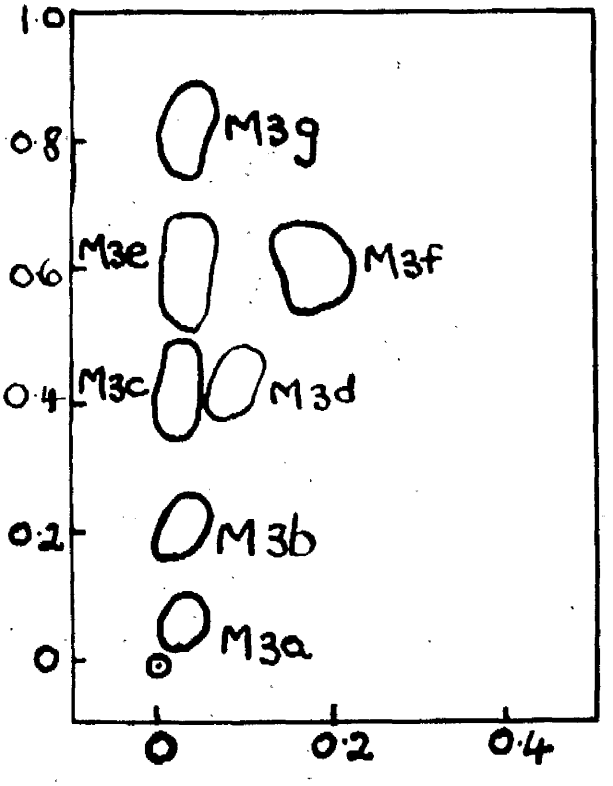


FIG. 24.

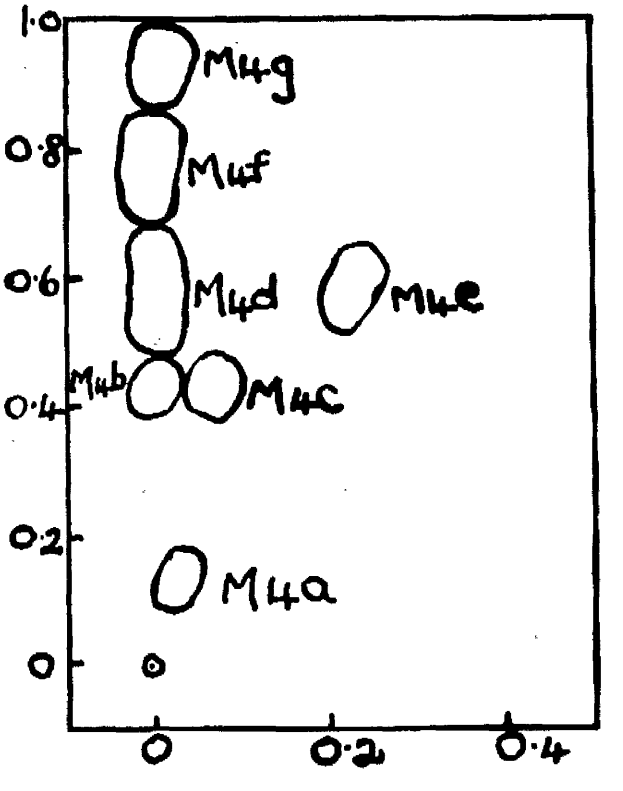


FIG. 25.

Fig. 26.

Chromatogram of fraction M5 & 6

Fig. 27.

Chromatogram of fraction T2.

Fig. 28.

Chromatogram of fraction T3.

Fig. 29.

Chromatogram of fraction T4.

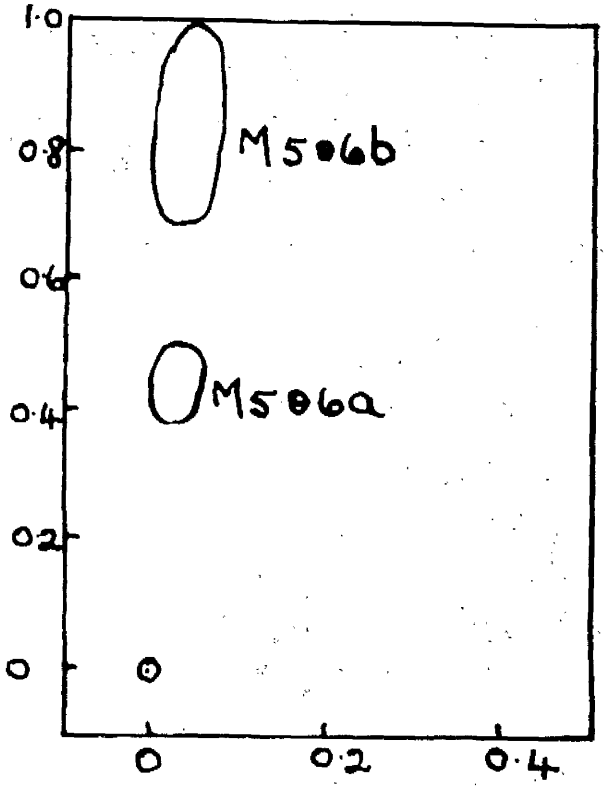


FIG. 26

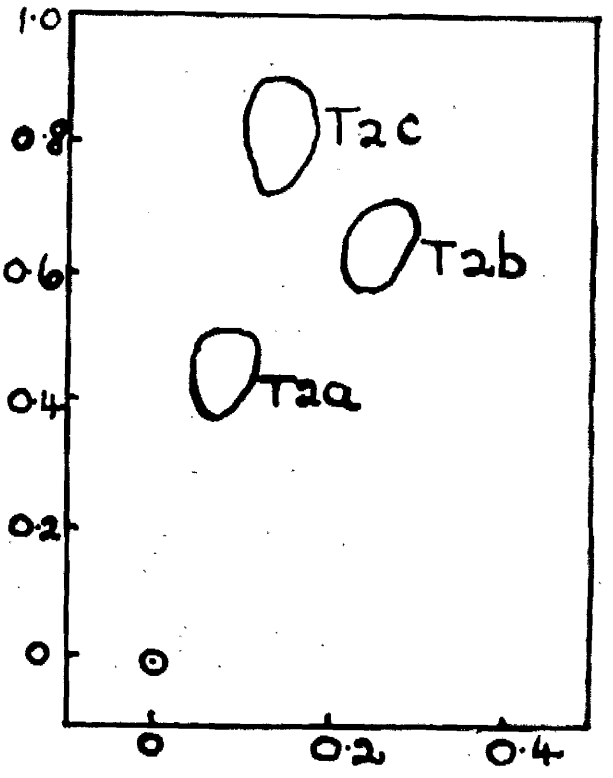


FIG. 27

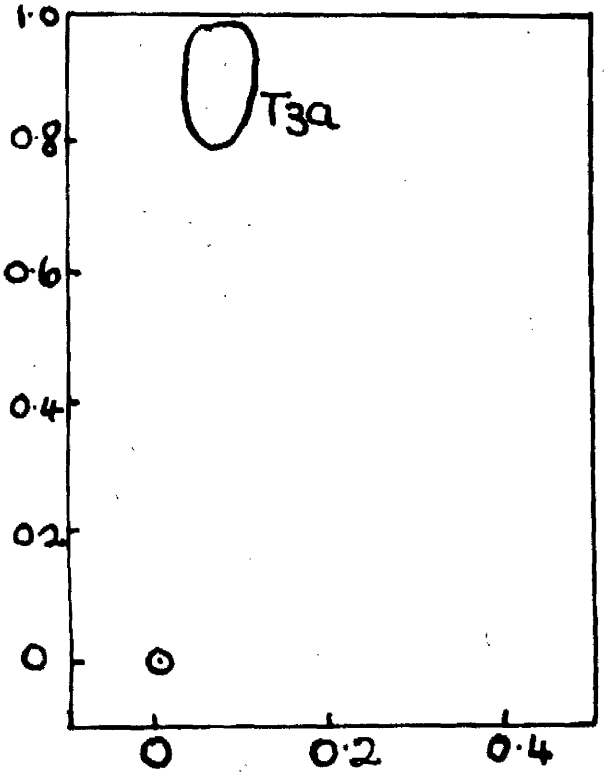


FIG. 28

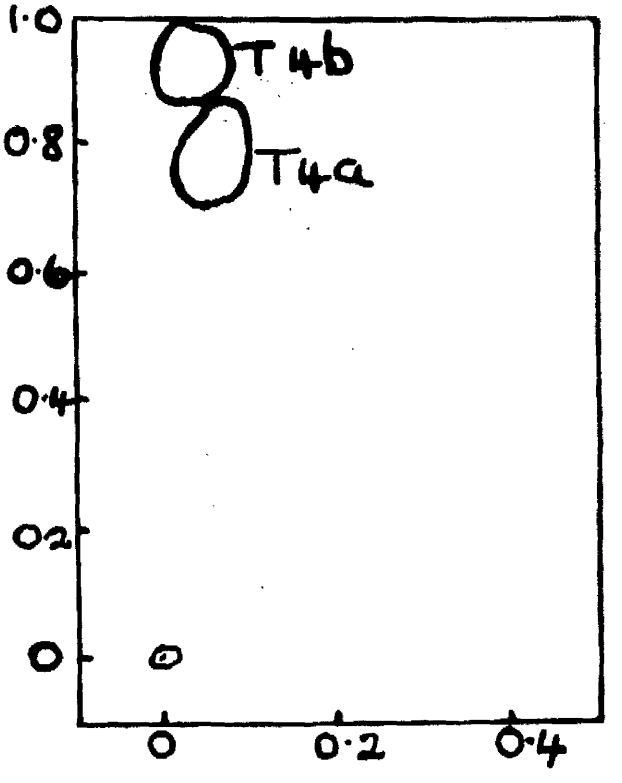


FIG. 29.

TABLE 19.

Peptides present in fraction P2.

Spot No.	Amino acids present.	Strength of Amino acid after Hydrolysis.	DNP Treatment.	Structure.
* P2a	Glu	+	+	[Glu, Asp]
	Asp ?	+	±	
P2b	Glu	+++		Glutamic acid.
* P2c	Glu	+	Neither spot detectable.	No decision.
	Gly	±		

* Hydrolysate fractionated on a one dimensional chromatogram.

TABLE 20.

Peptides present in fraction P3.

Spot No.	Amino acids present.	Strength of Amino acid after Hydrolysis.	DNP Treatment.	Structure.
	Glu	++	++	
* P3a	Gly	±	±	[Glu, Gly, $CySO_3H$]
	$CySO_3H$	+	±	
P3b	Glu	++		Glutamic acid.
P3c	Glu	+	+	Ala, Glu
	Ala	++	±	
* P3d	Thr	+		
	Gly	+		
	Glu	?		
P3e	Glu	++	++	[Glu, Val]
	Val	+++	++	

* Hydrolysate separated on a one dimensional chromatogram.

TABLE 21.

Peptides present in fraction P5.

Spot No.	Amino acids present.	Strength of amino acid after Hydrolysis.	Strength of amino acid after DNP Treatment.	Structure.
* P5a	Glu	?		[Glu,Gly,Thr]
	Gly	+		
	Thr	++		
P5b	Glu	±	Trace	[$CySO_3H$,Glu,Ala,Gly,Lys]
	$CySO_3H$	+	-	
	Ala	Trace	Trace	
	Gly	Trace	Trace	
	Lys	Trace	Trace	
P5c	Glu	+	+	Lys.[Glu,$CySO_3H$,Ala,Gly]
	$CySO_3H$	+	Trace	
	Ala	+	Trace	
	Gly	Trace	-	
	Lys	+	-	
* P5d	Glu	++	+	$CySO_3H$.[Glu,Gly,Ala,Val,Lys]
	$CySO_3H$	+	-	
	Gly	+	±	
	Ala	++	±	
	Val	++		
	Lys	++		
P5e	Gly	++		Glycine
* P5f	Gly	++	-	[Gly,Thr,Ala]
	Thr	++		
	Ala	±		
P5g	Ala	++		Alanine
* P5h	Glu	++	Trace	[Glu,Gly,Ala,Val,Lys]
	Gly	+	Trace	
	Ala	+	Trace	
	Val	++	Trace	
	Lys	±	-	

Table 21 continued.

Spot No.	Amino acids present.	Strength of amino acid after Hydrolysis.	Strength of amino acid after DNP Treatment.	Structure.
*P5k	Gly	±	-	
	Thr	+	-	
	Ala	±	±	Leu. [Val, Ala]
	Val	+	+	
	Leu	+	-	

* Hydrolysate fractionated on a one dimensional chromatogram.

TABLE 22.

Peptides present in fraction P6.

Spot No.	Amino acids present.	Strength of amino acid after Hydrolysis.	DNP Treatment.	DNP amino acid identified.	Structure.
P6a	No amino acids detectable.				
P6b, P6c	Lost.				
P6d	Glu	±	?		
	Ala	+	+		[Glu, Ala, Lys]
	Lys	+	-	ε-DNP-lys	

TABLE 23.

Peptides present in fraction P7.

Spot No.	Amino acids present.	Strength of amino acid after Hydrolysis.	DNP Treatment.	Structure.
P7a	Glu	Trace	?	
	$CySO_3H$	Trace	Trace	No decision
	Ala	Trace	-	
	Lys	Trace	-	
P7b	Gly	++	++	
	Ala	±	Trace	Lys. [Gly, Ala
	Lys	+++	±	

TABLE 24.

Peptides present in fraction P8.

Spot No.	Amino acids present.	Strength of Amino acid after Hydrolysis. DNP Treatment.	Structure.
P8a	Lys	+++	Lysine.
P8b	Lys	++	Lysine.

TABLE 25.

Peptides present in fraction Q1.

Spot No.	Amino acids present.	Strength of amino acid after Hydrolysis.	DNP Treatment.	DNP amino acid identified.	Structure.
Q1a	Glu	++	±	Glu	Glutamic acid
	Asp?	Trace	-	Asp ?	
Q1b	Glu	++			Glutamic acid

TABLE 26.

Peptides present in fraction Q2.

Spot No.	Amino acids present.	Strength of amino acid after Hydrolysis.	DNP Treatment.	DNP amino acid identified.	Structure.
Q2a	Glu	+	DNP hydrolysate lost.		No decision.
	Asp	±			
Q2b	Material lost				No decision.
Q2c	Glu	+			Glutamic acid
Q2d	Glu	++	++		
	Ala	++	±		Ala.Glu
Q2e	Glu	++	Only sufficient material for one chromatogram.		[Glu,Val]
	Val	++			

TABLE 27.

Peptides present in fraction Q3.

Spot No.	Amino acids present.	Strength of amino acid after Hydrolysis.	DNP Treatment.	DNP amino acid identified.	Structure.
Q3a	Glu	Trace			[Glu, $CySO_3H$, Gly, Ala]
	$CySO_3H$	Trace			
	Gly	Trace			
	Ala	Trace			
Q3b	Glu	+	+		[Glu, Asp, Gly, Ala, Lys]
	Asp	+	+		
	Gly	+	+		
	Ala	+	+		
	Lys	+	+		
Q3c	Glu	+	Trace		[Glu, Gly, Ala, Val, Lys]
	Gly	±	Trace		
	Ala	+	-		
	Val	+	Trace		
	Lys	++	-		
Q3d	Lys	++			Lysine
Q3e	Gly	+++			Glycine
Q3f	Thr	++			Threonine
Q3g	Ala	+++			Alanine
Q3h	Val	+	±		Leu.Val
	Leu	+	-	Leu	
Q3j	Val	+	±	Val	Leucine and Valine present as free amino acids.
	Leu	±	±	Leu	

TABLE 28.

Peptides present in fraction Q4.

Spot No.	Amino acids present.	Strength of amino acid after Hydrolysis.	Strength of amino acid after DNP Treatment.	DNP amino acid identified.	Structure.
Q4a	Gly	Trace	Trace		Lys.Gly
	Lys	Trace	-		
Q4b	Gly	±	±	ε-DNP-lys	No decision
	Lys	+	+		
Q4c	$CySO_3H$	+	?		Ala.[Lys,$CySO_3H$
	Ala	+	-		
	Lys	++	++		
Q4d	Lys	++			Lysine
Q4e	Glu	±	±		
	$CySO_3H$	±	±		Ala.[Glu,$CySO_3H$,Lys]
	Ala	+	-		
	Lys	++	++		

TABLE 29.

Peptides present on fraction Q5.

Spot No.	Amino acids present.	Strength of amino acid after Hydrolysis.	DNP Treatment.	DNP amino acid identified.	Structure.
	Glu	Trace	Trace		Lys. [Glu,Gly
Q5a	Gly	Trace	Trace		but
	Lys	Trace	-		doubtful.
	Gly	Trace	Insufficient material for DNP treatment		No decision
Q5b	Lys	±			
Q5c	Glu	±	±		
	Gly	+	+		
	Ala	Trace	Trace	bis-DNP-lys	Lys. [Glu,Gly Ala]
	Lys	++	±	ε-DNP-lys	
Q5d	Lys	++			Lysine

TABLE 30.

Peptides present in fraction Q6.

Spot No.	Amino acids present.	Strength of amino acid after Hydrolysis.	DNP Treatment.	DNP amino acid identified.	Structure.
Q6a	Gly	Trace			Glycine
Q6b	Gly	++	++	bis-DNP-lys	Lys.Gly
	Lys	++	±	ε-DNP-lys	
Q6c	Glu	±	Trace		
	Gly	±	Trace		No
	Ala	Trace	-		decision
	Val	Trace	-		
	Lys	++	Trace		

TABLE 31.

Peptides present in fraction Q7.

Spot No.	Amino acids present.	Strength of amino acid after Hydrolysis.	DNP Treatment.	DNP amino acid identified.	Structure.
Q7a	Lys	+++			Lysine
Q7b	Lys	+++	+	ε-DNP-lys	Lysine

TABLE 32.

Peptides present in fraction M3.

Spot No.	Amino acids present.	Strength of amino acid after Hydrolysis.	after DNP Treatment.	DNP amino acid identified.	Structure.
M3a	$CySO_3H$	Trace			[$CySO_3H$, Glu, Gly, Ala]
	Glu	Trace			
	Gly	Trace			
	Ala	Trace			
M3b	$CySO_3H$	±			[$CySO_3H$, Glu]
	Glu	+			
	Gly	Trace			
	Ala	Trace			
	Lys	Trace			
M3c	$CySO_3H$	+	±		[$CySO_3H$, Glu, Gly, Ala, Lys]
	Glu	++	±		
	Gly	Trace	Trace		
	Ala	+	±		
	Lys	++	±		
M3d	Gly	+			Glycine
M3e	$CySO_3H$	±	±		[$CySO_3H$, Glu, Ala, Val, Lys]
	Glu	+			
	Ala	+	-		
	Val	±	-		
	Lys	++	-		
M3f	Ala	+			Alanine
M3g	Glu	++	++		Val. [Glu, Ala, Lys]
	Ala	±	+		
	Val	+	-	Val	
	Lys	++	-	ε-DNP-lys	

TABLE 33.

Peptides present in fraction M4.

Spot No.	Amino acids present.	Strength of amino acid after Hydrolysis.	Strength of amino acid after DNP Treatment.	DNP amino acid identified.	Structure.
M4a	Glu	±			[Glu,Gly,Ala,Lys]
	Gly	±			
	Ala	±			
	Lys	±			
M4b	Glu	++	+		Val.[Glu,Gly, Ala,Lys]
	Gly	±	±		
	Ala	+	-		
	Val	±	±	Val	
	Lys	++		ε-DNP-lys	
M4c	Gly	±			[Gly,Ala,Lys]
	Ala	±			
	Lys	±			
M4d	Glu	Trace			No decision
	Ala	±			
	Val	Trace			
	Lys	Trace			
M4e	Ala	++			Alanine
M4f	Glu	+	±		Val.[Glu,Ala, Lys]
	Ala	±	-		
	Val	+	Trace	Val	
	Lys	++	+	ε-DNP-lys	
M4g	Glu	+			[Glu,Gly,Ala, Val,Lys]
	Gly	+	No amino		
	Ala	±	acid spots		
	Val	+	detectable		
	Lys	++		ε-DNP-lys	

TABLE 34.

Peptides present in fraction M5 &6.

Spot No.	Amino acids present.	Strength of amino acid after Hydrolysis.	DNP treatment.	DNP amino acid identified.	Structure.
M5 & 6a.	Glu	Trace			This spot consists mainly of Glycine
	Gly	±			
	Ala	Trace			
	Lys	Trace			
M5 & 6b.	Gly	++	DNP material lost.		[Gly, Lys]
	Lys	+++			

TABLE 35.

Peptides present in fraction T2.

Spot No.	Amino acids present.	Strength of amino acid after Hydrolysis.	DNP Treatment.	DNP amino acid identified.	Structure.
T2a	Glu	±			[Glu,Gly,Lys
	Gly	+			
	Lys	Trace			
T2b	Glu	+	+		[Val,Lys,Glu,Gly
	Gly	±	±		
	Val	±	-		
	Lys	+	-		
T2c	Glu	Trace			[Glu,Gly,Ala
	Gly	Trace			
	Ala	Trace			

TABLE 36.

Peptides present in fraction T3.

Spot No.	Amino acid present.	Strength of amino acid after Hydrolysis.	Strength of amino acid after DNP Treatment.	DNP amino acid present.	Structure.
T3a	Glu	++	++		
	Val	++	Trace	Val	Val.[Glu,Lys]
	Lys	++	+	ε-DNP-lysine	

TABLE 37.

Peptides present in fraction T4.

Spot No.	Amino acid present.	Strength of amino acid after Hydrolysis.	DNP Treatment.	DNP amino acid identified.	Structure.
T4a	Glu	+			
	Gly	+	DNP material lost.		[Glu,Gly,Lys]
	Lys	+++			
T4b	Gly	+++	+++	bis-DNP-lys	Lys.Gly
	Lys	+++	±	ε-DNP-lys	

Discussion.

Valine has been found to be the N-terminal residue of PMc. Peptide Q3h, however, has the structure Leu.Val.

It appears that the single preparation used in experiment Q had Leu.Val as its N-terminal sequence, but in all the other preparations valine is the N-terminal residue. It is possible that, in general, leucine has been split from the N-terminal end of the PMc amino acid chain.

The one dimensional chromatograms, which were run to separate the amino acids in hydrolysates of some of the peptides in experiment P, gave very unsatisfactory results. The reasons for these have already been given. It was therefore decided to neglect them when attempting to formulate the sequence of amino acids in PMc.

A number of peptides obtained from the partial hydrolysates were picked out as having structures which appeared to be definitely determined. P3c consists of Ala.Glu and this sequence is also found in Q2d. Another established dipeptide is Lys.Gly which is found in Q6b, Q4a and M5&6b. The same structure is also observed in T4b.

The presence of this structure in the last mentioned peptide is difficult to reconcile with the fact that trypsin is supposed to split only those bonds linking the carboxyl group of lysine with the amino group of another amino acid (Bergmann and Fruton, 1941). Histidine, however, was shown to be present in PMc by Leaf (1952). It could not be easily detected on two dimensional paper chromatograms by the Pauli

reaction when traces of phenol were present. It is possible that lysine and histidine were mistaken for each other after spraying with ninhydrin. On the small chromatograms used these two amino acids would not be separated from each other. Therefore T4b might be His.Gly which would explain its appearance among the products of tryptic hydrolysis.

T3a consists of Val.[Glu,Lys] and in combination with the dipeptide P3e, which is [Val,Glu], gives the sequence Val.Glu.Lys. If the dipeptide Leu.Val already mentioned is added to this, the sequence Leu.Val.Glu.Lys is obtained.

The two dipeptides Lys.Gly and Ala.Glu can be linked in the structure Lys.Gly.Lys.Ala which is found in P7b. The assumption must be made that there are two molecules of lysine in this peptide. This cannot be definitely proved as only very small amounts of material were available in these experiments, which made quantitative examination of each peptide impossible.

Under the conditions of manipulation in the experiments on PMc, cystine appears to be oxidised to cysteic acid. It is found as such in the peptides M3b (Glu.$CySO_3H$) and Q4e (Ala.[Glu,$CySO_3H$,Lys]). These can be fitted into the sequence Ala.Glu.$CySO_3H$.Lys.

Combination of the sequences so far described gives the peptide

Leu.Val.Glu.Lys.Gly.Lys.Ala.Glu.$CySO_3H$.Lys.

The peptides shown in Table 38 can all be fitted into this

TABLE 38.

The amino acid sequences of PMc.

Main Sequence.

Peptide.	Structure.
P3c	Ala. Glu
Q2d	Ala. Glu
Q6b	Lys. Gly
Q4a	Lys. Gly
M5 & 6b	Lys. Gly
T4b	Lys. Gly
P3e	Val. Glu
Q3h	Leu. Val
P7b	Lys. Gly. Lys. Ala
M3b	Glu. $CySO_3H$
Q4e	Ala. Glu. $CySO_3H$. Lys
T3a	Val. Glu. Lys
Q5c	Lys. Gly. Lys. Ala. Glu
M4b	Val. Glu. Lys. Gly. Lys. Ala
M3c	Gly. Lys. Ala. Glu. $CySO_3H$
P5b	Gly. Lys. Ala. Glu. $CySO_3H$
P5c	Lys. Gly. Lys. Ala. Glu. $CySO_3H$
P6d	Lys. Ala. Glu
Q2e	Val. Glu
Q3b	Glu. Lys. Gly. Lys. Ala
M4a	Gly. Lys. Ala. Glu
M4c	Gly. Lys. Ala
M4g	Val. Glu. Lys. Gly. Lys. Ala
Q3c	Val. Glu. Lys. Gly. Lys. Ala
T2a	Glu. Lys. Gly
T2b	Val. Glu. Lys. Gly
	Leu. Val. Glu. Lys. Gly. Lys. Ala. Glu. $CySO_3H$. Lys

TABLE 39.

The amino acid sequences of PMc.

Second Sequence.

Peptide.	Structure.
M3e	Val. [Lys, Ala, Glu] . $CySO_3H$
M3g	Val. [Lys, Ala, Glu] .
M4f	Val. [Lys, Ala, Glu]
	Val. [Lys, Ala, Glu] . $CySO_3H$

sequence. The peptides T2a, T2b and T4a can be assigned to this sequence although they should not have been formed if the digestion of PMc with trypsin had proceeded as expected.

Some peptides, however, cannot be fitted into the main sequence, the principal ones being M3e, M3g and M4f. These have been arranged in a separate sequence

$$Val.[Lys,Ala,Glu].CySO_3H$$

as shown in Table 39. The order of [Lys,Ala,Glu] cannot be deduced at the present stage.

Four peptides Q3a, Q4c, M3a and T2c still do not fit into either sequence. The first three appear to contain glycine, although there is only one molecule of this amino acid in PMc, and one ~~small~~ molecule of glycine has already been assigned to the main sequence. The spots from these peptides were detectable only in very small amounts. It was difficult to decide whether some of these were due to genuine amino acids or to the reaction of impurities in the paper with ninhydrin. These peptides have therefore been disregarded in attempting to establish the nature of the amino acid sequences in PMc.

It is not possible to decide whether both sequences described exist in the molecule of PMc as separate chains, because both appear to have valine as the N-terminal residue. It would be necessary to study partial hydrolysates of DNP-PMc to establish the N-terminal sequences

present, in order to settle this question.

In the experiments recorded no peptides were found which contained threonine. In experiment Q it was obtained as the free amino acid (spot Q3f). This is as expected, because the bonds in which the hydroxy amino acids are involved are the first to be split by acid hydrolysis. However, when a shorter time of hydrolysis was used in experiment M there was still no evidence of its occurrence in any peptides. Threonine was also not found in any of the peptides produced by the hydrolysis of PMc with trypsin.

It is possible that the two sequences, which have been postulated, are joined by one or perhaps two molecules of threonine. This would mean that PMc consists of one single peptide chain, instead of two separate ones.

There was evidence of the presence of phenylalanine in some of the PMc preparations examined and this amino acid may have been contained in the preparation on which a quantitative analysis was carried out. However, phenylalanine was not found in any of the PMc samples which were used for partial hydrolysis studies. It may be that phenylalanine is situated at one or other end of the PMc chain and is readily split off during preparation of the peptide. This would explain its presence in some and not in other preparations of PMc.

The two sequences which have been proposed account

for the other amino acids which are indicated by the quantitative amino acid composition of the molecule. No position has, however, been assigned to histidine, which was not definitely identified in any of the chromatograms. Four positions in the sequences have been allocated to lysine, although only three molecules of lysine appear to be present in PMc. Presumably one of these positions should, in fact, be assigned to histidine, but it is not possible to decide which one it should be. In future work it will be necessary to use methods which adequately distinguish between lysine and histidine.

Two molecules of cysteic acid are required by the sequences described, but only 0.17 moles of cystine were recovered in the quantitative analysis on PMc. It is possible that the two cysteine residues attaching the peptide chain to the prosthetic group have, to some extent, become oxidised to cysteic acid during the manipulations involved in the experiments. It is interesting that the peptides containing cysteic acid were obtained mainly from fractions which remained in the same region as the red-coloured material after ionophoresis of the products of hydrolysis. In the one instance in which the red-coloured material had moved away from the origin (fraction T1) no ninhydrin-positive material was detected in it. These results indicate that the prosthetic group may have been split off, leaving the cysteine residues attached to the

amino acid chain or chains.

Certain difficulties are introduced in working out the structures of proteins and peptides when enzymes are used as hydrolysing agents. Enzymes themselves are proteins, and sequences may be assigned to the molecule being studied which in actual fact are derived from the enzymes. In the present work two enzymes, pepsin and trypsin, have been employed. The possibility therefore arises that some amino acid sequences present in these enzymes have been mistaken for those in PMc. This is unlikely to occur in the case of pepsin, however, because a specific method for the isolation of PMc was used. Even if some material, arising from pepsin, was adsorbed on the PMc, it is almost certain to be removed on fractionation with dilute HCl.

Contamination of the peptides from PMc is also unlikely to be caused by trypsin. The ratio of enzyme to substrate in the digestion mixture was 1 to 8. The molecular weight of trypsin is approximately 34,000, while that of PMc is just over 2,000. Therefore any peptides originating from trypsin will contribute very little to the products of digestion obtained.

In the acid hydrolysis of a peptide, the equilibrium of the reaction lies far over on the side of hydrolysis. In enzymic hydrolysis, on the other hand, this is not necessarily the case. Trypsin has been shown to effect transpeptidations in certain circumstances (Waley and Watson, 1954), and it is possible that such reactions were occurring in experi-

ment T, although there is no direct evidence for these in this work.

The experiments on the amino acid sequences in PMc were of a preliminary nature, and a great deal of work remains to be done on this problem.

The interpretation of the results has been hampered by the small amount of PMc available for these experiments. Only 4 mg. of PMc were used as starting material in each case. The result was that some of the amino acid spots were detected only in traces on the chromatograms. It was therefore difficult to decide whether these spots were due to authentic amino acids or to interfering material reacting with ninhydrin on the paper. Because of these difficulties, some erroneous interpretations may have been made. For future work it is recommended that 50 - 100 mg. of pure PMc be used as the starting material. To cope with the larger amounts being handled, the peptides could be fractionated on an ion exchange column instead of by paper ionophoresis.

GENERAL CONCLUSIONS.

The elucidation of the quantitative amino acid composition of horse heart cytochrome c gives a limited amount of information concerning the intimate structure of the protein molecule. The very basic character of cytochrome c is reflected in its high content of lysine, and the fact that the number of free basic groups is far in excess of the number of free acidic groups.

Cytochrome c and haemoglobin have the same prosthetic group. Therefore their very different functions must be due to differences in the structures of their respective proteins as well as in the modes of linkage of the protein to the prosthetic group.

Although it is not possible to compare the detailed structures of the two proteins merely by examining their overall amino acid compositions, some general differences can be observed.

Tristram (1953) has reported the amino acid composition of horse haeoglobin. Comparison of this analysis with the values obtained for horse heart cytochrome c shows two main differences. Haemoglobin, unlike cytochrome c, does not contain isoleucine, while the reverse is found in the case of serine. Otherwise both proteins contain the same amino acids although not in the same proportions.

The properties of a protein are probably governed by both the arrangement of the amino acids in the molecule and

the nature of the side chains on its surface. It is therefore interesting to compare the distribution of the ionic groups in cytochrome *c* with the distribution of the ionic groups in horse haemoglobin as determined by Tristram (1953). The figures shown below are calculated as a percentage of the total groups present in the molecule.

	Free Anionic	Free Cationic	Amide	Excess Cationic
Haemoglobin	9.8	16.2	6.6	6.4
Cytochrome *c*	10.1	24.7	9.0	14.6

It can be seen that, although both are basic proteins, cytochrome *c* has substantially more free basic groups than haemoglobin. It is noteworthy that the basic character of haemoglobin is chiefly due to its high content of histidine, while lysine is responsible for the basic nature of cytochrome *c*.

From the present work it would appear that the protein of cytochrome *c* has a cyclic structure as it has no free *N*-terminal residues. Porter and Sanger (1948), on the other hand, found that horse haemoglobin has six *N*-terminal valine residues. This suggests that the protein consists of six peptide chains bound together by cross linkages.

No work has been reported so far on the amino acid sequences in haemoglobin, and the present studies on the sequences in cytochrome *c* have been of a preliminary nature, dealing only with a small part of the whole molecule. However, the results so far indicate that the structures of

haemoglobin and cytochrome c are very different, and that both must be derived from different sources. These findings are in agreement with the conclusions reached by Theorell and Åkeson (1941a).

No complete analyses of the compositions of catalase and peroxidase, which have the same prosthetic group as cytochrome c, have been published. It is, therefore, not possible to compare their structures with that of cytochrome c.

The work carried out in this laboratory tends to indicate that there are only small differences between the structures of beef and horse heart cytochrome c. More detailed studies of the two molecules may reveal differences which are not apparent at present.

The elucidation of the amino acid sequences in PMc is not complete and, as indicated in Section III, experimemts will have to be performed on a larger scale to enable quantitative estimations to be carried out. This is very important as some of the peptides, which have been postulated as occurring in partial hydrolysates of PMc, contain more than one molecule of lysine. We were unable to confirm if these were in fact the true structures of these peptides.

It is interesting that both PMc and cytochrome c appear to contain two molecules of valine. If this conclusion is true, valine should not be encountered in any of the other peptides which are presumably formed when pepsin acts on

cytochrome c.

After the structure of PMc has been confirmed, it will be necessary to study those colourless peptides which do not contain the prosthetic group. In this way a complete picture of the structure of the protein moiety of cytochrome c can be built up, which at the same time may reveal more definite information on the nature of the protein-prosthetic group linkage in the whole molecule.

SUMMARY.

1. Cytochrome c was prepared from horse heart according to the method of Keilin and Hartree (1945). The material obtained was chromatographed on a column of the cation exchange resin Amberlite IRC - 50, on which it separated into four fractions. The fastest moving fraction was found to consist of an iron-free protein. The remaining three fractions comprised a small amount of reduced cytochrome c, the main bulk of the cytochrome c in the oxidised state, and three narrow bands of the acid-modified haem-protein. The oxidised cytochrome c was found to have a purity comparable with that of electrophoretically pure cytochrome c obtained by other workers.

2. A quantitative amino acid analysis of cytochrome c was carried out after hydrolysates of the protein were fractionated on columns of potato starch and Dowex 50, according to the techniques introduced by Moore and Stein (1949, 1951) and Stein and Moore (1948).

3. An investigation was made of the N-terminal residues of cytochrome c, using the technique of Sanger (1945). Apart from ε-DNP-lysine, only traces of the DNP derivatives of alanine, serine and glutamic acid were observed. It is concluded that these traces were derived from impurities in the cytochrome c preparation. It therefore appears that cyto-

chrome c has no free N-terminal residues, and that it consists of a cyclic chain of amino acids.

4. An autoxidisable peptide, containing the haem prosthetic group, was isolated after digesting cytochrome c with pepsin according to the method of Tsou (1951a).

5. Valine was found to be the N-terminal residue of this peptide. Later experiments showed that leucine may be the next amino acid linked to the free amino group of the valine residue.

6. The peptide was partially hydrolysed by means of concentrated HCl at 37° and by digestion with trypsin. The products of hydrolysis were fractionated by paper ionophoresis followed by further separation on two dimensional chromatograms.

Studies were carried out on the structures of the small peptides obtained. From these the presence of two sequences in the autoxidisable peptide is postulated. These are

Leu.Val.Glu.Lys.Gly.Lys.Ala.Glu.$CySO_3H$.Lys.

and

Val.[Lys,Ala,Glu].$CySO_3H$.

These sequences may be linked by one or two molecules of threonine to form a single chain. Threonine was always identified as the free amino acid in the products of hydro-

lysis, even when hydrolysis with acid was reduced to 24 hours. Alternatively, the two sequences may be present as separate chains in the molecule bound together by inter-chain linkages involving the prosthetic group.

There is a certain amount of evidence to support the theory of Theorell and Åkeson (1941d) that cysteine residues link the protein to the prosthetic group in cytochrome c.

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