STUDIES IN THE CELL SPECIFICITY OF THE HISTONES

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CONTENTS

GENERAL INTRODUCTION

During recent years evidence has been accumulating in this laboratory in favour of the concept of the cell specificity of the histones.

The first experimental findings suggestive of such a concept date from the discovery that whilst the sperm heads of the salmon contain a protamine, the nuclei of the somatic cells, in particular the erythrocytes and liver cells, contain histones (Stedman and Stedman, 1944). In addition to this example of cell specificity, the same material provided evidence that the histones present in the erythrocytes and liver cells were also cell specific, for their arginine contents differed widely from one another.

In the course of further work directed towards extending and generalizing these results, Stedman and Stedman (1950; 1951) analysed the histones from the cell nuclei of various animal tissues. These were extracted by dilute mineral acids from preparations of the isolated cell nuclei and purified by submitting them to repeated alcoholic precipitation from their solutions in very dilute sulphuric acid.

When various histone preparations obtained in this way from calf-thymus nuclei were analysed for their tyrosine contents however, differing results were obtained and it became apparent that a partial fractionation was being effected by reprecipitation. One specimen of thymus histone sulphate was therefore separated into three fractions by precipitation from an aqueous solution by the successive addition of suitable volumes of ethanol. The fractions were dried with alcohol and ether and analysed for their total nitrogen, amide nitrogen, arginine nitrogen and tyrosine contents. The results are given in Table 1.

Table 1. Fractionation of crude calf-thymus histone sulphate.

Fraction	Total N%	Amide N%	Arginine N%	Tyrosine %
Most soluble	15.96	4.14	18.3	1.78
Middle	17.00	5.13	28.7	4.26
Least soluble	17.00	4.96	30.3	-

The histones which precipitated first on the addition of ethanol ('main' histones) differed considerably from those that precipitated after further additions ('subsidiary' histones). In many different tissues examined the main histones showed a considerably greater arginine and tyrosine content than was present in the subsidiary histones.

In one series of experiments, Stedman and Stedman (1951) compared preparations of the main and subsidiary histones which they had isolated from the nuclei of the erythrocytes and thymocytes of the fowl. The main components, after purification to constant composition by repeated precipitation, were shown to differ appreciably in their arginine contents and moreover, the erythrocyte nuclei were demonstrated to contain a subsidiary histone not present in the thymocytes. Despite this, and other examples of cell specificity however, it was not possible to demonstrate any chemical difference between the main histones from ox-thymocytes and liver cells. Later work in this laboratory by Cruft, Mauritzen and Stedman (1957) however, has shown the pH-mobility curves of these components to be appreciably different.

The finding that histone preparations contain a mixture of basic proteins has been confirmed by many later workers (Daly, Mirsky and Ris, 1951-52; Eadie and Leaf, 1952; Hamer, 1953; Davison and Butler, 1954; Daly and Mirsky, 1955; Davison and Shooter, 1956; Cruft, 1953; and Cruft, Mauritzen and Stedman, 1954).

These last three authors have, in this laboratory, made extensive electrophoretic and ultracentrifugal analyses of histones extracted from the cell nuclei of a wide variety of tissues and have shown them to be, in general, much more complex than appeared from the chemical fractionation procedures used in earlier work. Simple electrophoretic analysis of the unfractionated histones revealed many of them to consist of at least three components which Cruft et al. (1954) have designated as the α-, β- and γ-histones in descending order of mobility towards the cathode. Further work however has indicated that the α- and γ-histones are not necessarily homogeneous since, in some cases, the application of either chemical or ultracentrifugal fractionation procedures, or both, has shown the former to contain three components and the latter

two. The components of the α-fraction were designated by the symbols α_1-, α_2-, and α_3- in descending order of mobility whilst those in the γ-fraction are known, for the reasons given on p. 20 as the 0.8- and 1.6γ-components.

By means of techniques to be described later (pp. 23-25) Cruft et al. (1957) have been able to obtain highly purified preparations of the β- and 1.6γ-components from various histones and active work is now in progress comparing the amino acid analyses of these components from different tissues. In confirmation of the earlier results described however, the purified β-components from ox-thymocyte and ox-liver nuclei did not differ significantly in their quantitative amino acid composition and before the concept of cell specificity could be asserted to be a general one it was necessary to obtain chemical evidence of differences between these components. Part of the work to be described has therefore been concerned with the application of methods of terminal amino acid analysis, principally Sanger's (1945), in an attempt to establish these differences.

A further purpose was to attempt to fractionate the composite α-fraction of ox-thymus histone remaining after the precipitation of the β- and 1.6γ-components. This has been achieved, after the failure of various chromatographic techniques, by zone electrophoresis in starch blocks.

In 1951, Stedman and Stedman advanced the hypothesis that the basic proteins of cell nuclei are gene inhibitors, each histone or protamine being capable of suppressing the activity of specific groups of genes. Cell specificity follows as a necessary corollary from this hypothesis, which implies that the basic proteins have some specific function in initiating or controlling the character of the cell. A further corollary is that malignant cells would differ from the cells of their origin in the nature of the histone contained in their nuclei and this has been confirmed by physico-chemical measurements though not, as yet, chemically. Some specimens of tumour histones were therefore examined by Sanger's technique of N-terminal analysis.

Both by analogy with the nuclear heterogeneity of the histones from differentiated somatic tissues and in consideration of the fact of the apparent almost total inhibition of genetic activity in sperm heads until fertilization occurs, the question of the nuclear heterogeneity of the protamines assumes renewed importance. Several experiments bearing on the homogeneity or otherwise of carefully isolated preparations of clupeine and salmine were therefore performed.

ISOLATION AND FRACTIONATION OF THE BASIC PROTEINS

Introduction

Whilst the nuclear heterogeneity of the histones is now firmly established, the evidence for the sharing of this property by the protamines is unconvincing since it depends on the examination of protamine preparations which have been subjected to rather drastic treatment. Thus, Felix (1953) and his collaborators found their preparations of clupeine methyl ester hydrochloride to contain many components when examined by various techniques and Scanes and Tozer (1956), by chromatography on alumina and countercurrent distribution have shown a commercial sample of clupeine sulphate to contain three main components and a number of minor ones. In even more recent work Ando, Ishii, Yamasaki, Iwai, Hashimoto and Sawada (1957) have obtained evidence for the non-homogeneity of clupeine sulphate isolated under comparatively mild conditions, by countercurrent distribution and comparison of the relative amino acid contents of fractionated preparations. The heterogeneity disclosed however is much less marked than that found by the previous workers.

In this laboratory, protamines have been obtained under very mild conditions by extraction

of the sperm heads with cold 0.1 N acid and precipitation of the protamines with cold ethanol (Stedman and Stedman, 1947). In order to achieve their quantitative isolation from the sperm heads however, it was necessary to use cold 0.5 N acid, any deleterious effect of the greater acidity being reduced by using 0.5 N-hydrochloric acid in 50% aqueous-ethanol. The protamine was finally precipitated as the sulphate by the addition of dilute sulphuric acid and more ethanol.

The examination of such preparations should indicate whether the protamines are, in fact, heterogeneous, or whether this heterogeneity is artifactually produced.

Of the methods available for the isolation of the histones from animal tissues the most suitable, in our experience, has been that developed by the Stedmans since 1944 (Stedman and Stedman, 1944; 1947; 1951). By their procedure, using dilute acetic acid as lysing agent, pure preparations of cell nuclei have been obtained from a wide variety of tissues from which the histones were subsequently extracted with dilute acid. This method will be discussed later.

More recently, Mirsky and Pollister (1947), Butler, Davison, James and Shooter (1954) and

Bakay, Kolb and Toennies (1955) have investigated the histones which they isolated from nucleoprotein derived from calf-thymus tissue. Their method consisted essentially in homogenizing the whole glands in saline solutions followed by centrifugation in this medium to obtain the nucleoprotein. The histones were obtained either by extraction with dilute sulphuric acid or dissociation of the nucleoprotein complex with 10% saline. Such isolation procedures are subject to several criticisms. The implicit assumption is made that all the histone remains in combination with the nucleic acid. Furthermore, cytoplasmic contamination of an unknown degree cannot be avoided. Butler et al. (1954) believe that the heterogeneity of the isolated histone is due to the action of the intra-nuclear cathepsins discovered by Maver, Greco, Løvtrup and Dalton (1952).

In the light of the foregoing, the method of choice clearly involves the prior isolation of the cell nuclei in a pure state. For this purpose various techniques have been advocated amongst which adaptations based on Behrens' (1932) original method for the fractionation of cellular components in non-aqueous solvents (Murray-Luck,

Cook, Eldredge, Haley, Kupke and Rasmussen, 1956; Stern, Allfrey, Mirsky and Saetren, 1952) and processes involving homogenization and fractionation in sucrose solutions (Allfrey, Mirsky and Osawa, 1955), have been most widely used.

In this laboratory, nuclei have been obtained from various tissues by a method which has consistently given good results. It depends on the observation of Crossmon (1937) that when small pieces of tissues were placed in 5% citric acid solution, the nuclei were released from the cells. Subsequent development of this method by Stoneburg (1939) and then Marshak (1941) enabled them to isolate the nuclei from tissues such as muscle, tumour and perfused liver. Stedman and Stedman (1944) successfully used 4% acetic acid for this purpose and were able to obtain the cell nuclei from a wide variety of tissues.

There are several advantages of isolating nuclei in acid media. Thus, the necessity of vigorous grinding and homogenizing techniques in which the cells are disrupted purely mechanically is obviated. This is desirable because such processes, unless very carefully controlled, give preparations contaminated either by a considerable

proportion of whole cells or by much disrupted nuclear material. In contrast, the acid method gives good yields of clean nuclei under conditions which inhibit the action of any proteolytic enzymes (Dounce, 1943).

Criticisms have however, been levelled against the use of aqueous media for the isolation of nuclei on the basis of evidence suggesting considerable losses of nuclear protein during this procedure (Pollister and Leuchtenberger, 1949; Stern and Mirsky, 1953). Stedman and Stedman (1951) have seriously investigated these claims and their experiments leave little doubt that, at any rate the histone contents of the various nuclei are essentially unchanged by the isolation procedure.

The histones are obtained from the dried nuclei by exhaustive extraction with ice-cold 0.1 N-sulphuric acid and finally precipitated as the sulphates by the addition of excess ethanol or acetone.

Experimental

1. Protamines

(a) Isolation of Sperm Nuclei

These were obtained by Dr Stedman by a technique essentially similar to that employed by Miescher (1897).

The mature testes of West Coast herrings were coarsely minced and the pulp suspended in water. Salmon testes, being larger, were individually expressed to obtain the semi-fluid contents, which were suspended in five times their volume of water. The suspensions so obtained were vigorously stirred mechanically for 15 minutes, after which the products were strained through fine muslin. The milky fluid was rendered just acid to litmus with dilute acetic acid, and after standing for 15 minutes, centrifuged at 3000 r.p.m. for 15-20 minutes. The sperm heads, admixed with a little fibrous impurity, were resuspended in fresh dilute acetic acid and the sedimentations repeated until the supernatant was quite clear and the sperm heads appeared homogeneous and free from impurities when microscopically examined after staining with

methylene blue. The nuclei were finally dried and lipid material removed by suspending them in absolute alcohol overnight and finally washing on a Büchner funnel with ether.

(b) Extraction of the Protamines

In order to obtain quantitative extraction of the protamine from the sperm heads it was necessary to use ice-cold 0.5 N-hydrochloric acid in 50% aqueous ethanol. Three extractions were found to be sufficient and after centrifugation, the protamine was precipitated as the sulphate by the addition of dilute sulphuric acid and more ethanol. In the presence of water the protamine sulphates precipitated as oils. These however solidified on treatment with ethanol.

The crude sulphates were purified by dissolving them in water (1 g. protamine sulphate in 100 ml. of water), centrifuging to sediment any insoluble matter, and stirring the supernatant with 25 ml. of ethanol to reprecipitate the protamine sulphate. The clupeine sulphate used

had been submitted seven times to this procedure, and the salmine sulphate five times. The weight of protamine sulphate obtained in each case accounted for about 80% of the crude product. The final product in each case was a pure white powder.

2. Histones

(a) Isolation of Cell Nuclei

In the case of the larger animals (ox), the tissues were collected directly from the slaughter house. When smaller animals were involved (rat), the animals were sacrificed in the laboratory and the tissues dissected from the carcasses. In general, the tissues were worked up within a few hours of extirpation, but where this was impossible the material was stored frozen solid in a deep freeze. This procedure was necessary when only small amounts of the required tissue were available at a given time - for example, rat-hepatoma tissue.

The tissues were first trimmed to remove extraneous materials - e.g. fat, connective tissue, blood vessels - and cooled to 2°C, all subsequent work being done in a cold room below 2°C and in a

refrigerated centrifuge. The tissue was coarsely minced and the pulp immediately put through a fine pressure mincer, the product being collected in 4% acetic acid. After vigorous stirring, the mixture was allowed to stand for an hour or longer and, after a further period of stirring, filtered four times through one, two, four and five thicknesses of butter muslin. The suspension of nuclei was then freed from cytoplasmic contamination by repeated centrifugation in 1% acetic acid until microscopic examination of a sample of the sediment stained by methylene blue revealed freedom from cytoplasmic contaminants. The nuclei were defatted and dried by centrifuging with alcohol, alcohol-ether, and finally ether. The product was air dried and stored in this form. Thymus nuclei were obtained as a pure white powder whilst those from liver had a slight brown colour.

(b) Extraction of the Histones

In order to achieve thorough wetting of the nuclei by the extracting solvent it was necessary to pretreat them with ethanol. A weighed sample of nuclei was stirred with absolute ethanol to give a uniform suspension and the nuclei

sedimented by centrifugation. The ethanol was decanted and the residue washed three times with small quantities of water. The packed nuclei were then mixed with an equal volume of 0.1 N-sulphuric acid and the mixture stirred intermittently for half an hour. The mixture was then centrifuged, the supernatant decanted, and the histone sulphate precipitated by the addition of six volumes of acetone. Extractions were repeated, exactly as described, with further volumes of acid until the supernatant yielded no precipitate after the addition of six volumes of acetone.

The acetone precipitated extracts were bulked and the histone sulphates sedimented by centrifuging. They were finally isolated by washing with acetone, acetone-ether, ether, and air-dried.

Fractionation of Whole Histone

The scheme devised by Cruft et al. (1957) for the preliminary fractionation of whole histones depends on the observation that β-histones undergo aggregation in solutions of increasing pH and

ionic strength. This has been demonstrated by measurements of their diffusion and sedimentation coefficients.

After allowing the β-component to aggregate into high 'S_{20}' material it was removed by ultracentrifugation, the supernatant containing the (α + γ)-fraction. Electrophoretic analysis of this latter fraction by the moving boundary method indicated the presence of four components, namely, the α_1-, α_2-, α_3-, and γ-histones. The combined α-components accounted for 45% and the γ-fraction 55% of the total histone present. Analysis in the ultracentrifuge, on the other hand, indicated the presence of only two components with sedimentation coefficients of 0.8 and 2.0 x 10^{-13} in the proportions 70 and 30% respectively. The α-histones had previously been shown to have $S_{20} = 0.8 \times 10^{-13}$ and this apparent contradiction between the results from the sedimentation and electrophoretic experiments was finally resolved when it was found that there were two γ-components one of which had an S_{20} value close to that of the α-histones whilst the other had $S_{20} = 1.6 \times 10^{-13}$.

The fractionation of the (α + γ)-fraction depends on the observation that the solubility in

aqueous alcohol of α-histones increases as their isoelectric point is approached while the converse is true of 1.6 γ-histone. Controlled treatment with ethanol at a high pH thus yielded a fraction, contained in the supernatant, which possessed no material with a sedimentation coefficient greater than 0.8×10^{-13}. Electrophoretic analysis indicated it to contain the α_1-, α_2-, α_3- and 0.8 γ-components. This material is known as the α-fraction. The material with $S_{20} = 2.0$ which was precipitated by the ethanol treatment yielded, on further ultracentrifugation, an apparently pure γ-histone with $S_{20} = 1.6$, after the sedimentation of a small amount of aggregated material.

These observations formed the basis of the fractionation procedure, now to be described, used for the isolation of highly purified samples of the β- and 1.6 γ-components from various whole histones.

Fractionation of Crude Thymus Histone

A 2% solution of unfractionated ox-thymus histone was dialysed in Visking tubing for a

period of five days against two daily changes of veronal plus acetate buffer, pH 9.3, $\frac{\Gamma}{2}$ (ionic strength) = 0.5. This resulted in the β-component aggregating to give material with a sedimentation coefficient of about 270 x 10^{-13}. At 37,020 r.p.m. in Preparative Rotor K, sedimentation of this material was calculated to be complete in 25 minutes, a run of 2 hours being actually employed to ensure that all the aggregated material was sedimented and to pack the gel more firmly. When the run was completed, the colourless supernatant was decanted from the gel and dialysed against water for three days (2 changes daily). The solution was then made 0.1 N with respect to sulphuric acid and the (α + γ)-fraction precipitated by dialysis against 70% ethanol and subsequently absolute ethanol. Finally, six volumes of ethanol were added and the precipitate centrifuged off and dried with alcohol, alcohol-ether and ether.

The treatment of the gel for the preparation of the purified β-histone is described subsequently.

α-Fraction

A 2% solution of the (α + γ)-fraction was dialysed against veronal plus acetate buffer, pH

9.5, $\frac{\Gamma}{2}$ = 0.2, for three days. One fifth of its volume of ethanol was stirred in, and the mixture allowed to stand overnight. Next day the opalescent solution was centrifuged in Rotor K for 1 hour at 37,020 r.p.m. (S_{20} value 450 x 10^{-13}, sedimentation time 15 minutes). This yielded a water clear supernatant and a slightly opaque but colourless jelly. The former was decanted, dialysed against water, made 0.1 N with respect to sulphuric acid and precipitated and dried in the usual way. This constituted the α-fraction.

1.6 γ-Component

The sedimented component was dissolved in water, dialysed, acidified and precipitated as usual. This was then freed from traces of the α-components and 0.8 γ-histone by dissolving it in water, dialysing against veronal plus acetate buffer, pH 9.5, $\frac{\Gamma}{2}$ = 0.20, for three days and repeating twice the alcoholic precipitation procedure. The purified 1.6 γ-histone was then dissolved in a little water and dialysed against veronal plus acetate buffer, pH 8.5, $\frac{\Gamma}{2}$ = 0.20, for three days and the solution ultracentrifuged. A small amount of material sedimented rapidly

leaving the 1.6 γ-component in the supernatant. This was decanted, dialysed exhaustively against distilled water (7 days at 2°C) and freeze-dried. The product is known as 1.6 γ-histone sulphate.

β-Component

The gel of aggregated β-material from the first fractionation stage was dissolved in water, redialysed and sedimented as previously except that in this case the ionic strength of the buffer was increased to 1.0. The gel was dissolved in a little 0.1 N-hydrochloric acid and two volumes of 4.5 M-sodium chloride, $\frac{N}{20}$ with respect to sodium hydroxide were added. This resulted in immediate precipitation of the β-histone as a fine white granular precipitate. This was spun down at 1200 g, the sediment washed by stirring with 3 M-sodium chloride and spun down again.

After dissolving the precipitate in 0.1 N-hydrochloric acid, the above precipitation procedure was repeated three times. Finally, after solution in 0.5 N-hydrochloric acid and exhaustive dialysis of the solution against

distilled water (7-8 days at 2°C), the solution was freeze-dried. The light colourless material obtained is referred to as the β-component sulphate.

CHROMATOGRAPHY OF THE α-HISTONES

Chromatography on the Ion-Exchange Resin IRC-50

Introduction

The success which has attended the purification of basic proteins such as cytochrome c (Paleus and Neilands, 1950), ribonuclease (Hirs, Moore and Stein, 1953), lysozyme (Tallan and Stein, 1953), and chymotrypsinogen (Hirs, 1953) by the method of ion-exchange chromatography, and the reported fractionation of calf-thymus histone on the carboxylic acid resin IRC-50 (Crampton, Moore and Stein, 1955) suggested that a similar approach might permit a fractionation of the α-histones into their components.

In preliminary experiments, use of the sodium form of the resin was found to result in almost irreversible binding of the α-histones. The failure of whole histones to achieve a finite reversible distribution coefficient with this form of the resin had previously been noted by Crampton et al. (1955) and it was clear that the α-histones shared this property despite their much lower arginine contents. The above authors' modified procedure was therefore employed. In this, the barium form of the resin was used in

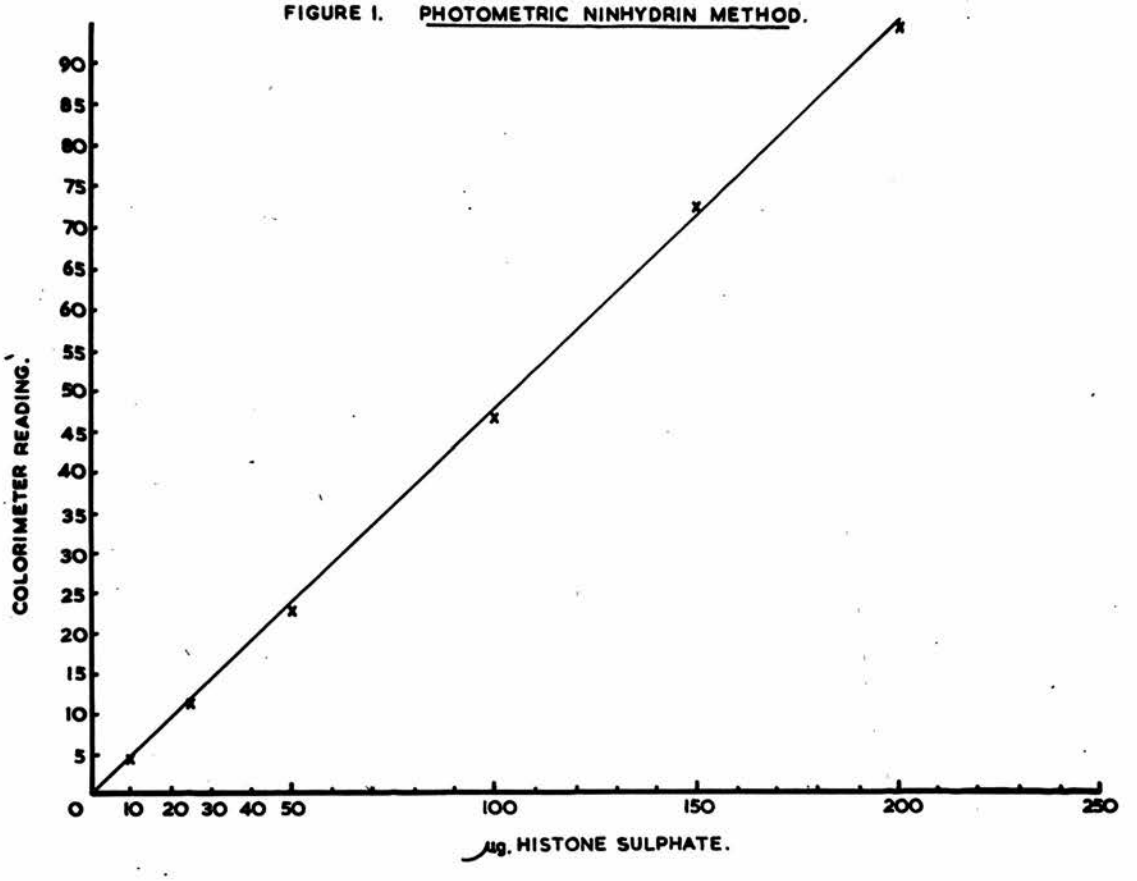
conjunction with barium acetate buffers of gradually increasing ionic strength and pH. In contrast to their finding that it was only possible to recover about half of the whole histone applied to the column, it was found possible to elute the α-histones in approximately 95% yield.

Though the mechanisms governing the distribution of histones between the resin and liquid phases are far from clear, the finding - described subsequently - of a reversible distribution of the α-histones between these two phases suggested the feasibility of their fractionation on ion-exchange columns.

Experimental

The detailed conditions for the preparation and equilibration of the barium form of the resin are set out in Appendix I. For the estimation of protein in the column effluents, the modified photometric ninhydrin method of Moore and Stein (1954) was used. A description of the method is given in Appendix II. Figure 1 indicates the

FIGURE I. PHOTOMETRIC NINHYDRIN METHOD.



sensitivity of the reaction using ox-thymus α-histone and demonstrates a linear relationship over the range 0-200 µg. histone sulphate.

Before any chromatography was attempted, it was necessary to determine whether a finite and reversible distribution of the histone occurred between the resin and liquid phases.

A series of graduated 10 ml. centrifuge tubes was set up and a slurry of resin, equilibrated in 0.1 M-barium acetate buffer, pH 6.73, was added to each tube such that after centrifuging 2.5 ml. of packed resin were present. Two tubes were run as blanks throughout the experiment. To each of the remaining tubes was added 1.0 ml. of a solution containing 2 mg./ml. of ox-thymus α-histone in 0.1 M buffer, pH 6.7, and the total volume adjusted to 5.0 ml. with buffer. Each tube was stirred with a glass rod for 2 minutes and a further 5 minutes, with intermittent stirring, allowed for equilibration. The tubes were centrifuged and 1.0 ml. of supernatant pipetted from each tube for estimation. The excess supernatant was decanted and fresh 0.1 M

buffer added to each tube up to the 5.0 ml. mark, and the same procedure repeated. The concentration of the eluting medium was then increased to 0.15 M, pH 6.70, and subsequently to 0.25 M, pH 6.70. From the amount eluted with each re-equilibration the amount of histone remaining on the resin could be calculated and the approximate distribution ratios obtained (Table 2).

Table 2. Distribution coefficients of ox-thymus α-histone.

Concentration of buffer	Distribution ratio $\frac{\text{Histone/ml. packed resin}}{\text{Histone/ml. effluent}}$
0.1 M	4.1
0.15 M	1.1
0.25 M	0.5

The 0.25 M buffer however, even after renewed re-equilibrations, failed to remove all the bound histone. Quantitative elution of the histone

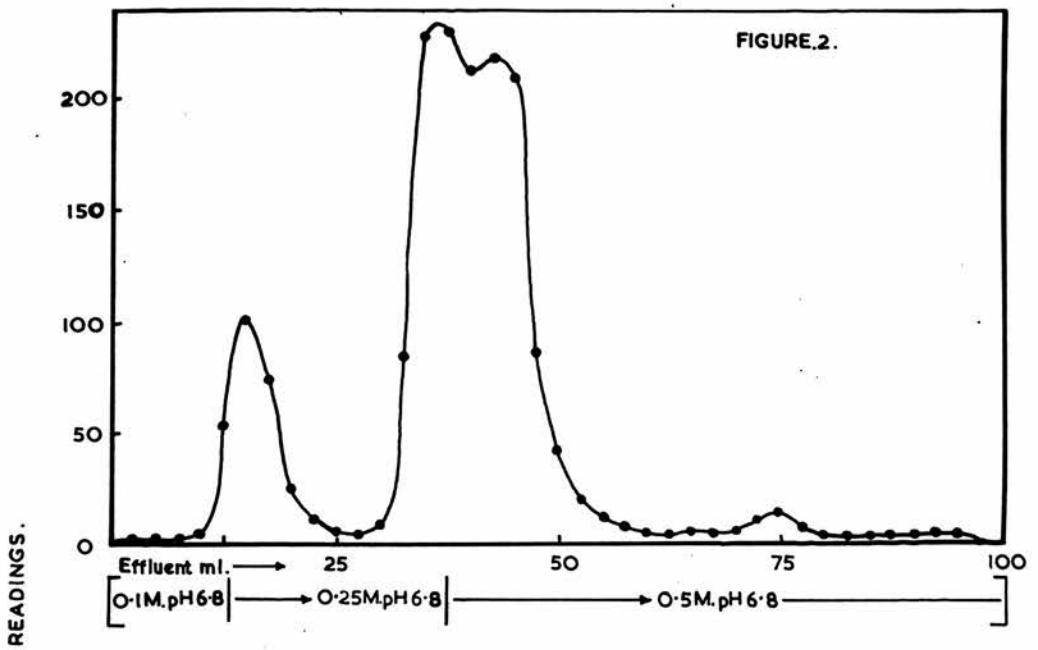
from the resin was, however, achieved by the use of 0.50 M buffer, pH 6.80.

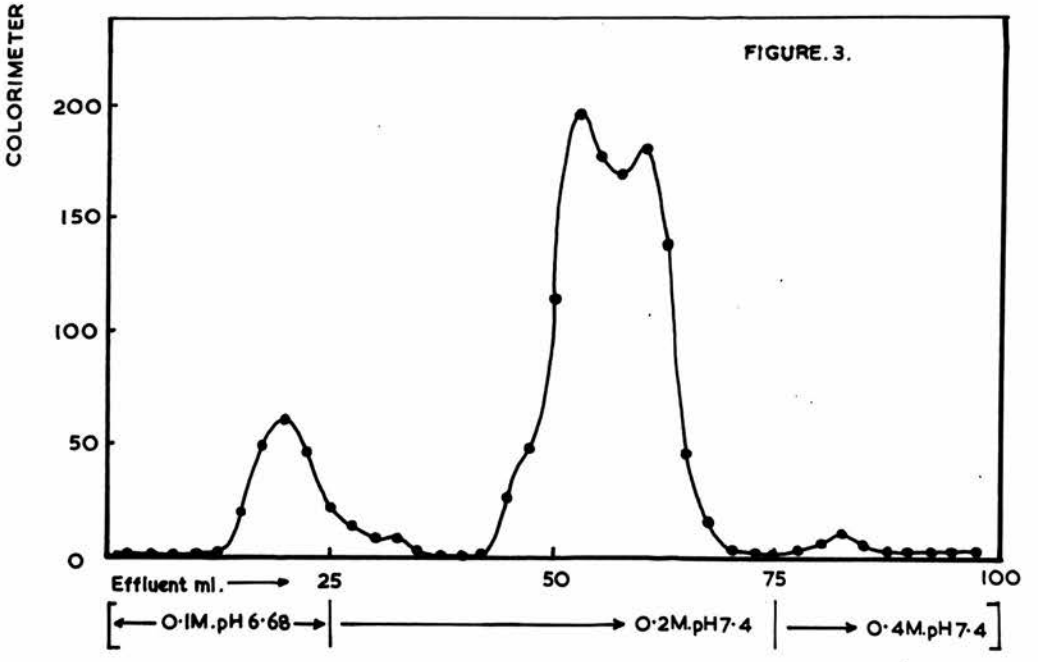
Column Chromatography

The analytical chromatograms were run using columns 0.9 cm. in diameter and 28-40 cm. high, fitted with sealed in sintered glass filter discs, and packed with resin as described in Appendix I. The histone (2-8 mg.) was dissolved in a few drops of water and diluted with buffer solution to a known volume (2-4 ml.), the barium sulphate precipitate being centrifuged down. An aliquot was placed on the column and carefully washed in with 2-3 ml. of buffer. A further portion was submitted to serial dilution and analysis by the ninhydrin method in order to permit subsequent calculation of the percentage recovery from the column.

Chromatography was carried out with eluents of gradually increasing ionic strength and pH by the use of a small magnetically stirred device (Crampton et al. 1955). The columns were operated at flow rates from 1.5-9 ml. per hour without affecting their performance. The effluent was

CHROMATOGRAPHY OF OX-THYMUS α-HISTONE ON IRC-50 RESIN.

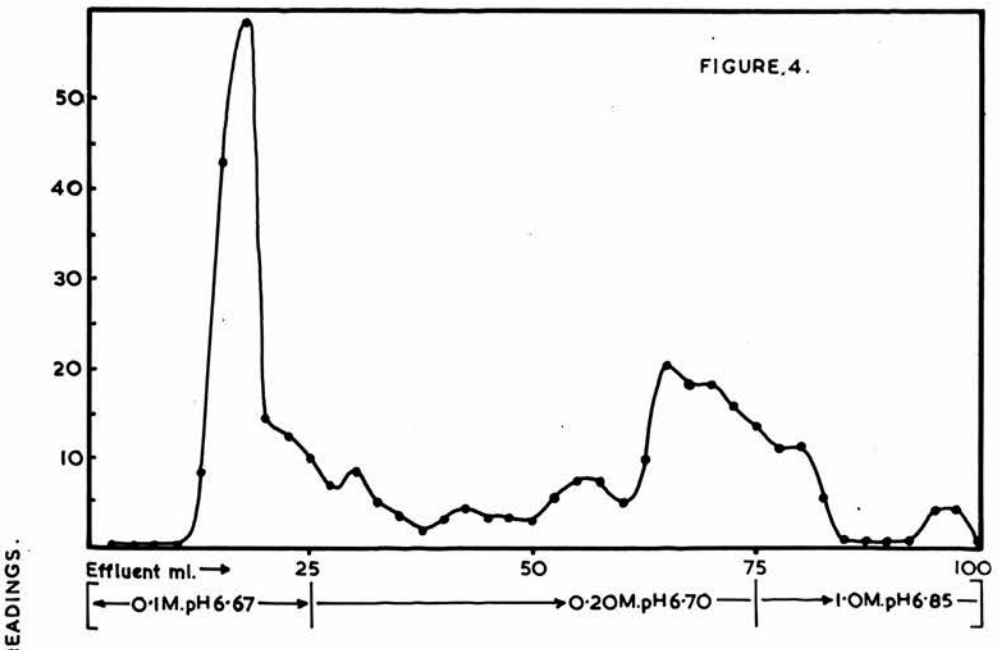


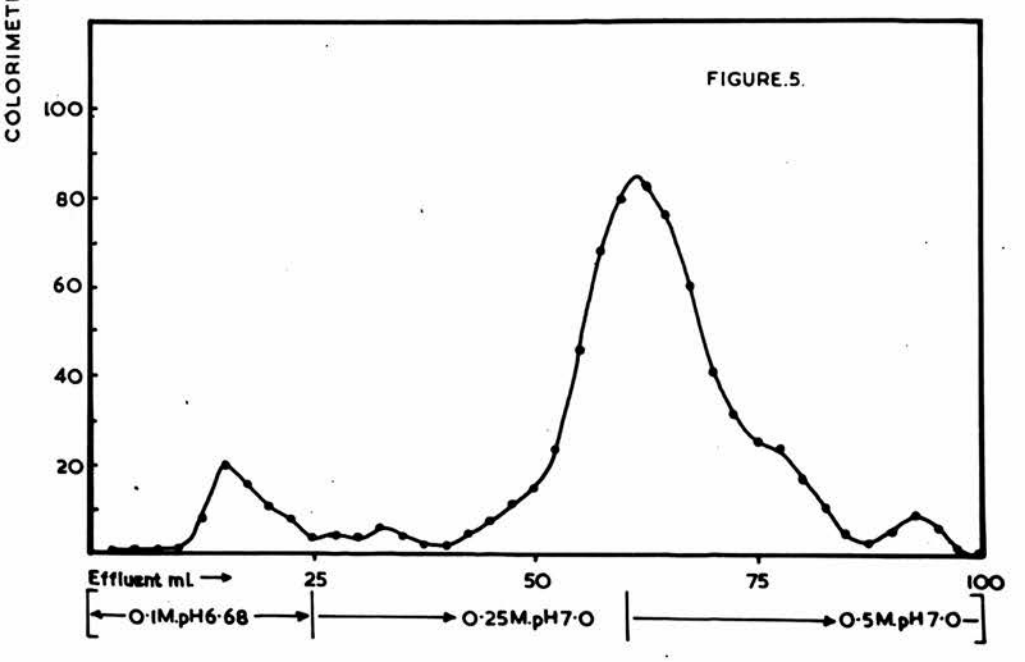


collected in 2.5 ml. portions by means of a siphon arrangement using an automatic fraction collector. These were analysed by the photometric ninhydrin technique, the blank readings gradually increasing as the barium acetate concentration in the effluent rose. The base line for each peak on the effluent curve was estimated from the blank readings preceding and following the peak.

In the first experiments 4-6 mg. of ox-thymus α-histone in 2.0 ml. of the initial buffer were applied to columns 30 cm. long. The flow rate was 6 ml./hour, and varying changes in the influent concentrations were made. Figures 2 and 3 reproduce the effluent curves obtained from two such experiments. The recoveries from the column, based on ninhydrin colour values, were 93 and 98% respectively. In both runs two main peaks and one very small one were obtained with some evidence of splitting of the centre peak. In order to try to increase the resolution of the fractionation of this peak, various buffer solutions operating at varying concentration gradients were employed. No improvement was

CHROMATOGRAPHY OF OX-LIVER α-HISTONE ON IRC-50 RESIN.





however obtained. Indeed, it appeared from some of these experiments that the relative sizes and shapes of the peaks depended on the elution conditions (Figure 4). Where the elution pattern departed from that shown in Figures 1 and 2 however, recoveries from the column were invariably down to 30-40%. The reason for this is not clear. Furthermore, it appeared that a small increase in pH was much more effective in removing bound histone than a small rise in the ionic strength of the influent. Davison and Shooter (1956) have fractionated whole histone on columns of carboxylated cellulose. They reported the dependence of the elution pattern on the pH of the buffer used. Their results, however, may perhaps be explained by the aggregation of the β- and γ-histones and their consequent immobilization on the columns.

Ox-liver α-histone gives the elution pattern shown in Figure 5. Its close similarity to the ox-thymus pattern is evident. Recovery from the column was 95%.

In other experiments with both ox-thymus and ox-liver α-histones, it was found that a low

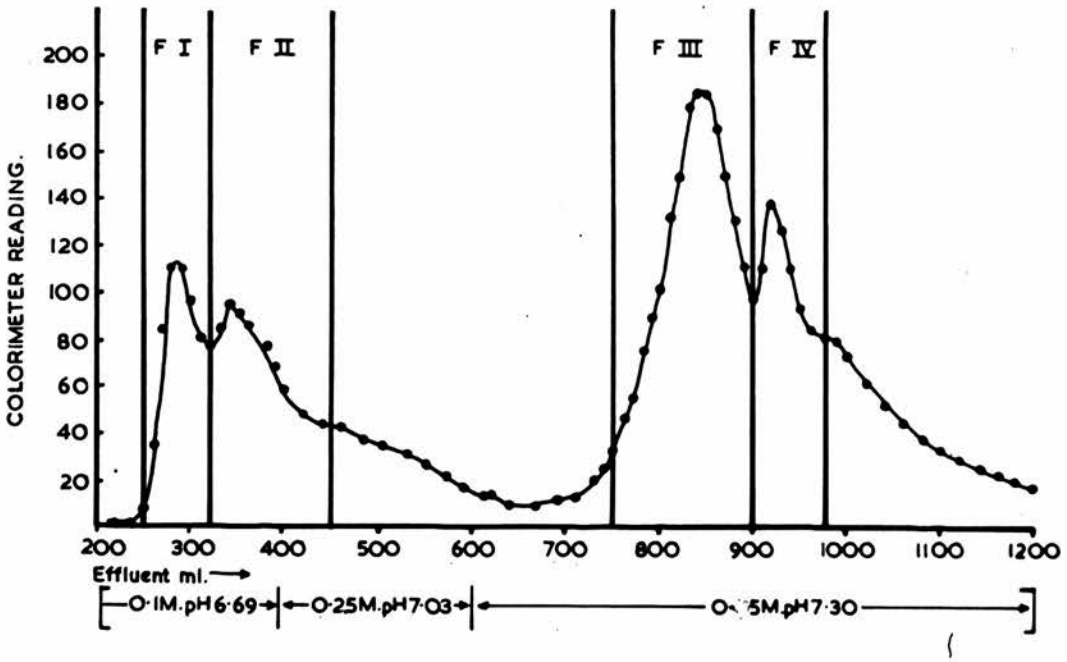
concentration gradient between the two main peaks gave a series of small bumps on the elution curve. With a higher gradient however, these did not appear. Other α-histone preparations, notably those from rat-liver and rat-hepatoma (azo-dye induced), showed similar elution patterns.

These results suggested that, though some unrecognized factors were involved, a genuine fractionation might be occurring. Accordingly, three preparative scale experiments were performed.

The columns used for these runs measured 4 cm. in diameter x 60 cm. long, and the elution gradient was provided by a 1 l. magnetically stirred mixing chamber. The temperature varied within the limits 17±2°C, and the histone used for each run was from the same batch. Further details of the setting-up of the columns are appended (Appendix I).

A solution of 300 mg. of the ox-thymus α-histone sulphate in 10 ml. of buffer was applied to the column which was run at a flow rate of 70 ml./hour. The first peak was brought off by 400 ml. of 0.1 M buffer, pH 6.69. 200 ml. of 0.25 M buffer, pH 7.03, were then run into the mixing chamber, followed by 700 ml. of 0.50 M buffer,

FIGURE .6. PREPARATIVE-SCALE CHROMATOGRAPHY OF OX-THYMUS α-HISTONE ON IRC-50 RESIN.



pH 7.30. About 18 hours were required for the run. The fractions were collected in 10 ml. cuts using a siphon and a fraction collector. From each tube 0.50 ml. portions were removed and analysed by the ninhydrin method. Figure 6 reproduces the elution pattern obtained.

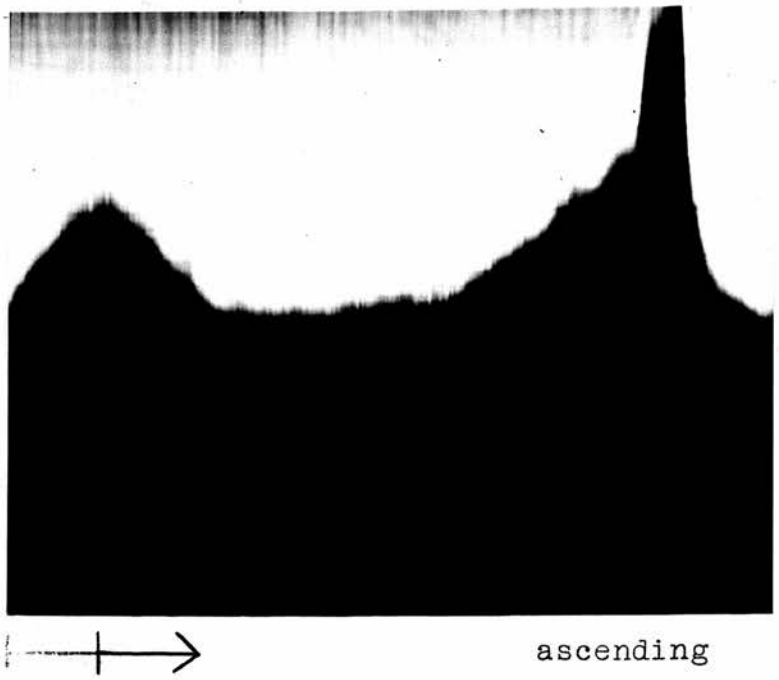
Each of the three runs was made under as nearly identical conditions as possible. The resin after each run was recycled through the hydrogen and sodium forms as described in Appendix I.

In order to isolate the fractionated material, the cuts indicated were bulked and stored frozen solid in polythene bottles until all the runs had been completed. The bulked fractions were then transferred to Visking cellophane dialysis tubing and dialysed against six changes of distilled water (50 hours) in a cold room at 2°C until a sample of the dialysate no longer gave a cloudiness with dilute sulphuric acid. The solutions were then made 0.1 N with respect to sulphuric acid and centrifuged to remove any dust particles etc.. The histone sulphates were finally precipitated by redialysis of the solutions against 70% ethanol and subsequently absolute ethanol. They were

Plate I

Ox-Thymus α-Histone - Fraction III

pH = 8.6, 0.8% solution, t = 2 hours, θ = 7

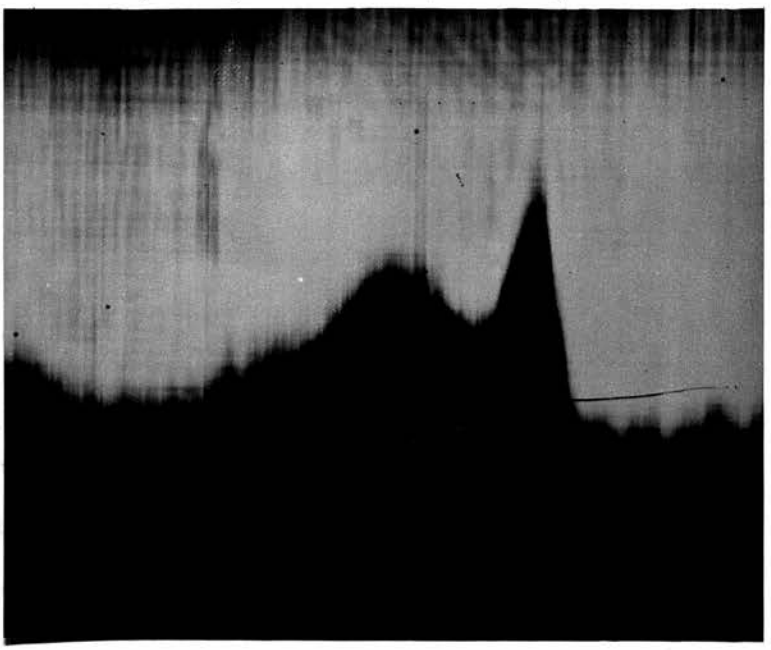


ascending

Plate II

Ox-Thymus α-Histone - Fraction IV

pH = 8.6, 0.8% solution, t = $1\frac{1}{2}$ hours, θ = 5



ascending

removed from the dialysis tubing with the aid of absolute ethanol, collected by centrifuging, and dried with alcohol-ether and ether. The air-dried products were thereby isolated as white powders.

Fraction I however, failed to yield any weighable material. The overall yields were:-

Fraction I	< 1 mg.
Fraction II	23 mg.
Fraction III	130 mg.
Fraction IV	100 mg.

Less than a third of the material applied to the columns was recovered despite the ninhydrin estimations indicating over 90% elution. Considerable losses were clearly associated with the dialysis.

Electrophoretic Behaviour

Fractions III and IV were studied electrophoretically in the Tiselius instrument. The method is described in Appendix III. The results shown in Plates I and II (ascending boundaries) indicate a wide heterogeneity of the isolated

materials. The partial overlapping of the peaks corresponding to Fractions III and IV would be expected to cause the appearance in the Schlieren photograph of a small quantity of a second component. The heterogeneity revealed, however, is much greater than can be accounted for by such contamination.

The nature of these fractions eluted from the resin columns is not known though they would appear to correspond to the A and B fractions isolated by Crampton et al. (1955). They clearly do not represent single components. After this work had been completed, Crampton, Moore and Stein (1957) reported on their further studies of calf-thymus histone. Their chromatographic findings almost duplicate those described above and furthermore, on studying the amino acid composition of histone material obtained from two portions of the same peak, they found considerable differences in the content of alanine, histidine, lysine and arginine. These results clearly support the heterogeneity indicated by electrophoresis.

Chromatography on Basic Alumina

Introduction

In view of some recent work in which the protamines clupeine and salmine were fractionated on basic alumina columns (Scanes and Tozer, 1956b), an attempt was made to apply this method to the α-histone fraction. This approach seemed reasonable since the α-histones, like the protamines, are highly basic proteins and also exhibit the same order of molecular weight.

Experimental

In all experiments Savory and Moore's Activated Alumina, 100/150 mesh, was used. Pretreatment of the alumina consisted only in stirring it with distilled water to remove air bubbles and decanting the supernatant to remove any finely divided material. The buffer solution was then added, the mixture stirred, and the supernatant decanted when the alumina, after slurrying with more buffer, was ready for use.

As in the case of the resin, the first

experiments were designed to investigate the conditions under which reversible adsorption of the histone on the alumina occurred. The centrifuge tube technique described previously was used. With 0.20 M-potassium hydrogen phosphate buffers at different pH's, and with a loading of 2.2 mg. histone sulphate per 2.5 ml. packed alumina, the results shown in Table 3 were obtained.

Table 3. Adsorption of ox-thymus α-fraction histone on alumina.

pH of buffer	9.5	10.0	10.5	11.0
% unadsorbed on alumina	8	10	16	50
% eluted with first eluting volume	5	6	11	25
% eluted with second eluting volume	2.5	3	9	25

Further experiments using 0.42 M-potassium hydrogen phosphate buffers at pH's ranging from 6-11 indicated very little adsorption onto the alumina. Furthermore, it became evident that

true completely reversible adsorption was not occurring since solvents, from which no histone was adsorbed by the alumina, failed to remove completely histone which had been adsorbed at a lower ionic strength.

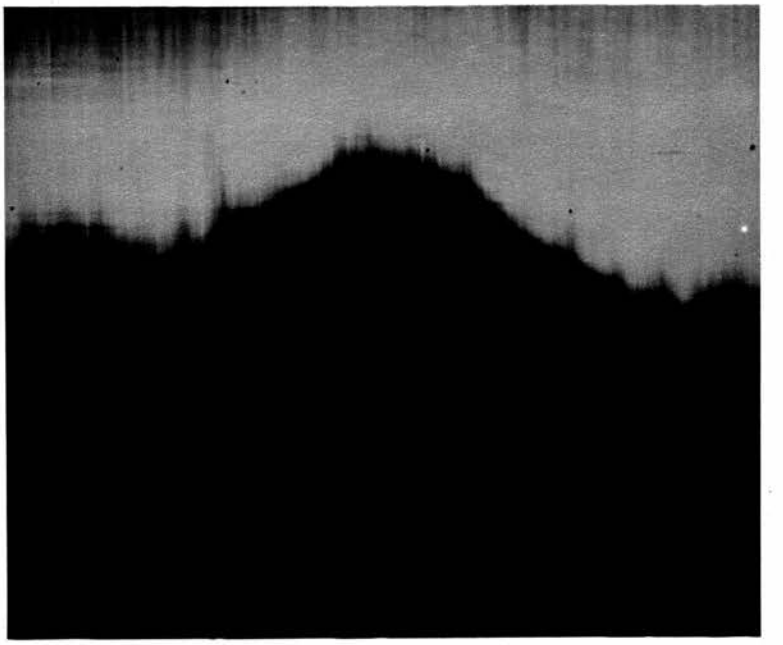
A number of analyses using columns 0.9 cm. in diameter x 40 cm. long were however run using different pH's and ionic strengths. The mixing chamber was dispensed with in these experiments, the increased concentrations of buffer being introduced directly into the reservoir funnel. Recoveries from the columns of ninhydrin positive material were about 70%, the same general elution pattern being obtained as from the ion-exchange columns. In these experiments, however, irregular results were found to depend on the attacking of the alumina by the buffers at the high pH's required to elute the histone.

When a large-scale preparative run was performed with a loading of 490 mg. ox-thymus α-histone and column 4 cm. in diameter x 58 cm. long, the dissolved alumina from the column reprecipitated as a gelatinous mass in the tubes collecting the later fractions of the column effluent. Ninhydrin positive material was eluted in 40% yield. The effluent pattern is

Plate III

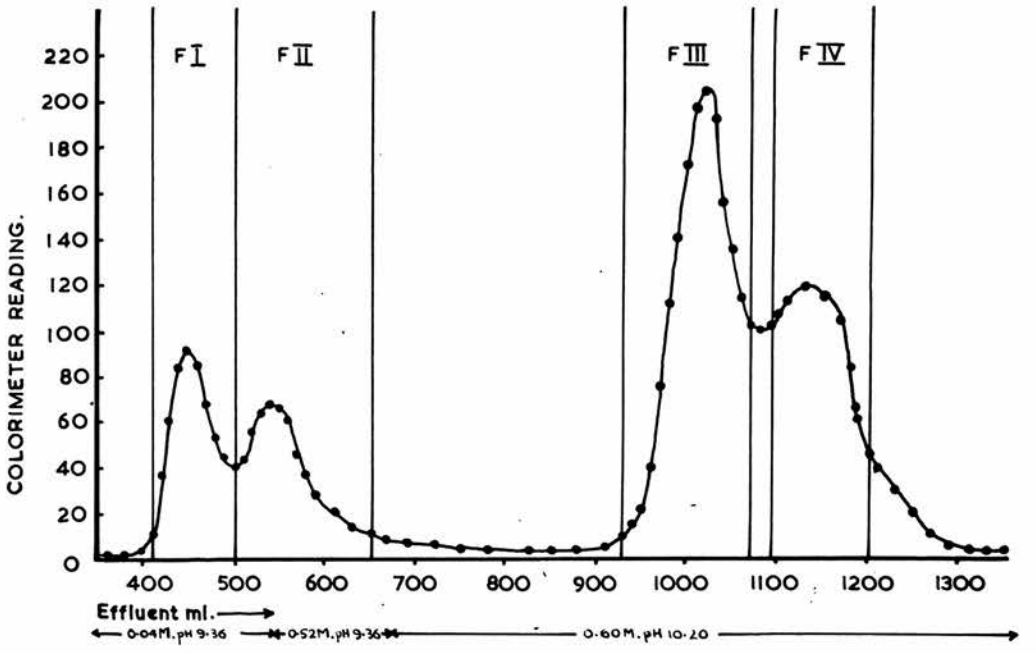
Ox-Thymus α-Histone - Fraction III

pH = 8.6, 0.8% solution, t = 2 hours, θ = 5



ascending

FIGURE.7. PREPARATIVE-SCALE CHROMATOGRAPHY OF OX-THYMUS α-HISTONE ON BASIC ALUMINA.



shown in Figure 7. The appropriate tubes were bulked as shown to give four fractions. These were stored frozen in polythene bottles. A second preparative run was performed under identical conditions and a similar effluent pattern was obtained. Subsequent dialysis, acidification and precipitation of the histones from the bulked fractions from both runs gave the following yields of dry histone sulphate:

Fraction I	No recoverable material
Fraction II	No recoverable material
Fraction III	92 mg.
Fraction IV	110 mg.

Fraction III was studied by moving boundary electrophoresis. Plate III shows the ascending boundary. The marked heterogeneity is evident.

Conclusion

The conclusion drawn from these studies is that chromatography of the subsidiary histones on the resin IRC-50 or basic alumina fails to produce a separation into electrophoretically distinct components.

ZONE ELECTROPHORESIS

Introduction

In view of the unsuccessful attempts to fractionate α-histones by chromatographic methods the use of another technique, not dependent on the same properties of the protein molecule as are involved in ion-exchange and adsorption phenomena, was desirable. The net charge existing on a protein molecule in solution at a particular pH is such a property. Electrophoretic analyses of α-histones by the moving boundary method (Cruft et al. 1954; 1957) have amply demonstrated that good separations can be obtained on this basis, and accordingly it was decided to use the technique of zone electrophoresis in an attempt to effect a separation.

Zone electrophoresis has been defined by Tiselius (1953) as the separation of materials in an electric field in the presence of some type of supporting medium in addition to the electrolyte solution. The possibilities of such a technique for the separation of closely related materials were fully appreciated by Consden, Gordon and Martin (1946) who used a silica-gel supporting medium to separate amino acids. Modifications of this procedure by Gordon (1950) also led to the separation of whole proteins.

Raacke and Li (1954) in their studies on crystalline ribonuclease showed that zone electrophoresis in starch was capable of revealing heterogeneity in cases where the moving boundary system failed to do so. It has been suggested in this connection that starch is not a completely inert support but exhibits some weak adsorptive capacity, especially towards basic proteins, and so causes an enhanced separation (Flodin and Porath, 1954; Kunkel, 1954).

The successful migration on starch of such basic proteins as lysozyme (Raacke, 1956) lent additional support to the applicability of such a method for the fractionation of the α-histones into their components though previous experience in this laboratory had shown a filter paper supporting medium to be quite unsuitable on account of heavy tailing.

Experimental

The first experiments were performed in order to find out whether any significant adsorption of ox-thymus α-histone occurred on starch. For this purpose the rate and extent of elution of the histone from columns of starch by various buffer

solutions was studied.

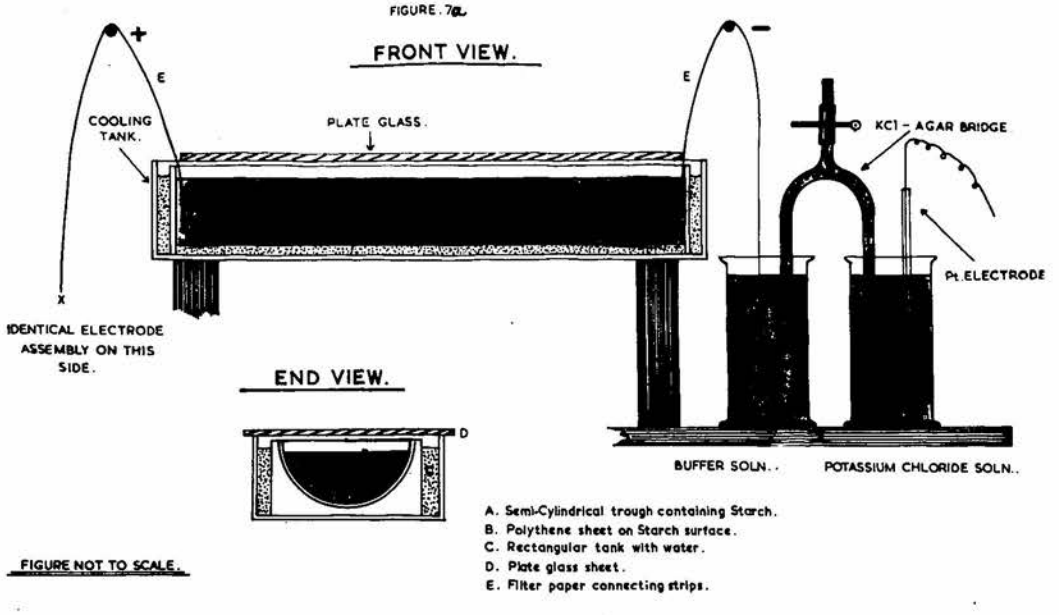
Ordinary potato starch, supplied by British Drug Houses Ltd., was thoroughly washed by repeatedly stirring it with warm distilled water, allowing it to settle, and decanting the supernatant until no cloudiness was evident in the latter after settling for half an hour. After stirring the washed starch with five volumes of the buffer solution to be used subsequently for elution, allowing it to settle, and discarding the supernatant, it was slurried with a little more buffer and poured into a 0.9 x 30 cm. column so as finally to give a starch column 30 cm. in height. The histone sulphate (2-5 mg.) was dissolved in 1 ml. of the appropriate buffer and an aliquot applied to the column which was run, under pressure, at a rate of 8 ml./hour. The effluent was collected in 1 ml. fractions and analysed by the ninhydrin technique. Using 0.03 M-phosphate buffer, pH 7.17, and also 0.1 M-veronal plus acetate buffers, pH 7.66 and 8.6, quantitative elution of the histone from the column was obtained in a single unretarded peak. Negligible adsorption is thereby indicated. Since moving boundary electrophoresis experiments with the α-histones had previously indicated that 0.1 M-veronal plus acetate buffer, pH 8.6 and ionic

strength 0.176, gave the highest degree of resolution, this buffer was adopted for the zone electrophoresis experiments.

All experiments were conducted in a cold room at 0-2°C. The preliminary analytical scale electrophoreses were performed in semi-cylindrical glass troughs 3.5 cm. in diameter x 30 cm. long, whilst for the later preparative experiments perspex troughs, 6.7 cm. in diameter x 65 cm. long, were used. These were constructed by cutting a perspex tube in half longitudinally and cementing on two end pieces.

The inert supporting medium was prepared as follows. Potato starch was stirred with about five times its weight of distilled water at 58°C, allowed to settle for half an hour and the supernatant decanted. This procedure was repeated twice with further quantities of hot distilled water followed by two washes with buffer solution, the first at 58°C and the second at 2°C. This gave a pure white starch preparation and a clear supernatant after settling for half an hour. The trough was filled to within 3 mm. of the top with a viscid mixture of the starch and buffer. After settling, the excess buffer was removed with

FIGURE. 7a.



A. Semi-Cylindrical trough containing Starch.
B. Polythene sheet on Starch surface.
C. Rectangular tank with water.
D. Plate glass sheet.
E. Filter paper connecting strips.

FIGURE NOT TO SCALE.

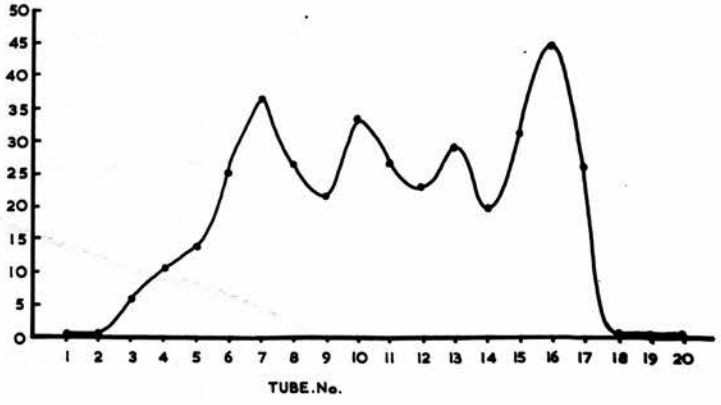
strips of thick filter paper. The trough, set in the horizontal plane, was contained in a larger rectangular tank filled with water in order to ensure adequate cooling. Electrical contact was made with strips of Whatman 3 MM filter paper (six thicknesses) dipping into jars containing the buffer which were in turn connected by means of potassium chloride-agar bridges to electrode vessels containing 5% potassium chloride solution into which dipped platinum electrodes. The experimental set-up is illustrated in Figure 7a. The starch block was allowed to stand overnight in order to achieve thermal equilibrium with the surroundings.

In the meantime the histone sample was prepared for introduction into the block. Ox-thymus α-histone sulphate (10-20 mg.) was dissolved in 0.5 ml. of buffer solution (300-400 mg. in 3-4 ml. for the preparative experiments) and dialysed for 36 hours against four changes of buffer. A segment of starch 0.5-0.6 cm. thick (1-1.5 cm.) was cut out from the block about one fifth of the distance from the anode end in such a way as to leave 2 mm. thickness of starch in contact with the trough. The segment so obtained

was sucked to near dryness in a Büchner funnel and the starch mixed with the dialysed solution to give a viscous mixture. This was transferred to the hole in the trough, filter paper being used to absorb any buffer solution displaced by the diffusion of the histone solution. After standing for 4 hours, the filter paper strips, moistened with buffer, were placed in contact with the block at each end of the trough. The block was then covered with a strip of polythene sheet laid directly onto the starch surface in order to prevent evaporation with consequent superficial distortion of the pattern of bands obtained, and finally covered with a sheet of plate glass. For electrophoresis in the smaller troughs a current of 10-20 mA. was passed for varying periods. The temperature of the starch did not rise appreciably. In the case of the preparative scale experiments a current of 40 mA. was used which gave a potential drop of approximately 2 volts/cm. along the trough. The heat generated was sufficient to keep the temperature of the starch 2°C above that of the surroundings. After a period of 80 hours the

FIGURE.8(a) DISTRIBUTION PATTERN OF OX-THYMUS $\mathcal{L}$-HISTONE AFTER ZONE ELECTROPHORESIS.

FIGURE.8(b)



fastest component had migrated a distance of about 40 cm.

Analytical Scale Electrophoreses

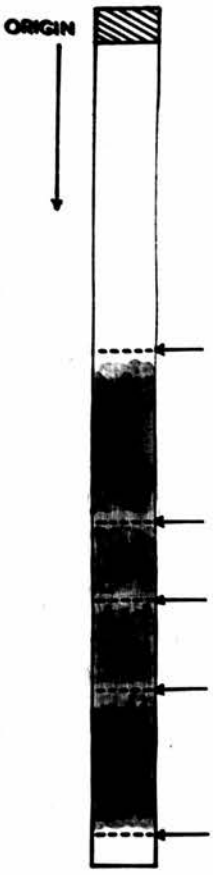
In a typical experiment, 16.0 mg. of ox-thymus α-histone were dissolved in 0.5 ml. of buffer solution and dialysed etc. as described. A current of 14 mA. was passed for 40 hours during which time a migration of 27 cm. was obtained. The distribution of the protein in the trough was localized by printing on strips of filter paper which were dried in an oven at 110°C and stained by immersion for 10 minutes in an 0.5% ethanolic solution of bromophenol blue saturated with mercuric chloride. After thorough washing with water, the histone distribution was indicated by the blue-dyed bands (Figure 8a). As a confirmation that the stained bands did, in fact, correspond to localizations of protein deeper in the block, the starch in the trough was sectioned into 1 cm. segments and each transferred individually into test tubes where they were extracted with 5.0 ml. of 0.1 N-sulphuric acid.

After centrifuging, 1 ml. portions of the supernatant were analysed by the photometric ninhydrin method. The distribution obtained is illustrated in Figure 8b. The positions of the peaks are seen to correspond closely to bands of maximum staining on the filter paper print. The necessity of cutting 1 cm. segments however, somewhat obscures the resolution revealed by printing.

Before these zones could be regarded as constituting the several components revealed by moving boundary electrophoresis (Plate VIII) it was necessary to isolate the histones from them and examine these further in order to determine their homogeneity and mobility. This work is described subsequently.

Since the mobility of an ion is inversely proportional to the square root of the ionic strength of the solution containing it, lowering the ionic strength would be expected to shorten the time required for a given degree of separation. Experiments made in this direction were unsuccessful, no separation into bands being obtained. This is presumably because at lower

FIGURE. 9.



DISTRIBUTION PATTERN OF OX-THYMUS α-HISTONE AFTER ZONE ELECTROPHORESIS.

LOADING	= 400 mg.
PERIOD OF RUN	= 80 HR.
CURRENT	= 38 mA
POTENTIAL DIFFERENCE ACROSS WHOLE ELECTRODE ASSEMBLY.	= 410 V.
DISTANCE OF MIGRATION OF FASTEST MATERIAL.	= 35 cm.

ionic strengths the histone molecules themselves contributed appreciably to the conductivity.

Preparative Electrophoreses

After the completion of each run a print was taken off the starch surface and stained to reveal the positions of the various fractions. A typical print obtained is illustrated in Figure 9. The block was then sectioned as indicated and the four sectors transferred to glass beakers of suitable sizes. The histones were recovered quantitatively from the starch by stirring with 0.1 N-sulphuric acid followed by filtration through two thicknesses of Whatman No. 1 paper on a Büchner funnel. The starch was washed four times with further small quantities of acid and sucked dry. The extracts were stored frozen solid in polythene bottles.

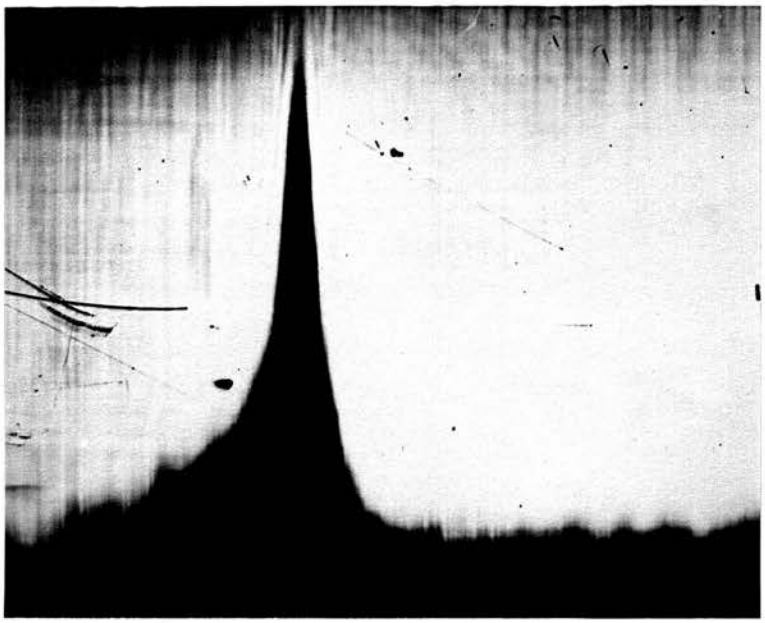
Five preparative experiments of the type described were performed and the total volume of bulked acid extracts amounted to 4.5 l. In order to avoid the considerable losses of α-histones which occur during dialysis of large volumes of dilute solutions the following technique was adopted.

The acidic solutions were first adjusted to pH 7-7.5 by the addition of 2 N-sodium hydroxide. The solutions obtained were transferred to standard blood transfusion bottles - two-thirds filling them - and freeze-dried. As much as possible of the dry white powders obtained was emptied into bags of Visking dialysis tubing and the residue in the bottles dissolved in 25 ml. portions of 0.1 N-sulphuric acid which were subsequently added to the powders in the dialysis tubing. Dialysis was performed against five changes of 0.1 N-sulphuric acid over a period of 60 hours, all the solid matter in the tubes dissolving during the first few hours of dialysis. The solutions were then transferred to centrifuge tubes and centrifuged at 1500 r.p.m. for 5 minutes to remove dust particles etc.. The histone sulphates were finally precipitated from the supernatants by the addition of twelve volumes of acetone and allowing the mixtures to stand over-night in a cold room (2°C). The white precipitates formed were centrifuged down and dried with acetone, acetone/ether and ether. The yields of dry histone sulphates obtained are shown in Table 4.

Plate IV

Ox-Thymus α-Histone - Fraction 1

pH = 8.6, 0.8% solution, t = $1\frac{1}{2}$ hours, θ = 6



ascending

Plate V

Ox-Thymus α-Histone - Fraction 2

pH = 8.6, 0.8% solution, t = $2\frac{1}{2}$ hours, θ = 5

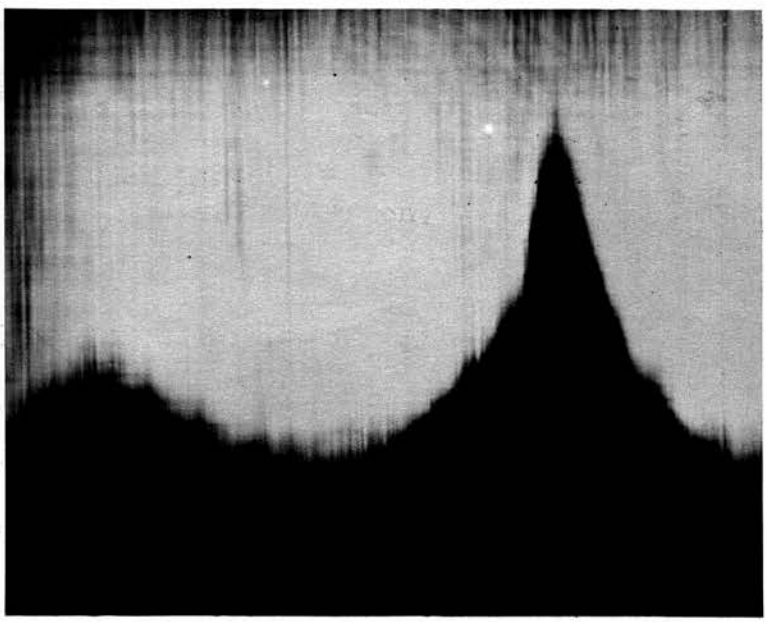


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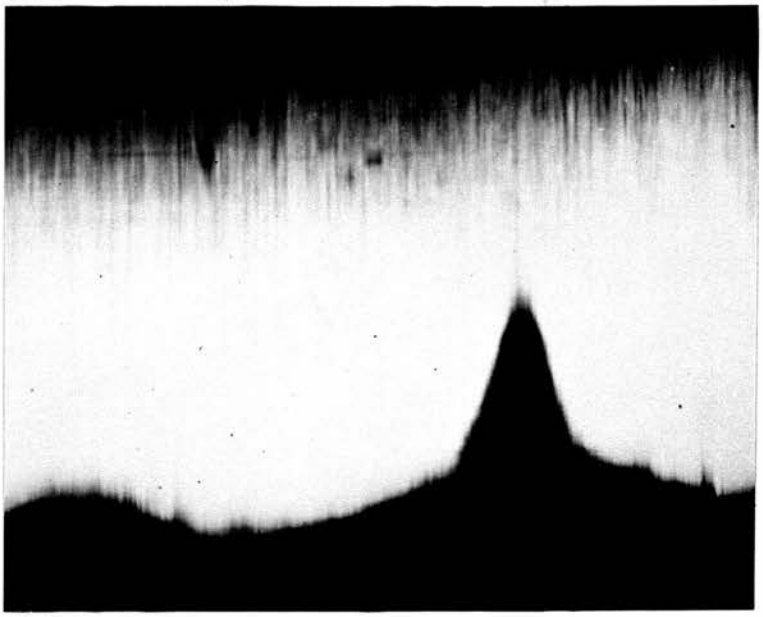
Plate VI

Ox-Thymus α-Histone - Fraction 3

pH = 8.6, 0.6% solution, t = 2 hours, θ = 5



ascending

Plate VII

Ox-Thymus α-Histone - Fraction 4
pH = 8.5, 0.8% solution, t = $1\frac{1}{2}$ hours, θ = 12

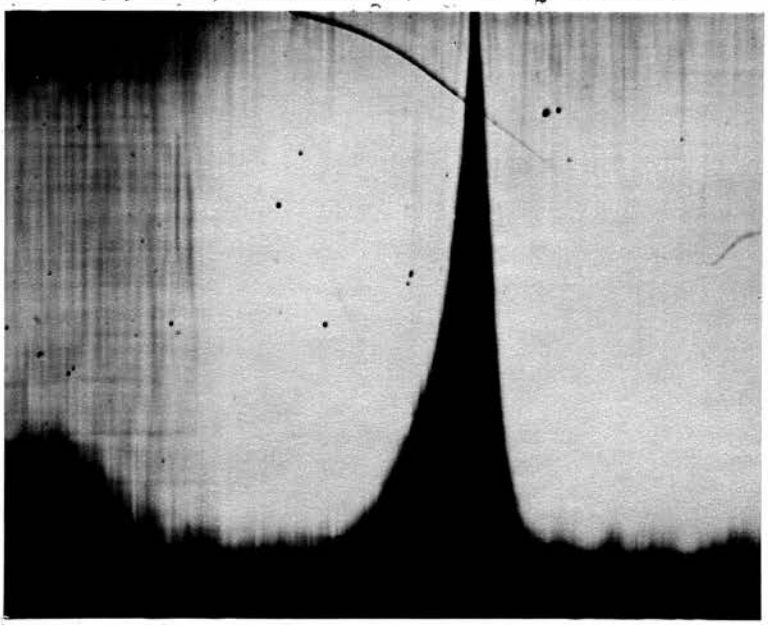


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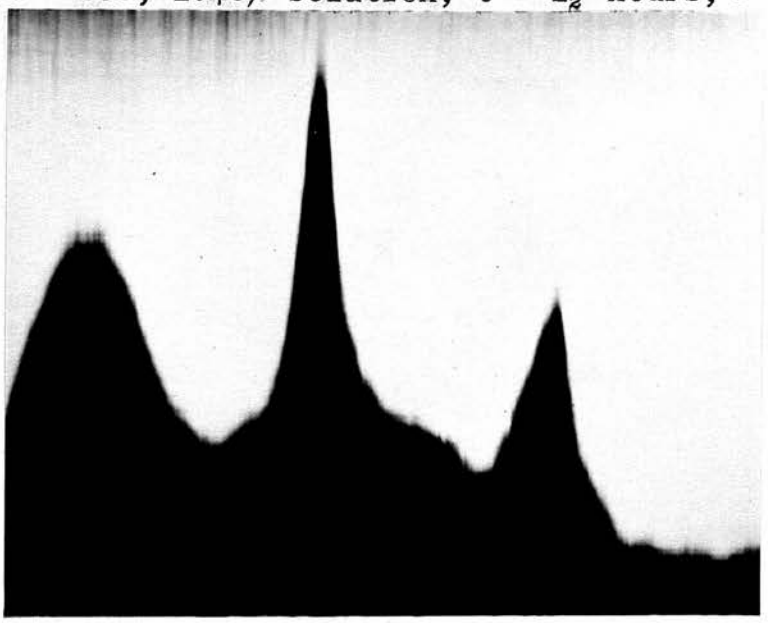
Plate VIII

Ox-Thymus α-Histone - Starting Material

pH = 8.6, 1.46% solution, t = 1½ hours, θ = 6



0.8γ- α_3- α_2 α_1

ascending

Table 4. Yields of fractionated material from starch blocks.

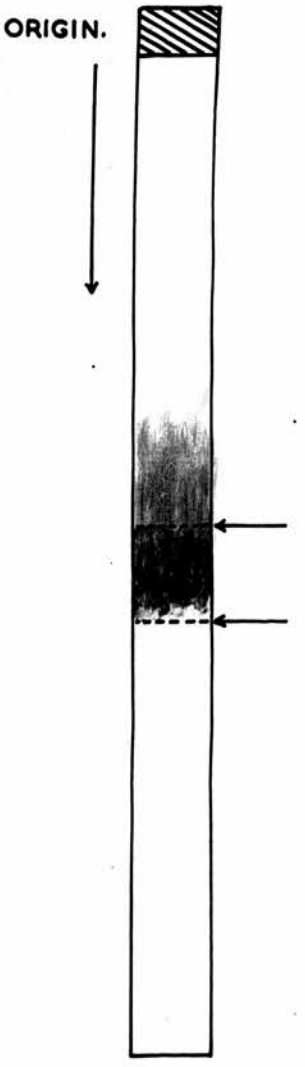
Fraction	Yield
1 (slowest)	600 mg.
2	190 mg.
3	80 mg.
4 (fastest)	125 mg.

Purity and Characterization of the Fractions

(a) Moving Boundary Electrophoresis

Approximately 50 mg. quantities of each fraction were dissolved in 7.0 ml. of veronal plus acetate buffer, pH 8.66, $\frac{\Gamma}{2} = 0.176$, and dialysed for three days against five changes of buffer. In addition, 146 mg. of the original ox-thymus α-fraction were dissolved in 7.0 ml. of the same buffer and dialysed for the same period, the volumes of the solutions being finally adjusted to 10 ml. by the addition of buffer solution. Plates IV, V, VI, VII and VIII reproduce the ascending boundaries, after the times shown, of fractions 1, 2, 3 and 4 and the starting material respectively.

FIGURE. 10.



DISTRIBUTION PATTERN OF OX-THYMUS 0·8 γ COMPONENT AFTER ZONE ELECTROPHORESIS.

LOADING	= 300 mg.
PERIOD OF RUN	= 90 HR.
CURRENT	40 mA.
POTENTIAL DIFFERENCE ACROSS WHOLE ELECTRODE ASSEMBLY.	= 435 V.
MEAN DISTANCE OF MIGRATION.	= 19 cm.

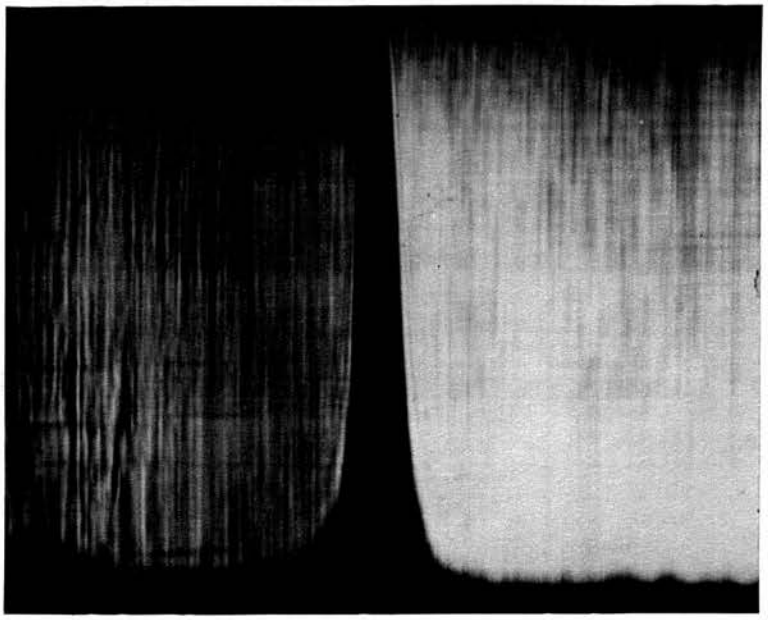
It is at once clear that zone electrophoresis has achieved a fractionation of ox-thymus α-histone into four distinct components. After allowing for the fact that the mobility of a particular component is affected by the presence of other components, close concordance was found between the mobilities of the isolated fractions and the corresponding components in the crude material. It was thus possible to identify fractions 1, 2, 3 and 4 as the 0.8γ, α_3-, α_2- and α_1-components respectively. Furthermore, the α_1-, α_2- and α_3-components would appear to be free from serious contamination.

In Plate IV the unsymmetrical broadening at the base of the peak suggests the presence of an even slower component contaminating the 0.8γ-component. Accordingly, the remaining 550 mg. of this histone were divided into two portions and re-run separately in starch blocks under the conditions used previously except for a slightly longer run of 90 hours. The filter paper print (Figure 10) confirms the suspicion that a small amount of a slower component is present. The starch block was sectioned where indicated and the histone

Plate IX

Ox-Thymus α-Histone - Purified 0.8 γ-component

pH = 8.6, 0.5% solution, t = 2 hr. 40 min., θ = 6



ascending

extracted with 0.1 N-sulphuric acid as described previously. The neutralized solution was lyophilized in a 500 ml. flask fitted with an acetone-dry ice cold finger arrangement using an oil pump. The residue, after dialysis, precipitation and drying, yielded 170 mg. of histone sulphate and it was clear that considerable losses were sustained during dialysis.

A sample of 80 mg. of the purified component was dissolved in veronal plus acetate buffer and dialysed as usual for moving boundary electrophoresis. Plate IX shows a single peak with only the normal spreading due to diffusion. The sample appears to be electrophoretically homogeneous.

(b) Ultracentrifuge Studies

The instrument used was a Spinco model E ultracentrifuge in conjunction with a 12 mm. synthetic boundary cell. All measurements were made from photographs taken at 59,780 r.p.m. using Analytical Rotor A.

Plate X

α_1-Component

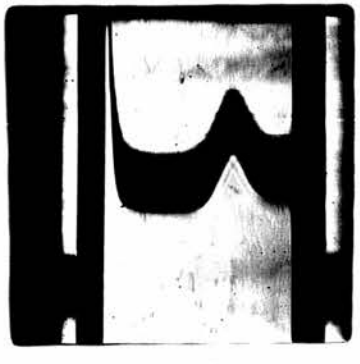
pH = 7.5, 0.2% solution

t = 128 min. t = 64 min. t = 0 min.

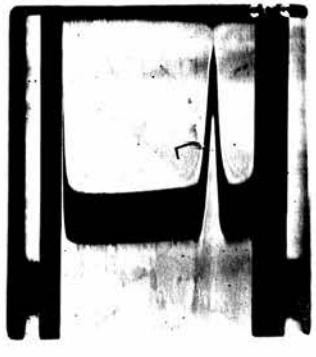
Plate XI

α_2-Component

pH = 7.5, 0.9% solution



t = 96 min.

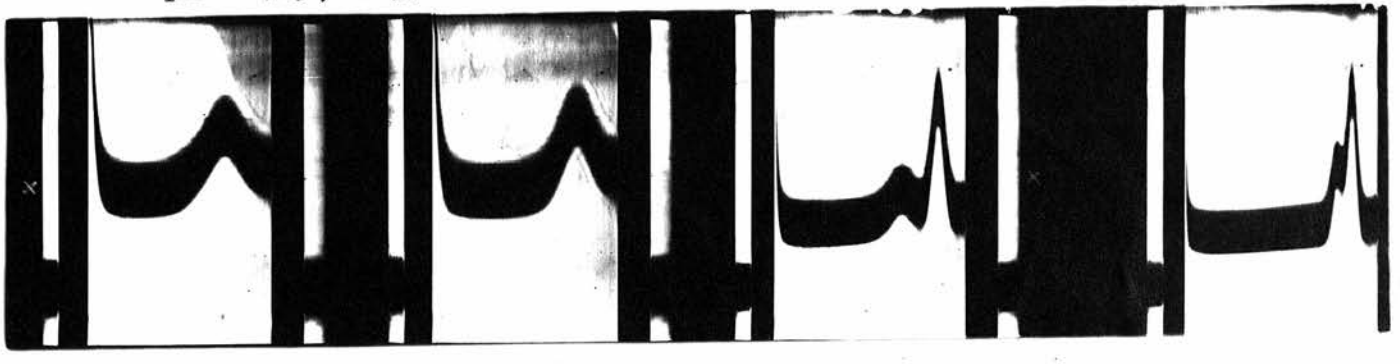


t = 0 min.

Plate XII

a_3-Component

pH = 7.5, 0.5% solution

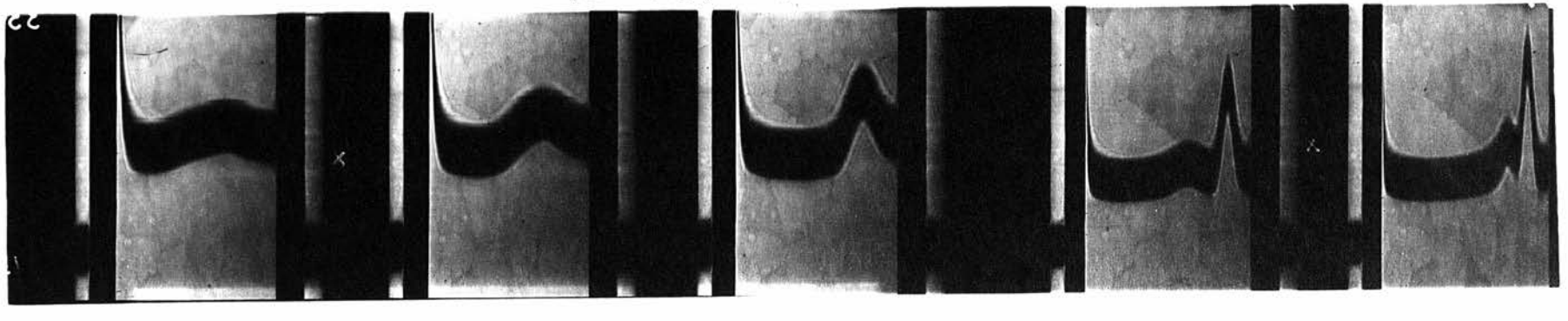


t = 104 min. t = 72 min. t = 8 min. t = 0 min.

Plate XIII

0.8 γ-Component

pH = 7.5, 1.2% solution



t = 184 min. t = 120 min. t = 56 min. t = 8 min. t = 0 min.

About 10-12 mg. of the α_1-, α_2-, α_3- and purified 0.8γ-components were dissolved in 0.5 ml. of veronal plus acetate buffer, pH 7.5, $\frac{\Gamma}{2}$ = 0.178, and the solutions dialysed for three days against five changes of the buffer. Plates X, XI, XII and XIII respectively reproduce the boundaries after the times shown.

It is immediately seen that, whilst the α_1- and α_2-components appear to be essentially homogeneous, the α_3- and 0.8γ-components are each associated with several per cent of a fast sedimenting material. The α_3-component after sedimentation of the high S_{20} material did not reveal any further heterogeneity. On the other hand, the 0.8γ-component was still not homogeneous and detailed analysis of the sedimentation diagrams indicated the presence of three components in all. At the very low S_{20} values involved in these experiments the resolving power of the ultracentrifuge is not good, the degree of separation between two components with sedimentation coefficients of 0.8 and 1.6 x 10^{-13} being largely obscured by diffusion of the relatively low molecular weight species. It was

established however that three components of sedimentation coefficients 0.8, 1.64 and 14.25 x 10^{-13} were present in 39, 31 and 30% amounts respectively. The fast sedimenting material associated with the α_3-component also accounted for 30% of the α_3-fraction obtained by zone electrophoresis. The sedimentation coefficients for the various components are shown in Table 5.

Table 5. Sedimentation coefficients.

Component	$S_{20} \times 10^{-13}$		
α_1	0.44		
α_2	0.82		
α_3	0.86		11.9
0.8γ	0.8	1.64	14.25

The presence of these extraneous materials associated with the α_3- and 0.8γ-components is readily explained on the basis of small quantities of the β- and 1.64γ-components remaining in the original α-fraction which was applied to the starch block. That the α_3- and β-histones, as well as the 0.8 and 1.64γ-histones, have almost if not identical mobilities, is evident from moving

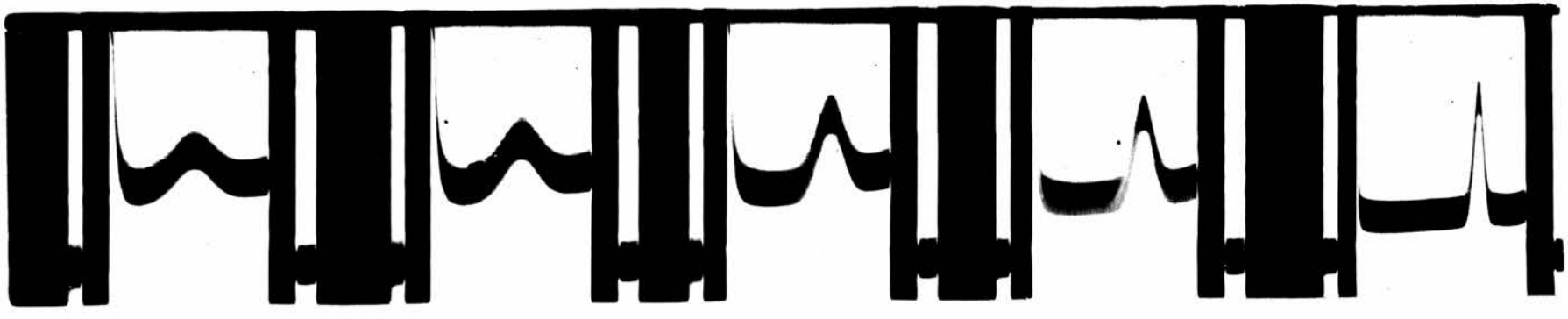
boundary experiments, and it appeared that the same was true during electrophoresis on a starch block. Aggregation of these components during the dialysis would give rise to the fast sedimenting material associated with the slower moving fractions.

The small amount of α_3 material available precluded any further attempts at its purification. Since 140 mg. of the 0.8γ-fraction were available however, it was decided to subject this to further fractionation by controlled treatment with ethanol (cf. pp. 23-24). The histone (together with that recovered from the moving boundary electrophoresis) was dissolved in 4 ml. of veronal plus acetate buffer, pH 9.3, $\frac{\Gamma}{2}$ = 0.176, and 1 ml. of ethanol added. This treatment should result in the precipitation of the 1.6γ-component. After standing for 24 hours the precipitate was centrifuged off and the supernatant dialysed against water for three days. Dilute sulphuric acid was then added to make the solution 0.1 N with respect to acid and the 0.8γ-component precipitated by the addition of an excess of acetone. The histone sulphate, after being collected and dried in the usual way, weighed 35 mg. Examination of 10 mg. of this product in

Plate XIV

Purified 0.8 γ-Component

pH = 8.5, 0.5% solution



t = 160 min. t = 128 min. t = 64 min. t = 32 min. t = 0 min.

the analytical rotor of the ultracentrifuge after 72 hours' dialysis against veronal plus acetate buffer, pH 8.6, $\frac{\Gamma}{2} = 0.176$, indicated the substance to be monodisperse with a sedimentation coefficient of 0.83×10^{-13} (Plate XIV). It was unfortunate that insufficient material was available for further electrophoretic analysis in the Tiselius instrument.

(c) Amino Acid Analysis of the Isolated Components

As a further means of characterizing the isolated histone components, quantitative amino acid analyses were performed. For this purpose a slight modification of the original chromatographic method of Moore and Stein (1951) was used. This is described in Appendix VI. Total nitrogen contents were determined, in duplicate, by the standard micro-Kjeldahl technique.

Results

Table 6 gives the complete amino acid analyses of the α_1-, α_2-, α_3- and 0.8γ-histone components.

Table 6. Amino acid analyses of the isolated components.

Amino acid	%N	% by wt. in α_1 (N = 12.9)	N as % total N α_1	% by wt. in α_2 (N = 14.4)	N as % total N α_2	% by wt. in α_3 (N = 15.4)	N as % total N (corr. for 30% β) α_3	% by wt. in 0.8γ (N = 15.5)
Lysine	19.2	33.1	49.3	24.2	32.2	15.9	23.6	17.5
Histidine	27.1	0	0	0.3	0.6	1.5	2.1	0.8
Arginine	32.2	0	0	4.7	10.5	7.9	10.4	6.8
Aspartic acid	10.5	0.4	0.3	2.1	1.5	4.4	2.8	4.9
Threonine	11.8	3.0	2.7	4.0	3.3	3.9	2.8	4.0
Serine	13.3	1.7	1.8	3.5	3.2	4.3	4.4	5.5
Glutamic acid	9.5	0.5	0.4	3.6	2.4	6.6	3.3	7.0
Proline	12.2	10.0	9.5	7.4	6.3	4.5	4.0	3.8
Glycine	18.7	1.6	2.3	4.4	5.7	5.0	6.5	5.2
Alanine	15.7	16.6	20.2	13.0	14.2	9.8	11.3	11.0
Valine	12.0	2.2	2.0	3.5	2.9	4.7	3.7	4.9
Leucine	10.7	0.5	0.4	3.8	2.8	7.1	4.4	7.3
isoLeucine	10.7	0	0	0.8	0.6	2.5	1.1	2.0
Methionine	9.4	0	0	0	0	0.4	0	0.7
Tyrosine	7.7	0	0	0.8	0.4	2.3	0.9	2.0
Phenyl-alanine	8.5	0	0	0.7	0.4	1.2	0.4	1.5
Total		69.6	88.9	76.8	87.0	82.0	81.7	84.9

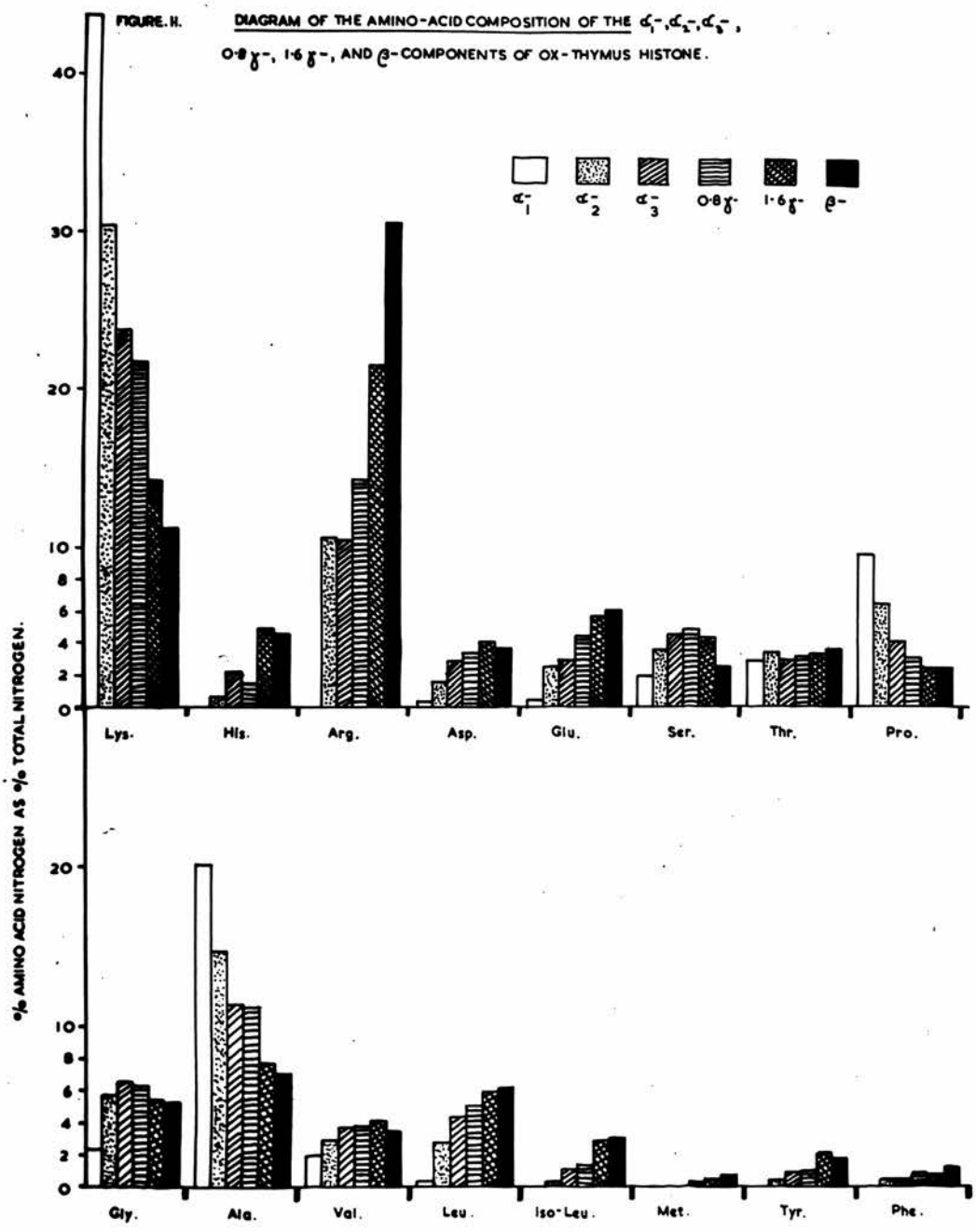
The results are expressed both as the percentage by weight of each amino acid in the histone and the amino acid nitrogen as a percentage of the total nitrogen.

In view of the fact that the α_3-component was contaminated with about 30% by weight of β, the figures shown in column 8 of the Table were obtained after correcting for this contamination by using the known analytical data for the purified β-component. In the case of the percentage by weight figures, the totals are considerably less than 100 on account of the sulphate present, whilst the failure of the figures in columns 4, 6, 8 and 10 to add up to this figure is probably due to contamination of the histone with veronal which was not totally removed during dialysis.

Discussion

The results shown in the Table, together with the corresponding data for the β- and 1.6γ-histones, are illustrated diagrammatically in Figure 11 which serves to emphasize several interesting features.

FIGURE. II. DIAGRAM OF THE AMINO-ACID COMPOSITION OF THE α_1-, α_2-, α_3-, 0·8 γ-, 1·6 γ-, AND β-COMPONENTS OF OX-THYMUS HISTONE.

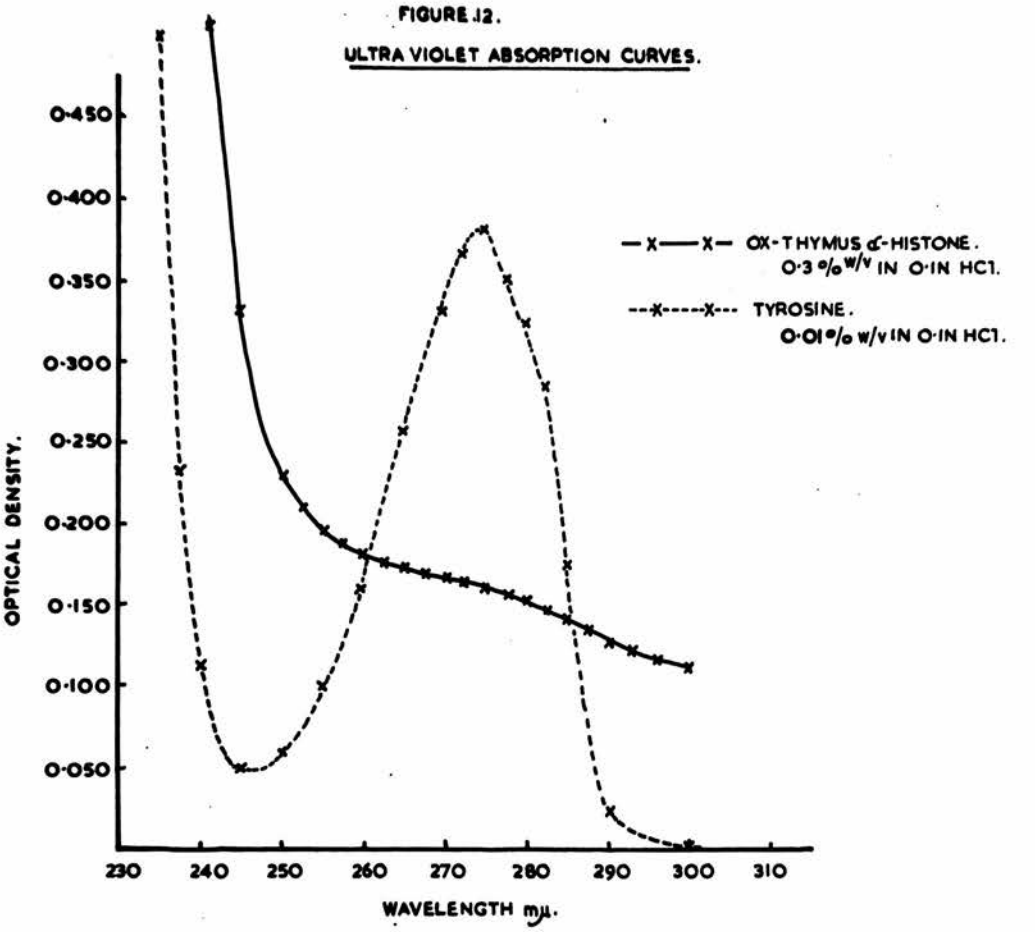


In the first place, the lysine, proline and alanine contents of the α_1-, α_2-, α_3-, 0.8γ-, 1.6γ- and β-components, in that order, show a marked progressive decrease whilst the corresponding figures for arginine, aspartic acid, glutamic acid, leucine, isoleucine and also methionine, tyrosine and phenylalanine show a similar increase. These results would appear to reflect a gradual increase in complexity from the α-, through the γ-, to the β-components.

A second item of note is the remarkable amino acid composition of the α_1-component. The only basic amino acid present is lysine, which accounts for nearly 50% of the total nitrogen content of the histone. Histidine and arginine are totally absent. This would appear to be the first reported instance of a protein containing no arginine. The very low sedimentation coefficient of this component (0.44×10^{-13}) corresponds to a molecular weight of about 4000 and this being so, the small quantities of aspartic and glutamic acids (0.3 and 0.4% respectively) and leucine (0.5%) present must be regarded as impurities. The absence of tyrosine, which is not well estimated by the Moore and Stein procedure, was also confirmed by finding a

FIGURE 12.

ULTRA VIOLET ABSORPTION CURVES.



negligible increase in the ultraviolet absorption of an 0.3% solution of the component in 0.1 N-hydrochloric acid at 275 mμ. (Figure 12), which is the maximum for tyrosine. The α_1-component is therefore comprised of the following seven amino acids: lysine, serine, threonine, proline, glycine, alanine and valine. The following tentative molecular formula is proposed:-

$$Lys_{15}\ Ser_1\ Thr_2\ Pro_7\ Gly_1\ Ala_{15}\ Val_1$$

The composition is seen to be very similar to that of the classical protamines except that lysine entirely replaces the arginine found in the latter. It is not possible, as yet, to comment on the significance of these findings in relation to the rôle of the histones in the cell nucleus.

TERMINAL AMINO ACID ANALYSIS OF THE HISTONES

Introduction

The demonstration of species- or cell-specificity amongst proteins necessitates their prior characterization. Electrophoretic and ultracentrifugal methods do not necessarily differentiate between closely related molecules and accordingly chemical methods have been sought.

Though quantitative amino acid analyses may distinguish between proteins homogeneous by physical techniques, the degree of reproducibility of these analyses is, in general, inadequate to establish small differences in the amino acid composition of very similar proteins.

At present, the most extensive means of characterizing a protein is the establishing of its amino acid sequence and the nature and positions of the interchain linkages, if any (Brown, Sanger and Kitai, 1955; Ryle, Sanger, Smith and Kitai, 1955; Li, Geschwind, Cole, Raacke, Harris and Dixon, 1955). A prerequisite to such sequence studies is the determination of the nature of the N- and C-terminal amino acids of the highly purified proteins. Such information alone,

however, may in some cases serve to demonstrate protein specificity (Porter and Sanger, 1948; Putnam, 1955; McFadden and Smith, 1955b; Niu and Fraenkel-Conrat, 1955b).

The considerable differences in physical properties between the β-histones from normal and malignant tissues have been described by Cruft (1953) and Cruft, Mauritzen and Stedman (1954). Furthermore, extensive experiments on the highly purified β-histones from ox-thymus and ox-liver cell nuclei have indicated small but consistent differences in their electrophoretic mobilities (Cruft, Mauritzen and Stedman, 1957). The methods of N- and C-terminal analyses appeared to provide a possible means of confirming these differences. Since histones as a group are heterogeneous, the end group method required separate application to each purified component. This need was emphasized by the finding described subsequently that in such components a plurality of end groups appears to exist.

N-Terminal analyses were performed by the 1-fluoro-2:4-dinitrobenzene (FDNB) method of Sanger (1945) and the phenylisothiocyanate (PTC) method of Edman (1950) was also used to

confirm some of the findings. C-Terminal analyses of ox-thymus and ox-liver β-histones were performed by the hydrazinolysis method of Niu and Fraenkel-Conrat (1955a).

N-Terminal Analysis by the Dinitrophenyl-(DNP) Method

Sanger (1945) showed that FDNB was capable of reacting under mildly alkaline conditions with the free amino, imino, and phenolic groups that might occur in proteins to yield their DNP-derivatives. Hydrolysis of these products yielded the variously labelled amino acids which he subjected to chromatographic identification on silica-gel columns. The main developments of this technique have been in the application of paper chromatographic methods to the separation and identification of the DNP-amino acids (Biserte and Osteux, 1951; Levy, 1954).

Most of the DNP-amino acids are easily extractable into ether from an acid hydrolysate. The exceptions are O-DNP-tyrosine, ε-DNP-lysine, Imidazole-DNP-histidine, DNP-arginine and DNP-cysteic acid. Di-DNP-histidine partitions

between the two phases but remains for the most part in the aqueous phase. During the acid hydrolysis of a DNP-protein the artifacts dinitrophenol and dinitroaniline appear to be invariably produced. The former is produced by the slight decomposition of many of the DNP-amino acids whilst the latter arises mainly from the breakdown of Im-DNP-histidine (Sanger, 1945).

For the paper chromatography of the ether soluble DNP-amino acids the two dimensional method of Levy (1954) was used utilizing the 'toluene' system of Biserte and Osteux (1951) in the first dimension followed by 1.5 M-phosphate buffer in the second. The water soluble DNP-amino acids were studied chromatographically in the tert.-amyl alcohol-phthalate system of Blackburn and Lowther (1951).

Experimental

A. Reaction with FDNB

The simplest procedure, similar to the method of Porter (1951) was employed.

The histone sulphate, 10-25 mg., was dissolved in 1-2 ml. of water in a 50 ml. glass stoppered

tube. Double its weight of sodium bicarbonate was added and allowed to dissolve. To this solution was added twice its volume of an ethanolic solution (5% v/v) of FDNB. This resulted in the immediate precipitation of the protein in a finely dispersed form. The mixture was shaken mechanically for 6 hours at 25°C in the dark. It was then acidified with 6 N-hydrochloric acid and shaken with several 25 ml. portions of peroxide-free ether which removed ethanol, excess FDNB, and dinitrophenol formed by the hydrolysis of the FDNB. The aqueous suspension of insoluble DNP-protein was filtered off on a Hirsch funnel and washed with water, alcohol, and ether. The yellow powder was air-dried in the dark.

B. Hydrolysis

Hydrolysis of the DNP-protein was, in general, performed with glass distilled 5.7 N-hydrochloric acid in sealed tubes at 105°C. Overnight hydrolysis (about 16 hours) was routinely employed. DNP-Proline, -glycine, and -cystine are, however, almost completely destroyed under

these conditions giving rise to dinitrophenol and the corresponding amino acids. DNP-Glycine is more stable in concentrated hydrochloric acid (Acher and Laurila, 1953) and accordingly 6 hour hydrolyses at 105°C in this medium were also performed. Under these conditions DNP-proline is almost completely destroyed, being converted to dinitrophenol and the two yellow artifacts identified by Scanes and Tozer (1956a)as DNP-chlorovaleric acids.

C. Extraction and paper chromatography of the ether-soluble DNP-amino acids.

The hydrolysate was transferred to a glass stoppered test tube, diluted to 1 N with respect to acid, and extracted with four 5 ml. portions of peroxide-free ether. In some experiments the ether extraction was followed by ethyl acetate extraction which would remove any small DNP-peptides and so give a yellow solution. Under the hydrolytic conditions used however, no DNP-peptides were detected and complete hydrolysis was assured.

The combined ether extracts were washed once with 0.1 N-hydrochloric acid and taken to dryness in an air stream. It was found advisable to remove most of the dinitrophenol from the residue by volatilization *in vacuo* at 50°C.

A sheet of Whatman No. 1 paper, 17 x 15 inches, was curled to form a cylinder 17 inches high and the overlapping margins (about 0.5 inch) stapled together. From the lower end of the cylinder was cut a 1 x 0.5 inch rectangle of paper such that the overlap did not quite reach to the bottom of the cylinder. The sample, dissolved in a little acetone, was applied with an Agla micrometer syringe to a point 2½ inches from one edge and 1 inch from the bottom of the cylinder.

The apparatus for chromatography in the first dimension consisted of a rectangular glass tank, 7 x 9 x 18 inches high, fitted with a ground glass lid. The paper cylinder, contained in a crystallizing dish 5 inches in diameter and 2 inches high, was placed in the tank which contained 250 ml. of a 0.8 N solution of ammonia, and the paper allowed to equilibrate in the dark

for 4 hours. At the same time 45 ml. of toluene, 14 ml. of pyridine and 27 ml. of 2-chloro-ethanol were mixed in a separating funnel and 27 ml. of 0.8 N-ammonia added slowly down the side of the funnel so as to avoid undue mixing of the two layers. The system was allowed to stand undisturbed for the duration of the equilibration period after which time the lower aqueous layer was withdrawn and discarded. The organic phase was introduced into the dish holding the paper cylinder by means of a funnel fitted with a long stem which was passed through a hole in the glass lid. The chromatogram was allowed to run overnight at a constant temperature of 24°C. During this time the solvent front rose to within about 2 inches of the top of the paper. The paper was then removed and dried for 4-6 hours in a stream of warm air. The paper cylinder was then unfolded and the positions of the yellow spots outlined in pencil, their pale yellow colour appearing as dark brown in ultraviolet light. Suitable controls (5-10 µl. of a 0.01 M solution of DNP-amino acid in acetone) were spotted alongside the unknowns and the chromatogram run in the second dimension for about 8 hours using 1.5 M-

phosphate buffer pH 6 as the irrigating fluid. In this case no period of equilibration was necessary; only a tray of water was placed at the bottom of the tank. The sheet was then removed, dried, and the spots outlined in pencil using an ultraviolet lamp.

D. Chromatography of the water-soluble DNP-amino acids.

The tert.-amyl alcohol-phthalate system of Blackburn and Lowther (1951) was used.

Sheets of Whatman No. 4 paper, 6 x 18 inches, were dipped in phthalate buffer pH 6 (50 ml. 0.1 M-potassium hydrogen phthalate + 45 ml. 0.1 N-sodium hydroxide) and allowed to dry. The sample for chromatography was prepared by evaporating to dryness in a vacuum desiccator the aqueous solution remaining after the ether extractions, and dissolving the residue in acetone containing a drop of water. The spots were applied and irrigation effected by the descending procedure using tert.-amyl alcohol saturated with

buffer. Water saturated with the alcohol was present in the bottom of the jar.

E. Preparation of DNP-amino acids.

The synthesis of control samples of DNP-amino acids was performed either by Sanger's procedure (1945) or by the method of Rao and Sober (1954).

Sanger's procedure is illustrated in the preparation of DNP-phenylalanine.

L-Phenylalanine (0.2 g.) and sodium bicarbonate (0.4 g.) were dissolved in 5.0 ml. of water. A solution of 0.4 g. (0.28 ml.) FDNB in 10 ml. of ethanol was added and the solution shaken at room temperature for 2 hours. The mixture was then extracted four times with 20 ml. portions of ether to remove ethanol and excess FDNB and the aqueous solution acidified when a yellowish oil separated which soon solidified. The 2:4-DNP-phenylalanine was filtered off on a Hirsch funnel and washed with water. The yellow compound was recrystallized twice from aqueous methanol, m.p. 186-7°C (lit. m.p. 186°C).

This method was also used for the preparation of DNP-glycine and α-DNP-arginine.

This latter compound was recrystallized by solution in dilute hydrochloric acid followed by neutralization with ammonia, with a final recrystallization from aqueous ethanol, m.p. 250-2°C (d).

This method had the drawback that yellow oils were formed which frequently proved difficult to crystallize, and the alternative method was found to be better. This is illustrated in the preparation of DNP-leucine.

DL-Leucine (0.65 g.) and anhydrous sodium carbonate (1.0 g.) were dissolved in 20 ml. of water. FDNB (0.92 ml.) was introduced and the mixture stirred vigorously with an air jet stirrer, the temperature being maintained at 40°C. After about half an hour the suspension of FDNB disappeared, the colour of the solution becoming deep orange. The solution was then acidified with 6 N-hydrochloric acid when DNP-leucine was precipitated as an oil which crystallized on rubbing. The product was recrystallized from aqueous acetic acid, m.p. 126°C (lit. m.p. 126°C).

Most of the DNP-amino acids were prepared by this method. In the case of histidine, lysine and tyrosine where bis- substitution occurs, twice

Table 7

DNP-Amino Acid	m.p.(found)°C	m.p.(lit.)°C[x]	Recrystall Solver
DL-Alanine	175	172-3,178,175	methanol/wa
L-Arginine	250-2(d)	252(d),260,276(d)	dil. HCl-an ethanol/wat
L-Aspartic acid	187-9	186-7	ethyl aceta
L-Cysteine	183	159	acetic aci
L-Cystine(di)	110-150(d)	109,118-21	acetic aci
DL-Glutamic acid	168-70	148-9, 155-62	ethyl acet
Glycine	203-4(d)	205,206,203-4,200(d)	acetic aci
L-Histidine(α:Im,di)	231-35(d)	250,228,232-4	acetic aci
DL-Leucine	126	203,132,126,130	acetic aci
DL-isoLeucine	166-7	166,168-72,174-5	acetic aci
L-Lysine(α:ε,di)	170-2(d)	146,173-4,174,170-72(d)	formic aci methanol/w
L-Lysine(ε,mono)HCl.H_2O	180-2	186	20% HCl
L-Phenylalanine	186-7	186,189,185-7	methanol/w
L-Proline	138	137,138-9	acetic aci
DL-Serine	197-9	199,186-8,200,200-2	methanol
DL-Threonine	149-52	152,177-8	methanol
DL-Tyrosine(O:N,di)	Could not be obtained crystalline		
DL-Valine	124	185,183,182-3	acetic ac

(d) - with decomposition

x - taken from the data collected by Fraenkel-Conrat, Harris and Levy (1954).

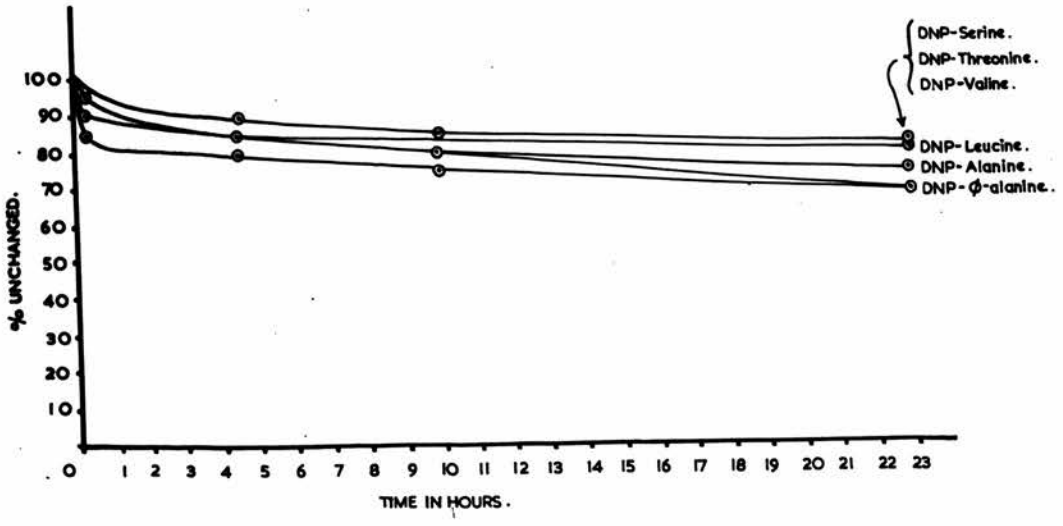
the amount of FDNB was taken with appropriate increases in the quantity of sodium carbonate. α:ε-bis-DNP-Lysine was crystallized from formic acid and recrystallized from aqueous methanol, m.p. 170-72°C (d) (lit. m.p. 173-4°C). ε -DNP-Lysine was prepared by dinitrophenylation of the copper derivative of lysine (Porter and Sanger, 1948).

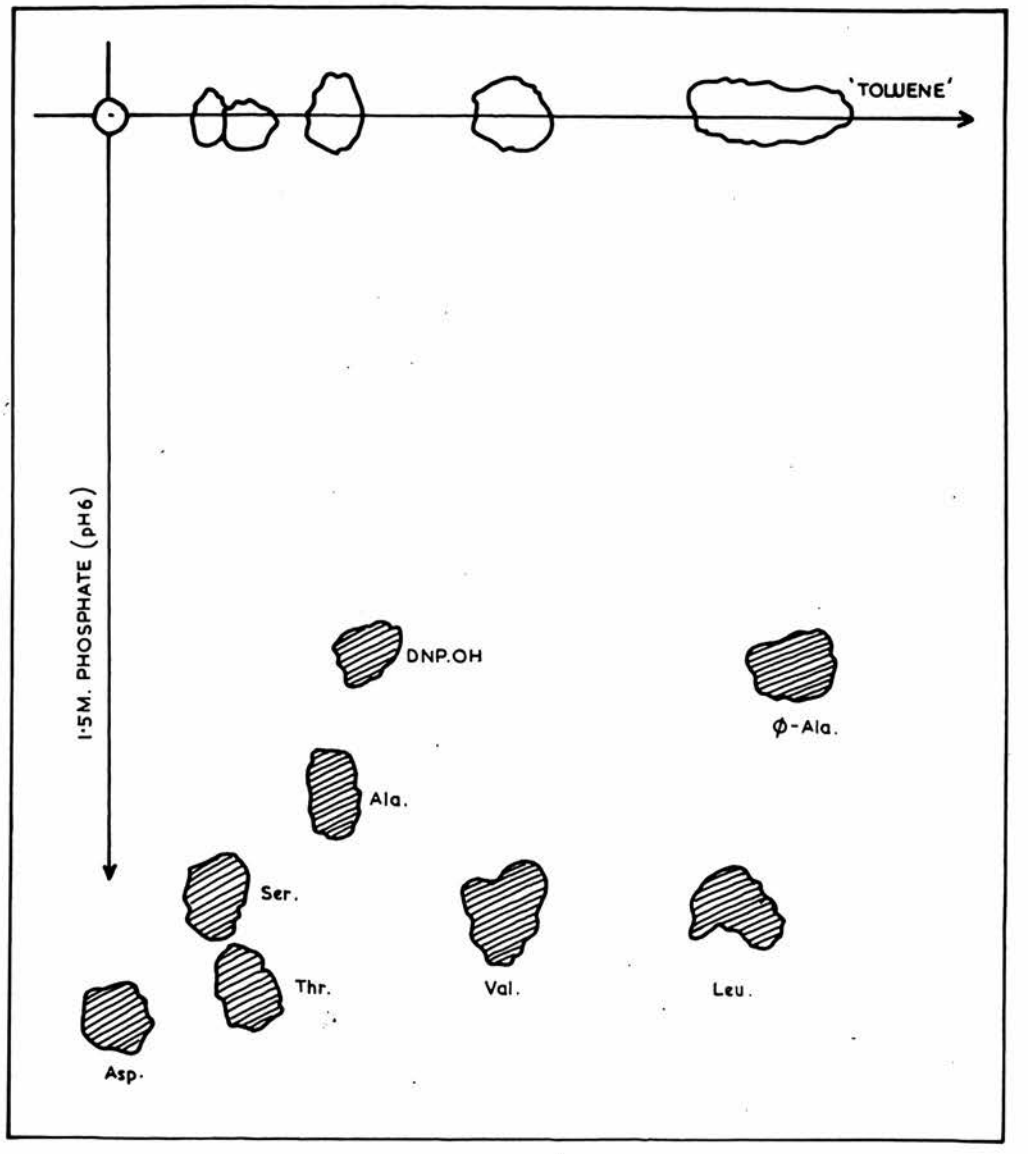
Table 7 lists the DNP-amino acids prepared together with their melting points and recrystallizing solvents.

F. Destruction of DNP-amino acids during hydrolysis.

Losses of N-terminal DNP-amino acids are encountered during hydrolysis. In order to determine the extent of this destruction a mixture of six DNP-amino acids - shown to be present as end groups in the DNP-histones - were submitted to the action of 5.7 N-hydrochloric acid at 105°C for varying times in the presence of added ox-thymus (β + γ)-histone. Figure 13 indicates the

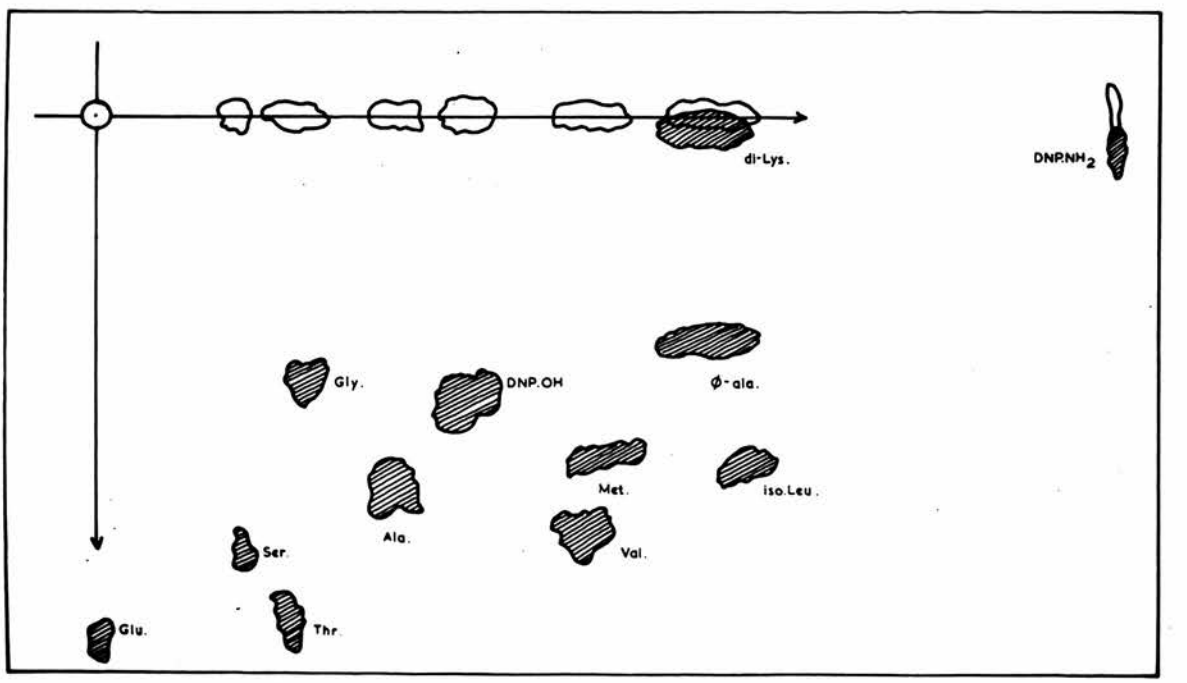
FIGURE . 13 STABILITY OF DNP-AMINO ACIDS TO ACID HYDROLYSIS (5·7 N. HCl.) FOR VARYING TIMES IN THE PRESENCE OF ADDED (β+γ) HISTONE.





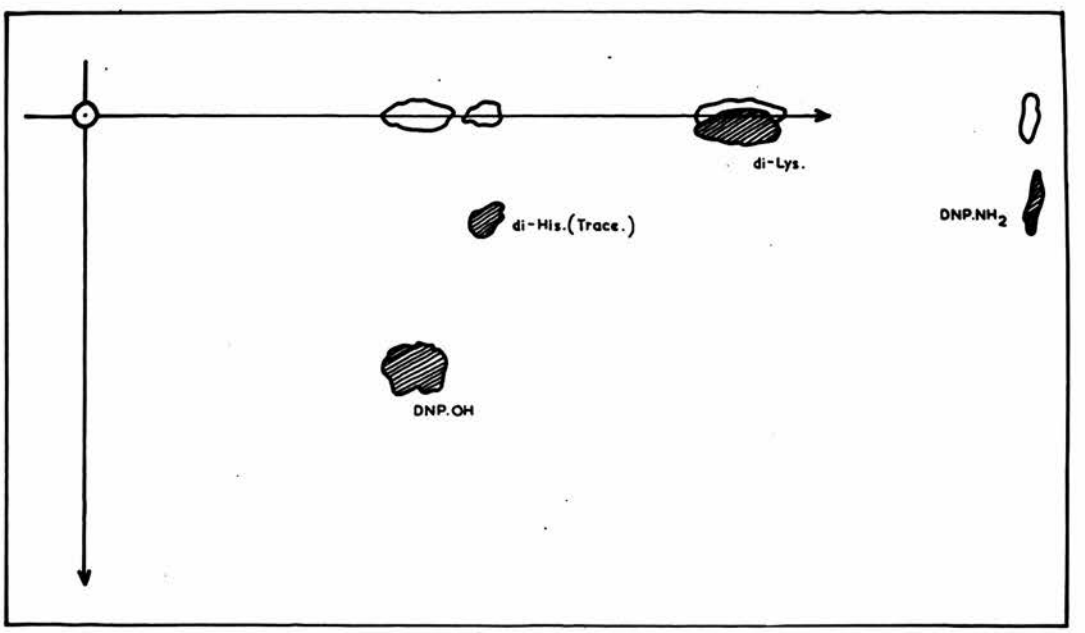
2-D CHROMATOGRAM OF A SYNTHETIC MIXTURE OF 7 DNP-AMINO ACIDS (APPROXIMATELY 0·2 μM OF EACH) AFTER 23 HR. REFLUXING WITH 5·7N. HYDROCHLORIC ACID.

FIGURE. 14.



CHROMATOGRAM OF A MIXTURE OF 10 DNP-AMINO ACIDS WITH ADDED DINITROPHENOL AND DINITROANILINE AFTER 12 HR. HYDROLYSIS IN 5·7N ACID.

FIGURE. 15.



CHROMATOGRAM OF THE ETHER-SOLUBLE DNP-DERIVATIVES OF THE BASIC AMINO ACIDS.

FIGURE. 16.

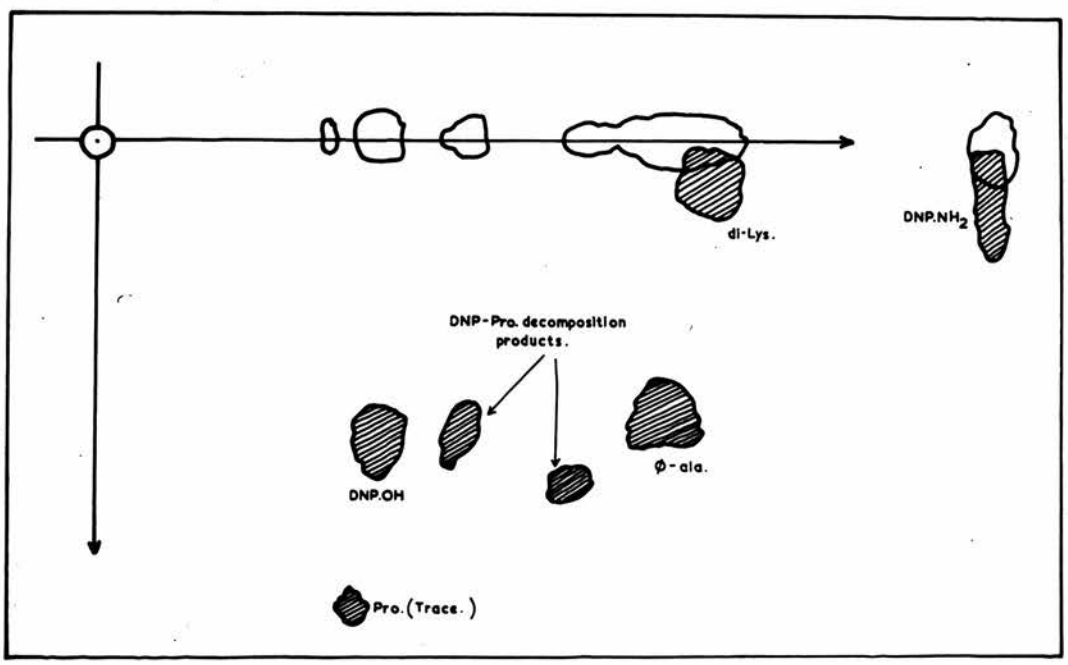
recoveries after different periods. It is seen that all the DNP-amino acids except di-DNP-lysine are relatively stable, over 70% being recoverable after 23 hours' refluxing.

Results

Figure 14 shows the chromatogram of the ethereal extract obtained after refluxing a mixture of DNP-aspartic acid, DNP-serine, DNP-threonine, DNP-alanine, DNP-valine, DNP-leucine and DNP-phenylalanine with constant boiling hydrochloric acid for 23 hours.

Figure 15 indicates the separation, after 12 hours' hydrolysis, of a mixture of DNP-glutamic acid, DNP-serine, DNP-threonine, DNP-alanine, DNP-isoleucine, DNP-glycine, DNP-valine, DNP-methionine, DNP-phenylalanine, α:ε-bis-DNP-lysine and dinitrophenol.

Figure 16 is the chromatogram of the ether-soluble DNP-amino acids obtained after 12 hours' refluxing with 5.7 N-hydrochloric acid of a mixture of α:ε-bis-DNP-lysine, bis-DNP-histidine, and DNP-arginine.

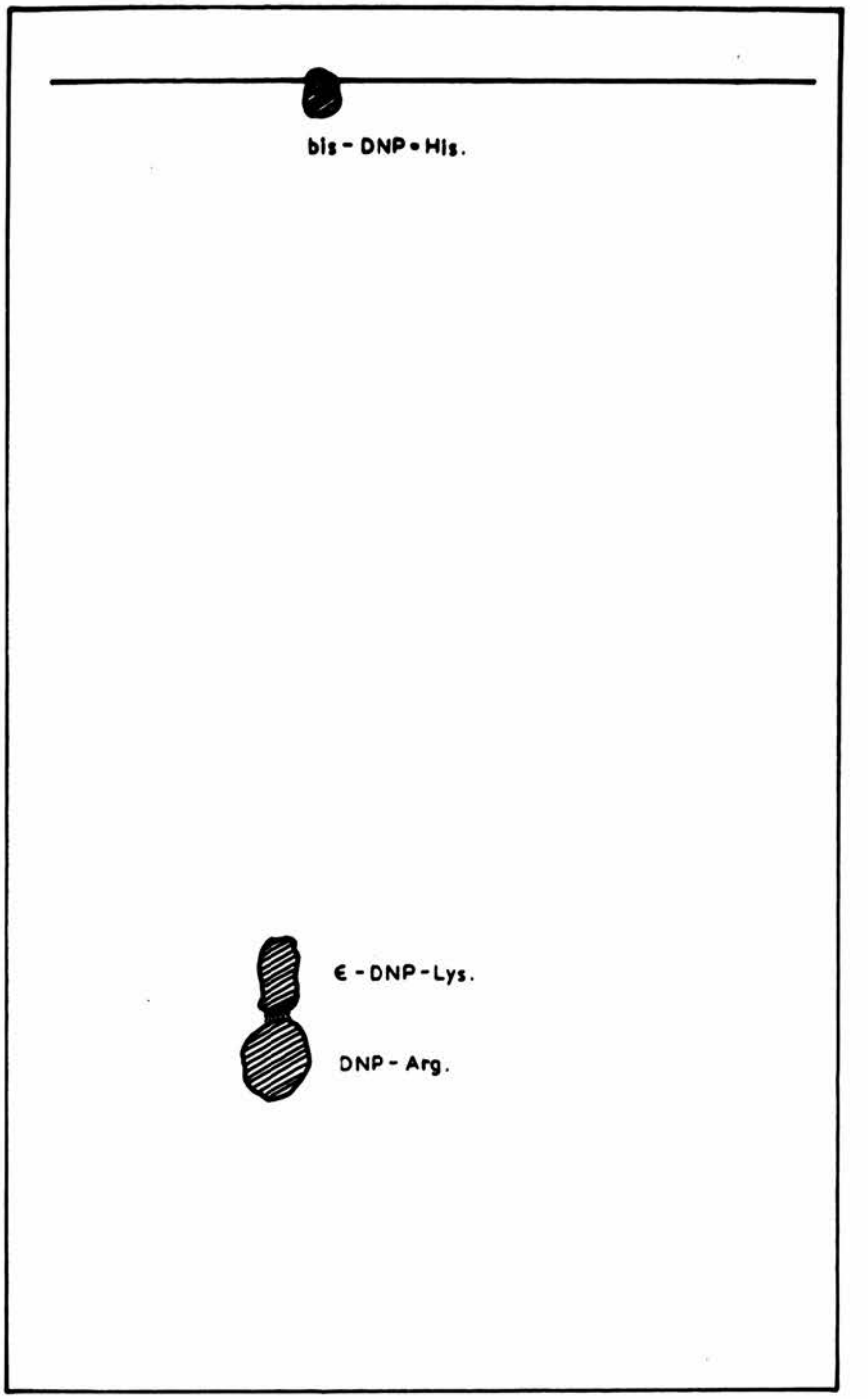


CHROMATOGRAM OF THE ETHER-SOLUBLE DNP-DERIVATIVES OBTAINED BY HYDROLYSIS OF DNP-Pro., DNP-Ø-ala., AND di-DNP-Lys., IN 12N ACID.

FIGURE. 17.

DNP-Proline is totally destroyed after 4 hours' refluxing with 5.7 N acid giving rise to dinitrophenol. Heating in a sealed tube with concentrated acid on the other hand resulted in the formation of the two yellow artifacts indicated in Figure 17. After 6 hours at 105°C in concentrated acid, a small amount of DNP-proline is still present.

These, and other chromatograms indicated that this method is capable - with the exception of leucine and isoleucine - of yielding clear, unequivocal separations of the various DNP-amino acids. Though the absolute R_F values were found to vary somewhat, the sequence of spots was quite reproducible. Acid hydrolysis, with the exception of DNP-proline, did not appear to result in the formation of any artifacts other than dinitrophenol and dinitroaniline. The dinitrophenol spot was decolourized by spraying the chromatogram with 6 N-hydrochloric acid, and this provided a useful reference point for the identification of the other spots.



CHROMATOGRAM OF WATER-SOLUBLE DNP-AMINO ACIDS SHOWN IN THE tert. AMYL ALCOHOL-PHTHALATE SYSTEM.

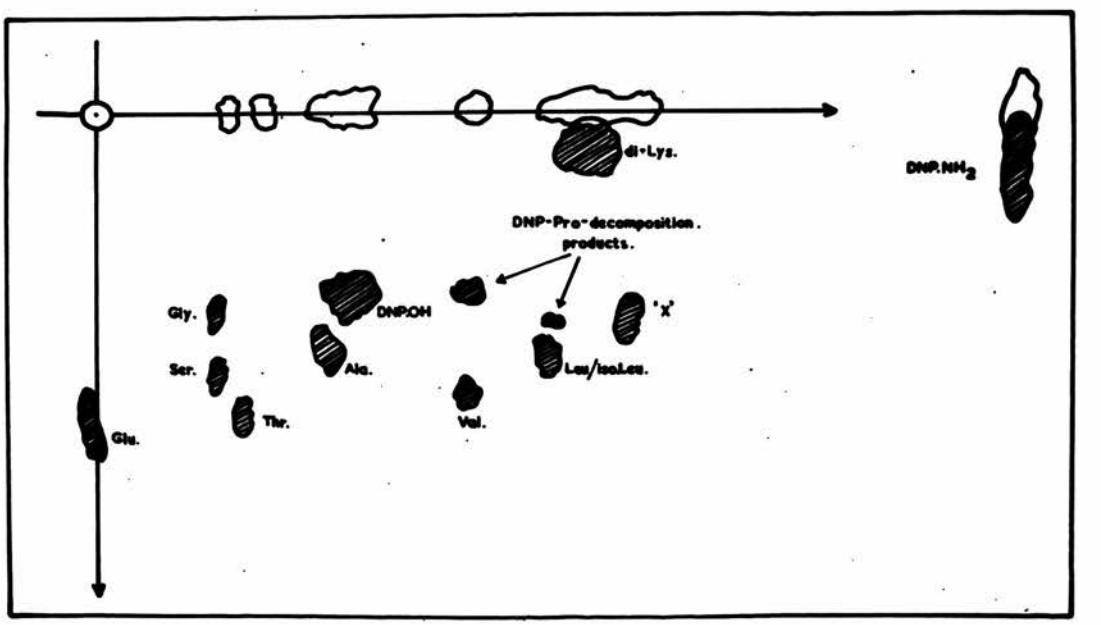
FIGURE. 18.

Figure 18 illustrates the separation of the water-soluble DNP-amino acids, α-DNP-arginine, α:Im-di-DNP-histidine and ε-DNP-lysine, in the tert.-amyl alcohol-phthalate system.

In order to verify that the technique was working satisfactorily it was applied in the first instance to a sample of crystalline insulin (Boots Pure Drug Co.). Hydrolysis of the DNP-insulin for 6 hours at 105°C with concentrated hydrochloric acid in a sealed tube yielded the ether-soluble DNP-derivatives of glycine and phenylalanine. These results are in accord with the findings of Sanger (1945), and indicate the method to be working satisfactorily.

The first experiments in applying this method to the histones were performed on samples of ox-thymus α- and (β + γ)-fractions, which had been prepared by the selective precipitation techniques described by Cruft et al. (1957).

Ox-thymus α-histone was labelled under the specified conditions for 4 hours. After acidifying and extracting with ether, the yellow DNP-protein was filtered off and hydrolysed. The filtrate from the DNP-protein however was found to be a pale yellow colour which may have been due to a DNP-amino acid or -peptide split off during the labelling. Accordingly, the filtrate was



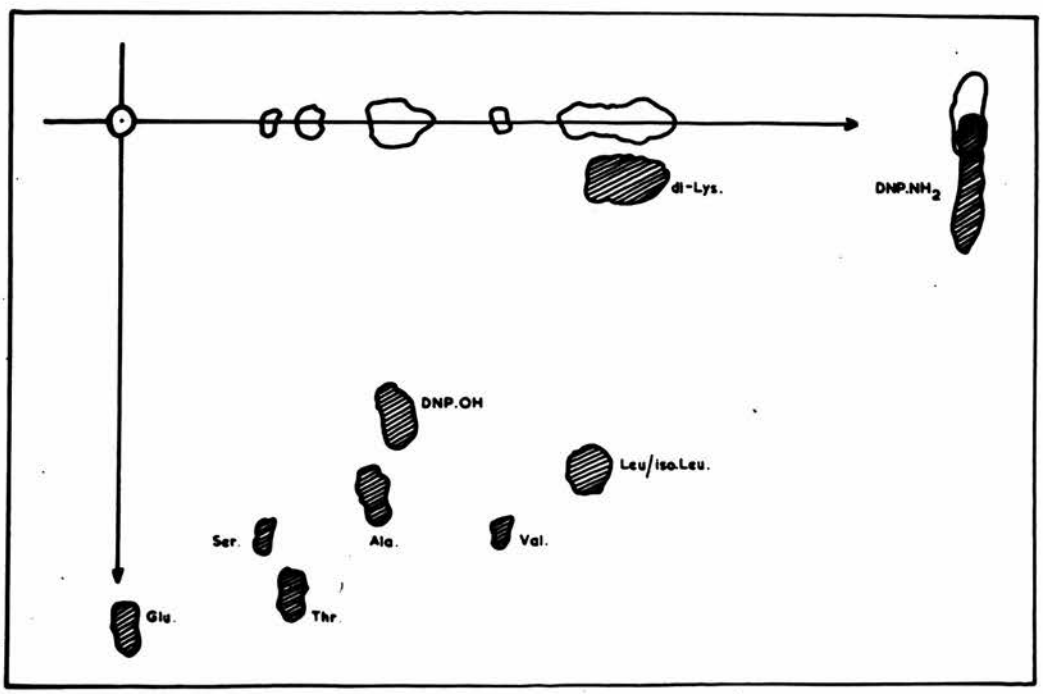
CHROMATOGRAM OF THE ETHER-SOLUBLE DNP-AMINO ACIDS FROM A 12N ACID HYDROLYSATE OF DNP-OX-THYMUS α-HISTONE.

FIGURE-19.

freeze-dried and a portion of the pale yellow residue applied to a paper chromatogram with aqueous acetone. The spot however, failed to move in the tert.-amyl alcohol-phthalate system indicating it to be a DNP-peptide. Hydrolysis in 5.7 N acid at 105°C for 15 hours yielded some ε-DNP-lysine and a faint spot - not identified with certainty - moving like DNP-alanine. The α-fractions from other histones similarly gave very pale yellow filtrates after labelling but their nature was not investigated further.

The chromatogram of the ether-soluble DNP-amino acids obtained by hydrolysis of the DNP-protein with concentrated acid is reproduced in Figure 19. The DNP-amino acids present are:- α:ε-bis-DNP-lysine and DNP-alanine in major quantities together with smaller amounts of DNP-glutamic acid, DNP-glycine, DNP-serine, DNP-threonine, DNP-valine, DNP-leucine, and traces of DNP-proline decomposition products. In addition, a spot (marked X) of unknown nature was present which travelled slightly in advance of α:ε-bis-DNP-lysine.

Hydrolysis of the DNP-protein with 5.7 N acid



CHROMATOGRAM OF THE ETHER-SOLUBLE DNP-AMINO ACIDS FROM A 5·7N ACID HYDROLYSATE OF DNP-OX-THYMUS ($\alpha + \gamma$) HISTONE

FIGURE 20

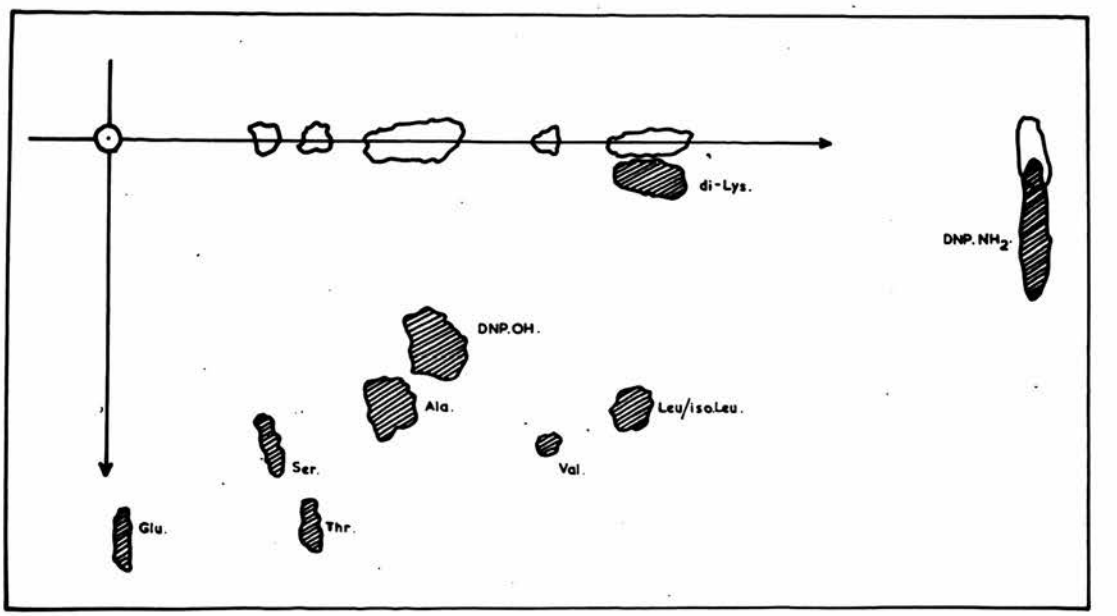
at 105°C for 16 hours gave similar results but with the absence of spots corresponding to DNP-glycine and the decomposition products of DNP-proline. Spot 'X' was also absent. This multiplicity of residues will be discussed later.

Examination of the aqueous phase revealed only the presence of ε-DNP-lysine.

DNP-Ox-thymus (β + γ)-histone gave the chromatogram reproduced in Figure 20 after hydrolysis in 5.7 N acid. The same residues are present as in the α-fraction but with a considerably increased amount of DNP-leucine. Hydrolysis with concentrated acid revealed the presence of a small quantity of DNP-glycine and the absence of DNP-proline decomposition products.

Examination of the aqueous phase only revealed ε-DNP-lysine.

The high degree of aggregation of the β-histones above pH 8 may result in the unavailability of some of the reactive sites for coupling with FDNB. An experiment was therefore performed in which the (β + γ)-histone was labelled in 0.4 M-phosphate buffer, pH 7.5 which contained 30% (w/w) guanidinium chloride as a dispersing agent. The chromatogram



CHROMATOGRAM OF THE ETHER - SOLUBLE DNP - AMINO ACIDS FROM A 5·7 N ACID HYDROLYSATE OF DNP - OX - THYMUS β - HISTONE.

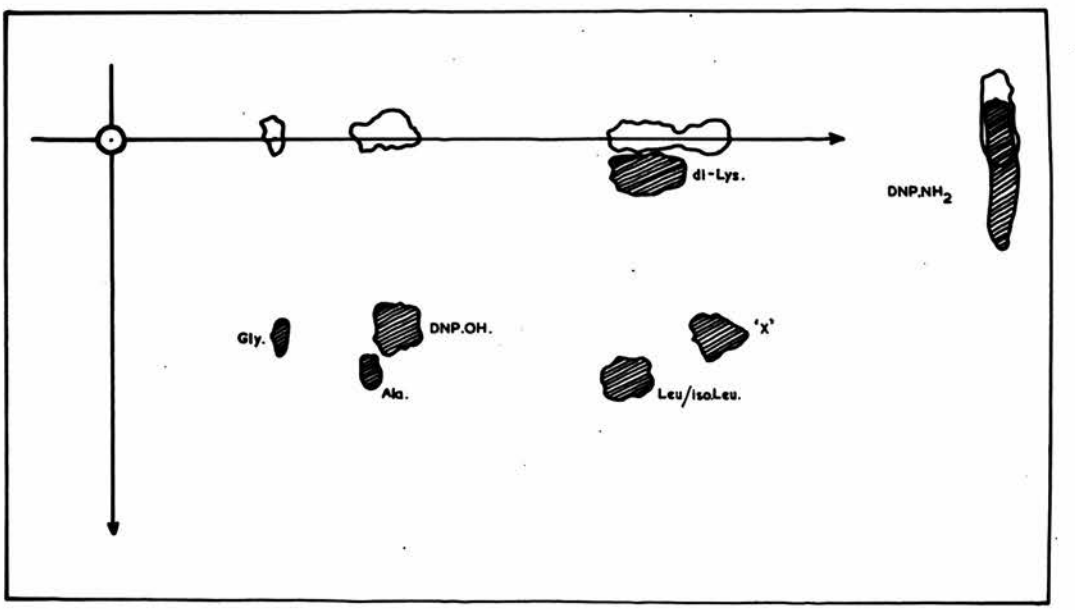
FIGURE. 21.

obtained was however similar in all respects to Figure 20 indicating that aggregation does not appear to hinder the reactivity of N-terminal amino acids.

The obvious explanation for this multiplicity of residues lay in the electrophoretically revealed heterogeneity of the histone samples. The methods for isolating highly purified samples of β- and 1.6 γ-histones were described on pp. 23-25 and these were next submitted to N-terminal analysis.

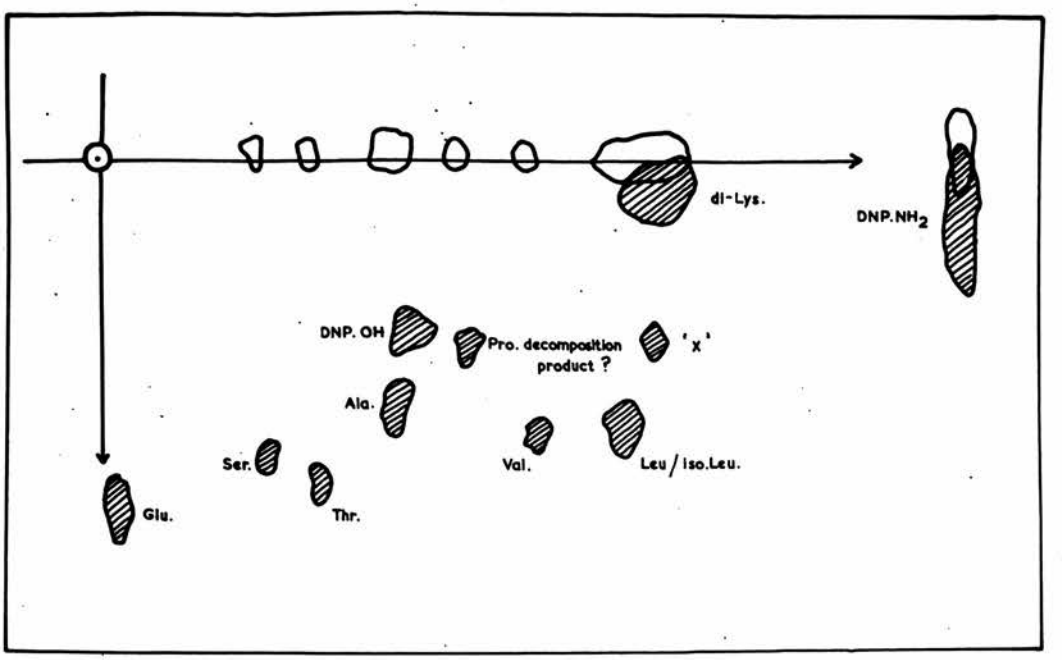
DNP-Ox-thymus β-histone after 16 hours' hydrolysis with 5.7 N acid yielded the ether-soluble DNP-amino acids illustrated in Figure 21. Alanine, leucine and lysine appear to be the main N-terminal amino acids together with smaller quantities of serine, threonine and faint traces of valine and glutamic acid.

A highly purified sample of ox-liver β-histone gave a very similar picture except that the trace amounts of DNP-valine and -glutamic acid were lacking.



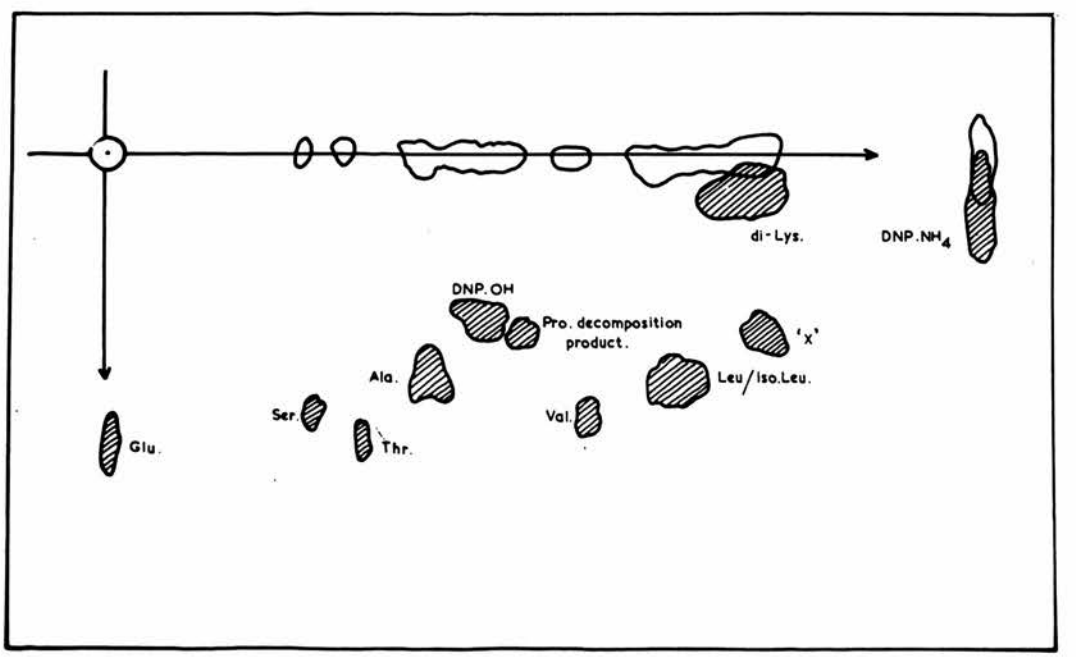
CHROMATOGRAM OF THE ETHER-SOLUBLE DNP-AMINO ACIDS FROM A 12N ACID HYDROLYSATE OF DNP-1·6 γ OX-THYMUS HISTONE.

FIGURE.22.



CHROMATOGRAM OF THE ETHER-SOLUBLE DNP-AMINO ACIDS FROM A 12N ACID HYDROLYSATE OF DNP-RAT-LIVER α-HISTONE.

FIGURE. 23.



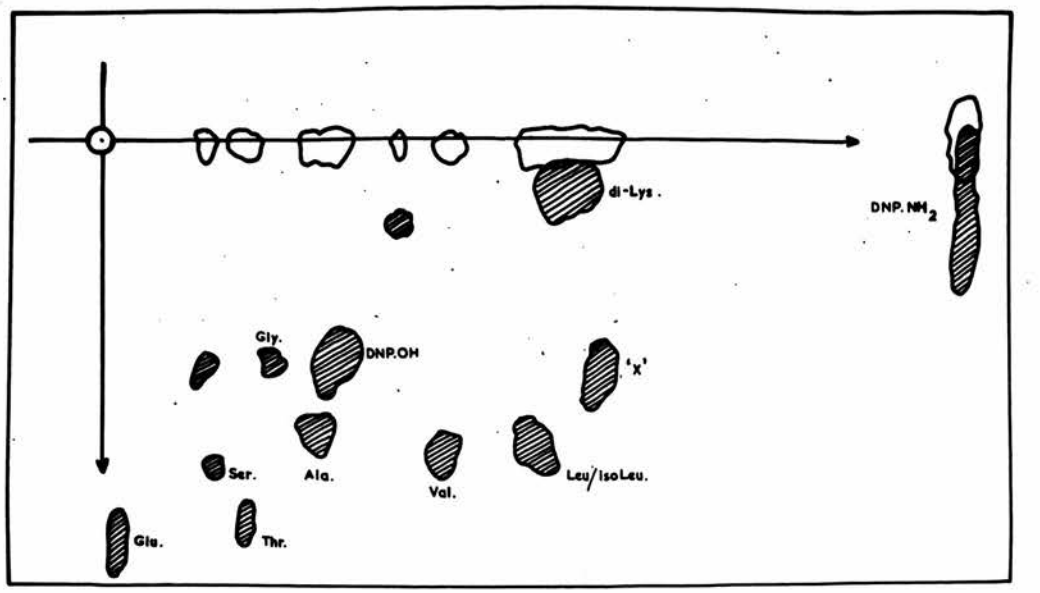
CHROMATOGRAM OF THE ETHER-SOLUBLE DNP-AMINO ACIDS FROM A 12N ACID HYDROLYSATE OF DNP-RAT-HEPATOMA α-HISTONE.

FIGURE. 24.

Ox-thymus 1.6 γ-histone gave the chromatogram reproduced in Figure 22. N-Terminal serine, threonine, valine, and glutamic acid are missing and alanine is present in reduced amount when compared with the β-histones. The main N-terminal amino acids are lysine and leucine with some alanine. From the examination of the aqueous phases, both β- and γ-histones were shown to be lacking in N-terminal arginine.

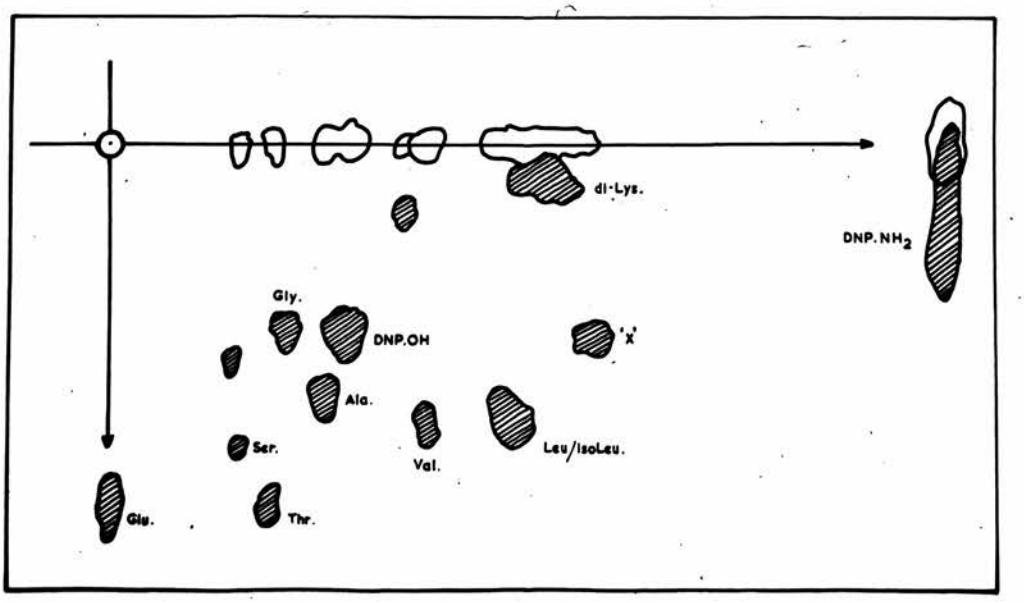
These results compare closely with those of Murray Luck, Cook, Eldredge, Haley, Kupke and Rasmussen (1956), who found the N-terminal groups of both their 'light' and 'heavy' ox-thymus histone fractions to be leucine, alanine, valine, glycine and valyl-lysine. Phillips (1957) has also reported on the N-terminal groups of partially fractionated thymus histones and finds proline to be a major end group in his lysine-rich fraction. Smaller quantities of N-terminal serine, alanine, and lysine were also present.

The α-component of both rat-liver and rat-hepatoma histones appears to be a homogeneous substance by both electrophoretic and ultra-centrifugal techniques. N-Terminal analyses (Figures 23 and24) do not disclose any detectable differences between the two histones. Lysine is



CHROMATOGRAM OF THE ETHER-SOLUBLE DNP-AMINO ACIDS FROM A 12N ACID HYDROLYSATE OF DNP-RAT-LIVER β-HISTONE.

FIGURE.25.



CHROMATOGRAM OF THE ETHER-SOLUBLE DNP-AMINO ACIDS FROM A 12N ACID HYDROLYSATE OF DNP-RAT-HEPATOMA β-HISTONE.

FIGURE.26.

the main N-terminal residue with leucine and alanine in smaller amounts. There are also the usual trace quantities of serine, threonine, valine and glutamic acid. In addition, small quantities of DNP-proline decomposition products and the unidentified DNP-compound 'X' were found, these being absent when the hydrolysis was performed in 5.7 N acid.

The purified rat-liver and rat-hepatoma β-histones also yield qualitatively identical chromatograms (Figures 25 and 26). The major end groups are lysine, leucine and alanine. Compound 'X' is again apparent in concentrated acid hydrolysates. Examination of the aqueous phase in all cases reveals only the presence of ε-DNP-lysine.

The α-component isolated by zone-electrophoresis from the ox-thymus α-fraction appeared, as has been seen, to possess a high degree of purity. Even so, N-terminal analysis yielded four ether-soluble DNP-amino acids. Lysine appeared to be the principal terminal amino acid with about half its quantity of alanine. DNP-Serine and DNP-threonine were also present.

ULTRAVIOLET ABSORPTION CURVES.

FIGURE 27. PTH FROM α-HISTONE.

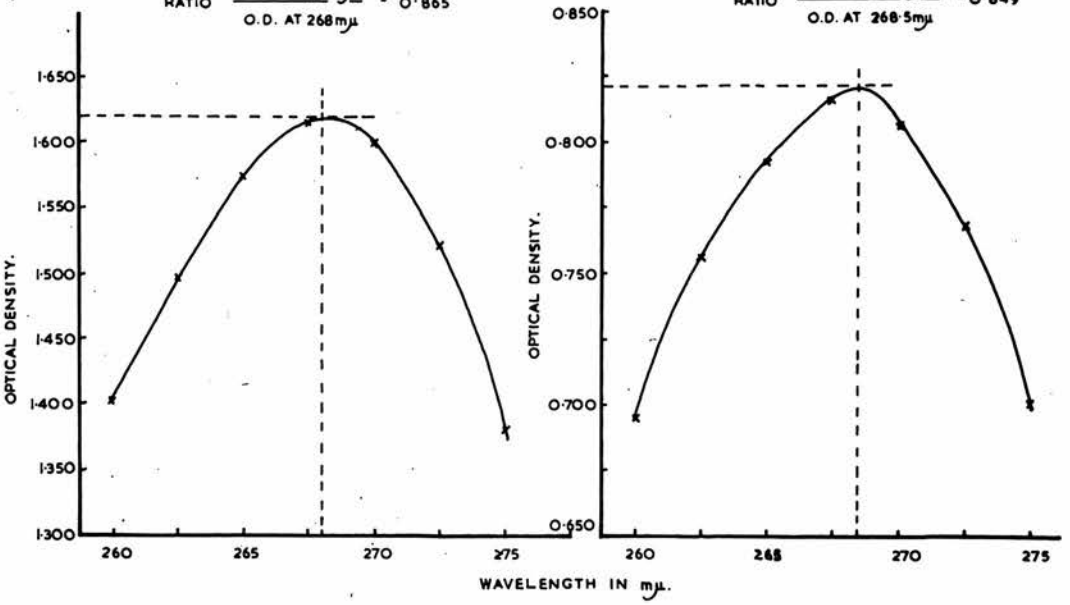
MAXIMUM AT 268 mμ.

RATIO $\frac{\text{O.D. AT 260 mμ}}{\text{O.D. AT 268 mμ}}$ = 0·865

FIGURE 28. PTH FROM β-HISTONE.

MAXIMUM AT 268·5

RATIO $\frac{\text{O.D. AT 260 mμ}}{\text{O.D. AT 268·5 mμ}}$ = 0·849



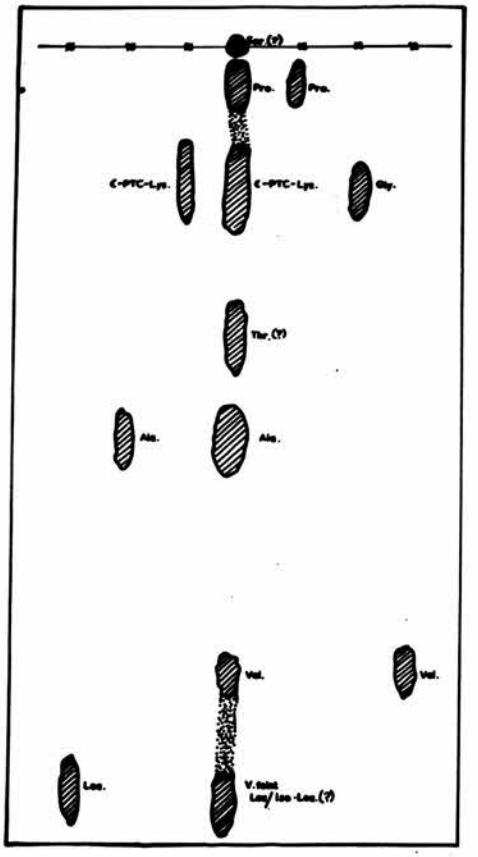
N-Terminal Analysis by the Phenylisothiocyanate (PTC) Method

Confirmation of the results obtained by the application of the DNP-method to histones would appear to be desirable, and the PTC-method of Edman was chosen for this purpose. The unidimensional paper chromatographic systems of Sjøquist (1953) were found to yield elongated spots, and were unsatisfactory for the unequivocal identification of spots when several were present on a single chromatogram. This method, though not used routinely, served to confirm the nature of the major N-terminal amino acids present in the ox-thymus α-fraction and the purified β-component.

The method is described in Appendix IV.

Results

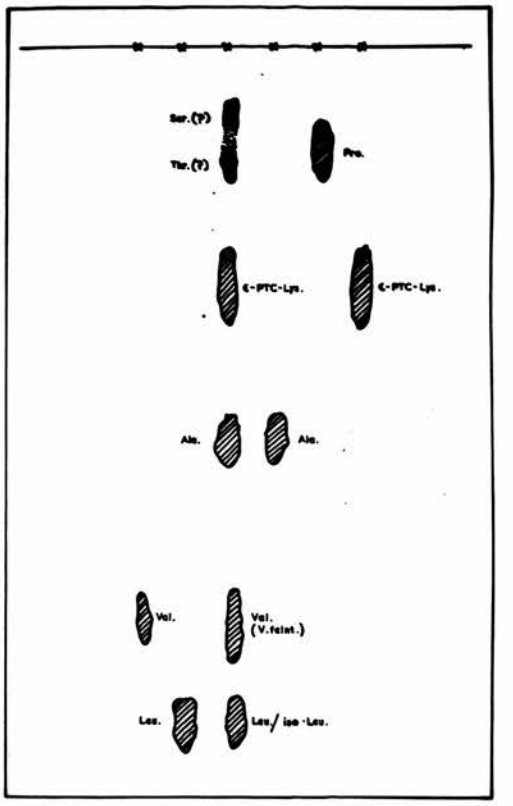
Figures 27 and 28 reproduce the absorption curves obtained from the PTH's of the N-terminal amino acids released from the α-fraction and β-component of ox-thymus histone. The optical



CHROMATOGRAM OF THE PTH-AMINO ACIDS LIBERATED FROM PTC-OX-THYMUS ε-HISTONE TOGETHER WITH SUITABLE CONTROLS.

SOLVENT SYSTEM A. (SEE APPENDIX).

FIGURE. 29.



CHROMATOGRAM OF THE PTH-AMINO ACIDS LIBERATED FROM PTC-OX-THYMUS β-HISTONE TOGETHER WITH SUITABLE CONTROLS.

SOLVENT SYSTEM B. (SEE APPENDIX).

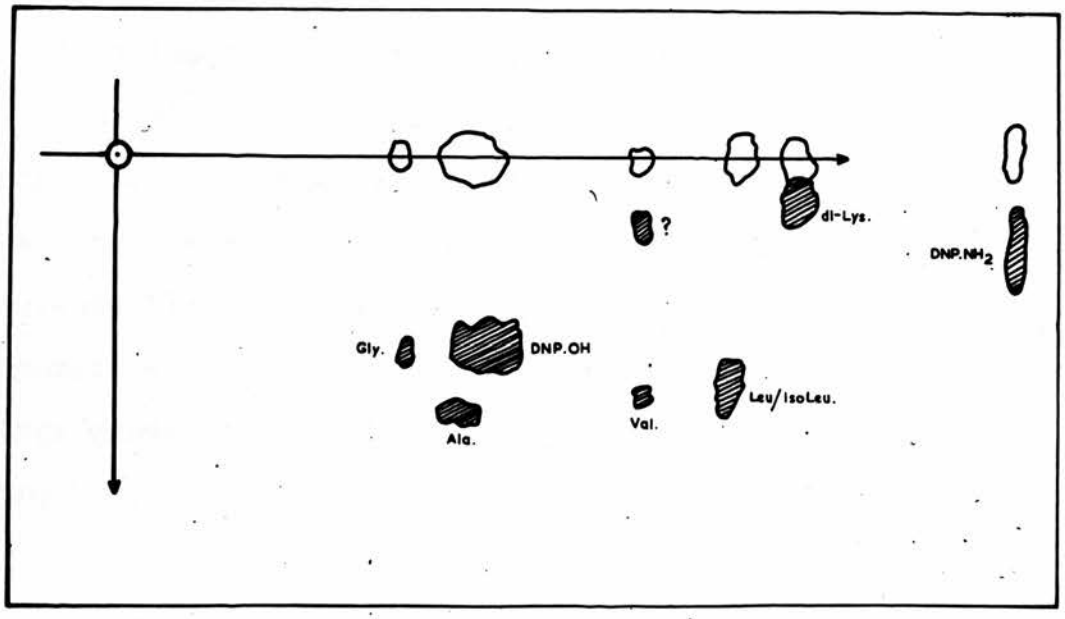
FIGURE. 30.

density ratio for the PTH's from the former is seen to be 0.865 - indicating slight contamination with noncyclized material. The latter gives a figure of 0.849, which indicates a relatively pure PTH preparation. Furthermore, the wavelengths corresponding to the absorption maxima indicate freedom from thiazolinone derivatives which have maxima at about 250 mμ. Figures 29 and30 reproduce the patterns obtained by direct chromatography of the PTH's with controls run alongside. Some unidentified spots are present, but otherwise the chromatograms confirm the presence of N-terminal lysine, alanine, valine and proline in ox-thymus α-histone and N-terminal lysine, alanine, valine and leucine in ox-thymus β-histone.

C-Terminal Analysis

C-Terminal amino acid analyses of the histones were performed using the hydrazinolysis technique of Niu and Fraenkel-Conrat (1955a). This is described in Appendix V.

The method was only applied to purified samples of the ox-thymus β- and ox-liver β-



CHROMATOGRAM OF THE ETHER-SOLUBLE DNP-AMINO ACIDS FROM THE C-TERMINAL GROUPS OF OX-LIVER β-HISTONE.

FIGURE. 31.

histones. No essential difference could be detected between the chromatograms of the DNP-derivatives of the C-terminal residues. Figure 31 reproduces the pattern obtained using ox-liver β-histone. Leucine appears to be the main C-terminal amino acid together with smaller quantities of lysine, glycine and alanine. A trace of valine is also present. Murray-Luck et al. (1956) using the carboxypeptidase technique reported the C-terminal amino acids in both 'light' and 'heavy' ox-thymus histone fractions to be leucine, alanine, valine and also tyrosine. The hydrazine method clearly confirms these findings except that lysine was found in place of tyrosine.

Discussion

A striking feature of the results obtained by N- and C-terminal analyses of the histones is the apparent multiplicity of their end groups. Even in the most highly purified preparations, which behave as homogeneous substances during electrophoresis at different pH's and in the ultracentrifuge, this feature is just as manifest. Similar behaviour on the part of other highly purified protein preparations has been reported in the literature. Thus Thompson (1953) studied the N-terminal sequence of five times recrystallized carboxypeptidase by the DNP-method. Labelling the protein under the usual conditions in bicarbonate solution gave a DNP-derivative which on hydrolysis yielded DNP-aspartic acid together with smaller quantities of DNP-glutamic acid, DNP-serine, DNP-threonine, DNP-valine and DNP-leucine. The author also found that DNP-asparagine and traces of DNP-serine and DNP-threonine were liberated into the aqueous medium during labelling and concluded on the basis of these findings that certain α-peptide linkages are particularly labile.

McFadden and Smith (1955a) examined various rabbit antibody globulins by the FDNB method and, in addition to finding N-terminal alanine and aspartic acid in major amounts, found serine, threonine, glutamic acid, valine and leucine to be present. Furthermore, these last six amino acids were partially liberated as their DNP-derivatives into the labelling medium.

The significance of these findings is not clear. Desnuelle and Casal (1948) and subsequent workers have commented on the particular lability of peptide linkages involving the amino groups of serine and threonine and this, together with an inherent instability of certain other types of α-peptide linkages as postulated by Thompson (1953), could well explain the release into the labelling medium of various DNP-amino acids and may also account for the soluble DNP-peptide released during the labelling of the α-histones. Burley and Solomons (1955) found the action of FDNB in an aqueous-alcoholic solution of sodium bicarbonate on the fish scale protein ichthylepidin to bring about the dissolution of more than half the protein yielding a complex mixture of DNP-peptides. This did not occur if FDNB was omitted from the mixture.

The above findings may also account for the number of DNP-amino acids found after hydrolysis. Thus, if it is assumed that a sequence of particularly labile α-peptide linkages is present in the molecule, then a given reaction period with FDNB would be expected to cause the splitting of only some of these linkages with subsequent labelling of the uncovered α-amino groups.

Certain types of chain branching could also account for the existence of several terminal amino acids in a single protein molecule. Leaving aside the question of ester bonds linking the hydroxyl-groups of serine and threonine to carboxylic acid residues, evidence is accumulating in the literature for the presence in proteins and peptides of several types of interpeptide linkages of unusual varieties. Thus in Bacitracin A, Lockhart and Abraham (1954) have demonstrated a peptide linkage involving the ε-amino group of lysine. Taken with the considerable lysine content of histones, the occurrence of such bonds in these proteins could cause extensive chain branching. Similarly, the occurrence of peptide linkages involving the ω-carboxylic acid groups

of aspartic and glutamic acids would result in chain branching with the creation of several C-terminal amino acids.

Without considerable further experimentation however, speculations such as these are unlikely to lead to any useful conclusions.

The purpose for which these end group experiments were undertaken was to seek for evidence of the cell-specificity of the histones. The method has not proved suitable for this purpose.

STUDIES ON PROTAMINES

Introduction

The protamines clupeine and salmine are responsible for over 80% of the protein found in the sperm heads of herring and salmon respectively. The almost quantitative isolation of these protamines by Dr Stedman, using the techniques described on pp. 15-17, has ensured that any protamine heterogeneity *in vivo* is preserved in the isolated preparations. The examination of these in the Tiselius apparatus and ultracentrifuge had previously suggested their homogeneity (Cruft, Mauritzen and Stedman, unpublished experiments), and it was of interest to determine whether these findings were confirmed by chromatography and N-terminal analysis.

The complete amino acid analyses of clupeine and salmine by several workers have demonstrated the absence of lysine, histidine, tyrosine and cysteine residues. Assuming these protamines to consist of single polypeptide chains, the only groups capable of substitution by FDNB are the N-terminal amino acids. It therefore seemed possible to apply the method of partial substitution by FDNB followed by fractionation of

the resultant derivatives, applied by Battersby and Craig (1951) to determine the molecular weight of insulin, to explore the homogeneity of clupeine and salmine and to determine their molecular weights.

Experimental

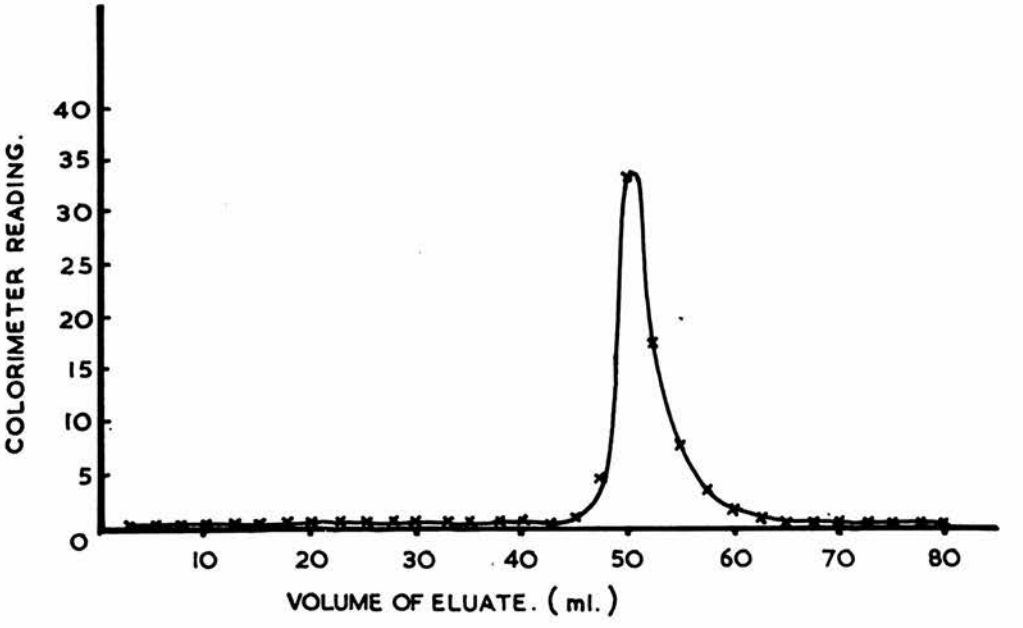
1. Chromatography of Clupeine Sulphate.

Scanes and Tozer (1956b) succeeded in separating a commercial clupeine sulphate preparation into several distinct fractions by chromatography on alumina using potassium phosphate buffers.

This method was accordingly used for analytical scale experiments designed to investigate the homogeneity of the protamine preparation.

Savory and Moore's alumina, 100/150 mesh, was stirred with water to remove air bubbles and any finely divided material. After decanting the supernatant the alumina was further stirred with some of the buffer solution selected for the experiment and the slurry poured into a column 0.9 cm. in diameter x 30 cm. long. This was

FIGURE. 32. CHROMATOGRAPHY OF CLUPEINE SULPHATE ON ALUMINA.

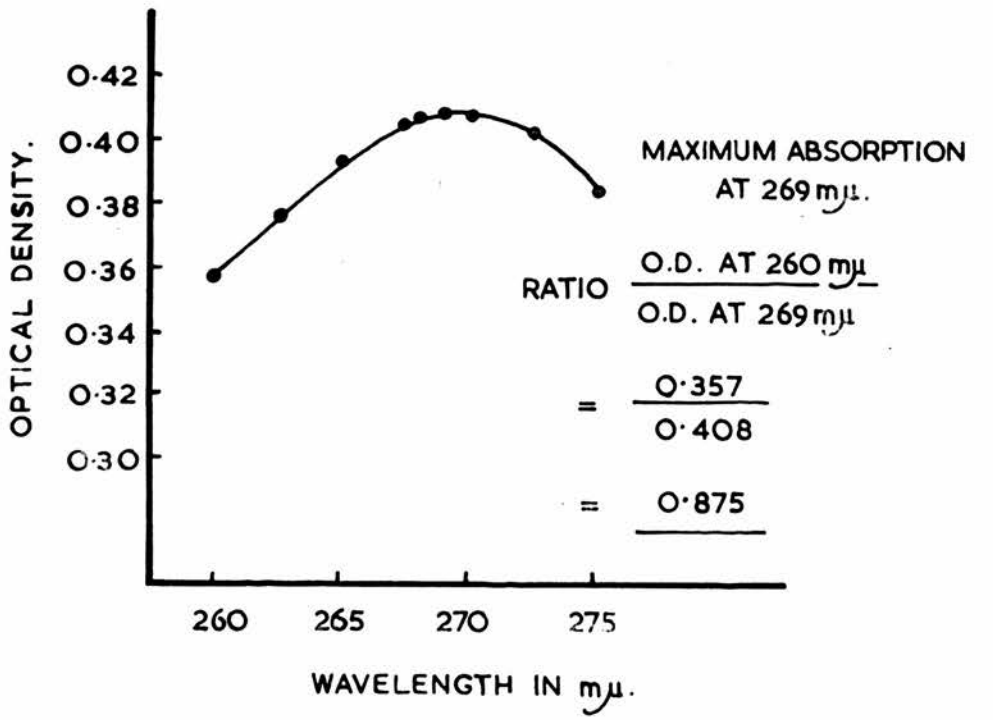


allowed to stand for several hours with a slow stream of buffer percolating through to ensure adequate packing.

Clupeine sulphate (5-10 mg.) was dissolved in 1-2 ml. of buffer and an aliquot applied to the column which was run at a rate of approximately 10 ml./hour. The effluent was collected in 2.5 ml. fractions and, after the addition of 0.5 ml. of 2 N-acetic acid to each, analysed by the photometric ninhydrin method. A few trial runs indicated that 0.43 M-dipotassium hydrogen phosphate was a suitable eluting agent and in a typical experiment 5.3 mg. clupeine sulphate applied to the column were eluted in 98% yield (Figure 32) as a single nearly symmetrical peak after the passage of about five hold-up volumes of the eluent. No evidence of heterogeneity other than a slight tailing of the peak could be found. This finding contrasts sharply with those referred to above in which clupeine sulphate was separated into three distinct fractions of differing composition. A possible explanation, referred to previously, is that the heterogeneity of these authors' preparations was artifactually produced during the isolation procedures.

FIGURE. 33.

ULTRAVIOLET ABSORPTION CURVE OF PTH FROM PTC-CLUPEINE.



2. N-Terminal Analysis

Clupeine sulphate was labelled with FDNB under the standard conditions (pp. 67-68). After acidifying the solution and extracting with ether all the DNP-clupeine dissolved in the aqueous phase which was then freeze-dried. The yellow residue was taken up in a few drops of 5.7 N-hydrochloric acid and hydrolysed in a sealed tube at 105°C for 16 hours. The only DNP-amino acid that could be detected in the hydrolysate was DNP-alanine.

The Edman PTC-method also indicated alanine as the only N-terminal amino acid. For this experiment the paper strip carrier technique of Fraenkel-Conrat (1954) was used.

In a semi-quantitative experiment, 6.50 mg. of clupeine sulphate, previously dried over phosphorus pentoxide, were dissolved in a few drops of 4% sodium bicarbonate solution and applied to seven paper strips. After the reaction sequence had been carried through the liberated PTH was extracted into 20.0 ml. of 50% ethanol-ether (v/v) and the ultraviolet absorption of the solution measured over the range 260-275 mμ. Figure 33 reproduces the curve obtained which shows an

optical density (O.D.) maximum of 0.408 at 269 mμ. The ratio O.D. at 260 m /O.D. at absorption maximum = 0.875 thus indicates a fairly pure PTH. This was identified by paper chromatography as alanine-PTH, a small spot remaining stationary at the origin probably being due to a trace of uncyclized PTC-alanine. Taking the molar extinction coefficient of alanine-PTH as 16,100 (Fraenkel-Conrat, Harris and Levy, 1954), a yield of 0.51 μM alanine-PTH was obtained. This gives the figure of 12,700 for the molecular weight of clupeine sulphate.

3. Ultraviolet Absorption Spectrum of DNP-Clupeine

Clupeine sulphate was thoroughly dried over phosphorus pentoxide and 40.5 mg., together with 65 mg. of A.R. sodium bicarbonate, were dissolved in 4.00 ml. of water. Three 1.00 ml. portions were pipetted into each of three tubes giving four tubes altogether. To three of them was added 2.0 ml. of a 5% ethanolic solution of FDNB, 2.0 ml. of ethanol only being added to the fourth tube. The tubes were shaken mechanically in the

FIGURE. 34. U.V. ABSORPTION CURVE OF DNP-CLUPEINE IN 1 % SODIUM BICARBONATE SOLN. AFTER 1·75, 4·5, AND 22 HOURS LABELLING.

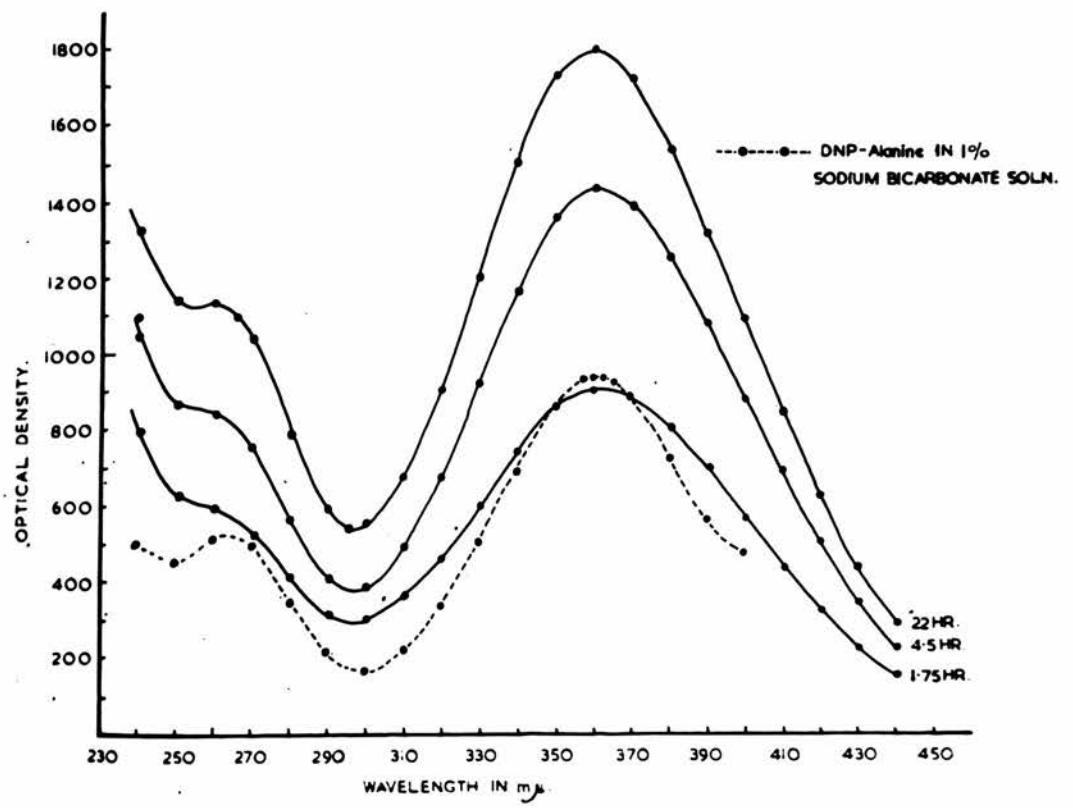
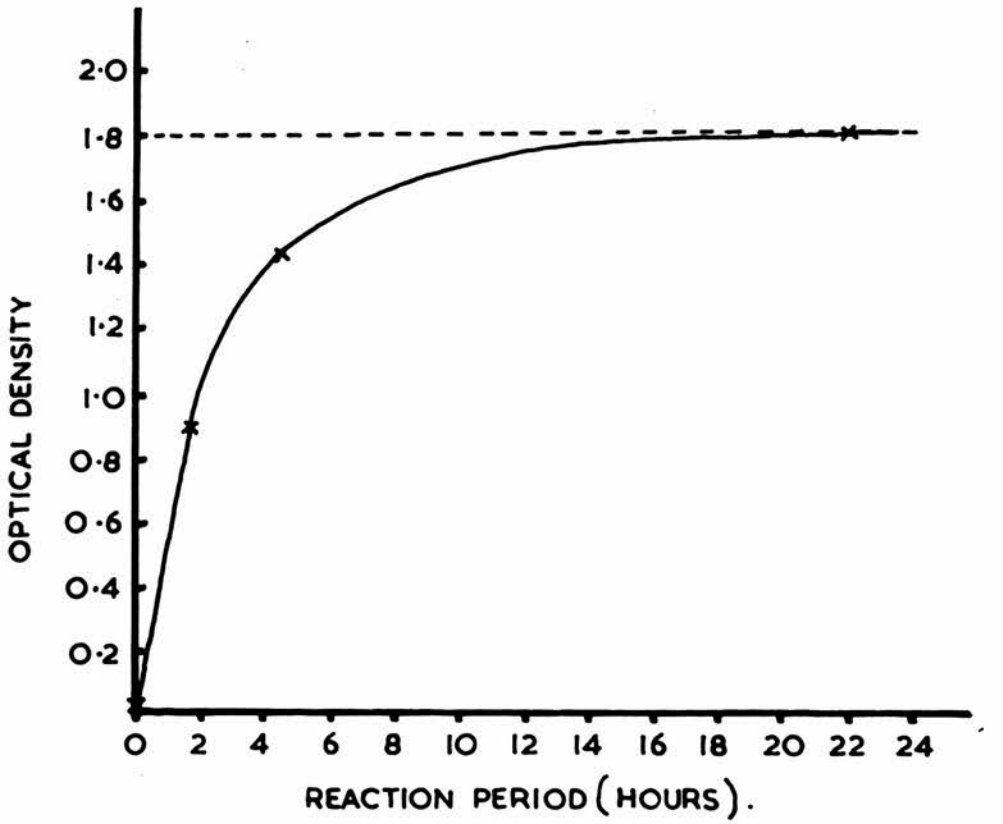


FIGURE. 35 REACTION OF CLUPEINE WITH FDNB.



dark at 18°C for 1.75, 4.5 and 22 hour periods, the reactions being stopped after the appropriate interval by the addition of 1 ml. of 0.5 N-hydrochloric acid. Excess FDNB, dinitrophenol, and ethanol were removed by five extractions with 20 ml. portions of peroxide-free ether, the last traces of ether being removed in a stream of filtered air. The pale yellow solutions were diluted to 12.5 ml. with freshly prepared 1% sodium bicarbonate solution and portions of these were transferred to 1 cm. quartz cells and their absorption measured over the range 230-420 mμ. against the prepared blank. Figure 34 shows the curves obtained together with that of DNP-alanine in 1% sodium bicarbonate solution. The optical densities at the absorption maxima plotted against the reaction times gave the curve shown in Figure 35.

Assuming the molecular extinction coefficient (ϵ) of DNP-clupeine to be identical with that of DNP-alanine and using Fletcher, Lowther and Reiths' (1954) value for the latter ($\epsilon_{\lambda max.} = 17.1 \times 10^3$), a molecular weight of 13,230 is indicated for clupeine sulphate.

Discussion

A reaction period of 1.75 hours is seen to give 50% substitution of the N-terminal group. If there were more than one reacting site in the molecule this period would be expected to result in differing degrees of substitution and the mono-, di- and other poly-DNP derivatives should be capable of separation. Neither adsorption chromatography on alumina nor partition chromatography on either starch or cellulose, using a 1% acetic acid-isobutanol system, disclosed any heterogeneity. Sluyterman (1955) claimed success in separating insulin, partially substituted by the DNP-method, into the mono- and di-substituted derivatives by filter paper electrophoresis using 33% acetic acid as the electrolyte. When this method was applied to the partially substituted clupeine two yellow bands were obtained. Electrophoresis of a larger quantity of the material in a cellulose block similarly gave rise to two well separated bands which subsequent isolation and analysis showed to possess identical compositions. They were therefore artifacts of separation due to the very low ionic

strength of the electrolyte employed. Use of 33% acetic acid, 0.05 M with respect to sodium chloride, consistently failed to give any separation, a single yellow band being obtained after several hours' electrophoresis.

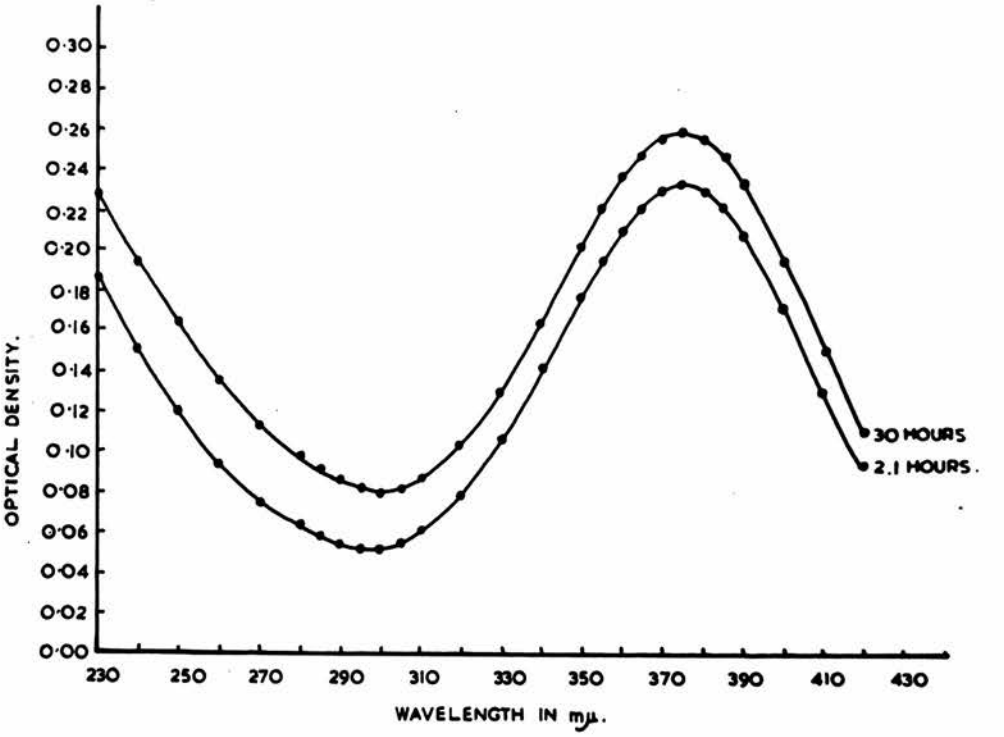
The conclusion drawn from all these experiments is that clupeine sulphate is a pure substance consisting of a single polypeptide chain with alanine as the N-terminal group and of molecular weight about 13,200.

4. Ultraviolet Absorption Spectrum of DNP-Salmine

DNP-L-Proline has been shown to possess a characteristic absorption spectrum in dilute alkaline solutions with a maximum at 386 mμ. (Schroeder, Honnen and Green, 1953). For a DNP-L-proline ester the position of the maximum is shifted to 375 mμ.

Felix (1953) and others have shown proline to be the principal and probably the only N-terminal group in salmine. Phillips (1955) following on this work, studied the absorption spectrum of DNP-salmine in dilute alkaline solution and found the maximum absorption to occur at 370 mμ. rather than at the expected 375 mμ.

FIGURE .36. U.V. ABSORPTION CURVE OF DNP-SALMINE IN 1% SODIUM BICARBONATE SOLUTION AFTER 2.1 AND 30 HOURS LABELLING.

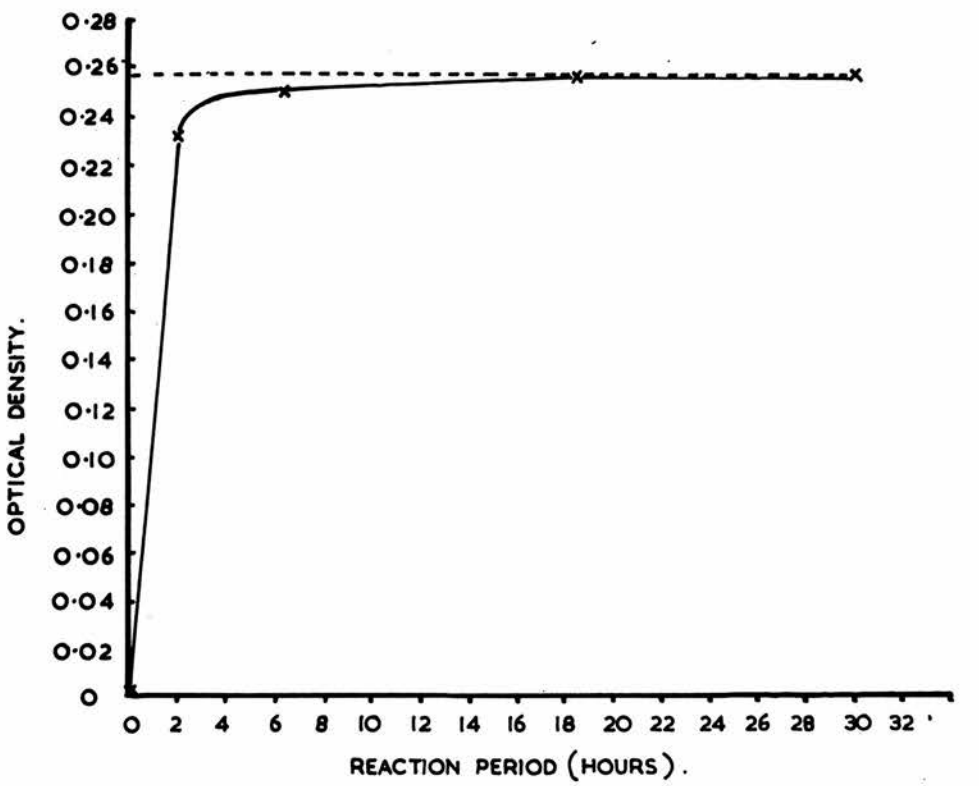


He explained this on the basis of contamination by small amounts of other DNP-amino acids which have their absorption maxima at about 362 mμ.

A sample of seven times reprecipitated salmine sulphate was dried in a vacuum desiccator over phosphorus pentoxide and 12.40 mg. weighed into a glass stoppered tube together with 21.2 mg. sodium bicarbonate, the mixture being dissolved in 5.00 ml. of water. Into each of four tubes were pipetted 1.00 ml. aliquots giving five tubes altogether. The reactions with FDNB etc. for different times were performed as with clupeine, except that the temperature was maintained at 24°C.

The DNP-salmine solutions resulting after acidification and extraction with ether were diluted to 25.0 ml. with 1% sodium bicarbonate solution and the absorption of the solutions measured over the range 230-420 m against the prepared blank as previously. Figure 36 shows the curves obtained for two samples after 2.1 and 30 hours' labelling respectively. The maxima in each case are seen to occur at 275 mμ. - the maximum for DNP-proline ester. No secondary labelling of any other residue, with the possible exception of proline, is thus occurring. Figure 37

FIGURE . 37 REACTION OF SALMINE WITH FDNB.



shows the time curve for the reaction. Its shape clearly indicates a monomolecular reaction which is virtually complete after 5 hours.

Assuming that the molecular extinction coefficients of DNP-salmine and DNP-L-proline are identical having $\epsilon_{\lambda max.} = 19.2 \times 10^3$, admittedly an unsound assumption since the maximum absorption occurs at different wavelengths, a molecular weight of 7,440 is obtained for salmine sulphate. After allowing for sulphate, a molecular weight of approximately 6000 is obtained for salmine.

Discussion

Unpublished experiments by Dr Stedman have indicated, on the basis of a single molecule of alanine per mole of salmine, a molecular weight of about 12,000 whilst the ultraviolet absorption measurements suggest a figure of 6000. This discrepancy is too great to be accounted for on the basis of differences in the molecular extinction coefficients of DNP-L-proline and DNP-salmine. Chain branching, with the existence of two N-terminal prolines, would serve to explain these results, but it is difficult to see how such

branching could occur unless ester linkages were involved. All attempts at the fractionation of partially substituted samples of salmine, by the methods described for DNP-clupeine, failed.

On the basis of these results the homogeneity of salmine, and consequently its molecular weight, must remain an open question.

APPENDICES

APPENDIX I

Preparation and Equilibration of Barium-IRC-50 Columns

The dry IRC-50 (XE-64) resin as supplied was screened through a 200 mesh sieve and the finely divided material stirred with five times its volume of water. After a settling period of 30 minutes any foam on the surface was withdrawn and the supernatant suspension decanted. This process was repeated five times until the supernatant liquid was clear after about 15 minutes' settling. The resin was then air-dried on a Büchner funnel and the filter cake added to five times its volume of acetone and the mixture stirred at intervals over a period of 4 hours. The resin was filtered and washed with acetone until the filtrate was clear. The air-dried resin was then resuspended in water and thoroughly washed until the last traces of acetone were removed.

The resin was cycled once through the sodium form before use. For this purpose, 1.5 l. of 10 N-sodium hydroxide were added, with stirring, to 1.5 kg. of the resin suspended in 5 l. of water.

The pH of the suspension rose to over 11 and stirring was continued until the evolution of heat subsided. The sodium salt of the resin was then washed by decantation with 3 l. portions of water until the supernatant had a pH of about 10. The resin was converted to the acid form by stirring with two 7 l. portions of 3 N-hydrochloric acid and finally washed with three 2 l. portions of water.

The original method described by Crampton et al. (1955) for the conversion of the acid form of the resin into the barium form (viz. washing on a filter with 2 M-barium acetate solution until the pH of the effluent rose to 6.5) was wasteful of barium acetate and the following technique was used instead. The acid form of the resin was suspended in 2 l. of 2 M-barium acetate solution and a saturated solution of barium hydroxide was added with constant stirring until the pH of the suspension rose to 6.6-6.7. The resin was then collected in a Büchner funnel and washed once with water followed by 4 l. of 0.1 M-barium acetate buffer, pH 6.7 (prepared by adding 0.67 ml. of glacial acetic acid to 10 l. of 0.1 M-barium acetate). The resin was then stirred with two volumes of 0.1 M buffer and the slurry poured into

a 4 x 60 cm. chromatograph tube. Final equilibration of the resin was achieved by allowing about 10 l. of the buffer to percolate through the resin bed over a period of 48 hours when the pH's of the influent and effluent were found to be identical. The final equilibration and the chromatography were performed at as constant a temperature as possible. Before re-use the resin was cycled through the acid and sodium forms.

The analytical chromatograms were performed with columns 0.9 cm. in diameter and of the desired height, prepared from suspensions of the resin in the given buffer by the procedure already described.

APPENDIX II

Photometric Ninhydrin Technique

Reagents

Ninhydrin

The ordinary grade reagent was recrystallized from hot water. A solution of 100 g. of ninhydrin in 250 ml. of hot water was stirred with 5 g. of decolourizing charcoal and filtered through three thicknesses of filter paper. The filtrate was allowed to stand at 4°C overnight. The crystalline ninhydrin was then filtered off on a Büchner funnel and washed four times with cold water. The air-dried crystals were stored in a dark glass bottle.

Hydrindantin

Recrystallized ninhydrin (20 g.) was dissolved in 500 ml. of water at 90°C. A solution of 20 g. of ascorbic acid B.P. in 100 ml. of water at 40°C was slowly added with constant stirring to the above solution. Crystallization of the hydrindantin started immediately. When complete the crystals were filtered off, washed with water, and dried over phosphorus pentoxide in a vacuum desiccator. They were stored in a dark glass bottle.

4 N-Sodium Acetate Buffer, pH 5.5

A.R. Sodium acetate trihydrate (544 g.) was dissolved in water with warming. After cooling to room temperature, 100 ml. of A.R. glacial acetic acid were added and the solution made up to 1 l. The pH was 5.5. It was stored at 4°C.

Reagent Solution

5 g. of ninhydrin and 0.75 g. of hydrindantin were dissolved in 187 ml. of peroxide free methyl cellosolve. Stirring was gentle to avoid air bubbles. To the solution was then added 4 N-sodium acetate buffer to give a volume of 250 ml. which was stored in a dark glass bottle under carbon dioxide for not more than one week.

Method

In the majority of analytical scale experiments the effluent was collected in 2.5 ml. cuts and the protein content of these estimated as follows: To each effluent fraction was added 2.0 ml. of the reagent solution and the mixture heated in a boiling water-bath for 20 minutes. After cooling, 6.0 ml. of 60% ethanol/water were added to each

tube and the solutions shaken vigorously to oxidize any remaining hydrindantin. The contents of each tube were then decanted, in turn, into a colorimeter tube and their relative colour intensities measured in an Eel colorimeter using a 626 filter against blanks prepared identically with the first few cuts from the column which contained no ninhydrin positive material. During the latter part of the runs when the pH of the effluent rose appreciably, it was necessary to adjust this to about pH 6 by addition of dilute acetic acid from a burette to each effluent fraction before the addition of the reagent solution. A corresponding allowance was made in the volume of diluent added before the estimations.

The analyses of 1.0 and 0.5 ml. volumes of column effluent were performed using 1.0 and 0.5 ml. portions of the reagent solution respectively. After heating for 20 minutes the volumes were made up to 7.0 ml. with the 60% ethanol/water diluent before reading the colour intensities as previously.

As pointed out by Crampton et al. (1955) the ninhydrin colour equivalents of each histone

fraction cannot be taken as identical. However, from the point of view of merely investigating the resolution on various columns and obtaining approximate recoveries it was not necessary to take these differences into account.

APPENDIX III

Moving Boundary Electrophoresis

The instrument used in these studies was that produced by Hilger and Watts Ltd., London, 1950, based on the apparatus of Professor A. Tiselius and the following standard procedure was carried out for all experiments.

The medium sized U-tube (10 ml.) was cleaned by removing grease with cotton wool dipped in ethanol and the bore of the tube cleaned with an ethanol soaked wad of cotton wool on the end of a wood splint. The whole assembly was rinsed with ethanol and finally ether and allowed to dry in a warm atmosphere.

The sliding surfaces were evenly covered with a thin layer of low temperature lubricant and the bottom two sections pressed together in alignment. These two sections were then filled, using a 10 ml. pipette, with the dialysed protein-buffer solution until the liquid meniscus rose just above the level of the top surface. Any visible bubbles inside the tube were removed with a piece of copper wire and the two sections placed in the U-tube holder.

The upper section was then slid on top but keeping the tube bores out of alignment, the surplus solution squeezed from the bottom section being absorbed by filter paper.

The upper limbs were filled with pure buffer solution in the same manner, and the top section was slid on and clamped into position with the centre guides fitted. Finally the electrode vessels were mounted on either side and filled with buffer solution to the level of the side limbs thus leaving room for 100 ml. of saturated potassium chloride solution to be added later.

This method invariably gave a leak-proof assembly.

The whole assembly was then placed in the refrigerated thermostat bath (0.5-0.7°C) and left for 2½ hours to achieve thermal equilibrium. The silver-silver chloride electrodes after rinsing in buffer solution were inserted into each vessel and 100 ml. of saturated potassium chloride, cooled to the bath temperature, were run to the bottom of each electrode vessel using a funnel attached to a long capillary tube.

The electrode leads from the power supply were

fitted and the solutions sucked up from each vessel into a Y-tube and left for half an hour for the liquid levels to reach equilibrium. After this period the top limbs were slid into alignment with the bottom section using an air plunger, and the liquid in the Y-tube was very slowly run back 3-4 cm. from the junction.

The boundaries were brought into view in the optical system with the compensator, which consisted of a small electrolytic device whereby buffer solution could be added to one limb in a slow and continuous fashion, the rate of flow being adjusted so that about 10 minutes were required to bring the sharp boundaries into view.

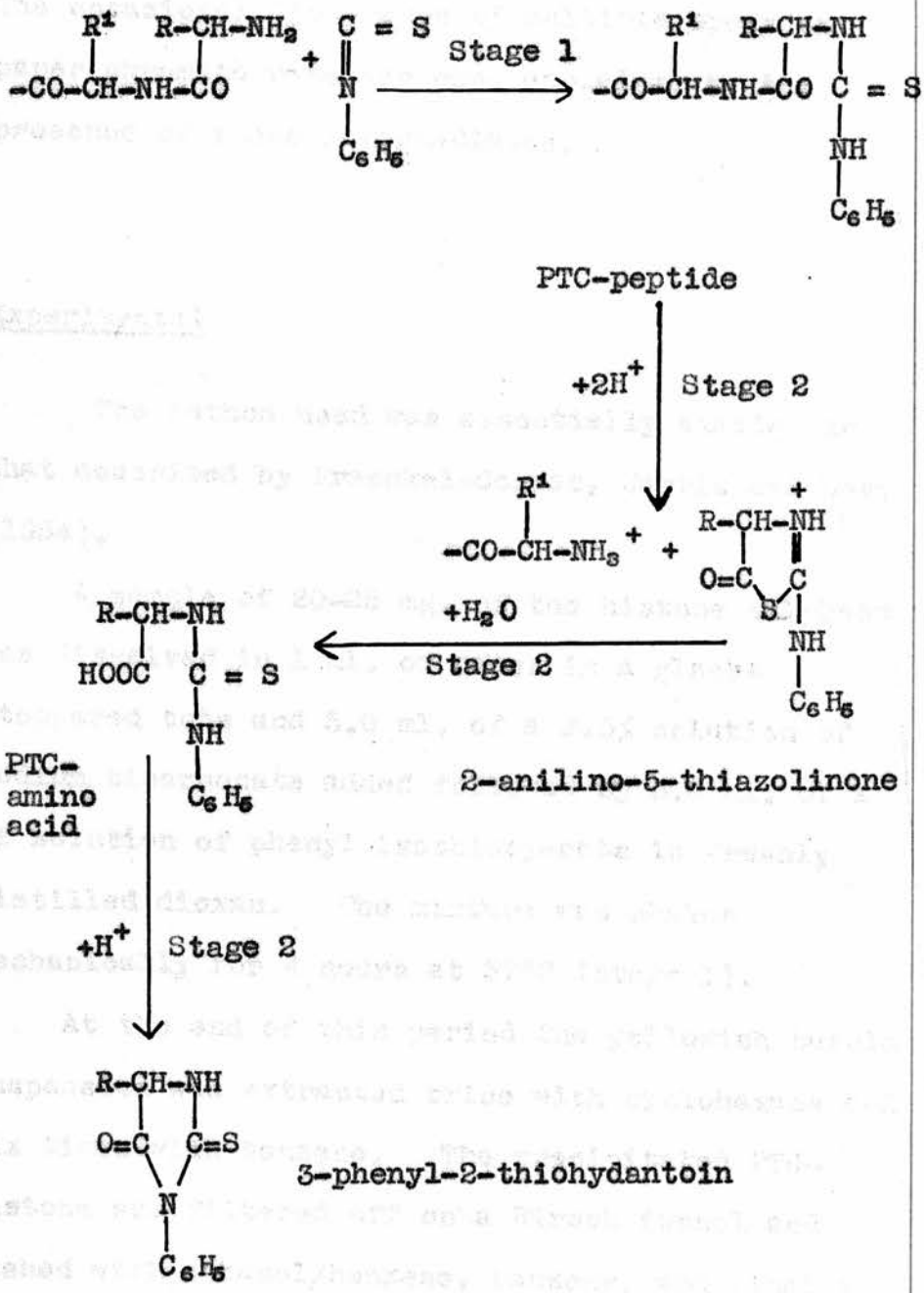
The current was switched on and gradually raised to 30 mA over a period of 3-4 minutes. The boundaries were then photographed at half hourly intervals over $2\frac{1}{2}$-3 hours, the current being checked periodically and adjusted if necessary.

APPENDIX IV

N-Terminal Analysis by the Phenylthiocarbonyl (PTC)-Method

This method depends on the discovery by Edman (1950) that with acid catalysis the phenylthiocarbamates of peptides rearrange readily to yield the phenylthiohydantoins of the N-terminal amino acids and the shortened peptides.

More recently Edman (1956) has shown that the compound first formed in the acid catalysed cleavage of a PTC-peptide is not the expected 3-phenyl-2-thiohydantoin derivative but the isomeric 2-anilino-5-thiazolinone. This is rather unstable and is readily converted to the PTC-amino acid which then slowly undergoes ring closure to the PTH as shown in the following reaction sequence.


Stage 1
PTC-peptide
+2H+
Stage 2
+H2O
Stage 2
2-anilino-5-thiazolinone
PTC-amino acid
+H+
Stage 2
3-phenyl-2-thiohydantoin

The occasional appearance of multiple spots on paper chromatograms has been explained by the presence of these intermediates.

Experimental

The method used was essentially similar to that described by Fraenkel-Conrat, Harris and Levy (1954).

A sample of 20-25 mg. of the histone sulphate was dissolved in 1 ml. of water in a glass-stoppered tube and 3.0 ml. of a 2.5% solution of sodium bicarbonate added followed by 3.0 ml. of a 5% solution of phenyl isothiocyanate in freshly distilled dioxan. The mixture was shaken mechanically for 4 hours at 37°C (Stage 1).

At the end of this period the yellowish turbid suspension was extracted twice with cyclohexane and six times with benzene. The precipitated PTC-histone was filtered off on a Hirsch funnel and washed with ethanol/benzene, benzene, and finally air-dried.

The second stage of the reaction involves the cyclization of the PTC-moiety with its concomitant release as the thiazolinone. For this purpose the PTC-

protein was heated for 1 hour in a boiling water-bath with 5 ml. of 1 N-hydrochloric acid. After cooling, the solution was extracted four times with 5 ml. portions of purified ethyl acetate and the bulked extracts taken to dryness in a stream of warm air. The PTH's were dissolved in a measured volume of absolute alcohol (5-10 ml.) and the ultraviolet absorption spectrum of the solution measured over the range 260-275 mμ. in 2.5 mμ. intervals. The characteristic absorption peak of most PTH's is at about 267.5 mμ. The ratio of the optical density of the solution at 260 mμ. to that at the maximum gives a measure of the purity of the preparation. A ratio of 0.85 or less indicates a relatively pure PTH, whilst a higher ratio indicates contamination with non-cyclized PTC-peptides, PTC-amino acids or other products.

The ethanol solution was then evaporated down to about 0.2 ml. for application to paper chromatograms.

Direct Identification of PTH's by Paper Chromatography

The procedure described by Sjoquist (1953) was used for this purpose despite its rather complicated nature.

Strips of Whatman No. 1 paper, 6 inches x 18 inches, were sprayed with 0.5% starch solution and allowed to dry. After the application of spots of about 0.03 to 0.1 μM of PTH the paper was conditioned in an atmosphere saturated with the solvent. With solvent mixture A (70 ml. of heptane and 30 ml. of pyridine) the solvent was allowed to travel down a blank sheet of paper for 2 hours prior to adding solvent to the experimental sheet. The descending run took about 4 hours. With solvent system B (upper phase of 40 ml. of heptane, 20 ml. of n-butanol and 40 ml. of 90% formic acid) overnight equilibration in a jar containing the solvent preceded the descending run which took about 6 hours.

After the papers had been dried they were sprayed with a fresh mixture of equal volumes of 0.01 M-iodine solution in 0.5 M-potassium iodide and 0.5 M-sodium azide solution. The PTH's were

indicated by bleached spots on a purple background, some of them showing a characteristic colour in the bleached area.

Preparation of PTH's from Amino Acids

The synthesis of the small quantities of amino acid PTH's needed as chromatographic standards was performed as follows: To 20-50 mg. of amino acid (about 0.3 mM) and an equimolar amount of sodium carbonate, dissolved in 4 ml. of 50% dioxan, was added a 20% excess of phenyl isothiocyanate (0.1 ml. = 0.84 mM) and the mixture vigorously stirred at 40°C for 2 hours. It was then extracted repeatedly with cyclohexane and benzene and aerated to remove traces of benzene. Hydrochloric acid was then added to about 1 N concentration whereupon the PTH's were often immediately precipitated. Holding the mixture at 40°C for 2-4 hours yielded crystalline products which were filtered off and recrystallized from ethanol-water mixtures.

In this way the PTH's of the following amino acids were prepared: threonine, glycine, alanine, phenylalanine, proline, valine, leucine, isoleucine and ε-PTC-lysine. The serine

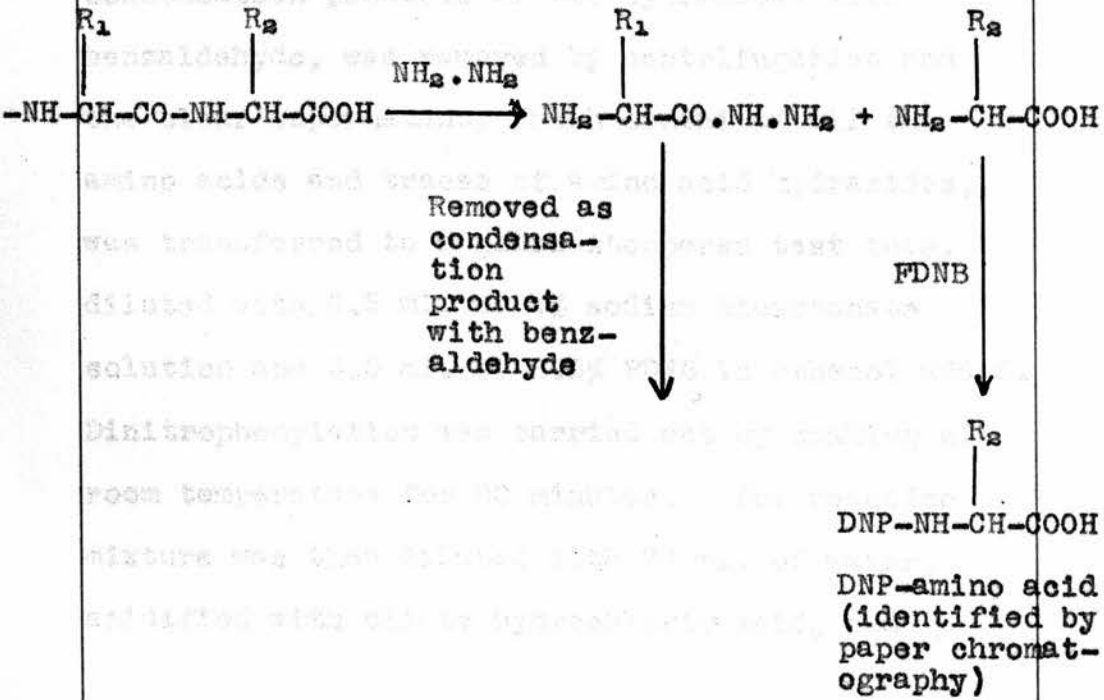
derivative did not move in either chromatographic system A or B, and it was presumed that dehydration and subsequent polymerization had occurred.

APPENDIX V

C-Terminal Amino Acid Analysis

Akaboni, Ohno and Narita (1952) proposed hydrazinalysis as a method for the identification of C-terminal amino acids. Ohno (1954) developed this method by using FDNB to label and thus identify the products, and Niu and Fraenkel-Conrat (1955a), after further modifications, used this method in an extensive series of investigations on various proteins. The procedure of these latter authors, without modification, was that applied to the histones.

The following sequence of reactions is involved:



Experimental

Samples of histone sulphate (10-15 mg.) were dried at 100°C for 2 hours and allowed to cool *in vacuo*. To these was added 0.5 ml. of anhydrous hydrazine (prepared by distilling hydrazine hydrate from excess quick lime) in dry tubes which were sealed and heated for 10 hours at 100°C. The excess hydrazine was removed in a vacuum desiccator containing concentrated sulphuric acid and the residue taken up in 1.0 ml. of water. About 0.2 ml. of benzaldehyde was added and the mixture shaken at room temperature for 2 hours. The benzaldehyde layer, together with the condensation products of the hydrazides with benzaldehyde, was removed by centrifugation and the clear supernatant, which contained all the amino acids and traces of amino acid hydrazides, was transferred to a glass-stoppered test tube, diluted with 0.5 ml. of 6% sodium bicarbonate solution and 3.0 ml. of 2.5% FDNB in ethanol added. Dinitrophenylation was carried out by shaking at room temperature for 80 minutes. The reaction mixture was then diluted with 20 ml. of water, acidified with dilute hydrochloric acid, and

extracted with 2 x 20 ml. of ethyl acetate. The ethyl acetate solution was then diluted with two volumes of ether and extracted with 2 x 50 ml. of 2% sodium bicarbonate solution which was washed once with 20 ml. of ethyl acetate. It was then acidified and extracted with 2 x 15 ml. of ethyl acetate. The solvent was removed in vacuo and much of the dinitrophenol sublimed from the residue at 60°C in vacuo. The non-volatile residue was dissolved in 0.2 ml. acetone and studied by two dimensional paper chromatography.

APPENDIX VI

Quantitative Amino Acid Analyses

These were performed by a slight modification of the chromatographic method of Moore and Stein (1951).

A sample of the histone sulphate was dried over phosphorus pentoxide in a high vacuum and 3-4 mg. accurately weighed into a small round-bottomed Quickfit flask. It was hydrolysed with 2.5 ml. of 5.7 N-hydrochloric acid by boiling gently under reflux for 30 hours.

At the end of this period the flask was allowed to cool and the condenser and neck of the flask carefully washed down with small quantities of water. The contents of the flask were then taken to dryness by heating the latter gently on a hot water-bath at reduced pressure. The condenser and the neck of the flask were again rinsed with a small quantity of water and again taken to dryness.

For the estimation of the non-basic amino acids, the residue obtained was dissolved in 1.00(0) ml. of 0.1 M-citrate buffer at pH 3.42. An aliquot (usually 0.80(0) ml.) of this

solution was applied to a column, 100 cm. x 0.9 cm., of Dowex-50 resin which had been equilibrated as prescribed (Moore and Stein, 1951). In order to perform the runs at the different temperatures required the column was jacketed with an electrical heating coil controlled by a Variac transformer.

At a column temperature of 37°C, approximately 200 ml. of 0.1 M-citrate buffer, pH 3.42, were run through at a rate of 4.0 ml./hour. The effluent was collected in 1 ml. aliquots by a drop-counter automatic fraction collector. The fractions were subsequently analysed by the photometric ninhydrin technique. When the peak corresponding to valine emerged the temperature was increased to 50°C and the influent buffer changed to 0.1 M-citrate, pH 4.25. This brought off the remaining monoamino acids in a series of well defined peaks which were estimated as previously. From the known ninhydrin colour equivalents of the various amino acids (Moore and Stein, 1948), the quantity of each amino acid was calculated and expressed as a percentage of the weight of the protein originally taken.

For the estimation of the basic amino acids the hydrolysis procedure and mode of application of the hydrolysate to the column was exactly as described for the non-basic amino acids. The column used in this case had, however, the dimensions 0.9 x 15 cm. and was similarly maintained at 25°C. Citrate buffer, 0.1 M pH 5.0, was run through the column overnight in order to elute all the non-basic amino acids which were discarded. The basic amino acids were then eluted with 0.1 M-citrate buffer, pH 6.5, in the order lysine, histidine and arginine. This contrasts with the original method where histidine and lysine were eluted, in that order, by phosphate buffer, pH 6.8 prior to elution of the arginine with the citrate buffer. The main advantage of this modification lay in the avoidance of using phosphate buffer which previous experience had shown to cause an increase in the blanks obtained from the column. The effluents were analysed as above and from their ninhydrin colour equivalents the amount of each amino acid was calculated. These were finally expressed as the percentage by weight of the protein sample hydrolysed.

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THE AMINO ACID COMPOSITION OF THE SIX HISTONES OF CALVES' THYMOCYTES

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Earlier work[1] from this laboratory established that the histones present in the cell nuclei of many species and tissues are composite in character, all of them containing a main and one or more subsidiary histones. The former, which constituted the major component, was characterized by a relatively small solubility in aqueous alcohol and a high arginine content; its general amino acid composition was, indeed, typical of the traditional histones of the older literature. The subsidiary histones were more soluble and, with one exception, were distinguished from the main component by their low content of arginine. Despite their deficiency in this amino acid they nevertheless exhibited strong basic properties. By examining an extensively fractionated specimen of a subsidiary histone from the thymocytes of the calf it was ascertained that, for this particular histone these strong basic properties were due to a very high content of lysine. It was also characterized by a high content of alanine and proline and by the absence of phenylalanine, tyrosine and histidine[2]. The indication that it contained a minute amount of methionine is now known to have

been incorrect, for this amino acid could not have survived the conditions under which the chromatogram in which it was detected was prepared.

The existence of subsidiary histones rich in lysine and alanine in the thymus gland of the calf has since been confirmed by several groups of workers[3-7]. In the meantime the results of our electrophoretic and ultracentrifugal studies of various histones had convinced us that all the subsidiary histones hitherto examined were actually mixtures of closely related proteins which had so far defied all attempts at complete separation. Such studies[8] had in fact shown that the total histone from the thymus gland of the calf and the liver of the ox consisted of six components which were designated α_1, α_2, α_3, β, 1.6 S and 0.8 S γ-histones. Of these, the first three replace the α-histone and the last two the γ-histone of our earlier nomenclature[9]. Such changes were rendered necessary by the discovery that the original α- and γ-components could be resolved into five histones. The three α's differed in their electrophoretic mobilities while the two γ's possessed nearly identical

electrophoretic mobilities although they differed in their sedimentation constants.

The physical characterization of these various components rendered possible attempts at their separation. The first step in this direction was the preparation from the total histones of the thymus gland of the calf of the crude α-fraction which, despite its name actually comprised the 3 α-histones and the 0.8 S γ-histone. The further stages of fractionation presented, however, many difficulties. For various reasons both ion exchange and adsorption (alumina) chromatography failed to achieve the desired results. Using the former method with the resin IRC-50[x] according to the procedure of Crampton, Moore & Stein[5], two fractions A and B, corresponding with their results, were obtained. But when fraction A was examined electrophoretically it was found to consist of a mixture of α_1 and α_2-histones. In their recent work[10] Crampton, Stein & Moore have also been led, on other evidence, to suspect the homogeneity of their preparations.

Much greater success in the separation of the α-fraction has been achieved by zone electrophoresis. Using a medium of starch with veronal buffers at pH 8.5

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[x] Generously donated by the Rohm and Haas Company, Philadelphia.

and of ionic strength 0.18 a substantial separation of the above α-fraction into the four components has been effected. In the procedure adopted, the influence on the results of the tailing observed during the electrophoresis of histones on paper or in a starch medium, which is caused by the binding of the positively charged histones to the negatively charged surfaces of the medium, was largely swamped by using high concentrations of the α-fraction. A current of 40 mA was passed through the starch in the trough, which had a cross sectional area of 17.6 cm.^{2}. The trough was immersed in ice cold water and kept in a cold room at 2°C. Electrophoresis was carried out for 80 hours, a time suggested by pilot experiments. A print on filter paper was then taken from the surface of the starch block. After staining this indicated the presence of four bands. Four sections corresponding with the positions of these bands were then cut from the starch block and extracted separately with 0.1 N-sulphuric acid. The extracts were then neutralized with sodium hydroxide and lyophilized. Of the four products so obtained the one with the smallest mobility (0.8 S γ) was submitted to a second run. The product was electrophoretically homogenous, but when

examined by sedimentation, was found to be contaminated with the 1.6 S γ-histone. This was accordingly removed by preparative ultracentrifugation in 20% aqueous alcohol at pH 9.0 and the 0.8 S γ-histone precipitated from the mother liquors with excess of alcohol. The product now sedimented as a monodisperse substance at pH 8.5 with a sedimentation coefficient of 0.83×10^{-13}. The quantities of the remaining three fractions available were unfortunately too small for further purification by zone electrophoresis. The four products were therefore dissolved separately in small volumes of water and dialysed in cellophane sacs successively against water and 0.1 N-sulphuric acid. The sulphates were then precipitated by addition of 12 volumes of acetone.

When examined in the Tiselius apparatus the three fastest fractions were found to possess mobilities corresponding with the α_1-, α_2-, α_3-histones although evidence of the presence of small amounts of the adjacent components was observed in each of them. It was calculated, for example, from the pattern of the α_1-component that the impurity amounted to 10-15% of α_2. Sedimentation coefficients were also determined in the Spinco ultracentrifuge. The values obtained are: α_1-histone, 0.45×10^{-13}; α_2, α_3 and 0.8 S γ-histones, 0.8×10^{-13}. Since the diffusion coefficient

of the crude α-fraction is about 8×10^{-7}, this suggests molecular weights for the α_2, α_3 and 0.8 S γ-histones of the order of 10,000, and of about half that figure for the α_1-histone. Of the four preparations, the α_3-histone proved to be the most heavily contaminated with impurity, for the sedimentation experiments revealed that this contained about 30% of β-histone. The presence of β-histone in this component and of the 1.6 S γ-component in the original 0.8 S γ-histone despite their preparation from material containing no detectable amounts of these histones was doubtless due to the large losses during dialysis of the α and 0.8 S γ-fractions. This, combined with the electrophoresis, resulted in the concentration of the negligible proportions of β and 1.6 S γ-histones in the original α-fraction into readily detectable amounts in the above preparations.

The four preparations described above were obtained in insufficient amount for further purification. They were, however, all analysed for amino acid composition. The results are presented graphically in Fig. 1[X] in which have also been included the figures obtained in unpublished work for pure β and 1.6 S γ-histones.

[X] For modified version see Figure 11 facing page 61.

In calculating the results of the analyses of the a_3-histone, we have corrected the values for the presence of 30% of β-histone.

Attention should be drawn to the fact that the ordinates in Fig. 1 represent amounts of α-amino nitrogen expressed as a percentage of the total nitrogen. This unusual method of recording the amino acid composition of proteins has been adopted in this case because it enables a direct comparison to be made between the number of cationic groups present in the various histones. Moreover, the figures are proportional to the number of mols of amino acid per mol of protein.

Consideration of the above results will show that they provide a complete confirmation of the previous work which has been carried out in this laboratory on the composite nature of the histones obtained from the thymocyte of the calf. In particular they confirm (1) the presence of the six components shown to be present by physicochemical methods[8]; (2) the strongly basic properties of all of them[8]; and (3) the presence of high quantities of lysine, alanine and proline[2] in many of the subsidiary histones. In addition to this, however, they serve to correlate the electrophoretic

mobilities of the histones with their amino acid composition. Thus, the number of cationic groups per 100 g. of protein N present in the six compounds, arranged in order of mobility, are: α_1 = 24.7; α_2 = 18.6; α_3 = 15.1; β = 14.3; 0.8 S γ = 14.2; 1.6 S γ = 13.9. It should be added that the two γ-components possess almost identical mobilities and that, while the β exhibits a greater mobility than the 1.6 S γ-histone despite their containing virtually identical numbers of cationic groups, this apparent slight discrepancy is explained by the greater number of free β-carboxyls present in the latter[8].

All the components resemble one another in being devoid of tryptophan, cysteine and cystine; the α's are, however, distinguished from the others by the absence of methionine. Of the six histones, α_1 is the simplest, containing only the seven amino acids lysine, alanine, proline, serine, threonine, glycine and valine. If one excludes simple peptides from the comparison, α_1-histone is probably unique amongst known proteins in containing no arginine. α_2 differs qualitatively from α_1 in containing the four additional amino acids arginine, leucine, glutamic and aspartic acids. The remaining components possess more complex compositions than do α_1 and α_2.

The conclusive demonstration in this and former communications from this laboratory of the composite nature of the histone present in the thymus gland of the calf clearly renders obsolete the many analyses of this protein recorded in the literature[11], for it is evident that the preparations to which these refer must have been unknown mixtures of two or more of the above components.

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