THE AMINO ACID COMPOSITION OF BASIC PROTEINS OF THE CELL NUCLEUS

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by

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INTRODUCTION

In 1951 Stedman & Stedman advanced the hypothesis that the basic proteins of cell nuclei are gene inhibitors, basing this view largely on examples of species specificity and cell specificity given in the same paper. The Stedmans also demonstrated at this time that it was generally possible to fractionate histones into two components, by alcohol precipitation of a subsidiary component from the mother liquors obtained during the purification of the main histones. Their evidence for species and cell specificity consisted of amino acid analyses of main histones prepared from cell nuclei from different organs from several species, and purified by the removal of the subsidiary component.

Other workers have published analytical data for histones from various sources, e.g. in 1933 Leipert & Leberl published results for a histone preparation obtained from fowl erythrocytes, and results have also been quoted for histones prepared from rat liver and calf thymus gland (Eadie & Leaf (1952)), for rat liver histone by Brunish, Fairley & Luck (1951), and for calf thymus histone by Hamer (1951) and by Daly, Mirsk & Ris (1951). In view of the Stedmans' discovery of a subsidiary histone fraction, none of these analyses are likely to have much significance in relation to cell or species specificity of histones./

histones.

Previous to this time, Stedman & Stedman (1944) had drawn attention to the existence of the phenomenon of cell specificity by proving that in salmon erythrocyte and liver cell nuclei the basic protein corresponding to the protamine of salmon sperm heads was a histone. With regard to species specificity, the homologous basic proteins salmine, clupeine, and sturine were regarded as being chemically distinct by Kossel (1928) and no reinvestigation of the chemical composition of these substances has produced evidence to the contrary.

Largely as a result of the Stedmans' investigations of the gross chemical composition of nuclei from different kinds of organs froma large number of species, the basic proteins are now generally regarded as universal components of the cell nuclei of animals, and probably also of plants. If this is the case, and there seems little reason for doubt, the phenomena of species specificity and cell specificity of basic proteins assume considerable biochemical importance.

The chromosome theory of inheritance is now supported by a considerable body of cytological evidence which has accumulated from studies of meiosis and mitosis, and since, according to this theory, the chromosomes are, apart from cases of polyploidy, identical in number and structure

and/

and therefore in composition in every cell of an organism, it is clear that such differences as exist between different kinds of living cells must be related to chemical differences in extra-chromosomal material.

One would, moreover, expect such material to be located in the nucleus rather than in the cytoplasm, for in the former position it would be in closer association with the genes and consequently more able to influence their activity. The phenomenon of the cell specificity of histones thus corresponds with deductions which can be made directly from the chromosome theory of inheritance.

Although genetics deals with rather superficial characteristics, such as eye colour and skin colour, it does not follow that only such characteristics come under chromosomal control. Because of the difficulty of crossing individuals from different species, genetical evidence is largely derived from studies of superficial character variations within particular species, and cannot, therefore, be expected to throw much light on the more fundamental aspects of cell function. However, in any biochemical interpretation of genetical control of even superficial cellular characteristics, one could not avoid associating the part played by the genes in controlling these with the control of the wider aspects/

aspects of cell function. Moreover, the number of characteristics now known to come under genetical control is so large that it seems evident that the only reason why genetical control of fundamental aspects of cell function has not been demonstrated is that genetical studies suffer from the inherent limitations already mentioned.

Since the publication of the Stedmans' hypothesis, Dr Stedman and Mr H. Cruft, working in this laboratory, have found that the main histones for which species and cell specificity had been demonstrated, actually consist of two components, distinguishable by different mobilities during electrophoresis. Generally, each main histone has been found to consist of a major component, and a minor component possessing a slower mobility, and these have been designated the 'main component' and the 'slow component' respectively. An account of this work has yet to be published.

It seemed possible that this discovery might obscure the significance of the Stedmans' demonstration of species and cell specificity since this had been based on analyses of unfractionated main histones. For example, it could be argued that the differences in amino acid composition observed were due to the presence of different proportions of the two component histones in the different kinds of cell nuclei/

nuclei studied. Thus it became necessary to fractionate several of the main histones into two electrophoretically homogeneous components and to carry out analyses of the pure components with a view to confirming the evidence of cell and species specificity presented in the Stedmans' (1951) publication. In view of the fact that the scope of the Stedmans' analyses was rather limited, the only amino acids estimated quantitatively being arginine and tyrosine, it was also considered necessary to use a method of analysis covering a larger number of amino acids. This thesis is an account of the development of such a method and its application to the analysis of histones.

The work falls naturally into several sections. MacPherson (1946) published details of a method of analysis of the three basic amino acids involving the separation of the basic fraction from whole protein hydrolysates as a preliminary step. The recoveries hes quoted were so good that it was decided to examine this method to see if it could be satisfactorily applied to the analysis of histones. The first section of this work is thus an account of experiments which were carried out using MacPherson's technique in a slightly modified form. It was concluded, as a result of these experiments, that MacPherson's electrodialysis technique was unsuitable for the author's purposes.

The/

The subsequent section deals with the development of a method of analysis which is similar to MacPherson's method in that the basic fraction is separated from the whole hydrolysate preliminary to the estimation of the basic amino acids. But it differs from this method in the important respect that an ion exchange resin, Amberlite IRC-50, is used for the separation of the basic fraction. MacPherson's actual analytical methods have been retained with minor modifications.

In Section III there is an account of the application of the method to the analysis of histones. Since the main purpose of the work is the confirmation of the phenomena of the species specificity and cell specificity of histones for electrophoretically homogeneous histone components, results for the analyses of the main component histones from different types of cells from two different species are presented. In addition, results of a number of analyses of unfractionated main histones, slow component histones, and the protamines salmine and clupeine, have been given.

Since the scope of the analytical method developed is still rather limited, some work has been carried out with a view to extending it to include the acidic amino acids. The final section has therefore been devoted to describing a few experiments/

experiments which were designed to separate the acidic amino acids from composite amino acid solutions using the anion exchanger, Amberlite IR-4B.

SECTION I

ELECTRODIALYSIS

INTRODUCTION

MacPherson (1946) claimed that he had satisfactorily demonstrated that an electrodialysis process he described was suitable for the quantitative isolation of the basic amino acids from hydrolysates of proteins, and that the final catholyte obtained by this procedure contained no other substance which would interfere with any of the analytical methods employed.

The contention of Albanese (1940) that all non-basic material could be eliminated by a single electrodialysis run at a selected pH had not been borne out by the work of Gordon, Martin & Synge (1941), and MacPherson published results indicating that at least three electrodialysis runs were necessary to remove all non-basic N from a composite amino acid solution containing the three basic amino acids and a mono-amino mono-carboxylic acid fraction.

In the procedure described by MacPherson, a protein hydrolysate or a test amino acid solution is subjected to four electrodialysis runs, the first serving merely to remove hydrochloric acid and the bulk of the non-basic N present, and the remainder, in each of which the pH is initially adjusted to 5.8, serving to remove the non-basic amino acids quantitatively. Results are quoted by/

by MacPherson for one test experiment with a composite amino acid solution containing tyrosine, cystine, glycine, arginine, histidine and lysine and the recoveries he claims after only three runs are 98.8%, 98.5% and 98.9% for arginine, histidine and lysine respectively. The corresponding recoveries after completion of a fourth electrodialysis run were only 97.2%, 96.4% and 97.3% respectively. A consideration of these figures makes it difficult to understand MacPherson's motive in including a fourth electrodialysis run in his experimental procedure, unless he suspected that his result for lysine, obtained by difference from the total catholyte N and the sum of the estimated arginine and histidine N values, was erroneously high owing to the presence of unremoved mono-amino mono-carboxylic acids. However, the recoveries of basic amino acids quoted above seemed sufficiently satisfactory to warrant applying MacPherson's analytical procedure to the analysis of histones. The analytical methods used by MacPherson are discussed in the appendix to this work.

Since the paper in question includes no satisfactory explanation of the inclusion of a fourth/

fourth electrodialysis run, it was resolved to try out MacPherson's procedure on a series of composite amino acid solutions of known composition, but to omit carrying out a fourth run for each analysis. Each run involved the risk of loss inherent in the quantitative transference of the contents of the cathode compartment of the electrodialysis apparatus into a flask, a concentration in vacuo, and the quantitative transference of the contents of the flask back into the centre compartment of the apparatus. Thus the total possible loss for a procedure involving even three electrodialysis runs seemed to the author considerable enough without the inclusion of an unnecessary run.

The Section which follows is an account of experimental work carried out on standard composite amino acid solutions with the object of obtaining recoveries of the basic amino acids of the same order of accuracy as that reported by MacPherson.

EXPERIMENTAL - ELECTRODIALYSIS OF AMINO ACID SOLUTIONS

Description of Apparatus

The apparatus was constructed of perspex, and consisted of three U-shaped perspex compartments, the central compartment having a capacity of 100 ml. and the two end compartments each having a capacity of about 60 ml. An exit tube was fitted to the bottom of each compartment so that its contents could be drained off completely when required. Each end compartment was cemented to a substantial perspex plate and the two plates were connected by four brass bolts passing two on either side of the three compartments. The bolts were threaded so that they could be tightened until the central compartment was held firmly in position by the pressure of the two end compartments.

Formolised gelatin was used for the anode membrane and vegetable parchment for the cathode membrane. It was found that when the membranes were inserted between the compartments, no matter how much the pressure on them was increased by tightening the bolts, the compartments could not be made water-tight, for liquid was able to leak vertically down the membranes. However, it was found possible to eliminate leaking by fitting the edges of each membrane into a slotted rubber gasket/

gasket shaped so as to fit between the two contiguous surfaces of the two appropriate compartments.

The electrodes used were of thin platinum foil and each had an area of 15 sq. cm.

General Procedure

The procedure followed was that described by MacPherson, except that only two electrodialysis runs were carried out at an initial pH of 5.8. During each run, the contents of the two outer compartments were kept cool by circulating cold water through filter tubes partially immersed in the liquid and the contents of the centre compartment were continuously stirred. A third run was not included, as the number of manipulative operations even in this simplified procedure is quite considerable, and it is not evident from MacPherson's results that the minute proportion of N removed during his final run with a standard amino acid solution was, in fact, non-basic N.

Experiment I - Electrodialysis of Composite Amino Acid Solution

It was intended to use the electrodialysis procedure to isolate the two acidic amino acids from protein hydrolysates as well as the basic amino acids. It was hoped that if this could be done quantitatively it would be a useful preliminary step in their estimation. It seemed feasible that within a certain range of pH on the acid side of neutrality and intermediate between the pI of glutamic acid and that of tyrosine, which is the most acidic of the mono-amino mono-carboxylic amino acids, the acidic amino acids would tend to migrate into the anode compartment while the mono-amino mono-carboxylic acid fraction would tend to migrate into the cathode compartment. For example, at pH 4, both aspartic and glutamic acids possess net negative charges, while at this pH all the mono-amino mono-carboxylic acids will possess net positive charges. Provided there was no loss of acidic amino acids during the first run, in which the pH is initially very low, the quantitative isolation of the acidic amino acid fraction uncontaminated by non-acidic material seemed to be a possibility, for after the removal of the basic fraction during the first run, the contents of the anode and centre compartments could be bulked and fractionation effected by electrodialysis/

Solution A (Volume 250 ml.)

Amino Acid	N% of Theoretical N	Dry Weight (mg.)	mg.N/50 ml.
Arginine	98.4	250.2	15.84
Histidine	99.6	72.2	3.90
Lysine .... (Dihydrochloride)	98.5	304.1	7.66
Glycine	99.0	263.9	9.75
Aspartic Acid	98.0	360.6	7.44
Glutamic Acid ..... (Monohydrochloride)	98.6	394.4	5.93
	Calculated Total N/50 ml.		50.52 mg.
	Calculated Basic N/50 ml.		27.40 mg.
	Calculated Acidic N/50 ml.		13.37 mg.

Estimations on Solution A (mg.N/50 ml. Solution A)

Arginine N = 15.71 mg.

Histidine N = 3.86 mg.

Total N = 50.08 mg.

Table 1.

electrodialysis at a suitable pH.

Procedure

Accordingly, an amino acid solution containing all the basic and acidic amino acids, and glycine to represent a mono-amino mono-carboxylic acid fraction was prepared to simulate a protein hydrolysate (Solution A). The weights of arginine and histidine were so chosen that the proportions of arginine N and histidine N would be approximately the same as those known to be present in hydrolysates of histones. The composition of this solution is given in Table 1.

Since the total N value quoted depends on micro-Kjeldahl estimations on all the individual amino acids employed in the preparation of the solution, this value was checked by carrying out micro-Kjeldahl estimations on aliquots of the final solution. The histidine and arginine contents of this solution were also checked by MacPherson's modification of the Pauly reaction and alkaline hydrolysis respectively, and the values found agreed within the limits of experimental error with the theoretical values.

Since, during electrodialysis of a protein hydrolysate, chlorine from the hydrochloric acid used for hydrolysis is liberated at the anode, 2 ml. N HCl were added to each aliquot of Solution A to/

Results;-

Aliquot 1. from Solution A

Estimated N (mg.)

Catholyte N	= 32.74
Centre N	= 3.23
Anolyte N	= 10.08
Total N accounted for	= 46.05

% N Recovery = 46.05/50.52 x 100%

= 92.1%

Table 2.

to be electrodialysed.

Aliquot 1. from Solution A:- On electrodialysis of one aliquot of Solution A, with a potential of 250 V. the current rose to 270 m.amp. at one stage during the first run, and finally fell to ca. 40 m.amp. after three and a half hours. The contents of each compartment were then collected separately and the washings added to each. The fractions were concentrated on the steam bath in vacuo, and the volume made to 50 ml. in each case. On estimating the N in each fraction, results were obtained as given in Table 2.

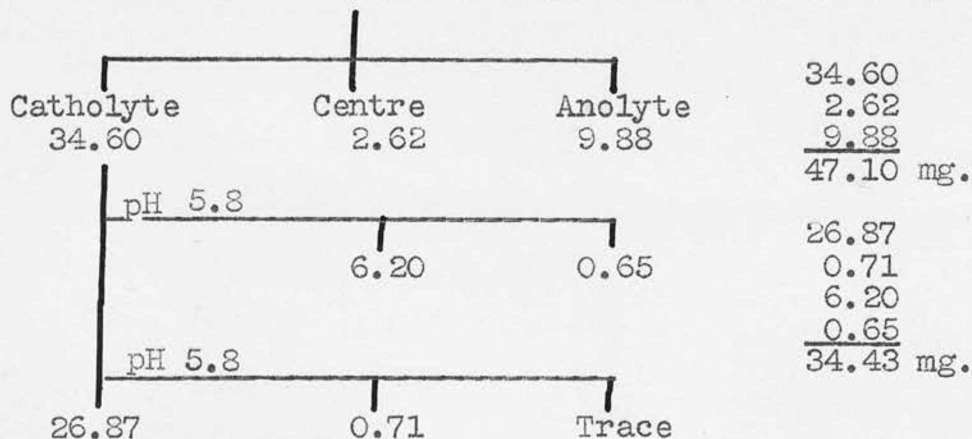
Aliquot 2. from Solution A:- A second 50 ml. aliquot of Solution A was acidified and electrodialysed in the same way, but during this run it was observed that the current rose to as high as ca. 500 m.amp. and finally fell to 40 m.amp. after two and a quarter hours. The contents of each compartment and washings were each adjusted to 50 ml. volume and two further electrodialysis runs carried out on a 40 ml. aliquot from the catholyte fraction, at an initial pH of 5.8. The pH adjustment was made by titrating with 0.04 N H_2SO_4, using bromocresol purple as an indicator. The contents of the anode and centre compartments after each of these runs, and the contents of the cathode compartment at the end of the third run, were each adjusted to 50 ml. volume/

Results:-

Aliquot 2. from Solution A

Estimated N (mg./50 ml. Solution A)

50.52 mg. Total N .... Basic N 27.40 mg.



34.60
2.62
9.88
47.10 mg.

26.87
0.71
6.20
0.65
34.43 mg.

Total N recovered after 1st. run = 47.10 mg.

% N Recovery = 47.10/50.52 x 100% = 93.1%

Total N recovered after 3rd. run = 34.43 mg.

% N Recovery = 34.43/34.60 x 100% = 99.5%

% Basic N Recovery = 26.87/27.40 x 100% = 98.0%

Estimated Arginihe N in final Catholyte = 14.85 mg., 14.72 mg.

% Arginine N Recovery = 93.75%, 92.9%

Table 3.

volume.

All the fractions were analysed for total N content and the diagram included in Table 3. indicates the N distribution found.

Discussion of Results

Apparently there is a considerable loss of N during the first electrodialysis run, for the sum of the N accounted for after the first run was considerably lower than the total N originally present for both aliquots. It seemed reasonable to assume that some decomposition had occurred owing to oxidation of the acidic amino acids in the anode compartment, where chlorine is liberated at the electrode surface during the first run. This assumption is wholly justified when it is considered that the diagram representing the N distribution found after three runs with aliquot 2. indicates a negligible loss of N during the second and third runs, during which there is no evolution of chlorine in the anode compartment.

It has been previously observed by Sperber (1946) that such oxidative decomposition does, in fact, occur and he has suggested a way of avoiding this difficulty. Sperber described a technique in which an ion exchange agent capable of absorbing the acidic amino acids (Amberlite IR-4B), was placed in the centre compartment together with the protein hydrolysate/

Results:-

Aliquot 3. from Solution A

Estimated N (mg./50 ml. Solution A)

Total basic N Recovery 26.63 mg.

% Basic N Recovery 26.63/27.40 x 100% 97.2%

Estimated Arginine N in final Catholyte 14.99 mg.

% Arginine N Recovery 14.99/15.84 x 100% 94.6

Table 4.

N Losses (mg./50 ml. Solution A)

Aliquot No.	Basic N	Arginine N
2.	0.53	0.99
3.	0.77	0.85

Table 5.

hydrolysate, thereby preventing migration of the acidic amino acids into the anode compartment. The acidic amino acids could be subsequently recovered by displacement from the resin with concentrated HCl. However, Sperber's recoveries of the three main fractions (basic, neutral and acidic) from synthetic hydrolysates were so low (94-95%) that it seemed to the author that there was little to be gained by adopting his modification of the electrodialysis procedure.

Estimations of arginine N in the final catholyte from aliquot 2. indicated a substantial loss of arginine during the electrodialysis process (6-7%), which was, at the time, rather surprising in view of the high apparent recovery of total basic N (98%).

This work was checked by electrodialysing a third 50 ml. aliquot of Solution A. in the same way and submitting the initial catholyte to two further runs at an initial pH of 5.8, when a similar poor recovery of arginine N was obtained.

Since the working loss of N involved in three electrodialysis runs must be appreciable, it is not easy to draw definite conclusions from the results from this experiment, but since the actual weight of basic N lost was in each case rather less than the corresponding loss of arginine N, it/

it can be said that the results are not inconsistent with the conclusion that part of the loss of basic N is due to the decomposition of arginine.

Accordingly, it was decided to electrodialyse a weighed sample of arginine, so that the arginine recovery could be studied independently of the other bases.

Results:-

	Estimated N (mg./50 ml.)	
	Arginine N	Kjeldahl N
Before Electrodialysis	21.83	21.77
After Electrodialysis	20.63	20.58
Recoveries	94.5%	94.5%

Table 6.

Experiment II - Electrodialysis of Standard Arginine Solution

Procedure

A convenient weight of arginine (ca. 140 mg.) was dried to constant weight over P_2O_5 and the volume made to 100 ml. with distilled water. Micro-Kjeldahl and arginine N estimations were carried out on aliquots of this standard solution and 50 ml. of the remainder electrodialysed in the usual way. The volume of the final catholyte was made to 50 ml. after concentrating in vacuo and micro-Kjeldahl and arginine N estimations again carried out.

Discussion of Results

The results indicate that electrodialysis of arginine alone does not appear to alter the ratio of arginine N to N as estimated by the Micro-Kjeldahl method, as one might expect to be the case were arginine being converted to ornithine with accompanying production of urea or ammonia. However, a possible explanation of these results might be that ornithine, being less basic than arginine, might be lost by diffusion from the cathode compartment to such an extent that the N loss occurring in this way would be sufficient to compensate for the ammonia loss responsible for the reduction of the arginine value. This would mean that during the whole electrodialysis process something/

something like one molecule of ornithine would be lost for every molecule of ammonia escaping, which does not seem very improbable.

Two other possibilities were that a substantial working loss had occurred during the course of this experiment or that the product of decomposition was non-basic. It seemed unlikely to the author that a non-basic compound could be produced as a result of electrolytic decomposition of arginine, and it scarcely seemed possible that the order of the working loss could be as high as 5% since considerable care had been taken with all the quantitative transferences. In addition, a total basic N recovery of 98% had been obtained in the previous experiment, where the experimental procedure was the same. Nevertheless, it was considered advisable to check the order of the working loss involved in the whole electrodialysis procedure by electrodialysing a standard solution of lysine.

Results:-

	Estimated N (mg./50 ml.)
Before Electrodialysis	17.56
After Electrodialysis	17.01
Recovery	96.9%

Table 7.

Experiment III - Electrodialysis of Standard Lysine Dihydrochloride Solution

Procedure

A convenient weight of lysine dihydrochloride, which was found to lose no weight on drying in vacuo over P_2O_5, was dissolved in water and the volume made to 100 ml. Micro-Kjeldahl estimations were carried out on suitable aliquots of this solution and 50 ml. of the remainder electrodialysed in the usual way. The volume of the final catholyte was made to 50 ml. after concentrating in vacuo and the N content again estimated.

Discussion of Results

A recovery of 96.9% of the original lysine N can probably be regarded as quantitative in view of the number of operations involved in the whole electrodialysis procedure, and this recovery is much the same as that reported by MacPherson after four electrodialysis runs with a synthetic hydrolysate containing lysine (97.3%). A certain loss of basic N by diffusion across the anode membrane is no doubt inevitable, and small losses are possible during the transference of the amino acid solution into the centre compartment of the electrodialysis apparatus, and on emptying the cathode compartment after each electrodialysis run. One concentration in vacuo is involved after each of/

of the three runs and bumping is difficult to avoid at these stages. This recovery of lysine N is of the same order as the recovery of the total bases obtained in the experiments on Solution A.

On the other hand, a recovery of only 94.5% of the original arginine N is significantly low, as were the recoveries of arginine N after electrodialysis of aliquots of Solution A. It was concluded that there had been some decomposition of arginine during the electrodialysis process, and it seemed probable that this decomposition was related to the high current densities obtaining during the first run.

DISCUSSION

Several conclusions can be drawn from the work reported in this Section and although most of these have already been adduced, some recapitulation is thought to be called for here.

The electrodialysis procedure described by MacPherson was not found to be suitable as a preliminary to the estimation of the basic amino acids because of appreciable destruction of arginine at the current densities involved; the loss occasioned in this way, in addition to a working loss of 2 or 3%, resulted in arginine recoveries lower than could be tolerated in the method of amino acid analysis sought by the author. In none of the experiments carried out was the recovery of arginine N considered sufficiently high to warrant using electrodialysis as a preliminary to the estimation of arginine in protein hydrolysates. Since the estimation of lysine in the method is by difference from the total basic N and the sum of the arginine and histidine N values, any error in the arginine N value would be reflected by an error in the dependent lysine value of a magnitude depending on the ratio of arginine N to lysine N in the protein analysed. Since this ratio is fairly high for histones it would be particularly unwise to use MacPherson's procedure in the analysis of these proteins.

It/

It was also found that a modification of the electrodialysis procedure of MacPherson, which the author had envisaged with a view to separating the acidic amino acids quantitatively, was impracticable owing to oxidative decomposition of the dicarboxylic amino acids during the course of the first run. An electrodialysis technique designed by Sperber (1946) in order to avoid such decomposition was ruled out because of the low recoveries of main fractions reported by that author.

Stein & Moore (1951) have reported that large losses of arginine occur during the electrolytic desalting process of Consden, Gordon & Martin (1947), and state that this loss is due to conversion of arginine to ornithine. This electrolytic process has the feature in common with electrodialysis that the current densities involved are of the same order of magnitude, so that loss of arginine in this way during electrodialysis is a probability.

For these reasons, it was decided to attempt to separate the basic amino acids from synthetic hydrolysates by a method in which electrolytic decomposition of arginine was not possible.

SECTION II

PRELIMINARY WORK WITH AMBERLITE IRC-50

INTRODUCTION

Block & Bolling (1951) report that if Amberlite IRC-50 is adjusted to pH 4.7 with molar acetate buffer, the basic amino acids may be quantitatively removed from a protein hydrolysate previously adjusted to pH 4.7 with an anion exchanger such as Amberlite IR-4B. The basic amino acids may then be quantitatively eluted from Amberlite IRC-50 with dilute mineral acid. Potentially, this appeared to be a much more eleagant method of separating the basic amino acids than electrodialysis.

Essential information, such as exhaustion flow rates, regeneration flow rate, and the capacities of the resin for arginine, histidine and lysine is not given by Block & Bolling. It was therefore decided to carry out several preliminary experiments to determine a set of conditions suitable for separating the basic amino acids quantitatively from a histone or protamine hydrolysate, or from a composite amino acid solution simulating a histone hydrolysate. This Section is essentially a record of these experiments.

After the complete experimental details of a method of separating and analysing the basic amino acids had been worked out further information regarding Amberlite IRC-50 became available from various sources. As a result, the experimental procedure/

procedure adopted in the earlier work has been slightly modified in the later experiments.

Owing to a misconception of the nature of the ion exchange mechanism of Amberlite IRC-50 it was not initially appreciated that the buffering capacity of the resin itself was adequate for stabilising the pH of the ambient phase. The initial experiments were, in fact, based on the assumption that the exchange of the amino acids would be with the hydrogen form of the resin, and that hydrogen ions would be liberated into the solution during the exchange process. With a view to stabilising the pH in the ambient phase, it was therefore decided to wash the amino acid solutions through the columns with N acetate buffer pH 4.7. This practice was subsequently found considerably to reduce the capacity of the resin for the basic amino acids. Nevertheless, the method proved quite satisfactory for the object in view, namely the quantitative separation of the basic amino acids and it was, in fact, used with satisfactory results in the analysis of several histones. During the course of the work, it was found convenient to reduce the concentration of acetate buffer used for washing in order to minimise the solid content of the buffer wash fractions. When, later, it was discovered that the use of 0.1 N acetate buffer for/

for washing was superfluous it was replaced by water.

EXPERIMENTAL

Experiment I - Retention of Arginine by Amberlite IRC-50

It was decided first to test the capacity of columns of Amberlite IRC-50 of various dimensions in order to find the minimum size of column which could be depended on to retain the maximum weight of basic amino acids likely to be contained in the protein hydrolysates to be employed for analysis. A rate of flow of 20 ml./hr. was arbitrarily selected for the experiment to be described.

Since the presence of arginine can easily be detected, even in trace quantities, by the Sakaguchi reaction, the first experiment was designed to test the ability of columns of various dimensions to retain arginine.

Procedure

Four columns of the following dimensions were employed:-

(i) 10 x 1 cm.

(ii) 15 x 1 cm.

(iii) 15 x 1.5 cm.

(iv) 20 x 1.5 cm.

The volume of resin in the smallest column (i) was measured and found to be approximately 2.5 ml., so the exhaustion flow rate for the resin in this column corresponding to a flow rate of 20 ml./hr. for/

for the column as a whole, is 0.134 ml./min./ml. of resin.

The Rohm & Haas Company, Philadelphia, PA., have quoted equilibrium flow rates of 0.134 ml./min. /ml. and 0.268 ml./min./ml. respectively, for the free acid and sodium forms of the resin. Thus the flow rates for all the columns used in this experiment are comfortably less than the equilibration flow rates for both forms of the resin. The volume of resin in the largest column (iv) was found to be 13 ml.

Each column was filled to 2.5 cm. from the top with the resin and washed with 200 ml. N acetate buffer pH 4.7, by siphoning the buffer slowly through the column. The resin was then left in contact with the buffer solution overnight.

Since it was intended to use ca. 100 mg. of histone for each analysis, it was necessary to prepare a solution of arginine of such a concentration that the weight of arginine in the volume of solution to be tested would be in excess of that found in a hydrolysate from 100 mg. histone. The arginine solution prepared contained ca. 250 mg. arginine in 100 ml. solution and 25 ml. aliquots of this solution were used for each column. Since 100 mg. histone will usually contain 10-15 mg. arginine, the solution prepared contained more than/

than four times the amount of arginine likely to be encountered in a hydrolysate from 100 mg. histone, for each 25 ml. of solution. Since, in the hydrolysis of 100 mg. quantities of histone, 7 ml. volumes of 7N HCl are used, 28 ml. of 7N HCl were added to the arginine solution before making to the 100 ml. volume.

The procedure followed for each column was as follows:- A 25 ml. aliquot of the arginine solution was concentrated in vacuo nearly to dryness two times, the solution transferred into a 50 ml. beaker, and the pH adjusted to 4-6 by adding successive small quantities of Amberlite IR-4B previously converted into its basic form by washing with NaOH and distilled water. Changes in pH were followed by removing small volumes of solution with a capillary tube and testing with 'Universal' indicator paper. The solution was then decanted into a 50 ml. separating funnel containing a glass wool plug and connected to the top of the column by means of a 'Quickfit' joint. The residual resin was washed with successive small volumes of N acetate buffer, pH 4.7, and washings transferred to the funnel.

The solution was then washed through the column with N acetate buffer at a rate of flow of ca. 20 ml./hr. until 200 ml. of effluent had been collected./

Results:-

Runs with 25 ml. Volumes of Arginine Solution containing ca. 2.5 mg. Arginine/ml.

Sakaguchi Reactions

Legend:
- Strongly positive +++
- Positive ++
- Trace +

	Column Dimensions (cm.)			
Fractions (50 ml.)	(i) 10 x 1	(ii) 15 x 1	(iii) 15 x 1.5	(iv) 20 x 1.5
Buffer Wash 1.	+++	+	−	−
" " 2.	++	+++	++	−
" " 3.	+	+++	+	−
" " 4.	−	+	−	+
N HCl Eluate 1.	+	+++	+++	+++
N HCl Eluate 2.	−	−	+	++

Table 8.

collected. The column was finally eluted with 100 ml. N HCl at the same rate of flow. The buffer wash from the column was collected in four successive 50 ml. fractions and the eluate in two 50 ml. fractions. Each fraction was concentrated in vacuo and its volume adjusted to precisely 25 ml., a procedure which has been adhered to in all subsequent work. On testing the various fractions for the presence of arginine by the Sakaguchi reaction and comparing the colours produced with a reagent blank, results were obtained as shown in Table 8.

Discussion of Results

The results obtained indicate that only the columns (iii) and (iv) contained a sufficiently large volume of resin to retain the bulk of the arginine. The results for columns (i) and (ii) clearly indicate that the bulk of the arginine has migrated down the columns at the buffer wash stage, and in all the columns there has evidently been continuous displacement of a substantial proportion of the arginine, the remainder being more or less firmly held until the elution stage. The effect of increasing column size was not to eliminate displacement of arginine but merely to delay it by increasing the length of the resin column relative to the volume of the buffer wash collected. The fact that the results for columns (iii) and (iv) indicate that there was migration of a smaller proportion/

proportion of the arginine at the buffer wash stage than occurred with columns (i) and (ii) suggests that the amount of arginine firmly held by the resin is directly related to the resin volume, so that the amount of arginine available for displacement is less in the case of the two larger columns. The results certainly demonstrate that only a proportion of the arginine is displaceable at the exhaustion flow rates used, which, as has already been pointed out, are well within the equilibration flow rates for both the sodium salt and free acid forms of the resin.

The fact that the first 50 ml. fractions of the eluates from columns (iii) and (iv) contained most of the arginine in the total eluates seemed to indicate that 100 ml. N HCl was adequate to elute all the arginine from the columns.

Since for column (iv), the fraction of the arginine moving down the column at the buffer wash stage was only represented by a trace Sakaguchi reaction in the fourth buffer wash fraction, a further experiment was designed as follows to confirm that migration of arginine was actually taking place in this column.

Experiment II - Retention of Arginine by Amberlite IRC-50

The procedure for this experiment was essentially a repetition of the procedure of the previous experiment for the run with column (iv), but in this case a fifth 50 ml. fraction of buffer wash was collected and 0.1 N HCl was used in place of N HCl. It was hoped that by using a greater dilution of HCl, the distribution of the arginine in the eluate fractions would be related to the arginine distribution at the buffer wash stage, and hence confirm the displacement of arginine at that stage.

Procedure

Column (iv) was washed with a further 50 ml. of N HCl and this wash was concentrated in vacuo and the volume adjusted to 25 ml. On testing the wash by the Sakaguchi reaction, a negative result was obtained, so the resin was apparently free from arginine. The pH of the column was then readjusted to pH 4.7 by washing with 200 ml. of N acetate buffer as before.

A second arginine solution of approximately the same concentration as before (2.5 mg./ml.) was prepared and the procedure followed in the previous experiment repeated, with the difference that in this case, five 50 ml. fractions of buffer wash/

Results:-

Run with 25 ml. Volume of Arginine Solution containing ca. 2.5 mg. arginine/ml.

Rate of Flow - ca. 20 ml./hr.

Sakaguchi Reactions

Fractions (50 ml.)		Column (iv) (13 ml. resin)
Buffer Wash	1.	—
" "	2.	—
" "	3.	—
" "	4.	—
" "	5.	+++
0.1 N HCl Eluate	1.	+++
"	2.	++
"	3.	+++
"	4.	+
"	5.	+

Table 9.

wash were collected. The column was then eluted with 250 ml. of 0.1 N HCl and the eluate collected in five successive 50 ml. fractions.

Discussion of Results

The fact that all the arginine appeared to be eluted from the column by 200 ml. of 0.1 N HCl corresponded with a statement by the Rohm & Haas Company that two milliequivalents of 0.1 N HCl/ml. of resin effect complete displacement of arginine, histidine, and lysine.

It was evident from the results presented in Table 9. that a proportion of the arginine had been tracking down the column during the course of the buffer wash stage. The beginning of the arginine front was indicated by the intense positive Sakaguchi reaction obtained with the fifth buffer wash fraction. The bulk of the remainder of the arginine displaced by the sodium ions in the mobile phase* appeared to be present in the first eluate fraction and the firmly held arginine was distinguished from the arginine which is displaceable by/

*It was not appreciated at the time this experiment was carried out that this displacement was due to the presence of sodium ions in the mobile phase.

by sodium ions by the fact that there was evidently considerably less arginine in the second eluate fraction than in the first and third fractions.

Despite the movement by displacement of a proportion of the arginine down this column, it seemed likely that it would be feasible to effect a separation of arginine from the non-basic amino acids with a column of this size, for it seemed possible that 150 ml. of buffer wash would be sufficient to wash all the non-basic amino acids from the column.

Accordingly, in the next experiment with a composite amino acid solution containing the three basic amino acids and a non-basic fraction, column (iv) was used and elution carried out after the collection of only 150 ml. of buffer wash.

Column (iv), or a column of identical dimensions, has been used in all subsequent work with Amberlite IRC-50.

Experiment III - Retention of Basic Amino Acids by Amberlite IRC-50

In the preparation of a composite amino acid solution to be used to test the retention of the basic amino acids by Amberlite IRC-50, it was thought advisable to use concentrations of basic amino acids greater than those likely to be encountered in hydrolysates of histones, assuming an approximate weight of 100 mg. histone per analysis.

It was found convenient to use the following approximate weights of amino acids for each 25 ml. of composite amino acid solution prepared, 25 ml. being the volume of composite solution used for each run on the column.

ca. 30 mg. arginine
ca. 25 mg. lysine dihydrochloride
ca. 5 mg. histidine
ca. 50 mg. glycine

The corresponding weights of basic amino acids present in 100 mg. histone are:-

ca. 11 mg. arginine
ca. 17 mg. lysine dihydrochloride (The equivalent)
ca. 2 mg. histidine

Procedure

A composite amino acid solution (volume 100 ml.) was prepared containing approximately the above weights of amino acid per 25 ml. solution. Before making/

making to volume, 28 ml. of 7N HCl were added to represent the HCl used in the hydrolysis of histones.

A 25 ml. aliquot of this solution was concentrated in vacuo three times and the pH adjusted to 4-6 with Amberlite IR-4B. The solution was then introduced into a dropping funnel leading into the column, and washings of the residual resin with 0.1 N acetate buffer, pH 4.7, added. A rate of flow of **ca.** 20 ml. /hr. was used as in previous experiments, and three 50 ml. fractions of 0.1 N acetate buffer wash collected, i.e. sufficient 0.1 N acetate buffer was used to wash the residual Amberlite IR-4B to make the total buffer wash volume 150 ml. It was thought inadvisable to collect a fourth 50 ml. fraction of buffer wash in view of the fact that a trace of arginine had been detected in the fourth buffer wash fraction in Experiment I. The Sakaguchi reaction is so very sensitive that the amount of arginine present in this particular fraction must have been very small, possibly quantitatively insignificant, but at the time this experiment was carried out, it was considered necessary to use a smaller volume of buffer for washing since the amino acid solution used contained the less strongly basic histidine. In actual fact, as Experiment VI will later show, it would have been quite safe to collect up to 200 ml. of 0.1 N buffer wash as the reduction/

reduction of the normality of the acetate buffer used for washing in this experiment greatly reduces the displacement of the basic amino acids by sodium ions.

Although the previous experiment indicates that 0.1 N HCl is too dilute for use as a convenient eluting agent (200 ml. of 0.1 N HCl were required to elute arginine quantitatively), 0.1 N HCl was used to elute in this experiment because it was again required to study the behaviour of arginine and histidine at the buffer wash stage. The use of N HCl for elution would have caused most of the arginine and histidine to be eluted in the first 50 ml. of eluate.

Five 50 ml. fractions of 0.1 N HCl were collected and it was found that both the arginine and histidine were distributed among the first four fractions of the eluate. It was assumed that the distributions found were a reflection of the corresponding distributions of arginine and histidine at the buffer wash stage.

As in previous experiments, each 50 ml. fraction of buffer wash and eluate was concentrated in vacuo and the volume adjusted to 25 ml. before testing for arginine, but since, in this experiment histidine was tested for by MacPherson's modification of the Pauly reaction, and this method is sensitive to variations/

Results:-

Run with 25 ml. Volume of Composite

Amino Acid Solution

Rate of Flow - ca. 20 ml./hr.

Colorimetric Reactions with Amino Acids

Fractions (50 ml.)	Arginine Sakaguchi	Histidine Pauly	Glycine Ninhydrin
0.1 N Buffer Wash 1.	−	−	+++
" " " 2.	−	−	+++
" " " 3.	−	−	+
0.1 N HCl Eluate 1.	++	+	+
" " " 2.	+	++	−
" " " 3.	+	++	−
" " " 4.	+++	+++	−
" " " 5.	−	−	−

Table 10.

variations in pH, each fraction was adjusted to pH 5-7 by titrating with 0.5 N NaOH before making to volume. On testing the buffer wash fractions for arginine, histidine and glycine, results were obtained as given in Table 10.

Details of the amino acid tests carried out are included in the Appendix.

Discussion of Results

The results indicate that 150 ml. of buffer wash seems to be sufficient to wash all the glycine off the column, since most of the glycine appears in the first two 50 ml. fractions of the wash. Also, since neither arginine nor histidine was detected in the first 150 ml. of buffer wash, it appears that it is possible to separate these amino acids quantitatively from glycine by collecting only 150 ml. of buffer wash. However, since there was a trace ninhydrin reaction given by the first fraction of the eluate only, and no corresponding reaction with any of the other eluate fractions, some of which undoubtedly contained higher concentrations of the basic amino acids, it seemed likely that this trace ninhydrin reaction was due to a little unremoved glycine. It was hoped that the amount of glycine involved would be quantitatively insignificant.

Experiment V, which includes micro-Kjeldahl estimations of N on the eluate fraction was designed largely/

largely to resolve this difficulty.

Results:-

Run with 25 ml. Volume of

Composite Amino Acid Solution

Rate of Flow - ca. 20 ml./hr.

Colorimetric Reactions with Amino Acids

Fractions (50 ml.)		Arginine Sakaguchi	Histidine Pauly	Glycine Ninhydrin
0.1 N Buffer Wash	1.	−	−	+ +
" " "	2.	−	−	+ + +
" " "	3.	−	−	+
N.HCl Eluate	1.	+ + +	+ +	+
" "	2.	+ + +	+ + +	−
" "	3.	−	−	−
" "	4.	−	−	−

Table 11.

Experiment IV - Retention of Basic Amino Acids

It was decided to confirm the findings of the previous experiment that no arginine or histidine were lost from the column in the first 150 ml. of buffer wash, by repeating the procedure up to the elution stage with a second 25 ml. volume of composite amino acid solution, and then eluting with N HCl. Four N HCl eluate fractions were collected in successive 50 ml. portions.

Discussion of Results

The results confirm that 100 ml. N HCl is sufficient for quantitative elution of the basic amino acids, and, as in the previous experiment, a trace ninhydrin reaction was given by the first eluate fraction.

The behaviour of lysine on the column was not followed because there is no convenient way of testing for lysine colorimetrically, but it was presumed that, as the isoelectric point of lysine (9.7) is close to that of arginine (10.8) and greater than that of histidine (7.6), its behaviour on the column will be similar to the behaviour of arginine. It is not an unreasonable assumption that any experimental procedure suitable for the quantitative separation of both arginine and histidine from the non-basic amino acids will also be suitable for the quantitative separation of lysine.

Experiment V - Separation and Recovery of Basic Amino Acids from Composite Amino Acid Solution

This experiment was designed to confirm that the conditions used in Experiment IV could be used for the separation of the basic amino acids from glycine. Presumably, if the basic amino acids can be separated from glycine under these conditions, they will also be separated from the acidic amino acids, as well as from the other mono-amino mono-carboxylic acids contained in any protein hydrolysate.

The methods of estimation of the basic amino acids employed are those described by MacPherson, except that Stedman's (1951) modification of Plimmer's method is used in the estimation of arginine.

Procedure

The column was adjusted to pH 4.7 in the usual way, and washed with 150 ml. of 0.1 N acetate buffer, pH 4.7, to simulate the conditions obtaining during a buffer wash. The column was then washed with 100 ml. of N HCl and the acid wash concentrated to 25 ml. Micro-Kjeldahl estimations on 2 ml. aliquots of this solution indicated that no appreciable amount of N was washed off the column by 100 ml. of N HCl, the N in each aliquot corresponding to only about 0.03 ml. of 0.01 N HCl. The/

Solution 1. (Volume - 100 ml.)

Amino Acid	N% of Theor. N	Dry Weight (mg.)	Mg.N/25 ml.
Arginine	98.4	118.0	9.34
Histidine	99.6	32.4	2.19
Lysine Dihydrochlor.	98.5	92.2	2.90
Glycine	99.0	ca. 200	———
	Total Basic N/25 ml. aliquot		14.43 mg.

Estimations on Solution 1. (mg. N/25 ml. Solution 1.)

Arginine N = 9.3

Histidine N = 2.18

Table 12.

The pH of the column was then readjusted to 4.7 with N acetate buffer.

A standard composite amino acid solution, volume 100 ml., was prepared from the dry weights of amino acids given in Table 12. 28 ml. of 7 N HCl were added as before, in order to represent the HCl used in the hydrolysis of an amount of histone roughly corresponding to the amino acid content of the solution.

The composition of this amino acid solution was checked by estimation of histidine, arginine and total N, and a 25 ml. aliquot of the solution was adjusted to pH 4-6 with Amberlite IR-4B and run through the column using essentially the same conditions as in Experiment IV. Three 50 ml. fractions of 0.1 N buffer wash were collected and the column was finally eluted with 100 ml. of N HCl. Before readjusting the pH of the column to 4.7 in readiness for further use, the column was washed with a further 50 ml. of N HCl. The eluate and this final acid wash were then concentrated in vacuo and the pH of each adjusted to 5-7 before making to 25 ml. volume.

As in previous experiments, the buffer wash fractions were each tested for the presence of arginine, histidine and glycine, and the same tests were applied to the final acid wash.

Arginine,/

Results:-

Runs with 25 ml. Aliquets of Composite

Amino Acid Solution 1.

Rate of Flow - ca. 20 ml./hr.

Colorimetric Reactions with Amino Acids

Fractions (50 ml.)	Arginine Sakaguchi	Histidine Pauly	Glycine Ninhydrin
0.1 N Buffer Wash 1.	−	−	++
" " " 2.	−	−	+++
" " " 3.	−	−	+
Column eluted with 100 ml. N HCl	Eluate untested		
N HCl Wash	−	−	−

Estimation of Basic Amino Acids

on Eluate Fractions (mg. N/25 ml.)

Estimation	Aliquot 1.	Aliquot 2.
Total Basic N	14.9	15.4
Arginine N	-	9.6
Histidine N	-	2.17
Lysine N	-	3.6

Table 13.

Arginine, histidine and total N were estimated in the eluate and the values obtained for the three basic amino acids on the basis of these estimations compared with the quantities present in the original solution. The entire process was repeated with a second 25 ml. aliquot of Solution 1.

Discussion of Results

When the total N of the eluate from the first aliquot from Solution 1. was estimated it was found to be considerably higher than the original basic N content. It was assumed that the extraneous N was due to the presence of unremoved glycine and the rest of the eluate was discarded; thus individual results for the basic amino acids are not quoted for the first aliquot from Solution 1. When a high recovery of total basic N was obtained for the second aliquot, it was evident that something was seriously wrong with the experimental procedure. However, estimations of arginine and histidine were proceeded with in the hope that the results would throw some light on the source of the extraneous N. High recoveries of arginine N were obtained, although histidine appeared to be recovered quantitatively.

Since in Experiments III and IV a trace ninhydrin reaction had been obtained for the first 50 ml./

50 ml. fraction of the eluate it seemed a possibility that the high values obtained for basic N recovered in the eluate fractions were partly due to the presence of unremoved glycine. It was clear, however, that the high value for arginine N recovered could not be accounted for in this way, since glycine is not decomposed under the conditions used in the estimation of arginine by alkaline hydrolysis.

Results:-

Run with 25 ml. Aliquot of composite

Amino Acid Solution 1.

Rate of Flow - ca. 20 ml./hr.

Colorimetric Reactions with Amino Acids

Fractions 0.1 N Buffer Wash		Arginine Sakaguchi	Histidine Pauly	Glycine Ninhydrin
Volume (ml.)	No.			
100	1.	−	−	+++
50	2.	−	−	+++
20	3.	−	−	−
20	4.	−	−	−
20	5.	−	−	−
Column eluted with 100 ml. N HCl		Eluate untested		
N HCl Wash		−	−	−

Table 14.

Experiment VI - Separation and Recovery of Basic Amino Acids

Although the high values of basic N recovered obtained in the previous experiment could not have been entirely due to the presence of unremoved glycine, it was resolved to attempt to eliminate all risk of incomplete removal of glycine in the buffer wash by collecting a greater volume of 0.1 N buffer wash, so that the glycine would be more effectively washed off the column.

Procedure

The column was prepared in the usual way and a third 25 ml. aliquot of Solution 1. used for a further run at pH 4.7. In this experiment, 210 ml. of buffer wash were collected in all, in five fractions as indicated in Table 14., the last 60 ml. of this volume being collected in three successive 20 ml. fractions. The column was then eluted with N HCl, washed with a further 50 ml. N HCl, and its pH adjusted to 4.7 in readiness for further use.

The fractions collected were made to 25 ml. volume and separately tested for the presence of arginine, histidine and glycine in the usual way. In addition, arginine N, histidine and total basic N estimations were carried out on suitable aliquots of the eluate. The results obtained are included in the summary of results obtained for the three separate/

Summary of N Recoveries obtained for Solution 1.

(mg. N/25 ml. Solution 1.)

Estimation	Solution 1.		Recovered Aliquot No.		
	Calculated	Estimated	1.	2.	3.
Total Basic N	14.43	-	14.9	15.4	15.4
Arginine N	9.34	9.3	-	9.6	9.9
Histidine N	2.19	2.18	-	2.17	2.11
Lysine N	2.90	-	-	3.6	3.4

Table 15.

separate runs so far carried out on three 25 ml. aliquots of Solution 1., which is given in Table 15.

Discussion of Results

The results obtained in this experiment confirm the conclusion that the presence of unremoved glycine in the eluate fractions could not be entirely responsible for the high values obtained for basic N recovered, for the results in Table 14. indicate that all the glycine was removed in the first 150 ml. of the buffer wash. The recoveries of basic N and arginine N are so high as to be well outside the limits of experimental error, and since similar high recoveries of basic N were obtained in the two previous runs on different aliquots of Solution 1. it is probable that for the two previous aliquots, the bulk of the extraneous N was not due to unremoved glycine. Nevertheless, it was decided to, continue to collect 200 ml. buffer wash in future work in order to ensure complete removal of the non-basic fraction.

The only conceivable source of extraneous N now seemed to be the resin Amberlite IR-4B which was used to adjust the pH of each aliquot to 4-6 before introduction into the column. In order to determine whether this resin was the real source, a quantity of Amberlite IR-4B, approximately equal to the quantity used in adjusting the pH of 25 ml. Solution 1./

Solution 1., was washed with 25 ml. N HCl, the resin filtered off, and the volume taken to 25 ml. Micro-Kjeldahl estimations on 2 ml. aliquots of this solution indicated the presence of a substantial amount of N derived from the resin, e.g. when 10 g. of Amberlite IR-4B were stirred with 25 ml. N HCl for five minutes, 1.7 mg. N was recovered from the filtrate.

Thus concentrations of N derived from Amberlite IR-4B were likely to be large in relation to the amino acid N concentrations present. Accordingly, it was decided to discontinue using Amberlite IR-4B for pH adjustment, and to use in its place 0.5 N NaOH. This modification suffers from the disadvantage that additional sodiumnions are introduced into the system during the buffer wash stage. However, if the aliquot of standard amino acid solution or protein hydrolysate is twice concentrated in vacuo almost to dryness, the volume of 0.5 N NaOH required to adjust the pH to 4-6 amounts to only a few ml. Certainly, the sodium ion concentration in the resulting solution will not approach 0.5 M,* so, since Experiment II, which involved washing with N acetate buffer in which the sodium ion concentration is approximately 0.5 M, indicated no appreciable loss of arginine in the buffer wash, the increased sodium ion concentration occasioned by this modification/

in experimental procedure would not be likely to affect the separation adversely. Nevertheless, this has not been taken on trust, and in the following experiment involving pH adjustment with 0.5 N NaOH, all the N in 25 ml. aliquots of a standard composite amino acid solution is finally accounted for as the N of its particular amino acid.

*In all experiments in this work involving pH adjustment of protein hydrolysates with 0.5 N NaOH the volume of alkali used has been noted. In no case did the volume exceed 20 ml. Subsequent procedure invariably consisted in adjusting the volume to 30 ml., 40 ml., or 50 ml. and taking a 25 ml. aliquot of the resulting solution for the column runs. Thus the maximum concentration of sodium ions which could be present at the buffer wash stage in any run is 10 x 25/30 milliequivalents in 25 ml., i.e. M/3.

Solution 2. (Volume 100 ml.)

Amino Acid	% of Theor. N	Dry Weight (mg.)	Mg. N/25 ml.
Arginine	98.4	89.3	7.03
Histidine	99.6	34.8	2.35
Lysine .... (Dihydrochlor.)	98.5	101.0	3.18
Glycine	99.0	193.4	8.93
	Calculated Total N/25 ml.		21.49
	Calculated Basic N/25 ml.		12.56

Estimations on Solution 2. (mg. N/25 ml.)

Arginine N = 6.95

Histidine N = 2.35

Total N = 21.4

Table 16.

Experiment VII - Recovery of Basic Amino Acids from Composite Amino Acid Solutions

In this experiment it was decided to attempt to account for all the N present in the original 25 ml. aliquots of amino acid solution by analysing all the buffer wash fractions for N, in addition to carrying out all the tests and estimations included in previous experiments on the eluate fractions.

Procedure

Another standard composite amino acid solution was prepared from known dry weights of amino acids. Since it was intended to account for glycine N quantitatively, the glycine component was, in this case, weighed exactly before making the solution to volume.

As in the preparation of Solution 1., 28 ml. of 7 N HCl was added to represent the HCl of hydrolysis. The pH of a 25 ml. aliquot of Solution 2. was adjusted to 4-6 by concentrating in vacuo twice and titrating with 0.5 N NaOH. The volume was made to ca. 30 ml. and the solution introduced into the column. Four 50 ml. fractions of buffer wash were collected and 100 ml. N HCl was used for elution followed by a further 50 ml. N HCl. Arginine, histidine and glycine were tested for in the four buffer wash fractions and micro-Kjeldahl estimations were also carried out on suitable aliquots of each fraction (2 ml.) after it had been made to 25 ml. volume./

Results:-

Run with 2nd 25 ml. Aliquot of composite

Amino Acid Solution 2.

Rate of Flow - ca. 20 ml./hr.

Colorimetric Reactions with Amino Acids

Fractions (50 ml.)	Arginine	Histidine	Glycine	Mg. N
0.1 N Buffer Wash 1.	—	—	+ +	0.55
" " " 2.	—	—	+++	5.84
" " " 3.	—	—	++	1.76
" " " 4.	—	—	+	0.23
Column eluted with 100 ml. N HCl	Eluate untested			12.43
N HCl Wash	—	—	—	—

Total Glycine N accounted for 8.38 mg.

Table 17.

volume.

The basic amino acids were estimated in the eluate in the usual way, and the whole procedure repeated with a second 25 ml. aliquot of Solution 2., but ommitting the tests for arginine, histidine and glycine. Since, when these tests were applied to the buffer wash fractions corresponding to the first aliquot of Solution 2., the expected results were obtained, it was considered unnecessary to repeat the tests in the work on the second aliquot.

Discussion of Results

Although both the values obtained for total glycine N (Tables 17. & 18.) recovered are considerably low, these low recoveries were not considered to be of much significance. The possible experimental error involved in arriving at these values was undoubtedly high, for each value depended on four individual estimations of N (on the four buffer wash fractions), and the quantities of N present in some of these fractions were so low as to make their accurate estimation impossible. It is evident from the results however, that the glycine was distributed throughout the buffer wash in much the same way in the two runs carried out. In each case, nearly all the glycine N was found to be present in the second buffer wash fraction and only a trace was present in the fourth and final fraction.

The/

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Results:-

Run with 2nd 25 ml. Aliquot of composite Amino Acid Solution 2.

Fractions (50 ml.)	Mg. N
0.1 N Buffer Wash 1.	1.19
" " " 2.	7.24
" " " 3.	0.07
" " " 4.	0.04

Total Glycine N accounted for = 8.54 mg.

Total Basic N accounted for = 12.43 mg.

Table 18.

The recoveries of arginine N and total basic N are satisfactory, the losses being within 1%. Recoveries of histidine N are less satisfactory, but such is the slope of the calibration curves used in determining histidine, that the method is probably not capable of accuracy within 2%. Lysine N values are obtained by difference from the total basic N, but since the amount of lysine N present is only about a quarter of the total basic N, the lysine values calculated in this way will carry about four times the percentage error of the total basic N estimation as well as the errors of the arginine N and histidine estimations on which the lysine N values depend.

Van Slyke Nitrogen Distribution Method:- As a check on the accuracy of histidine estimations on basic fractions it was resolved to make use of the Van Slyke Nitrogen Distribution Method. This method has been out of vogue for many years largely because it has been previously applied to analyses of basic amino acid fractions separated from protein hydrolysates by phosphotungstic acid precipitation, and precipitation of the basic fraction is incomplete when this reagent is used. In addition, cystine is precipitated along with the bases and its presence in the basic fraction is a serious drawback. The fact that the significance of amino N values obtained/

Results:-

Summary of N Recoveries obtained

for Solution 2. (mg. N/25 ml. Solution 2.)

Estimation	Solution 2.		Recovered Aliquot No.	
	Calculated	Estimated	1.	2.
Total Basic N	12.56	-	12.4	12.4
Arginine N	7.03	6.95	6.89	6.99
Histidine N	2.35	2.35	2.27	2.30
Lysine N	3.18	-	3.3	3.1
Glycine N	8.93	-	8.4	8.5

Table 19.

obtained for phosphotungstic acid precipitates is further reduced because of decomposition of cystine during acid hydrolysis makes the Van Slyke Nitrogen Distribution Method of little value when applied to basic fraction obtained in this way.

The quantitative separation of the basic amino acids uncontaminated by cystine makes their accurate estimation by the Van Slyke Method a possibility, and since the results obtained in Experiment VII indicate that this may be readily achieved by using a column of Amberlite IRC-50, it was decided to use the Van Slyke Method as a check on the values for lysine and histidine obtained by the methods already described.

The details of experimental work carried out with the Van Slyke apparatus and a discussion of the N Distribution Method as applied in analyses described in this work are given in the Appendix.

The next Experiment, which is the last in this Section, is essentially a repetition of Experiment VII with the difference that estimations of amino N are carried out on the original composite amino acid solution and the Van Slyke N Distribution Method is applied to the eluate fraction.

Solution 3. (Volume 100 ml.)

Amino Acid	N% of Theor.N	Dry Wt. (mg.)	Mg.N/25 ml.	Mg. amino N/25 ml.
Arginine	98.4	103.0	8.11	2.03
Histidine	99.6	38.0	2.56	0.85
Lysine .... (dihydrochlor.)	98.5	90.7	2.85	2.85
Glycine	99.0	177.8	8.21	8.21

Calculated Total N/25 ml. = 21.73 mg.

Calculated Total Amino N/25 ml. .... 13.94 mg.

Calculated Basic N/25 ml. = 13.52 mg.

Calculated Basic Amino N/25 ml. .... 5.73 mg.

Estimations on Solution 3.

Arginine N = 8.18

Amino N = 14.11 (Method of Kendrick & Hanke (1937))

Total N = 21.55

Table 20.

Experiment VIII - Recovery of Basic Amino Acids from Composite Amino Acid Solutions

Procedure

A new standard composite amino acid solution was prepared and a run carried out on a 25 ml. aliquot of this solution in the usual way.

In this experiment, the testing of successive buffer wash fractions for arginine, histidine and glycine was ommitted and 200 ml. of buffer wash was collected in a single fraction. It was considered that the evidence of previous experiments justified the assumption that separation of the basic fraction was quantitative under the conditions of the runs.

Histidine estimations by MacPherson's Method were not carried out, the histidine N values being determined by the Van Slyke N Distribution Method.

Discussion of Results

The fact that the N contained in the buffer wash agrees within the limits of experimental error with the calculated value for glycine N confirms previous observations that the collection of 200 ml. buffer wash will ensure the quantitative removal of glycine from the basic amino acids. The recoveries of arginine and histidine can be considered as quantitative, the N losses in both cases being within the limits of the experimental error possible at the titration stage in the estimations. The lysine N recovery is

over/

Summary of N Recoveries obtained for

Solution 3. (mg. N/25 ml. Solution 3.)

Estimation	Solution 3.		Recovered
	Calculated	Estimated	
Total Basic N	13.53	-	13.7
Basic Amino N	5.74	-	5.9
Arginine N	8.11	8.18	8.14
Lysine N	2.85	-	3.0
Histidine N	2.56	-	2.58
Glycine N	8.22	-	8.3

Table 21.

over four per cent high but as the lysine N value carries all the error of the Micro-Kjeldahl estimations on the eluate fraction, this result was not considered to be out of the way.

The recoveries obtained appeared to be sufficiently good to warrant applying this method of separating the basic amino acids to the analysis of hydrolysates of histones.

Experiment IX - Testing the Capacity of Amberlite IRC-50 for Arginine

The capacity of arginine of Amberlite IRC-50 buffered to pH 7 is reported by the Rohm & Haas Company to be 150 mg./ml. resin. The capacity of the resin under the conditions described in this Section was found to be very much less than this; e.g. in Experiment I, where the runs were carried out by washing an arginine solution through the columns with molar acetate buffer, the capacity of the resin for arginine is only 5-6 mg./ml. resin. This low capacity is of a different order of magnitude from what might be expected at pH 4.7 on the basis of the capacity at pH 7 as reported by the Rohm & Haas Company.

It was decided to determine the order of magnitude of the arginine capacity of the resin using conditions prescribed by the Rohm & Haas Company, that is to say the column was washed free of buffer after the adjustment of the pH to 4.7 and a standard arginine solution was washed through the column with water in place of acetate buffer.

Procedure

A column, 20 x 1.5 cm., containing approximately 13 ml. of Amberlite IRC-50 was buffered to pH 4.7 by washing with 200 ml. of N acetate buffer, and the excess buffer was removed by washing with 200 ml. water./

water. 500 ml. of an arginine solution containing ca. 1.5 mg. arginine/ml. was then passed through the column at a rate of flow of 50 ml./hr.* The effluent was collected in ten successive 50 ml. fractions and each was tested for arginine by the Sakaguchi method. A negative reaction was obtained in every case.

The column was finally eluted with 100 ml. N HCl and the eluate collected in two 50 ml. fractions. On testing these for arginine a strongly positive reaction was obtained for each fraction.

Discussion of Results

The resin capacity is evidently greater than 750/13 ≡ ca. 60 mg. arginine/ml. under these conditions, i.e. the capacity of the resin for arginine is increased about ten timesnby eliminating sodium ions from the ambient phase.

Clearly, the presence of sodium ions is undesirable in the ambient phase , although quantitative separation of the basic amino acids from solutions containing glycine as well as the bases has been found to be possible in the presence of concentrations of up to 0.5 M with respect to sodium ions.

*The equilibtration flow rates of the free acid and/

and sodium forms of Amberlite IRC-50 are reported as being 0.134 ml./min./ml. and 0.268 ml./min./ml. respectively (Rohm & Haas Company). Since a rate of flow of 50 ml./hr. for a column containing 13 ml. of resin corresponds to 0.064 ml./min./ml., the flow rate used is well within the equilibration flow rates for both forms of the resin.

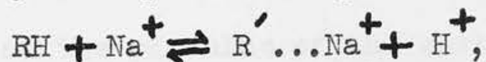
DISCUSSION

The whole purpose of the experiments which have been described in this Section was to develop a simple method of separating the basic amino acids from histone hydrolysates quantitatively, and uncontaminated by any non-basic material.

The development of the method necessitated carrying out a number of experiments in which an initial experimental procedure was gradually modified on the basis of the observations made during the course of the work, until a method was finally evolved apparently suitable for application to the analysis of histones. This method is described in its final form in the Appendix with the difference that water is used for washing the non-basic amino acids off the column instead of 0.1 N acetate buffer. pH 4.7.

The following argument provides an explanation of why this modification was made:-

According to Partridge & Westall (1949), the reaction between sodium ions and a cation exchange resin may be represented by

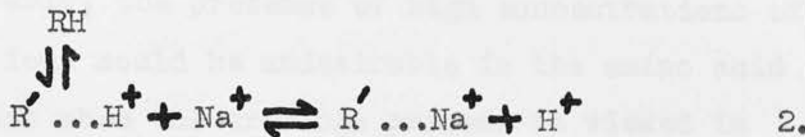


$$RH + Na^{+} \rightleftharpoons R'...Na^{+} + H^{+},$$

where RH is the hydrogen form of the resin and the symbol $R'...Na^{+}$ represents the dissociated resin salt in which movement of the cation Na^{+} is restricted by the effect of the negative charges on/

on the resin.

Since the affinity of Amberlite IRC-50 for hydrogen ions is very mch greater than its affinity for sodium ions, the extent to which this reaction will proceed to the right will depend on the concentration of dissociated carboxyl groups on the resin surface, so the above equation may be rewritten



$$\begin{array}{c} RH \\ \Downarrow\!\Uparrow \\ R'...H^{+} + Na^{+} \rightleftharpoons R'...Na^{+} + H^{+} \end{array} \qquad 2.$$

where $R'...H^{+}$ represents the dissociated acid form of the resin. The concentration of the dissociated acid form will depend, in turn, on the pH of the solution, so that the quantity of sodium ions taken up by the resin will be limited by the pH. It follows that if the resin is in contact with an acetate buffer at any given pH, there will be a characteristic ratio of the sodium salt form of the resin to the undissociated acid form and , that if we make the simple assumption that adsorption is governed solely by electrostatic forces, this ratio will be determinable by the application of the Henderson-Hasselbalch equation to the system.

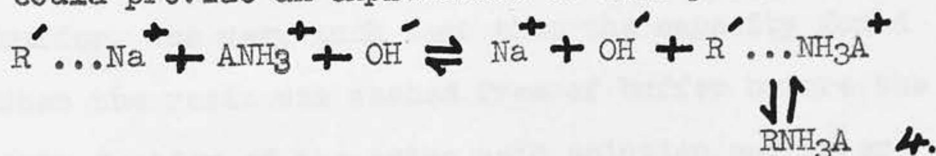
Since the pK value of the carboxylic acid group of Amberlite IRC-50 is between 5 and 6, Kunin & Meyers (1950), at pH 4.7 the number of dissociated acid groups present, $R'...H^{+}$, will be very small relative/

relative to the number of groups associated with sodium ion, $R' \ldots Na^{+}$, so that the retention of amino acids on a column of Amberlite IRC-50 at this pH may be considered to be essentially due to exchange with the dissociated sodium salt form of the resin and may be represented:-

$$R' \ldots Na^{+} + ANH_3^{+} + OH' \rightleftharpoons RNH_3A + Na^{+} + OH' \qquad 3.$$

Clearly, the presence of high concentrations of sodium ions would be undesirable in the amino acid solutions when the exchange process is viewed in this light, since the progress of the exchange reaction would be hindered by a direct mass action effect.

The results for Experiment I of this Section indicate that during runs with arginine solutions, in the presence of high concentrations of sodium ions a proportion of the arginine is continually displaced down the column at the buffer wash stage. The expansion to the following form of Equation 3. could provide an explanation of this phenomenon.



$$R' \ldots Na^{+} + ANH_3^{+} + OH' \rightleftharpoons Na^{+} + OH' + R' \ldots NH_3A^{+} \;\; \updownarrow \;\; RNH_3A \qquad 4.$$

The amino acid exchange reaction will take place, if the above reasoning is valid, with the dissociated salt form of the resin, $R' \ldots Na^{+}$, so that the resin-amino acid complex will be formed initially/

initially as the dissociated salt form on the resin surface. The progress of the formation of this form, $R^{\prime}...\overset{+}{N}H_3A$ will be hindered by the presence of the sodium ions in the mobile phase, so that to explain the firm retention of a proportion of the arginine in this experiment, where the concentration of sodium ions in the mobile phase was approximately 0.5 M, it is necessary to assume the slow formation of an undissociated resin-amino acid complex, RNH_3A, which cannot react directly with sodium ions, and which is consequently only influenced indirectly by the presence of sodium ions in the ambient phase.

That equation 3. is, in fact, a description of the amino acid exchange reaction is borne out by the fact that the capacity of Amberlite IRC-50 for arginine which was found in this Section, where the amino acid solutions were introduced directly into columns still in contact with N acetate buffer and subsequently washed through the columns with N or 0.1 N acetate buffer, was very much less than the capacity found when the resin was washed free of buffer before the introduction of the amino acid solution and water used subsequently in place of buffer. In the experiment described at the end of the Section, Experiment IX, results were obtained indicating that the resin has a capacity for arginine greater than 50 mg. arginine/ml. resin when sodium iohs

were/

were absent from the ambient phase.

By contrast, the capacity of the resin for arginine under the conditions described in the Experimental part of this Section was very much less; probably of the order of a few mg. arginine /ml. of resin.

Although most of the work presented in this Section was based on the misconception that the amino acid exchange reaction is with the hydrogen form of the resin, it has been presented here because certain conclusions can be drawn from some of the experimental results regarding the mechanism of the exchange reaction, and because, in spite of the initial faulty premises, the method evolved on their basis has proved to be quite satisfactory for the separation of the basic fractions from histone hydrolysates.

Moreover, although the use of acetate buffer to wash glycine off the columns was based, in the first instance, on an error of judgment, it has proved to be a singularly fortunate error. The fact that Amberlite IR-4B turned out to be unsuitable for the adjustment of the pH of protein hydrolysates made it necessary to employ NaOH for this purpose. This necessitated the introduction of considerable concentrations of sodium ions into the amino acid solutions, the which modification would have been impossible/

impossible had not the experimental conditions been determined for runs involving washing with N acetate buffer in the first place.

SECTION III

ANALYSES OF HISTONES & PROTAMINES

INTRODUCTION

The following section includes the results of analyses performed on (i) histones prepared by Dr Stedman in this Department by the methods described by Stedman & Stedman (1951), (ii) "main histones" prepared by the methods referred to above and partially purified by the removal of a proportion of a slow component exhibiting a slower rate of migration during electrophoresis, and (iii) main component histones more or less free from all but a trace of the slow component. Results of analyses of two slow component histones have also been given.

The electrophoretic homogeneity of all the single component histones has been checked by Tiselius runs and photographs relating to these are included in the Appendix. Some of the photographs suggest there is trace contamination with the other histone component, but since the contaminating component has been found to be similar in composition and the extent of contamination is in every case small, in no case could the analytical results be significantly affected.

Since the main purpose of this thesis is the demonstration of cell and species specificity for electrophoretically pure main component histones, most of the histones analysed belong to this category, and analysis are presented for main component/

component histones prepared from nuclei from two types of cells from two different species -- ox livêr and ox thymus cell nuclei, and fowl erythrocyte and fowl thymus cell nuclei.

All the analyses of histones and protamines for which results are presented have been carried out on the histone and protamine sulphates, and this has been assumed to be understood in the phrasing of the text.

EXPERIMENTAL

Preliminary Analysis - Ox Thymus Histone 111L1

Thymus histone 111L1 was a specimen of main histone prepared from calf thymus nuclei and it was decided to use this to conduct a trial analysis using the technique evolved in Section II.

Procedure

The procedure described in the Appendix was used. About 100 mg. of the dry protein was used for hydrolysis and the volume was adjusted to ca. 25 ml. after adjustment of the pH to 4-6. The hydrolysate was then introduced into the column in toto.

The eluate and buffer wash fractions were each brought to 25 ml. volume, and micro-Kjeldahl estimations carried out on aliquots from each fraction. In addition, amino N, arginine N and amide N estimations were carried out on the eluate.

In this particular analysis, aliquots were taken from the eluate for the various estimations as follows:-

10 ml. - amide N
5 ml. - arginine N
2 x 2 ml. - amino N
2 x 2 ml. - total N

All results were expressed as the N equivalent to that originally present in the hydrolysate,

and/

Results:-

Ox Thymus IIILI

Dry weight of Histone = 90.9 mg.

% N recovered in Histone = 17.06
(Buffer Wash N Eluate N)

	Weight N recovered (mg.)	Equivalent % Recovery
Amide N	0.82	5.3
Basic N	7.28	46.9
Non-basic N	7.41	47.8
Arginine N	4.01	25.8
Histidine N	0.63	4.0
Lysine N	2.64	17.0

Table 22.

and the corresponding percentages of the total N recovered in the buffer wash and the eluate were calculated. The amino N estimations were carried out in the Van Slyke apparatus using a reaction time of one hour, and the results for histidine and lysine obtained by applying the N Distribution Method to the mean of the two results.

Discussion of Results

An amide N estimation on 10 ml. of the buffer wash fraction gave the blank value, so all the amide N was apparently present in the eluate fraction. Thus the amino N results included N derived from the amide ammonia. Provided reaction of ammonia with nitrous acid was quantitative under the conditions of the estimation, the accuracy of the results for histidine and lysine would not be affected, since these results depend on the non-amino N arrived at by difference from the total basic N and amino N values. In Van Slyke's (1911) original paper it is stated that ammonia requires 1.5 - 2 hr. to react quantitatively and that 60% of ammonia reacts to give free nitrogen in the Van Slyke apparatus in ten minutes. It was assumed, therefore, that treatment with nitrous acid for a period of one hour would ensure that a sufficiently high proportion of the ammonia present would react to give fairly accurate results. Since the amide N in/

in the eluate represented only about one quarter of the amino N present, fairly accurate values for histidine and lysine, especially the latter, could be obtained by the N Distribution method even if the recovery of amide N in the Van Slyke apparatus were not quite quantitative under these conditions.

It has since been found that Van Slyke estimations of basic amino N give erratic results when carried out in the presence of amide ammonia, although approximate values for histidine and lysine can be obtained. For this reason, the values for histidine N and lysine N quoted here are to be regarded as approximate.

An interesting feature of this analysis is that the value for arginine N found is approximately 5% less than the value recently reported by Hamer (1951), despite the fact that Hamer's total basic amino acid N recovery of 46.3% agrees closely with the basic N value found of 46.9%. The value found for arginine N was also ca. 5% lower than values previously found by Stedman (1951) for the same specimen of histone (29.1, 29.8%). The real discrepancy between the latter results and the authors must have been actually greater than 5% for a correction factor of 1.024 was used only in deriving the present result.

This substantial differenc could only have

been/

been caused by the loss of arginine or by the removal of an alkali labile amino acid during the fractionation process. It seemed a safe assumption to make that no overall working loss of arginine could account for a difference of this order and that if all the arginine originally present in the hydrolysate were not in the eluate fraction, it must have been present in the buffer wash fraction. However, when the Sakaguchi reaction was applied to a few ml. of the buffer wash fraction a negative result was obtained, conclusively establishing the absence of all arginine from the buffer wash fraction. Thus it seemed to the author that the low arginine N recovery could only have been caused by the presence of some alkali labile amino acid other than arginine in the whole histone hydrolysate and that this alkali labile material must be present in the non-basic fraction.

A second preliminary analysis was carried out on another sample of histone in order to check this conclusion.

Preliminary Analysis - Ox Liver Histone I (Main Component)

It was resolved to compare the value for arginine N found after separation of the basic amino acids with a corresponding arginine estimation of the original hydrolysate, for it seemed probable, in view of the results from the previous analysis, that during the six hours alkaline hydrolysis of arginine in whole histone hydrolysates some non-basic amino acids were being decomposed with liberation of ammonia. If this were the case, all values for arginine N previously determined by Plimmer's method or any modification thereof would really represent the true arginine N plus twice the nitrogen corresponding to the ammonia liberated from the alkali labile amino acids. Since these alkali labile amino acids should be present in the buffer wash after fractionation of a histone hydrolysate, it was decided to test the buffer wash fraction obtained in this analysis by applying the alkaline hydrolysis method to a suitable aliquot from it.

Procedure

In this analysis the hydrolysate volume was adjusted to 50 ml. and a 25 ml. aliquot from this solution used for the column run. Estimations were carried out on the remaining solution as follows:-/

follows:-

10 ml. amide N

5 ml. arginine N

2 x 2 ml. total N

The eluate was concentrated in vacuo two times and its pH adjusted to 5-7 before making to volume, 25 ml. Estimations were carried out on the eluate and buffer wash as follows:-

Eluate:- 10 ml. - amide N

5 ml. - arginine N

2 x 2 ml. - total N

1 ml. - colorimetric histidine

Buffer Wash:- 10 ml. - amide N

5 ml. - pseudo-arginine N

5 ml. - Sakaguchi reaction

2 x 2 ml. - total N

Discussion of Results

Amino N in Presence of Amide Ammonia:- The two Van Slyke estimations of amino N yielded rather erratic results but the mean result for histidine N as determined by the N Distribution method (3.3%) was of the same order as the colorimetric value of 3.7%. Since it seemed that the Van Slyke N Distribution Method was rather undependable when applied to solutions of the basic amino acids in presence of amide ammonia the results for lysine N and histidine N quoted for this analysis are those depending/

Results:-

Ox Liver Histone I

% N in Histone = 17.0

Weight N/25 ml. Hydrolysate = 16.30 mg.

Arginine N Recoveries

	Weight N (mg.)	Equivalent N% Recovery
Apparent Arginine N ...... (Hydrolysate)	5.04	30.9
Arginine N .... (Eluate)	4.30	26.4
2 x Pseudo-Arginine N .... (Buffer Wash)	0.88	5.4
2 x Pseudo-Arginine N .... + Arginine N	5.18	31.8

N Recoveries

	Weight N (mg.)	Equivalent N% Recovery
Amide N	0.69	4.2
Basic N	7.56	46.4
Non-basic N	8.10	49.7
Total N Recovery	16.35	100.3
Arginine N	4.30	26.4
Histidine N	0.59	3.6
Lysine N	2.67	16.4

Table 23.

depending on the colorimetric histidine estimation.

Pseudo-Arginine N:- It is evident from the results quoted in Table 23 that there is a considerable difference between the values for arginine N as determined directly on the hydrolysates and the corresponding value found for the arginine N in the eluate fraction. Since these differences could only have been due to the presence of alkali labile material in the buffer wash fraction, it was not surprising that an "arginine estimation" on the buffer wash fraction accounted for the missing N. This N was designated the "pseudo-arginine N", and it was found that the sum of twice the pseudo-arginine N value and the eluate arginine N value agreed roughly with the apparent arginine N value. Rather more N was found in the buffer wash than accounted for the difference between the other two values but this discrepancy was attributed to the high possible percentage error inherent in the estimation of the small quantity of pseudo-arginine N. The approximate possible error involved in the titrations on which the pseudo-arginine N value depended was ± 5%.

This work was taken to demonstrate conclusively that arginine N cannot be estimated accurately by Plimmer's method or any simple modification of it when the method is applied to whole hydrolysates of/

of histones, and that accurate values can only be obtained by this method when a partial separation of the amino acid components has been effected prior to the estimation of arginine.

Modifications in Analytical Technique

The experience of these two preliminary analyses suggested that it might be profitable to make a few minor modifications in the analytical procedure. For example, the difference between the "apparent arginine N" and the "true arginine N" values already demonstrated, made it advisable to obtain results for both types of estimation for some of the analyses at any rate. This could easily be done by making the hydrolysate volume to 50 ml. and pipetting out a 25 ml. aliquot of this solution for the column run, estimations being made on the remaining solution as follows:-

10 ml. - amide N

5 ml. - apparent arginine N

2 x 2 ml. - total N

This procedure involves a certain amount of wastage of valuable material since the total hydrolysate volume is not utilised in carrying out all the necessary estimations. Moreover, it seemed to the author unnecessary that apparent arginine N estimations should be carried out for all analyses, it having been clearly demonstrated that results obtained in this way have no absolute value.

For these reasons, it was decided to use smaller volumetric flasks -- either 30 ml. or 40 ml. volume/

volume -- for analyses of histones which were in particularly short supply, and to omit carrying out apparent arginine N estimations for these analyses. When the 30 ml. volumetric flask was employed it was also necessary to omit carrying out an amide N estimation on the hydrolysate, and hence for such analyses no amide value is quoted.

The amide N is, of course, present in the eluate fraction after the column run, but agreement between amide N estimations carried out on eluate fractions and corresponding estimations on hydrolysate fractions was generally found to be rather unsatisfactory. Since there was always a certain risk of loss of amide N during the concentration in vacuo of the eluate (vide - Appendix), it was obviously desirable that all amide N values should be obtained from direct estimations on hydrolysates.

There being no necessity to preserve amide N in eluate fractions, it was resolved to eliminate it as far as possible, by adjusting the pH of the eluate fraction to 9-10 after the removal of excess HCl by an initial two concentrations in vacuo almost to dryness. A further concentration in vacuo almost to dryness should then ensure complete removal of amide ammonia. However, for all analyses, an estimation of amide ammonia was carried out on a 5 ml. aliquot of the eluate fraction to confirm quantitative/

quantitative elimination of ammonia.

It was hoped that the elimination of amide ammonia would make the accurate estimation of histidine and lysine by the Van Slyke Nitrogen Distribution Method a practical proposition. This hope has not materialised for the reasons discussed in the Appendix.

In all the analyses of histones following in this Section, the following estimations were carried out on the buffer wash (or water wash) and eluate fractions:-

Buffer Wash:-	5 ml.	- pseudo-arginine N
	5 ml.	- Sakaguchi reaction
	2 x 2 ml.	- total N
Eluate:-	5 ml.	- amide N
	5 ml.	- arginine N
	2 x 2 ml.	- amino N
	2 x 2 ml.	- total N
	1 ml.	- colorimetric histidine

For several of the analyses additional colorimetric estimations of histidine were carried out in place of Van Slyke estimations of amino N as the latter method was finally abandoned owing to the erratic nature of the results obtained.

Analyses of Ox Thymus Histones

The histones analysed were Thymus IIILI, a main histone of the type for which analytical results have already been published by Stedman & Stedman (1951), two main component histones, here designated 'Thymus II' and 'Thymus III', fractionated by electrophoresis from thymus main histone, and the corresponding slow component histone -- 'Thymus Slow.' Tiselius runs carried out with both the main component histones indicated that they were, to all intents and purposes, electrophoretically pure. Duplicate analyses are presented for Thymus IIILI and Thymus II.

Procedure

The column runs for these analyses were carried out using the procedure described in the Appendix, and for all the analyses the modifications just described were incorporated in the experimental procedure. This is to say that the amide N results were obtained by direct estimation on the hydrolysates. A single exception is one of the duplicate analyses carried out on Thymus II; in this case the amide N was determined on the eluate fraction, the hydrolysate volume having been made up to only 30 ml. in the first instance.

Discussion of Results

Amide N:- The low amide N recovery of 0.7%, obtained/

Results:-

	Ox Thymus Histones (N% of total N)					
	Main IIILI (i)	Main IIILI (ii)	Main Comp. II (i)	Main Comp. II (ii)	Main Comp. III	Slow Comp.
Amide N	5.1	6.3	0.7	5.6	4.6	4.7
Basic N	47.0	45.8	45.7	45.5	45.4	46.3
Non-basic N	49.6	49.0	48.8	48.5	48.6	48.8
N% Recovery	101.7	101.1	95.2	99.6	98.6	99.8
Arginine N	27.0	27.5	26.2	26.0	25.9	27.3
Histidine N .... (Van Slyke)				3.0 2.8	-	-
Histidine N .... (Colorimetric)	4.2	4.0	3.7	3.8	3.3	3.6
Lysine N	15.7	14.3	15.8	15.8	16.2	15.5
Pseudo-Arginine N .	4.1	2.8	3.5	2.7	3.0	3.4
Apparent Arginine N	31.7	31.4	-	-	-	-

N Content (% N on basis of dry weight)					
Main IIILI (i)	Main IIILI (ii)	Main Comp. II (i)	Main Comp. II (ii)	Main Comp. III	Slow Comp.
16.6	16.6	16.4	16.6	16.8	16.3

Table 24.

obtained in the analysis just referred to, is obviously due to loss of ammonia during concentration in vacuo, since the sum of all the N recoveries for this analysis falls short of 100% by approximately the same percentage as the amide N value is less than the corresponding value for the duplicate analysis

Generally, the results for amide N seem to be somewhat variable, even when estimations are carried out directly on hydrolysate solutions. For example, the two amide N values obtained for the analyses of Thymus IIILI of 5.1% and 6.3% are in poor agreement, and it seems probable that this estimation may be considerably affected by slight variations in hydrolysis conditions.

Pseudo-Arginine N:- The values for pseudo-arginine N vary to an even greater extent, and the difference between the pseudo-arginine N values found for the two analyses of Thymus IIILI (4.1%, 2.8%) is much greater than could be accounted for by any experimental error. The possible error involved in the titrations from which the pseudo-arginine N results are derived is undoubtedly high (ca. ±5%), for the net titre is ca. 0.60 ml. of 0.01 N HCl as a rule, but the variation in pseudo-arginine N results is of a much higher order than this.

There is, nevertheless, fairly good agreement between/

between the two apparent arginine N results found for the two analyses of Thymus IIILI.

Arginine N:- By contrast with the amide N and pseudo-arginine N estimations the arginine N estimations yielded results for duplicate analyses of the same histone which were in excellent agreement, e.g. the values for Thymus II were 26.2% and 26.0%, and for Thymus IIILI, 27.0% and 27.5%. For this reason greater significance has been attached to arginine N values than to any other kind of analytical result.

Histidine N and Lysine N:- The agreement between the histidine N results for duplicate analyses was considered to be satisfactory in view of the shortcomings of colorimetric methods of estimation. The agreement between lysine N values was less satisfactory, but this was expected since the lysine N values carry all the error of the total basic N estimations. Since the total basic N values are generally about three times as great as the corresponding lysine N values, a threefold magnification of percentage error is inherent in the calculation of lysine N by difference in this way.

Chemical Relationship between Thymus Histones:- Bearing all the above considerations in mind, a comparison of the analytical results for the thymus histones was made, and it seemed to be clear that, despite/

despite their different behaviour during electrophoresis, the slow and main component histones were closely related chemically. As might be expected, the main histone from which these two components were derived, appeared to be very similar in composition to both components.

The arginine N values for the analyses of Thymus IIILI are somewhat higher than the values for the analyses of the two main component histones, so a consideration of the results of these analyses alone would suggest that the slow component would contain a rather higher arginine content than either the parent main histone of the main component. However, the single analysis of the slow component which was carried out actually indicates an arginine content which is indistinguishable from the arginine content of Thymus IIILI, so it may be that there is no real difference between the arginine content of the slow and main component histones. The alternative possibility is that such difference in arginine content as exists between them is of the same order of magnitude as the total experimental error.

The histidine N and lysine N values for the three types of histones analysed are also such that neither can a definite distinction be made between the main component histones and the slow component nor between either component and the parent main histone.

The/

The analysis of the slow component yielded a rather low result for histidine N as compared with the values obtained for Thymus IIILI, it is true, but little significance was attached to this since the histidine N values for the main component histone analyses were also lower than the values for Thymus IIILI.

The general conclusions which have been drawn from the results of all these analyses are that main histones consist of two components which are very similar in their amino acid composition as far as it has been investigated by the methods used here, and that such differences as may exist between these components are within the limits of the experimental errors of the analytical methods used, and were, as a consequence, undetectable.

Analyses of Ox Liver Histones

Two main component histones which had been checked by Tiselius runs and found to be virtually electrophoretically pure, were analysed and these are here designated 'Liver II' and 'Liver III'. Duplicate analyses were carried out on Liver II and the results of all three analyses are presented opposite. The results for the preliminary analysis of another preparation, Liver I, which have already been quoted and discussed in this Section, are also included opposite, since this histone is also a main component ox liver histone.

Procedure

The procedure was that described for the analyses of the ox thymus histones. In one of the duplicate analyses of Liver II, shortage of material made it necessary to make the hydrolysate volume to 30 ml. only. Thus for this analysis, it was not practicable to carry out an amide N estimation on the hydrolysate, a 25 ml. aliquot being employed for the column run.

An apparent arginine N estimation was included in the analysis of Liver I.

Discussion of Results

Since these histones are all different preparations of the same compound and are therefore chemically identical, the variations in corresponding analytical results furnish some indication of the order of variation/

Results:-

Ox Liver Histones (N% of total N)

	Main Comp. I	Main Comp. II (i)	Main Comp. II (ii)	Main Comp. III
Amide N	4.2	4.5	-	4.9
Basic N	46.4	46.9	46.6	45.2
Non-basic N	49.7	49.9	50.0	48.9
N% Recovery	100.3	101.3		99.0
Arginine N	26.4	26.4	27.0	26.4
Histidine N .... (Van Slyke)	3.3	3.8	3.3	2.8 3.0
Histidine N .... (Colorimetric)	3.6	3.7	3.6	3.4
Lysine N	16.4	16.8	16.0	15.4
Pseudo-Arginine N ...	2.7	3.2	2.9	2.3
Apparent Arginine N .	31.1	-	-	-

N Content (% N on basis of dry weight)

Main Comp. I	Main Comp. II (i)	Main Comp. II (ii)	Main Comp. III
17.0	16.5	16.6	16.8

Table 25.

variation which can be expected for the analytical methods employed.

It will be observed that the differences between corresponding results for these four analyses are of the same order as the differences observed between the three kinds of thymus histone analysed. A general comparison of the results for these analyses with those for ox thymus histones indicates no marked difference between the two types of histone. There is a general tendency for arginine N results to be slightly higher than the arginine N results for the thymus histone analyses, but a much larger number of analyses would require to be performed before it could be determined if this difference is of any significance.

The result obtained for the estimation of apparent arginine N (31.1%) is in satisfactory agrrement with the values quoted by Stedman (1951) for ox liver main histone (30.4%, 29.5%) when it is borne in mind that Stedman's values were uncorrected for incomplete hydrolysis of arginine. This suggests that ox liver main component histone is similar in composition to ox liver main histone. By analogy with the conclusions drawn from the analyses of ox thymus histones one would expect there to be no change in the amino acid composition, with respect to/

to the amino acids estimated, on the removal of the slow component from ox liver main histone.

Analyses of Fowl Erythrocyte Histones

Analyses were carried out on a sample of erythrocyte main histone (Erythrocyte IIIFI), a specimen of main histone which had been partly purified by removal of a proportion of the slow component (Erythrocyte A), and a main component histone which had been found to be electrophoretically pure.

An electrophoretically pure sample of the slow component was also analysed and it was hoped from these analyses that it would be possible to demonstrate some difference in amino acid composition between the slow and main components. Duplicate analyses of Erythrocyte IIIFI and Erythrocyte A were carried out.

Procedure

The procedure was as already described. For all analyses amide N was directly estimated on the hydrolysate with the exception of the analysis of the slow component. In this case, shortage of material made it necessary to restrict the hydrolysate volume to 30 ml.

Discussion of Results

Although the agreement between most corresponding results for the two pairs of duplicate analyses carried out was rather disappointing, good agreement was obtained between the corresponding arginine results./

Results:-

Fowl Erythrocyte Histones (N% of total N)

	Main IIIFI (i)	Main IIIFI (ii)	Main A (i)	Main A (ii)	Main Comp.	Slow Comp.
Amide N	5.9	5.6	4.9	5.1	5.0	-
Basic N	42.7	44.2	44.6	41.8	43.3	46.0
Non-basic N	51.0	50.3	49.7	52.3	52.6	35.8
N% Recovery	99.6	100.1	99.2	99.2	100.9	
Arginine N	23.6	23.7	23.8	23.3	23.4	27.4
Histidine N ..... (Van Slyke)	1.4	2.6	-	3.0 2.2	-	-
Histidine N ..... (Colorimetric)	3.4	3.3	4.4	4.3	4.1	4.1
Lysine N	16.2	17.2	16.4	14.2	15.8	14.4
Pseudo-Arginine N	2.8	3.5	2.5	3.7	4.7	4.1

N Content (% N on basis of dry weight)

Main IIIFI (i)	Main IIIFI (ii)	Main A (i)	Main A (ii)	Main Comp.	Slow Comp.
16.4	16.6	16.8	16.7	15.1	15.3

Table 26.

results. The arginine values for Erythrocyte IIIFI and Erythrocyte A were also in good agreement, and a general comparison of the results for these two histones indicates that no detectable change in arginine N content occurs on removal of a proportion of the slow component from the main histone. This conclusion is supported by the fact that the analytical results for the main component histone, containing only a trace of the slow component as a contaminant, are also indistinguishable from the results for these analyses.

Thus the fact that the arginine N value for the slow component was found to be markedly different from the other arginine N values appeared to conflict with the results of the other analyses. Although the proportion of the slow component originally present in the unfractionated main histone was small, and its removal would not make a large difference in the arginine content of the main component, one would certainly expect there to be a detectable difference. The difference between the arginine N content of the slow component and that found for any of the other histones in this group certainly seems to be well outside the limits of experimental error and the reliability of the result appears to be confirmed by the fact that the total basic N content reflects the arginine N difference, but since it is difficult

to/

to reconcile such a high arginine N value with the excellent agreement between the arginine N values for the main component and the parent main histone, it may be that the results for this analysis are rather unreliable.

It is particularly regrettable that it was net possible to include a direct estimation of amide N in this analysis since the N recoveries for the main fractions indicate an unusual amide N value of ca. 18% if 100% recovery of N is assumed. No amide N value of this order has been found in any of the other analyses and this also suggests that something has gone wrong with this analysis.

However, the analytical results for the slow component have been given because this compound has never been analysed before, and because the results, although possibly inaccurate, at least indicate that the slow component is a histone which is similar chemically to the others analysed in this work.

main component histones from the same gland in two unrelated species no doubt indicates that there is a close relationship between amino acid composition of histones and cell function.

Analyses of Salmine

Recoveries of Arginine

Since the buffer wash fractions from histone hydrolysates had been found to contain alkali labile material, it seemed a possibility that the non-basic fraction from hydrolysates of protamines would also yield a certain amount of ammonia on prolonged treatment with sodium hydroxide. It was therefore decided to separate the basic fraction from a hydrolysate of salmine and compare the result of an arginine N estimation on the basic fraction with the result of a direct estimation on the hydrolysate. It was hoped that it would be possible to account for any difference found between these results as pseudo-arginine N.

The procedure followed was similar to that used previously for the histone analyses and is detailed below.

Procedure

About 80 mg. of salmine are dried to constant weight over P_2O_5 and hydrolysed in the usual manner. The hydrolysate volume is adjusted to 50 ml. and a 25 ml. aliquot is used for the column run.

Estimations are carried out on aliquots of the various fractions as follows:-

Hydrolysate - 10 ml. - amide N

5 ml. - apparent arginine N

2 x 2 ml. - total N

over/

Results:-

% N in Salmine (Sample 1.) = 23.9

Total Weight N/25 ml. Hydrolysate = 10.33

% N in Salmine (Sample 2.) = 24.1

Total Weight N/25 ml. Hydrolysate = 8.15

Arginine N Recoveries

	Weight N (mg.)		Equivalent N% Recovery	
	Samp. 1.	Samp. 2.	Samp. 1.	Samp. 2.
Apparent Arginine N ... (Hydrolysate)	9.56	7.56	92.6	92.7
Arginine N .... (Eluate)	9.07	7.23	87.8	88.7
2 x Pseudo-Arginine N . (Buffer Wash)	0.30	0.42	2.9	5.2
2 x Pseudo-Arginine N . + Arginine N	9.37	7.65	90.7	93.9

Amide N Recoveries

	Weight N (mg.)		Equivalent N% Recovery	
	Samp. 1.	Samp. 2.	Samp. 1.	Samp. 2.
Hydrolysate	0.09	0.09	0.8	1.1
Eluate	0.14	0.04	1.3	0.5
Buffer Wash	0.02	0.02	0.2	0.3

N Recoveries - Main Fractions

	Weight N Recovered (mg.)		Equiv. N% Recovery	
	Samp. 1.	Samp. 2.	Samp. 1.	Samp. 2.
Total Basic N	9.43	7.27	91.3	89.2
Non-basic N	0.92	0.87	8.9	10.7
Total N Recovery	10.35	8.14	100.2	99.9

Table 28.

Buffer Wash - 10 ml. - amide N
5 ml. - Sakaguchi reaction
5 ml. - pseudo-arginine N
2 x 2 ml. - total N
Eluate - 10 ml. - amide N
5 ml. - arginine N
2 x 2 ml. - total N

The whole procedure was repeated with a second sample of salmine.

Discussion of Results

The results for the analysis of the first sample indicate that the arginine N found in the eluate fraction is considerably less than the arginine N apparently present in the hydrolysate. The pseudo-arginine N value found did not account for all of this difference, the sum of twice the pseudo-arginine N value and the eluate arginine N value (9.37 mg.) being substantially less than the apparent arginine N (9.56 mg.). On the other hand, for the analysis of the second sample of salmine, the pseudo-arginine N value is considerably higher than would be expected on the basis of the difference between the arginine N values. It seemed evident that these discrepancies were mainly due to the unreproducibility of the pseudo-arginine N values which is so well illustrated by the results which have already been given for the analyses of histones.

An interesting feature of the results for the analysis/

analysis of the first sample is that there is a small but appreciable increase in amide N after fractionation. Virtually all the amide N was recovered in the eluate fraction although there seemed to be a trace present in the buffer wash fraction.

An increase in amide N could be caused either by deamination of a proportion of the arginine or by absorption of basic material from the atmosphere. The latter source of error can be checked, to a certain extent, for the sum of the N values for the main fractions can be compared with the total N of the hydrolysate. Deamination of the arginine at the α amino position would not be a serious difficulty, for it would not interfere with the accuracy of the arginine N estimations on basic fractions, all arginine N values being corrected for any amide N found present. On the other hand, any decomposition at the guanidine group of arginine would lead to low recoveries of arginine in eluate fractions. The fact that the arginine N recovery for the first sample (87.8%) is rather low as compared with the corresponding value for the second sample (88.7%), for which there was no observed increase in amide N after fractionation, therefore suggests a certain amount of decomposition at the guanidine grouping.

It is rather an interesting coincidence that these/

these results for arginine N are in fairly good agreement with the value quoted for salmine by Stedman & Stedman (1951) (88.9%), despite the fact that the results were arrived at in very different ways. No doubt this agreement is due to the fact that the small amount of pseudo-arginine N estimated as arginine N in the Stedmans' analysis rather more than compensated for the fact that they did not use a correction factor to allow for incomplete hydrolysis of arginine. The author's results are also in fair agreement with a value quoted by Tristram (1947) of 89%.

Results:-

% N in Clupeine = 24.1

Total Weight N/25 ml. Hydrolysate = 10.18

Arginine N Recoveries

	Weight N (mg.)	Equiv. N% Recovery
Apparent Arginine N ..... (Hydrolysate)	9.29	91.3
Arginine N .... (Eluate)	8.92	87.6
2 x Pseudo-Arginine N ... (Buffer Wash)	0.48	4.7
2 x Pseudo-Arginine N ... + Arginine N	9.40	92.4

Amide N Recoveries

	Weight N (mg.)	Equiv. N% Recovery
Hydrolysate	0.08	0.8
Eluate	0.13	1.3
Buffer Wash	0.03	0.3

N Recoveries - Main Fractions

	Wt. N Recovered	Equiv. N% Recovery
Total Basic N	9.30	91.4
Non-basic N	1.12	11.0
Total N Recovery	10.42	102.4

Table 29.

Analysis of Clupeine

Recoveries of Arginine

In their (1951) publication the Stedmans reported slightly differing arginine N contents for clupeine (87.6%, 87.9%) and salmine (88.9%). The Stedman's demonstration of the distinction between salmine and clupeine did not depend on this difference but on the identification of the various amino acids present in these two protamines by paper partition chromatography, and the observed difference in arginine content was therefore in the nature of confirmatory evidence. Since the results of the analyses of salmine indicate that direct estimation of arginine by alkaline hydrolysis does not necessarily give an accurate result for the arginine N content of protamines, an analysis of clupeine was obviously called for.

Procedure

The analytical procedure was that used for the analyses of salmine.

Discussion of Results

Again in this analysis the amide N in the eluate fraction was slightly higher than the corresponding value found for the hydrolysate. The result for arginine N found in the eluate (87.6%) is therefore comparable with the result found for salmine under the same circumstances (87.8%), and since these agree/

agree within the limits of experimental error, these two protamines may actually possess the same true arginine N content. The apparent arginine N content (91.3) however, is significantly lower than the corresponding value for salmine (92.6%) in accordance with the Stedmans' findings.

On the other hand, a comparison between the clupeine arginine N value and the arginine N result for the duplicate analysis of salmine (88.7%) would suggest that the difference already observed between the apparent arginine N values has been reproduced in the true arginine N values. The results of these analyses are therefore inconclusive in that they do not make it clear whether or not there is a difference in true arginine N content between salmine and clupeine.

DISCUSSION

Some of the results of the analyses reported in this Section provide confirmation of cell and species specificity for electrophoretically homogeneous histones, which was the main objective of this work. The discussion of the wider significance of the results therefore properly belongs to the General Discussion af the end of the thesis, and there it has been included. All that it is proposed to do here is to summarise the main conclusions arrived at on the basis of the results reported in this Section.

One of the most important findings has been that the alkaline hydrolysis method for arginine will yield erroneously high results when it is applied directly to hydrolysates of histones owing to the presence of alkali labile material in the non-basic fraction. The N equivalent to the ammonia evolved from non-basic amino acids has been designated the pseudo-arginine N, and the protamines, clupeine and salmine, have been shown to possess a small proportion of such N.

In the case of histones, the amount of non-basic alkali labile material in relation to the true arginine N content is substantial, so that the separation of the basic fraction frem the whole hydrolysate is an essential preliminary if the arginine is to be estimated/

estimated by a method involving alkaline hydrolysis.

In the case of protamines, on the other hand, the proportion of alkali labile material other than arginine is smaller, the amount of pseudo-arginine N being of the same order of magnitude as half the N correction employed by the author to allow for incomplete hydrolysis of arginine. Since, in the calculation of apparent arginine N values, the pseudo-arginine N is doubled, there are two compensating errors in the estimation of the arginine N content of salmine or clupeine when the estimation is carried out by alkaline hydrolysis of an aliquot from the hydrolysate, the error caused by liberation of ammonia from amino acids other than arginine being roughly equivalent to the error due to incomplete hydrolysis of arginine. This, no doubt, explains why the arginine N content of salmine found by the author (88.7%) agrees so well with that reported by the Stedmans (88.9) for a specimen prepared in the same way.

The differences in apparent arginine N content between the homologous basic proteins salmine and clupeine, have been confirmed by the analyses of these substances, and it appears probable that there is a corresponding difference in true arginine N content, although the author has not succeeded in demonstrating /

Summary of Analytical Results for Main Component Histones
(N% of Total N)

	Ox Thym. III	Ox Liver III	Fowl Eryth.	Fowl Thym.
Amide N	4.6	4.9	5.0	3.7
Basic N	45.4	45.2	43.3	47.9
Non-basic N	48.6	48.9	52.6	50.2
N% Recovery	98.6	99.0	100.9	101.8
Arginine N	25.9	26.4	23.4	27.9
Histidine N .... (Colorimetric)	3.3	3.4	4.1	3.1
Lysine N	16.2	15.4	15.8	16.9
Pseudo-Arginine N .	3.0	2.3	4.7	3.6

N Content (% N on basis of dry weight)

Ox Thym. III	Ox Liver III	Fowl Eryth.	Fowl Thym.
16.8	16.8	15.1	15.4

Table 30.

demonstrating this conclusively.

The analyses of the main component histones from the thymus glands of the ox and the fowl have provided a good example of species specificity. Although these histones appear to be similar in amino acid composition, it is possible to distinguish between them on the basis of the results from their analyses. For example, the difference between the arginine N value found for the fowl thymus histone (27.9%) and the highest arginine N value for the main component histone from ox thymus gland (26.2%), is clearly outside the limits of experimental error.

The Stedmans' analyses failed to distinguish between the main histones prepared from ox thymus gland and ox liver although their results did suggest that the ox liver histone might possess a slightly higher arginine content. The relationship between the corresponding main component histones has been found to be similar. These appear to be almost identical with respect to the content of every amino acid which has been estimated, with the exception that the arginine N content is generally found to be slightly higher for the ox liver histone. Unfortunately, this difference is not sufficiently great to allow one to postulate a definite difference in amino acid composition between the two histones, although it certainly suggests that such a difference exists/

exists.

The difference between the two types of histone from the fowl is more marked, and there is a particularly pronounced difference between the arginine N contents in this case. Indeed, the main component histone prepared from fowl erythrocytes appears to be quite distinct, with respect to its arginine content, from any of the other main component histones analysed.

Thus the analyses of main component histones from two different kinds of cells from two species have provided one example of species specificity, the difference between the thymus histones from the ox and the fowl, and one clear-cut example of cell specificity, the difference between the histones from fowl thymus gland and fowl erythrocytes.

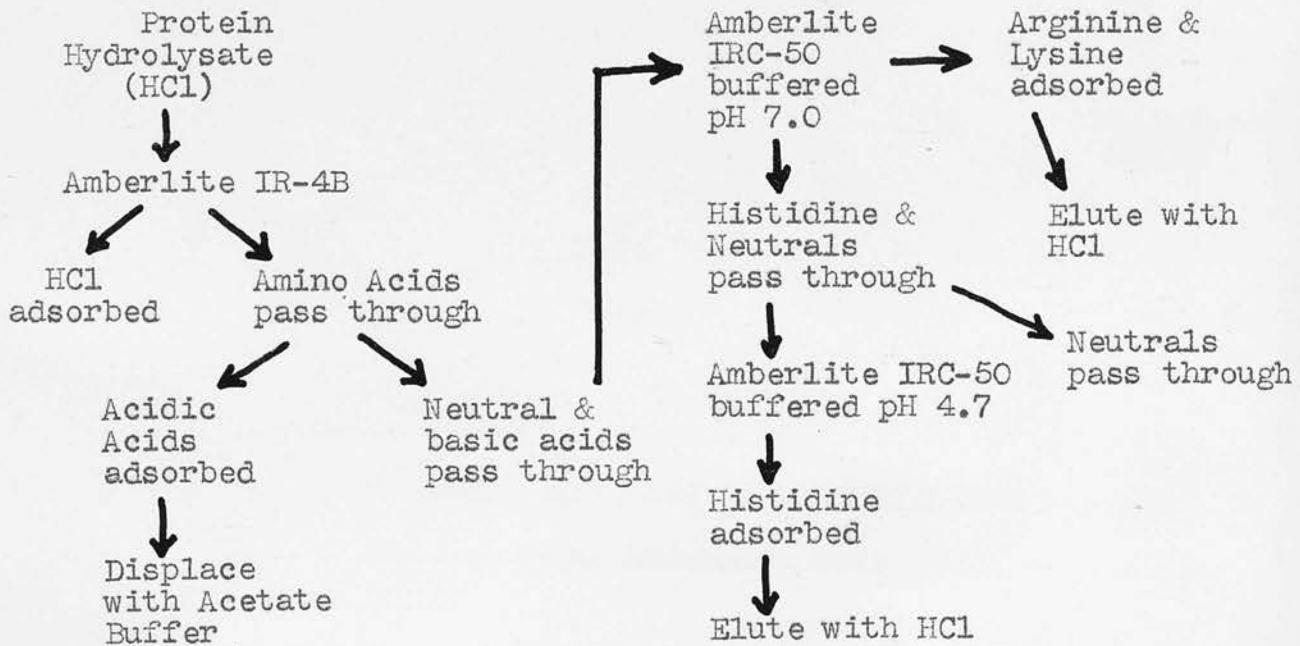
Two slow component histones were analysed, from fowl erythrocytes and ox thymus gland, but since these were prepared from different kinds of cells from different species, they are not comparable. No doubt cell and species specificity exists for the slow component, as well as the main component, histones, but the demonstration of this phenomenon will have to await further investigation. The main purpose of this work has been the study of differences between comparable main component histones and the analyses of the slow components were only carried out incidentally.

The/

The chemical relationships between the slow components and the corresponding main components, prepared from the same kind of cells, have already been discussed under the appropriate groups of analyses.

SECTION IV

SEPARATION OF ACIDIC AMINO ACIDS WITH AMBERLITE IR-4B


Protein Hydrolysate (HCl)
Amberlite IR-4B
HCl adsorbed
Amino Acids pass through
Acidic Acids adsorbed
Displace with Acetate Buffer
Neutral & basic acids pass through
Amberlite IRC-50 buffered pH 7.0
Arginine & Lysine adsorbed
Elute with HCl
Histidine & Neutrals pass through
Neutrals pass through
Amberlite IRC-50 buffered pH 4.7
Histidine adsorbed
Elute with HCl

INTRODUCTION

Because of the obvious limitations of a method of amino acid analysis covering only the three basic amino acids it was necessary to make some attempt to extend the scope of the method to include other amino acids. The comprehensive method of Moore & Stein (1951) is, of course, eminently suitable for the purposes the author had in mind at the time this work was embarked upon, but this method had not been evolved at that time. The author's method of analysis was capable of producing results for the basic amino acids at least as accurate as the method of Moore & Stein and rather than abandon it in favour of the latter method, it seemed more profitable to work on the basis of what success had been achieved with columns of Amberlite IRC-50.

After the quantitative separation of the basic fraction from a protein hydrolysate all the neutral and acidic amino acids are contained in the water wash, and there seemed no reason why this should not be further fractionated into an acidic and a neutral fraction by using a weak base exchanger in a way analogous to the initial fractionation involving the weak acid exchanger Amberlite IRC-50.

On the basis of a method suggested by Cannan (1946), Winters & Kunin (1949) have described an ion exchange procedure for fractionating protein hydrolysates/

hydrolysates into the three main groups, acidic, neutral and basic. The essentials of their scheme are represented in the diagram opposite.

Winters & Kunin admit that this scheme suffers from the disadvantage that quantitative separation of the various amino acids is not effected since 'the weak base anion exchanger will not remove all of the glutamic and aspartic acids present in a mixture containing arginine, histidine and lysine.' This scheme suffers from the additional disadvantage that treatment of a strongly acid protein hydrolysate with Amberlite IR-4B liberates substantial amounts of colouring matter and nitrogenous material from the resin. (Consden, Gordon & Martin (1948)).

An obvious way of overcoming the former disadvantage of the method of Winters & Kunin is to remove the bases from the hydrolysate prior to the removal of the acidic amino acids, and, in a method described by Cannan (1944), this is done by precipitation with phosphotungstic acid. As it is well known that phosphotungstic acid precipitation of the basic amino acids is incomplete, Cannan's procedure could not be used satisfactorily in a method of analysis including estimation of the bases. In the method envisaged by the author, the water wash from a column run with Amberlite IRC-50 would be used for further fractionation into a neutral and an acidic fraction./

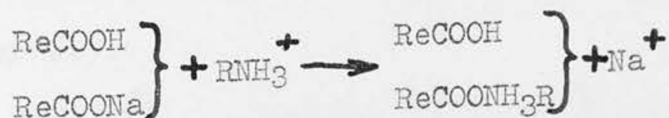
fraction. Thus the estimation of both the acidic and the basic amino acids would be made on two fractions quantitatively separated from a single aliquot of the protein hydrolysate. This Section is, for the most part, a description of a few experiments which were carried out with this object in view.

In 1948, Consden, Gordon & Martin published details of a chromatographic method for the separation of glutamic from aspartic acid in which the acidic amino acids were separated from the protein hydrolysate by putting the resin Amberlite IR-4B into the chloride form with HCl, washing off the excess with water, and introducing the hydrolysate at ca. pH 4. At this pH the resin was supposed to be capable of competing with the basic amino acids for the acidic amino acids. The latter were finally removed from a column of the resin by elution with HCl. However, Consden, Gordon & Martin did not claim that quantitative recovery of the acidic amino acids could be obtained in this way, but simply that the separation of the acidic amino acids from the rest of the protein hydrolysate was complete. A few experiments were carried out in this laboratory using this technique with test amino acid solutions containing only glycine and the acidic amino acids, bu the capacity of the chloride form of the resin for the acidic amino/

amino acids was found to be very low, and quantitative retention of them on a column of Amberlite IR-4B could not be achieved. This was attributed to the fact that the dissociation of the carboxyl groups of aspartic acid (pK_1 1.88, pK_2 3.65) and glutamic acid (pK_1 2.16, pK_2 4.32) will be considerably repressed at pH 4, and it was decided to devise experiments using columns of Amberlite IR-4B buffered to a higher pH.

In the case of the weakly acidic resin Amberlite IRC-50, the buffering action of the column depends on the fact that washing with, say, acetate buffer converts the resin into a buffer system in itself, in equilibrium with the buffer solution used at its particular pH. The resin acquires a certain ratio of salt to free acid form characteristic of the pH of the wash solution, and in the ideal case where the exchange equilibrium is regarded simply as a double decomposition reaction, this ratio would be defined by the Henderson-Hasselbalch equation.

The exchange reaction can be regarded as being with the sodium form of the resin as follows:-

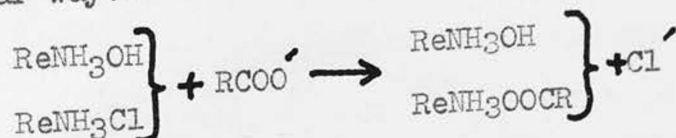


$$\left.\begin{matrix} ReCOOH \\ ReCOONa \end{matrix}\right\} + RNH_3^+ \longrightarrow \left.\begin{matrix} ReCOOH \\ ReCOONH_3R \end{matrix}\right\} + Na^+$$

The exchange of a basic amino acid molecule, ionised as a base, for a sodium ion will tend to increase/

increase the pH in the mobile phase but this will be compensated for by dissociation of additional carboxyl groups, which is simply to say that the resin surface can be regarded as analogous to an ordinary weak acid/strong base buffer system.

Now if we consider the resin, Amberlite IR-4B, in order to arrive at a system strictly comparable to that described above and suitable for reaction with the acidic amino acids, we would have to wash the resin with a buffer solution of the weak base/strong acid type. An ammonium chloride/ammonium hydroxide system could be used for this purpose but this would be ruled out in practice because of the necessity of keeping extraneous N out of the columns. An alternative would be to wash a column of the resin in its basic form with a solution of NaCl until the effluent was neutral. This should convert the resin into a combined salt/free base form which would buffer at the pH of the effluent, i.e. at pH 7. Such a weak base/strong acid system would be strictly analogous to that described above and the exchange reaction could be represented in a similar way:-



$$\left.\begin{matrix} ReNH_3OH \\ ReNH_3Cl \end{matrix}\right\} + RCOO' \longrightarrow \left.\begin{matrix} ReNH_3OH \\ ReNH_3OOCR \end{matrix}\right\} + Cl'$$

A different approach would be to convert the

resin/

resin into a weak base/weak acid buffer system by washing with an acetate buffer. Experiments have been carried out involving both types of system and these are described in the experimental part of this Section which follows.

EXPERIMENTAL

Experiment I - Retention of Acidic Amino Acids by Amberlite IR-4B

Procedure

A column of the same dimensions as that used in the experiments with Amberlite IRC-50 (20 x 1.5 cm.) was employed and a slurry of the resin with water was introduced until the resin occupied two thirds of the column volume. It was necessary to leave one third of the column empty at this stage to allow for the swelling which takes place when the resin is converted from the free base to the salt form. The resin was then washed by siphoning 200 ml. of 0.5 N NaOH through the column at a rate of flow of ca. 20 ml./hr. and this was followed by a washing with 200 ml. of water to remove excess NaOH.

0.5 N NaCl was then siphoned through the column at a rate of flow of about 60 ml./hr. until the pH of the effluent was 7-8. This required several days and about 1,500 ml. of NaCl solution was used in the process. The excess NaCl was removed by washing with 200 ml. of water and the effluent from this wash was collected in two 100 ml. fractions. The N content of the second fraction was estimated in order to ascertain how much N was derived from the resin at this stage.

A test amino acid solution (Solution P) was prepared/

Solution P. (Volume - 100 ml.)

Amino Acid	N% of Theor. N	Dry Wt. (mg.)	Mg. N/25 ml.
Glycine	99.0	196.2	9.06
Glutamic Acid	98.0	214.4	4.01
		Total N Content/25 ml. =	13.07

Micro-Kjeldahl Estimations on Solution P.

(mg. N/25 ml.)

Total N = 13.00

Table 31.

Results:-

Run with 25 ml. Aliquot of Solution P.

Rate of Flow - ca. 20 ml./hr.

Fractions	Fraction Vol.	Total N Content (mg.)	
Water Wash 1.	150 ml.	8.96	9.22
" " 2.	50 ml.	0.26	
Eluate 1.	100 ml.	3.59	4.56
" 2.	100 ml.	0.97	
Total N recovered	=	13.78	

Table 32.

prepared of composition as indicated in Table 32, and a 25 ml. aliquot from this used for the column run. The amino acid solution was washed through the column with 175 ml. of water and the water wash collected in two successive fractions, of volumes 150 ml., and 50 ml., in that order.

The column was finally eluted with 200 ml. of 0.25 N acetate buffer, pH 4.0, and the eluate collected in two 100 ml. fractions. It was hoped that by using a buffer solution for elution, removal of extraneous N from the resin would be avoided at this stage.

Discussion of Results

The total N recovery in the water wash roughly corresponds to the glycine N content of the solution and the fact that it is rather higher is almost certainly due to the presence of N derived from the resin. The total N found in the second 100 ml. of water used to wash the resin immediately before the start of the column run, was about 0.15 mg. (it is, of course, difficult to make an accurate estimation of such a low concentration of N) and the difference between the water wash N content and the glycine N can be accounted for in this way. The separation of glycine and glutamic acid is apparently complete, for the quantity of N in the second water wash fraction (50 ml.) is comparitively very small.

There/

There is a much larger discrepancy between the eluate N content and the glutamic acid N of the solution and it may be that large quantities of nitrogenous material are inevitably washed out of the resin whenever it is converted into the salt form. A large amount of extraneous N was not expected in the eluate, since according to Consden, Gordon & Martin (1944) the amount of N liberated from the resin can be considerably reduced by reducing the concentration when HCl is used for elution. It was therefore thought that the amount of N liberated from the resin would be a function of pH and that by using a buffer solution with as high a pH as 4.0, it would be possible to prevent N loss from the resin on a scale which would interfere with the estimation of the amino acids by the micro-Kjeldahl method.

Since the high NaCl wash requirement in this experiment was highly inconvenient, in the next experiment the resin is converted into a different kind of buffer system by washing with an acetate buffer.

Solution Q. (Volume - 100 ml.)

Amino Acid	N% of Theor. N	Dry Wt. (mg.)	Mg. N/25 ml.
Glycine	99.0	208.3	9.62
Glutamic Acid .... (hydrochloride)	98.0	267.4	5.03
		Total N Content/25 ml.	14.65

Micro-Kjeldahl Estimations on Solution Q.

(mg. N/25 ml.)

Total N = 14.77

Table 33.

Results:-

Run with 25 ml. Aliquot of Solution Q.

Rate of Flow - ca. 20 ml./hr.

Fractions	Fraction Vol.	Total N Content (mg.)	
Water Wash 1.	100 ml.	9.62	10.08
" "	100 ml.	0.46	
Eluate	100 ml.	0.43	

Total N Recovered = 10.51 mg.

Table 34.

Experiment II - Retention of Acidic Amino Acids by Amberlite IR-4B

The acetate buffer range does not extend as high as pH 7.0 so a buffer solution was prepared with a pH near the top of the range (pH 6.03) and at this pH the acidic amino acids should be satisfactorily retained by Amberlite IR-4B (Cannan (1944)).

Procedure

The same column was washed with 300 ml. molar acetate buffer, pH 6.03, and the excess buffer removed by washing with 200 ml. water. A second test amino acid solution, 'Solution Q', was prepared and a 25 ml. aliquot of this introduced into the column. The amino acids were then washed through the column with 175 ml. water and the water wash collected in two 100 ml. fractions. The column was finally eluted with 100 ml. 0.25 N acetate buffer pH 4.0.

The buffer wash and eluate fractions were concentrated and the volume of each adjusted to 25 ml. Micro-Kjeldahl estimations were carried out on each fraction.

Discussion of Results

The results in Table 34 again indicate satisfactory retention of glutamic acid by the resin, and complete separation from the glycine. Unfortunately, during the elution with 0.25 N acetate buffer, the resin swelled/

swelled to such an extent that flow from the column was virtually stopped, and it was not possible to collect more than 100 ml. of effluent at this stage. The eluate N recovery is so low that it is evident that the wash with 100 ml. 0.25 N acetate buffer has removed only a small proportion, if any, of the glutamic acid from the column, in contrast with the previous experiment, where most of the glycine was recovered in the first 100 ml. of 0.25 N acetate buffer wash.

It appeared that it would be necessary to use some other eluting agent in order to recover the glutamic acid from the column when it was buffered in this way.

Experiment III - Retention of Acidic Amino Acids by Amberlite IR-4B

This experiment was designed to confirm the findings of Experiment II, and to modify the procedure so that the glutamic acid could be recovered conveniently from the column.

Procedure

In order to minimise the effect of resin expansion in reducing the rate of flow through the column, it was decided to increase the free space surrounding the resin particles by rejecting all the particles fine enough to pass through a 60 mesh sieve. The remainder of the resin was used to repack the column.

The resin was regenerated by washing with 200 ml. of 0.5 N NaOH and then buffered by washing with 300 ml. of N acetate buffer, pH 6.03. The rest of the procedure was the same as for the previous experiment up to the elution stage, when again the rate of flow was considerably reduced because of the expansion of the resin.

After collecting 100 ml. of effluent from the wash with 0.25 N acetate buffer, it was found necessary to stir up the resin with a glass rod in order to keep up the rate of flow. The elution was then continued by washing with 100 ml. N acetic acid.

Discussion of Results

On/

Results:-

Runs with 25 ml. Aliquots of Solution Q.

Rate of Flow - ca. 20 ml./hr.

Fractions	Fraction Vol. (ml.)	Total N Content (mg.)	
		Aliquot 1.	Aliquot 2.
Water Wash 1.	100 ml.	9.62	9.57
" " 2.	100 ml.	0.46	0.19
Eluate, 0.25 N Acetate Buffer	100 ml.	0.43	0.43
Eluate	100 ml.	-	5.52

Table 35.

On estimating the N content in the buffer wash and eluate fractions, the results given in Table 35 were obtained. The results from the previous experiment are also reproduced in the table since the two experiments are strictly comparable up to the final stage.

The presence of little more than the blank N value in the second water wash fraction again indicates complete separation of glycine from glutamic acid. The fact that this value is considerably lower than the corresponding value for Aliquot 1. is interesting and is no doubt due to the less finely divided state of the resin in this experiment. The finding of the previous experiment that 0.25 N acetate buffer, pH 4.0, is apparently unsuitable for elution when the resin has been previously buffered with acetate buffer at pH 6.03, has been confirmed. By contrast N acetic acid seems to be quite an efficient eluting agent since practically all of the glutamic acid N has been recovered in the acetic acid wash.

DISCUSSION

Although the work presented in this Section is incomplete, sufficient progress has been made to indicate that it should not be a difficult matter to fractionate a protein hydrolysate into the acidic, basic and neutral fractions completely, and without loss of material. It is true that the few experiments carried out in this Section have only demonstrated that it is possible to separate glycine from glutamic acid quantitatively by using a buffered column of Amberlite IR-4B, and that one would not be justified in assuming that aspartic acid would behave similarly to glutamic acid simply on the ground that aspartic acid is slightly more acidic.

However, it is clear from the studies of other workers on the retention of the acidic amino acids on columns of Amberlite IR-4B, that aspartic acid does not behave in any way abnormally, and that the outstanding difficulty when such methods are applied to protein hydrolysates has been the competitive interference of the basic amino acids. Thus there seems little reason to doubt that the fractionation of more complex solutions could be effected using the same procedure as in Experiment III. If this assumption can be made, and the procedure in Experiment III used to fractionate non-basic fractions derived from hydrolysates of proteins, the/

the work described in this thesis has gone a long way towards eliminating one of the principal disadvantages of the scheme of separation propounded by Winters & Kunin (1949) -- that a weak base anion exchanger will not remove all of the glutamic acid and aspartic acid present in a mixture containing the basic amino acids.

GENERAL DISCUSSION

Although the principal object of this work has been to confirm the differences between several of the histones analysed by the Stedmans for electrophoretically homogeneous histones, some of the findings made incidentally during the course of the author's investigations are of value in relation to other problems. Those findings which are, in the view of the author, of general importance, as distinct from findings of significance only in relation to these investigations, are summarised briefly in this discussion.

In Section I it was observed that there was appreciable decomposition of arginine during electrodialysis of the basic amino acids and it was consequently concluded that, because of this and the general inelegance of the method, electrodialysis is unsuitable as a means of separating basic fractions from protein hydrolysates when this is a preliminary step in their analysis. If the method of analysis of MacPherson (1946) is used, electrodialysis is particularly unsuitable since the lysine N values, which are calculated by difference from the total basic N content and the sum of the arginine N and histidine N values, will also be affected.

In Section II it was discovered how to separate the basic amino acids on a column of Amberlite IRC-50 in the presence of fairly high concentrations of sodium/

sodium ions in test amino acid solutions, thus making it possible to make adjustments in the pH of protein hydrolysates by titration with NaOH.

In Section III it was observed that the alkaline hydrolysis method of estimating arginine, originally designed by Plimmer (1916) gave erroneously high results when applied directly to hydrolysates of histones owing to the decomposition of non-basic material, and it is a fair assumption to make that many other proteins will also contain appreciable amounts of non-basic material yielding ammonia on prolonged treatment with alkali.

Finally, in Section IV a problem which has occupied the attention of Cannan (1944) and Winters & Kunin (1949), that the presence of basic amino acids in protein hydrolysates prevents the quantitative adsorption of the acidic amino acids by Amberlite IR-4B, has been resolved.

Because of the many disagreements in the literature in relation to ion exchange phenomena, no attempt has been made to make an independent study of the physico-chemical properties of ion exchange resins, and most of the trial experiments carried out with resin columns are purely empirical in character.

As has been pointed out by Boyd (1951), ion exchange equilibria have been interpreted in many different ways and the various approaches may be divided/

divided into several broad classes as follows. 'The exchange equilibrium has been regarded (a) as analogous to a reversible double decomposition reaction to which the mass action law may be applied, (b) as analogous to an ionic adsorption reaction, capable of being described by the Langmuir isotherm for a mixture of adsorbates, (c) as a problem of ions at a charged surface or (d) as a Gibbs-Donnan distribution between two homogeneous phases.' The fact that so many interpretations are possible illustrates the complexity of ion exchange phenomena and justifies the empirical nature of the author's experiments. However, whenever the author has attempted to give a theoretical interpretation to the results obtained with ion exchange columns, the first of these approaches has been used.

The principal object of this work, which was the demonstration of species and cell specificity for main component histones, has been achieved. There were several important reasons why such a demonstration became necessary at the time the work was commenced. In papers published in 1944 and 1947 the Stedmans drew the conclusion from their extensive studies on the composition of cell nuclei that the basic proteins could not provide the material of which the genes were composed and they advanced cogent arguments in support of this view. The essence of the Stedmans'

case/

case at this time was as follows:- The fact that basic proteins are universally present as major components of cell nuclei indicates that their function must be connected with the control of hereditary characteristics by the genes with which they are intimately associated in the nucleus. However, histones or protamines cannot constitute the actual material of the chromosomes, for it would indeed be remarkable if the tremendous differences in form and behaviour found amongst the higher animals could be the result of differences rung on the histone or protamine molecule. If these substances did constitute the material of the chromosomes, one would expect to find that some sort of relationship existed between species possessing cell nuclei containing protamines, and that such species would be very much simpler in their structure and organisation than species containing histones in their sperm heads. As it is, the herring and the salmon do not particularly resemble each other and it cannot be imagined that the differences between a herring and a cod could be correlated with the chemical differences between clupeine and the histone from cod sperm heads. Nor, for that matter, could it be imagined that the differences between the individual genes in any species of fish could be rung on a simple protamine molecule.

The/

The fact that nuclei of the somatic cells of the salmon have been shown to contain a histone in place of the expected protamine (Stedman & Stedman (1944)) is a piece of conclusive evidence against histones or protamines being constituents of the chromosomes. The chromosomes of all the cell nuclei in any given organism must be chemically identical, unless we are to discount the chromosome theory of inheritance, and it is not conceivable that the chromosomes of the somatic cells of the salmon could consist of a histone while the chromosomes of the sperm cells consisted of protamine.

The presence of a protamine in salmon sperm heads and histones in salmon somatic cells was in itself an example of cell specificity, and the fact that there was also a difference in amino acid composition between the histone from salmon liver cell and salmon erythrocyte nuclei suggested that cell specificity might be a general phenomenon. By 1951 the Stedmans had widened the scope of their analyses of histones prepared from various kinds of cells to such an extent that they were able to claim that this was the case, and to advance the hypothesis that the basic proteins of cell nuclei are gene inhibitors.

The findings of this work are in accordance with this hypothesis, and in addition they make it clear that the differences observed by the Stedmans were not/

not simply due to the presence of different proportions of the two component histones in the different kinds of nuclei studied. Analyses of main component histones from two types of cell from two species have provided one example of species specificity, the difference in amino acid composition observed between the thymus histone from the ox and the fowl, and one example of cell specificity, the difference between the composition of the histones from fowl thymus gland and fowl eryhtrocytes.

If the Stedmans' hypothesis is assumed to be correct, in any given type of cell, say, a liver cell, all the genes will be prevented from exercising their functions, or inhibited, by the presence of the histones characteristic of liver cells, with the exception of those genes which are responsible for the peculiar characteristics of liver cells. Since the proportion of the total number of genes actively controlling the characteristics of any specific type of cell will be minute, it follows that the histones in the different types of cells in an organism will be inhibiting much the same assortment of genes. So on the basis of the hypothesis, one would expect that histones from the different types of somatic cells from any organism would closely resemble each other in chemical compesition; indeed, one would only expect differences in chemical composition to/

to be demonstrable in a few cases. The fact that there was no obvious difference observed between the main component histones from ox liver and ox thymus gland is therefore not in any way inconsistent with the Stedmans' hypothesis.

In the germ cells, where none of the genes is exercising its function, all the genes will be inhibited so that, in general, one would expect more marked differences in composition between basic proteins prepared from germ cells and somatic cells than between histones prepared from different kinds of somatic cells. This is certainly true in the case of the salmon as has already been pointed out.

ACKNOWLEDGEMENTS

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APPENDIX

METHODS

MICRO-KJELDAHL ESTIMATIONS

All micro-Kjeldahl estimations referred to in this work were carried out using the method described by Chibnall, Rees & Williams (1943), but with the following single modification. The ammonia was absorbed in 10 ml. 2% boric acid for each determination and estimated by direct titration with 0.01 N HCl.

Whenever possible, aliquots were so chosen that the equivalent HCl titre was approximately 5 ml. For the 5 ml. micro-burette employed for all titrations, the volume of a drop was ca. 0.025 ml. so that an error of a single drop in the titration was, for most estimations, equivalent to an error of 0.5%. The end-point in the titration, in which the indicator is a mixture of five parts 0.1% bromo-cresol green to one part 0.1% methyl red in 95% alcohol, is such that a N difference corresponding to less than a drop of 0.01 N HCl cannot be detected visually; there is thus a possible experimental error inherent in all the micro-Kjeldahl results quoted of $\pm$ 0.5%.

It was found convenient to leave aliquots for estimation to digest overnight, the estimations of ammonia being invariably carried out the following morning. Each aliquot thus received at least 15 hours digestion, the risk of incomplete digestion discussed by Chibnall, Rees & Williams being thereby/

Results:-

N Contents of Amino Acids

Amino Acid	Source	N% of Theoretical N
Arginine	Roche	98.4, 98.25, 98.1
Histidine	Roche	99.6, 99.1
Lysine .... (dihydrochloride)	Roche	97.2, 98.5
Glycine	B. D. H.	99.2, 99.0
Aspartic acid	B.D.H.	98.0
Glutamic acid ...... (hydrochloride)	B.D.H.	98.6

Table 36.

thereby avoided.

All micro-Kjeldahl estimations were carried out in duplicate. The figures reported are the mean values from duplicate results so obtained. A reagent blank was included in each batch of aliquots for digestion.

Micro-Kjeldahl Estimations of N Contents of individual Amino Acids

As composite amino acid solutions simulating protein hydrolysates were used throughout this work it was necessary to test the purity of the amino acids to be employed. A convenient way of doing this was to compare the percentage N as determined by the micro-Kjeldahl method with the percentage N theoretically present for each amino acid.

Standard solutions of arginine, histidine, lysine dihydrochloride, glycine, aspartic acid and glutamic acid were prepared and micro-Kjeldahl estimations carried out on suitable aliquots from each solution. The percentage of the theoretical N was calculated in each case and the results obtained are presented in Table 36.

Each amino acid was dried to constant weight in vacuo over P_2O_5 before the preparation of the standard solutions, but it was found that there was only a significant reduction in weight in the case of arginine. The arginine required several days drying/

drying before constant weight was attained and it lost approximately 10% of its original weight.

If it is assumed that any impurity present in these amino acids is non-nitrogenous, i.e. that their N contents are due entirely to the particular amino acid present, then these percentages will represent the percentage purity of the amino acids. All trial experiments with standard amino acid solutions have been based on this assumption for the foregoing percentages have been used as a basis for calculation of the N content of the various solutions employed.

Results:-

Estimations on Standard Arginine Solutions

	N% of Theoretical N
	96.1
	95.7
	95.9
	96.0
	95.5
	95.7
Mean Value	95.8

Table 37.

ESTIMATION OF ARGININE BY ALKALINE HYDROLYSIS

The method used was an adaptation of the original method of Plimmer & Rosedale (1925), designed by Stedman (1951) for use on a semi-micro scale. This method differs only in detail from that described by MacPherson (1946), and both workers are agreed that recovery of arginine does not appear to be quantitative under the conditions of the determination; e.g. MacPherson has reported a recovery of 97.0% of the theoretical N on alkaline hydrolysis for four hours of a specimen of arginine monohydrochloride presumed to be absolutely pure. On the basis of this recovery, MacPherson applies the correction factor 1.03 to all results for arginine N obtained by the alkaline hydrolysis method.

The author was not fortunate enough to possess a specimen of arginine completely free from impurity but examination of a 10 g. sample purchased from Roche Products Ltd. indicated a fairly high degree of purity, and this sample has been used in all trial experiments described in this thesis. The results listed in Table 37. are N percentage recoveries of the theoretical N , found by alkaline hydrolysis of portions of this sample for a period of six hours. The recovery is in every case more than 2% less than that already found by micro-Kjeldahl estimation (98.25%), so that these findings substantiate/

substantiate MacPherson's claim that arginine is not completely hydrolysed under the conditions of the method. However, it seemed to the author that one would only be justified in applying the appropriate correction factor if the impurities responsible for the difference between the Kjeldahl N and the theoretical N values were known to be non-nitrogenous. It seemed reasonable to assume this in view of the remarkably close agreement between the recovery obtained by the author (97.6% of Kjeldahl N) and that reported by the Stedmans (1951), (97.7%), for a different arginine specimen. Hence all values for arginine N obtained by the alkaline hydrolysis method which are quoted in this work, have been previously corrected by multiplying by the factor, 1.024.

This correction factor is somewhat smaller than that that used by MacPherson (1.03), presumably because a six instead of a four hour period is used for hydrolysis and the alkali employed is rather more concentrated. Under these conditions one would expect hydrolysis to be more complete than under the rather milder conditions used by MacPherson.

The application of the correction factor has been to a certain extent justified during the course of the work by the excellent recoveries of arginine N obtained in Experiment VII of Section II from a synthetic hydrolysate (99.0%), for it seems unlikely that such recoveries could have been obtained if the difference/

Results:-

Weight N/25 ml. Standard Arginine Solution (mg.)

	Arginine N (Uncorrected values)	Kjeldahl N	N% of Kjeldahl N accounted for
Standard Arginine	7.38	7.51	98.2
Eluate	7.36	7.50	98.1

Table 38.

difference between the Kjeldahl N and arginine N values had been caused wholly by the presence of a non-basic nitrogenous contaminant. However, the question could only be settled conclusively by studying recoveries of arginine from a specimen free of nitrogenous impurity. It was therefore resolved to clarify the issue by purifying the arginine specimen already referred to by the application of the technique evolved in Section II for separation of the basic fraction from histone hydrolysates.

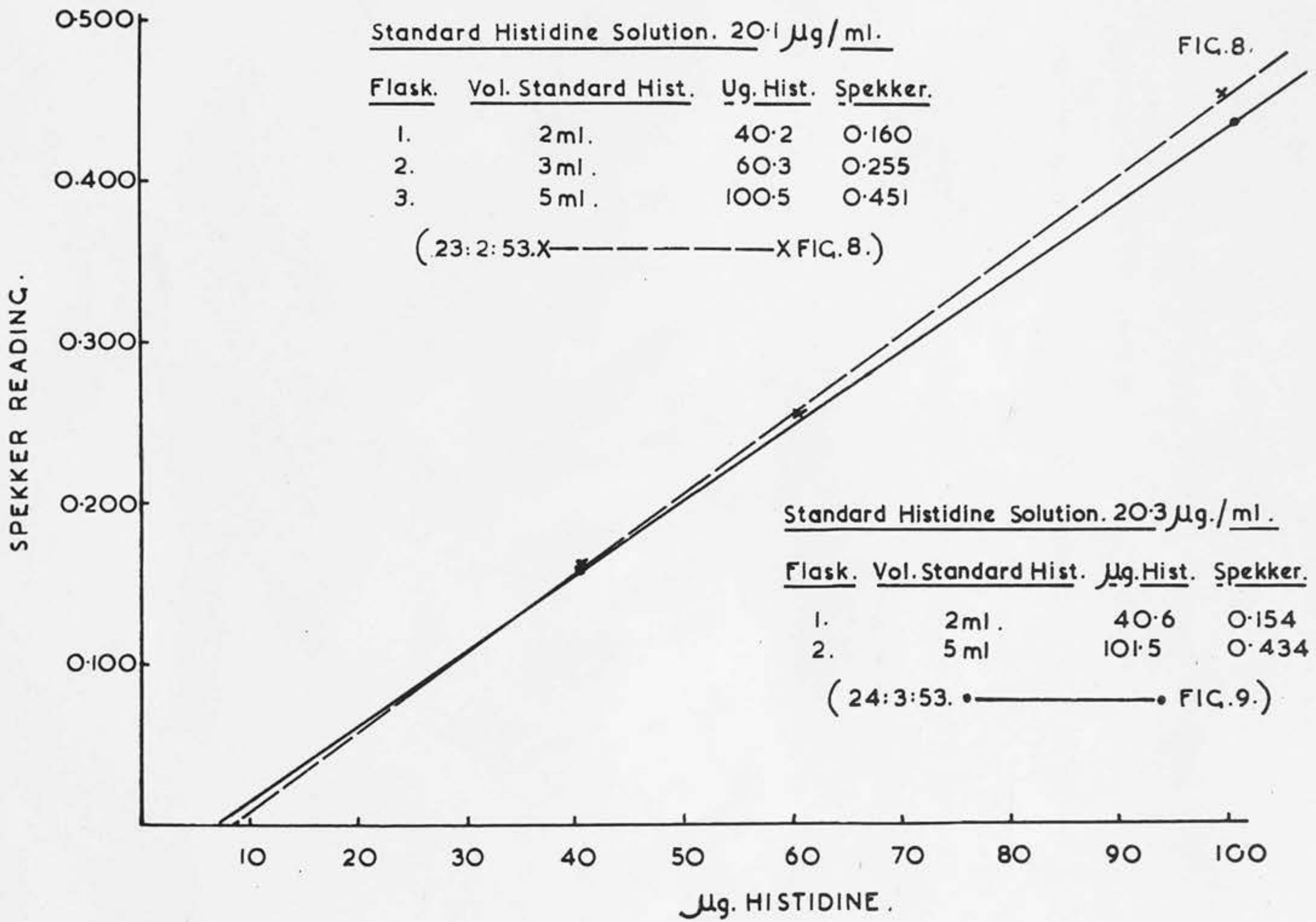
<u>Purification of Arginine using Amberlite IRC-50</u>

The procedure used for purification was that already described in Section II for synthetic protein hydrolysates. A standard arginine solution was prepared of concentration ca. 1 mg./ml. and micro-Kjeldahl and arginine N estimations carried out on aliquots of this solution. A 25 ml. aliquot of this solution was introduced into a column of Amberlite IRC-50 which had been prepared in the usual manner, and the column was washed with 175 ml. water and finally eluted with 100 ml. N HCl. Micro-Kjeldahl and arginine N estimations were carried out on the eluate and results were obtained as given in Table 38.

Since the ratio of arginine N, as estimated by alkaline hydrolysis, to the N as estimated by the micro-Kjeldahl/

micro-Kjeldahl method is not significantly altered on purifying the arginine by this method, the results of this experiment are again consistent with MacPherson's statement that low recovery of arginine is due to incomplete hydrolysis, although, of course, it could be argued that the discrepancy was due to the presence of a basic amino acid contaminant.

In this experiment, the percentages of the Kjeldahl N accounted for are rather higher than have been found previously, but the difference between these results and the expected proportion of 97.6% is less than 1% in each case and was not considered to be significant.



CALIBRATION CURVE - ESTIMATION OF HISTIDINE.
Standard Histidine Solution. 20·1 μg/ml.
Flask. Vol. Standard Hist. Ug. Hist. Spekker.
1. 2ml. 40·2 0·160
2. 3ml. 60·3 0·255
3. 5ml. 100·5 0·451
(23:2:53. X——————X FIG. 8.)
FIG. 8.
Standard Histidine Solution. 20·3 μg./ml.
Flask. Vol. Standard Hist. μg. Hist. Spekker.
1. 2ml. 40·6 0·154
2. 5ml 101·5 0·434
(24:3:53. •——————• FIG. 9.)
SPEKKER READING.
0·500
0·400
0·300
0·200
0·100
10
20
30
40
50
60
70
80
90
100
μg. HISTIDINE.

THE COLORIMETRIC ESTIMATION OF HISTIDINE

The modification of the Pauly reaction described by MacPherson (1946) was used throughout this work.

During preliminary work on histidine estimations it was found that the gradient of the calibration curve changed when determined at different times although the same set of reagents was used. For this reason, it was decided to plot a new calibration curve each time histidine estimations were made, and all histidine results quoted have been determined in this way. A freshly prepared histidine solution was invariably used as a standard.

The two typical curves given opposite, which were determined with the same set of reagents, illustrate the change in gradient with time.

Most of the estimations of histidine carried out in this work involved the determination of ca. 70 μg. histidine. The error for the corresponding reading of the Spekker absorptiometer is of the order of $\pm$1%. There is thus in the colorimetric estimation of histidine an intrinsic error of ca. $\pm$ 1% involved in the reading of the instrument alone.

THE SAKAGUCHI REACTION

The following modification of the Sakaguchi reaction (Sakaguchi (1925)) was used in testing for arginine throughout this work. 2 ml. of 5% NaOH are added to 5 ml. of the solution to be tested and this is followed by two drops of a 1% solution α naphthol in alcohol, and a single drop of freshly prepared 10% sodium hypochlorite. On shaking a bright-red colour quickly develops in the presence of arginine in dilutions up to 1 : 10^6.

THE NINHYDRIN REACTION

The ninhydrin reaction was used in Section III of this work to test for the presence of glycine in the presence of lesser concentrations of the basic amino acids. 5 drops of freshly prepared 0.2% ninhydrin solution were added to each 5 ml. of the solution to be tested and on boiling for two minutes the colour developed in the presence of glycine in dilutions up to 1 : 10^4.

In the experiments in Section III where qualitative amino acid tests were applied to the various fractions from column runs with test amino acid solutions, the maximum concentration of any of the basic amino acids which could possibly be present in any of the buffer wash fractions tested was estimated at 1 : 3 x 10^3, and at this concentration a negative result was obtained on testing solutions of the basic amino acids under the conditions described above. Thus the ninhydrin reaction could be used to test for the presence of glycine in buffer wash fractions without interference from any basic amino acids which might also be present.

ANALYSIS OF HISTONES

Hydrolysis

100-200 mg. portions of histone sulphate were used for each hydrolysis. The protein was dried to constant weight and hydrolysed by refluxing it with 7 ml. 7 N HCl for 30 hr. Before commencing hydrolysis the protein was brought into solution by warming it with the hydrochloric acid on the water bath for a few minutes. The hydrolysate was concentrated in vacuo at least two times, the pH adjusted to 4-6 by titration with 0.5 N NaOH, and the volume of the solution made to the required standard volume -- 30, 40, or 50 ml.

Column Runs with Amberlite IRC-50

The resin was invariably prepared for use by siphoning 200 ml. of N acetate buffer slowly through the column, which contained about 13 ml. of resin (20 x 1.5 cm.). The column used was fitted with a levelling device designed to maintain the surface of the liquid in the column at a level just above that of the resin. Thus the buffer solution could be allowed to siphon through the column overnight without risk of the column running dry. The excess buffer was finally removed by washing the column with 200 ml. water. The column was then ready for use and a 25 ml. aliquot of the histone hydrolysate was introduced. When this volume had run into the column/

column the non-basic amino acids were washed through with 175 ml. water. The 200 ml. effluent containing the non-basic amino acids was designated the water wash.* The basic amino acids were then eluted from the column with 100 ml. N HCl and the column prepared again for further use by washing with N acetate buffer. The water wash and eluate fractions were then concentrated in vacuo and their volumes adjusted to 25 ml. in each case. The rates of flow from the column were invariably kept below 20 ml. / hr.

*For some of the earlier analyses the non-basic amino acids were washed of the column with 0.1 N acetate buffer, pH 4.7, but this practice was eventually discontinued for the reasons given in the Discussion at the end of Section II.

VAN SLYKE METHOD OF NITROGEN DISTRIBUTION

The details of the method are given in Van Slyke's original paper (1911) on the subject, and a comprehensive set of instructions for carrying out amino N determinations is included in "Quantitative Clinical Chemistry (Methods)" by Peters & Van Slyke (1932).

For a solution containing the three basic amino acids the difference between the total N content and the amino N content of the amino acids represents the non-amino N content, and since when treated with nitrous acid for one hour, lysine gives off all, arginine one fourth, and histidine one third of its content of N,

Non-amino N = 3/4 x arginine N + 2/3 x histidine N

i.e. Histidine N = 3/2(non-amino N + 3/4 x arginine N)

In Van Slyke's original paper on the nitrous acid method for determining amino N it was stated that cystine and glycine react peculiarly in that they evolve, under the conditions of the determinations, 6-8% more N than the theoretical results. Other naturally occurring amino acids were found to react quantitatively with their α amino groups only, with the exception of lysine which reacted also at the ω position on treatment with nitrous acid for one hour.

Kendrick & Hanke (1937) have published details of a modification of the Van Slyke method which yields/

yields theoretical results for cystine and glycine.

It is reported by Schmidt (1938) that, although the Van Slyke method is supposed to give fairly reliable results for arginine, when the basic amino acids are treated with nitrous acid for 30 minutes some of the nitrogen from the quanidine grouping of arginine will be liberated. Since basic fractions from hydrolysates of histones invariably contain a much larger proportion of arginine than of histidine, any decomposition of the guanidine group of arginine could seriously reduce the accuracy of histidine results obtained in this way.

The following experiment was designed to ascertain the degree of accuracy of Van Slyke estimations of arginine amino N.

<u>Estimations of Amino N with the Van Slyke Apparatus</u>

Since 0.6 mg. amino N is the maximum weight of N which can be conveniently estimated in the Van Slyke apparatus, and this weight is equivalent to 400 mm. Hg at a volume of 2 ml., it was decided that wherever possible, either the volume of the aliquot taken for estimation or the dilution of the solution to be analysed, would be adjusted so that the amount of amino N was equivalent to between 200 and 400 mm. Hg. Since the manometer scale can be read easily to 0.5 mm., the corresponding maximum possible error attributable/

attributable to faulty reading of the manometer scale is only 0.25%.

(i) Estimations on Standard Arginine Solution

A standard amino acid solution, volume 100 ml., was prepared from 64.7 mg. of dry arginine, and one estimation of amino N carried out on a 5 ml. aliquot of this solution. A reaction time of 1 hour was used.

The results obtained was somewhat higher than the amino N content calculated on the basis of micro-Kjeldahl estimations on the same arginine sample.

Results:-

N % of Theoretical N = 98.4

Dry Weight of Arginine = 64.7 mg.

Weight of Arginine/5 ml. = 3.23 mg.

≡ 0.256 mg. amino N

Van Slyke Estimation of Arginine Amino N

(5 ml. aliquot) 1 hr. reaction time

Amino N .... 0.260 mg.

The fact that this result is 1.5% higher than the value expected considerably reduces the value of the Van Slyke method as applied to basic fractions from hydrolysates of histones. In the histone analyses reported in this work the weight of arginine N is usually about seven times the weight of histidine present, so that an error in arginine amino/

amino N values will give rise to aproportionately greater one in the dependent histidine values.

The values for histidine N which have been obtained by the Van Slyke Nitrogen Distribution Method have not been corrected to allow for decomposition of arginine at the guanidine group, and the fact that such values are almost invariably low as compared with the corresponding values obtained by the colorimetric method therefore suggests that there is some decomposition at this position.

It is, unfortunately, impossible to be certain that the disparity between the Van Slyke and the colorimetric histidine values is due entirely to such decomposition. A correction factor of 1.024 is applied to the arginine N values used in the calculation of histidine N, and, as has already been pointed out, the difference between arginine N values obtained by the alkaline hydrolysis and micro-Kjeldahl methods may not be entirely due to incomplete hydrolysis of arginine.

It is certainly interesting that when arginine N values which have not been corrected to allow for incomplete hydrolysis are used in calculating histidine values, much better agreement is obtained. This is illustrated by a comparison of the following values for histidine N, calculated on the basis of corrected and uncorrected arginine N values, with the/

the colorimetric value.

Main Component Ox Liver Histone III

Histidine N (% of total N)

Van Slyke	Colorimetric
3.00, 2.80 (arginine corrected)	3.41
3.61, 3.41 (arginine uncorrected)	

However, when the possibility exists of some decomposition of arginine at the guanidine position on treatment with nitrous acid for one hour, one would not be justified in employing uncorrected arginine values. Thus, even when the Van Slyke method is applied to the basic fractions quantitatively separated from whole hydrolysates, there are still serious disadvantages. There is the possibility of decomposition of arginine at the guanidine group on treatment with nitrous acid, there is the problem of deciding whether or not the correction of arginine N values to allow for incomplete hydrolysis is justified, and there is the considerable magnification of all the experimental errors inherent in the Van Slyke Method of Nitrogen Distribution when applied to basic fractions in which the proportion of histidine is small.

For these reasons little significance has been attached to values for histidine N obtained by the Van/

Van Slyke method and such histidine values are quoted only because it is considered that the fact that it is possible to obtain even approximate values for histidine N in this way, testifies to the purity of the basic fractions.

(ii) Estimations on Standard Lysine Dihydrochloride Solution

A standard solution of lysine dihydrochloride was prepared containing 60.4 mg. dry solid in 100 ml. solution and two estimations were carried out on two separate 5 ml. portions of the solution. Both results obtained agree within 1% with the theoretical amino N value calculated on the basis of Kjeldahl estimations on the same lysine preparation.

Results:-

% of Theoretical N = 98.5

Dry Weight of Lysine dihydrochloride = 60.4 mg.

Weight of Lysine dihydrochloride/5 ml. = 3.02 mg.

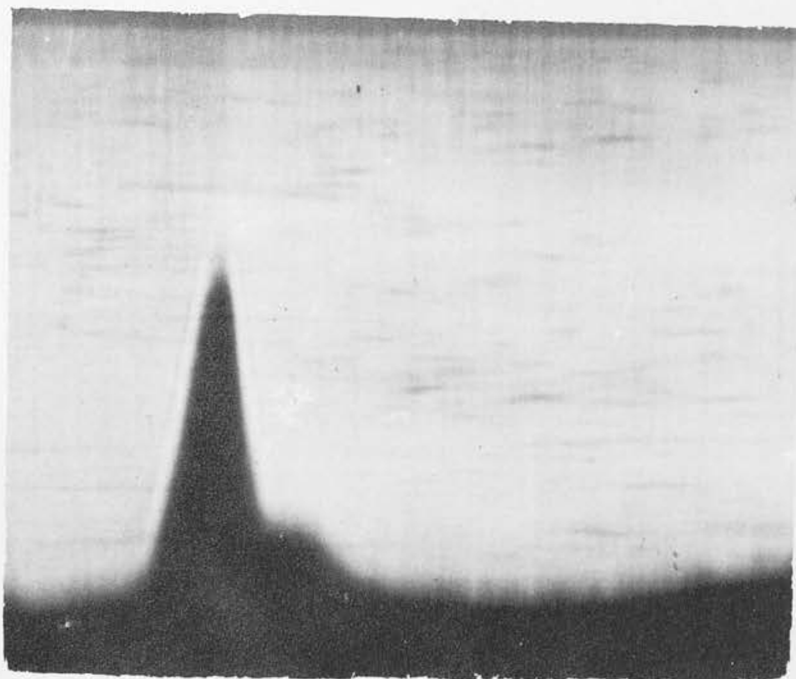
≡ 0.380 mg. amino N

Van Slyke Estimations of Lysine dihydrochloride

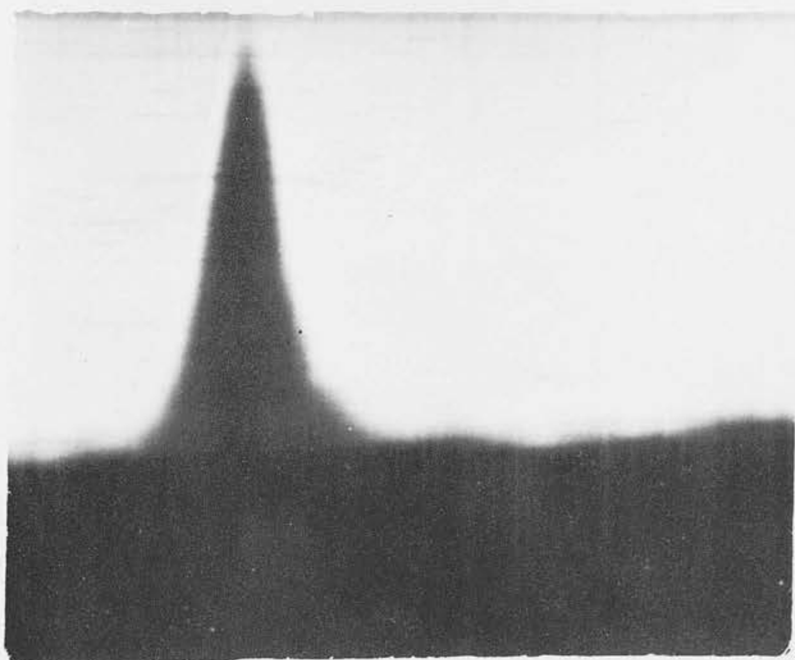
Amino N (5 ml. aliquots) 1 hr. reaction time

Amino N (mg.)

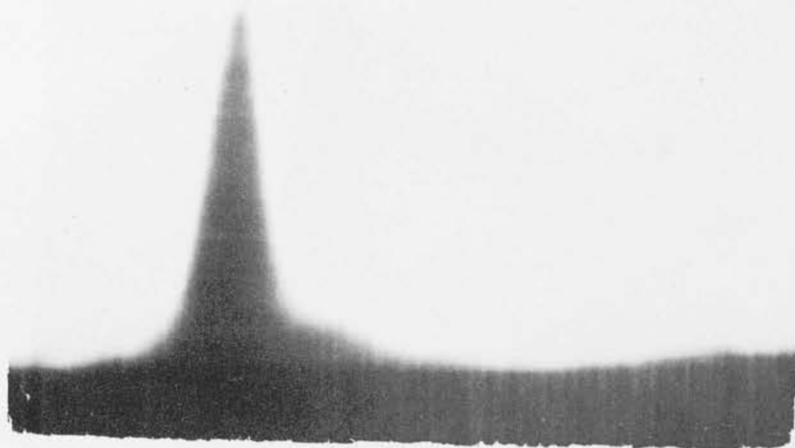
0.377, 0.380



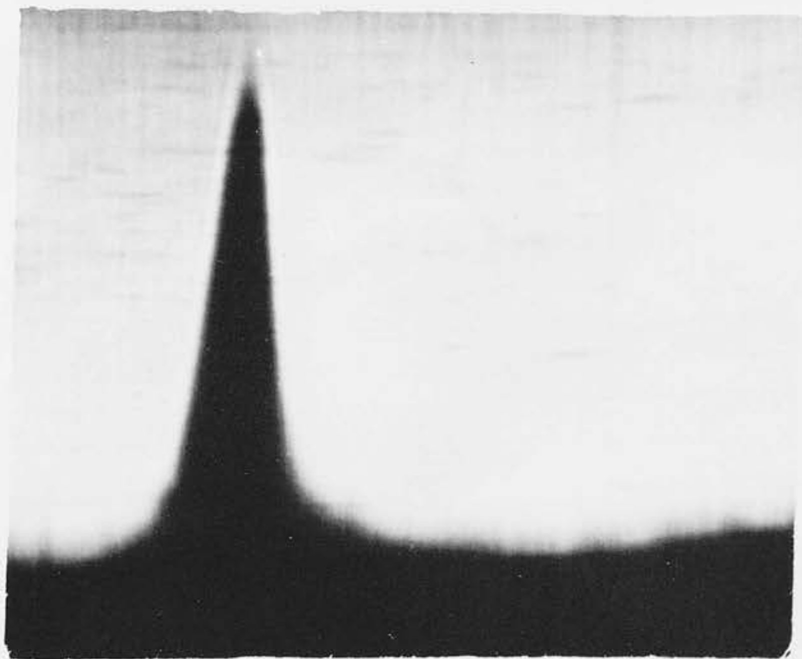
Ox Thymus Histone - IIILI



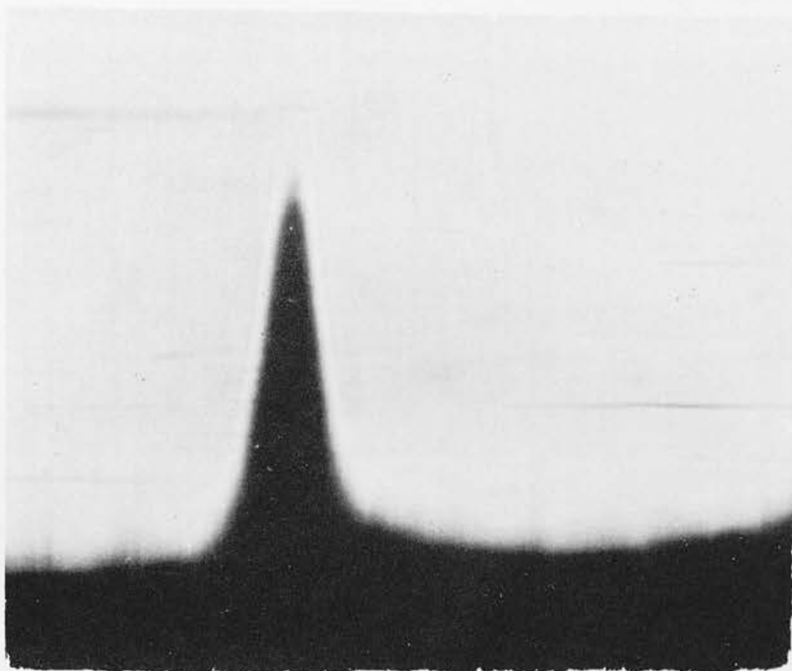
Ox Thymus Histone - Main Component III



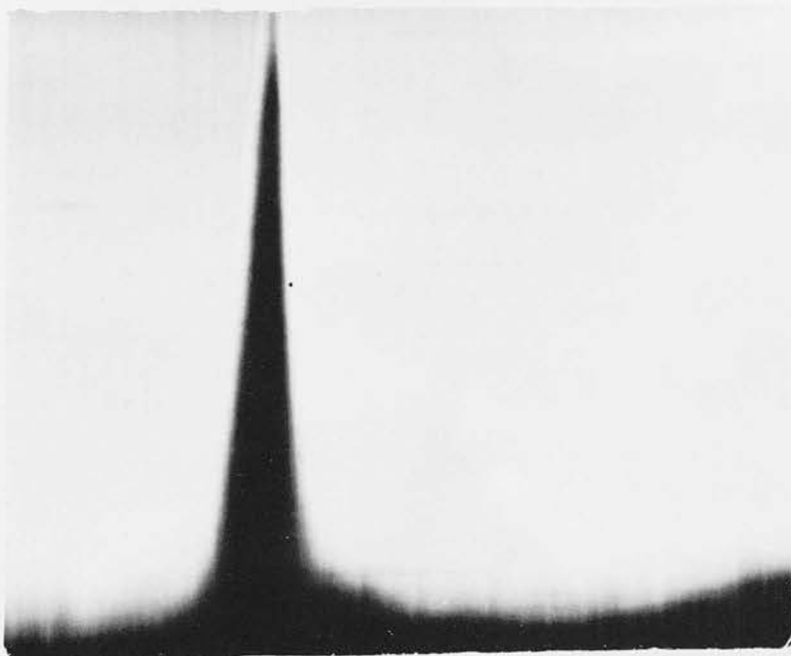
Ox Liver - Main Histone



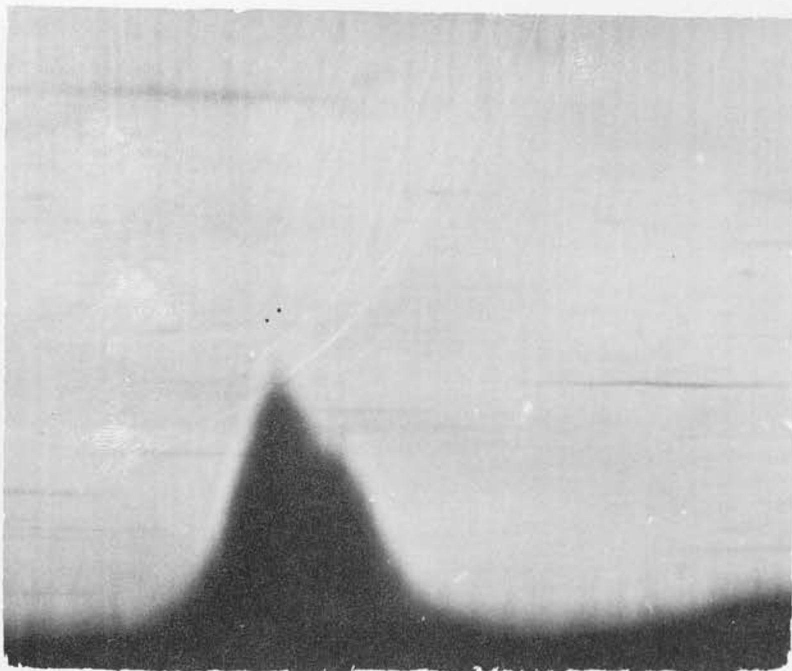
Ox Liver - Main Component Histone III



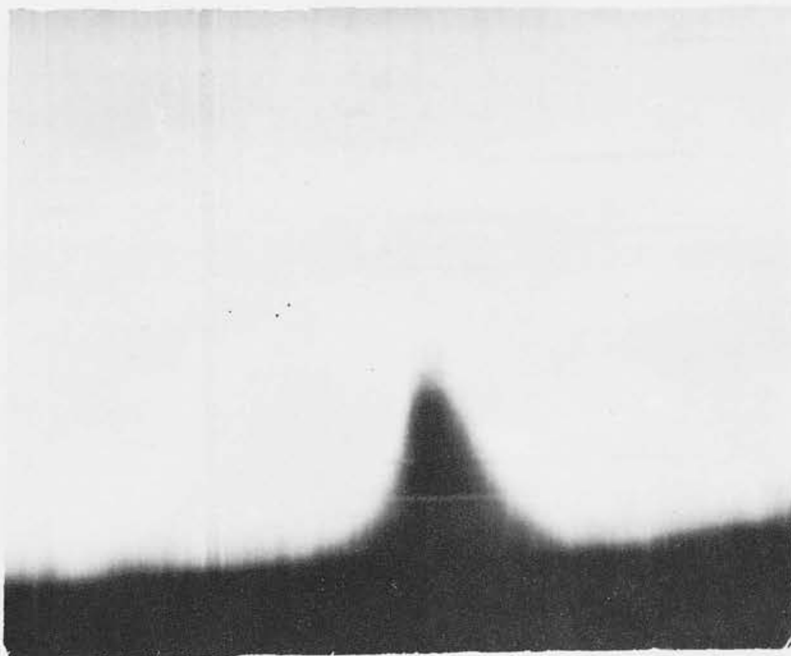
Ox Liver Histone - Main Component II



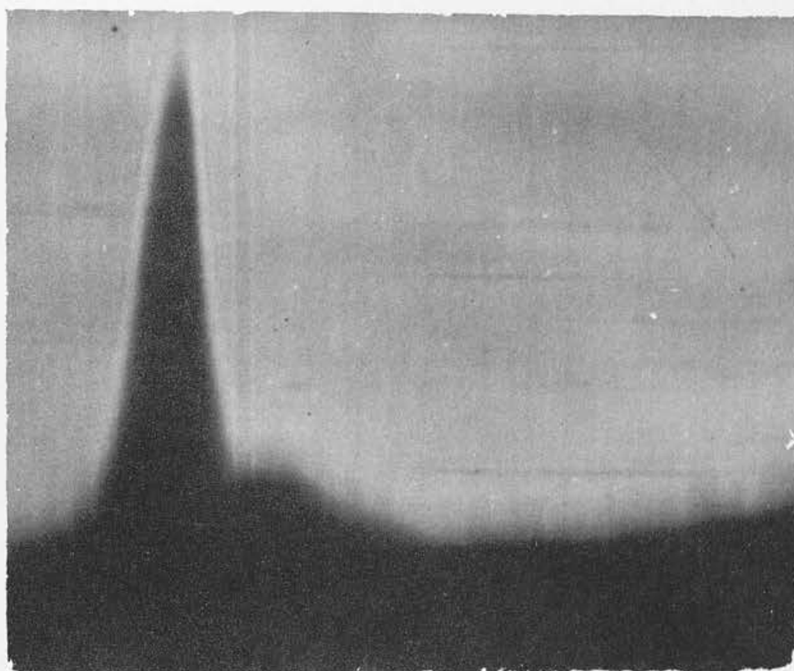
Ox Thymus Histone - Slow Component



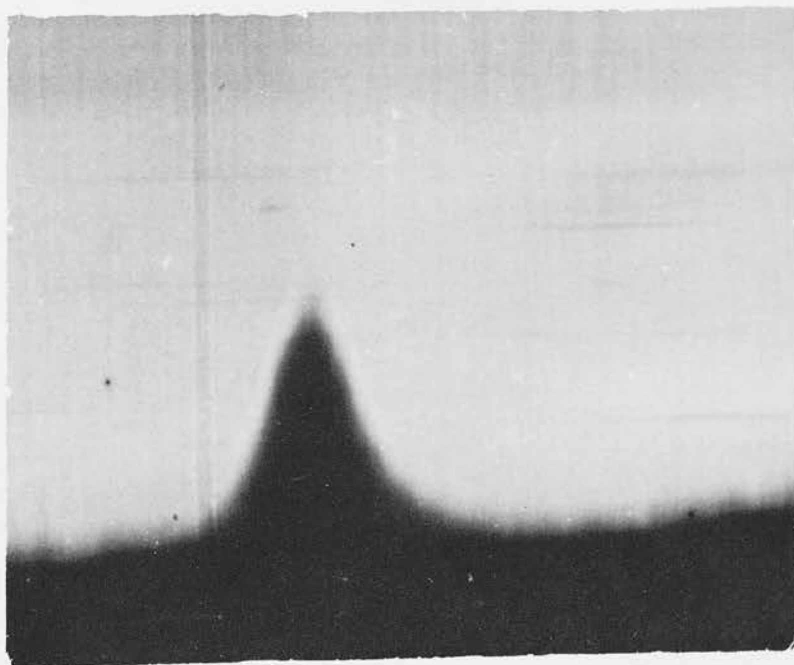
Fowl Erythrocyte Histone - IIIFI



Fowl Erythrocyte Histone - Main Component



Fowl Thymus Histone - Main Component



Fowl Erythrocyte Histone - Slow Component

REFERENCES

Albanese, A.A. (1940). J. biol. Chem., 134, 467.

Block, R.J. & Bolling, D. (1951). The amino acid composition of proteins and foods. Springfield : Thomas.

Boyd, G.E. (1951). Ann. Rev. Phys. Chem., 2, 309

Brunish, R., Fairley, D. & Luck, J.M. (1951). Nature, 168, 83.

Cannan, R.K. (1944). J. biol. Chem., 152, 401.

Cannan, R.K. (1946). N.Y. Acad. Sci., 47 (2), 154.

Chibnall, A.C., Rees, M.W. & Williams, E.T. (1943). Biochem. J., 37, 354.

Consden, R., Gordon, A.H., & Martin, A.J.P. (1947). Biochem. J., 41, 590.

Consden, R., Gordon, A.H. & Martin, A.J.P. (1948). Biochem. J., 42, 443.

Daly, M.M., Mirsk, A.E. & Ris, H. (1951). J. gen. Physiol., 34, 439.

Eadie, E.J. & Leaf, G. (1952). Biochem. J., 50, xxxiv.

Gordon, A.H., Martin, A.J.P., & Synge, R.L.M. (1941). Biochem. J., 35, 1369.

Hamer, D. (1951). Nature, 167, 40.

Kendrick, A.B. & Hanke, M.E. (1937). J. biol. Chem., 117, 161.

Kossel, A. (1928). The protamines and histones. London : Longmans.

Kunin, R. & Myers, R.J. (1950). Ion exchange resins. New York : Wiley.

Leipert, T. & Leberl, E. (1933). Biochem. Zeitschrift, 265, 113.

MacPherson, H.T./

MacPherson, H.T. (1946). Biochem. J., 40, 470.

Moore, S. & Stein, W.H. (1951). J. biol. Chem., 192, 663.

Partridge, S.M. & Westall, R.G. (1949). Biochem. J., 44, 418.

Peters, J.P. & Van Slyke, D.D. (1932). Quantitative clinical chemistry (Methods). London : Bailliere.

Plimmer, R.H.A. (1916). Biochem. J., 10, 115.

Plimmer, R.H.A. & Rosedale, J.L. (1925). Biochem. J. 19, 1020.

Sakaguchi, S. (1925). J. Biochem., Japan, 5, 143, 159.

Schmidt, C.L.A. (1938). The chemistry of the amino acids and proteins. Baltimore : Bailliere.

Sperber, E. (1946). J. biol Chem., 166, 75.

Stedman, E. & Stedman, E. (1944). Biochem. J., 38, xxvi.

Stedman, E. & Stedman, E. (1947)a. Symp. Soc. Exp. Biol., 1, 232.

Stedman, E. & Stedman, E. (1947)b. Cold Spr. Harb. Symp. Quant. Biol., 12, 224.

Stedman, E. & Stedman, E. (1951). Phil. Trans. (b) 235, 583.

Stein, W.H. & Moore, S. (1951). J. biol. Chem., 190, 106.

Tristram, G.R. (1947). Nature, 160, 637.

Van Slyke, D.D. (1911). J. biol. Chem., 10, 15.

Winters, J.C., & Kunin, R. (1949). Ind. Eng. Chem., 41, 460.