CCXXXVII. EXPERIMENTS ON AMINO-ACIDS

II. AN EXPERIMENT ON THE SEPARATION OF AMINO-ACIDS BY MEANS OF THEIR N-ACETYL DERIVATIVES

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THE partition coefficients of acetamino-acids between water and chloroform have been shown in Paper I of this series to be distributed over a wide range. It should therefore be possible to make use of this property for the separation of amino-acids. By constructing an extraction train in which water and chloroform flow counter-current at a controlled flow-ratio without serious entrainment it should be possible to achieve separation of a mixture of acetamino-acids by injecting it at the middle of the train. The theory of the separation is identical with that of fractional distillation, and concrete problems lend themselves to graphical treatment on the McCabe-Thiele diagram [McCabe & Thiele, 1925]. The possibilities and advantages of extractional fractionation have been stressed by Cornish *et al.* [1934], who describe an all-metal apparatus for the purpose. Dr A. J. P. Martin (unpublished work) designed and put into operation at the Dunn Nutritional Laboratory, Cambridge, an all-glass apparatus, working with the phases of a petroleum-methyl alcohol mixture. He and I have jointly been engaged on the design and construction of a train capable of handling chloroform-water phases, but this is not completed, and the present paper is concerned with the preliminary chemical problem of converting an acid hydrolysate of a protein into a mixture of those acetamino-acids which, on the basis of the figures given in Paper I of this series, might be expected to be fractionated in such a train.

Before describing the procedure adopted, it is desirable to state the reasons why acetyl derivatives rather than other acylamino-acids are used. On general grounds, it is likely that the smaller the acyl group that is introduced into the molecule, the less will it dominate the physical properties of the resulting compound, and the greater will be the differences between the partition coefficients of successive members of a homologous series of acylamino-acids. The smallest available acyl group is formyl, but formylation of amino-acids is not a convenient or gentle process, since it requires heat and dehydrating conditions. The next smallest group, acetyl, may be conveniently introduced in aqueous solution at low temperature, and involves the use of readily available reagents. For the above reasons it was not thought very hopeful to explore the use of other acyl groups for this type of separation.

Neuberger [1938, 2] showed that in the course of acetylating arginine and histidine by ketene and NaOH in aqueous solution, considerable racemization occurs. Dr Neuberger and I accordingly investigated the action of these reagents at 0° on *l*-glutamic acid, acetyl-*l*-glutamic acid, *l*-methionine, *d*-leucine, *N*-formyl-*d*-leucine, acetyl-*d*-leucine, and *d*-norleucine. We observed in each case considerable racemization. Further details of these experiments need not be given here,

since Cahill & Jackson [1938], Jackson & Cahill [1938] and Cahill [1939] have studied the phenomenon more closely.

Du Vigneaud & Meyer [1932] have shown that acetic anhydride and NaOH used in aqueous solution at 0° acetylate without appreciable racemization. As this procedure had given uniformly satisfactory results in the acetylation of individual amino-acids, it was decided to try to apply it to a known mixture of amino-acids.

It was found, by Van Slyke amino-N determinations, that a single treatment of a solution of mixed amino-acids with acetic anhydride and NaOH resulted in the acetylation of 80–95 % of the amino groups present. The problem was to obtain from an acid hydrolysate of a protein by acetylation a reasonably high yield of those acetamino-acids which, from the evidence presented in Paper I of this series, might be expected to be capable of fractionation in a suitable chloroform-water counter-current train. It is clearly desirable that this mixture should be free from salt and from non-acetylated amino-acids. It was found that this could be satisfactorily accomplished by acidifying the acetylation mixture and subjecting it to prolonged extraction by chloroform in the Neuburger [1938, 1] extractor. The mixed acetylated and non-acetylated amino-acids remaining in the aqueous layer after extraction could be separated nearly completely from the large quantities of salt present by evaporation to dryness *in vacuo*, followed by extraction with hot alcohol. They were then acetylated again, as before, and again subjected to continuous extraction with chloroform. The whole process was repeated once more.

In the experimental part of this paper the behaviour of a known mixture of amino-acids under this treatment is described in detail. The mixture was intended to simulate a gelatin hydrolysate, and lacked cystine and tryptophan. It would obviously be desirable to repeat the experiment with a mixture containing these amino-acids, as they are responsible for much secondary change when such a mixture is boiled in acid solution.

EXPERIMENTAL

All samples used in analysis were aliquots; allowance has been made for the samples removed at each stage for analysis, and all figures given in the experimental part of the paper are therefore to be referred to the original quantities used, as detailed in Table I.

Table I. *The mixture of amino-acids used in the experiment*

Compound	N (g.)	Amino-N (g.)	N as % of total N
Glycine	1.170	1.170	31.4
<i>DL</i> -Alanine	0.354	0.354	9.5
<i>L</i> -Valine	0.062	0.062	1.7
<i>L</i> -Leucine	0.189	0.189	5.1
<i>DL</i> -Serine	0.065	0.065	1.7
<i>L</i> -Phenylalanine	0.036	0.036	1.0
<i>L</i> -Methionine	0.048	0.048	1.3
<i>L</i> -Proline	0.308	0.000	8.3
<i>L</i> -Aspartic acid	0.107	0.107	2.9
<i>L</i> -Glutamic acid	0.140	0.140	3.8
<i>L</i> -Histidine (as hydrochloride)	0.075	0.025	2.0
<i>L</i> -Tyrosine	0.076	0.076	2.0
<i>L</i> -Arginine (as hydrochloride)	0.762	0.190	20.4
<i>L</i> -Lysine (as dihydrochloride)	0.330	0.330	8.9
Total	3.722	2.792	100

The mixture of amino-acids shown in Table I was dissolved in 200 ml. 10 *N* HCl, and refluxed for 5 hr. The resulting solution was evaporated to dryness *in vacuo*. The residue was taken up in water, and again evaporated. This was repeated once more. The product was then taken up in water, and treated with strong aqueous NaOH at 0° until just alkaline to thymolphthalein. The solution was then concentrated to a medium syrup *in vacuo*, and was acetylated with 400 ml. 2*N* NaOH and 40 ml. acetic anhydride, added in small portions in the course of 45 min. The mixture was well shaken and cooled in ice during the acetylation. The resulting mixture was alkaline to thymolphthalein, and was kept at 0° overnight, to make certain that any *O*-acetyl linkage that might have been formed should be broken. (Cf. note on *ON*-diacetyl-*dl*-tyrosine in Appendix to Paper I of this series.) A Van Slyke amino-N determination indicated that 80 % of the amino groups originally present were acetylated, and this figure was unchanged by storage at 0° overnight in alkaline solution.

The mixture was then brought to pH 5 by addition of 6*N* H₂SO₄, and was concentrated *in vacuo* to about 400 ml. Further 6*N* H₂SO₄ was added until the mixture was just acid to thymol blue. The volume was now approximately 500 ml. The mixture was transferred quantitatively to the Neuberger extractor, and extracted with chloroform (Extract 1, Table II). The aqueous solution was concentrated to dryness *in vacuo*, and the residue was thoroughly extracted with 800 ml. of boiling absolute alcohol, used in five successive portions. The salt residue (Residue 1, Table II) was dissolved in water, and its N content was determined on an aliquot. The alcoholic extract was concentrated to dryness *in vacuo*, the residue was dissolved in water, made alkaline and acetylated as before. The mixture was kept alkaline at 0° overnight as before. This now also served to break down any ethyl ester groupings that had resulted from the extraction with hot absolute alcohol under acid conditions [cf. Hollander & du Vigneaud, 1931]. The degree of acetylation achieved corresponded this time to acetylation of 98 % of the amino-N present in the original mixture, including that already removed in extract 1. The solution was acidified and extracted as before; this time the chloroform was changed once in the receiver of the Neuberger apparatus, so two extracts resulted. These are referred to as extracts 2A and 2B in Table II. The process of extraction occupied 3 days, as the apparatus was turned off at night, and by the end of this period an amino-N determination showed that slight hydrolysis had occurred in the aqueous layer, the degree of acetylation now corresponding to 94 % of the amino-groups of the original mixture.

The whole process was repeated once more, giving rise to Residue 2 and Extract 3. 98 % acetylation was again obtained. The final aqueous layer was freed from salt as before, giving rise to Residue 3 and "Unextracted N compounds". Direct N determinations (Kjeldahl) were carried out on all these fractions, and are given in Table II as percentages of the original total N of the mixture. 99.3 % of the original total N was thus accounted for. The number of volumes of chloroform used for each of the four extractions is also given.

Table II. *Scheme of fractionation of the mixture*

N (as % of total N)	Vol. CHCl ₃	Extract	Residue	N (as % of total N)
20.1	19	1	↔ 1	0.1
13.2	47	2A	↔ 2	0.3
3.6	21	2B	←	
5.3	50	3	↔ 3	0.3
			↓	
			"Unextracted N compounds"	56.4

Investigation of the extracts

Each chloroform extract was evaporated to dryness *in vacuo*, and the residue was dissolved in water. In no case was $\text{SO}_4^{=}$ detectable in the resulting solution. In view of the fact that the aqueous layer of the extractor was in each case a nearly saturated solution of Na_2SO_4 this demonstrates the practically complete absence of entrainment in the Neuburger apparatus. This fact has been referred to in Paper I of this series. In this case the absence of $\text{SO}_4^{=}$ from the extracts made possible the use of S balance to determine the distribution of methionine in the extracts, as this was the only other S-containing substance present.

The results of Van Slyke amino-N determinations on the aqueous solutions of the extracts are shown in Table III. This indicates that prolonged boiling of acetamino-acids in chloroform in the presence of acetic acid brings about hardly any hydrolysis.

Table III. *Amino-N determinations on the extracts*

Extract	Amino-N as % of total N of each extract
1	0.06
2A	<0.01
2B	<0.01
3	0.32

An aliquot of the aqueous solution of each extract was hydrolysed at 100° in 2N HCl until the Van Slyke amino-N (as determined after neutralization of free HCl with excess of sodium acetate) reached a constant value. In Table IV the total amino-N of each extract is shown, compared with the total N of each extract. "Non-amino-N" of each extract is found by subtracting the former of these values from the latter. It will be seen that the combined "non-amino-N" totals 354 mg., in good agreement with the proline-N of the original mixture—308 mg.

The only other "non-amino-N" present in the original amino-acids is in the form of arginine and histidine. The former of these was practically absent from the extracts (see below).

Table IV. *Amino-N of extracts after acid hydrolysis*

Extract	Amino-N mg.	Total N mg.	"Non-amino-N" mg.
1	422	716	294
2A	442	469	27
2B	118	127	9
3	166	190	24
Total	1148	1502	354

S was determined on aliquots of the extracts by the method of Bailey [1937]. The results are shown in Table V. They indicate 80 % recovery of methionine in the combined extracts.

Table V. *Total S of extracts*

Extract	S as N (mg.)
1	25.6
2A	13
2B	0
3	0
Total	38.6

(Original methionine-N of mixture = 48 mg.)

The cumulative "total N", "methionine-N" and "proline-" values of the extracts are plotted against total volume of chloroform used for extraction in Fig. 1. The line AA' represents the total N of leucine, phenylalanine, proline, methionine, valine and alanine present in the original mixture. It will be seen that the "extracted N" reaches a value considerably above this. This is due to the extraction of considerable quantities of acetylglycine, which crystallized out in the receiver of the extractor during extractions 2B and 3. The amount extracted is much larger than would be expected from the partition coefficient

Fig. 1. Total N, non-amino-N, and S (as N) extracted from aqueous phase by chloroform after acetylation of amino-acid mixture.

determined for this substance in Paper I of this series. This is undoubtedly due to the high concentration of Na_2SO_4 in the aqueous layer of the extractor, which has a strong "salting-out effect" and is thus very favourable to the extraction, particularly as none of the salt passes into the extract by entrainment. Tyrosine was detected in all the extracts (Millon test, and yellow colour with nitrous acid). Experiments with *N*-acetyl-*L*-tyrosine showed that the "salting-out effect" with this compound is very marked, as has already been noted in Paper I of this series. The possible presence of acetyl-*L*-glutamic acid in the extracts has not been investigated.

Arginine and serine in the extracts

The acid hydrolysate of Extract 1 was tested colorimetrically for arginine by the Sakaguchi method. The test was completely negative. Addition to the test solution of arginine corresponding to the presence in Extract 1 of 0.02 % of the arginine originally present in the mixture resulted in a positive test. From this it is argued that Extract 1 (which contained 20.1 % of the original total N of the mixture) contained less than 0.02 % of the original arginine.

Dr D. D. Woods, while working in this laboratory, observed that *Clostridium Welchii* decomposes serine under anaerobic conditions, liberating H_2 . This does not occur with any of the other compounds present in the mixture of Table I.

Dr Woods carried out bacterial tests on the acid hydrolysate of Extract 1, after it had been neutralized to pH 7 with NaOH. He showed manometrically that no gas evolution occurred, and that addition to the test solution of an amount of *dl*-serine corresponding to the presence in Extract 1 of 1 % of the serine originally present in the mixture resulted in a significant evolution of gas. From this it is argued that < 1 % of the serine originally present found its way into Extract 1. This is in agreement with the experiments on *N*-acetyl-*dl*-serine referred to in Paper I of this series.

SUMMARY

1. The possibility of separating acetamino-acids by making use of their different partition coefficients between chloroform and water is discussed.
2. In order to make this technique available for protein analysis it would first be necessary to acetylate the amino-acids of the protein hydrolysate, and to free the resulting acetyl compounds from free amino-acids and from salt.
3. A technique for carrying out this operation is outlined, and an experiment with a known mixture of amino-acids is described. Evidence is presented that those amino-acids that would be susceptible to separation by extractational fractionation are obtained in high yield in the desired state.

I wish to express my thanks to Dr K. Bailey for instructing me in the technique of S determination, to Mr N. W. Pirie for a gift of *l*-methionine, and to Dr D. D. Woods for carrying out the manometric tests for serine.

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