12. SEPARATION OF THE HIGHER MONOAMINO-ACIDS BY COUNTER-CURRENT LIQUID-LIQUID EXTRACTION: THE AMINO-ACID COMPOSITION OF WOOL

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Introduction

THE possibility of employing counter-current liquid-liquid extraction for the separation of amino-acids in the form of acyl derivatives from protein hydrolysates was briefly discussed by Synge [1939, 1–4], who described preliminary experiments on the separation of some of the naturally occurring 'monoamino-acids'. In the present paper we review the literature on counter-current liquid-liquid extraction, discuss the mathematical and physical basis of the separation of acylamino-acids by liquid-liquid extraction, and describe the construction, operation and testing of a multi-plate chloroform-water counter-current extraction train. We then describe the use of this machine in a control analysis of a known mixture of amino-acids for such components as methionine, valine, proline, the leucines and phenylalanine, together with the application of the new technique to an analysis of wool for these amino-acids. In conclusion we compare the results with those obtained by other methods, and summarize existing knowledge of the amino-acid composition of wool.

Historical

The development of a mathematical treatment of the problems of continuous fractional distillation has made clear the analogy that exists between all counter-current processes. Graphical methods are generally employed, two of the most important being those of Ponchon [1921; cf. Thiele, 1935] and of McCabe & Thiele [1925]. Randall & Longtin [1938, 1–3; 1939, 1–3] have pointed out that the McCabe-Thiele diagram is a point-line contact transformation of the Ponchon diagram, which latter is for many purposes more convenient. These authors have generalized the method of Ponchon for dealing with counter-current processes of all kinds, including liquid-liquid extraction.

Although extraction by immiscible solvents has long been one of the standard purification techniques of the organic chemist, the advantages offered by the application of counter-current principles to this process are only at the present day beginning to be recognized. Nearly all development in this field has so far been in connexion with industrial processes.

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The advantages of this technique, as against distillation, are chiefly these:

(1). Non-volatile and thermolabile materials may be fractionated.

(2) Very small quantities of material may be fractionated by employing dilute solutions. In distillation this is impossible unless a suitable 'carrier' substance is available.

(3) In extractional fractionation partition coefficients—a function of the balance of polar and non-polar groups in the molecule—are the determining property. In distillation, volatility determines the separation. This renders, in many cases, fewer theoretical plates necessary for extractional than for distillatory separation.

Lewis [1916] discussed the application of counter-current principles to the extraction of solids and gases by liquids. Hunter & Nash [1932] applied the McCabe-Thiele diagram to counter-current liquid-liquid extraction. The analogy between this process and distillation was also recognized by Saal & van Dijck [1933], who applied the method of Ponchon to the usual triangular phase diagram for ternary systems. This was also done by Hunter & Nash [1934]. Evans [1934] and Thiele [1935] have discussed the analogy between distillation and extraction, and reviewed previous theoretical treatments of extraction processes. Thiele points out the analogy between extraction columns with successive mixing and settling chambers and plate distilling columns, and shows how continuous-contacting extraction columns (packed or spray types) can be treated by making use of the H.E.T.P. concept, as for packed distilling columns.

During this period counter-current liquid-liquid extraction has become of great importance in the refining of lubricating oils; extraction by liquid sulphur dioxide, nitrobenzene and cresylic acid-liquid propane ('duo-sol' process) have been employed. The efficiency of packed and spray towers has been accurately determined (cf. 'Symposium on Absorption and Extraction', *J. industr. Engng Chem.* **29** (1937), and elsewhere in the same journal).

So far the industrial applications of liquid-liquid extraction have not necessitated columns of very high 'plate number', and in most cases spray or packed columns (which have much greater H.E.T.P. than when used for distillation), or a few mixing and settling units in series have been employed.

The only published work describing a 'multi-plate' column suitable for laboratory use is by Cornish *et al.* [1934], who constructed a counter-current column of 210 units, each with a plate efficiency of about 50 % under the conditions of operation. The apparatus had alternate stirring and settling chambers, and was made of nickel. It was used for the fractionation of fat-soluble vitamins between such solvents as octane and methyl alcohol. The procedure employed was to inject the material to be fractionated at the middle of the column at the beginning of the run, and at the end of the run to collect the two fractions that had flowed out at either end and that which was left in the column.

This procedure differs from all the industrial processes described above, which are essentially continuous. Batch operation necessitates a different theoretical treatment, and Cornish and co-workers obtained an expression for the proportion of material left in the column after a given time of running where the number of theoretical plates, rates of liquid flow and volume of solvents per theoretical plate are given, where the solute is distributed between the two phases according to Henry's distribution law, and where the flow ratio of the two solvents is the inverse of the partition coefficient of the solute under consideration. Under these circumstances the movement of the solute is equally towards each end of the column, and is directly analogous to the flow of heat along a bar.

In connexion with the present work, where a similar mode of operation was adopted, Daniels & Martin, in a forthcoming paper, have generalized and extended the theoretical treatment to deal with solutes obeying Henry's law, but whose partition coefficients differ from the inverse flow ratio of the solvents. They have incidentally shown that the distribution of solute that emerges at opposite ends of the column after an infinite time of running is the same as that which would be obtained under conditions of continuous operation, as calculated from the McCabe-Thiele or similar diagram.

One of us (A. J. P. M.) (unpublished work carried out at the Dunn Nutritional Laboratory, Cambridge) has constructed a glass multi-plate column working with the phases of a petroleum-methyl alcohol mixture, and has applied it to the purification of vitamin E.

Physical chemistry of the separation

In the present work we have separated acetamino-acids by partition between chloroform and water phases. The reasons for using the acetyl derivatives, and some data on their distribution between immiscible solvents, have been given by Synge [1939, 1, 2]. We have used the solvent pair chloroform-water because of the considerable differences between the partition coefficients of acetamino-acids given by these solvents. In addition, the absolute values of these partition coefficients (which determine the flow ratios employed in the column) lie in a suitable range. This is not the case for carbon tetrachloride and water, between which solvents *N*-acetyl-*dl*-leucine has $P=130$ (37°, $c=3{\cdot}2$).

Here and subsequently,

c = concentration of solute in aqueous phase, expressed as mg. per ml.,

$$P=\frac{\text{Concentration of solute in aqueous phase}}{\text{Concentration of solute in organic solvent phase}}.$$

An investigation of the distribution of acetamino-acids between petroleum-methyl alcohol phases showed very little 'spread' of the partition coefficients, and these had a very high temperature coefficient. It appeared that the acetamino-acid distribution followed very closely the distribution of methyl alcohol between the phases.

The use of chloroform-water had other secondary advantages: the phases separate readily, having a large difference in density and a high surface tension. In addition, B.P. chloroform, containing a trace of alcohol, does not readily undergo decomposition; we have never detected any liberation of Cl^- into the aqueous phase. Chloroform is non-inflammable. The operation of the column is not affected by atmospheric moisture, as when an anhydrous solvent pair is employed.

In view of the considerable temperature coefficient of the partition coefficients, it was necessary to maintain the column at a constant temperature. The highest practicable working temperature (37°) was employed, as this renders the acetamino-acids more readily extractable into chloroform, and thus allows the column to be operated at convenient flow ratios.

In practice, the acetamino-acids have always been present in dilute solution, and since the relative volumes of chloroform and water phases are known to be scarcely affected by the addition of acetic acid up to a concentration of 5%, we conclude that the same holds good for acetamino-acids, and in practice have found this conclusion justified. This makes possible treatment of the equilibria of each ternary system on a simple two-coordinate diagram, and renders the usual triangular diagram with tie-lines unnecessary. The coordinates

employed have been concentration of solute (mg. per ml.) in the chloroform and water phases respectively.

The equilibria concerned have not been investigated experimentally in great detail, although there has been some addition to and revision of the data given by Synge [1939, 1]. This is appended to the present section.

It is clear that, at a given temperature, two distinct factors will cause the equilibrium curve for a carboxylic acid in chloroform-water to depart from the straight line obtaining under Henry's law. These are (1) ionic dissociation of the solute in the aqueous phase, and (2) association of the solute to double molecules in the chloroform phase.

Smith & White [1929] have investigated the distribution of various carboxylic acids between organic solvents (including chloroform) and water, and have shown that the experimentally obtained curves agree very closely with those calculated by applying the mass action law to the dissociation in the aqueous phase and association in the organic solvent phase, on the assumption that unionized single molecules are distributed between the phases according to Henry's law.

In the present investigation we have studied the distribution of acetyl-*dl*-leucine between chloroform and water phases at 37°.

Assuming an ionization constant of $2{\cdot}3 \times 10^{-4}$ (as recorded for acetylglycine in Landolt-Börnstein's 'Tabellen') it is possible to calculate the concentration of unionized acetylleucine in the aqueous phase from its total concentration, according to Ostwald's dilution law. It will be seen from Table 1 that the ratio

$$D = \frac{\text{Concentration of unionized solute in aqueous phase}}{\text{Concentration of solute in chloroform phase}},$$

remains, within the experimental error, constant over the range studied. No accurate value can therefore be given from these data for the association constant for acetylleucine in chloroform.

Table 1. *Distribution of acetyl*-dl-*leucine between chloroform and water at 37°*

Acetylleucine in water phase millimol./ml.	Acetylleucine in $CHCl_3$ phase millimol./ml.	Percentage ionization	Unionized acetylleucine in water phase millimol./ml.	D
0·0442	0·00462	7·2	0·0410	8·9
0·0242	0·00227	10·0	0·0218	9·6
0·0200	0·00210	10·7	0·0179	8·5
0·0118	0·00104	14·0	0·0102	9·8
0·0055	0·00048	20·5	0·0044	9·2

From this it is clear that in dilute chloroform solution association of acetylleucine to double molecules is insignificant. This is not necessarily the case for other acetamino-acids, although Smith & White have shown (Table 2) that for the series of fatty acids in chloroform the association equilibrium constant is of the same order throughout. In this table

$$K = \frac{(\text{Conc. single molecules})^2}{\text{Conc. double molecules}} \text{ as mg.mol. per ml.}$$

Table 2. *Association of fatty acids in chloroform at 25°*

Acid	K	Acid	K
Propionic	15·3	—	—
Butyric	10·1	*iso*Butyric	9·1
Valeric	10·1	*iso*Valeric	7·6
Hexoic	17·0	*iso*Hexoic	9·2

It is interesting to note that Smith & White found K for α-bromopropionic acid to be 3900, i.e. that association to double molecules is much inhibited by the presence of the α-bromo group. It is possible that the α-acetamino group has the same effect.

We have compared the partition coefficients of optically active and racemic acetamino-acids in one case only (Table 3). Here no difference was observed.

Table 3. *Distribution of acetyl*-d(+)-*leucine between chloroform and water at 37° (cf. Table 1)*

Acetylleucine in water phase millimol./ml.	Acetylleucine in $CHCl_3$ phase millimol./ml.	Percentage ionization	Unionized acetylleucine in water phase millimol./ml.	D
0·0132	0·00125	13·8	0·0114	9·1
0·0069	0·00068	18·5	0·0056	8·3

We considered at one stage the possibility of using acetic acid as a solvent for acetamino-acid mixtures during injection into the chloroform-water extraction train. A preliminary experiment on the effect of acetic acid on the distribution of acetylleucine between chloroform and water phases showed that it caused the acetylleucine to enter the chloroform more readily. The results of this experiment are summarized in Table 4.

Table 4. *Effect of acetic acid on the distribution of acetyl*-dl-*leucine between chloroform and water phases at 37°*

The system contained 10 ml. water, 30 ml. chloroform and 56·2 mg. acetyl-*dl*-leucine, to which was added before equilibration the amount of acetic acid shown in the first column of the table.

Acetic acid added mg.	Acetylleucine in water phase mg./ml.	Acetylleucine in chloroform phase mg./ml.	P
0	4·18	0·393	10·6
50	4·20	0·497	8·45
150	3·98	0·500	7·96
400	3·58	0·700	5·11

This effect is presumably due to the formation of the double molecule ($CH_3.COOH.(CH_3)_2.CH.CH_2.CHNHAc.COOH$) in the chloroform phase.

The presence of considerable amounts of acetic acid in the extraction of acetylated protein hydrolysates and amino-acid mixtures by chloroform in the Neuberger [1938, 1] apparatus [cf. Synge, 1939, 2] is thus seen to be of assistance to the extraction, which is also aided by the large quantities of salt present in the aqueous phase.

In applying these equilibrium data to mixtures of acetamino-acids, the following points must be made:

(1) The degree of ionization in the aqueous phase will depend on the total concentration of acetamino-acid present in that phase. If the ionization constants of all the acetamino-acids present are the same, the degree of ionization of each component of the mixture will be the same, and will depend on the total molar concentration of the components. Thus the ratio of the partition coefficients will be unaffected by dilution, although the absolute value will change.

(2) Association to double molecules in the chloroform phase will, where it occurs, render the substances more readily extractable into chloroform. In mixtures, 'mixed double molecules' may be expected, and the equilibrium

constant for their formation is not predictable. Since the effect seems likely to be small compared with the ionization effect under the conditions used by us, we have not subjected this question to experiment.

In the light of these equilibrium data, it is possible to calculate what degree of separation may be expected when a mixture of two acetamino-acids is fractionated in a multi-plate chloroform-water extraction train. In designing the column described in the present paper we employed the McCabe-Thiele diagram for estimating the number of plates necessary to give the required separation. The following example will illustrate its use.

A column of forty theoretical plates with central feed of an undiluted 1 : 1 mixture of acetyl-*dl*-leucine and acetyl-*l*-proline hydrate is assumed, as this has some bearing on our own experiments. The column is operated at 37°. At this temperature, D for acetyl-*dl*-leucine is 9·2, for acetyl-*l*-proline 17·2 (mean value of determinations). The equilibrium curves for these two substances are plotted separately (Figs. 1 and 2), assuming the ionization in the aqueous phase to follow Ostwald's dilution law, and to have the ionization constant $2{\cdot}3 \times 10^{-4}\,M$.

The flow ratio employed is the geometric mean of the two values of D, i.e. $\frac{\text{Rate of flow of chloroform}}{\text{Rate of flow of water}} = 12{\cdot}58$, and is taken as constant throughout the column.

McCabe-Thiele diagrams are constructed for each solute separately (Figs. 1 and 2). The location of the operating lines is determined as follows:

(1) Both lines (rectifying and analysing) are parallel, and have a slope of 1/12·58.

(2) The operating lines cut the axes at the concentration of solute in the chloroform and water outflows respectively.

(3) Twenty theoretical plates may be drawn between each operating line and the equilibrium line from the point at which the operating line cuts the axis to the point on the equilibrium line corresponding to the feed-plate.

In Figs. 1 and 2 the separation of the two solutes is treated individually. In Fig. 3 the individual and total concentrations of the two solutes on each plate, as given by Figs. 1 and 2, are plotted.

It will be seen that the curvature of the equilibrium lines in very dilute solution causes the acetylproline to flow out almost completely in the water outflow, whereas only 97·6 % of the acetylleucine flows out in the chloroform, and the rest in the water.

Since, however, the degree of ionization will depend on the total concentration of acid in each plate, it will be seen from Fig. 3 that this does not fall below $0{\cdot}02\,M$, and the degree of ionization will therefore at no point of the column be greater than 10 %. The equilibrium lines will therefore approximate more closely to straight lines through the origin, and the separation will be more nearly that calculated on the assumption that Henry's law operates, i.e. 99·6 % of each solute will emerge from the column at one end and 0·4 % at the other.

Under conditions of batch operation, where the mixture to be fractionated is added in one lot initially at the middle of the column, the separation cannot be so readily predicted, although the work of Daniels & Martin suggests that it should not differ widely from that obtained under conditions of continuous operation.

In batch operation, dilution will cause any component of the mixture to move more readily towards the water outflow end of the column; complete removal will therefore be more readily obtainable for the components of the mixture flowing out largely in the water than for those flowing out largely in the chloroform. We therefore intend in future to operate the column in a manner

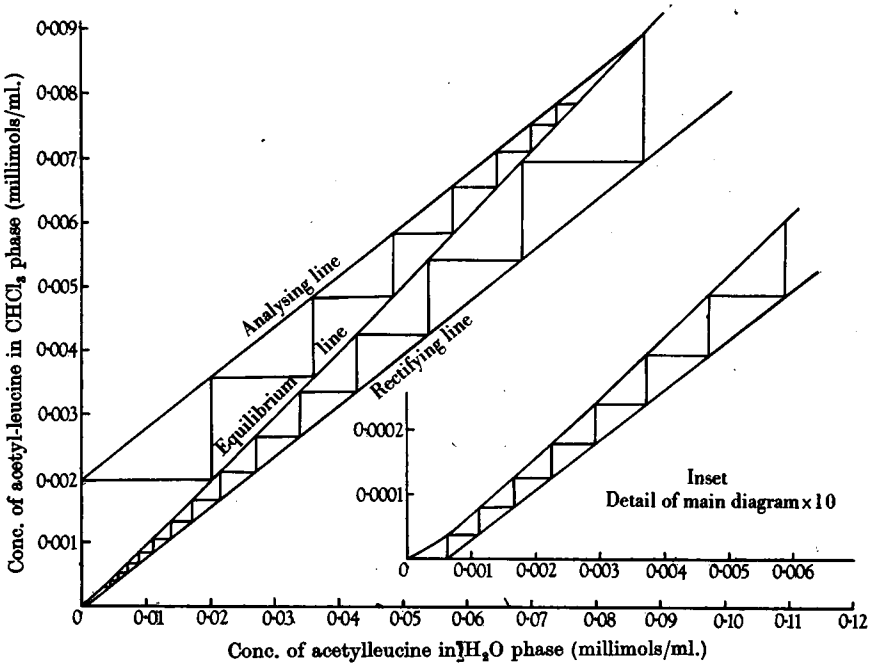


Fig. 1. McCable-Thiele diagram showing distribution of acetylleucine.

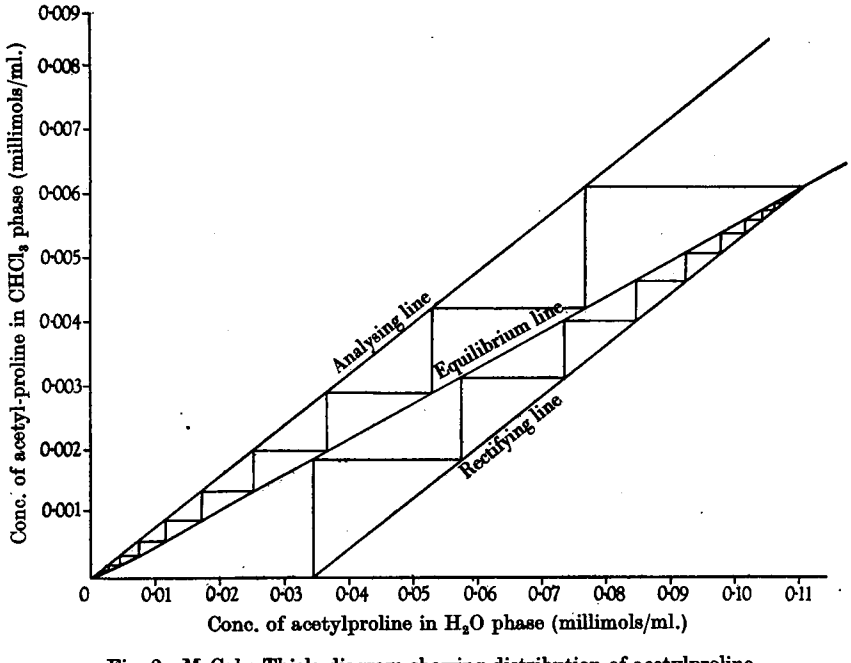


Fig. 2. McCabe-Thiele diagram showing distribution of acetylproline.

directly analogous to batch fractional distillation, i.e. by adding the mixture initially at the chloroform outflow end of the column, and maintaining its concentration there by evaporation of solvent without withdrawal of solution. Gradual increase of the rate of water flow through the column should then lead to sharp fractionation of the material obtained in the water outflow from the

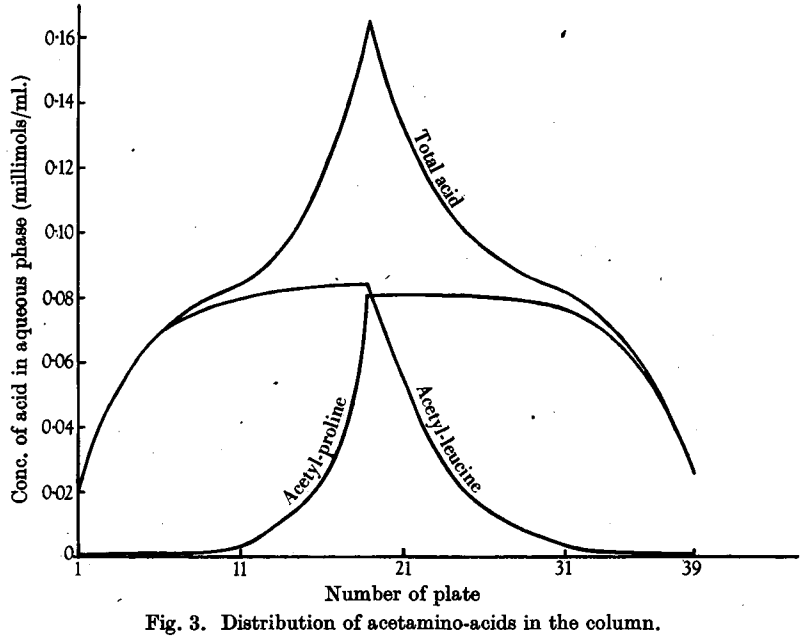


Fig. 3. Distribution of acetamino-acids in the column.

column. In addition, the effective length of the column would be twice that under the conditions of operation described in the present paper, since the column would be completely rectifying, whereas under the present conditions each half of the column serves as analyser for the rectifying action of the other half.

Table 5. *Partition of acetamino-acids between chloroform and water phases at 37°*

Compound	Conc. in water phase mg./ml.	Conc. in chloroform phase mg./ml.	P
Acetyl-*l*-phenylalanine	1·44	0·37	3·9
	0·76	0·18	4·2
Acetyl-*d*-*nor*leucine	1·74	0·27	6·45
	0·94	0·13	7·2
Acetyl-*d*-*iso*leucine	1·86	0·25	7·4
	0·98	0·11	9·0
Acetyl-*d*-leucine	2·28	0·22	10·5
	1·20	0·12	10·0
Acetyl-*l*-valine	3·3	0·22	15
Acetyl-*l*-proline (hydrate)	3·03	0·16	18·9
	1·55	0·09	17·2
Acetyl-*l*-methionine	3·04	0·11	27·6
	1·46	0·045	32·4
Acetyl-*dl*-α-aminobutyric acid	3·42	0·05	68
Acetyl-*dl*-alanine	4·74	0·022	216

New data on the partition of acetamino-acids between chloroform and water

In connexion with the present work it was desirable to redetermine and supplement some of the data for partition coefficients given by Synge [1939, 1]. The figures given in Table 5 were obtained by gravimetric determination of the solute in each phase after equilibration in a separating funnel.

The main discrepancy between the present figures and those previously given is in the figure for valine. The actual separations described below gave valine a place intermediate between proline and methionine. It is possible that the partition coefficient of its acetyl derivative alters more rapidly on dilution than that of proline.

Description of the machine

In the apparatus employed for the present work, the column in which water and chloroform phases flow counter-current consists of 40 units in series. Fig. 4 illustrates a single unit. The vertical or 'stirring' tubes (1, Figs. 4 *B*, 5, 6, 7) and

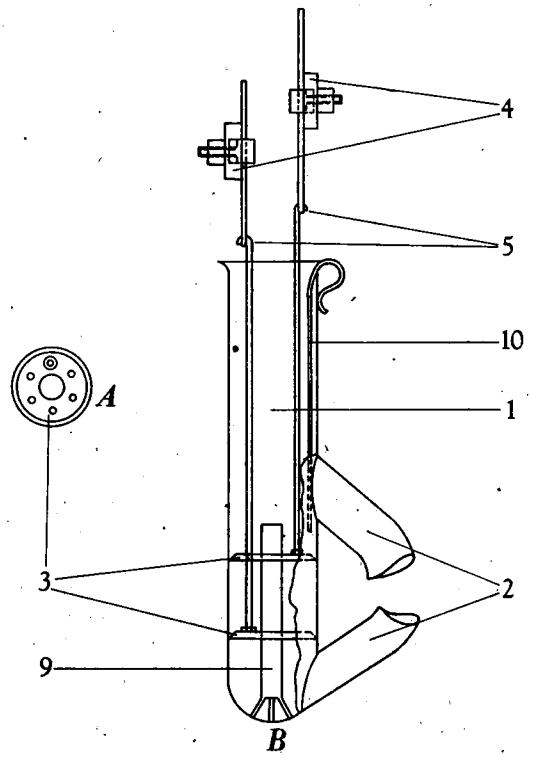


Fig. 4. Single unit of column.

the inclined or 'settling' tubes (2, Figs. 4 *B*, 5, 6, 7) connecting successive stirring tubes are made of pyrex glass. Each stirring tube holds a pair of silver stirrers (3, Fig. 4), each being a perforated dished disk of 0·017 in. silver sheet riveted to hard-drawn 16 s.w.g. silver wire. (Silver, unlike nickel and silver solder, is not attacked under the conditions of operation of the column.)

Each stirrer is attached to a grid (4, Figs. 4 *B*, 5, 6, 7) through a universal joint (5, Figs. 4 *B*, 7). The two grids, and thus each pair of stirrers, are given equal and opposite reciprocating motions by a system of links (Watt's parallel motion) (6, Figs. 5, 6, 7). This is driven through a variable-throw crank (7, Fig. 8) and sprocket gear by a 1/4 H.P. induction motor (8, Fig. 8) mounted below the table.

Fig. 5.

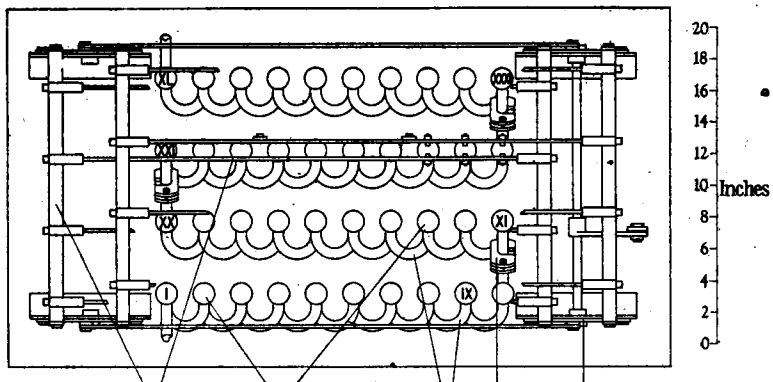


Fig. 6.

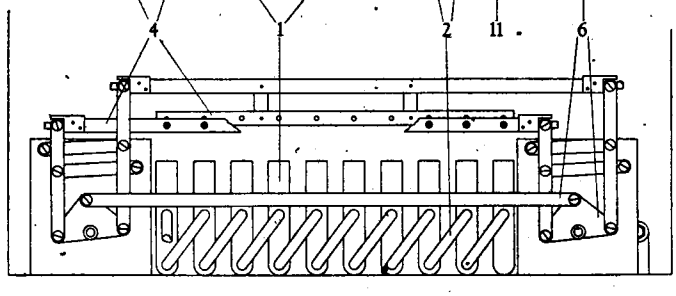


Fig. 5. Plan of column. Fig. 6. Side elevation of column.

A vertical glass tube (9, Fig. 4 *B*), open top and bottom and having three feet, rests on the bottom of each stirring tube and passes through the central perforation of each stirrer. A baffle plate (10, Fig. 4 *B*) of silver sheet is clipped to the rim of each stirring tube, and covers the mouth of the settling tube.

The stirring tubes are numbered 1–40 as shown in Fig. 5, and are sealed together in four banks of ten elements each. Successive banks are connected through a ground-glass flange joint, having a cellophane washer and held in position by an ebonite clamp (11, Figs. 5, 7). The chloroform and water entries (12 and 13) and exits (14 and 15) are arranged as shown in Figs. 7 and 8.

The column rests on a stoutly built table, and is held in position by light clips (not shown in drawing). The link-gear and the whole column (except for the water and chloroform exits) is enclosed in a wooden casing (16, Figs. 7, 8) resting on the table. The interior of this is maintained at 37° by thermostatically controlled electric lamps. The air is kept in circulation by an electric fan. The casing is provided with a lid (17, Fig. 8), windows (18, Fig. 8) and a small door near to tube 20.

On leaving the column, chloroform flows from the adjustable level outflow (14, Figs. 7, 8) through a tube provided with a tap for sampling (19, Fig. 8) to the chloroform recovery still, a 2 l. flask (20, Fig. 8) which is immersed in an

electrically heated water bath (1 kW.). The vapour from the still passes through the cyclone trap (21, Fig. 8), which eliminates entrainment of drops of liquid and, after condensation in the reflux condenser (22, Fig. 8), the chloroform falls to the constant level vessel (23, Fig. 8). The reflux arrangement is adopted so that the chloroform shall enter the constant level vessel hot, and thus free from dissolved air. This prevents the formation of bubbles in the flowmeter.

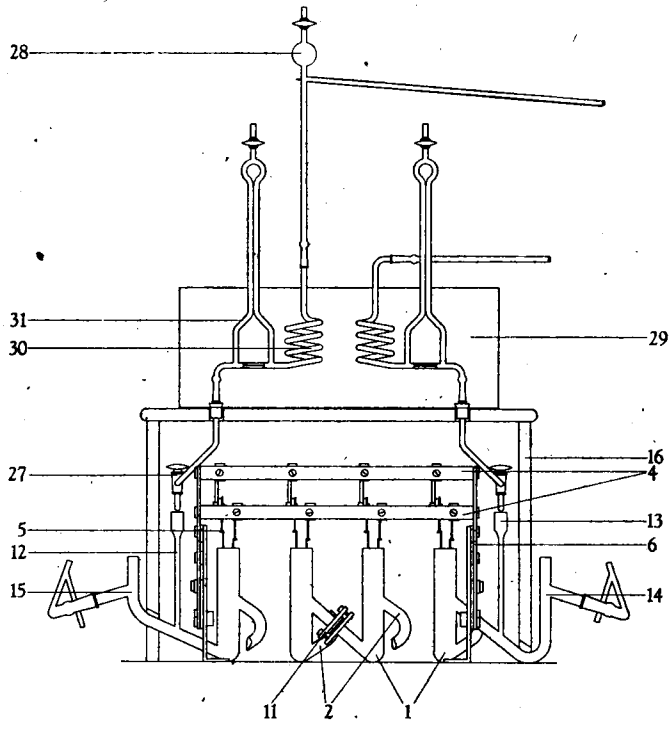


Fig. 7. End elevation of column.

The level of liquid in the constant level vessel is adjusted by raising or lowering the air inlet tube of the 10 l. Mariotte bottle (24, Fig. 8) which acts as the chloroform reservoir. The constant level vessel is connected to the reservoir and to the flowmeter through siphon tubes (25, Fig. 8); the siphons are maintained by differential pressure provided by a column of chloroform in the wash-bottle (26, Fig. 8) through which air is withdrawn from the Mariotte bottle. Chloroform is admitted to the column through the tap (27, Fig. 7). The rate of admission of chloroform is controlled by adjustment of the tap and of the chloroform level in the constant level vessel. Any water separating from the chloroform on its way to the column as a result of cooling is removed in the trap (28, Figs. 7, 8) below which the chloroform supply tube enters the water bath (29, Figs. 7, 8), which is thermostatically maintained at 37°. The chloroform passes through the heating coil (30, Fig. 7) and the capillary flowmeter (31, Figs. 7, 8) before passing to the column.

The same arrangement (not shown in drawing) is used for supplying water to the column; here de-aeration is effected by an electric immersion heater (60 W.) placed in the constant level vessel.

The gear for driving the stirrers was constructed for us by Unicam Instruments, Ltd., Cambridge, and the glass part of the column was made by Messrs A. Gallenkamp, London. We are grateful to their glass-blower, Mr J. Holme, for the efficient execution of this work. We wish also to thank Messrs H. Hall and E. Benton, of the Biochemical Laboratory, Cambridge, and Mr S. Dilworth of Torridon for their help in building the apparatus.

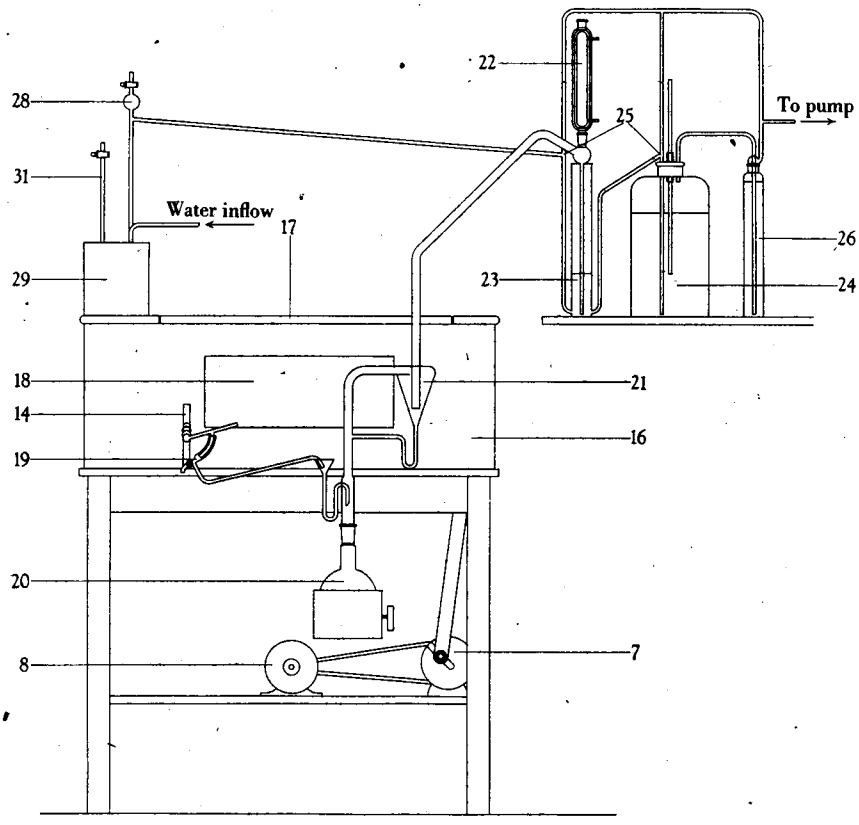


Fig. 8. General view of apparatus.

Operation of the column

When the column is in action, the motion of the stirrers maintains in each stirring tube an emulsion of which the aqueous is the disperse phase. Drops of water thus rise through the settling tube which leads to the next stirring tube. In this way a continuous stream of water passes along the column from tube 1 to tube 40. At the same time, chloroform flows in the opposite direction; under constant conditions the flow of each solvent will be uniform along the whole length of the train, and will depend on the rate at which the solvents are admitted to the column. The solvents leave the column by the adjustable-level outflows.

It will be appreciated that in addition to their normal function of contacting the liquids, the stirrers serve to pump the liquids along the column by continually displacing, in the stirring tubes, the chloroform and water phases from the

positions that they would occupy at rest, thus providing gravitational potential energy for the settling process. This feature of design makes it unnecessary for the column to be tilted [cf. Cornish *et al.* 1934] and thus makes possible the present compact arrangement.

Although the stirrers in each tube are always moving equally in opposite directions, they tend to have, in addition to their stirring action, a 'dynamic' pumping action, which depends on:

(1) The orientation of the dished rim of the stirrers, as there is greater resistance to the motion of the stirrer in one direction than in the other.

(2) The asymmetry of motion of the link gear: the motion is not simple harmonic, and the stirrers approach each other at a different speed from that at which they separate. This effect can be reversed by attaching each stirrer to the other bar.

(3) The length of stroke and velocity of the stirrers, being greater for long strokes and high velocities.

(4) Possible differences in the viscosity of the emulsion at the different levels in the tube at which each stirrer is situated.

This pumping effect, being additive from tube to tube, can, if present in the same sense in a succession of tubes, so alter the level of liquid at different points in the column as to render it inoperative. It is greatly reduced by the central glass tubes, which prevent the establishment of serious pressure differences across any pair of stirrers, and are included for this reason as well as for convenience in emptying the column.

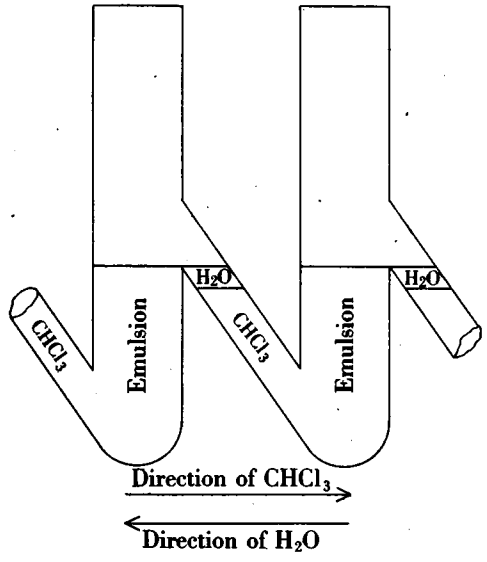


Fig. 9. Normal running of column.

If, as is the case when water is the disperse phase, the density of the emulsion in a stirring tube is less than that of the emulsion in the settling tube, there will be a tendency to 'static' pumping of liquid along the column towards tube 1. The level of the top of the emulsion in each stirring tube will thus tend to its low limit, i.e. the level of the lip of the settling tube leading out of it, as shown in Fig. 9. In practice, at low flow rates of chloroform, the surface can fall lower

than this by splashing of chloroform over the lip into the settling tube. In this way the surface level of the liquid may fall to the level of the top stirrer, and splashing then becomes so bad that liquid is actually lost from the column. This is prevented by placing a silver baffle plate in front of the mouth of each settling tube, which checks the passage of liquid by splashing, and at the same time serves to stop 'entrainment' of chloroform by back-mixing between successive units. Thus, at low flow rates of chloroform, each settling tube will have a layer of water at its top and each stirring tube will be full to the lip of the settling tube (Fig. 9), so that the average density of liquid in each limb is equal.

Under these conditions, the level of liquid in each stirring tube will be at its low limit, and this is the most desirable condition in which to operate the column. Stirring is less efficient when the level of the liquid in the stirring tubes rises, as is bound to happen on increasing the rate of chloroform flow, owing to the head required for overcoming the resistance of the column to flow. This rate can, however, be increased without impairing the operation of the column until it cancels out the effect of the 'static pumping', i.e. until there is no separated layer of water at the top of the settling tubes.

The rate of passage of water out of each stirring tube, and thus along the column, is normally determined by the bottom aperture of the corresponding settling tube, by the opposed rate of flow of chloroform (increase of which decreases the speed of settling), and by the concentration and state of division of the water phase at the bottom of the stirring tube. These depend on the efficiency of the stirring and on the surface tension of the emulsion, which may be lowered by the presence of dissolved substances. If, as occasionally happens, the presence of these causes excessive emulsification, the rate of flow of solvents may be determined entirely by the rate of settling in the settling tubes.

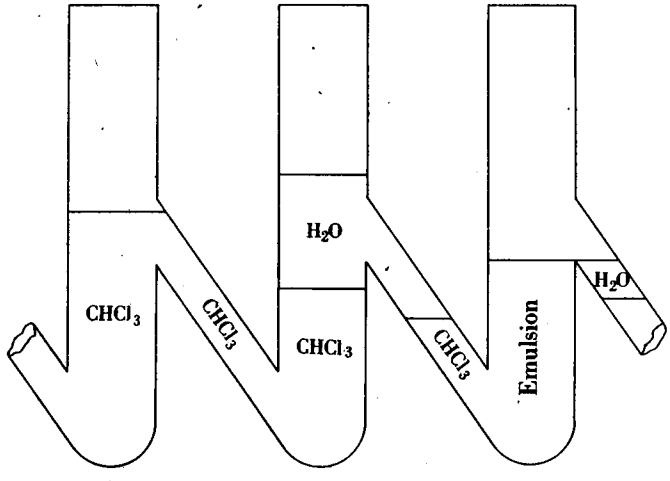


Fig. 10. Water blockage of column.

In order to get efficient stirring throughout the tube, the vertical position of the stirrers must be carefully adjusted. This is particularly necessary when the stirrers are operating on a short stroke (at correspondingly greater speed); these are the most favourable conditions for the elimination of 'dynamic' pumping and splashing.

When more water enters a stirring tube than it is capable of passing, water collects at the top of the tube, raising the level in the tube and preventing the flow of chloroform. This causes accumulation of chloroform between the defective unit and the chloroform inflow, as shown in Fig. 10. This type of blockage is more difficult to deal with than a block due to excessive flow of chloroform, as the column responds more slowly to changes in the rate of water inflow than to changes in the rate of chloroform inflow. Moreover, in the course of eliminating a 'water block' (and sometimes in ordinary operation, when water-flow is being established through the column) the column may exhibit a cyclical instability, since the removal of the 'water block' may be followed by a period of intense 'static pumping', followed by the re-establishment of the block farther along the column. These phenomena involve fluctuations in the flow ratio at different points of the column, and are avoided as far as possible by working with a low level of liquid in the column and moderate rates of solvent-flow.

Working and testing the machine

In working the machine, we proceed as follows. The water and air thermostats being at 37°, chloroform is admitted until the column is full. The rate of admission of chloroform to the column is then adjusted, and the stirrers are set in motion (stroke 0·75 cm. at 390 vibrations per min.). The required flow of water is then started. After running in this manner for some hours, constant conditions will be established throughout the column. Since, however, the addition of the material to be analysed lowers the surface tension between the phases, thereby increasing emulsification and the rate of flow of water, the material is usually added to the column at tube 20 as soon as the advancing stream of water has reached tube 27–29. This immediately liberates water from the middle part of the column, which rapidly finds its way towards tube 40, and an approximately steady state is thus established before water begins to leave the column.

At the end of the run, the material that has emerged in the chloroform is collected from the flask of the chloroform recovery still, while that which has emerged in the water is obtained by concentration *in vacuo*. The fraction remaining in the column is obtained by emptying the column and concentrating separately the two phases so obtained.

Although Cherbuliez & Wahl [1925] have found benzamino-acids to be volatile in steam, we have examined for N the distillates obtained in the concentration of solutions of acetamino-acids with negative results.

The solvent volumes in the column are nearly independent of the flow ratio employed. 3·0–3·5 l. of chloroform and 150–350 ml. of water are the usual amounts.

Satisfactory rates of solvent flow for the range of flow ratios employed are shown in Table 6.

Table 6. *Solvent rates for different flow ratios in the column*

Flow ratio	Chloroform rate ml./min.	Water rate ml./min.
68	22·5	0·33
20·8	18·75	0·86
13·9	19	1·37
8·0	8·0	1·00

Since on the average each molecule of chloroform or water will spend about 3 min. in each unit of the column, and as intimacy of mixing in the stirring tubes is greater than in the ordinary laboratory separating funnel, it seems likely that each unit will correspond nearly to one theoretical plate, assuming no entrainment to occur. By the use of red ink, the pigment of which stays completely in the water phase, we have never detected any entrainment of water, while the use of Sudan III, which stays completely in the chloroform, has shown that entrainment of chloroform is only detectable at the lowest rate of chloroform flow employed; under these circumstances it is not great, and only occurs in the absence of the baffle. The main factor vitiating the performance of the machine would seem to be local variations in the flow ratio, particularly those occurring at the beginning of the run.

Although precautions were taken against loss of chloroform by evaporation by sealing cracks, etc. in the walls of the air-thermostat box with plasticine, by packing wool around the entry of the driving-arm to the box, and by providing a rubber seating for the box lid, they were not completely successful, the total loss of chloroform on a 24 hr. run amounting to about 1·5 l.; this is not surprising when it is remembered that the equilibrium weight of chloroform vapour in the box at 37° is about 500 g. This loss corresponds to about 1 ml. per min. of chloroform, and most of it must be assumed to take place along the length of the column. Its effect on the operation of the column is greatest at low flow ratios, and is to produce a continuous change of flow ratio along the length of the column, amounting, at flow ratio 8, to a change of 12·5 %.

In operating the machine in most of this work we placed the material to be analysed in the middle of the column in solid form in one batch. Larger quantities could be added in a continuous manner; the machine is just as capable of continuous as of batch operation. For this purpose tube 20 is connected through a capillary to an electrically driven glass syringe. The problem is to find a suitable solvent for the acetamino-acids which will permit the slow injection of a concentrated solution. The mixtures obtained from proteins crystallize from concentrated aqueous solution, and glacial acetic acid, though a good solvent, distils with the chloroform, and thus accumulates in the column, seriously affecting the equilibria, as already pointed out. This effect is strikingly demonstrated by comparing the results of tests 1 and 2 given below.

Test 1 is also of interest because the time course of emergence of the substances to be separated was studied. In other experiments we have only collected the three fractions at the end of the run.

Possibly alcohol would prove a more desirable solvent for injection.

Tests. For testing the machine, a 1 : 1 mixture of acetyl-*l*-proline hydrate [du Vigneaud & Meyer, 1932, 1, 2] and acetyl-*dl*-leucine [Fischer, 1901] was employed. These substances have the partition coefficients given in Tables 1 and 5, which are sufficiently close to give a good test of the machine, and in addition small amounts of each in the presence of the other can be conveniently detected, the former by its large negative rotation, and the latter by determination of amino-N after acid hydrolysis.

Test 1. The column was allowed to reach a steady state at flow ratio 13·9 (solvent rates as in Table 6). A solution of 4·5 g. of acetyl-*l*-proline hydrate and 4·5 g. of acetyl-*dl*-leucine in 25 g. of glacial acetic acid was then injected at a steady rate, 12 hr. being required for complete injection. The chloroform and water effluents were collected at the intervals shown in Table 7, which shows cumulatively the amount of each solute (as % of amount finally injected) which had emerged at different times.

Table 7

Time from commencement of injection hr.	In chloroform effluent		In water effluent	
	% of acetyl-leucine	% of acetyl-proline	% of acetyl-leucine	% of acetyl-proline
4	0·46	0·0	0·00	1·0
6	2·7	0·1	0·02	1·8
8	14·0	0·6	0·07	3·2
10	40·4	3·4	0·13	5·1
12	65·9	9·3	0·24	6·7
15	92·4	25·2	0·38	8·7
19	98·8	41·2	0·50	11·5

Test 2. Column as in test 1. A mixture of 1·04 g. acetyl-*dl*-leucine and 1·02 g. acetyl-*l*-proline hydrate was added in the solid form, and the machine was allowed to run for 11 hr. At the end of this time there had emerged in the chloroform 26·5 % of the acetylleucine and 0·60 % of the acetylproline. In the same time there had emerged in the water 4·36 % of the acetylleucine and 48·7 % of the acetylproline.

The wide divergence between tests 1 and 2 may be ascribed to the presence of acetic acid in test 1. The accumulation of this has the effect of rendering both solutes more readily extractable by chloroform.

These tests gave a rough idea of what degree of separation was to be expected from the machine, and we were able on this basis to use it for the separation of more complex mixtures.

Separation of a known mixture of amino-acids

The possibility of using liquid-liquid extraction for separating and estimating some of the monoamino-acids occurring in protein hydrolysates was briefly discussed by Synge [1939, 2], who described a procedure for obtaining a good yield of the required amino-acids in the form of their acetyl derivatives and free from salt. Such a mixture is suitable for fractionation in the machine which we describe in this paper.

Before attempting to use the new technique for the analysis of a protein it was considered advisable to control it by experiment with a known mixture of amino-acids.

The preliminary fractionation of such a mixture was described by Synge [1939, 2], who stated: '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.'

The present investigation has been conducted with special reference to wool, in which both these amino-acids are present, cystine being a major constituent. A mixture was made up containing all the amino-acids recorded in the literature as constituents of wool, with the exception of methionine and phenylalanine. The former was omitted because its behaviour had already been studied [Synge, 1939, 2] and its absence permitted a study of the behaviour of cystine during the fractionation by means of total S determinations.

The amino-acids were in roughly the proportions recorded for wool.

All samples used for analysis were aliquots; allowance has been made for the samples removed at each stage for analysis, and all figures given are on the basis of the original mixture, as detailed in Table 8.

All evaporations were carried out *in vacuo* below 40°.

The values given for 'amino-N' refer to amino-N determined by the Van Slyke procedure on samples hydrolysed with 2*N* HCl for 3 hr. at 100°, and subsequently neutralized with excess of sodium acetate. Where the material had been dissolved in alcohol, great care was taken to remove this completely before determining amino-N.

Table 8. *The mixture of amino-acids used in the experiment*

Compound	N (g.)	N as % of total N
Glycine	0·290	12·8
dl-Alanine	0·137	6·0
dl-Valine	0·091	4·0
l-Leucine	0·206	9·0
dl-Serine	0·070	3·1
l-Proline	0·128	5·6
l-Aspartic acid	0·053	2·3
l-Glutamic acid	0·190	8·3
l-Histidine (as hydrochloride)	0·048	2·1
l-Arginine (as hydrochloride)	0·482	21·2
dl-Lysine (as dihydrochloride)	0·109	4·8
l-Tyrosine	0·083	3·6
l-Cystine	0·242	10·7
l-Tryptophan	0·026	1·1
Ammonium chloride	0·124	5·4
Total	2·279	100

The mixture shown in Table 8 was dissolved in 200 ml. 6*N* HCl, and was refluxed for 24 hr. The resulting solution was concentrated and acetylated exactly as described by Synge [1939, 2], using 400 ml. 2*N* NaOH and 40 ml. of acetic anhydride. After keeping overnight, the mixture was acidified as described, transferred to the Neuberger extractor, and extracted with approximately 30 vol. of chloroform. The aqueous residue was freed from salt and re-acetylated exactly as described, acidified, and again extracted with 30 vol. of chloroform in the Neuberger extractor. The two chloroform extracts were combined and evaporated to dryness; the residue was made up to standard volume in water (fraction E).

This fraction contained 24·7 % of the total N of the mixture.

It was completely free from SO_4^{--}, and contained 42 mg. S (7·6 % of the cystine-S of the mixture). We are grateful to Mr W. R. Cuthbertson for this S determination.

Fraction E was evaporated to dryness in a vacuum desiccator, and the resulting toffee-like solid was fractionated in the machine for 24 hr. at flow-ratio 68, the geometric mean of the partition coefficients of acetylalanine and acetylproline. In this and subsequent fractionations the solvent rates employed were those given in Table 6.

Table 9 shows the distribution of N, S and amino-N in the three resulting fractions.

Table 9

Fraction	N as % of total N	S as % of original cystine S	Amino-N as % of total N
68_c (in chloroform)	17·9	2·2	12·7
68_m (in column)	0·8	—	—
68_w (in water)	5·9	5·0	—

Fraction 68_w was evaporated to dryness. It was obviously a complicated mixture, and crystallized very slowly. By successive crystallization from ethyl

acetate and acetone a small amount of acetylglycine was isolated. The fraction might be expected to contain also acetylalanine, *N*-acetyltyrosine, diacetylcystine, diacetyllysine and possibly other constituents. It was not further investigated.

Fraction 68_m was not further investigated.

Fraction 68_c was evaporated to dryness, to give a practically completely crystalline residue, coloured pale yellow. It was fractionated in the machine for 24 hr. at flow-ratio 13, giving the fractions shown in Table 10.

Table 10

Fraction	N as % of total N	Amino-N as % of total N
68_c13_o	2·7	—
68_c13_m	3·2	—
68_c13_w	10·2	5·8

68_c13_m and 68_c13_c both obviously consisted almost completely of acetylleucine, and were therefore combined before working up.

The material consisted of crystals accompanied by a brown oil, which was later found always to be produced in the course of fractionation in the machine. Its nature is discussed in the succeeding section dealing with the analysis of wool. It could readily be separated from the crystals by washing them with chloroform or ligroin. By subsequent recrystallization from chloroform and from water, three crops were obtained:

Crop I. 1·25 g. M.P. 182°. $[\alpha]_D^{24°}$ −23·5° (alcohol, $c=3·3$). (Found: C, 56·2; H, 8·54; N, 8·23 %. Calc. for $C_8H_{15}O_3N$: C, 55·5; H, 8·67; N, 8·1 %.)

Crop II. 0·070 g. M.P. 155–160°. $[\alpha]_D^{24°}$ −5·2° (alcohol, $c=1·0$). (Found: C, 55·9; H, 8·53; N, 7·8 %.)

Crop III. 0·162 g. Not further investigated.

Synge [1939, 1] records acetyl-*d*-leucine as having M.P. 186–188°; $[\alpha]_D^{22°}$ +23·2° (alcohol). Fischer [1901] records acetyl-*dl*-leucine as having M.P. 161°.

From this it seems that the crystalline part of this fraction consists of acetyl-*l*-leucine which has undergone about 15 % racemization.

89 % of the N of fractions 68_c13_m and 68_c13_c is thus accounted for as crystalline acetylleucine.

68_c13_w. On concentrating an aqueous solution of this fraction, the first crop of crystals (0·353 g.) had M.P. 180° and this was not depressed on admixture of acetyl-*l*-leucine from the other two fractions.

The next crop obtained melted at 130–137°, and the next at 110–140°. From these it proved possible, by repeated crystallization from water, to isolate 0·269 g. of a product having M.P. 147–148°. This was not depressed by admixture with authentic acetyl-*dl*-valine [Synge, 1939, 1]. (Found: C, 52·5; H, 8·22; N, 8·90 %. Calc. for $C_7H_{13}O_3N$: C, 52·8; H, 8·17; N, 8·8 %.)

Since it was clear that the mother liquors contained a mixture of acetylleucine, acetylvaline and acetylproline, and since acetylproline had already been satisfactorily isolated in the analysis of wool (see below), this fraction was not further investigated. Its proline content could be judged by the difference between total and amino-N, shown in Table 10.

We draw the following conclusions from this experiment with a known mixture of amino-acids:

(1) Fraction E, on fractionation in the machine at flow-ratio 68, yields in fraction 68_c the amino-acids leucine, proline and valine in about 95 % yield. It

will be noted that the ratio of amino-N to total N for fraction 68_c is 0·71. Calculated from the original mixture, $\frac{\text{Leucine-N + valine-N}}{\text{Leucine-N + valine-N + proline-N}} = 0{\cdot}704$. Methionine, leucine isomers and phenylalanine may be expected to behave similarly.

(2) Racemization to the extent of about 15% is to be expected. This probably occurs during acetylation, although the conditions of acetylation are so chosen as to reduce it to a minimum [cf. du Vigneaud & Meyer, 1932, 1]. The possibility of racemization of the free amino-acids on prolonged boiling with acid cannot be ignored. With cystine, this is known to be considerable, and Arnow & Opsahl [1940] and Johnson [1940] report slight racemization of glutamic acid under these conditions.

(3) 2–3% of the S of the cystine in the mixture is carried into fraction 68_c. This cannot be in the form of diacetylcystine [cf. Synge, 1939, 1]. The state of combination of this S is discussed in the following section on the analysis of wool under fraction 68_m.

(4) Further fractionation of 68_c at flow ratio 13 largely freed about 70% of the leucine from valine and proline, the bulk of which remained in fraction 68_c13_w. It might be expected that repeated fractionation in the machine at different flow ratios would achieve better separation. This was attempted with some success in the course of the work on the monoamino-acid fraction of wool described below.

Investigation of the higher monoamino-acids of wool

The literature on these constituents of wool is meagre. Abderhalden & Voitinovici [1907] investigated a wool hydrolysate by the ester distillation procedure of Fischer and reported figures for valine, proline and 'leucine' which are of interest in connexion with the present investigation. No attempt was made to decide whether *iso*leucine was present with the leucine. Phenylalanine could not be detected in the fraction in which it was expected to occur. Mueller [1923] described the isolation of methionine in small quantity from wool, and Barritt [1934] employed the volatile iodide method of Baernstein [1932] for its determination. Block [1939] reported a colorimetric determination of phenylalanine in wool. These analyses are compared at the end of this section with the results of the present investigation.

Commercial Merino 64*s* dry-combed top was extracted in a Soxhlet apparatus with benzene and alcohol successively. It was then washed several times with distilled water.

An amount containing 3·310 g. of N (approx. 20 g. of wool) was refluxed on an air bath with 200 ml. of 6*N* HCl for 25 hr. [cf. Marston, 1928].

In describing the subsequent analysis, allowance has been made for the aliquots removed at each stage. Wherever possible, amounts are expressed in N as % of total N of the original wool. Where absolute quantities are mentioned, they are to be referred to the original wool employed, containing 3·310 g. of N. In the later stages of the analysis the amounts of material actually employed corresponded to about 10 g. of wool.

Total N determinations on the various fractions were carried out by the Kjeldahl technique. N determinations on solid material were by the Dumas technique, and were carried out by Dr G. Weiler, Oxford, who is also responsible for the C, H and S determinations.

In the course of the acid hydrolysis a small amount of partly crystalline water-insoluble material was observed to collect in the condenser. It could be

recrystallized from ether, in which it was very soluble. Suspecting the presence of a volatile organic acid, we diluted the hydrolysate when cool to 500 ml. and extracted it with 5 vol. of chloroform in the Neuberger extractor. The material which had collected in the condenser was combined with the hydrolysate before extraction.

An aliquot of the chloroform extract (which was free from Cl^-) was shaken with water, and the mixture was titrated to phenol red with NaOH. The alkali required was equivalent to 0·204 milliequiv. of acid in the whole extract.

Another aliquot of the extract was evaporated at 37° in the open in a tared dish. The material not volatile under these conditions corresponded to 83 mg. in the whole extract.

The rest of the extract was evaporated at atmospheric pressure; the non-volatile residue from this was suspended in water and subjected to steam distillation until 500 ml. of distillate had been collected. The acid not distillable under these conditions amounted to 0·08 milliequiv.

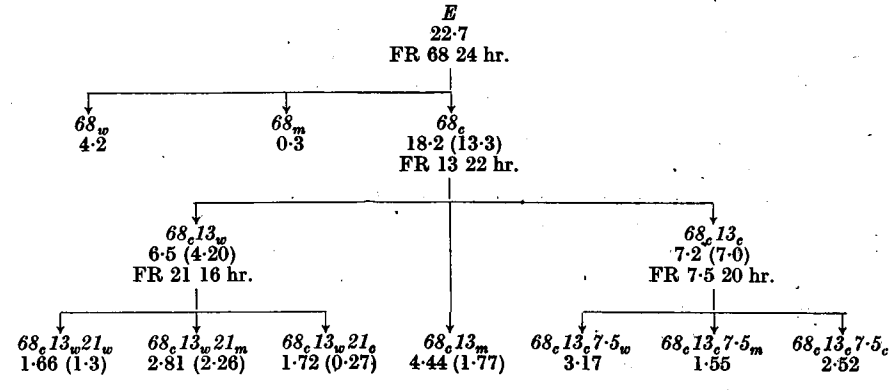
Steam distillation of the extract after neutralization to *p*H 7 with NaOH gave a small amount of water-insoluble material in the distillate.

In view of the insignificant quantities concerned, these fractions were not further investigated. We have indications that the amount of material extractable by chloroform from an acid hydrolysate of wool is increased by increasing the time and temperature of hydrolysis. Most of the material in question is therefore probably a product of secondary change.

After this extraction by chloroform, the hydrolysate was concentrated to dryness *in vacuo*. Two successive acetylations and extractions were carried out exactly as described above for the known mixture, and the combined extracts constituted fraction E, containing 22·7 % of the total N and 54·6 mg. of S (about 8 % of the total S of the wool).

Fraction E was then fractionated and sub-fractionated in the machine according to the scheme shown in Table 11. Between fractionations it was

Table 11. *Sub-fractionation of fraction E*



found convenient to handle the fractions by solution in alcohol. The nomenclature of the fractions is that employed in the previous section. The figures given immediately after each fraction in the table show its N content as % of total N, and the figures in brackets its amino-N content on the same basis, determined as in the previous section.

The subsequent fractionation to which a fraction was subjected is shown in the table thus: *FR 68 24 hr.*

This means that the material in question was fractionated in the machine at flow ratio 68 (with solvent rates as given in Table 6) for 24 hr., after which the three resulting fractions were collected.

Non-amino-N of the fractions. This, the difference between total and amino-N, sets an upper limit to the proline present. It is calculated for the relevant fractions as shown in Table 12. In view of the good yields of crystalline acetyl-proline obtained in the investigation of the end-fractions, there is no reason for postulating the presence of any non-amino-N-containing substance other than proline.

Table 12. *Non-amino-N of the fractions*

Fraction	Non-amino-N as % of total N of wool
$68_c13_w21_w$	0·36
$68_c13_w21_m$	0·55
$68_c13_w21_c$	1·45
68_c13_m	2·67
68_c13_c	0·20
Total	5·23
Cf. 68_c	4·9

Methionine determinations on the fractions. These were kindly carried out for us by Mr G. Lindley, by the 'volatile iodide' method of Baernstein [1932; 1936]. The results are given in Table 13.

Table 13. *Methionine of the fractions*

Fraction	Methionine-N as % of total N of wool
68_c	0·52
$68_c13_w21_w$	0·31
$68_c13_w21_m$	0·09
$68_c13_w21_c$	0·01

Great care was taken to remove alcohol completely from the samples before subjecting them to analysis, since this would appear as volatile iodide in the course of the determination. There remained the possibility that handling the fractions with alcohol had induced partial esterification.

The distribution of volatile iodide in the end-fractions, however, makes it clear that acetamino-acid ethyl esters are not involved, since these are readily extractable from water by chloroform. It is however possible that 25% of the volatile iodide determined on fraction 68_c may be due to this cause.

The final distribution of volatile iodide in the end-fractions is roughly what would be expected from a knowledge of the partition coefficient of acetyl-methionine (cf. Table 5).

Detailed investigation of the end-fractions.

68_w. This did not readily crystallize, and being obviously a complex mixture was not further investigated.

68_m. No acetamino-acid could be crystallized from this fraction. It is in this fraction that α-aminobutyric acid [cf. Vickery & Schmidt, 1931] would be expected to occur, if present (cf. Table 5). We conclude from the low N content of this fraction that α-aminobutyric acid is absent from, or at most a very minor constituent of, wool.

In this and all other 'M' fractions we obtained by crystallization from alcohol, followed sometimes by recrystallization from ether, small quantities (never more than 20 mg. from a fraction) of a substance having M.P. 120–121°. (Found: C, 35·2; H, 5·65; S, 59·8 %; N absent. $C_3H_6S_2$ requires C, 34·0; H, 5·67; S, 60·4 %.)

This substance was insoluble in water and extremely soluble in chloroform and ligroin. It was accompanied by a brown oil, which remained insoluble in water on boiling with mineral acid. The oil from fraction $68_c13_c7{\cdot}5_m$ after boiling with 2*N* HCl had S, 5·9 %.

A compound of the same composition and M.P., to which the formula $C_6H_{12}S_4$ was ascribed, was reported by Tucker & Reid [1932] to result in low yield from the interaction of CH_2Cl_2 with $CH_2SH.CH_2SH$ (formed by the interaction of H_2S with $CH_2Cl.CH_2Cl$). They postulated a cyclic polymethylene sulphide structure.

Although it is hard to see how the same compound could have resulted under the conditions employed by us, it seems reasonable to suppose that the present sulphur-rich bodies may be formed by the interaction of H_2S, etc. (resulting from the breakdown of cystine) with halogenated hydrocarbons. We are informed by Dr K. Bailey that 3–4 % of the S of wool is to be found in an acid hydrolysate as elementary S.

It is difficult to see why substances so soluble in chloroform and insoluble in water should remain in the column of the machine, rather than leave it in the chloroform effluent. Perhaps they are strongly adsorbed by the chloroform-water interface, the area of which is considerable while the machine is in operation.

$68_c13_w21_w$. In view of the high estimated methionine content of this fraction (19 % of N of fraction) it was subjected to immediate acid hydrolysis, with a view to carrying out the $HgCl_2$ precipitation described by Pirie [1932]. Possibly because of the small total quantity of material, this procedure did not give a satisfactory precipitation. The mixture was accordingly treated with H_2S to remove Hg, Ag_2O to remove Cl^- and H_2S to remove Ag. The filtrate from Ag_2S was evaporated to dryness. The crystalline residue was refluxed with alcohol, and after cooling was filtered off and washed with alcohol. The alcohol dissolved 216 mg. of non-volatile matter, which was not further investigated. The alcohol-insoluble material (308 mg.) had C, 47·7; H, 9·22; N, 7·55 %.

Some of this material was benzoylated by the method of Fischer [1900]. A 15 % yield of crystalline benzoylated product was obtained, which on recrystallization from water had M.P. 127°, not depressed by admixture with authentic benzoyl-*l*-valine, for which Karrer & van der Sluys Veer [1932] record M.P. 127°. Karrer & Schneider [1930] record benzoyl-*l-nor*valine as having M.P. 95°, and as forming a hydrate of M.P. 64°.

$68_c13_w21_m$. This fraction crystallized readily from a small volume of chloroform. The crystals, after filtering off and washing with chloroform, weighed 594 mg. and had M.P. 156°, which was not depressed on admixture with authentic acetyl-*l*-valine. $[\alpha]_D^{23°}+2{\cdot}5°$ (alcohol, $c=2$). (Synge [1939, 1] records acetyl-*l*-valine as having M.P. 157–158°. $[\alpha]_D^{20°}+5{\cdot}8°$.) (Found: C, 52·7; H, 7·84; N, 8·3 %. Calc. for $C_7H_{13}O_3N$: C, 52·8; H, 8·17; N, 8·8 %.) A further 19 mg. of the same material were obtained on crystallization of the material in the mother liquors from chloroform-ether. No further crystals resulting, the material in the mother liquors was hydrolysed with H_2SO_4, freed from $SO_4^=$ with $Ba(OH)_2$ and extracted with alcohol as described for the previous fraction. The resulting alcohol-insoluble material (104 mg.) had C, 50·4; H, 8·89; N, 9·3 %. Valine

$C_5H_{11}O_2N$ has C, 51·2; H, 9·41; N, 11·98%. A 1% solution in water showed no optical activity in a 2 dm. tube.

The alcohol extract yielded 302 mg. of non-volatile matter which did not crystallize readily, and has not been further investigated.

$68_c 13_w 21_c$. This fraction gave no crystals from chloroform, but crystallized slowly from moist ether. The product, on recrystallization from moist chloroform ligroin, weighed 532 mg. (air-dry). It had M.P. 77°, and did not depress the M.P. of authentic acetyl-*l*-proline hydrate (see under fraction $68_c 13_m$).

The material in the mother liquors was hydrolysed and extracted with alcohol as previously described. Only a very small amount remained insoluble in alcohol, and neither this nor the alcoholic extract has been further investigated.

$68_c 13_m$. This fraction yielded as crystals from chloroform a first crop of 473 mg., M.P. 183–184°, not depressed on admixture with authentic acetyl-*l*-leucine (cf. corresponding fraction from known mixture). $[\alpha]_D^{24°} -22·6°$ (alcohol, $c=3·3$). (Found: C, 55·0; H, 8·94; N, 7·64%. Calc. for $C_8H_{15}O_3N$: C, 55·5; H, 8·67; N, 8·1%.)

The mother liquors from this material, by successive crystallization from ligroin and moist ether-ligroin, yielded 615 mg. of material having M.P. 78° (115° after drying *in vacuo*). $[\alpha]_D^{22°} -114·0°$ (water, $c=4·2$) (rotation calculated for anhydrous compound). Du Vigneaud & Meyer [1932, 1, 2] record for acetyl-*l*-proline M.P. 81–82° (hydrate), 116–117° (anhydrous) $[\alpha]_D^{27°} -106·7$, $-107·1°$ (water, $c=0·5$). (Found: C, 52·9; H, 7·02; N, 8·62%. Calc. for $C_7H_{11}O_3N$: C, 53·5; H, 7·01; N, 8·91%.)

A second crop of the same material (M.P. 77°), weighing 203 mg., was obtained.

After this removal of acetylproline, it was possible to obtain a further crop of acetylleucine from a small volume of chloroform. 181 mg. $[\alpha]_D^{21°} -14·5°$ (alcohol, $c=1$). M.P. 155–169°. (Found: C, 55·5; H, 8·92; N, 6·4%.)

The small amount of material still remaining in the mother liquors has not been further investigated.

$68_c 13_c 7·5_w$. This fraction crystallized readily from water, and seemed nearly completely crystalline, although the later crops crystallized very slowly.

Crop I (852 mg.) had M.P. 175–179°. $[\alpha]_D^{20°} -13·0°$ (alcohol, $c=1·5$). (Found: C, 55·2; H, 8·63; N, 6·6%.)

Crop II (176 mg.) had M.P. 143–158°. $[\alpha]_D^{20°} -4·5°$ (alcohol, $c=1·3$). It contained 4% of incombustible residue, and after 'correction' for this, had elementary composition: C, 54·2; H, 8·13; N, 7·0%. The residue (275 mg.) has not been further investigated.

$68_c 13_c 7·5_m$. Crystallization from chloroform, which was very slow, yielded 180 mg. of material having M.P. 179–181° (crop I). $[\alpha]_D^{20°} -8·4°$ (alcohol, $c=1$). (Found: C, 54·8; H, 8·50; N, 7·6%.)

Crystallization of the residue in the mother liquor from chloroform-ligroin gave crystalline material melting at 133–148° (crop II). Repeated crystallization of this yielded crystals (34 mg.) having M.P. 153–163°. These were not further investigated. The remaining material of crop II had $[\alpha]_D^{20°} -1·7°$ (alcohol, $c=1$). (Found: C, 53·4; H, 7·87; N, 7·0%.)

The material in the mother liquors from crop II was hydrolysed with 2*N* HCl at 100° for 3 hr., and on cooling the aqueous phase was separated from the oil (see under fraction 68_m) and evaporated to dryness several times with water. The residue was taken up in water, and brought to *p*H 6 with LiOH. The mixture was again evaporated to dryness, and extraction with alcohol yielded 38 mg. of insoluble material, which has not been further investigated.

$68_c 13_c 7{\cdot}5_c$. This fraction did not readily give crystals from chloroform. Crystallization from ether-ligroin, followed by recrystallization from chloroform, gave 536 mg. of material having M.P. 141–143°, whose M.P. was not depressed on admixture with authentic acetyl-*dl*-phenylalanine of the same M.P. Despite reports in the literature of higher M.P. (151° [Knoop & Blanco, 1925], 150–151° [du Vigneaud & Meyer, 1932, 1], 148° [Neuberger, 1938, 2]), we agree with Jackson & Cahill [1938] in having been unable to prepare synthetic acetyl-*dl*-phenylalanine of M.P. higher than 143°. $[\alpha]_D^{20°}+2{\cdot}4°$ (alcohol, $c=1{\cdot}7$). For acetyl-*l*-phenylalanine Jackson & Cahill [1938] record $[\alpha]_D^{25°}+47{\cdot}6°$ (alcohol), while for the *d*-isomer du Vigneaud & Meyer [1932, 1] record a corresponding figure of $-51°$. (Found: C, 63·1; H, 6·20; N, 5·9%. Calc. for $C_{11}H_{13}O_3N$: C, 63·7; H, 6·28; N, 6·77%.)

A further crop of 112 mg. of the same material was obtained.

As the residue would give no more crystals, it was hydrolysed and neutralized with LiOH in the same manner as the previous fraction. Precipitation by alcohol from concentrated aqueous solution yielded 144 mg. of material having $[\alpha]_D^{20°}$ $-4{\cdot}9°$ (water, $c=0{\cdot}8$). (Found: C, 60·1; H, 8·01; N, 7·6%.) This elementary composition is intermediate between that of phenylalanine and leucine (isomers) of which this material is presumably a mixture.

Isolation of hydroxyamino-acid fraction from wool hydrolysate

The aqueous solution resulting from the second extraction of the acetylated hydrolysate in the Neuberger apparatus was evaporated to dryness and the acetamino-acids were freed from salt by extraction with alcohol [cf. Synge, 1939, 2]. The product resulting from evaporation of the alcohol was refluxed for 4 hr. with 450 ml. of $3N$ H_2SO_4. This solution, when cool, was treated with 75 g. of phosphotungstic acid at a final volume of a litre. After storing for 15 hr. the precipitate was filtered off, and washed with a 1% solution of phosphotungstic acid in 1% H_2SO_4. The precipitate was found (by difference) to contain 29·3% of the total protein-N.

The combined filtrate and washings from this were treated with $Ba(OH)_2$ at 100° until just permanently alkaline to thymol blue. The resulting precipitate was thoroughly extracted with hot water, being maintained at the same *p*H by addition of $Ba(OH)_2$ when necessary; the filtrate and washings were then combined, and concentrated to a medium syrup. This was subjected to the acetylation-benzoylation procedure described by Synge [1939, 3], employing for the acetylation 200 ml. of $2N$ NaOH and 20 ml. of acetic anhydride, and for the benzoylation 185 ml. of $2N$ NaOH and 23 ml. of benzoyl chloride.

After the saponification of the *O*-benzoyl groups by $Ba(OH)_2$ as described Ba was removed exactly before the chloroform extraction, after which the aqueous layer was concentrated to dryness, giving the hydroxyamino-acids as their *N*-acetyl derivatives.

This fraction contained 5·9% of the protein-N (5·6% amino-N), in good agreement with the results obtained on wool by Synge [1939, 3] without previous removal of the higher monoamino-acids. The *N*-acetyl-hydroxyamino-acids formed a nearly colourless glass, which showed no sign of crystallization after storage in a desiccator for 6 months.

In a forthcoming paper we describe the further investigation of this fraction.

Isolation of the hydroxyamino-acids by acetylation-benzoylation after removal of the higher monoamino-acids by extraction of their acetyl derivatives into chloroform appears to be preferable to the direct procedure, since it minimizes the possibility of contamination of the fraction by monoacetamino-

acids, which pass readily into chloroform from strong salt solution during the extraction of the *N*-acetyl-*O*-benzoyl-hydroxyamino-acids, but may not be so readily extractable by chloroform from the salt-free solution resulting from the saponification of the *O*-benzoyl derivatives.

Discussion

The valine fractions. Abderhalden & co-workers [1930–37] have reported the occurrence of *l-nor*valine with valine in globin, casein, tussore silk, steer-horn and yeast proteins. We have obtained no positive evidence for its occurrence in wool, about two-thirds of the possible valines in fractions $68_c13_w21_w$ and $68_c13_w21_m$ having been identified as *l*-valine in the form of its acetyl and benzoyl derivatives.

The leucine fractions. The rotations and M.P. of the various preparations isolated having the composition of acetylleucine are summarized in Table 14.

Table 14. *Acetylleucine isomers from wool*

Fraction	Crop	Wt. (mg.)	M.P.	$[\alpha]_D$ (alcohol)
68_c13_m	I	473	183–184°	−22·6°
	II	181	155–169°	−14·5°
$68_c13_c7{\cdot}5_w$	I	852	175–179°	−13·0°
	II	176	143–158°	− 4·5°
$68_c13_c7{\cdot}5_m$	I	180	179–181°	− 8·4°
	II	34	153–163°	—
	III	241	133–148°	− 1·7°

From the data provided by Synge [1939, 1] it may be inferred that acetyl-*l*-leucine has M.P. 186–188°, $[\alpha]_D-23{\cdot}2°$ (alcohol), and acetyl-*l-iso*leucine, M.P. 150–151°, $[\alpha]_D+15{\cdot}6°$ (alcohol).

Acetyl-*l*-leucine does not on admixture depress the M.P. of acetyl-*l-iso*leucine or of acetyl-*dl*-leucine. Acetyl-*dl*-leucine on admixture depresses the M.P. of acetyl-*l-iso*leucine. It seems therefore unnecessary to postulate the occurrence of *nor*leucine in wool in order to account for the M.P. and rotations of the acetyl-leucine fractions isolated. Acetyl-*d-nor*leucine [Synge, 1939, 1] has the low M.P. 112–114° and an insignificant rotation in alcohol.

It has been shown in the control experiment described above that the procedure employed racemizes leucine to the extent of about 15%. In the case of *l-iso*leucine, a corresponding epimerization to *d-alloiso*leucine may be expected. Since *d-alloiso*leucine has $[\alpha]_D-14{\cdot}2°$ (water), $-36{\cdot}8°$ (20% HCl) [Ehrlich, 1907], which is nearly equal and opposite to that of *l-iso*leucine ($[\alpha]_D+11{\cdot}3°$ (water), $+37{\cdot}4°$ (20% HCl) [Locquin, 1907]), it seems likely that the same may be true for the corresponding acetyl derivatives. Assuming this to be the case, and that 15% racemization (epimerization) has occurred, the calculated leucine : *iso*-leucine ratio in the isolated crops of acetylleucine isomers would be 4 : 1. The proportion of *iso*leucine left in the mother liquors would be greater than this, on account of the greater solubility of its acetyl derivative.

The phenylalanine fraction. No isolation of phenylalanine has been previously reported from wool, although Block [1939] reports a colorimetric determination, giving the phenylalanine-N as 2·2% of the total N of the wool. Abderhalden & Voitinovici [1907] obtained in the ester fraction in which phenylalanine was to be expected a product having the elementary composition of leucine. In the present work we have isolated 1·32% of the N of wool as a product agreeing in its properties with acetyl-*dl*-phenylalanine and having only

4·7 % of the optical activity of acetyl-*l*-phenylalanine. Moreover, since acetyl-*l*-phenylalanine is less soluble and crystallizes more readily than the racemic compound, there is no reason to suspect significant quantities of it in the mother liquors. The possibility of extensive racemization in the course of acetylation may be ruled out, since the procedure employed gave, in the hands of du Vigneaud & Meyer [1932, 1], a 92 % yield of acetyl-*l*-phenylalanine from *l*-phenylalanine, and we can confirm this from our own experience. Moreover, the other amino-acids in the same hydrolysate were not seriously racemized.

It does not seem to us necessary to postulate the occurrence of phenylalanine residues in wool other than in the *l* form. Levene & Steiger [1930] have shown that treatment of *d*-phenylalanine for 36 hr. at 100° with 20 % HCl induces no detectable racemization, whereas in glycyl-*d*-phenylalanine and the corresponding diketopiperazine up to 20 % racemization of the phenylalanine was observed under the same conditions. Little, if any, other work has been published on the racemization of amino-acid residues during the treatment of peptides etc. with mineral acids, although the question has assumed importance in connexion with the recent controversy over the occurrence of partially racemic glutamic acid in tumour hydrolysates. The earlier stages of this controversy have been reviewed by Pirie [1939; cf. also Graff *et al.* 1940, and Chibnall *et al.* 1940]. These last workers observe: 'Protein chemists have so far given but little attention to the occurrence of racemized amino-acids in protein hydrolysates, and it may well be that they are more widespread than has been imagined, for the possibility that racemization accompanies the splitting of certain peptide bonds during hydrolysis of the protein molecule must not be lost sight of.'

It seems to us possible that the position occupied by the phenylalanine residues in wool may be such as to render them more liable than other residues to racemization in the course of acid hydrolysis. In this connexion it is interesting that Jensen & Evans [1935], by subjecting insulin to treatment with phenyl*iso*-cyanate, followed by acid hydrolysis, isolated a yield of the phenylhydantoin of *dl*-phenylalanine which suggested that a substantial proportion of the amino groups of this protein belong to phenylalanine residues.

It is possible that the present phenomenon may not be restricted to wool: Sharp [1939] isolated very considerably racemized phenylalanine from a myosin hydrolysate by ester distillation, and Baptist & Robson [1940] have commented on the high degree of racemization recorded in the literature for phenylalanine preparations from proteins.

Summary of present analysis

In Table 15 we summarize our analytical data on the seven end-fractions which we have investigated. In this table, each amino-acid is expressed in N as % of total N of the wool. Under each amino-acid col. I records material isolated and formally identified, while col. II refers to 'unidentified N'. In the cases of methionine and proline, these figures are based respectively on the 'volatile iodide' and 'non-amino-N' figures for each fraction. In the cases of valine, leucine isomers and phenylalanine, the figures in col. II are based on the residual amino-N of the fraction, and are allocated, in the case of fraction $68_c 13_c 7{\cdot}5_c$, on the basis of the elementary composition of the material isolated by acid hydrolysis from the mother liquors of acetylphenylalanine.

The total N of the end-fractions (17·87 % of total N) is in good agreement with the N figure for fraction 68_c (18·2 %), showing that the loss of material in the course of analysis is slight.

Table 15. *The higher monoamino-acids of wool*

(N as % of total N)

Fraction	N of fraction	Methionine I	Methionine II	Valine I	Valine II	Proline I	Proline II	Leucines I	Leucines II	Phenylalanine I	Phenylalanine II
$68_c13_w21_w$	1·66	—	0·31	0·17	0·92	—	0·26	—	—	—	—
$68_c13_w21_m$	2·81	—	0·09	2·01	0·16	—	0·55	—	—	—	—
$68_c13_w21_c$	1·72	—	0·01	—	0·13	1·29	0·16	—	0·13	—	—
68_c13_m	4·44	—	—	—	—	1·97	0·70	1·60	0·17	—	—
$68_c13_c7{\cdot}5_w$	3·17	—	—	—	—	—	—	2·52	0·65	—	—
$68_c13_c7{\cdot}5_m$	1·55	—	—	—	—	—	—	1·11	0·44	—	—
$68_c13_c7{\cdot}5_c$	2·52	—	—	—	—	—	—	—	0·58	1·32	0·62
Total	17·87	—	0·41	2·18	1·21	3·26	1·67	5·23	1·97	1·32	0·62
Combined totals		0·41		3·39		4·93		7·20		1·94	

In Table 16 the present analysis is correlated with the more important published data on the amino-acid composition of wool. In this table the amount of each amino-acid is expressed as N in % of total N of wool. Where authors have not recorded the N content of the wool analysed, it has been assumed to be 16·75 %. Barritt [1928] found the N content of a very wide variety of white wools to lie between 16·50 and 17·07 %.

The high amino-N content of the hydroxyamino-acid fraction from wool makes the presence of hydroxyproline unlikely.

This is in agreement with the failure of Abderhalden & Voitinovici [1907] to isolate hydroxyproline from wool or from sheep-horn where they made a special search for it.

Table 16. *Summarized data on the amino-acid composition of wool*

(N as % of total N)

Author ...	Abderhalden & Voitinovici [1907]	Marston [1928]	Block [1939]	Other authors	Present investigation
N content of wool %	—	17·8	15·4	—	—
Glycine	0·65	—	7·87	—	—
Alanine	4·13	—	—	—	—
Valine	2·0	—	—	—	3·4
Leucine isomers	7·35	—	—	—	7·2
Phenylalanine	—	—	2·2	—	1·9
Proline	3·20	—	—	—	4·9
Methionine	—	—	—	0·3 Barritt [1934]	0·4
Cystine	5·08	8·59	9·93	—	—
Serine, etc.	0·05	—	—	7·5 Martin & Synge	—
Threonine	—	—	—	4·7 (to be published)	—
Tyrosine	1·34	2·08	2·26	—	—
Tryptophane	—	1·39	0·62	—	—
Aspartic acid	1·45	—	—	4·56 Speakman &	—
Glutamic acid	7·34	—	—	8·68 Townend	—
Ammonia	—	6·74	—	8·18 [1937]	—
Arginine	—	18·42	18·16	20·2 Vickery [1940]	—
Histidine	—	10·50	1·23	—	—
Lysine	—	5·02	3·12	—	—

It will be seen from Table 16 that more than 90 % of the N of wool has now been reasonably accounted for in the form of ultimate hydrolytic products. The corresponding figure for gelatin, based on the figures of Dakin [1920], as modified by Bergmann & Stein [1939] for glycine and proline and by Synge [1939, 3] for hydroxyamino-acids other than hydroxyproline, is 92·5 %.

The serine and threonine values given in Table 16 are taken from our forthcoming paper.

Criticism of the new analytical procedure

In appraising the analytical value of the technique described in this paper, we wish to emphasize that the procedure is still in a crude state and is capable of technical development. Thus it is clear, from the consistent change of rotation of successive acetylleucine fractions shown in Table 14, that a longer extraction column, operating in a more controlled manner, could give practically complete separation of the components of this mixture.

It is natural to compare the present procedure with the Dakin butyl alcohol extraction and Fischer ester distillation procedures, to which its two stages are directly analogous, and which are the only methods for separating this group of amino-acids in general use among protein chemists.

The present procedure is not suited to deal with glycine or alanine. For methionine, valine, proline, leucine isomers and phenylalanine it has definite advantages. The procedure leading to fraction 68_c gives these amino-acids in 95% yield, and substantially free from amino-acids of other groups. Butyl alcohol extraction, according to Sharp [1939], may give significant amounts of basic and dicarboxylic amino-acids together with the monoamino-acids. In subsequent fractionation, the present procedure leads to a strikingly better separation of valine from leucine than those recorded for ester distillation in the literature. It should, however, be remembered that modern distillation technique has so far not been applied to the Fischer distillation. The degrees of racemization induced by the two procedures seem to be similar. The acetamino-acids have the advantage over the free amino-acids of a lower tendency to form mixed crystals, and therefore may prove of use for 'isotope dilution' analysis [Rittenberg & Foster, 1940] where high purity is called for, irrespective of yield. The acetamino-acids have definite melting points, which is useful for rapid identification. The quantity of protein required for an analysis (10 g.) by the present procedure is much smaller than that required for an ester distillation. Finally, the residues from the first extractions may advantageously be used for the isolation of hydroxyamino-acids by acetylation-benzoylation, as described above, and it is possible that other analytical procedures would benefit from a preliminary removal of the higher monoamino-acids.

Summary

1. The history of the development of purification processes based on liquid-liquid extraction is reviewed.

2. The possibility of separating certain amino-acids as their acetyl derivatives by extractional fractionation in a chloroform-water counter-current train is discussed. The physical chemistry of the separation is outlined, and some new experimental data are provided on the equilibria concerned.

3. The design, construction and operation of a 40-unit counter-current liquid-liquid extraction train is described, together with tests of the efficiency of this machine in separating acetamino-acids.

4. On this basis an analytical procedure for the determination and isolation of methionine, valine, proline, leucine isomers and phenylalanine in protein hydrolysates is developed, and is tested on a complex known mixture of amino-acids. The results show that the new procedure has some advantages over the use of butyl alcohol extraction (Dakin) followed by ester distillation (Fischer).

5. The new procedure is applied to the analysis of a wool hydrolysate.

6. The results of the present investigation are summarized together with the published information on the amino-acid composition of wool.

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