Table 1. *Examination of bushy stunt virus in the ultracentrifuge*

Run	Index no.	Type of product	Conc. %	pH	$S_{20}^{20} \times 10^{-13}$	Fraction in boundary
270	381 J	Unheated	0.25	4.7	135.0	0.87
269	368 G	Unheated	0.25	4.7	130.3	—
274	381 K	Unheated	0.25	4.7	133.8	—
275	431 B	Heated after purification	0.25	4.7	130.1	—
273	381 M	Heated after purification	0.25	4.7	133.4	—
					225	ca. 0.05
266	363 G	Prep. from heated sap	0.25	4.7	130.8	0.93
267	363 G	Prep. from heated sap	0.25	6.8	135.9	—
268	350 B	Material inactivated by lying in sap	0.35	4.7	130.2	0.89
297	531 A	Inactivated by freezing	0.25	4.7	132.7	—
298	531 B	Inactivated by freezing	0.25	4.7	131.1	—

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Partition Chromatography in the Study of Protein Constituents

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In the present paper we report new applications and developments of partition chromatography* [Martin & Synge, 1941*b*] in the study of amino-acids and peptides. We have further developed the technique, aiming first at the isolation and identification of protein constituents, both amino-acids and peptides, and secondly, at a reliable micro-analytical procedure for the determination of as many different amino-acids as possible in the same sample of a complete hydrolysate of a protein or peptide.

Here we describe the more technical aspects of the work and give a detailed account of the micro-method for monoamino-acids at its present stage of development, together with the results obtained with it on wool and gelatin hydrolysates. The two following papers illustrate further the application of our new methods to particular problems in protein chemistry. Throughout the present work we have employed, as previously, the acetyl derivatives of the amino-acids and peptides.

* We employ the term 'partition chromatography' at the suggestion of Dr E. Lester Smith, to distinguish it from the classical adsorption chromatography. Our earlier term 'liquid-liquid chromatography' was liable to confusion with the fractional elution procedure sometimes called 'liquid chromatography'.

EXPERIMENTAL

Preparation of silica gel for chromatography

Adsorption by the silica of the substances undergoing analysis could seriously alter their behaviour from that to be expected were their partition between the two liquid phases the only determining factor. There must be an adequate concentration of anti-adsorbent substance in the solvent employed [Martin & Synge, 1941*b*]. Silica gel prepared in different ways has very different adsorption properties, and the more adsorbent gels may require unsuitably high concentrations of alcohol before they are useful. Adsorption by the silica may manifest itself as 'tailing' of the solutes undergoing analysis, and results in imperfect separation. This is the behaviour to be expected for adsorption of the 'Freundlich' type, where, for low concentrations, the adsorption is relatively much greater than for high ones. 'Ageing' of the freshly precipitated gel under dilute acid before drying it is essential for the production of a suitable colour with methyl orange (MO), but if ageing is continued for much longer than prescribed, a serious increase in adsorbing power may result. Deviations from the precipitation procedure given below (order of addi-

tion, concentration, temperature, etc.) result in less satisfactory products. Thorough acidification during precipitation and ageing is essential. Otherwise, low recoveries of the first acid band moving down the column will result. This is presumably due to neutralization by retained Na^+ ions, and explains the low recovery of acetylphenylalanine noted previously.

Detailed procedure. The silica gel is prepared according to Martin & Synge [1941b, footnote]. We give here a fuller description.

Commercial water-glass (140°Tw. -Jos. Crosfield, Ltd., Warrington) is diluted to 3 vol. with distilled water containing a little MO. $10N$ HCl is added in a thin stream with vigorous stirring, addition being interrupted at intervals and stirring continued to get efficient mixing. The solution changes first slowly and then rapidly to a thick porridge and all but the smallest lumps are broken up by stirring. When the mixture is permanently acid to thymol blue, addition of HCl is stopped and the mixture is kept 3 hr. It is filtered on a Buchner funnel and washed with distilled water (approx. 2 l./250 g. dry gel) without allowing the precipitate to crack. The gel is then suspended in $N/5$ HCl and aged 2 days at room temperature. It is again filtered and washed in the same way with distilled water (approx. 5 l./250 g. dry gel) until the washings are free from MO. Finally, the gel is crumbled and dried at 110° in an air oven. With such a preparation the addition of 53% w/w of indicator solution to the dry gel should be satisfactory with the butanol-chloroform or propanol-cyclohexane mixtures. The dry gel can be stored in a closed vessel for long periods without deterioration.

Indicators

MO has been employed throughout these experiments for indicating the presence of the acetyl derivatives. Most of the usual indicators of suitable pH range which we have studied are very readily extracted from the aqueous phase by the developing solvent. MO is not free from this defect, and is slowly removed by the solvent, particularly if the alcohol content of the latter is high. When this happens, the column becomes colourless from above downwards, and is useless for the visual separation of solutes whose bands move much slower than that of acetyl glycine.

The anthocyanin series promises to yield suitable indicators. Of those tested, an aqueous solution (0.05%) of pelargonin chloride (prepared from petals of the scarlet dahlia 'Coltness Gem' [cf. Willstätter & Mallison, 1915]) has proved useful. It is not readily 'leached' by solvent mixtures quite rich in alcohols; its sensitivity to acid is not (like that of MO) markedly depressed by alcohols; and it is free from N and S.

The presence of MO in the fractions obtained from the chromatograms has not interfered with the titration of the acetyl derivatives described below, but the indicator has sometimes been difficult to eliminate in the course of preparative work.

The 'background' colour of the MO in the chromatograms is greatly affected by the nature of the developing solvent, as well as by the silica, and may vary from bright pink to bright yellow. The solvents whose use we describe below all give usable orange background colours, although 30% propanol-cyclohexane gives a rather yellow and insensitive column. Good lighting is essential for making the best 'cut' when the bands are not widely separated. In the absence of bright daylight we have used a 80W B.T.H. fluorescent lamp with success.

Behaviour of amino-acid and peptide derivatives in different solvent systems

We have studied in detail three different solvent systems in which the acetyl derivatives of the mono-amino-acids and some of their peptides have suitable distributions, viz.: (1) chloroform-*n*-butanol-water, (2) cyclohexane-*n*-propanol-water, (3) ethyl acetate-water. Tables 1 and 2 give the band-rates of a number of those compounds in chromatograms employing these solvent systems. R has the same significance as before [Martin & Synge, 1941b], and may be related to the partition coefficient by the same formula [*loc. cit.*]. Since R depends on a number of variable factors, the values which we give must be regarded as a rough guide only. The ratios between these values for different compounds in the same column are, however, relatively constant.

Here and subsequently such an expression as 1% butanol-chloroform refers to a solution in chloroform of butanol, 1% v/v, subsequently saturated with water.

(1) *Acetamino-acids.* While in general the acetamino-acids with longer and less polar 'side-chains' move fastest on the chromatograms, there are some striking differences in their sequence in the different solvents. Thus while acetylphenylalanine travels faster than acetylleucine in chloroform-butanol-water, it is not separable from it in cyclohexane-propanol-water. Acetyltryptophan, acetylmethionine and acetylproline are also relatively retarded in the latter solvent. The same is generally true for this 'more polar' class of acetamino-acids in ethyl acetate-water as against chloroform-butanol-water. In ethyl acetate-water, however, *N*-acetyltyrosine is considerably accelerated.

In general, the less polar solvent (cyclohexane) discriminates more between these classes of compound than does the more polar solvent (CHCl_3). However, a single parameter such as 'polarity' probably cannot be employed for giving a complete

Table 1. *Band rates of acetamino-acids with various solvents*(Figures are values of *R*)

Developing solvents	Butanol-chloroform			Propanol-cyclohexane		Ethyl acetate
	1%	3%	17%	5%	30%	
Acetylphenylalanine	0.5	0.9	Fast	0.3	Fast	Fast
Acetylnorleucine	0.4	—	Fast	—	—	—
Acetylleucine	0.3	0.6	Fast	0.3	Fast	Fast
Acetylisoleucine						
Acetyltryptophan	0.3	0.6	Fast	0.15	Fast	—
Acetylvaline	0.15	0.3	Fast	0.15	Fast	0.9
Acetylnorvaline						
Acetylmethionine	0.15	0.3	Fast	0.09	0.7	0.8
Acetylproline	0.15	0.3	Fast	0.04	0.3	0.2
α -Acetaminobutyric acid	0.07	—	—	—	—	0.5
Acetylalanine	0.025	0.04	0.35	0.04	0.3	0.2
<i>N</i> -Acetyltyrosine	0.02	0.04	0.7	—	0.6	Fast
Acetylglycine	Slow	Slow	0.15	Slow	0.15	0.1
Acetylaspartic acid	Slow	Slow	0.1-0.2	—	—	—
Acetylglutamic acid						
<i>NN'</i> -Diacetylcystine						
<i>NN'</i> -Diacetyllysine						
<i>N</i> -Acetylhydroxyproline	Slow	Slow	0.07	Slow	0.1	0.06

theoretical description of such systems. Our experience suggests that new and useful solvent mixtures having specific effects with particular side-chains remain to be found.

We have not yet effected a satisfactory separation of leucine from isoleucine, or of valine from norvaline, and are now unable to confirm the separation of the former using 0.5% butanol-chloroform previously reported [Martin & Synge, 1941b]. We have not observed any separation of an optically active substance from the corresponding racemic compound in the course of this work.

(2) *Acetylpeptides*. The evidence presented in Table 2 suggests that acetylpeptides travel in general at rates intermediate between those of the acetyl

matograms packed with carbon and developed with aqueous solutions show a considerable difference in sequence from that obtained with our systems. Use of the two methods in succession might lead to highly effective separations of peptides. However, before Tiselius's chromatograms can be used for the isolation of substances it will be necessary to eliminate 'tailing' of the bands, which the adsorption data given by Cheldelin & Williams [1942] lead us to expect [cf. Wachtel & Cassidy, 1942]. Tiselius's use of 'moving boundary' observation methods with a chromatogram continuously fed with the mixture to be investigated is not interfered with by 'tailing'—which, indeed, increases the sharpness of the front boundary of each solute. The disadvantages of this procedure for isolative work are manifest, only a small portion of the least adsorbed component being readily obtainable in a state of purity.

Preparation and properties of acetyl derivatives studied

The compounds referred to in Tables 1 and 2 were described by Synge [1939] with bibliography, except for the following. Compounds reported here for the first time are indicated by an asterisk. All m.p.'s are given uncorrected unless otherwise stated. *N* determinations were by the Kjeldahl procedure. *C* and *H* determinations were carried out for us by Dr G. Weiler, Oxford.

(1) *Acetyl-L-valine*. The earlier preparation made by acetylation of *l*(+)-valine ('*d*-valin' of Hofmann La Roche) proved on chromatographic investigation to be a mixture containing at least 15% of impurity, probably an acetyl-leucine isomer. This would account for the deviations of the analytical values from the theoretical recorded by Synge [1939].

derivatives of their constituent amino-acids, and this is confirmed by experiments on the products of the partial hydrolysis of gelatin [Gordon, Martin & Synge, 1943b].

The data of Tiselius [1942] on the behaviour of free amino-acids and peptides in adsorption chro-

Table 2. *Band rates of acetyldipeptides with various solvents*(Figures are values of *R*)

Developing solvents	Butanol-chloroform		Propanol-cyclohexane		Ethyl acetate
	1%	17%	5%	30%	
Acetylleucyl-leucine	1.0	Fast	—	—	Fast
Acetylleucyl-glycine	0.04	0.6	0.04	1.0	0.5
Acetylglycyl-leucine					
Acetylglycyl-glycine	Slow	0.04	—	—	0.06

Our present material was prepared by resolution of acetyl-*dl*-valine [cf. Synge, 1939] obtained by acetylation of a specimen of *dl*-valine which we had prepared by a malonic ester synthesis from isopropyl bromide [Marvel & du Vigneaud, 1931; Marvel, 1940]. The acetyl-*dl*-valine was resolved directly as its brucine salt. A number of intermediate stages that would have been necessary had we employed Fischer's [1906] resolution were thus avoided.

3 g. of acetyl-*dl*-valine and 7.5 g. of brucine were dissolved in a minimum of ethanol, and ether was added to turbidity. Slow crystallization resulted, which in later preparations could be accelerated by seeding and stirring. The crystalline brucine salt was filtered off, washed with ether, and recrystallized in the same way from ethanol-ether. The recrystallized product weighed 4.2 g. Brucine was removed from the salt by treatment with NaOH in aqueous solution at 0°. After filtration of the precipitated brucine, the solution was extracted with CHCl₃ to remove the part remaining in solution. Dilute H₂SO₄ equivalent to the NaOH employed was then added, the mixture was evaporated to dryness *in vacuo*, and the solid residue was thoroughly extracted with boiling ethanol. On evaporation of the combined alcoholic extracts, crystalline acetyl-*l*-valine resulted, which was recrystallized from water until its optical rotation and m.p. were constant. Yield 0.9 g., having m.p. 164°, (α)_D¹⁹ + 4.0° (ethanol, *c* = 2). The preparation was chromatographically homogeneous. These physical data supersede those given for acetyl-*l*-valine by Synge [1939].

(2)* *Acetyl-dl-norvaline*. This was prepared from a specimen of *dl*-norvaline (manufactured by Fränkel and Landau, and kindly given to us by Prof. H. A. Krebs) in the way described by Synge [1939]. The product on recrystallization from water had constant m.p. 111–112.5°. It proved, however, on chromatographic investigation to contain an impurity (at least 15%), probably an acetyl-leucine isomer. This suggests the occurrence of mixed crystals. As the preparation was required for qualitative chromatographic study, the presence of this impurity was not an obstacle, and we have not yet purified the compound. The elementary composition of the preparation is in agreement with that expected from the chromatographic data. (Found: C, 53.5; H, 8.27; N, 8.67%. C₇H₁₃O₃N requires: C, 52.8; H, 8.17; N, 8.8%. C₈H₁₅O₃N requires: C, 55.5; H, 8.67; N, 8.1%.)

(3)* *NN'*-Diacetyl-*dl*-lysine [cf. Synge, 1939; Kolb & Toennies, 1942]. Synthetic *dl*-lysine was acetylated in aqueous solution with NaOH and acetic anhydride as usual. After acidification with H₂SO₄, the aqueous solution was extracted repeatedly with ethyl acetate. The syrupy residue from evaporation of the combined extracts crystallized after keeping for several weeks. The product was readily recrystallized from ethyl acetate, in which it is rather insoluble. m.p. 140–141°. (Found: C, 52.0; H, 7.62; N, 12.1%; acid equiv. wt. 232. C₁₀H₁₈O₄N₂ requires: C, 52.2; H, 7.82; N, 12.2%; acid equiv. wt. 230.)

(4)* *Acetyl-l-aspartic acid*. This was prepared from *l*-aspartic acid by acetylation and extraction from acid solution with ethyl acetate as for the previous compound. The product was syrupy.

(5) *Acetyl-l-tryptophan*. This was prepared according to du Vigneaud & Sealock [1932] from *l*-tryptophan. Our product had m.p. 185–6°, (α)_D^{21.5°} + 27.6° (water containing 1 equiv. of NaOH, *c* = 1), (α)_D^{19°} + 26.0° (ethanol, *c* = 1.1).

Du Vigneaud & Sealock record m.p. 189–190° (corr.), (α)_D^{31°} + 29° (water containing 1 equiv. of NaOH, *c* = 1). From CHCl₃ the compound was found to crystallize with 1 mol. of solvent. This was lost only slowly in a vacuum desiccator at room temperature, but was readily removed by drying *in vacuo* at 100°. Under these conditions the specimen lost 30.2% of its weight. (Loss of wt. calc. for C₁₃H₁₄O₃N₂·CHCl₃ is 32.7%.)

(6) *Acetyl-glycyl-dl-leucine*. Prepared according to Steiger [1934] from glycyl-*dl*-leucine [cf. Fischer & Warburg, 1905]. m.p. 176–177°. Steiger records 178–180° (corr.). (Acid equiv. wt.: Found, 223. Calc. for C₁₀H₁₈O₄N₂, 230.)

(7) *Acetyl-dl-leucylglycine*. Prepared according to Steiger [1934] from *dl*-leucylglycine [cf. Fischer & Brunner, 1905]. When crystallized from acetone containing 5% water the compound crystallized with $\frac{2}{3}$ mol. of acetone, which could be removed by drying over P₂O₅ *in vacuo* at 100°. The undried compound had acid equiv. wt. 270, and lost 14.4% of its weight on drying. (Calc. for C₁₀H₁₈O₄N₂, $\frac{2}{3}$ (C₃H₆O): acid equiv. wt. 268.6; loss of weight on drying, 14.3%.) The dried material regained the lost weight in an atmosphere saturated with acetone vapour, but not in an atmosphere saturated with water vapour. The dried material had m.p. 178°. Steiger records m.p. 182–183.5° (corr.). He does not mention the occurrence of acetone of crystallization.

(8) *Acetyl-glycylglycine*. Prepared from glycylglycine [cf. Fischer & Fourneau, 1901] by Steiger's [1934] acetylation procedure. Recrystallized from ethanol, the product had m.p. 178–179°. Fischer & Otto [1903] record m.p. 184–186°. (Acid equiv. wt.: Found, 175. Calc. for C₆H₁₀O₄N₂, 174.)

(9)* *Acetyl-dl-leucyl-dl-leucine*. This was prepared from the racemic leucylleucine [cf. Fischer, 1904] subsequently named leucylleucine 'A' by Fischer & Koelker [1907] and shown by them to be the racemic compound involving *d*-leucyl-*l*-leucine as opposed to *d*-leucyl-*d*-leucine. We acetylated the dipeptide with acetic anhydride and excess NaOH in aqueous solution as usual, and on acidification of the reaction mixture the acetyl compound crystallized out in 71% yield. Recrystallization from ethyl acetate yielded a product having m.p. 200°. (Found: C, 58.6; H, 9.02; N, 9.4%; acid equiv. wt. 278. C₁₄H₂₆O₄N₂ requires: C, 58.7; H, 9.09; N, 9.8%; acid equiv. wt. 286.)

Micro-determination of monoamino-acids

The method of analysis to be described is a development of that given by Martin & Synge [1941*b*], and is based on the observations reported above. By the use of additional solvent mixtures it is now possible to determine the following amino-acids and groups of amino-acids in a protein hydrolysate, viz. phenylalanine, leucine + isoleucine, valine (+*nor*-valine, if present), methionine, proline, alanine, and tyrosine. Tryptophan may also be determined semi-quantitatively if it survives the treatment employed for hydrolysing the specimen [cf. Gordon *et al.* 1943*a*]. Also in certain special cases [cf. Gordon *et al.* 1943*a, b*] glycine has been determined semi-quantitatively by this procedure. A satisfactory determination of lysine as its diacetyl derivative could be developed on these lines for use with basic amino-acid fractions prepared by electrodialysis or

precipitation with phosphotungstic acid. The need for a direct determination of this amino-acid has long been felt.

This extension of the range of the method is due to two novel features:

(1) After the faster moving constituents of the mixture undergoing analysis have left the column, some of the remaining slow-moving constituents are accelerated and run out by the use of a higher percentage of alcohol in the developing solvent. This has brought alanine, tyrosine, etc., within the scope of the method.

(2) By subjecting each fraction obtained from the butanol-chloroform chromatogram to further fractionation on a propanol-cyclohexane chromatogram, it is possible first to separate the (proline + valine + methionine) fraction into its three constituents, and secondly to remove a number of impurities (probably decomposition products resulting from prolonged treatment of the mixture with hot acid) which affect significantly the accuracy of analyses by the procedure originally described.

It is sometimes advantageous to change the order in which the chromatograms are used [cf. Gordon *et al.* 1943*a*], or to adopt different solvent mixtures. The present scheme, however, is likely to be a useful and routine approach where little is known about the constituents of the mixture for analysis. With the method described here, the amino-acids in question may in the first instance be determined on the hydrolysate of a single sample of protein with an accuracy of $\pm 5\%$. It may then be possible to devise control experiments [cf. Gordon *et al.* 1943*a*] for increasing the accuracy of the determination.

These analytical results are not affected by the degree of racemization of the amino-acids. As pointed out by Moore, Stein & Bergmann [1942], this is an inherent defect of their 'solubility product' techniques.

Our new procedure yields results of accuracy comparable with that of the better routine amino-acid determinations in current use, and furthermore covers a group of amino-acids for which analytical methods have in the past been inadequate. Since, in addition, it is on the microchemical scale (about seven different components being determinable on a 25 mg. sample of protein), it may help to satisfy a long-felt need.

Preparation of material for chromatography. Further work on the acetylation and extraction procedure described by Martin & Synge [1941*b*] has led us to introduce some improvements and simplifications. The method at present in use is as follows. The HCl hydrolysate to be acetylated (corresponding to about 25 mg. of protein) is evaporated *in vacuo* to dryness in a 100 ml. Erlenmeyer flask. 2 ml. of 2*N* NaOH are added and then 0.4 ml. of 6*N* NaOH. The solution is then cooled to 0°, and 0.2 ml. of acetic anhydride is immediately introduced, after which the flask is shaken and again cooled. Four further additions of 2 ml.

of 2*N* NaOH, followed by 0.2 ml. of acetic anhydride, are made at 2 min. intervals and are accompanied by cooling and shaking. Thymolphthalein (as an external indicator) is used to ensure that the solution remains alkaline throughout. The solution is then allowed to stand for 10 min., care being taken to see that it remains alkaline. If this is not done anomalous bands appear which are probably due to *ON*-diacetyl hydroxyamino-acids which are formed if insufficient NaOH is present [cf. du Vigneaud & Meyer, 1932]. The *O*-acetyl groups are readily saponified by alkali in the cold. At the end of the 10 min. period, the solution is made acid (red) to thymol blue by the addition of 10*N* H₂SO₄. Next it is transferred to a 60 ml. separating funnel and extracted with five successive 50 ml. portions of 17% butanol-chloroform. The extracting solvent is used to rinse out the flask after transferring the mixture to the separating funnel. The CHCl₃ extracts are filtered through a dry filter paper (Whatman no. 2) into a Claisen flask from which the solvent is subsequently evaporated *in vacuo*, the temperature being allowed to rise to 100°. Finally, the extracted acetamino-acids are transferred in ethanol to a small flask, the ethanol is removed *in vacuo*, and the residue kept overnight in a vacuum desiccator over soda-lime and H₂SO₄.

Chromatographic procedure. 3 g. of silica gel (prepared as described above) are saturated with methyl orange solution and suspended in 3% butanol-chloroform. This is introduced into a chromatogram tube having an internal diameter of 1 cm. [Martin & Synge, 1941*b*]. When the column is ready, as much as possible of the acetylated mixture is transferred to it by repeated (hot) extraction with small portions (1 ml. or less) of the solvent mixture. Each portion is allowed to drain into the gel before the next is added. After about five successive extractions, the flask containing the unextracted material is put aside for the second stage of the analysis, and the chromatogram tube is filled with the solvent mixture for development (3% butanol-chloroform).

The bands corresponding to phenylalanine (1), (leucine + isoleucine) (2), and (proline + valine + methionine) (3), are allowed to run out and are collected separately in pyrex flasks. The surface of the solvent is then allowed to sink until it is level with the top of the silica gel. At this point the acetylated material remaining in the flask, previously dissolved by boiling with about 1 ml. of 17% butanol-chloroform, is run on to the column. This is allowed to sink in, and after the flask has been washed out three times as before, development of the chromatogram is continued with the new solvent. This causes the bands corresponding to tyrosine and alanine, which have up to now been moving at nearly the same rate (ahead of the glycine, cystine, lysine, glutamic acid, etc.), to move faster, and at the same time draw apart from one another, the tyrosine band (4) running approximately twice as fast as the alanine band (5). These fractions are allowed to run out, and are collected separately.

Fractions 1-5 are freed from butanol-chloroform by evaporation on the water-bath *in vacuo*. Frac-

tions 1 and 2 are further analysed exactly as before, on columns containing 2 g. of silica, but using 5% propanol-cyclohexane instead of butanol-chloroform. The main band from each chromatogram is run out and collected (1.1 and 2.1, Table 3) free from any minor contaminants moving at different rates. Fraction 3 is similarly fractionated with the same solvent mixture on a rather longer column

gives rather poor separations between bands 1, 2 and 3. Thus the low recovery of phenylalanine and the excess of leucine no doubt result from the collection of some of the former material with the leucine band. Similarly, some of the leucine was collected in the proline-valine-methionine band, but this does not affect the accuracy of the analysis because, although inseparable from phenylalanine,

Table 3. *Scheme of fractionation*

employing 3 g. of silica. Here the first band to be collected corresponds to valine (3.1). When this has run out, the solvent mixture is changed to 30% propanol-cyclohexane, and the bands corresponding to methionine (3.2) and proline (3.3) are collected separately. Fractions 4 and 5 are fractionated on columns containing 2 g. of silica and developed with 30% propanol-cyclohexane. These result in the main fractions 4.1 and 5.1. Table 3 illustrates this fractionation.

Titration of resulting fractions. The seven final fractions are freed from solvent by evaporation *in vacuo* on the water-bath; the residue in each flask is dissolved in 3 ml. of water (by heating if necessary) and titrated against $N/100$ $\text{Ba}(\text{OH})_2$ using bromthymol blue as internal indicator. This is used in preference to phenolphthalein because the indicator blank is smaller and it is not affected by room concentration of CO_2 ; further, it gives with N -acetyltyrosine accurate titrations of the $-\text{COOH}$ group only. With phenolphthalein the phenolic group of this compound titrates partially, giving a high result with an unsatisfactory end-point.

For all the amino-acids determined at present except tryptophan, 1 ml. of $N/100$ $\text{Ba}(\text{OH})_2$ corresponds to 0.14 mg. of N; for tryptophan it corresponds to 0.28 mg. of N.

Control experiments on procedure. The mixture of amino-acids and their acetyl derivatives shown in Table 4, which simulates in amino-acid composition approximately 25 mg. of wool, was refluxed in an excess of 6 N HCl for 24 hr., and was then analysed as above.

In this experiment 3% butanol-chloroform was used for the initial fractionation because it causes more rapid movement of the bands, even though it

leucine can easily be separated from proline, valine and methionine on the propanol-cyclohexane column. As previously reported [Martin & Syngé, 1941*b*], good separations of bands 1, 2 and 3 can be obtained by using 1% butanol-chloroform, and unless a longer column is used, this solvent is recommended for development until bands 1 and 2 have been run out of the column.

Table 4. *Control analysis on an amino-acid mixture*

	Taken (mg. N)	Found (mg. N)	Recovery (%)
Acetyl- <i>dl</i> -phenylalanine	0.0954	0.088	92
Acetyl- <i>d</i> -leucine	0.2420	0.310	104
Acetyl- <i>d</i> -isoleucine	0.0566		
Acetyl- <i>dl</i> -valine	0.1637	0.156	95
<i>l</i> -Methionine	0.0226	0.021	93
Acetyl- <i>l</i> -proline hydrate	0.1799	0.189	105
Acetyl- <i>dl</i> -alanine	0.1700	0.165	97
<i>l</i> -Tyrosine	0.0795	0.082	103
Acetyl- <i>l</i> -tryptophan	0.0376	—	—
Glycine	0.5330	—	—
<i>l</i> -Cystine	0.3455	—	—
<i>l</i> -Glutamic acid	0.2555	—	—
<i>l</i> -Aspartic acid	0.1295	—	—
<i>dl</i> -Serine	0.2478	—	—
<i>dl</i> -Threonine	0.1928	—	—
<i>l</i> -Arginine monohydrochloride	0.9280	—	—
<i>dl</i> -Lysine dihydrochloride	0.1330	—	—
<i>l</i> -Histidine monohydrochloride	0.0417	—	—
Ammonium sulphate	0.3415	—	—
Total	4.1956		

No tryptophan survived the conditions to which the mixture was subjected—had any done so it would have been detected during the further fractionation of fraction 2 with propanol-cyclohexane. The work reported in our paper on gramicidin

[Gordon *et al.* 1943*a*] shows that substantial recoveries of acetyltryptophan result when tryptophan is present, with simpler amino-acids, in large amount, and when the hydrolysis is conducted with exclusion of air. Even here, however, some destruction of tryptophan occurs.

In the control experiment here reported, the following 'artefact bands' were noted during refractionation of the materials from the first column with propanol-cyclohexane. All were present in small amount.

Fraction 1. (a) A band travelling twice as fast as the phenylalanine band. (b) A band remaining near the top of the chromatogram.

Fraction 3. (a) A band travelling between the methionine and proline bands. (b) A band travelling just ahead of the proline band, but so close that its adequate elimination proved difficult.

Fraction 4. (a) A band moving at about half the rate of the tyrosine band.

It seems possible from the high value of the tyrosine recovery, that a contaminant not separable from it by this procedure is present. Although none of these artefact contaminants is present in very large amount, they render quite inaccurate the single fractionation procedure originally given by Martin & Synge [1941*b*]. Moreover, as they are probably formed by alteration of the original components of the mixture, this may explain the lower accuracy of the present determinations where a known complicated mixture was heated with acid in the open, as compared with those reported by Gordon *et al.* [1943*b*] for a much simpler known mixture heated *in vacuo*. These considerations are important and introduce a factor of uncertainty, stressed by Moore *et al.* [1942], into the process of relating the results of analysis of a protein hydrolysate to the amino-acid composition of the intact protein.

Analysis of wool and gelatin

Wool (Merino 64's) and gelatin (from the same batch of cow-hide gelatin studied by Gordon *et al.* 1943*b*) were subjected to analysis by the procedure given above. The results of the analysis

(uncorrected) are shown in Table 5, together with previous data on wool. The analysis of gelatin is correlated with the data of other workers in an accompanying paper [Gordon *et al.* 1943*b*].

Before acetylation the wool hydrolysate had been extracted with CHCl_3 to remove non-amino-acid constituents [cf. Martin & Synge, 1941*a*]. The artefact contaminants noted in the control analysis were all present also in the wool hydrolysate, but one additional artefact was noted, which appeared as a slow-moving band in fraction 2.

In the gelatin hydrolysate, contaminants 1(a), 3(a), 3(b) and 4(a) were absent, but two faint bands (one ahead of and one behind the leucine band) were noted during the fractionation of band 2. A faint band running at about half the rate of the proline band was noted in the fractionation of band 3. Some of these differences may be attributed to the absence of tryptophan from gelatin; the absence of cystine is probably also of significance.

The most striking feature of the analysis of wool is the phenylalanine value, which has been much lowered by the elimination of 'artefacts'. The possibility that aromatic constituents as yet unknown are present is suggested by Block's [1939] higher value, obtained by the not very specific colorimetric procedure.

Otherwise the results are in substantial agreement with those previously recorded [cf. Martin & Synge, 1941*a*, for bibliography]. The value for alanine is the first to be reported for wool since Abderhalden & Voitnovici's [1907] ester analysis, and is in substantial agreement with their figure.

SUMMARY

1. Technical and theoretical aspects of partition chromatography are discussed in relation to the separation of amino-acids and peptides in the form of their acetyl derivatives.

2. New data are presented on the behaviour of a number of these compounds in different solvent systems.

3. A micro-analytical procedure is described for the simultaneous determination of phenylalanine,

Table 5. *Analyses of wool and gelatin*

(N as % of total N)

	Gelatin Present detn.	Wool		
		Present detn.	Martin & Synge [1941 <i>b</i>]	Other detns.
Phenylalanine	0.4	0.8	1.9	1.9
Leucine-isoleucine	3.9	6.2	7.4	7.2
Valine	1.7	3.9	8.2	3.4
Methionine	0.3	0.2		0.4
Proline	9.6	5.2		4.9
Tyrosine	? Trace	2.8	—	2.1 Marston [1928] 2.3 Block [1939]
Alanine	8.3	3.9	—	4.1 Abderhalden & Voitnovici [1907]

leucine isomers, valine, methionine, proline, alanine and tyrosine in the hydrolysate of a single sample of protein.

4. Determinations of these constituents of wool and cow-hide gelatin are reported.

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The Amino-Acid Composition of Gramicidin

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The bactericidal substance gramicidin, isolated by Dubos *et al.* from cultures of various bacteria [for bibliography cf. Dubos & Hotchkiss, 1942] is of polypeptide character, yielding on acid hydrolysis a mixture of amino-acids, some of which possess the 'unnatural' *d*-configuration [Hotchkiss, 1941; Lipmann, Hotchkiss & Dubos, 1941; Christensen, Edwards & Piersma, 1941].

We have subjected acid hydrolysates of gramicidin to analysis by our new techniques [Gordon, Martin & Synge, 1943a] and have been able to confirm and extend most of the data of these authors.

Taken in conjunction with the analytical results of Hotchkiss [1941], our analyses suggest that the gramicidin molecule contains 24 amino-acid residues or some integral multiple thereof. These are made up as shown in Table 1.

The presence of leucine and tryptophan residues in gramicidin has already been established by isolation and characterization [Hotchkiss, 1941; Christensen *et al.* 1941]. Christensen *et al.* also claim to have isolated alanine through its dioxypyridate and

Table 1. *Suggested composition of minimum molecule of gramicidin*

	No. of residues	No. of atoms N
Leucine	6	6
Tryptophan	6	12
Valine	5	5
Alanine	3	3
Glycine	2	2
Hydroxyamino compound	2	2
Total	24	30

phosphotungstate, although no experimental details are given. They obtained a negative result for glycine, using the colorimetric method of Patton [1934]. We have established the occurrence in the hydrolysate of alanine, valine and glycine by isolation and characterization as the acetyl derivatives, and have confirmed in the same way the presence of leucine and tryptophan.

Our technique was not suitable for the isolation of the hydroxyamino compound [Hotchkiss, 1941]. We have, however, established by control experiments (see Experimental Section) that serine is