SUMMARY

Gramicidin has been subjected to acid hydrolysis, and the presence in the hydrolysate of *d*-leucine, *l*-tryptophan, *dl*-valine, *l*-alanine and glycine has been established.

A quantitative determination of these components has been made, and from this, in conjunction with the data of other authors, it seems probable that the gramicidin molecule contains the following 24 residues: 6 leucine, 6 tryptophan, 5 valine,

3 alanine, 2 glycine and 2 of an unknown hydroxy-amino compound.

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A Study of the Partial Acid Hydrolysis of Cow-hide Gelatin

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In a recent paper [Gordon, Martin & Synge, 1941] we described preliminary experiments aiming at the characterization of products of partial hydrolysis of proteins and through this at the elucidation of structural sequences in the intact protein molecule.

In the present paper we report further work in this direction, using partition chromatography [cf. Martin & Synge, 1941*b*; Gordon *et al.* 1943*a, b*]. This procedure has made possible the semi-quantitative isolation of a number of peptides in the form of their acetyl derivatives. Peptide isolations have usually depended on the chance insolubility of a particular peptide or its derivative, and have given unknown yields. The new, more refined methods make possible a study of all the peptides of a given class occurring in a partial hydrolysate of a protein,

and thus provide a more extensive picture than was formerly possible. The chromatographic procedure has been used simultaneously for rapid determination, on the micro scale, of the amino-acid composition of the peptide fractions isolated, an essential step too frequently neglected by workers in this field.

Apart from its value for the study of protein structure, this work may have applications in the field of serology, since Landsteiner [1942] has shown that the products of the partial hydrolysis of an antigenic protein can specifically inhibit its precipitin reactions with a homologous antiserum.

The present work was carried out with gelatin, since the absence of tryptophan, tyrosine and cystine from this material considerably simplifies the isola-

tive part of the investigation. This afforded us simultaneously a relatively simple check on current speculations as to the sequence of amino-acids in gelatin. The bearing of our experimental data on these speculations is discussed at the end of this paper.

Commercial gelatins are derived from the skin or bones of a number of species of animal, and are often mixtures in the biological sense. Because the parent collagens show significant differences in X-ray diffraction properties [cf. Astbury, 1940], it seemed particularly desirable to work with gelatin derived from a single source. The material used in the present work was a sample of cow-hide gelatin kindly placed at our disposal by Messrs Ross (Gelatines), Ltd., of Newlay, Leeds.

EXPERIMENTAL

(1) Analytical methods

Determinations of N (Kjeldahl), amino-N, free amino-acids (by ninhydrin) and tyrosine were carried out as previously [cf. Gordon *et al.* 1941]. The procedure used for determining monoamino-acids was as follows:

Determination of monoamino-acids. The chromatographic method previously described [Martin & Syngé, 1941*b*] had, for the present work, been extended to deal with alanine, and in certain circumstances with glycine. It had further proved possible to differentiate valine and methionine from proline by determination of amino-N. The analysis was carried out as before [Gordon *et al.* 1943*a*] except that the series of refractionations with propanol-cyclohexane was omitted, the material from the butanol-chloroform column being titrated directly. 1% butanol-chloroform, followed by 17% butanol-chloroform after band 3 had run out, was used for development of the chromatograms. Phenolphthalein was used as indicator for the titrations.

The acetylalanine band was followed by that of acetyl-glycine. This was run out and titrated in the same way. As explained elsewhere [Gordon *et al.* 1943*a*], this titration represents glycine only if cystine, lysine, glutamic and aspartic acids are absent, otherwise, under these conditions, the acetyl derivatives of those acids move at about the same rate as acetyl-glycine. When the glycine band has moved some way down the column, any hydroxyproline present appears as a more slowly moving band. No attempt was made to run this out, but semi-quantitative estimates could be made on the basis of the colour and breadth of the band.

After titration with $\text{Ba}(\text{OH})_2$ the proline-valine-methionine fraction was hydrolysed by refluxing for 5 hr. in 6*N* HCl at a volume of 10 ml. After this de-acetylation, excess HCl was removed *in vacuo*, and it was neutralized with Na acetate. Amino-N was then determined on the fraction. This made it possible to differentiate the proline from the valine and methionine. In the control experiment described below, where proline was separated from a mixture of amino-acids not containing valine or methionine, 0.232 mg. of proline-N in the fraction gave only 0.007 mg. amino-N. Similar experiments showed that under the same conditions valine and methionine gave 85–95% recovery of amino-N. This imperfect recovery, and the fact that in many experiments only very small amounts of valine and methionine

were present, make it necessary to interpret the relevant figures with circumspection.

Influence of indicators. Methyl orange, phenolphthalein and baryta were always present in the final fractions prepared for amino-acid analysis (cf. § 6). As it was possible that these substances might give rise during hydrolysis to acid degradation products which might interfere with the chromatography, control experiments with regard to their decomposition were undertaken.

Methyl orange degradation products. 100 mg. of methyl orange were refluxed for 24 hr. in 6*N* HCl. Excess HCl was removed *in vacuo*. The resultant material was acetylated, and extracted with 17% butanol-chloroform as usual and one-fifth of this extract was taken for the chromatogram. This was run first with 1% and then with 17% butanol-chloroform as described above. During the first stage no acid band appeared. On development with 17% butanol-chloroform a faint band was seen to move down slightly behind the front of the new solvent. This was followed by a second very faint band which was estimated to move at a rate between that characteristic of glycine and that of hydroxyproline. Neither of these bands was strong enough to give a significant titration.

Phenolphthalein degradation products. 10 mg. of phenolphthalein were refluxed with acid, acetylated and extracted as above, and the whole of the extract put on the column. On development with 1% butanol-chloroform, a faint band moved down the column having $R=1.0$. A second band was observed near the top of the column. On development with 17% butanol-chloroform this band moved down at $R=0.15$. A third faint band moved at $R=0.065$. Again none of these bands was of significant intensity.

Percentage recovery of amino-acids in a known mixture. Thus it appeared that indicators did not interfere with the analysis and a control experiment on amino-acid recovery was carried out. The amino-acids in the proportions shown in Table 1, together with 1 ml. of a saturated aqueous solution of methyl orange, 0.5 ml. of a 1% solution of phenolphthalein in ethanol and 0.33 ml. of $N/3 \text{ Ba}(\text{OH})_2$ were made up to 10 ml. in 6*N* HCl. The solution was refluxed for 36 hr. and after removal of excess HCl *in vacuo* the mixture was acetylated and extracted as described above.

One-tenth of the extract (corresponding to 1.10 mg. N in the original amino-acid mixture) was put on a column as above. The resultant analysis led to the recoveries shown in Table 1. The low

Table 1. Percentage recovery in a known mixture of amino-acids

Amino-acid	N as % of total N of mixture		% recovery
	Taken	Found	
<i>dl</i> -Phenylalanine	15.5	11.5	74
<i>dl</i> -Leucine	15.3	15.3	100
<i>l</i> -Proline	14.9	14.3	96
<i>dl</i> -Alanine	19.1	18.1	95
Glycine	16.6	13.6	82
<i>l</i> -Hydroxyproline	18.6	—	—
	100		

recovery of acetylglycine is explained by the incomplete extraction of this acid from the aqueous acetylation mixture. All figures given for glycine in the present paper have therefore been corrected assuming 82% recovery.

The low recovery of phenylalanine was due to the use of gel containing traces of alkali. This question is dealt with by Gordon *et al.* [1943*a*]. The unsatisfactory gel used in the present experiment was also used in analysing the 'neutral peptide' fractions from the 4-day gelatin hydrolysate. Elsewhere in the present work gel giving satisfactory phenylalanine recoveries was used.

The above control experiment gives an indication of the accuracy of the analytical operations described in this paper.

(2) Analysis of gelatin

Monoamino-acids. Since the present investigation was concerned mainly with peptides of the monoamino-acids, an analysis for the total of each of these components was first carried out on a complete hydrolysate.

The gelatin was hydrolysed by refluxing for 24 hr. in 6*N* HCl. A colorimetric tyrosine determination on the hydrolysate showed some colour-producing substance to be present—this, however, was mainly associated with the small amount of insoluble humin in the hydrolysate—and no band corresponding to tyrosine was ever noticed on the chromatograms [cf. Gordon *et al.* 1943*a*].

The hydrolysate was analysed for constituent monoamino-acids. The results are shown in Table 2, together with our later figures for the same sample of gelatin [Gordon *et al.* 1943*a*] and those of the best previous determinations on gelatin. In converting these earlier figures to a N basis it was assumed that the N content of the gelatin (not recorded by Dakin or by Bergmann & Stein) was 18.3% [cf. Beach & Teague, 1942].

Table 2. *Monoamino-acids of gelatin*

(N as % of total N).				
Author ...	(1)	(2)	(3)	Others
Phenylalanine	1.0	0.4	0.6	—
Leucine isomers	3.9	3.9	4.1	—
Valine	1.5	1.7	0	—
Methionine		0.3	—	0.4 Beach & Teague [1942]
Proline	9.7	9.6	7.5	11.6 (4)
Alanine	9.0	8.3	7.5	—
Glycine	—	—	26.0	27.0 (4)
Hydroxyproline	—	—	8.2	8.4 Bergmann [1935]

(1) Present determination.

(2) Gordon *et al.* [1943*a*].

(3) Dakin [1920].

(4) Bergmann & Stein [1939].

The difference between the phenylalanine value obtained here, and that obtained by our later procedure, shows clearly the necessity for the second fractionations with propanol-cyclohexane. In other cases our two analyses are in substantial agreement.

Methionine. Determinations on the present sample of gelatin, kindly carried out by Dr S. Blackburn of Torridon by the Baernstein volatile iodide procedure, gave a corresponding figure of 0.67%. After the gelatin had been hydrolysed by 10*N* HCl at 37° for several weeks, this value had fallen to 0.14%, suggesting demethylation. The possibility that methionine was oxidized to its sulphoxide by the H₂O₂ used in the manufacture of the gelatin should not be ignored.

Valine. The isolation, from the present sample of gelatin, of valine, in the form of its acetyl derivative, is described below (§ 6*d*). This is the first time valine has been shown by isolation to be a constituent of gelatin. Fischer, Levene & Aders [1902] obtained some evidence for its occurrence but left the question open pending more detailed investigation. Dakin [1920] asserted that it was absent. Dr R. Block (private communication) has obtained evidence for its occurrence in gelatin, using a modification of the procedure of Fromageot & Heitz [1939] [cf. Block & Bolling, 1941: we have not had access to this publication].

(3) Course of hydrolysis

The gelatin was hydrolysed in the same manner as before [Gordon *et al.* 1941], by allowing it to stand in 10*N* HCl at 37°, samples being removed at intervals for estimation of amino-N and free amino-acids. Fig. 1 shows a plot of these results. The large

Fig. 1. Course of hydrolysis of gelatin in 10*N* HCl at 37°.

amount of glycine in gelatin makes it necessary to treat the amino-N figures with reserve. No correction for the glycine can be made because, although

the proper factor for the free amino-acid is well known, this is not the case when it is still in peptide linkage [cf. Van Slyke, 1911].

That the figure for free amino-acids had reached only a fraction of its final value when the figure for amino-N had almost reached 100% seems to indicate that the imino groups of proline and hydroxyproline are present, as previously suggested [Gordon *et al.* 1941], in peptide bonds resistant to acid hydrolysis at 37°.

The partial hydrolysates used for analysis were removed after 4 and 19 days. Further hydrolysis was stopped by removal of HCl by repeated evaporation with water *in vacuo*. The mixture was then diluted with water and stored at 0°. Fig. 1 shows

tive amounts of the various molecular species left in the middle compartment, are to be expected.

In the case of the 'basic' fraction [cf. Gordon *et al.* 1941], the recovery should be quantitative.

The distribution of total N, amino-N, and free amino-acids in the partial hydrolysates and the fractions derived from these by electro dialysis is shown in Table 3. Amino-N and free amino-acids were determined before and after completely hydrolysing the material by refluxing for 24 hr. in 6*N* HCl. Comparison of these figures gives a measure of the amount of peptide material present in the different fractions.

The further analyses of the 'basic' and 'neutral' fractions are described separately below.

Table 3. *Analysis of fractions obtained by electro dialysis from partial hydrolysates of gelatin*

(N as % of total N of unfractionated gelatin)		
Unfractionated hydrolysate:	4 days' hydrolysis	19 days' hydrolysis
Amino-N before complete hydrolysis	40.7	59.9
Amino-N after complete hydrolysis	67.0	67.0 (assumed)
Free amino-acids before complete hydrolysis	24.7	60.5
Free amino-acids after complete hydrolysis	87.0	87.0 (assumed)
Basic fraction:		
Total N	22.0	22.5
Amino-N before complete hydrolysis	6.3	7.6
Amino-N after complete hydrolysis	10.5	9.6
Free amino-acids before complete hydrolysis	2.3	5.5
Free amino-acids after complete hydrolysis	9.6	8.4
Neutral fraction:		
Total N	51.6	62.6
Amino-N before complete hydrolysis	25.3	37.8
Amino-N after complete hydrolysis	38.2	45.1
Free amino-acids before complete hydrolysis	16.2	45.7
Free amino-acids after complete hydrolysis	50.0	60.7

that in the 4-day hydrolysate approximately one-third, and in the 19-day hydrolysate approximately two-thirds, of the residues had been converted to free amino-acids.

(4) *Preparation of 'basic' and 'neutral' fractions*

The electro dialysis of the partial hydrolysates was conducted in a diaphragm cell as previously described [Gordon *et al.* 1941], except that, in addition to the basic fraction, a neutral fraction was prepared. This consisted of the contents of the middle compartment of the cell after the first electro dialysis at pH 6. To ensure complete removal of basic and acidic material, the current was switched on again for a few minutes after the end compartments had been washed out, and refilled with distilled water. This was repeated a second time. Since some neutral material diffused out of the middle compartment during the preparation, the recovery of neutral amino-acids and peptides in this fraction was not quantitative. The amounts of the different substances lost depended on their different rates of diffusion, but only very slight changes in the rela-

The above figures for free amino-acids are residues (1 mol. CO₂ evolved in presence of ninhydrin) expressed as N (1 atom) as % of the total N of the original unfractionated gelatin.

(5) *Investigation of the 'basic' fractions*

The 'basic' fractions obtained from the 4- and 19-day hydrolysates were analysed for monoamino-acids after complete hydrolysis, by the chromatographic method described above (§ 1). This experiment was similar to the analysis of the 'basic' fractions derived from various proteins [cf. Gordon *et al.* 1941], but owing to improved technique it could be carried out quantitatively. The amounts of the various amino-acids found are shown in Table 4. Assuming that these monoamino-acids are all linked as dipeptides with basic amino-acids, the yields from the 19-day hydrolysate account nearly quantitatively for the residues present in peptide linkage as determined by the ninhydrin procedure. Note also that the total amount of phenylalanine, leucine, valine and methionine residues bound in basic peptides after 4 days' hydrolysis is substan-

tially the same as after 19 days' hydrolysis. This is not, however, true for proline and alanine residues, and suggests that the bonds involving the former amino-acids are much more resistant to such hydrolysis than are those involving others such as proline and alanine.

Table 4. Analysis of 'basic' fractions

(N as % of total N of unfractionated gelatin)

Amino-acid	4 days' hydrolysis	19 days' hydrolysis
Phenylalanine	0.05	0.04
Leucine	0.29	0.29
Valine + methionine	0.22	0.26
Proline	0.96	0.35
Alanine	0.77	0.30
Total	2.29	1.24
Residues in peptide linkage (moles as N by difference of free amino-acid determinations: cf. Table 3)	7.29	2.9

(6) Investigation of the 'neutral' fractions

(a) *Chromatographic fractionation.* Preliminary experiments with synthetic acetyldipeptides [Gordon *et al.* 1943a] showed that these compounds are as readily separable by partition chromatography as are acetamino-acids, and that in general, for a given solvent pair, the acetylpeptides travel at rates intermediate between those for the acetyl derivatives of their constituent amino-acids.

In the present work on neutral peptides from gelatin, we have used successive fractionation on ethyl acetate-water and chloroform-butanol-water columns. This gives specially sharp separations of proline, whose acetyl derivative moves at the same rate as acetylvaline in chloroform-butanol, but moves more slowly than acetylalanine in ethyl acetate. The proline peptides in partial hydrolysates of gelatin behave similarly, and are therefore specially amenable to study by the technique described here.

The 'neutral' fraction resulting from electrodialysis of the partial hydrolysate (corresponding to about 0.8 g. of gelatin) was evaporated to dryness *in vacuo* below 40°, acetylated, acidified and extracted with 17% butanol-chloroform as in the case of the monoamino-acid determination. The 4-day hydrolysate had 73 mg. N in the solution taken for the acetylation. In the 19-day hydrolysate 78.5 mg. N was present, 73% of which was obtained in the extract by this procedure.

The extracted material was fractionated on an ethyl acetate-water column of diameter 4.5 cm. Where the band was relatively fast-moving, and had 'run out' at the bottom of the column, the fraction was collected by evaporation of the solvent *in vacuo*. To obtain the slower moving fractions the column was pushed out of its tube after the developing solvent had run through, and each 'band' cut out immediately with a palette knife. Each fraction was immediately

Table 5. Fractionation of 'neutral' material from 4-day hydrolysate

Fractionations					
<i>R</i> _{ethylacetate}		<i>R</i> _{butanol-chloroform}		Final fractions	
		1% butanol	17% butanol		
Acetylated extract	>1 0.9	→ {	0.5	—	I
			0.3	—	II
			0.15	—	III
			0.1	—	Discarded
	0.52	→ {	0.07	—	Discarded
			0.5	—	Discarded
			0.15	—	IV
			0.05	—	V
	0.3*	→ {	—	0.24	Discarded
			—	1.2	VI
	0.15	→ {	—	0.4	VII
			—	0.7	VIII
			—	0.45	IX
			—	0.23	X
	<0.08*	→ {	—	0.6	XI
			—	0.4	XII
			—	0.13	XIII

* These fractions left a residue insoluble in hot 17% butanol-chloroform which was not further fractionated.

Table 6. Fractionation of 'neutral' material from 19-day hydrolysate

Fractionations				
<i>R</i> _{ethylacetate}	<i>R</i> _{butanol-chloroform}		Final fractions	
	1% butanol	17% butanol		
Acetylated extract	1.5 →	0.5 —	1	
	1.35 →	0.5 —	2	
		0.25 —	3	
	0.9 →	0.15 —	Discarded	
		0.10 —	4	
		0.02 —	5	
	0.6 →	0.15, 0.08 —	6	
		— 0.4	Discarded	
	0.26 →	0.7 —	7	
		0.4 —	8†	
	0.16 →	— 0.9	9	
		— 0.3	10†	
		— 0.1	11	
	0.13 →	— 0.8	12	
		— 0.15	13†	
	0.03 →	— 0.8	Discarded	
		— 0.5	14	
		— 0.32	15	
		— 0.19	16	
	0.05 →	— 0.51	17	
		— 0.42	18	
— 0.30		Discarded		
— 0.18		19		
— 0.13		20		
0.04 →	— 0.07	21		
	— 0.4	22†		
Slowest →	— 0.07	23		
	— 0.46	24		
	— 0.34	25		
	— 0.17	26		
	(< 0.12	Not analysed		

eluted from the gel by thorough washing with alcohol on a Buchner funnel and the eluate evaporated to dryness *in vacuo*.

Every fraction so obtained was further fractionated on a chloroform-butanol-water column of suitable size and developed successively with solvent containing 1 and 17% of butanol v/v. The final fractions were collected in exactly the same way. For details see Tables 5 and 6.

In Tables 5 and 6 the final fractions, to each of which a number has been assigned, are tabulated. Several fractions were discarded, as too small. The symbol $\frown$ between two fractions indicates grossly incomplete resolution of the corresponding bands; $\cdots$ indicates less serious mutual contamination, judged visually from the separation of the bands; while $\dagger$ indicates that the corresponding band was obviously due to more than one substance, being either very diffuse, or having the point of maximum concentration not near the leading edge of the band. Further fractionation on different chromatograms would have led to purer products, but was omitted because the present work was intended mainly as a preliminary survey.

The fractionation of the material from the 19-day hydrolysate was carried out in much greater detail than the fractionation of that from the 4-day hydrolysate, because during the initial fractionation on an ethylacetate column, the former resolved into a greater number of separate bands, between which 'cuts' could be made. Presumably the 19-day hydrolysate is a less complicated mixture. As seen in Table 5, the 4-day hydrolysate gave no resolution of bands having $R_{\text{ethylacetate}} < 0.08$, the column above this level being uniformly pink. In the 19-day hydrolysate, a number of satisfactory 'cuts' could be made, and thus fractions 14-26 in the 19-day hydrolysate correspond to fractions XI-XIII in the 4-day hydrolysate. This in part explains the impossibility of interpreting stoichiometrically the amino-acid figures for fractions XI-XIII.

(b) *Amino-acid analysis of the final fractions.* Each of the final fractions was dissolved in ethanol, and a suitable aliquot was taken for amino-acid analysis. After evaporation of ethanol *in vacuo*, the material was dissolved in a little water and titrated with $N/100 \text{ Ba(OH)}_2$, using phenolphthalein as indicator. This titration gave a measure of the number of equivalents of acetylpeptide and/or acetamino-acid present, and served as a check on the subsequent amino-acid analysis, thus:

HCl was then added to the titration mixture so that the final volume (10 ml.) contained 6N HCl. The mixture was then refluxed for 24 hr. to effect complete hydrolysis. Monoamino-acids were determined as described in § 1 (see Table 7).

In any given fraction, the total quantity of each amino-acid is expressed as the percentage of the total N in the 'neutral' fraction of the corresponding partial hydrolysate of gelatin. The acid equivalents in the fraction before hydrolysis are expressed in the same units. The symbol * attached to an analytical figure indicates that in the fraction concerned the amino-acid in question occurred as its acetyl derivative and not as a peptide, and hence could be assumed to be in the free form in at least this amount in the partial hydrolysate. This is deduced from the analytical figures for the fraction in question, and is confirmed by (a) chemical characterization (see § 6d) and (b) the fact that the rate of travel on the chromatogram agrees with that expected for the particular acetamino-acid [cf. Gordon *et al.* 1943a].

The analyses showed no bands other than those corresponding to the known acetamino-acids and 'indicator artefacts', suggesting that the treatment with acid resulted in complete hydrolysis of all peptide material. In a few cases the 'glycine' band appeared to be complex, suggesting that lysine, glutamic or aspartic acids might be present. Peptides of lysine with either glutamic or aspartic acids should occur in the 'neutral' fraction resulting from electrodialysis.

The symbols 'ftr' (faint trace), +, etc., in Table 7 are relative to the total amount of material in the fraction submitted to analysis, and not with respect to the original gelatin.

Apart from fractions marked with an asterisk, in which single acetamino-acids predominate, the analyses in Table 7 suggest that the final fractions are still, in the majority of cases, complicated mixtures of acetylpeptides. Speculation as to the peptides present must be based, first, on the correspondence between the rates of travel of the band corresponding to the fraction on the different chromatograms and those of known acetylpeptides and acetamino-acids, and secondly on stoichiometry. Serine and threonine, for which the fractions have not been analysed, may be present in some of them, and may make interpretation yet more difficult.

The following fractions seem the only ones of reasonably obvious composition:

4-day hydrolysate

Fraction V. Mainly dipeptide glycine-leucine. The corresponding fraction 6 from the 19-day hydrolysate probably contains the same substance though more heavily contaminated.

Table 7. *Amino-acid analysis of final fractions*
 N as % of N of 'neutral' fraction of corresponding gelatin hydrolysate

Fraction no.	Acid equivs. before hydrolysis (as N)	Phenyl-alanine + leucine + valine + methionine + proline	Phenyl-alanine	Leucine	Valine + methionine	Proline	Alanine	Glycine	Hydroxy-proline
4-day hydrolysate									
I	0.09	0.09	0.09*	0	0	0	0	0	0
II	0.40	0.40	0	0.40*	0	0	0	0	0
III	0.77	0.70	0	fttr	0.63*	0.07	0.04	0.04	0
IV	0.25	0.28	0.14	0.12	0.02	—————→	0.05	0.16	0
V	0.79	0.66	0.08	0.58	fttr	—————→	0.08	0.59	+
VI	3.54	3.76	0.86	0	0.78	2.12*	0.28	0.50	++
VII	4.90	0.87	0	0.29	0.58	—————→	3.85*	0.82	0
VIII	0.36	0.41	0	0.18	0.05	0.18	tr	0.13	0
IX	0.23	0.23	0	0.08	0.04	0.11	0.10	0.07	0
X	11.7	—	fftr	fttr	fftr	—————→	0.61	11.0*	0
XI	0.60	0.78	0	fttr	0.12	0.66	0.23	0.23	++
XII	4.10	4.20	0.70	0	1.05	2.45	1.45	3.38	++
XIII	5.37	2.02	0	0.29	0.25	1.48	1.94	2.34	+++*
19-day hydrolysate									
1	0.29	0.24	0.24*	tr	fttr	—————→	fttr	tr	fttr
2	0.47	0.41	0.41*	fftr	tr	—————→	fttr	tr	fttr
3	1.99	1.7	fttr	1.7*	fftr	—————→	fftr	fttr	fftr
4†	0.46	0.37	fftr	tr	0.36*	0.01	tr	tr	tr
5	0.32	0.18	0.06	0.07	0.05	fttr	0.06	0.11	0
6	1.13	1.06	0.17	0.67	0.14	0.08	0.21	0.61	++
7	0.42	0.42	0.04	0.20	0.17	0.01	0.12	0.11	++
8	10.4	—	0	fftr	tr	—————→	9.0*	1.15	fftr
9	8.15	6.79	0	fftr	0.39	6.4*	0.52	tr	++
10	0.56	0.30	0	0	0.20	0.10	0.17	0.21	++
11	0.64	0	0	0	0	0	0.05	0.64*	+
12	1.29	1.14	0	0	0.06	1.08*	fttr	tr	tr
13	23.3	—	0	0	fftr	—————→	tr	23.6*	0
14	0.19	0.12	0.02	0.03	0.04	0.03	0.02	0.04	+++
15	0.21	0.03	0	fttr	0.03	—————→	0.05	Lost	++
16	0.60	—	fttr	fftr	fttr	—————→	0.04	0.20	+++
17	0.06	0.06	0	tr	0.01	0.05	0.04	0.05	+
18	0.28	0.28	fftr	fttr	0.03	0.25	0.26	0.03	+
19	0.23	—	0	fttr	tr	—————→	0.07	0.06	+++
20	0.90	0	0	0	0	0	0.11	0.70	+++
21	1.17	—	0	0	fftr	—————→	0.06	fttr	+++*
22	1.20	1.05	0	0	0.09	0.96	0.22	0.81	+
23	2.76	—	0	fftr	tr	—————→	fttr	tr	+++*
24	0.14	0.18	0	0	0	0.18	tr	0.02	+++
25	0.27	0.22	fttr	0	0.03	0.19	0	tr	++
26	0.53	0.51	0	0	0.08	0.43	0.03	0.14	+++

† Minimum figures, as loss of unknown amount occurred in working up this fraction.

19-day hydrolysate

Fraction 17. Mainly tripeptide proline-alanine-glycine.

Fraction 18: Mainly dipeptide proline-alanine.

Fraction 22. A mixture of dipeptides proline-alanine and proline-glycine, with the latter predominating. The resolution of these substances seemed to have been achieved on the original ethylacetate-water chromatogram, with the result that fraction 18 was free from proline-glycine dipeptide, so some of fraction 22 was further fractionated on an ethylacetate-water column. Two bands were obtained. By cutting between these, two fractions were obtained—22a ($R_{\text{ethylacetate}} = 0.063$) and 22b

($R_{\text{ethylacetate}} = 0.050$). The band corresponding to 22b was much the stronger. Resolution of the bands did not appear to be perfect, and on amino-acid analysis of 22b it was found that 10% of alanine-proline dipeptide was still present with the glycine-proline dipeptide.

Fraction 24. Perhaps dipeptide proline-hydroxyproline.

(c) *Determination of order of amino-acid residues in peptides.* Recrystallization and formal identification of the various acetyl peptides described above was impracticable owing to the small amounts at our disposal. The fractionations shown in Tables 5 and 6 were, for the sake of rapid working, each carried out on hydrolysates of less than 1 g. of gelatin.

We have, however, obtained some evidence as to the order of arrangement of the amino-acid residues in some of the peptides, a point on which the analyses given above yield no information. The following is a preliminary description of the chromatographic micro-method which we are developing for this purpose.

When an acetyldipeptide is subjected to partial hydrolysis, the initial splitting can occur in one of two ways, of which one leads to the formation of an acetamino-acid:

Thus acetylglucylleucine on partial hydrolysis should yield acetylglucine but no acetylleucine, and acetylleucylglycine *vice versa*. These acetamino-acids may be separated from the hydrolysis mixture by extraction and chromatography, and quantitatively determined.

Experiments were accordingly undertaken on synthetic specimens of these two acetyldipeptides [Gordon *et al.* 1943a], and in the light of the results of these, some of the fractions derived from gelatin were investigated.

(1) Acetyl-*dl*-leucylglycine was hydrolysed for 36 hr. in 10*N* HCl at 37°. An aliquot of this partial hydrolysate, corresponding to 7 mg. of acetyldipeptide was evaporated to dryness *in vacuo* below 40°, and stored overnight in a vacuum desiccator over H₂SO₄ and soda-lime to effect complete removal of acetic acid and excess HCl. The product was then analysed on a chromatogram, giving:

Acetylleucine	0.14 mol. per mol. of starting material
Acetylleucylglycine	0.13 mol. per mol. of starting material
And no acetylglucine	

(2) A similar experiment with acetylglucyl-*dl*-leucine gave:

No acetylleucine	
Acetylglucylleucine	0.40 mol. per mol. of starting material
Acetylglucine	0.26 mol. per mol. of starting material

(3) On the basis of these experiments a portion of fraction V (glycine-leucine dipeptide) was subjected to the same procedure, and gave:

Acetylleucine	0.10 mol. per mol. of starting material
Starting material	0.22 mol. per mol. of starting material
Acetylglucine	0.15 mol. per mol. of starting material

This suggests that the peptides glycylleucine and leucylglycine are simultaneously present in the partial hydrolysate.

(4) A similar experiment was carried out with fraction 22b. This gave:

No acetylproline	
Starting material	0.14 mol. per mol. of starting material
Acetylglucine	A faint trace

Exp. 4 suggests that we are dealing with acetylglucylproline, but is inconclusive owing to the small amount of acetylglucine formed. Exp. 2 shows that acetylglucine is comparatively resistant to hydrolysis under these conditions—and could therefore not have been formed in significant amount at any stage. The most plausible explanation is that the hydrolysis proceeds almost entirely at the bond between the acetic acid and glycine residues, thus rendering the method inapplicable. This result accords with the view of enhanced stability to acid hydrolysis of peptide bonds involving proline imino groups which we have postulated above (§ 3) to explain the course of hydrolysis of gelatin itself under the same conditions. We have not yet conducted experiments using synthetic acetylglucylproline to check this hypothesis.

The method described in outline above may prove of value for the location of free amino groups in more complicated peptides and in proteins. Extraction from acid aqueous solution by suitable organic solvents of the partial hydrolysis products of acetylated proteins or peptides should yield a mixture of acetylated amino-acids and peptides. Amino-acid analysis of a complete hydrolysate of this extract should give information as to the terminal amino-acids of the peptide chains and in the case of a substance consisting of a single unbranched acetylated peptide chain, e.g. Ac. $x_1.x_2.x_3...$ should give the order of the residues directly, the quantities found being in the order $x_1 > x_2 > x_3$, etc.

The hydrolytic agent need not be an acid, but could be alkali, or, if rearrangement is out of the question, an enzyme. Other acyl groups than acetyl may also prove of value for the method.

(d) *Chemical identification of monoamino-acids as acetyl derivatives.* The amounts of acetamino-acids, as opposed to acetylpeptides, resulting from the fractionations described above, were adequate for complete chemical characterization. All were obtained as the *l*(+) optical stereoisomer.

All m.p.'s are given uncorrected. N determinations were by the Kjeldahl procedure. C, H determinations were done for us by Dr G. Weiler, Oxford.

Acetyl-l-phenylalanine. Crystallization from CHCl₃ of a fraction obtained from 10 g. of gelatin in the same way as fraction I yielded 22 mg. having m.p. 167–168° (not depressed on admixture of authentic specimen), (α)_D¹⁸ + 45.6° (ethanol, *c* = 0.5). Jackson & Cahill [1938] record m.p. 170–171°, (α)_D²⁵ + 47.6° (ethanol, *c* = 1). (Found: C, 63.4; H, 6.08; N, 6.84%; acid equiv. wt. 208. Calc. for C₁₁H₁₃O₃N: C, 63.7; H, 6.28; N, 6.77%; acid equiv. wt. 207.)

This isolation of phenylalanine in the optically active state by an acetylation and extraction procedure provides a check on the isolation of the racemic material from a wool hydrolysate [Martin & Synge, 1941a] confirming that racemization did not

take place in the course of isolation. Phenylalanine in wool must therefore exist in the racemic state, or be very readily racemized during hydrolysis by boiling acid.

Acetyl-l-leucine. Crystallization from CHCl_3 of a fraction obtained from 10 g. of gelatin in the same way as II yielded 40 mg. having m.p. 180° (not depressed on admixture of authentic specimen), $(\alpha)_D^{18.5^\circ} - 20.0^\circ$ (ethanol, $c=1.3$). Martin & Syngé [1941a] record m.p. $183-184^\circ$, $(\alpha)_D^{24^\circ} - 22.6^\circ$ (ethanol, $c=3.3$). (Found: C, 56.2; H, 8.37; N, 8.0%; acid equiv. wt. 180. Calc. for $\text{C}_8\text{H}_{15}\text{O}_3\text{N}$: C, 55.5; H, 8.67; N, 8.1%; acid equiv. wt. 173.)

If isoleucine were also present in gelatin, acetyl-isoleucine should be found in the mother liquors from the above crystallization, which we have not further investigated. Isoleucine was stated by Dakin [1920] to be absent from gelatin.

Acetyl-l-valine. Crystallization from ethylacetate-benzene of a fraction obtained from 10 g. of gelatin in the same manner as III yielded 13 mg. having m.p. $155-156.5^\circ$ (not depressed on admixture of authentic acetyl-l-valine), $(\alpha)_D^{20^\circ} + 2^\circ$ (ethanol, $c=0.4$). Gordon *et al.* [1943a] record m.p. 164° , $(\alpha)_D^{19^\circ} + 4.0^\circ$ (ethanol, $c=2$). The present material was evidently racemized to a considerable extent. (Found: C, 52.7; H, 8.00%; acid equiv. wt. 165. Calc. for $\text{C}_7\text{H}_{13}\text{O}_3\text{N}$: C, 52.8; H, 8.17%; acid equiv. wt. 159.) Our original N determination was lost and material did not suffice for accurate redetermination.

Acetyl-l-proline (monohydrate). Crystallization from wet ethylacetate of material from fractions 9 and 12 yielded 50 mg. having m.p. 80° (not depressed on admixture of authentic specimen), $(\alpha)_D^{19.5^\circ} - 113.4^\circ$ (water, $c=1.3$, -rotation calc. for anhydrous compound). Martin & Syngé [1941a] record m.p. 78° , $(\alpha)_D^{22^\circ} - 114.0^\circ$ (water, $c=4.2$), and give references to previous determinations. (Found: C, 53.7; H, 7.07% (on anhydrous material.) Calc. for $\text{C}_7\text{H}_{11}\text{O}_3\text{N}$: C, 53.5; H, 7.01%.) (Found: N, 8.1; water of crystallization, 10.8%; acid equiv. wt. 170 (on hydrated material). Calc. for $\text{C}_7\text{H}_{11}\text{O}_3\text{N} \cdot \text{H}_2\text{O}$: N, 8.0; water of crystallization, 10.3%; acid equiv. wt. 175.)

Acetyl-l-alanine. Crystallization from ethylacetate-benzene and ethylacetate successively of material from fraction 8 yielded 40 mg. having m.p. $126-127^\circ$, $(\alpha)_D^{19^\circ} - 65.0^\circ$ (water, $c=0.7$), $[\alpha]_D^{18.5^\circ} - 36.9^\circ$ (ethanol, $c=0.4$). Karrer, Escher & Widmer [1926] record m.p. 116° , $(\alpha)_D^{16^\circ} - 45.6^\circ$ (water, $c=5$). Their material must have been grossly racemized as would be expected from their mode of acetylation. The acetyl-l-leucine described in the same paper was prepared in the same way, and was also largely racemic. (Found: C, 45.8; H, 7.00; N, 10.4%; acid equiv. wt. 136. Calc. for $\text{C}_5\text{H}_9\text{O}_3\text{N}$: C, 45.8; H, 6.87; N, 10.7%; acid equiv. wt. 131.)

Acetyl-glycine. Crystallization from ethanol of material from fractions 11 and 13 yielded 78 mg. having m.p. $202-203^\circ$ (not depressed on admixture of authentic specimen). Curtius [1884] records m.p. 206° . (Found: C, 41.3; H, 6.01; N, 12.1%; acid equiv. wt. 120. Calc. for $\text{C}_2\text{H}_5\text{O}_2\text{N}$: C, 41.0; H, 5.98; N, 12.0%; acid equiv. wt. 117.)

(e) *Liberation of free amino-acids during the partial hydrolysis of gelatin.* From the figures marked

with asterisks in Table 7 it is possible to deduce minimum figures for the amounts of free neutral amino-acids present in the hydrolysate after 4 and 19 days. Although losses of unknown extent occurred in the preparation by electrodialysis, of the 'neutral' fractions, they were probably similar for both the 4- and 19-day hydrolysates, and the relative amounts of the different amino-acids present were probably little affected in the course of isolation.

The figures are shown in Table 8, expressed as percentages of the number of residues of the same species present in the original gelatin. We base these latter figures on our present determinations shown in Table 2, and employ in addition the mean figure for glycine given in that table.

Table 8. *Free amino-acids in partial hydrolysates of gelatin*

(As % of total residues of same species present)

Amino-acid	4-day hydrolysate	19-day hydrolysate
Basic amino-acids*	32	78
Phenylalanine	5	40
Leucine	5	27
Valine + methionine	21	†
Proline	11	48
Alanine	23	63
Glycine	21	57

* The free basic amino-acids have been calculated from the data in Table 3, and are expressed as a percentage of the total arginine + histidine + lysine residues determined by Dakin [1920], assuming for gelatin an N content of 18.3%. No loss is associated with these figures for basic amino-acids, since the 'basic' fraction is obtained quantitatively by electrodialysis (cf. § 4).

† Not calculated owing to loss involved in isolation of fraction 4 (cf. Table 7).

It will be seen that the different amino-acids are liberated at significantly different rates as the hydrolysis proceeds.

DISCUSSION

Earlier work on the isolation of the products of partial hydrolysis of gelatin and other proteins will shortly be reviewed by one of us (R. L. M. S.). Here it will suffice to mention the isolation by previous workers of diketopiperazines corresponding to some of the dipeptides isolated in the present study. These former resulted either from enzymic hydrolysis or from treatment with hot dilute acids. Our hydrolysis with cold concentrated acid did not give rise to such products, which, had they been present, would have been isolated, since they are extractable by organic solvents [cf. Gawrilow & Lawrowsky, 1927], and would have raised the amino-acid content of the final fractions far above the acid equivalent determined by titration. We deduce that

these diketopiperazines are artefacts arising by condensation from the corresponding dipeptides.

Glycine-proline anhydride was isolated from enzymic digests by Levene [1910] and from acid hydrolysates by Gawrilow & Lawrowsky [1927] and Konuma [1938]. Gawrilow & Lawrowsky also obtained evidence for the occurrence of glycine-leucine anhydride. Dakin's [1920] proline-hydroxyproline anhydride more probably arose from condensation of free amino-acids in butanol than from a corresponding dipeptide, although the present work gives indications of a dipeptide of proline and hydroxyproline (fraction 24).

The results we have obtained are not inconsistent with the structure suggested by Astbury [1940] (cf. Bergmann [1935]) for gelatin. One-eighteenth of the total residues have not been allotted a definite place in this structure, and such peptides as we have found (leucylglycine and glycyllucine and peptides of bases with neutral amino-acids) which could not be fitted into the main arrangement do not total one-eighteenth of the whole. To reconcile our data with Astbury's structure for gelatin it is necessary to make the assumption that the acid hydrolysis of bonds involving these 'anomalous' residues proceeds more slowly than for the others, and we believe that the structure will be found to be much more complicated than Astbury suggests.

Apart from the arrangement of the small number of peptides for which we have obtained definite evidence, we have noted what may be an underlying structural principle concerning the arrangement of the amino-acid residues in the peptide chain; i.e. monoamino-acids with longer fatty side-chains (phenylalanine, leucine, proline, valine and methionine) apparently are not linked to one another in gelatin. This seems to be established: (1) by the almost complete absence of acetylpeptides giving bands running faster than acetylvaline on the chromatogram. From the behaviour of synthetic acetyl-leucylleucine [cf. Gordon *et al.* 1943*a*] we expect that bands due to this type of peptide will move faster than that of acetylvaline; (2) by the fact that in no fraction does the sum of the phenylalanine + leucine + proline + valine + methionine equivalents (cf. Table 7) exceed the number of acid equivalents before hydrolysis, as would be the case if two resi-

dues from this group of acids were in the same molecule.

Being a negative principle, it is less easy to establish, and we are tentative in drawing conclusions. But it seems that this spacing out of fatty residues along the peptide chain may determine some of the peculiar physical properties of gelatin, and further that the principle of distribution of amino-acids by physical classes [cf. Astbury, 1942] may be of general significance with regard to protein structure.

SUMMARY

1. An investigation has been made of the partial hydrolysis of cow-hide gelatin by 10*N* HCl at 37°.
2. 'Basic' and 'neutral' fractions have been isolated from the resulting mixtures by electro dialysis at pH 6.
3. Analysis of the 'basic' fractions strongly suggests that residues of basic amino-acids occur in gelatin directly linked to residues of the higher monoamino-acids.
4. Analysis of the 'neutral' material by acetylation followed by fractionation on silica gel chromatograms has led to the isolation of a number of lower peptides in the form of their acetyl derivatives.
5. By similar means the gelatin has been analysed quantitatively for its constituent monoamino-acids, and these have been formally identified in the form of their acetyl derivatives. *L*-Valine has been shown to be present, and the rates of liberation of the various amino-acids during the partial hydrolysis have been studied.
6. A chromatographic micro-method has been developed for the determination of the order of amino-acid residues in acetylpeptides, etc.
7. The relation of the present study to previous work on gelatin, and to current theories of gelatin structure is discussed. It is suggested that in gelatin the higher monoamino-acids do not occur in juxtaposition along the peptide chain.

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Fatty Acid Dehydrogenase in Adipose Tissue

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It has been established in recent years that adipose tissue in the body is not an inert site of fat deposition but a tissue with specific metabolic functions. This is proved by its respiratory activity [Mirski, 1942] and by its ability to deposit glycogen under suitable conditions [Hoffmann & Wertheimer, 1927; Tuerkischer & Wertheimer, 1942]. Further proof is given by experiments showing the dependence of various adipose functions upon innervation [Hausberger, 1937].

It might be assumed, in view of these results, that adipose tissue plays an important part in the deposition and mobilization of fat. Investigation of the fat metabolism of this tissue may therefore well prove of interest for the understanding of the deposition and mobilization processes.

The present investigation arises from the observation that aqueous extracts of adipose tissue decolorize methylene blue, the speed of the decolorization being enhanced when fatty acids are added. It was proposed, therefore, to prepare an active fatty acid dehydrogenase from adipose tissue and to study the properties of the isolated enzyme.

Towards the conclusion of this work, abstracts of papers by Lang [1939], Lang & Mayer [1939] and Lang & Adickes [1939], appeared in which the demonstration of fatty acid dehydrogenase in various sources is reported. As the original papers are not at present available to us, no comparison of methods is possible. The authors report that the dehydrogenase action is dependent on the availability of muscle-adenylic acid as coenzyme and that the activity is greater to high than to low fatty acids.

METHODS

Preparation of enzyme solution. Adipose tissue from the leg region and the abdominal cavity of rats is cut into small pieces with scissors and crushed with twice its weight of sand. The pulp thus obtained is extracted with 2 volumes of distilled water

for $\frac{1}{2}$ hr. at room temperature and centrifuged. The supernatant is freed of fat by filtration. The liquid is then dialysed through cuprophane for 6–20 hr. against running tap water. Adipose tissue of rabbits did not yield active extracts.

Boiled tissue juice was prepared by boiling 2 g. of adipose tissue pulp with 5 ml. water in a water bath for 30 min. The juice is filtered while hot.

Crude cozymase preparation was prepared according to Euler [1931]. Solutions obtained after cleavage of the lead precipitate with H_2S were used for the experiment.

Dehydrogenase activity estimations were carried out according to Thunberg. Test mixtures consisted of 0.1 ml. *M*/2 phosphate buffer pH 8.0, 0.1 ml. methylene blue (1/5000), with the following additions: 0.5 ml. enzyme solution, 0.1 ml. substrate (*M*/20) and 0.1 ml. coenzyme. When one of these constituents was not added it was replaced by distilled water. Fatty acids in the experimental mixture were neutralized with NaOH and brought to pH 8.0. In some cases, dehydrocholic acid was used for dispersion, as it had no influence on the dehydrogenase activity.

EXPERIMENTAL

As is shown in Table 1, extracts dialysed for 20 hr. did not cause decolorization of methylene blue even after the addition of fatty acids. However, when boiled tissue juice was added decolorization took place. The speed of decolorization was markedly enhanced by addition of fatty acids. Crude cozymase instead of tissue juice may be used with even better results. Neither tissue juice nor cozymase preparation caused decolorization when added to fatty acids but without enzyme. When enzyme solution boiled for 5 min. was used decolorization failed to take place even in the presence of coenzyme and substrate. The enzyme nature of the dehydrogenase is thus established.