A Further Study of Hydrolysates of Gramicidin

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Most of the following experiments had been done before the author learnt that Gregory & Craig (1948) had shown that gramicidin, as previously prepared and studied, was a mixture of substances. This work seems, nevertheless, to be worth reporting, since a statement of technical successes and failures and of results obtained may prove helpful for future studies of individual gramicidins. Furthermore, evidence was secured which was very difficult to reconcile with the assumption that gramicidin is homogeneous, and which may throw some light on the nature of the differences between individual gramicidins.

The present work is a continuation of work with acid hydrolysates of gramicidin previously published (Gordon, Martin & Syngé, 1943; Syngé, 1944, 1945*a, b*). The main object was to ascertain the numbers, sequence and optical configuration of the different amino-acid residues in the molecule.

Amino-acid composition: slow liberation of valine in acid hydrolysis

In the first place, acid hydrolysates prepared at high temperatures were analyzed for amino-acids by the procedure of Gordon *et al.* (1943) and with closely similar results (see Table 2, p. 545). In all, five different preparations of gramicidin were compared, and no definite differences in amino-acid composition were observed. One of the specimens (13941) crystallized readily from aqueous ethanol, giving chunky, well-formed crystals. Of all specimens previously studied, only one had ever yielded such crystals, and it had proved impossible to reproduce the phenomenon. Crystalline and mother-liquor fractions obtained from 13941 by crystallization from aqueous ethanol did not differ significantly from the parent material in amino-acid composition or optical rotation. A countercurrent extraction of 13941 carried out by Dr Craig confirmed, however, that the material was heterogeneous (though containing a proportion of gramicidin A rather higher than usual). Thus crystallization from aqueous ethanol is no more capable of effecting separation of the different gramicidins than the usual crystallization from acetone.

On comparing the amino-acid data for different conditions of hydrolysis, it is seen that consistently more valine is obtained with more vigorous hydrolysis (Table 2). Previous valine figures must therefore be

regarded as too low. Prolonging the period of hydrolysis to 48 hr. led to a marked diminution in the amount of the second of the two 'anomalous' bands noted in purifying the leucine fraction (cf. Gordon *et al.* 1943). It is reasonable to suppose that the last valine to be liberated is derived from the somewhat slow hydrolysis of valylvaline (Christensen 1943, 1944) and that the second 'anomalous' band is due to acetylvalylvaline. Dr Gregory (personal communication) states that he, too, has isolated valylvaline after vigorous acid hydrolysis of gramicidin. He also states that phenylalanine is present as a component of gramicidin B; it is presumably a small amount of acetylphenylalanine from this source that is responsible for the first 'anomalous' band. The finding of substantial amounts of unhydrolyzed peptides after performing hydrolysis under such conditions emphasizes the importance, in amino-acid analysis of proteins, of demonstrating rather than assuming completeness of hydrolysis. The revision upwards of the figure for the valine content of gramicidin leads to a satisfactory account for its entire carbon content, and abolished the need for postulating hitherto unrecognized residues (cf. Syngé, 1945*b*).

The valine isolated after hydrolysis with hydrochloric acid at 110° for 48 hr. was found to be racemic, whereas with hydrolysis for 10 days at 37° followed by 48 hr. at 110° there was a marked preponderance of D-valine, as in previous work (Syngé, 1944).

Further study of partial acid hydrolysate: identification of dipeptides

The faster running fractions from the starch *n*-butanol-water chromatogram of a 10-day acid hydrolysate of gramicidin (Syngé, 1944) were subjected to further qualitative study by published methods (Consden, Gordon & Martin, 1944, 1947; Sanger, 1945; Porter & Sanger, 1948). It was established that, in addition to the valylglycine (fractions 26-31) found previously (Syngé, 1944), fractions 21-19 were a mixture of free valine, leucylglycine and alanylvaline (and not, as had previously been suggested on inadequate grounds, a tripeptide of leucine, valine and glycine). Fractions 18-14 contained free tryptophan and free leucine, fractions 13-11 alanylleucine and fraction 10 material apparently chromatographically homogeneous with all solvent systems tried; this last fraction could have been

alanylvalylleucine, alanylleucylvaline or a mixture of these. The three newly recognized dipeptides were further identified by comparison on mixed chromatograms with synthetic D-leucylglycine, L-alanyl-D-valine and L-alanyl-D-leucine. The synthesis of the two latter compounds is described in the Experimental section.

Table 1 shows the sequence of the different peptides and amino-acids on emerging from the original starch chromatogram. It should be remembered that the substances fractionated were originally in the form of their hydrochlorides, which may run in a different sequence from free amino-acids and peptides (Synge, 1944; Moore & Stein, 1948).

peptides (tripeptides, etc.) of leucine, tryptophan and valine.

It is clear that mixtures of peptides of this kind (which represent by far the greater part of the hydrolysate) cannot be satisfactorily fractionated on ordinary silica, paper or starch partition chromatograms. Attempts, following the suggestion of Boscott (1947), to hold the less polar phase stationary with cellulose acetate as a supporting structure (either in the form of Celanese satin or of fully acetylated filter paper (cellulose triacetate)) gave disappointing results with simple amino-acids and peptides. Although it was clear from the relative rates of movement of the different substances that the less

Table 1. *Products of partial hydrolysis of gramicidin so far identified*

(The compounds are listed in the sequence in which they emerged from the original *n*-butanol-water-starch chromatogram (Synge, 1944). Fraction numbers and *R*_f values refer to this chromatogram.)

Fraction no.	Approx. <i>R</i> _f value	Compound	Amount (N of compound isolated as % of total N of gramicidin)	Reference
37-40	0.10	Alanine	2.5	Synge (1944)
25-31	0.3	L-Valylglycine*	Approx. 7	" "
17-22	0.5	Valine	0.45	Present work (see also Synge, 1944)
17-22	0.5	L-Alanyl-D-valine*	0.45	" "
17-22	0.5	D-Leucylglycine*	1.0	" "
14-18	0.6	Tryptophan	—	" "
12-16	0.75	Leucine	—	" "
11-13	0.9	L-Alanyl-D-leucine*	Little	" "
9-11	1.1	?(Alanylvalylleucine or alanylleucyl-valine)	Little	" "
4-10	1.4	?(Dipeptides of higher amino-acids, perhaps including L-valyl-L-valine* and D-valyl-D-valine*)	Much 2.6 2.6	" Christensen (1944)
1-4	2.0	?(Tripeptides, etc. of higher amino-acids)	Much	Present work

† See Martin & Synge (1941).

* These peptides have been identified conclusively.

Attempted fractionation of peptides present in faster running fractions

It did not prove easy to identify or isolate individual peptides from fractions faster than fraction 10. Repeated attempts were made at further fractionation on paper chromatograms, but the materials either ran very fast in the more polar solvents such as phenol and collidine or else tailed very badly or did not run at all in the less polar solvents and solvent mixtures used (light petroleum, carbon tetrachloride, etc.). Some of the materials also gave very poor colours with ninhydrin. This last behaviour is typical of higher peptides, but also occurs with tryptophyltryptophan (Synge & Tiselius, 1949). An impression was gained by hydrolysis of some of the fractions, and by comparison with synthetic model substances, that the ninhydrin and Ehrlich peak around fraction 6 was primarily due to dipeptides such as leucylleucine, leucyltryptophan, etc., while that around fractions 1 and 2 was due to higher

polar phase was being held stationary in the chromatograms, excessive 'tailing' occurred, which may be attributed to adsorption effects on the cellulose acetate in the presence of such solvents as butanol and collidine. Perhaps better results may be obtained with rubber as a stationary phase as in the latex-impregnation technique of Belding (1948).

Another promising approach to this problem is to use bulk countercurrent liquid extraction as used by Craig and his co-workers. This may largely eliminate undesirable effects due to adsorption, and permits working with partition coefficients more favourable to the less polar phase. The use of charcoal-adsorption methods may also facilitate separation of the peptides according to their tryptophan content, for which partition methods with the usual solvents are not very suitable (see Synge & Tiselius, 1949). Ionophoresis (Consden, Gordon & Martin, 1946) of the peptides, their acyl or *N*-2:4-dinitrophenyl derivatives in gels shows promise as a method for achieving fractionation according to ionic mobility,

which is an inverse function of molecular size. Thus leucine could be separated from leucylleucine or leucyltryptophan by ionophoresis of the dinitrophenyl derivatives in silica gel containing 0.2M-sodium acetate buffer at pH 6. The dinitrophenyl-peptides moved towards the anode at about half the rate of the dinitrophenylleucine. The true difference in ionic mobility is not of course so great since the substances were migrating against the electro-osmotic stream, and adsorption effects may also have contributed.

Optical form of amino-acids from different peptide fractions: an ultramicro-method for its determination

In view of the interest attaching to the optical form of the amino-acids isolated after acid hydrolysis of gramicidin and other bacterial substances, it seemed important to ascertain the configuration after hydrolyzing further the individual partial hydrolysis products.

A semi-quantitative micromethod was developed based on the specificity of the D-amino-acid oxidase of kidney. Spots of an individual amino-acid, after separation on a paper chromatogram from other components of the mixture, were sprayed with a solution of the purified enzyme, and the extent of the attack on the amino-acid was gauged by comparing the spot with a number of control spots of optical antipodes of the same amino-acid mixed in varying proportions. The method requires as little as 30 μ g. of the amino-acid under examination. Jones (1948) has mentioned its use in a study of the hydrolysis products of 'aerosporin' (polymyxin). In the present work it has given satisfactory results with leucine and valine. It is not suitable for detecting small proportions of D-isomer mixed with excess of L-isomer, for which purpose L-amino-acid oxidases may in future prove useful. Obviously, the spraying of paper chromatograms with enzyme solutions is capable of innumerable other applications.

The experiments on the complete acid hydrolysates of the leucylglycine, alanylvaline and alanylleucine isolated as described above indicated, as expected, that the leucine from the leucylglycine and alanylleucine was largely the D-form. The valine from the alanylvaline proved to be predominantly the D-isomer. All these products were, however, to a considerable extent racemic (see Table 4). It was confirmed by the new method that the valine from the valylglycine (Synge, 1944) was predominantly the L-form. Alanine appeared to be predominantly the L-isomer.

Thus different partial hydrolysis products have been isolated yielding respectively an excess of L-valine (L-valylglycine by Synge, 1944; and L-valyl-L-valine by Christensen, 1944) and of D-valine (L-alanyl-D-valine in the present work and D-valyl-D-valine by Christensen, 1944). If one starts from

conventional premises it may be argued that valine residues of each of the two antipodal forms exist preformed in one or more of the molecular species constituting 'gramicidin'. However, a more cautious interpretation seems advisable in view of the fact that most of the products, whose optical character has been determined so far, are partly racemic. As previously implied in a footnote (Synge, 1944) and more explicitly stated by Neuberger (1948) there is no reason why a process similar to that which induces simple racemization in a free amino-acid in the presence of mineral acid should not, when the same residue still exists in peptide linkage with other optically active amino-acid residues, cause epimerization or inversion of the residue in question. It is not justifiable, therefore, in the present state of our knowledge to deduce rigorously from the optical configuration of free amino-acids found in hydrolysates the configuration of the corresponding residues in the intact molecule. Extensive model experiments with optically active peptides are required.

Conclusion

When it appeared reasonable to suppose that gramicidin was a single homogeneous substance, it was argued that, since the molecule could not contain more than two glycine residues, and since more than half the total glycine had actually been isolated as valylglycine, all the glycine residues must occur linked to valine through their amino groups (Synge, 1944). This view became untenable when leucylglycine was found to occur in the same partial hydrolysate with valylglycine. Gregory & Craig's (1948) demonstration of the heterogeneity of gramicidin resolves this difficulty. It would be the part of wisdom to expect similar anomalies in studies of partial hydrolysis of 'pure' proteins as evidence accumulates that calls for scepticism as to their homogeneity.

At the present time it is impossible to put forward any extensive hypothesis as to the structure of individual species of gramicidin on the basis of partial hydrolysis studies made with the mixture. All that can be done is to list the peptides so far identified in partial hydrolysates of mixed material, with their probable optical configurations. This is done in Table 1, where those peptides that have been conclusively identified are marked with an asterisk. Future studies of partial hydrolysates of individual gramicidins will be required for further progress towards determining sequences of amino-acid residues.

EXPERIMENTAL

Melting points are uncorrected. Optical rotations were determined in a 0.5 dm. tube. Carbon and hydrogen determinations were by Drs Weiler and Strauss, Oxford.

Materials

Gramicidin specimens W_2 and R_3 (Synge, 1944, 1945*a*, *b*; Synge & Tiselius, 1947) were studied together with a batch of acetone-recrystallized gramicidin (no. 13941) kindly supplied by the Wallerstein Company, New York, during 1945. As already mentioned, this material differed from previously studied preparations in crystallizing fairly readily from aqueous ethanol, on allowing slow evaporation of the solution. Specimens of the resulting crystals (13941 X) and mother liquor (13941 ML) were separated after about half of the material had crystallized. Optical rotations (c , 1.5 in 94.5% (w/v) aqueous ethanol) were as follows: W_2 , $[\alpha]_D^{21.5} + 3^\circ$; R_3 , $[\alpha]_D^{27} + 5^\circ$; 13941, $[\alpha]_D^{23} + 6^\circ$; 13941 X, $[\alpha]_D^{24} + 4^\circ$; 13941 ML, $[\alpha]_D^{24} + 2^\circ$ (calculated for materials dried to constant wt. in a vacuum desiccator over H_2SO_4 and soda lime at room temperature).

The fractions obtained (Synge, 1944) by starch partition chromatography (*n*-butanol-water) from a hydrolysate of gramicidin R_3 were further studied. Hydrolysis had been in equal volumes of 10*N*-HCl and glacial acetic acid for 10 days at 37°. Fractions are numbered as in the original paper.

Optical rotation of valine specimens

Acetylvaline, isolated by partition chromatography as above from hydrolysates of gramicidin (13941), was hydrolyzed with 6*N*-HCl for 24 hr. at 110°. The product was evaporated to dryness and weighed. From the weight of valine HCl found, the weight of valine present was calculated. The optical rotation of the product was determined. Results are shown in Table 3.

Table 3. *Optical rotation of valine isolated from gramicidin after hydrolysis*

(Hydrolysis in acetic acid + 3.5 vol. of 6*N*-HCl. $[\alpha]_D$ measured in 6*N*-HCl.)

Duration and temperature of hydrolysis	$[\alpha]_D$ (°)	Temp. (°)	c
24 hr. at 110°	-1	19	0.7
48 hr. at 110°	± 0	19	1.7
10 days at 37° followed by 48 hr. at 110°	-6	17	1.2

Study of fractions obtained from n-butanol-water starch partition chromatogram of partial hydrolysate (Synge, 1944)

In general, small portions of these fractions were subjected to further study on paper chromatograms. Amino-acids and peptides were made visible by spraying with ninhydrin, tryptophan compounds by Ehrlich's reagent (cf. Synge & Tiselius, 1949). In addition, observation of the chromatograms in ultraviolet light showed the presence of a variety of intensely fluorescent substances that did not colour either with ninhydrin or Ehrlich's reagent. They 'tailed' considerably and amino-acids and peptides either displaced them or quenched their fluorescence. These fluorescent spots thus served as markers for cutting the chromatograms. They were thought to be breakdown products of tryptophan. After hydrolysis of peptide material, constituent amino-acids were detected routinely on *n*-butanol- H_2O one-dimensional chromatograms with Munktel OB paper. All five amino-acids could readily be identified in this way, tryptophan falling conveniently between leucine and valine.

Table 2. *Amino-acid analyses of gramicidin hydrolysates, with varying hydrolysis conditions*

(Amino-acid figures are N as % of total N of gramicidin (Kjeldahl-Friedrich).)

	Syngé (1944)		Gordon <i>et al.</i> (1943)		Present work															
Hydrolysis mixture ...	0.8 ml. acetic acid 3.0 ml. 5 <i>N</i> -HCl		0.8 ml. acetic acid; 3.0 ml. 6 <i>N</i> -HCl		0.8 ml. acetic acid; 3.0 ml. 6 <i>N</i> -HCl															
Temperature ...	100°		110°		110°															
Duration (hr.) ...	48		24		24				48											
Specimen ...	R ₃	W ₂	Various		R ₃	R ₃	W ₂	W ₂	R ₃	R ₃	W ₂	W ₂	13941	13941	13941 X	13941 X	13941	ML	13941	ML
Leucine N	23.5*	25.2*	20.0-21.6		20.0	21.0	20.0	20.3	19.8	20.4	20.8	20.6	19.6	19.7	20.3	20.3	20.4		20.6	
Valine N	13.9	15.7	15.6-17.0		15.9	16.4	16.9	15.8	18.3	18.1	18.7	18.6	17.9	18.3	18.4	18.5	18.9		17.3	
Tryptophan N	38.4	41.1	33.5-44.8		37.5	38.0	40.0	40.5	32.8	35.1	40.5	39.7	35.4	36.3	36.6	35.7	31.6		33.6	
Alanine N	10.1	11.7	9.8-11.5		10.4	9.9	10.4	10.3	10.0	10.1	10.4	10.4	9.9	9.8	9.7	10.0	10.0		10.2	
Glycine N	7.4	7.8	5.0-8.0		5.5	5.5	5.6	5.1	5.5	5.2	5.3	4.5	5.2	5.2	5.3	5.4	6.0		6.5	

* Before chromatography with chloroform.

Amino-acid analysis of gramicidin specimens

Specimens were hydrolyzed in hot HCl-acetic acid, acetylated and analyzed for component amino-acids by partition chromatography on silica gel exactly as described by Gordon *et al.* (1943). The results are shown in Table 2 corrected (as previously) by the recovery factors for a known mixture of acetamido-acids, subjected to exactly the same procedures and reagents. As before, the same anomalous bands were noted in the $CHCl_3$ chromatography of the leucine fraction. In the 48 hr. hydrolysates, the anomalous band running behind the leucine band had become very much fainter.

Fractions 21-19. These fractions had already been pooled (Synge, 1944) and were known to yield valine, leucine and glycine, with smaller amounts of alanine on complete acid hydrolysis. The material in the pooled fractions seemed best further fractionated with collidine. Paper chromatography with this solvent revealed free valine and, ahead of it, two spots very close to one another. These were so close that it was not easy to cut them apart. Accordingly, the two compounds were isolated together by running several one-dimensional chromatograms in parallel, and eluting them with water after cutting away from free valine, etc. The extract was subjected to hydrolysis, with and without

previous deamination by nitrous fumes according to Consden *et al.* (1947). The hydrolysate showed leucine and glycine as main and alanine and valine as subsidiary components. Deamination abolished the leucine and alanine. This suggested that the major component of the mixture was leucylglycine and the minor component alanylvaline. The identification of the residues bearing the free amino groups was confirmed by treatment of the material with 1:2:4-fluorodinitrobenzene according to Sanger's (1945) techniques. After acid hydrolysis *N*-2:4-dinitrophenylleucine and *N*-2:4-dinitrophenylalanine were identified qualitatively by mixed chromatography on narrow silica gel columns. Finally, the identity of the peptides was confirmed by running mixed paper chromatograms with synthetic *D*-leucylglycine (Hofmann-La Roche) and *L*-alanyl-*D*-valine (see below) with *n*-butanol and *s*-collidine as solvents. At the same time it was established that in collidine leucylglycine is the faster moving of the two peptides. From the amino-acid analysis of the material in fractions 19-21 (Synge, 1944) it is reasonable to deduce that the free valine embodies 0.45 % of the N of the original gramicidin, the leucylglycine 1.0 % and the alanylvaline 0.45 %.

Fractions 18-14. Paper chromatography with *n*-butanol, collidine, etc. of test portions of these fractions before and after acid hydrolysis showed the presence of free leucine and free tryptophan, the leucine predominating in the earlier fractions (14-16) and the tryptophan in the later fractions (17-18). There was also some overlap of alanylleucine (see p. 547) into the earlier fractions and of leucylglycine into the later fractions. No other components were recognized.

Fractions 13-11. Paper chromatography with *n*-butanol (after neutralization with NH_3 vapour) showed a common constituent in all these fractions. In fraction 13 it was contaminated with free leucine (which ran somewhat faster than the unknown compound) and in fraction 11 it was contaminated with the faster-running peptide material also present in fraction 10. From Fraction 12, however, only a single spot resulted with a variety of solvents. Fraction 12 was accordingly studied without further fractionation. On acid hydrolysis, the material yielded alanine and leucine only. With previous deamination, the alanine spot was weakened, though not abolished. However, the fluorodinitrobenzene procedure yielded only dinitrophenylalanine and no dinitrophenylleucine. Accordingly the material was compared by mixed paper chromatograms using both *n*-butanol and *s*-collidine with synthetic *L*-alanyl-*D*-leucine (see p. 547) with which it appeared to be identical.

Fraction 10. The material in this fraction gave only a single spot with ninhydrin on paper chromatography with a very wide variety of solvent mixtures. On acid hydrolysis, it showed alanine, valine and leucine as spots of roughly equal intensity. Previous deamination relatively weakened, but by no means abolished the alanine spot. The fluorodinitrobenzene procedure yielded only dinitrophenylalanine. Since the material could not be alanylleucine or alanylvaline, both of which, as hydrochlorides, had run more slowly on the original starch chromatogram and, as free peptides, on paper with *n*-butanol, it is suggested that it may be alanylvalylleucine or alanylleucylvaline or a mixture of both these peptides.

Synthesis of L-alanyl-D-valine

Carbobenzyl-L-alanine was prepared (Bergmann & Zervas, 1932) from *L*-alanine (made by resolution of DL-

alanine according to Pope & Gibson (1912; cf. Fischer, 1899). Carbobenzyl-L-alanine (0.645 g.) was allowed to react in 6 ml. dry ether with 0.5 g. PCl_5 at first at 0° and then at room temperature until all the PCl_5 had dissolved. The resulting solution was washed quickly with ice water twice, and the ethereal solution of the acid chloride was then allowed to react for 2 hr. at room temperature with 0.55 g. *D*-valine methyl ester in the presence of saturated aqueous KHCO_3 . The ester (not characterized) had been prepared from *D*-valine exactly as described by Synge (1948) for *L*-valine methyl ester. The ether layer was then washed successively with dilute HCl and KHCO_3 and water, dried lightly over Na_2SO_4 , and on evaporation to dryness yielded a residue crystallizing readily from ether-light petroleum. Melting point and rotation were constant after one recrystallization (yield of recrystallized product, 0.3 g.).

Carbobenzyl-L-alanyl-D-valine methyl ester had m.p. $89-90^\circ$, $[\alpha]_D^{21.5} + 1^\circ$ (*c*, 1.4, ethanol). (Found: C, 60.7; H, 7.2; N (Kjeldahl) 8.5. $\text{C}_{17}\text{H}_{24}\text{O}_5\text{N}_2$ requires C, 60.7; H, 7.1; N, 8.3 %.)

This compound was saponified by dissolving 0.27 g. in 3 ml. dioxan and adding 2 ml. 1.2*N*-NaOH. The mixture was gently shaken until homogeneous, and kept for a further 0.5 hr. at room temperature. The mixture was then largely diluted with water, acidified with 0.5 ml. 10*N*-HCl (no precipitation occurred) and extracted with ether. The residue from evaporation of the ether extract (carbobenzyl-L-alanyl-*D*-valine) could not be brought to crystallization, and was accordingly reduced with Pd-H_2 in the usual way, after dissolving in a mixture of 10 ml. methanol, 1 ml. glacial acetic acid and 2 ml. water. After removal of Pd and repeated evaporation with water there was obtained a colourless, glassy residue (0.20 g.) of *L*-alanyl-*D*-valine. The product would not crystallize from water-ethanol-ether, nor on keeping in a desiccator, but crystallized slowly in rosettes (evidently with water of crystallization) on keeping in the laboratory air. Paper chromatography indicated that the material was contaminated with a trace of free alanine, but was otherwise homogeneous. R_F values, comparable with the data of Consden *et al.* (1947), were, for *n*-butanol-water (with HCN) 0.21, and for *s*-collidine-water, 0.53. $[\alpha]_D^{19.5} + 35^\circ$ (*c*, 2.8, water) (calculated, from N content of solution, for the anhydrous peptide.) Air-dry crystals were analyzed. (Found: C, 47.1; H, 8.8; N (Kjeldahl), 13.6; amino N (Van Slyke, 4 min.) 52 % of total N. $\text{C}_8\text{H}_{16}\text{O}_3\text{N}_2 \cdot \text{H}_2\text{O}$ requires C, 46.6; H, 8.7; N, 13.6 %; amino N 50 % of total N.)

Synthesis of L-alanyl-D-leucine

Carbobenzyl-L-alanyl chloride prepared from 0.7 g. carbobenzyl-L-alanine was allowed to react in 6 ml. of washed ether solution in the presence of saturated aqueous KHCO_3 with *D*-leucine methyl ester that had been liberated with K_2CO_3 in the usual way from 0.8 g. of its hydrochloride (Smith & Brown, 1941). After 1.5 hr. at room temperature the ether layer was separated, washed successively with dilute HCl and KHCO_3 solutions and with water, dried lightly over Na_2SO_4 and on evaporation yielded 0.87 g. of syrup which could be crystallized from ether-light petroleum. The resulting carbobenzyl-L-alanyl-*D*-leucine methyl ester after two crystallizations (yield 0.71 g.) had m.p. $71-73^\circ$, $[\alpha]_D^{20.5} + 10^\circ$ (*c*, 1.6, ethanol). (Found: C, 61.6; H, 7.5; N (Kjeldahl) 7.9. $\text{C}_{18}\text{H}_{26}\text{O}_5\text{N}_2$ requires C, 61.8; H, 7.4; N, 8.0 %.)

This compound was saponified by dissolving 0.37 g. in 3 ml. dioxan and adding 3 ml. *N*-NaOH. The mixture was kept for 0.5 hr. at room temperature after it had become homogeneous: 30 ml. water was then added, followed by 4 ml. *N*-HCl. An oil precipitated which crystallized on keeping at 0°. Recrystallization to constant m.p. and rotation was effected from methanol-water. The product (0.22 g.), characterized after drying in a vacuum desiccator at room temperature, appeared to be a hydrate of *carbo-benzyloxy-L-alanyl-D-leucine*: m.p. 57–59°; $[\alpha]_D^{19.5} \pm 0^\circ$ (c, 1.9, methanol). (Found: C, 58.1; H, 7.4; N (Kjeldahl), 7.6%; acid equiv. wt., 348. $C_{17}H_{24}O_5N_2 \cdot H_2O$ requires C, 57.7; H, 7.4; N, 7.9%; acid equiv. wt. 354.)

This material (0.17 g.) was reduced with $Pd-H_2$ as above. The crystallization behaviour of the resulting glassy *L-alanyl-D-leucine* was similar to that of *L-alanyl-D-valine*, the peptide crystallizing over aqueous acetic acid, but not in the laboratory air nor in the desiccator. The material appeared homogeneous by partition chromatography on paper. R_F values comparable with those given by Consden *et al.* (1947) were, for *n*-butanol-water (with HCN), 0.33 and for *s*-collidine-water 0.60; $[\alpha]_D^{21} + 50^\circ$ (c, 1.9, water) (calculated for the anhydrous peptide from N content of solution). Air-dry crystals were analyzed. The data suggest the presence of both acetic acid and water of crystallization. (Found: C, 43.7; H, 8.1; N (Kjeldahl), 9.5%; amino N (Van Slyke, 4 min.), 49% of total N. $C_9H_{18}O_3N_2 \cdot C_2H_4O_2 \cdot 2H_2O$ requires C, 44.3; H, 8.7; N, 9.4%; amino N, 50% of total N.)

Ultramicrodetermination of the optical configuration of valine and leucine: results with peptides from gramicidin

In preliminary trials it was found essential to purify the *D*-amino-acid oxidase considerably before use, in order to eliminate impurities giving colours with ninhydrin. It was also found necessary to allow the enzyme reaction to proceed on paper that is thoroughly wet. The time of reaction should therefore be limited, since the amino-acids in the spots diffuse freely under these conditions.

The following procedure was found satisfactory. The kidneys of freshly slaughtered sheep were worked up for *D*-amino-acid oxidase according to Negelein & Brömel (1939).

level directly proportional to the amount of solution subjected to test. This indicates a yield of enzyme closely similar to that described by Negelein & Brömel.

For carrying out the assay, the peptides to be tested were hydrolyzed in excess of 6 *N*-HCl in sealed evacuated tubes for 48 hr. at 110°. The hydrolysate was evaporated to dryness *in vacuo*, and the residue was dissolved in such a volume of water as would give a 0.2–0.4% (w/v) solution of the amino-acid to be assayed. Spots of this solution (each 5 μ l.) were placed on at least two different sheets of filter paper. Beside the spot on the first sheet were placed 5 μ l. spots of solutions of different strengths of the amino-acid undergoing assay. The spots were developed (after neutralization with NH_3 vapour) as parallel one-dimensional chromatograms with *n*-butanol-water. On subsequent colouring with ninhydrin, the concentration of the test solution in respect of the amino-acid being studied could be assessed. Solutions of the same amino-acid were then made up, having the same total amino-acid concentration, but different proportions of the *D*- and *L*-isomers. Solutions used were pure *D*-, 25% *L*-, *DL*-, 75% *L*-, and pure *L*-. 5 μ l. spots of each of these solutions were then placed beside the spot of test solution on the second sheet, and the chromatograms were developed with *n*-butanol-water after neutralization with NH_3 as above. Care was taken to develop far enough to get wide separation of the leucine and valine spots. After development, the sheet of paper was dried in the air, and sprayed as evenly as possible with the enzyme solution (pH 8.3). It was important to wet the whole of the sheet during the spraying, otherwise the enzyme solution moved from one part of the paper to another, carrying the amino-acid spots with it. The wet sheet was hung up at 37° in an atmosphere of O_2 rendered moist by bubbling through the *M*/15 pyrophosphate buffer. After 2.5 hr. the sheet was dried at 37°, and sprayed with 0.1% (w/v) ninhydrin in a mixture of 4 vol. *n*-butanol and 1 vol. acetic acid. The sheet was then hung in the hot room (37°) for the colour to develop (1–2 hr.). Spots of pure *D*-leucine and *D*-valine were completely abolished by this treatment, whereas the *L*-isomers survived. It was possible to form rough estimates of the proportion of the isomers by comparing the test spot with the control spots. It was felt that estimates where *L*-isomers predominated were less accurate than those where *D*-isomers predominated. Results with the hydrolysates are shown in Table 4.

Table 4. Proportions of *D*- and *L*-isomers of leucine and valine in hydrolysates of peptides from gramicidin

Fraction no.	Peptide present	Leucine		Valine	
		% <i>D</i> -	% <i>L</i> -	% <i>D</i> -	% <i>L</i> -
26–30 (Synge, 1944)	Valylglycine	—	—	10	90
19–21 (peptides only)	Alanylvaline, leucylglycine	65	35	80	20
12	Alanylleucine	90	10	—	—
10	(?Alanylvalylleucine, ?Alanylleucylvaline)	90	10	60	40

The preparation was taken to 'Schritt 5'. The material precipitated at this step was not, however, dried, but was dialyzed free of $(NH_4)_2SO_4$ against several changes of Negelein & Brömel's pyrophosphate buffer (*M*/15, pH 8.3). The product from five kidneys, on diluting the bag contents with the same buffer solution to 50 ml. and adding a saturating amount of coenzyme (flavin-adenine-dinucleotide) was used for spraying the paper chromatograms: 0.1 ml. of this solution gave, in Negelein & Brömel's manometric test with *DL*-alanine, an uptake of 34 μ l. O_2 in 10 min., the rate of uptake being at this

SUMMARY

1. Quantitative amino-acid analysis of hydrolysates of gramicidin has shown that valine is liberated excessively slowly in acid hydrolysis. Previous valine figures are too low; with higher valine figures the data obtained with the fission products account satisfactorily for the elementary composition of gramicidin.

2. Further examination of the partial hydrolysate of gramicidin studied by Syngé (1944) has led to isolation and recognition of the dipeptides D-leucylglycine, L-alanyl-D-valine and L-alanyl-D-leucine. The synthesis of the two last compounds is described.

3. Technical problems of fractionating the more complicated peptides in the partial hydrolysate are discussed, and some possible new approaches to the problem are suggested.

4. An ultramicro-method for assessing the optical configuration of individual amino-acids has been developed and applied to leucine and valine. The amino-acids are separated on paper chromatograms, which are then sprayed with a solution of D-amino-acid oxidase from kidney.

5. The partial hydrolysis products of gramicidin so far recognized, and the optical form of the amino-acids isolated from them and from gramicidin, are discussed with reference to Gregory & Craig's (1948) demonstration of the heterogeneity of gramicidin and to the problem of racemization, epimerization and inversion in the hydrolysis of peptides.

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A Study of the Acidic Peptides Formed on the Partial Acid Hydrolysis of Wool

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It has been accepted for many years that proteins consist of chains of amino-acids in peptide linkage, and that the partial hydrolysis products of proteins consist of a complicated mixture of amino-acids and peptides of various lengths. However, very few studies of the actual peptides present in the partial hydrolysis product of any given protein have, in fact, been made (see Syngé, 1943). This has been due to the lack of methods for the separation and analysis

of complicated mixtures of peptides. Recently Consden, Gordon & Martin (1947) have developed such methods, and Consden, Gordon, Martin & Syngé (1947) have applied these methods to the mixture formed by the partial acid hydrolysis of gramicidin S; many of the products in that mixture were identified.

The study reported here is of the vastly more complicated mixture produced by the acid hydrolysis of wool. The problem is confused in the case of wool by uncertainty as to the number of molecular species which are, in fact, present in the wool. Thus, wool has been broken up into fractions containing widely varying proportions of various amino-acids (for

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