The Amino-acid Composition of Tyrocidine

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Tyrocidine hydrochloride, a crystalline antibacterial substance obtained, with gramicidin, from cultures of certain bacteria [Hotchkiss & Dubos, 1941] has been shown [Hotchkiss, 1941; Christensen, Edwards & Piersma, 1941] to be of polypeptide nature. A limited study of the constituent amino-acids has been made by these authors, and by Lipmann, Hotchkiss & Dubos [1941]. Dubos [1942] and Dubos & Hotchkiss [1942] have reviewed its biological properties.

We have studied the products of acid hydrolysis of tyrocidine by partition chromatography [Gordon, Martin & Syngé, 1943 *a, b*]. The extraction of the acetylation mixture with organic solvents has been rendered almost quantitative, and the use of peltanin chloride instead of methyl orange has practically eliminated 'leaching' of indicator, facilitating continuous observation of the more slowly moving 'bands'. These changes have extended the quantitative applicability of the method to acetamino-acids, whose bands move more slowly than that of acetylalanine, and although further investigation is necessary before the method can be used for the simultaneous determination of the eight common amino-acids of this group, the present procedure has proved useful with tyrocidine which seems to contain only three of these. Using a variety of solvent systems, we have not yet achieved a satisfactory chromatographic separation of the acetyl derivatives of glutamic and aspartic acid, and have made use of the differential evolution of CO₂ in the presence of ninhydrin from the corresponding free amino-acids [Van Slyke, Dillon, MacFadyen & Hamilton, 1941] for estimating their relative proportions.

From tyrocidine hydrolysates we have isolated and identified as their acetyl derivatives, phenylalanine, leucine, valine, proline, tyrosine, glutamic acid and ornithine, and have obtained further evidence for the presence of tryptophan and aspartic acid [Hotchkiss, 1941]. Alanine, glycine, lysine and hydroxyproline appear to be absent. Christensen *et al.* [1941] claim to have isolated alanine (as its dioxypyridate) but give no details.

All these amino-acids have been obtained predominantly as the *L*-isomers, except phenylalanine, which we find predominantly *D*-, and tryptophan, whose optical configuration was not determined. Many of the specimens isolated were considerably

racemized. Our estimate of the total amount of *D*-amino-acids in the hydrolysate is consistent with that of Lipmann *et al.* [1941]. This seems to be the first report of *D*-phenylalanine in nature. The simultaneous incorporation by the same organism of *D*-leucine residues (in gramicidin) and *L*-leucine residues (in tyrocidine) has biological interest.

Ornithine was isolated as its di-acetyl derivative which proved identical with that obtained from *L*-ornithine, described here for the first time. Ornithine has previously been isolated only from hydrolysates of protein or polypeptide material that had been treated in such a way as probably to modify the guanidino group of arginine residues [Kossel & Weiss, 1913]. The negative Sakaguchi reaction of tyrothricin [Hotchkiss & Dubos, 1941] excludes arginine as an immediate precursor, although it must be borne in mind that tyrothricin is obtained from autolysed cultures. The presence of both proline and ornithine in the hydrolysate suggested that citrulline might be a common precursor for both [Wada, 1933], particularly since tyrocidine, like most proteins, gives a positive Fearon [1939] reaction. However, *L*-citrulline, when subjected to the same conditions of acid hydrolysis, acetylation and chromatographic analysis, gave at most 8% of a mol. of proline and 22% of a mol. of ornithine. Since under the same conditions of acid hydrolysis citrulline evolved no CO₂, while urea, another possible product of the reaction postulated by Wada, evolved 42–53% of a mol. of CO₂, and also in view of his inadequate characterization of his product, we disbelieve Wada's statement that proline is the main product formed by boiling citrulline with acid. We believe that ornithine and proline residues exist in the intact molecule of tyrocidine, although the presence of some citrulline is not excluded. Further work on the Fearon reaction of proteins is desirable.

The satisfactory results with the estimation of ornithine given below confirm our view [Gordon *et al.*, 1943 *a*] that partition chromatography should provide a satisfactory micro-analytical procedure for the direct determination of lysine.

Quantitative analyses of tyrocidine hydrolysates were carried out and controlled by parallel analyses of a known mixture of the constituent amino-acids in similar quantity and proportion (Table 1). We were struck by the very low and variable recovery

of tryptophan, as in similar control experiments with a different amino-acid mixture [Gordon *et al.*, 1943*b*] higher and less variable recoveries of tryptophan were obtained. Homer [1915] and Onslow [1921, 1924] suggested that other amino-acids might

some component not present in our first mixture. Gortner and collaborators (for references cf. Gortner & Norris [1923]) studied the role of tryptophan in humin formation. In our experiments humin formation is negligible although destruction of

Table 1. *Analysis of a known mixture which had been heated with acid under the conditions of hydrolysis employed with tyrocidine*

Known mixture		Analytical results (recovery %)	
Substance present	Amount mg. N	Exp. 1 (batch 'E' gel)	Exp. 2 (batch 'F' gel)
Acetyl- <i>L</i> -phenylalanine	0.348	106	101
Acetyl- <i>D</i> -leucine	0.290	98.5	105
Acetyl- <i>L</i> -valine	0.248	102	102
Acetyl- <i>L</i> -tryptophan	0.430	7	42
Acetyl- <i>L</i> -proline hydrate	0.158	91	93
Acetyl- <i>L</i> -tyrosine	0.167	99	104
Total dicarboxylic acids	0.343	79.5	96
<i>L</i> -Glutamic acid HCl	0.172	45	98 (by diff.)
<i>L</i> -Aspartic acid	0.171	113	94 (by ninhydrin)
Diacetyl- <i>L</i> -ornithine	0.493	93.5	96
Ammonium sulphate	0.349	—	—

Table 2. *Analysis of a glutamic acid-tryptophan mixture which had been heated with acid in the presence of, or in the absence of, air*

Known mixture		Air present during heating ml. at N.T.P.	Analytical results		Carbon dioxide formed mg.
Substance present	Amount mg.		Amino-acids		
			Found mg.	Recovery %	
<i>L</i> -Glutamic acid HCl	2.9	0.6	2.46	84	0.14
Acetyl- <i>L</i> -tryptophan	6.7		4.05	60	
<i>L</i> -Glutamic acid HCl	2.9	0.0	2.67	92	0.00
Acetyl- <i>L</i> -tryptophan	6.7		4.69	70	

Table 3. *Amino-acid analysis of tyrocidine*

(N as % of total N unless otherwise stated)

Present results					
Preparation analysed	...	A	A	B	
		I	II	—	
mg. N taken for analysis	...	1.56	3.12	2.47	Hotchkiss [1941]
Phenylalanine		12.2	14.8	12.7	—
Leucine		8.6	7.4	8.7	—
Valine		7.1	8.0	6.5	—
Tryptophan		9.5	0.9	1.8	11.7, 15.9*
Proline		6.4	6.6	7.1	—
Tyrosine		6.4	6.0	6.8	—
Total dicarboxylic acids		—	12.2	14.1	16.5
Glutamic acid (by diff.)		—	6.1	7.2	—
Aspartic acid (ninhydrin)		—	6.1	6.9	4.5
Ornithine		—	9.3	13.2	—
Ammonia		—	—	—	12.0*, 13.3
Batch of gel used in analysis		E	E	F	Christensen Edwards & Piersma [1941]

* Determined after brief hydrolysis.

increase the destruction of tryptophan in hot acid, while Holm & Gortner [1920] studied the destruction of tryptophan heated alone with acid. All these workers allowed free access of air during hydrolysis, which is known to influence the destruction of tryptophan. In our experiments air was excluded, and we provisionally attribute the increased destruction to the interaction of tryptophan with

tryptophan is almost complete. If this loss is due to interaction with other amino-acids, some destruction of these might be expected, and the results in Table 1 suggest that proline or glutamic acid might be involved. Preliminary experiments described below with tryptophan-glutamic acid mixtures were not decisive, although the evolution of CO₂ when air was present (Table 2) is of interest,

since proteins as usually hydrolysed yield significant quantities of CO_2 [Dunn, 1925]. The hydrolysis of tyrocidine, with exclusion of air, in our hands yielded no CO_2 .

The accuracy of the analyses for the other amino-acids appears similar to that previously observed [Gordon *et al.*, 1943*a*]. Some of the minor differences between analyses 1 and 2 (Table 1) are undoubtedly due to differences in the adsorptive properties of the two batches of silica gel employed.

On applying the same analytical procedure to tyrocidine hydrolysates (cf. Table 3) we obtained very variable figures, the extent of the fluctuations being much greater than the experimental error of the method as established by the control experiments. Destruction of tryptophan was with one exception practically complete. We conclude that there are real differences in the composition of different hydrolysates. Incomplete hydrolysis seems an unlikely explanation, since bands corresponding with acetylpeptides should have been seen [Gordon *et al.*, 1943*c*], whereas the appearance of the chromatograms in the analysis of tyrocidine was in no way qualitatively different from that in the control experiments, exactly corresponding 'artefact bands' being observed. Diketopiperazines, however, if present, would not have been observed. We tentatively attribute the discrepancies to the different course which the interaction of tryptophan and other amino-acids may take under the conditions of hydrolysis, when these substances are present in the form of polypeptides rather than as free amino-acids or their simple derivatives.

Further research is required to elucidate the behaviour of tryptophan during acid hydrolysis of proteins. If the existence of interaction with other amino-acids or NH_3 is established, this introduces

yet a fresh uncertainty into the deduction of the amino-acid composition of intact molecules from the composition of hydrolysates as usually prepared. With the average protein, of low tryptophan content, the percentage errors introduced in this way would not be as great as with tyrocidine. Nevertheless, they might invalidate stoichiometric calculations for a large molecule.

Whatever general significance for amino-acid analysis our observations may have, the errors encountered have entirely prevented the stoichiometric calculation of a 'minimum molecule' of tyrocidine hydrochloride, since molecules of a composition within the accuracy of our determinations can easily be postulated having two or any larger number of Cl' groups, while we cannot rigidly exclude a molecule having only one Cl' group.

Nevertheless, if we combine Hotchkiss's tryptophan and NH_3 figures with those now obtained for other constituents, we can say that 88–105% of the N of tyrocidine has been identified in its hydrolysate. A reliable estimate of the molecular weight by physical means might throw considerable further light on the data presented here.

EXPERIMENTAL

General analytical procedure

[cf. Gordon *et al.*, 1943*a, b*]

Unless otherwise stated, preparations were hydrolysed by heating for 24 hr., at the temperature of boiling 6*N* HCl, with 0.8 ml. acetic acid and 3.0 ml. 6*N* HCl in sealed tubes which had been freed from air by boiling the mixture *in vacuo* before sealing [Gordon *et al.*, 1943*b*].

The acetylation and extraction procedure has been modified as follows: 5 ml. 4*N* NaOH were used in place of 10 ml. 2*N* NaOH. After acidification the solution was immediately absorbed in 16 g. silica gel. After thorough

The dotted lines in the diagram correspond to the 'artefact bands' observed during the fractionation.

mixing this was made to a slurry with 17% butanol- CHCl_3 (half-saturated with water), and made into a column in the usual way. No indicator was used. The vessels were washed through three times with small quantities of solvent, which were then added separately to the top of the column and allowed to drain in. Finally, fresh solvent to a total of 1 l. was run through the column. This multi-plate 'chromatographic' extraction utilizes solvent much more efficiently than the single-plate separating funnel procedure previously employed.

The scheme of fractionation shown above was employed.

The initial fractionation with 17% butanol- CHCl_3 was introduced to ensure complete solution of the material for analysis, which does not occur when 1% butanol- CHCl_3 is used first. The silica was saturated with a 0.05% aqueous solution of pelargonin chloride [Gordon *et al.*, 1943*a*].

In calculating N values from the titrations, the following factors were used: ornithine and tryptophan, 2 atoms N; glutamic and aspartic acids, $\frac{1}{2}$ atom N; other fractions 1 atom N per acid equivalent.

Determination of glutamic and aspartic acids

After titration, fraction (2.1 + 3.1) was hydrolysed with 6*N* HCl for 6 hr. at 100°, evaporated to dryness *in vacuo* and dissolved in water. A suitable portion was taken for ninhydrin- CO_2 determination by the method of Christensen, West & Dimick [1941] with 10 min. heating. Calibration experiments showed that under these conditions glutamic acid evolved 1.02 mol. CO_2 , and aspartic acid 1.88 mol. CO_2 , and the relative amounts of the two amino-acids present were calculated from this.

CO_2 evolved during hydrolyses

Before analysis of their contents the hydrolysis tubes were connected (still sealed) to the Van Slyke-Neill manometric apparatus through pressure tubing; the tip of the tube was then broken off inside the rubber, the CO_2 transferred to the chamber and determined as described by Van Slyke *et al.* [1941]. Control experiments showed that no CO_2 was evolved by the HCl-acetic acid mixture during 24 hr. heating.

Preparation of tyrocidine hydrochloride

The material studied was obtained from the residues of our preparation of gramicidin from tyrothricin [Gordon *et al.*, 1943*b*]. Preparation A was obtained by three recrystallizations according to Hotchkiss & Dubos [1941]. After the 1st recrystallization, the material had $(\alpha)_D^{17.5-18.5} = -98^\circ$, after the 2nd, -100° , and after the 3rd, -100.5° (95% ethanol, $c=1.1-1.3$). Prep. A had $N=14.5\%$ (Kjeldahl, with or without P-HI pretreatment). Prep. B was obtained from A by one further recrystallization.

Characterization of tyrocidine constituents

244 mg. tyrocidine HCl (Prep. A) was hydrolysed and prepared for chromatography as described above, using double the quantity of each reagent. After making the hydrolysate alkaline, NH_3 was removed *in vacuo* before addition of acetic anhydride. The chromatographic fractionation was as above, with columns of suitably enlarged diameter. Pelargonin chloride was used as indicator throughout. Significant quantities of this were eluted by

30% propanol-cyclohexane though not by 17% butanol- CHCl_3 . In these cases the contaminated fraction, dissolved in a little 17% butanol- CHCl_3 , was filtered through a layer of 2 g. silica gel saturated with water. By washing with a suitable volume of fresh solvent the acetamino-acid was eluted free from pelargonin.

Acid equivalent weight (A.E.W.) and N (Kjeldahl) determinations were carried out as before [Gordon *et al.*, 1943*b*]; in the former, the first faint persistent pink of phenolphthalein was the end-point. C and H determinations were made by Dr G. Weiler, Oxford. Melting-points are given uncorrected unless otherwise stated. Optical rotations were observed in a 2 dm. tube.

1.1.1. Acetyl-D-phenylalanine. Crystallization from CHCl_3 gave a product (m.p. 160°) which on recrystallization yielded 39 mg. having m.p. 164°, which was depressed to 150–158° on admixture of authentic acetyl-L-phenylalanine (m.p. 169–171°). $(\alpha)_D^{19.5} = -37.8^\circ$ (ethanol, $c=1.1$). Du Vigneaud & Meyer [1932] record for acetyl-D-phenylalanine m.p. 172° (corr.), $(\alpha)_D^{26} = -51^\circ$ (ethanol). (Found: C, 63.6; H, 6.48; N, 6.53%; A.E.W. 211. Calc. for $\text{C}_{11}\text{H}_{13}\text{O}_3\text{N}$: C, 63.7; H, 6.28; N, 6.77%; A.E.W. 207.)

1.2.1 + 1.3.1. Acetyl-L-leucine. Crystallization from CHCl_3 yielded 17 mg. having m.p. 180–181°, not depressed on admixture of authentic acetyl-L-leucine. Admixture of acetyl-D-leucine depressed the m.p. to 160°. $(\alpha)_D^{20.5} = -19.0^\circ$ (ethanol, $c=0.5$). Martin & Synge [1941] record for acetyl-L-leucine m.p. 182°, $(\alpha)_D^{24} = -23.5^\circ$ (ethanol, $c=3.3$). (Found: C, 55.7; H, 8.93; N, 7.9%; A.E.W. 175. Calc. for $\text{C}_8\text{H}_{13}\text{O}_3\text{N}$: C, 55.5; H, 8.67; N, 8.1%; A.E.W. 173.)

1.2.2. Acetyltryptophan would be expected in this fraction [Gordon *et al.*, 1943*b*]. Crystallization from CHCl_3 -cyclohexane followed by recrystallization from CHCl_3 yielded 4.7 mg. having m.p. 170–190°, not depressed on admixture of acetyl-L-tryptophan. $(\alpha)_D^{20.5}$ slightly positive ($< +3^\circ$) (ethanol, $c=0.15$). After evaporation from ethanol solution N content was 9.1% (P-HI pretreatment), and A.E.W. = 205. (Calc. for $\text{C}_{13}\text{H}_{14}\text{O}_3\text{N}_2$: N, 11.4%; A.E.W. 246.) Perhaps this fraction was a mixture of acetyl-leucine and acetyltryptophan. In two of the three quantitative analyses described below this fraction was also very small; in the other a substantial acid fraction was found.

1.3.2. Acetyl-L-valine. Crystallization from CHCl_3 yielded 13 mg. having m.p. 158°, not depressed on admixture of authentic acetyl-L-valine. The crystals were contaminated by a water-insoluble gummy material, possibly derived from tryptophan, which proved difficult to eliminate. $(\alpha)_D^{19} + 2 \pm 1^\circ$ (ethanol, $c=0.35$). Gordon *et al.* [1943*a*] record for acetyl-L-valine m.p. 164°, $(\alpha)_D^{19} + 4.0^\circ$ (ethanol, $c=2$). (Found: C, 54.8; H, 8.22; N, 8.0%; A.E.W. 176. Calc. for $\text{C}_7\text{H}_{13}\text{O}_3\text{N}$: C, 52.8; H, 8.17; N, 8.8%; A.E.W. 159.)

1.3.3. Acetyl-L-proline (monohydrate). Crystallization from wet ethylacetate yielded 23 mg. having (air-dry) m.p. 76–78°, not depressed on admixture of authentic acetyl-L-proline hydrate. $(\alpha)_D^{19} = -112.5^\circ$ (H_2O , $c=0.7$) (rotation calc. for anhydrous compound). Martin & Synge [1941] record m.p. 78°, $(\alpha)_D^{23} = -114.0^\circ$ (H_2O , $c=4.2$). Analyses were on the air-dry hydrate. (Found: C, 47.2; H, 7.26; N, 7.7%; A.E.W. 184. Calc. for $\text{C}_5\text{H}_{11}\text{O}_3\text{N} \cdot \text{H}_2\text{O}$: C, 48.0; H, 7.43; N, 8.0%; A.E.W. 175.)

1.4.1. Acetyl-L-tyrosine. Treatment with ethanol-light petroleum eliminated much gummy material, after which

were slowly deposited 17 mg. crystals (slightly contaminated by gum) having m.p. 148°, not depressed on admixture of authentic acetyl-*l*-tyrosine. ($\alpha_D^{19.5} + 41^\circ$ (H₂O, $c=0.4$). Du Vigneaud & Meyer [1932] record m.p. 152–154° (corr.), ($\alpha_D^{26} + 47.5^\circ$ (H₂O, $c=2$). (Found: C, 57.6; H, 5.53; N, 5.9%; A.E.W. 252. Calc. for C₁₁H₁₃O₄N: C, 59.3; H, 5.83; N, 6.3%; A.E.W. 223.)

2.1+3.1. Dicarboxylic amino-acids. A preliminary determination on a portion of this fraction (*R* (chromatogram band rate) = 0.2 (approx.) on both 17% butanol-CHCl₃ and 30% propanol-cyclohexane columns [Gordon *et al.*, 1943a]) showed exactly 2 acid equiv./atom N, suggesting the presence of dicarboxylic acids only. Since attempts at crystallization led only to mixed products, the material was reprecipitated on a 17% butanol-CHCl₃ chromatogram, and the front and back portions of the resulting broad band were collected separately. Crystallization of the former material from ethanol-CHCl₃ yielded 6 mg. having m.p. 190°, not depressed on admixture of authentic acetyl-*l*-glutamic acid. Bergmann & Zervas [1928] record m.p. 199° (corr.). (Found: C, 44.9; H, 5.71; N, 8.4%; A.E.W. 91. Calc. for C₇H₁₁O₅N: C, 44.4; H, 5.82; N, 7.4%; A.E.W. 94.5.) The material available did not suffice for optimum analytical accuracy.

In a second analysis, the corresponding fraction from the hydrolysate of 210 mg. of Prep. A was hydrolysed (6 hr. in 6*N* HCl at 100°), and evaporated to small volume. The dicarboxylic acids were separated in the usual way. Saturation with HCl gas at 0° gave slow deposition of glutamic acid HCl crystals (16 mg.), having ($\alpha_D^{18.3} + 13^\circ$ (H₂O, $c=0.3$) (rotation calc. for glutamic acid). Wood [1914] records for *l*-glutamic acid in water + 1 mol. HCl ($\alpha_D^{25} + 27.9^\circ$. (Found: N, 7.3%; A.E.W. 93. Calc. for C₅H₉O₄N.HCl: N, 7.6%; A.E.W. 92.)

The mother liquors from the above, after most of the HCl had been removed *in vacuo*, were treated with copper acetate in 2 ml. water. The crystalline Cu salt deposited was filtered off and decomposed in the usual way with H₂S, yielding material (5.2 mg.) which did not readily crystallize from water, having N content (7%) lower than the theoretical for aspartic acid. After treatment with excess HCl followed by evaporation to dryness, the material had ($\alpha_D^{18} + 13^\circ$ (H₂O, $c=0.2$) (rotation calc. for dry wt. before HCl treatment). Wood [1914] records for *l*-aspartic acid in H₂O + 0.94 mol. HCl ($\alpha_D^{25} + 18.4^\circ$. A ninhydrin-CO₂ determination showed the same CO₂/N ratio as aspartic acid (see above). From this identity, the insolubility of the Cu salt, the —COOH/N ratio, and the fact that the rate and appearance of the dicarboxylic acid chromatogram band from tyrocidine hydrolysates could be closely simulated by mixtures of acetylglutamic and acetylaspargic acids, we conclude that the fraction under consideration was an impure specimen of aspartic acid, and that tyrocidine hydrolysates contain *l*-glutamic and *l*-aspartic acids, the former considerably racemized.

3.2. NN'-diacetyl-*l*-ornithine. Crystallization from ethylacetate and from ethanol-ether successively yielded 25 mg. needles having m.p. 156°, not depressed on admixture of authentic diacetyl-*l*-ornithine (see below). ($\alpha_D^{18} + 4^\circ$ (ethanol, $c=0.4$). (Found: C, 50.6; H, 7.77; N, 12.3%; A.E.W. 224.) On 17% butanol-CHCl₃ and 30% propanol-cyclohexane chromatograms the band-rate *R* was approx. 0.1, and the material was chromatographically indistinguishable from authentic diacetyl-*l*-ornithine on a butanol-

CHCl₃ column, while readily separable from diacetyl-*dl*-lysine [cf. Gordon *et al.*, 1943a].

Acetylation of *l*-ornithine

l-Ornithine HCl prepared from *l*-arginine HCl by the action of liver arginase [Hunter, 1939] and having ($\alpha_D^{18} + 10.8^\circ$ (H₂O, $c=5$) was subjected to acetylation and extraction as described above, and yielded material similar in appearance and crystallization properties to the product from tyrocidine, having m.p. 156° (constant on recrystallization), ($\alpha_D^{15} + 6.3^\circ$ (ethanol, $c=0.8$). (Found: C, 49.9; H, 7.68; N, 12.7%; A.E.W. 218. C₉H₁₆O₄N₂ requires: C, 50.0; H, 7.40; N, 13.0%; A.E.W. 216.)

Quantitative analyses of tyrocidine hydrolysates, etc.

Three analyses were performed, according to the general scheme given above, on 10–20 mg. tyrocidine HCl with 3 g. silica gel in 1 cm. tubes for the butanol-CHCl₃, and 2 g. for the other fractionations. Methyl orange was substituted for pelargonin in those columns where the development was begun with 1% butanol-CHCl₃ or 5% propanol-cyclohexane, because of its superior sensitivity.

Control analyses on a known mixture after heating in acid are shown in Table 1. Similar experiments on simple mixtures of glutamic acid and acetyltryptophan were carried out in the presence and absence of air. In the former analysis air was readmitted to the 20 ml. tube to a pressure of 4 cm. Hg after the tube had been completely evacuated. The analytical procedure was simplified by omitting the propanol-cyclohexane chromatograms. Table 2 indicates the slightly higher recoveries found in the absence of air and gives the CO₂ evolution measured as described above. No CO₂ was evolved by tyrocidine or by the control mixture or by 4.05 and 6.1 mg. *l*-citrulline (Hofmann La Roche—kindly provided by Prof. H. A. Krebs) when heated in acid under the same oxygen-free conditions. 1.65 and 2.5 mg. of urea similarly treated yielded 0.565 and 0.98 mg. CO₂ respectively. After 4 mg. citrulline had been treated as above the resulting material was analysed in the same way as the glutamic acid-tryptophan mixtures, yielding at most 8% of a mol. of acids having *R* > 0.5 in 17% butanol-CHCl₃ (which would include acetylproline), and 22% of a mol. of diacetylornithine. These substances were not identified; the figures are based on titration of bands having corresponding rates. (For recovery of proline and ornithine from known mixture cf. Table 1.)

Table 3 shows the results (uncorrected) of our analyses of tyrocidine together with the figures obtained by other workers.

SUMMARY

1. Technical modifications have extended quantitative analysis by partition chromatography to a further group of amino-acids.

2. From an acid hydrolysate of tyrocidine, phenylalanine, leucine, proline, valine, tyrosine, ornithine and glutamic acid have been isolated by partition chromatography, and characterized as their acetyl derivatives. Additional evidence has been obtained for the occurrence of tryptophan and

aspartic acid. This appears to be the first isolation of ornithine by acid hydrolysis of an intact protein or polypeptide.

3. The phenylalanine isolated had mainly the *d*-configuration; the other amino-acids were mainly *l*-.

4. 88–105 % of the N of tyrocidine has now been accounted for, but the variability of the analytical

figures has not permitted stoichiometric calculations. The implications of this variability, which exceeds the analytical errors, are discussed.

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The Structure of the Galactose-phosphate Present in the Liver During Galactose Assimilation

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In this paper further support is provided for the view [Kosterlitz, 1937; 1938; 1939b] that in the galactose-phosphate present in the liver during galactose assimilation the phosphate group is attached to carbon atom 1. It has not yet been possible to separate this ester from the accompanying glycerophosphate, and from small quantities of glucose-1-phosphate and an unidentified ester. But the facts that reducing hexosemonophosphates can be removed by alkaline hydrolysis, and that Ba glycerophosphate is very resistant to acid hydrolysis and has a specific rotation of 0°, have made possible the approximate determination of the rate of acid hydrolysis of the galactose-phosphate and of its specific rotation. Since the ester is non-reducing, resistant to alkaline hydrolysis and very labile to acid hydrolysis, it is concluded that the first carbon atom is substituted. The velocity constant (*k*) of hydrolysis of the natural ester in 0.25 *N* HCl at 25° is 0.91×10^{-3} , while that of synthetic galactose-1-phosphoric acid is 0.89×10^{-3} .

Natural Ba galactose-1-phosphate has $[\alpha]_{5461} + 113^\circ$ while the synthetic ester has $[\alpha]_{5461} + 109.5^\circ$. These values strongly suggest that the natural and synthetic esters are identical. It is of interest to note that Wolfrom & Pletcher [1941] and Wolfrom, Pletcher & Brown [1942] have succeeded in finally proving that the glucose-1-phosphate of Cori, Colowick & Cori [1937] has the structure of phosphoryl α -mono-*d*-glucopyranoside. Since the principle of the synthesis of galactose-1-phosphate is analogous to that devised by Cori *et al.* [1937] for glucose-1-phosphate, the findings of Wolfrom *et al.* [1942] give further support to the view expressed on other grounds [Kosterlitz, 1939a] that the synthetic galactose-1-phosphate is phosphoryl α -mono-*d*-galactopyranoside.

EXPERIMENTAL

The general methods were the same as those described in previous papers [Kosterlitz, 1937; 1939a]. The Ba salts of the phosphoric esters were obtained