dark CoA samples gave a heavy, black precipitate on acidification; during reduction, this dissolved and most of the color disappeared. After 30 minutes the suspension of zinc was filtered or centrifuged with a minimum exposure to air, and 2 ml. of 20% mercuric acetate in 2% acetic acid was added with stirring. The resulting tan precipitate was centrifuged and washed with a small volume of water.

Hydrogen sulfide was bubbled for several hours through a suspension of the precipitate in 5 ml. of water, and the mercuric sulfide removed and washed with a little water. The solution and washing (strongly acidic) were combined and aerated briefly to remove most of the hydrogen sulfide. At this stage about 5,000 coenzyme units remained.

The solution was further acidified (if necessary) to below *p*H 1 and applied to a 5 × 185 mm. column of Duolite CS-100 resin (100–200 mesh; reusable repeatedly), which had been washed with 0.2 *N* hydrochloric acid. The sample was followed by acid of this concentration and the optical density of the effluent at 260 mμ examined. A large amount of absorbing impurity appeared quickly, and when the density had fallen to approximately $d = 1$ (about 25 ml.), the eluant was changed to water. As soon as the effluent *p*H rose to 2 or higher, the CoA emerged, and 30 ml. later had virtually ceased. The fractions with coenzyme activity were combined and freeze-dried, leaving a white, cottony product assaying in various runs from 330 to 384 units per mg. The yield was 15–20% of the units in the starting material. The analysis of such a compound, containing 25.6% pantothenic acid, has been reported[19]; this material, on the basis of the figures given above, is about 90% pure. Hydrolysis and paper chromatography gave a pattern as in Fig. 1, sample 2, showing the complete absence of cystine.

(19) J. D. Gregory, G. D. Novelli and F. Lipmann, THIS JOURNAL, **74**, 854 (1952).

BOSTON 14, MASSACHUSETTS

[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

The Chemistry of Tyrocidine. I. Isolation and Characterization of a Single Peptide

BY ALAN R. BATTERSBY[1] AND LYMAN C. CRAIG

RECEIVED FEBRUARY 15, 1952

Crystalline tyrocidine hydrochloride has been fractionated by countercurrent distribution and shown to contain three major components. One of these, tyrocidine A, has been purified to the stage where it probably represents a single substance. It has been shown to contain only amino acids and ammonia all of which have been isolated and characterized. An empirical formula $C_{66}H_{87}O_{13}N_{13}{\cdot}HCl$ has been derived for the molecule. It has been shown to be a cyclic peptide containing one primary amino group, one phenolic hydroxyl group and two primary amide groups. The amino acid residues have been found to be: three of phenylalanine and one each of leucine, valine, tyrosine, proline, ornithine, glutamic acid and aspartic acid.

Introduction

Attempts to fractionate the higher, naturally occurring polypeptides by countercurrent distribution, CCD,[2] have shown that in this class of substances even the first step in structural study, the isolation of a single pure compound to use as a starting point, has seldom been achieved. However, an automatic 220 tube CCD[3] apparatus has now been constructed and it is of considerable interest to determine whether or not a pure polypeptide can be isolated with the aid of this instrument. Tyrocidine has been chosen as a suitable test material.

Tyrocidine was isolated from autolyzed cultures of *B. brevis* by Hotchkiss and Dubos[4] in 1940. It was soon shown to be a basic polypeptide. Since that time it has interested a number of chemists, whose work has been reviewed by Hotchkiss[5] and by Synge.[6] In this early work samples of the peptide were recrystallized until there was no observable change in properties. The criterion of purity then available was thus satisfied. Later these preparations were shown to be mixtures by frontal analysis[7] and by CCD.[2]

The isolation of a pure polypeptide from the family of closely related substances present in tyrocidine has been the first objective in the researches reported here. The second objective has been the characterization of the isolated material, determination of its molecular weight and sufficient structural study to give adequate support to the thesis of purity. The molecular weight studies are reported in an accompanying contribution.[8]

Experimental

We are indebted to the Wallerstein Company for the tyrocidine used in this work. A system made from a mixture of methanol, chloroform and 0.1 *N* hydrochloric acid, volume proportions 2,2,1, appeared to be a satisfactory system for the preliminary fractionation. A 5-g. sample after 673 transfers gave the pattern shown in Fig. 1. Weight determinations were made by the method of Craig, *et al.*[9] Ultraviolet absorption measurements were made directly in the appropriate layer with the Beckman quartz spectrophotometer.

Attention was first focused on the large band, which may be called tyrocidine A, on the left of the pattern of Fig. 1 and an attempt was made to purify it further. For this purpose a second distribution was made with twice the starting charge, 10 g., scattered in 15 tubes. This gave pattern 2a, Fig. 2, at 760 transfers for the part remaining in the train. After removal of the tail material in tubes 0–150 the apparatus was set for recycling.[3] At 1600 transfers pattern 2b was obtained. The experimental band was noticeably too broad but determinations of the partition ratio at various points on the pattern did not show impurity. Six fractions were taken as shown at the top of 2b and separately examined. When solutions of fractions 2–5 in the minimum quantity of methanol were treated with ether until incipiently turbid, beautiful colorless rods separated, total weight 1.9 g. Fractions 1 and 6 yielded no crystalline material.

The recrystallized material from fractions 3 and 4 of

(1) On leave of absence as a Commonwealth Fund Fellow from the University of St. Andrews, Scotland.

(2) L. C. Craig, J. D. Gregory and G. T. Barry, *Cold Spring Harbor Symposia Quantitative Biol.*, **14**, 24 (1949).

(3) L. C. Craig, W. Hausmann, E. H. Ahrens, Jr., and E. J. Harfenist, *Anal. Chem.*, **23**, 1236 (1951).

(4) R. D. Hotchkiss and R. J. Dubos, *J. Biol. Chem.*, **132**, 791 (1940).

(5) R. D. Hotchkiss, *Advances in Enzymology*, **4**, 153 (1944).

(6) R. L. M. Synge, *Quarterly Reviews*, **3**, 245 (1949).

(7) R. L. M. Synge and A. Tiselius, *Acta Chem. Scand.*, **1**, 749 (1947).

(8) A. R. Battersby and L. C. Craig, THIS JOURNAL, **74**, 4023 (1952).

(9) L. C. Craig, W. Hausmann, E. H. Ahrens, Jr., and E. J. Harfenist, *Anal. Chem.*, **23**, 1326 (1951).

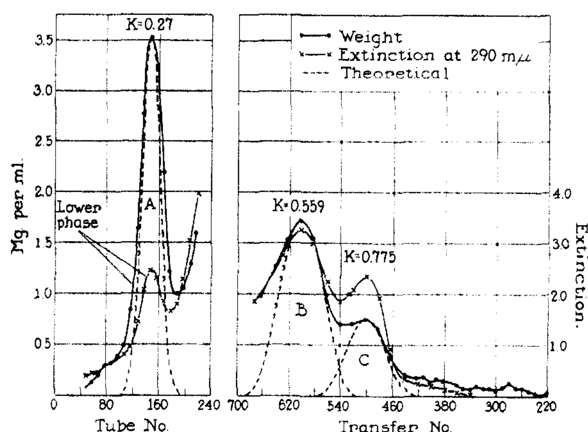

Fig. 1.—Countercurrent distribution patterns of crude tyrocidine.

pattern 2b, with that from other identical experiments, total weight 4.2 g., was distributed in a different system made from methanol, benzene, chloroform and 0.1 N hydrochloric acid in the volume proportions 23, 10, 20, 7. This gave pattern 2c at 793 transfers. The tail portions were removed and the apparatus was set again for recycling. At 2140 transfers pattern 2d was obtained.

The material from pattern 2b was found to give a positive tryptophan test with the procedure of Spies and Chambers.[10] Such tests were also made on various tubes in pattern 2c and 2d. The results are given above the patterns. Pattern 2d shows that the tryptophan containing impurity had moved mostly to the right, away from the main band. The solute in tubes 190 to 275 was therefore collected, 2.91 g., and regarded as the pure sample of tyrocidine A.

On recrystallization from methanol-ether it melted at 240–242°. It was dried at 110° for analysis.

Anal. Calcd. for $C_{66}H_{87}O_{13}N_{13}\cdot HCl$: C, 60.64; H, 6.79; N, 13.93; Cl, 2.71. Found: C, 60.61; H, 6.82; N(Kj.), 13.7; Cl, 2.81; $[\alpha]^{25}D$ −111° (c 1.37 in 50% ethanol).

The salt was insoluble or sparingly soluble in chloroform, water, acetone or ether, fairly soluble in methanol or ethanol, readily soluble in aqueous methanol or ethanol. Tyrocidine A free base was precipitated by making an aqueous alcoholic solution of the hydrochloride alkaline with ammonia or sodium carbonate, but redissolved when sodium hydroxide was added.

The determination of free amino nitrogen was carried out in the following way to avoid the formation of a precipitate which occurred when the standard conditions were used. A solution of 50 mg. of potassium iodide in 5 cc. of dioxane, 2.5 cc. of glacial acetic acid and 1 cc. of water was allowed to stand for 30 min. The tyrocidine A hydrochloride (21.90 mg.) was suspended in 0.75 cc. of water in a 5-cc. graduated flask and diluted to the mark with the dioxane-glacial acetic acid mixture to give a clear solution. A 4-cc. sample of the latter was used in a 60-min. reaction; ornithine was found to give the correct value under these conditions. Found: amino-N, 1.16. Calcd. for a single primary amino group in a molecule of weight 1307, 1.07%.

Nitrogen as $-CONH_2$ was determined by hydrolyzing tyrocidine A hydrochloride (15.26 mg.) in 1.5 cc. of 6 N HCl at 110° for 20 hr. The clear solution was then washed into a Kjeldahl apparatus and the usual procedure followed with the omission of the digestion. Found: amide-N, 2.01. Calcd. for 2-$CONH_2$ in molecule of weight 1307, 2.14%.

The ultraviolet absorption of tyrocidine A was determined with the Beckman quartz spectrophotometer in a solution made by dissolving 12.8 mg. of the hydrochloride in a mixture of 7.5 cc. of methanol and 5 cc. of water followed by 12.5 cc. of 0.2 N aqueous sodium hydroxide. Water was added to bring the volume to exactly 25 cc. The solution of tyrosine used for comparison was made in the same way. The results are given in Fig. 3.

Isolation of Ammonia from the Hydrolysate of Tyrocidine A.—A solution of 3 g. of tyrocidine A hydrochloride in 100 cc. of 6 N hydrochloric acid was heated under reflux for 20 hours. It was evaporated to about 20 cc. and treated with a large excess of 50% aqueous sodium hydroxide. The alkaline solution was gradually heated to boiling whilst air was bubbled through the apparatus into 2 N hydrochloric acid. Evaporation of the acid solution left a white residue which was sublimed at 110° and 0.1 mm. for analysis. A rough microtitration by the iodate method for Cl was made.

Anal. Calcd. for NH_4Cl; Cl, 66. Found: Cl, 68.

Hydrolysis of Tyrocidine A and Separation of the Amino Acids Formed.—A solution of 1.51 g. of tyrocidine A hydrochloride, in 300 cc. of approximately 6 N hydrochloric acid was heated under reflux in nitrogen for 24 hours. The hydrolysate was evaporated to dryness *in vacuo* and the residue kept over potassium hydroxide in a vacuum for 24 hours. This mixture of amino acid hydrochlorides was distributed in a solvent system made by equilibrating 3000 cc. of an aqueous solution of 300 g. of ammonium acetate and 300 cc. of ammonium hydroxide (sp. gr. 0.88) with a mixture of 900 cc. of n-propanol and 1800 cc. of 2-butanol. The mixture of solutes was scattered in the first 10 tubes. At 1410 transfers pattern a of Fig. 4 was obtained. Since a residue of ammonium acetate requires more time than the solvents themselves for volatilization, the residues for weight analysis were dried in high vacuum for 10 min. at 100° except in the region of tubes 120–190 of the fundamental series and transfer No. 1050 to 1410 of the withdrawn pattern.

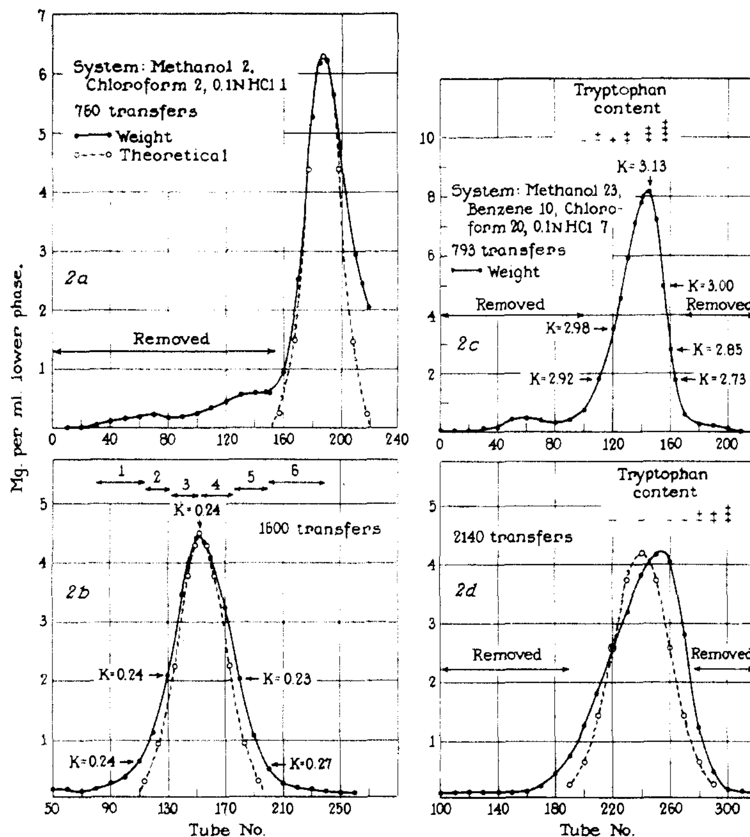

Fig. 2.—Purification of tyrocidine A by countercurrent distribution.

(10) J. R. Spies and Dorris C. Chambers, *Anal. Chem.*, **21**, 1249 (1949).

TABLE I

ANALYTICAL DATA FOR AMINO ACIDS ISOLATED FROM HYDROLYSATE OF TYROCIDINE A

	Carbon, %		Hydrogen, %		$[\alpha]^{25}D$		
	Calcd.	Found	Calcd.	Found	Literature	Found	Solutions used
DL- and D(+)-Phenylalanine	65.43	65.35	6.71	6.88	+35.0°	+ 8°	c 1.11 in H_2O
L-Leucine	54.93	55.05	9.99	9.92	+13.9 (in 4.5 N HCl)	+10	c 1.42 in 6 N HCl
L-Tyrosine	59.65	59.57	6.12	5.99	−12.4	−11	c 1.82 in 1 N HCl
L-Valine	51.26	51.33	9.46	9.30	+28.8	+31	c 1.17 in 6 N HCl
L-Proline	52.13	52.16	7.88	7.88	−85	−84	c 1.02 in H_2O
L-Ornithine monohydrochloride	35.60	35.90	7.78	7.95	+11.0	+11	c 2.17 in H_2O
L-Glutamic acid hydrochloride	32.71	32.89	5.36	5.55	+31.9	+31	c 0.97 in 6 N HCl
L-Aspartic acid hydrochloride	...	...	..	..	+24.0 (in 6 N HCl)	+22	c 0.89 in 10% HCl

The latter contained ammonium chloride and a 30-minute drying period was required to remove this salt.

Since the proline band contained ammonium chloride, it was determined by a colorimetric method[11] developed by Chinard, as well as by weight. The method depends on the color produced when ninhydrin reacts with proline in phosphoric acid at a *p*H of 1; the absorption is measured at 490 mμ.

Paper chromatography showed the first band of pattern a to be an overlapping one of aspartic acid, glutamic acid and ornithine. It was resolved by recovery of the mixed solutes and redistribution in the system *t*-amyl alcohol/5% hydrochloric acid.

Pattern b of Fig. 4 was obtained at 720 transfers. Ammonium chloride was found in the region of tubes 100–140. The contents of tubes 30–60 and of 86–100 were removed. The well separated band of ornithine was also removed for isolation.

The apparatus was then permitted to operate by recycling until 1690 transfers had been reached. For weight analysis, a longer period of drying, 30 minutes, was again employed to remove the ammonium chloride. Pattern c of Fig. 4 was obtained. Paper chromatography confirmed the degree of separation represented by the overlapping theoretical curves.

A cut of tubes 330–360 was taken for isolation of glutamic acid as the hydrochloride. Upon evaporation, the residue was recrystallized from hydrochloric acid. No difficulty was experienced in getting an analytically pure sample.

More difficulty was experienced in the case of aspartic acid because it would not crystallize as the hydrochloride. The cut of tubes 260–300 was therefore evaporated to dryness and made up to a standard solution on which $-NH_2$ nitrogen, carboxyl nitrogen and the optical rotation were determined. The ratio of amino nitrogen to carboxyl nitrogen was here used as the confirming analytical figure.[12] Aspartic acid is the only amino acid which gives the 1:2 ratio found in this analysis.

Ornithine was isolated as the monohydrochloride by crystallization from aqueous ethanol containing pyridine and was recrystallized from water.

Proline was crystallized from the residue obtained on evaporation of the appropriate band and further purified for analysis by sublimation at 140° and 0.001 mm. pressure.

The other amino acids, except valine, were obtained in analytically pure form by crystallizing the residues obtained directly from the bands. The crude valine contained a small amount of halogen which was removed by silver acetate and H_2S prior to crystallization.

Analytical data for the various amino acids isolated are collected in Table I.

O,N-Diacetyltyrocidine A.—A suspension of tyrocidine A hydrochloride (74 mg.) in 1 cc. of pyridine and 0.5 cc. of acetic anhydride, was warmed at 40° for a few minutes until a clear solution was obtained. This was allowed to stand at room temperature for 24 hr. and then ether was added until no further precipitate was formed. The precipitate was dissolved in the minimum quantity of methanol, reprecipitated with ether and rubbed with this solvent until very finely divided. It was washed twice with water and finally recrystallized twice from methanol as colorless needles (34 mg.) m.p. 289–292° slight dec. It was dried at 110° over phosphoric oxide *in vacuo*. *Anal.* Calcd. for $C_{70}H_{91}O_{15}N_{13}$: C, 62.09; H, 6.77. Found: C, 61.95; H, 6.74.

(11) F. P. Chinard, unpublished method.

(12) D. D. Van Slyke, *J. Biol. Chem.*, **83**, 425 (1929); D. D. Van Slyke, R. T. Dillon, D. A. Macfadyen and P. Hamilton, *ibid.*, **141**, 627 (1941).

Discussion

Figure 1 shows clearly that crystalline tyrocidine hydrochloride is in fact a family of polypeptides with the three major components. An undetermined number of minor peptides are also present. The major peptides have been called tyrocidine A, tyrocidine B and tyrocidine C according to their partition ratios in the solvent system, as shown in Fig. 1. From the relation of the weight pattern to the extinction pattern (at 290 mμ), part of the differences between the peptides may be ascribed to differences in tryptophan or tyrosine content. It is indicated that tyrocidine

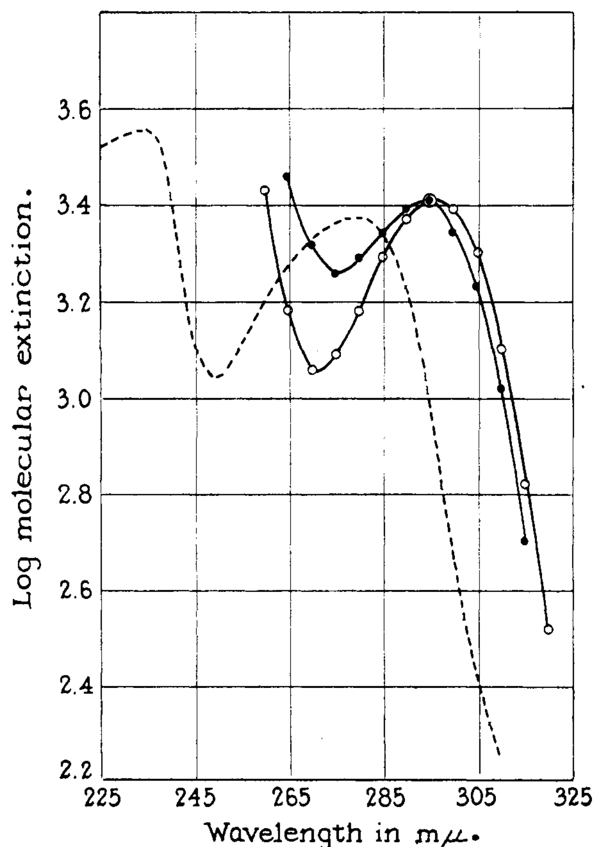

Fig. 3.—Absorption spectrum curves: -----, tyrosine in neutral solvent; O—O, tyrosine in alkaline solution; ●—●, tyrocidine A in alkaline solution.

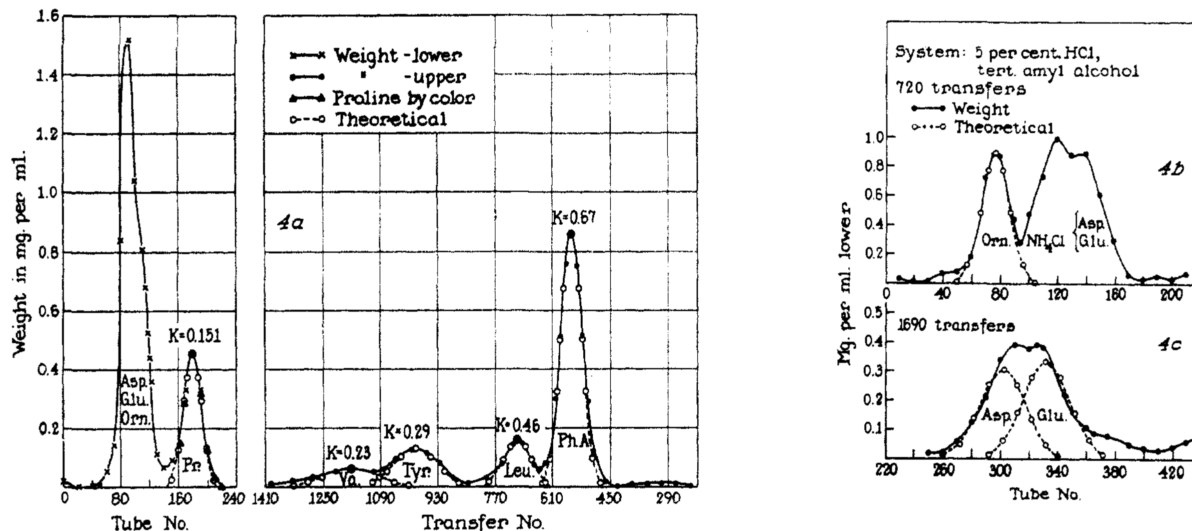

Fig. 4.—Distribution of hydrolysis products of tyrocidine A.

A contains little if any tryptophan, whilst tyrocidine C probably has the highest tryptophan content. This indication was readily confirmed when portions of the three components were hydrolyzed and the hydrolysate was studied by paper chromatography. All three gave distinct spots corresponding to phenylalanine, leucine, tyrosine, valine, proline, ornithine, glutamic acid and aspartic acid. In addition B and C gave strong spots corresponding to tryptophan. Tyrocidine A gave only a very faint spot in this position.

Tyrocidines B and C will not be treated further in this report, but will be taken up in a later study. Only tyrocidine A has been exhaustively purified thus far.

Purity studies by fractional crystallization and further distribution of the A band seemed to show that it was not yet entirely homogeneous. Hydrolysis and careful paper chromatography again showed a faint tryptophan spot. The presence of tryptophan was confirmed by the colorimetric method of Spies and Chambers.[10] Quantitatively this method showed the presence of about 2% of tryptophan, which on the basis of one residue of this amino acid per molecule would correspond to a molecular weight in the order of 10,000 for the polypeptide. A value of this magnitude appeared very unlikely and the presence of an impurity containing tryptophan was suspected. Since fractional crystallization did not remove the impurity, distribution in a different system was tried.

The result is shown in Fig. 2, patterns c and d. Although a skewed distribution resulted, the tryptophan-containing material was slowly separated from the main band and by 2140 transfers the desired result had been obtained. It was then possible to remove the impurity which was apparently present only in small amount and must have had a high tryptophan content. The main part of the band of pattern d was regarded as a single substance. This conclusion has been supported by amino acid determinations and analytical data, and by the molecular weight studies reported in an accompanying paper.[8]

The only amino acid present which could cause the type of ultraviolet absorption spectrum noted in tyrocidine A is tyrosine. It is evident from Fig. 3 that the presence of one residue of tyrosine in a minimum molecular weight of 1270 can account completely for the absorption of the polypeptide.

The relative proportions of the amino acids in tyrocidine A were determined by complete hydrolysis and separation of the products by CCD as shown in patterns a, b and c of Fig. 4. The weights of the different amino acids were calculated from the areas under the curves. In the cases where the amino acids were recovered as hydrochlorides, a correction factor was applied since the salt partially dissociates under the conditions of drying. Table II gives the amount of each amino acid obtained and the theoretical quantity, calculated on the basis of a molecular weight of 1270 for tyrocidine A free base. The optical activity of the isolated amino acids showed that the L-form had been obtained in all cases except that of phenylalanine. Here the specific rotation corresponded approximately to a mixture of two moles of the D-, with one of the L-isomer. Until further work proves otherwise, it is assumed that these occur preformed in the molecule of tyrocidine A.

TABLE II

AMINO ACID COMPOSITION OF TYROCIDINE A

	Wt. of amino acid recovered in g.	Theoretical amount in g.	Molar proportions found
Phenylalanine	0.590	0.573	3.09
Tyrosine	.198	.209	0.94
Valine	.121	.135	0.90
Leucine	.145	.152	0.95
Proline	.155	.133	1.16
Ornithine monohydro chloride	.195	.195	1.00
Glutamic acid hydrochloride	.188	.212	0.89
Aspartic acid hydrochloride	.182	.196	0.93

Although in several cases the amounts of the amino acids indicated by the curve were less than the theoretical, no others could be detected by

CCD or paper chromatography. Proline is slightly high perhaps due to the overlapping NH_4Cl band. The primary objective in the experiments summarized in Table II was that of isolation and the necessary careful standardization of the analytical procedure which would give a closer approximation to theoretical recoveries was not done. The figures for the hydrochlorides resulted from a re-run and were expected to be somewhat low.

The hydrolysis products of tyrocidine A contain a volatile base in addition to amino acids. This base has been isolated as the hydrochloride and proved to be ammonia. Quantitatively, two ammonia molecules result from the minimum molecule of tyrocidine A.

Evidence against the presence of a free carboxyl group in tyrocidine A was obtained by a study of the corresponding acetyl derivative. The latter was prepared in the crystalline state and was found to be neutral in reaction. Hence there can be no free carboxyl group in the original polypeptide. The fact that tyrocidine A is insoluble in excess aqueous sodium carbonate but readily soluble in aqueous sodium hydroxide is consistent with the presence of a free phenolic hydroxy group but no carboxyl group in the molecule.

The molecular weight of tyrocidine A has been shown in an accompanying paper[8] to approximate 1270. This value is in agreement with the amino acid analyses of Table II and with the other analytical data reported in this paper. The peptide is entirely built up of amino acids or their primary amides. Furthermore, the following paper[8] reports that there are no free α-amino groups in tyrocidine A which is basic by virtue of the free δ-amino group of the ornithine residue. No free carboxyl group is present. It must therefore be a cyclic peptide with the γ- and β-carboxyl groups of glutamic and aspartic acids in the form of primary amides. A large ring with 20 carbon atoms and 10 nitrogen atoms appears most probable.

The amino acid residue formula of tyrocidine A can be written (D-phe.)$_2$(L-phe.), (L-val.), (L-tyr.), (L-leu.), (L-pro.), (L-ornithine), (L-glutamine) and (L-asparagine) which corresponds to the empirical formula $C_{66}H_{87}O_{13}N_{13}$. This formula has been confirmed by the preparation of derivatives.[8]

It would thus appear that the first objective stated in the introduction, the isolation of a pure polypeptide, has now been reached in a satisfactory manner. Also the question raised as to whether the 220 tube distribution apparatus is adequate for such a problem has been answered in the affirmative.

Acknowledgments.—We are indebted to Mr. D. Rigakos for the microanalyses and to Dr. J. R. Weisiger and Miss E. Jacobs for the amino nitrogen and amino carboxyl determinations. The technical assistance of Miss D. McNamara is also acknowledged.

NEW YORK, N. Y.

[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

The Chemistry of Tyrocidine. II. Molecular Weight Studies

BY ALAN R. BATTERSBY[1] AND LYMAN C. CRAIG

RECEIVED FEBRUARY 15, 1952

A general approach to the determination of the molecular weights of polyfunctional substances is suggested. The method should prove particularly useful for the characterization of higher molecular weight substances and for additional criteria of purity. The procedure has been applied to tyrocidine A and has shown the value 1270 which is the minimum molecular weight derived by amino acid analysis, to be the true molecular weight.

An unexpected result was obtained when attempts were made to purify tyrocidine A by fractional crystallization from methanol containing dry HCl. The sample being studied had already been purified by countercurrent distribution (CCD)[2] until it gave a single band. However, on redistribution of the recrystallized material two well separated bands were obtained, one of which corresponded to tyrocidine A.

It was soon found that the new band contained a transformation product of tyrocidine A formed by conversion of $-CONH_2$ groups to $-COOCH_3$ groups. The conclusion that only *one* of the possible $-CONH_2$ groups present per molecule was involved could be deduced from interpretation of the distribution pattern. This result suggested a possible means of deriving the molecular weight of tyrocidine A and in fact a general approach to the derivation of molecular weights of polyfunctional substances. This paper presents the molecular weight studies with tyrocidine A.

(1) On leave of absence as a Commonwealth Fund Fellow from the University of St. Andrews, Scotland.

(2) A. R. Battersby and L. C. Craig, THIS JOURNAL, **74**, 4019 (1952).

Experimental

Partial Methanolysis of Tyrocidine A.—A solution of 1.09 g. of tyrocidine A hydrochloride in 12 cc. of methanol was cooled to 0° and a slow stream of dry HCl was passed into the ice-cold solution for about 10 sec. The solution, which was now about 0.1 *N* with respect to HCl, was concentrated *in vacuo* at a low temperature to about 7 cc., when crystallization began. It was permitted to stand at room temperature for 4 hr. after which 0.70 g. of colorless rods was collected.

At the time of the experiment this product was considered to be tyrocidine A of the highest purity but upon distribution of a 400-mg. portion of it to 720 transfers, pattern a of Fig. 1 was obtained. The system methanol, chloroform, 0.1 *N* HCl, volume proportions 2,2,1, was used.

The material in the right peak was recovered by evaporation of the solutions *in vacuo* at less than room temperature. The crystalline residue of tyrocidine A hydrochloride, 203 mg., was recrystallized from methanol–ether. It melted at 240–242° with decomposition. It was dried for analysis at 110° in high vacuum.