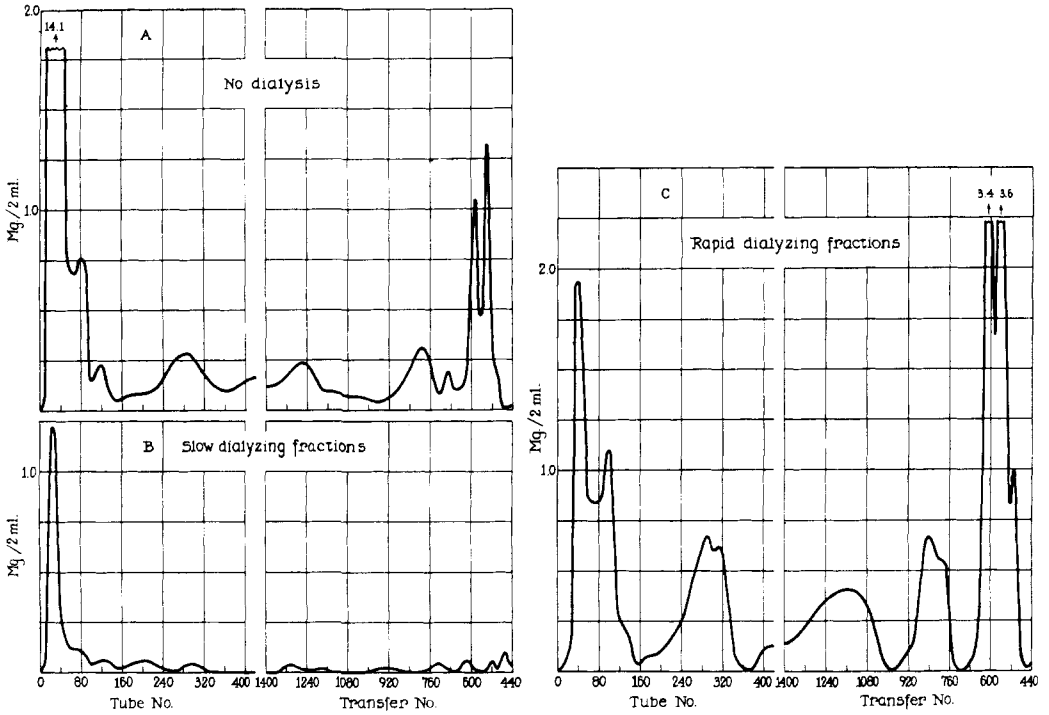

Fig. 5.—Comparative countercurrent dialysis patterns of hydrolysates of tyrocidine treated in different ways.

polypeptides by multiple dialysis. Data are given for partial hydrolysates of tyrocidine B which show that the molecular sizes in a desired fraction can be considerably restricted. The procedure has been shown to be a worthwhile supplementary method to the more precise fractionation tools such as countercurrent distribution, chromatography and zone electrophoresis.

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The Chemistry of Tyrocidine. IV. Purification and Characterization of Tyrocidine B

BY T. P. KING AND L. C. CRAIG

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In a previous paper from this Laboratory[1] crude tyrocidine, an antibacterial agent from cultures of a soil micro-örganism, *B. brevis*, was shown by countercurrent distribution (C.C.D.) to be a mixture of at least three major components. One of these, called tyrocidine A,[2] was isolated in a state of high purity, and its molecular weight determined. It was conclusively shown by amino acid sequence studies[3] to be a large cyclic peptide formed by joining the ten amino acid residues in simple peptide linkages.

The B component was found to differ qualitatively from the A by giving tryptophan on hydrolysis. It appeared of interest to learn whether other structural differences also could be found. A chemical study of tyrocidine B has, therefore, been undertaken. The present paper will report the further purification, molecular weight determination and amino acid composition of tyrocidine B.

Experimental

During the course of the structural studies with tyrocidine A[1] a number of preparative C.C.D. runs on crude tyrocidine had been made. The cuts containing the B component had been evaporated and the residues put aside for the present study.

This solid residue was recrystallized from methanol by addition of isopropyl ether, and 2.2 g. of residue gave a total of 1.56 g. of crystalline peptide hydrochloride when several successive crops were combined. This material was then redistributed in the same system used for tyrocidine A.[1] It contained chloroform, methanol and 0.1 *N* HCl in the volume proportions of 2,2,1. The 200-tube automatic distribution apparatus[4] was used for the distribution. After recycling to 591 transfers, analysis by optical density at 290 mμ gave pattern A as shown in Fig. 1.

The solutes from the three fractions were recovered by evaporation in the rotary evaporator.[5] They were analyzed after drying at 100° under vacuum.

Anal. Calcd. for $C_{68}H_{89}O_{13}N_{14}Cl$: C, 60.68; H, 6.67; N, 14.58; Cl, 2.64; N (2 amides), 2.08. Calcd. for $C_{69}H_{90}O_{14}N_{13}Cl$: C, 60.89; H, 6.67; N, 13.38; Cl, 2.61; N (1 amide), 1.03; OCH_3, 2.28.

Found:	C	H	N	Cl	N (amide)	OCH_3
Cut 1	60.74	6.63	13.18		1.08	1.13
Cut 2	60.65	6.71	13.55		1.10	1.76
Cut 3	60.56	6.52	14.38	2.59	1.77	0

Amide nitrogen was determined on hydrolysates prepared by heating a sample in 6 *N* hydrochloric acid in an evacuated tube for 20 hours at 108°. The liberated ammonia was then estimated using the Conway micro-diffusion technique.

When the material was distributed directly without the attempt to purify it by fractional crystallization, analysis at 210 transfers gave pattern B. Recycling the main band,

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

(2) A. R. Battersby and L. C. Craig, *ibid.*, **74**, 4023 (1952).

(3) A. Paladini and L. C. Craig, *ibid.*, **76**, 688 (1954).

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

(5) L. C. Craig, J. D. Gregory and W. Hausmann, *ibid.*, **22**, 1462 (1950).

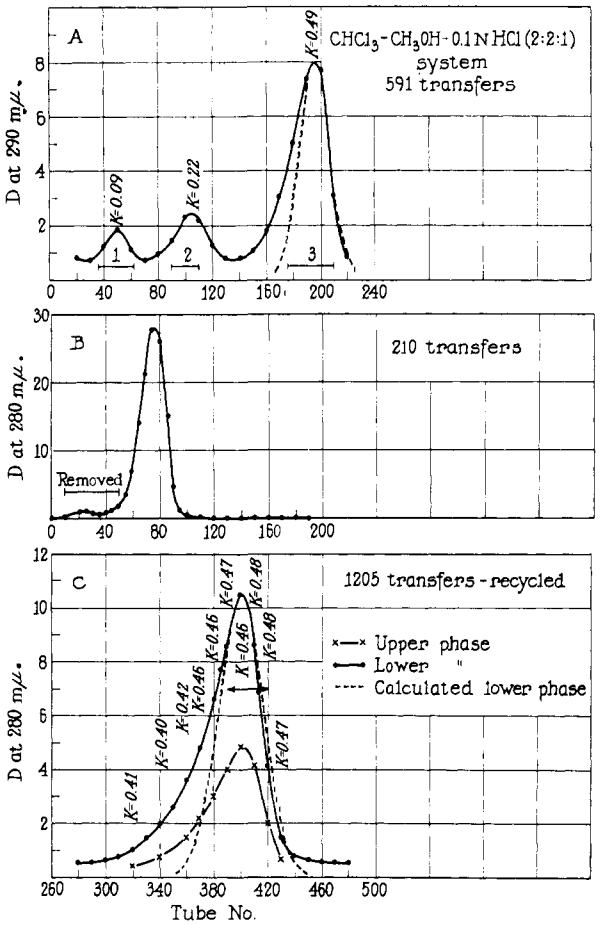

Fig. 1.—Distribution patterns of crude tyrocidine B.

tubes 50–100 to 1205 transfers gave pattern C. A cut of tubes 390–420 from pattern C was recovered by evaporation in the rotary evaporator. This was the material considered to be tyrocidine B of sufficient purity for the present study.

Isolation of the Amino Acids from a Total Hydrolysate.—A sample was hydrolyzed by refluxing under nitrogen a solution containing 910 mg. of the peptide in 200 ml. of constant boiling hydrochloric acid for 24 hours. The slightly colored solution was evaporated to dryness under vacuum in the rotary evaporator. The residue was stored in a vacuum desiccator over solid potassium hydroxide for 24 hours to further reduce the hydrochloric acid content. This residue was distributed in a system made by equilibrating 3 volumes of 90% aqueous phenol with 2.3 volumes of 2% aqueous hydrochloric acid. The automatic C.C.D. apparatus used contained 420 tubes. The phase volumes in each cell were 5 ml. of the aqueous phase and 10 ml. of the heavier phenol phase. The sample was initially scattered in 8 tubes.

At 410 transfers a very small brown colored band which had scarcely moved was removed from the train and discarded since this material appeared to be composed of humin or other ill-defined transformation products. The distribution was continued to 853 transfers. At this point weight analysis gave the pattern shown in Fig. 2. The right half of the pattern is from the aqueous effluent phases.

The amino acid hydrochlorides were recovered from the various cuts shown in the pattern by extracting the phenol with chloroform and evaporating of the aqueous acid solution. Leucine and proline were only partly separated as can be seen from the overlapping band under cut 3. The combined band in the cut was separated by a distribution to 64 transfers in a system made by equilibrating 2-butanol, *n*-butyl alcohol and 5% hydrochloric acid in 5, 5, 10 volume proportions. In this system the K's for proline and leucine were 0.25 and 0.94, respectively.

The mixed band of valine and tyrosine from cut 4 was

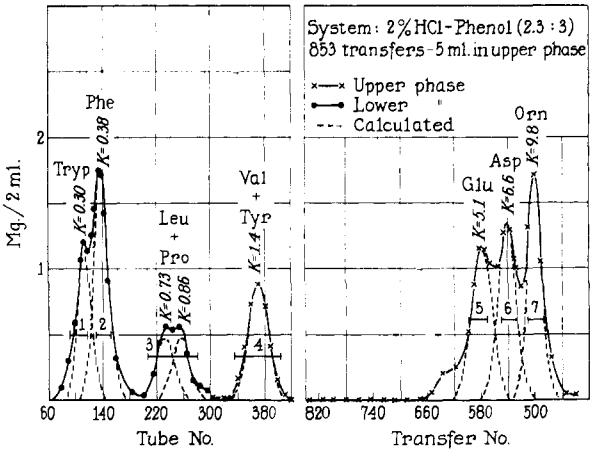

Fig. 2.—Distribution pattern of a complete hydrolysate of tyrocidine B.

separated by fractional crystallization from solutions buffered with pyridine. No difficulty was experienced in getting the slightly soluble tyrosine free from the valine. The valine in the mother liquor was then separated from the residual tyrosine by sublimation at 145° and under 0.2 mm. pressure.

Phenylalanine from cut 2, leucine, aspartic acid from cut 6 and ornithine from cut 7 (as the monohydrochloride) were isolated by crystallization from mixtures of water, ethanol and pyridine. Tryptophan from cut 1 was isolated more satisfactorily following addition of sufficient 0.01 N sodium hydroxide to give a pH of 6. After filtration through a little bone black addition of ethanol caused crystallization.

The residue from the proline band of the redistribution of cut 3 crystallized well from the mixture of water, ethanol and pyridine when sufficiently concentrated but did not give the correct carbon analysis. It was converted to the 2,4-dinitrophenyl derivative by the usual procedure, and this derivative recrystallized from the mixture of acetic acid and water. Glutamic acid from cut 5 was isolated as the hydrochloride by recrystallization from 6 N hydrochloric acid.

The analytical data for the purified amino acids are given in Table I. Optical rotations were taken in a 5-cm. tube. Optical rotations on the crude amino acid residues were also taken and are given together with the yields in Table II.

Partial Substitution of Tyrocidine B with 2,4-Dinitrofluorobenzene.—A solution containing 101 mg. of tyrocidine B hydrochloride (0.075 mmole), 5 ml. of absolute ethanol and 5 ml. of water was treated with 44 mg. of 2,4-dinitrofluorobenzene (0.24 mmole) in 5 ml. of absolute ethanol. An alkaline pH was reached by addition of 0.1 ml. of triethylamine. After 105 minutes at room temperature the mixture was quickly evaporated to dryness and the residue was triturated with isopropyl ether. The solid was filtered and washed with isopropyl ether. The yellow solid weighed 125 mg. It was distributed in a system made from benzene, methanol, chloroform and 0.01 N hydrochloric acid in the volume proportions of 20, 23, 10, 7. At 198 transfers analysis by weight and optical density at 350 mμ gave the pattern shown in Fig. 3.

The material in the three major bands was separately recovered. Weights of 30.5, 36.0 and 13 mg. from the bands, respectively, from left to right were obtained.

Discussion

In the attempt to prepare pure tyrocidine B it soon became apparent that while tyrocidines A and C would be separated readily from B by C.C.D. there remained another complication to be overcome. C.C.D. with the system used in the earlier work for preliminary separation did not lead to a distribution pattern under the more refined conditions which would agree with a calculated curve. Moreover, an attempt to reach a preliminary separation by fractional crystallization gave pat-

TABLE I

ANALYTICAL DATA FOR AMINO ACIDS OF TYROCIDINE B

	Carbon, % Calcd.	Found	Hydrogen, % Calcd.	Found	Nitrogen, % Calcd.	Found	$[\alpha]^{25}D$ Reptd.	Found	Solutions used	Ref.
L-Aspartic acid	36.09	36.18	5.30	5.46	10.52	10.44	+25.4°	+25.3°	*c* 1.66, 5 *N* HCl	6
L-Glutamic acid hydrochloride	32.71	32.93	5.49	5.40	7.63	7.90	+31.8	+31.2	*c* 1.76, 5 *N* HCl	6
L-Leucine	54.93	54.85	9.99	9.87	10.68	10.76	+22.5	+22.4	*c* 1.70, HOAc	6
L-Ornithine monohydrochloride	35.60	35.75	7.78	7.75	16.62	16.78	+28.4	+28.8	*c* 1.51, 5 *N* HCl	6
D-Phenylalanine	65.43	65.38	6.71	6.67	8.48	8.72	+35.0	+31.7	*c* 1.65, H_2O	7
L-Proline as DNP derivative	46.98	47.19	3.94	3.96	14.98	15.06	−674	−620	*c* 0.83, HOAc	
L-Tryptophan	64.69	64.42	5.92	6.03	13.72	13.69	−34.0	−21.2	*c* 0.85, HOAc	6
L-Tyrosine	59.65	59.75	6.12	6.30	7.73	7.90	−10.0	− 8.3	*c* 3.37, 5 *N* HCl	6
L-Valine	51.22	51.16	9.46	9.29	11.96	11.74	+62.1	+58.2	*c* 0.69, HOAc	6

TABLE II

THE RECOVERIES AND OPTICAL ACTIVITIES OF THE CRUDE AMINO ACID HYDROCHLORIDES OF TYROCIDINE B

	Molar ratio	Calcd. yield,[a] mg.	Recovd., mg.	$[\alpha]^{25}D$ Reptd.	Found	Solutions used	Ref.
L-Aspartic acid	1	113	102	+25.3°	+10 ± 2°	*c* 1.26 in 6 *N* HCl	8
L-Glutamic acid	1	122	105	+31.6	+22 ± 2	*c* 1.30 in 6 *N* HCl	8
L-Leucine	1	102	90	+15.2	+17 ± 1	*c* 1.76 in 6 *N* HCl	8
L-Ornithine	1	114	103	+28.4	+26 ± 2	*c* 1.30 in 5 *N* HCl	6
D-Phenylalanine	2	273	249	− 4.5[b]	+ 6.1 ± 0.7	*c* 3.18 in 5 *N* HCl	6
L-Proline	1	102	78	−60.5	−24 ± 2	*c* 0.84 in 5 *N* HCl	6
L-Tryptophan	1	163	140	−13.5	− 1 ± 1	*c* 1.49 in H_2O	9
L-Tyrosine	1	147	120				
L-Valine	1	104	80				

[a] Calculated for 910 mg. of peptide hydrolyzed. [b] For L-phenylalanine.

tern A, Fig. 1, obviously indicating even a more complicated mixture than the original. Apparently, the two smaller bands were formed by a transformation during the recrystallization since they are nearly absent from pattern B. The analytical data obtained on the material isolated from the two small bands, cuts 1 and 2 of pattern A, indicate replacement of an amide nitrogen by a methoxyl as was previously found with tyrocidine A.[2]

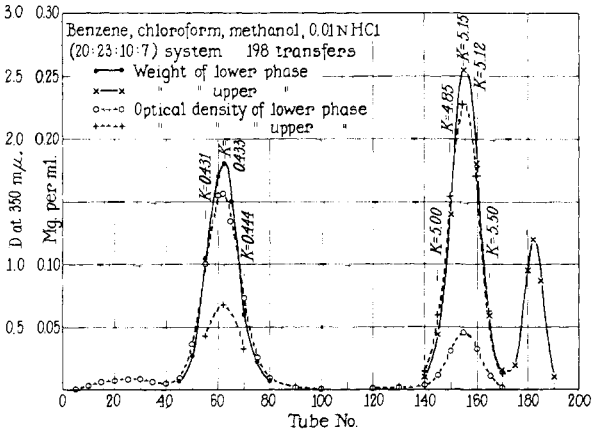

Fig. 3.—Distribution pattern of the partial substitution products of tyrocidine B.

From the earlier experience with tyrocidine A it was anticipated that a certain degree of skewing due to concentration effects would be expected. However, determination of partition ratios at various points across the main band of pattern A indicated this not to be the cause of the divergence from the calculated curve. A certain degree of transformation also during the run seemed the only possible explanation.

That this was in fact the case was shown by amide determinations made on material recovered from the trailing part of the main band. This material contained less amide nitrogen and showed a lower partition ratio than did material isolated from the right side of the band. On the other hand, the partition ratios from tube 190 to 220 pattern A were essentially constant.

In attempting to purify tyrocidine A by fractional crystallization from methanol acidified with dry HCl it was found that extensive methanolysis occurred.[2] An amide on either the glutamic acid or aspartic acid residues was replaced by a methoxyl. The present experience seems to indicate that the loss of amide is due to the same cause since simple hydrolysis would give a free carboxyl group and this derivative would be more hydrophilic than B instead of hydrophobic. The trailing position of the transformation product together with the analytical data indicates the latter. Apparently tyrocidine B undergoes methanolysis even more readily than A. The two small bands in pattern A could arise, one from methanolysis of the asparagine residue and the other from the glutamine residue.

In the final purification of tyrocidine A it was found difficult to remove a small fraction of a peptide with tryptophan content. A likely explanation of this can now be found in the B transformation products. The data of pattern A, Fig. 1, indicate that the product nearest the main band would have a partition ratio similar to that of tyrocidine A in the system used.[1]

(6) J. P. Greenstein, J. M. Birnbaum and M. C. Otey, *J. Biol. Chem.*, **204**, 307 (1953).

(7) E. Fischer and W. Schoeller, *Ann.*, **357**, 1 (1907).

(8) C. H. W. Hirs, S. Moore and W. H. Stein, *J. Biol. Chem.*, **195**, 669 (1952).

(9) H. Fischer, *Z. physiol. Chem.*, **55**, 76 (1908).

Irrespective of the problem of the loss of ammonia it appeared likely that the material in tubes 390 to 420 of pattern C of Fig. 1 represented material of high purity. Evidence supporting this view was obtained in the experiment designed to establish the molecular weight by the method of partial substitution.[2]

This experiment gave only two major yellow bands in addition to the colorless band of the unchanged peptide. All three of the bands were in close agreement with their respective calculated curves. A small amount of material, only a little over 2% of the whole, was to be found in the minor band in the region of tubes 20–35 of Fig. 3. Total hydrolysis of this latter material and two dimensional paper chromatography showed the same spots as a similar experiment with tyrocidine B, except for tyrosine and ornithine. Spots corresponding to δ-DNP-ornithine and O-DNP-tyrosine were obtained. The labile amide could again explain the minor band.

The band occurring in the region of tubes 178–188 was found to contain unchanged peptide. The solute in the one next to it in the region of tubes 145 to 165 was shown by hydrolysis and paper chromatography to be a mono-DNP derivative with the DNP group substituted on the δ-amino group of the single ornithine residue. A yellow spot corresponding to the position of δ-DNP ornithine was obtained instead of the ornithine spot shown by the hydrolysate of the unchanged peptide. All other spots were the same in both cases.

The solute in the band, tubes 50 to 75, was found to be a di-DNP derivative. Hydrolysis and paper chromatography showed spots in the region of δ-DNP-ornithine and O-DNP-tyrosine.

When the molecular weight was calculated from the data given in Fig. 3 for the mono-DNP derivative using a molecular extinction coefficient of 14500[2] for the wave length of 350 mμ a value of 1660 was obtained. Similarly, calculation of the molecular weight from the di-DNP derivative using a molecular extinction of 17000[2] gave a figure of 1890.

When a sample of the hydrolysate of tyrocidine B was studied by two dimensional paper chromatography, spots corresponding to ornithine, leucine, phenylalanine, proline, tryptophan, aspartic acid, glutamic acid, tyrosine and valine were found. No other spots were found. In this study the solvent systems aqueous ammonia, 2-butanol and aqueous formic acid, 2-butanol were used.[10]

Following this study each of the amino acids was isolated in a state of analytical purity by a combination of C.C.D., fractional crystallization and sublimation. From the data obtained it was possible to calculate the molar ratios to within about 15% as given in Table II. All were present in equimolar proportions except phenylalanine which was present in double the molar amount.

Tyrocidine B was found not to contain a free carboxyl group. When studied by the Sanger DNP procedure[11] only two functional groups were found which would react with 2,4-dinitrofluorobenzene. These were found by hydrolysis and paper chromatography to be the δ-amino group of the single ornithine and the hydroxyl group of the tyrosine. Tyrocidine B, like tyrocidine A, is therefore probably a cyclic ten amino acid residue polypeptide.

When the empirical formulas of the ten amino acid residues mentioned above together with two ammonia molecules to form the amides are added with the loss of 12 molecules of water, a formula $C_{68}H_{85}O_{13}N_{14}$ is obtained. The over-all analytical results obtained with the hydrochloride are in good agreement with this formula.

The molecular weight of the proposed formula is 1346. The mono-DNP derivative would have the molecular weight 1512 and the di-DNP derivative 1678. The molecular weights calculated for these two derivatives from weight–optical density ratios are 1660 and 1890, respectively. These values come within a figure believed to be the experimental error of the method.[2]

From the data given in Table I it can be seen that hydrolysis of tyrocidine B gives amino acids of the levo configuration except for the two residues of phenylalanine. These are of the dextro configuration. The tryptophan, proline and aspartic acid residues appear from the data in Table II to emerge partly racemized under the hydrolysis conditions used.

Summary.—Tyrocidine B has been purified and more accurately characterized by determination of its amino acid composition. The molecular weight has been determined by the method of partial substitution.

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(10) W. Hausmann, THIS JOURNAL, **74**, 3181 (1952).
(11) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

The Chemistry of Tyrocidine. V. The Amino Acid Sequence of Tyrocidine B

BY T. P. KING AND L. C. CRAIG

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A preceding paper[1] has shown that tyrocidine B, one of the bactericidal polypeptides produced by *B. brevis*, has the empirical formula $C_{68}H_{88}N_{14}O_{13}$. This formula can be derived by joining in amide linkages the amino acids and two moles of ammonia obtained on complete hydrolysis. These amino acids were L-aspartic acid, L-glutamic acid, L-tyrosine, L-valine, L-ornithine, L-leucine, L-proline, L-tryptophan and two moles of D-phenylalanine. The formulation indicates a cyclic peptide, a conclusion supported by the absence of a free carboxyl group or α-amino group. The only functional groups present are the δ-amino group of the ornithine and the hydroxyl group of the tyrosine. These groups were shown to be present by the preparation of DNP derivatives. Two moles of ammonia liberated in the amide determination would account for the two extra carboxyl groups of aspartic and glutamic acids.

This paper will describe experiments which have

(1) T. P. King and L. C. Craig, THIS JOURNAL, **77**, 6624 (1955).