2,3,5-Trimethoxybenzonitrile (III).—This compound was obtained from 3 g. of the corresponding oxime by dehydration with acetic anhydride[4]; b.p. 132° (0.2 mm.), m.p. 66–67°.

Anal. Calcd. for $C_{10}H_{11}NO_3$: C, 62.16; H, 5.74; N, 7.25; CH_3O, 48.19. Found: C, 62.35; H, 5.49; N, 7.35; CH_3O, 48.24.

2,3,5-Trimethoxyacetophenone (IV).—One gram of the above nitrile III was treated with methylmagnesium iodide as described by Baker, *et al.*[4] After recrystallization from petroleum ether the m.p. was 60–62°. Mauthner described a product of m.p. 101–102° which he considered to be IV.[10] However, his product was probably VIII which has a m.p. 101–102°.[9]

Anal. Calcd. for $C_{11}H_{14}O_4$: C, 62.84; H, 6.71; CH_3O, 44.29. Found: C, 62.68; H, 6.78; CH_3O, 44.36.

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Some Dialysis Experiments with Polypeptides

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In the last few years considerable progress has been made in determining the amino acid sequence in polypeptides and a few proteins. The approach has involved partial hydrolysis followed by isolation of a sufficient number of interlocking peptides and determination of their structure so that a unique over-all sequence is revealed. At best it is a laborious procedure requiring much fractionation with ion exchange chromatography, countercurrent distribution, zone electrophoresis and paper chromatography or preferably by all of these techniques since they have different selectivities.

Ion exchange chromatography and zone electrophoresis depend primarily on the differences in charge for their separation while chromatography depends on a combination of properties involving polarity and relative adsorbability. Countercurrent distribution depends on a combination of properties also largely involving polarity or something related to solubility as expressed by the relative preference at equilibrium of a solute for two phases in contact.

It would be of great help in the fractionation of these complex mixtures if even a rough separation could be made solely on the basis of molecular size or weight. The advantages of this with partial hydrolysis are obvious not only from the standpoint of separation but also from the standpoint of attaining higher yields of peptides of a desired size. Milder conditions or shorter hydrolysis times could be used initially and the smaller peptides separated from those of large size. The latter could then be repeated several times with removal of the small peptides after each treatment. With these considerations in mind a number of experiments have been made in the use of partial dialysis.

The rate of escape of solutes through a sintered glass diaphragm has been used for some time[1] for estimating the relative molecular size of molecules. There are many accounts in the literature of the correlation of molecular size with the rate of dialysis through a semi-permeable membrane.[2–4]

Interesting attempts also have been made by Signer, *et al.*,[5] to use the differences in the relative rates of passage through a semi-permeable membrane for the separation of complex mixtures of smaller molecules. In the latter work a train of dialysis cells has been set up so that solution and dialysate flow countercurrently to each other.

A few preliminary experiments have been made in this Laboratory with a series of dialysis units which can give a result strictly on a discontinuous basis in a manner entirely analogous to the approach of countercurrent distribution.[6] The dialysis is performed in such manner that it can be interrupted at each stage when a desired percentage of a given solute has escaped from the solution into the dialysate.

Experimental

The type of dialysis unit chosen for the study is shown in Fig. 1 where A is a cell made from 4.8 cm. glass tubing, 11 cm. in length, supported by a buret clamp. The support B for the dialysis sac is made by cutting a 16 mm. test-tube 60 mm. from the top and fire polishing the lower end. A length of Visking cellophane dialysis tubing #20/32 is then cut and one end closed by tying a knot in the wet casing. The open end is pulled over the glass support B until it reaches the flared upper end of the glass tube. The clamp C closes around the cellophane and the glass tube B and holds both in place. The cellophane sac is cut of such length that 10 cm. of the sac extends below the glass tube B.

D is a glass tube 9 mm. in diameter and approximately 27 cm. in length. It is partially closed on the bottom and serves as a bubble tube for stirring the solution in the dialysis sac. It is supported by the clamp E. A rubber tube connected to its upper end supplies the nitrogen. D is of the size given so that when nitrogen has displaced all the solution from its lower end and with 12 ml. of solution the level of the solution outside D but inside the sac will rise to within 1 cm. of the glass support B. Since the dialysis tubing approximates 5 cm. in circumference approximately 45 sq. cm. of dialyzing surface is afforded.

One hundred-twenty ml. of solvent is placed in the cell A outside the sac. This volume will fill it to a point level with the solution inside the sac. It was found that good stirring resulted from quite slow bubbling of nitrogen through a tube F which extends to the bottom of the cell A. Even with surface active solutes which tended to foam the rate of bubbling could be reduced and effective results still obtained. In this case a drop of octanol was of great help. It was found experimentally that reduction of the rate of bubbling to practically nothing from the tube D inside the sac did not strongly affect the rate of escape of solute through the membrane.

The dialysate could be removed easily by lowering A from the apparatus. The solution could be removed conveniently through the top of D by a polyethylene tube attached to a 20-ml. hypodermic syringe. This disturbed as little as possible the fragile sac so that it could be used repeatedly. A sac was always tested for leakage immediately before use.

Rate studies were made at room temperature with many different solutes. The experiment with phenylalanine will be taken as an example. A sample of 120 mg. of the amino

(1) J. H. Northrop and M. L. Anson, *J. Gen. Physiol.*, **12**, 543 (1929).

(2) A. Bethe, H. Bethe and Y. Terada, *Z. physik. Chem.*, **112**, 250 (1924).

(3) H. Brintzinger and W. Brintzinger, *Z. anorg. u. allgem. Chem.*, **196**, 33 (1931).

(4) M. Kamateka and K. Hatakayama, *Tôhoku J. Agr. Research*, **3**, 101 (1952).

(5) R. Signer, H. Hänni, W. Koestler, W. Rottenberg and P. Von Tavel, *Helv. Chim. Acta*, **29**, 1984 (1946).

(6) L. C. Craig and O. Post, *Anal. Chem.*, **21**, 500 (1949).

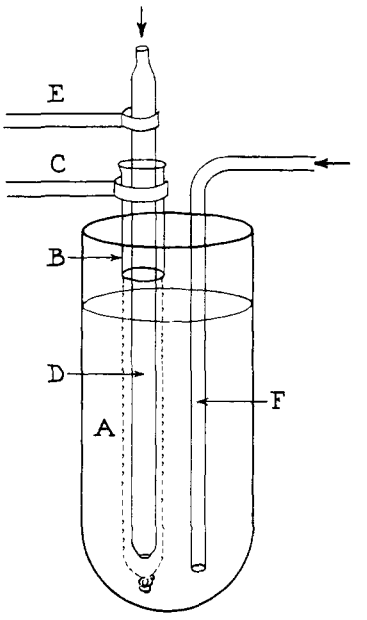

Fig. 1.—Schematic drawing of dialysis unit.

acid in 12 ml. of 0.1 *N* acetic acid was placed in the dialysis sac. The cell A contained 120 ml. of 0.1 *N* acetic acid. Slow bubbling of nitrogen was started. At recorded intervals 1 ml. of the solution in A was withdrawn for weight analysis.[7]

In Fig. 2 is shown a plot of % of solute escaped from the sac against time. These curves were found to be very reproducible and with pure solutes were always found to have the same general shape. Curve 1 is that obtained with sodium chloride, curve 2 with phenylalanine and curve 3 with sucrose.

Obviously at complete equilibrium 90.9% of the solute will have escaped from the sac since the outside volume is ten times that of the inside. However, for example, with a mixture of equal parts of sodium chloride and sucrose it is possible, assuming no solute interaction, to interrupt the dialysis at an optimum time for the separation of the two solutes. From the curve in Fig. 2 this would be at 20 minutes when 74% of the sodium chloride would be in the dialysate and 26% would remain in the sac. At that time only 20% of the sucrose would be in the dialysate but 80% would remain in the sac.

In order to make these data analogous with the basic data required in countercurrent distribution[6] one can assume that for this particular time the fraction in the dialysate divided by that in the sac is a constant. Thus K_d for NaCl is 2.84 while K_d for sucrose is 0.25.

If a series of such dialysis units numbered 0, 1, 2, 3...*n* is set up, the above separation could be the first transfer in the run. The second transfer is accomplished by recovery of the solute from the dialysate of unit 0 and placing it inside the sac of unit 1 together with 12 ml. of the solvent. The solutions inside the sacs of both 0 and 1 are again dialyzed against 120 ml. of the solvent for the optimum time of 20 minutes. Both dialysates are separately evaporated and transferred to the sacs of units 1 and 2, respectively. The third transfer is accomplished by separately dialyzing all three against 120 ml. of the solvent for 20 minutes. Obviously this process can be expanded indefinitely.

Discussion

It is plain that a stepwise procedure as outlined in the Experimental section will be laborious if many steps are involved. The first point to be discussed by way of reducing the number of stages is therefore the selectivity of each stage or the question of how much difference can be expected in the rate of dialysis with solutes of different size and shape.

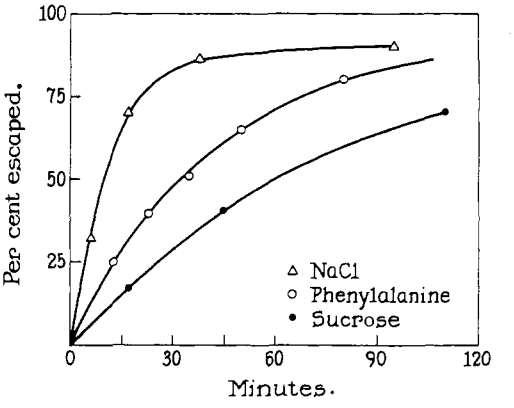

Fig. 2.—Escape rate curves.

After studying the escape rates for a number of solutes it seemed best to compare them on the basis of the time required for 50% of the solute to have escaped from the dialysis sac under conditions described in the Experimental part. Table I gives part of the data thus far obtained together with the molecular weights of the substances.

Since many derivatives of the polypeptides are not soluble in water the effect of the use of aqueous mixtures of organic solvents was also investigated. This was found to greatly retard the rate of escape of any given solute. Ethanol and acetic acid were found to have a similar effect, but since glacial acetic acid is a better general solvent for peptides and their derivatives it was studied more thoroughly than was ethanol. The cellophane sac was not altered by the acetic acid even when glacial acetic acid was used. Table I includes comparative data for a mixture of 1:1 glacial acetic acid–water.

Although the data in Table I show that the escape rates decrease generally as the molecular weight increases individual selectivities based on structural differences are also apparent. Thus aspartic acid has a slower rate than ornithine. This may be due to the fact that some acetic acid molecules are adsorbed on the cellophane making the pores repel the acidic molecules but attracting the basic ornithine molecules. That this may be true is supported by the fact that the difference is even greater in 1:1 aqueous acetic acid.

A striking difference is also found in polymyxin B or polypeptin as compared to bacitracin A. The same explanation can be given here since the first two peptides[8,9] are cyclic with 5 and 3 amino groups, respectively, but no carboxyl groups while bacitracin is a nearly neutral peptide with two carboxyl groups.[10]

This explanation, however, cannot account for the slow rate of escape of tyrocidine A in 1:1 glacial acetic acid. It has no carboxyl groups and only one amino group, yet bacitracin A with a somewhat larger size shows an escape rate more than three times as great. Other causes such as

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

(8) W. Hausmann and L. C. Craig, THIS JOURNAL, **76**, 4892 (1954).

(9) W. Hausmann and L. C. Craig, *J. Biol. Chem.*, **198**, 405 (1952).

(10) J. R. Weisiger, W. Hausman and L. C. Craig, THIS JOURNAL, **77**, 3123 (1955).

TABLE I

COMPARATIVE DIALYSIS RATES OF A NUMBER OF SOLUTES

Substance	Mol. wt.	50% escape time, min. In 0.1 *N* aq. HAc	In 1:1 H_2O–HAc
Sodium chloride	58	10	37
Urea	60	10	
Phenylalanine	165	33	85
Aspartic acid	133	40	2.5 hr.
Ornithine monohydrochloride	132	26	58
Glucose	180	35	60
Sucrose	342	60	3.2 hr.
Raffinose	504	190	4 hr.
DNP-glycine[a]	241		1.6 hr.
DNP-Gly-Leu[a]	354		4 hr.
DNP-Orn.ILeu.Phe[a]	558		5.5 hr.
Polymyxin B hydrochloride	1220	60	
Polypeptin hydrochloride	1150	66	
Tyrocidine A hydrochloride	1270		29 hr.
Bacitracin A	1500	2.7 hr.	8.7 hr.
Subtilin	3300	8.4	
Gramicidin A[a]	3800		76 hr.
Insulin	6000	No detectable	
Ribonuclease	13900	escape	

[a] Experiment made with a few mg. of solute and concentration in dialysate determined spectrophotometrically.

that of the viscosity of the solution may play an important role.

Other smaller selective effects could be pointed out in Table I and it is obvious that changes in molecular weights in general produce an effect considerably greater than would be expected if free diffusion were the sole rate controlling factor. These observations indicate that fractional dialysis could be made a very selective separation tool indeed if it would lend itself to the application of many transfers as does simple extraction in the form of countercurrent distribution.

However, it is not at present obvious how this can be accomplished and for the time being attention should be given to useful separations that can be accomplished with only a few transfers and with the proper systematization.

Two systematizations have long been used in multiple extractions and have more recently been called "Alternate Withdrawal"[6] and "The Diamond Pattern."[11] Their use in discontinuous partial dialysis where labor is a large factor would appear to be obvious.

The rate at which a single stage can be accomplished is made slow by the time required for dialysis and by the time required for evaporation of the separate dialysates. The dialysis time can be reduced to one-half or one-third by increasing the dialysis area. This has been accomplished by increasing the depth of the dialysis cell A in Fig. 1 and making it correspondingly more narrow so that it holds the same volume of solution. Similarly the dialysis sac was made longer but the solution caused to rise correspondingly higher by use of a larger tube D inside the sac. Escape rates for the larger solutes determined in the longer dialysis sacs were found to be higher in direct proportion to the length of the sac.

Evaporation of the multiple dialysates can be done with the least labor by multiple lyophilization. Where only a few dialysates are involved the rotary evaporator[12] used with an oil-pump and Dry Ice cooling around the condenser is much more rapid. A lengthened condenser will permit complete evaporation of 120 ml. of an aqueous solution in 20–30 minutes even when the heating bath in which the flask containing the solution rotates is maintained at a temperature not above 35°.

It is to be expected that solute concentration will have an effect on the escape rate particularly with those solutes which tend to associate strongly. Several experiments with simple solutes did not reveal a striking effect when the initial concentration inside the sac was not greater than a few per cent. The apparatus described in Fig. 1 can be used equally well for smaller concentrations. In this case the analysis can be made by withdrawing aliquots for weight analysis at given times from inside the sac rather than from outside.

A practical example of the use of dialysis experiments in partial hydrolysis experiments was found in studies with tyrocidine B. Rather extensive partial hydrolysis studies with tyrocidine A[13] had led to the establishment of the complete amino acid sequence. It was thought that little difficulty would be found with B since it differs from A only by a single tryptophan residue. However, this did not prove to be the case. The hydrolysis conditions which were satisfactory for A were not at all satisfactory for B.[14]

A sample of tyrocidine B was hydrolyzed with 100 times its weight of concentrated hydrochloric acid at 80° for 1 hour in an evacuated sealed tube. The acid was removed by evaporation at 30° in the rotary evaporator and a dialysis rate study made on the residue in 0.01 *N* hydrochloric acid. This gave curve 2 of Fig. 3. Under these conditions intact tyrocidine B was found to have escaped from the dialysis sac to an extent not more than 2% after 2.5 hours as shown by curve 1. Obviously considerable change in structure has taken place but comparison of the 50% escape time with the data in Table I would indicate the average size to be still too large. A 3-hour hydrolysate on the other hand gave curve 3. Curve 4 was obtained from a 6-hour hydrolysate. Curve 5 was obtained with aspartic acid and is given for comparison. The 50% escape time for the 3-hour hydrolysate was 60 minutes which corresponded to that obtained with sucrose (Table I). This would indicate that the average molecular size approximated that of a tripeptide. This evaluation has been very well substantiated by a survey of the isolation work reported in an accompanying paper.[14]

A sample of tyrocidine B was hydrolyzed for 4 hours and the residue recovered. It was subjected to a 2-stage "Diamond" pattern similar to the procedure given below for a 3-stage preparative

(11) M. T. Bush and P. M. Densen, *Anal. Chem.*, **20**, 121 (1948).

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

(13) A. Paladini and L. C. Craig, THIS JOURNAL, **76**, 688 (1954).

(14) T. P. King and L. C. Craig, *ibid.*, **77**, 6624 (1955).

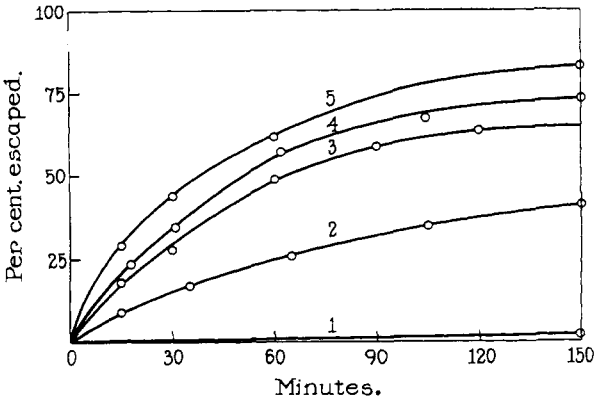

Fig. 3.—Escape rate curves used in the study of the hydrolytic behavior of tyrocidine B.

run. Each of the four fractions was then studied by paper chromatography. The most rapidly dialyzing fractions showed strong spots corresponding to all the amino acids of tyrocidine B. Tryptophan was weaker than the others partly due to decomposition. On the other hand, the slowest dialyzing fraction did not show any spots corresponding to amino acids although there were a number of spots of various intensity obviously due to peptides. However, the intensity of the spots was no indication of the amount of peptidic material because many of these peptides give poor olor responses.

A confirmation of the effect was obtained as follows: A 3-hour hydrolysis was repeated on a preparative sample (1.8 g.) and the peptides recovered. A countercurrent dialysis procedure was then applied according to the "Diamond" pattern as shown in Fig. 4. This involved the use of three dialysis units. The first stage was done in 2 portions to avoid too high a concentration level. A dialysis time of 2 hours at each stage was used. The fractions moving toward the right are the dialysate. Those moving toward the left are the solutes in the dialysis sac. The dialysate was set aside but the 600 mg. of material recovered from the sac was given a further 3-hour hydrolysis treatment. The procedure given in Fig. 4 was then repeated. This gave an additional 420 mg. of dialysate which was combined with the first dialysate. The material recovered from the sac now weighed 180 mg.

The value of the dialysis experiments became apparent when comparative attempts were made to isolate individual peptides from the above dialysate, the slow dialyzing material and a 2-hour hydrolysate in which no preliminary dialysis separation had been made. Figure 5 shows the preliminary countercurrent distribution patterns on the three preparations. These patterns represent only the preliminary separation into groups but already show the dialysis mixture to be much simpler than that receiving no dialysis treatment. The further separation work reported in the accompanying paper[14] contrasted the two even much more sharply. It was considerably easier to isolate individual peptides from cuts taken from the material which had undergone preliminary separation by partial dialysis.

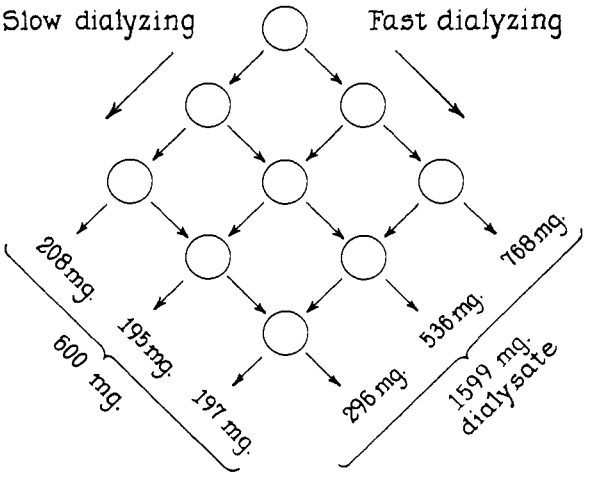

Fig. 4.—Countercurrent dialysis scheme used in separating peptides from partial hydrolysis of tyrocidine B.

For example, material isolated from the region of tubes 260–300 in pattern A, Fig. 5, showed nine distinct bands by paper electrophoresis and paper chromatography. One of these was found to be a pentapeptide Glu.Tyr.Val.Orn.Leu. On the other hand, material from tubes 260–300 of pattern C gave only three components, phenylalanine, Orn. Leu.Phe. and Phe.Asp.Glu.

The largest peptide which could be isolated from any of the cuts of pattern C was a tetrapeptide. This contrasts with our experience with partial hydrolysates of bacitracin, tyrocidine A and others in which dialysis had not been used and from which hexa-, hepta- and octapeptides as well as amino acids had been isolated.

The material which remained in the dialysis sac proved to be very different from that which had escaped. This is emphasized by the distribution pattern of this material shown in pattern B of Fig. 5. Closer examination of this material revealed it to be composed of humin, larger peptides containing most of the amino acids of tyrocidine B and tetra- or pentapeptides containing phenylalanine, tryptophan, leucine and proline. The smaller peptides and amino acids which occur in the central and left hand part of pattern C of the dialysate were practically absent from pattern B. It would seem certain that with the dialysis steps a 3-hour hydrolysis time is too long. A 2-hour time would have given less of the free amino acids and higher yields of the desired di- and tripeptides.

When the over-all result of the experiments thus far made are considered it is apparent that the additional time required for the partial dialysis studies is well worth while. This viewpoint is supported by the experience of Dr. Hausmann of this Laboratory in structural studies with the polymyxin group of peptides and soon to be reported.

The implications of the above general approach in the study of enzymatic splitting of proteins and peptides is obvious. Work along this line is planned.

Summary.—Apparatus and procedures have been described for fractionating partial hydrolysates of

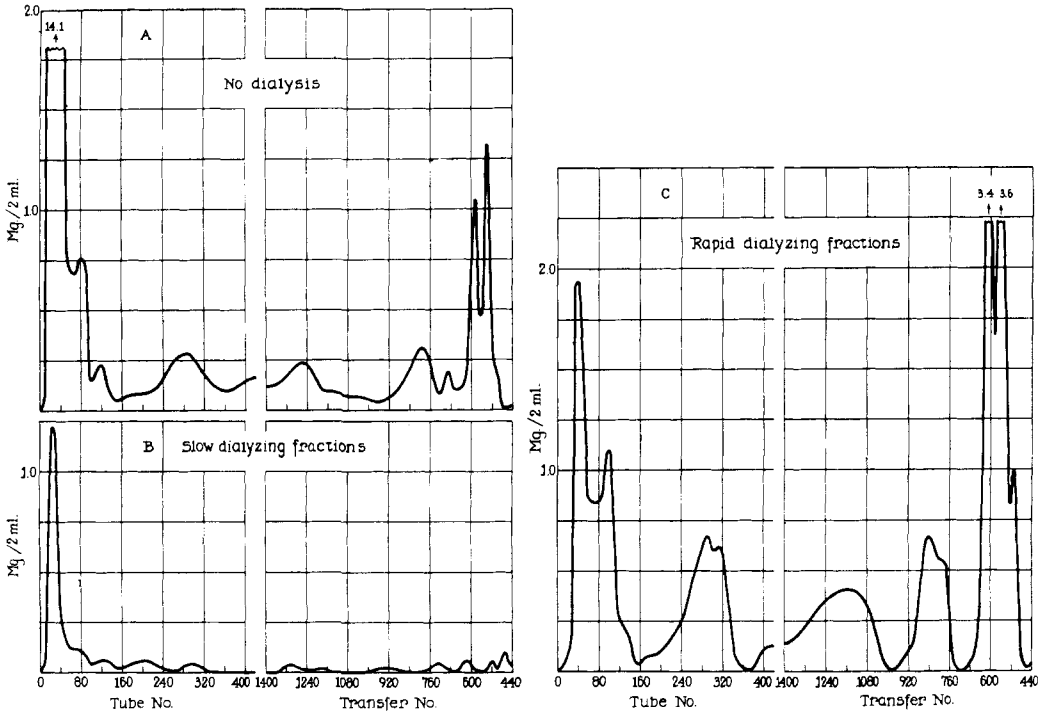

Fig. 5.—Comparative countercurrent dialysis patterns of hydrolysates of tyrocidine treated in different ways.

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).