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Adsorption Experiments with Gramicidin and Related Substances

The Heterogeneity of Tyrocidine

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The experiments described here were begun with a view to finding out whether adsorption chromatographic methods could be of use as an additional criterion of homogeneity for the antibacterial peptides gramicidin, tyrocidine and 'gramicidin S'. It was also hoped that they might throw light on the nature of tyrothricin, the crude material from which gramicidin and tyrocidine are isolated, since it is not known what other substances it may contain. Hotchkiss [1] suggests that the non-crystallizing residue of tyrothricin consists of substances not greatly differing from gramicidin and tyrocidine, and the results of the present work tend to support his conclusion.

The main evidence of homogeneity previously available for gramicidin and tyrocidine was the constancy of their physical and chemical properties on repeated crystallization from one or two different solvents (*cf.* Hotchkiss [1], Synge [2]). In the case of gramicidin, physical and chemical characterization of a fraction purified chromatographically had also suggested homogeneity (Gordon, Martin and Synge [3, 4]). For gramicidin S, in addition to constancy of properties on recrystallization, the simple picture revealed by study of the products of complete and partial acid hydrolysis suggested homogeneity (Synge [5]; Consden, Gordon, Martin and Synge [6]). Nevertheless, particularly in view of experience in the ergot alkaloid series (Stoll and Hofmann [7]; Stoll, Hofmann and Becker [8]), it still seemed possible that the products might consist of two or more different peptides crystallizing together in proportions unchanged or not markedly changed by recrystallization. Further tests of homogeneity were therefore desirable, and adsorption analysis, particularly 'front analysis' (Tiselius [9, 10, 11, 12, 13]), seemed promising for the purpose. At the

same time it was hoped that experience of the adsorption behaviour of these fairly well-defined peptides having molecular weights of the order of a few thousand might be a useful orientation for future work with other compounds in this class, since the range of methods available for purifying and characterizing such substances is at present very limited.

MATERIALS

Gramicidin. Specimen R3 (Synge[2]) and a specimen supplied by the Wallerstein Company, New York, during 1945.

Tyrocidine hydrochloride. A specimen, thrice recrystallized, prepared in the same way and from the same material as that studied by Gordon, Martin and Synge[4] and a specimen supplied by the Wallerstein Company during 1945.

Gramicidin S (hydrochloride). Specimen I (Synge[5]) and the specimen studied by Consden *et al.*[6]

Tyrothricin. A 'de-fatted' specimen supplied by the Wallerstein Company during 1945.

No significant differences were observed at any time between the behaviour of the different specimens of the same peptide.

METHODS

Adsorption experiments. Most of the adsorption runs were made with optical control in the interferometric apparatus of Tiselius and Claesson[14] (see Claesson[15]). The general procedure was as given by Claesson[15]. Of the pure solvents available in bulk only the lower alcohols were satisfactory solvents for all the materials studied. Anhydrous ethanol was accordingly used as solvent unless otherwise stated. Before use the solvent was de-aerated by shaking *in vacuo* for a few minutes at room temperature. Heating was usually required for effecting solution; the solutions were cooled before use, and invariably remained clear. The adsorbent was packed in ordinary 'front analysis' filters, which were supplied with liquid from syringes of the type described by Claesson[15]. Where elution or displacement analysis was employed, a measured volume of the solution for analysis was forced into an ordinary 'front analysis' filter (previously washed with fresh solvent) from a micro-burette by means of compressed air. The filter was then without delay connected to a syringe filled with solvent or displacing solution, the whole fitted to the interferometer and operated as described by Claesson. All the experiments were done at 25°.

All retention volumes, and sometimes the volumes on the abscissae of front analysis plots are given 'corrected' — *i. e.* the volume of solvent initially present in the pores of the filter has been *subtracted from*, and the volume of solvent (1.2 ml) that had to flow at the beginning of a run to fill the outlet tube has been *added to* the volume present in the receiver at the moment the optical reading was taken. The volume between the interferometer cuvette and the end of the outlet tube was 1.5 ml. This means that, if

mixing in the outlet tube is ignored, the optical reading at any moment corresponds to the material flowing into the receiver when 1.5 ml more has entered it. Accordingly a cut is shown on the diagrams opposite the optical reading taken when the receiver contained 1.5 ml less than when the cut was made.

The refractive index increments($\Delta\mu_0 10^5$) extrapolated for 1 % *w/v* solutions in ethanol of the substances studied were found to be for gramicidin S, 165; tyrocidine hydrochloride, 180; tyrothricin, 183; and gramicidin, 197.

The charcoal specimens used as adsorbents were from batches of material prepared, used, and fully described by Claesson[15].

Partition chromatographic identification of amino-acids was done by the filter-paper methods of Consden, Gordon and Martin[16]. Filter-paper of the grade 'OB' manufactured by Munktell, Grycksbo, Sweden was employed.

Tryptophan colour reaction. An amount of peptide corresponding to not more than 0.1 mg of tryptophan was dissolved in 2.5 ml acetic acid, and 2.5 ml 10 *N* HCl containing 0.1 % *w/v* *p*-dimethylaminobenzaldehyde was added. The resulting mixture was kept in a dark place at room temperature for the colour to develop (*cf.* Synge[2]).

Tyrosine was determined in acid hydrolysates by the colorimetric method of Arnow[17].

Ethanolamine and NH_3 determinations were done in Conway units on solutions made from HCl-acetic acid hydrolysates of the peptides that had been evaporated to dryness[18].

Optical Rotations were determined in a 1 dm tube on material that had been dried to constant weight in a vacuum desiccator at room temperature.

Evaporation was done *in vacuo* below 40° unless otherwise stated.

RESULTS

Front analyses were done as the first stage of investigating all the materials studied, and it was only when evidence had been obtained in this way of the heterogeneity of tyrocidine that elution and displacement experiments were done with it. Accordingly the work with front analysis of the various substances is described first, and there follow accounts of the fractionation of tyrocidine by other adsorption methods and of the characterisation of the fractions isolated from tyrocidine.

Front analysis work

Choice of adsorbents. Alumina Cγ 'according to Brockmann' (Savory & Moore, London) showed some adsorption of tyrocidine and gramicidin S. The diagram with gramicidin S (0.1 % *w/v*) showed a large step followed by a small one at retention volumes 8 and 14 ml per g adsorbent respectively. On testing

the effluent with $AgNO_3$ it was seen that Cl^- was absent from the first step material, but present in the second. Thus with this adsorbent ion exchange effects seem to exist in addition to 'ordinary' adsorption, which complicate interpretation of the data.

Tyrocidine hydrochloride, analysed in the same way, gave about the same degree of adsorption but no well-defined fronts — not a surprising result if ion-exchange effects are superimposed on the behaviour of the mixture of peptides that tyrocidine appears to be. Gramicidin showed very little adsorption on the alumina. Tyrocidine showed little adsorption on two samples of silica differing in their adsorptive capacity for *N*-2 : 4-dinitrophenyl-amino-acids.

With charcoals no significant interference by ion exchange effects was detected. Tested with 0.23 % *w/v* solutions of tyrothricin, the charcoals *Carboraffin* (unwashed)., *Eponit 3n*, *Norit* SA 30 and *Carbo activ* III (*cf.* Claesson [15]) gave front analysis diagrams all having the same general form, but differing in the retention volumes for the different components. Carbo activ III, although not the most strongly adsorbing of the charcoals tested, showed the greatest relative differences for the different components, and was therefore used in all the work described below.

Gramicidin. Front analysis on Carbo activ III showed a single step. The retention volume for a 0.1 % *w/v* solution was 35 ml and for a 0.2 % *w/v* solution 20 ml per g adsorbent. In the latter experiment the properties of the material coming through the filter at different stages were compared. Fractions corresponding roughly to the first, second and third 20 mg portions of material to emerge from a filter packed with 0.45 g of charcoal were collected separately. No significant differences between the fractions, or divergences from the data for unfractionated gramicidin were noted. $[\alpha]_D^{18-19°}$ lay in the range + 7.8° to + 8.6° (94 % *w/v* aqueous ethanol, c = 1.5) (*cf.* Synge [2]). No noticeable difference appeared in the colours given by the different fractions in the tryptophan reaction. The fractions were hydrolysed for 24 h at 110° with 0.4 ml glacial acetic acid and 1.5 ml 6 *N* HCl. Ethanolamine N in the hydrolysates lay in the range 3.8—4.7 % of total N (*cf.* Synge [18]). No obvious differences in the amino-acid composition were observed when paper-strip chromatograms (*n*-butanol-H_2O) were run on the hydrolysates of the different fractions.

Gramicidin S (hydrochloride). Front analysis on Carbo activ III showed a single step. The retention volume for a 0.1 % *w/v* solution was 7.5 ml per g adsorbent. In view of the physical and chemical data indicating that gramicidin S is a homogeneous substance (see below) it did not appear necessary to subject the effluent from the filter to further analysis.

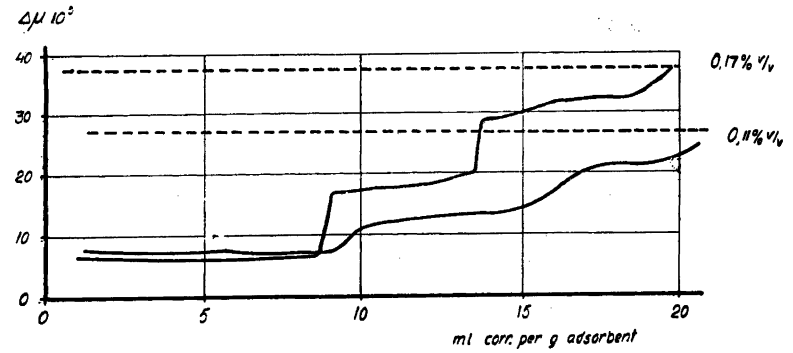

Fig. 1. Front analysis of tyrocidine hydrochloride solutions in ethanol on Carbo Activ III (dotted lines correspond to the solutions used).

Tyrocidine hydrochloride. Front analysis on Carbo activ III immediately showed a number of components to be present. Fig. 1 shows typical front analysis diagrams for 0.11 % and 0.17 % *w/v* solutions. From the increasing fluorescence of the effluent solution in ultra-violet light it was immediately suspected that the successive components had differing tryptophan contents. This was confirmed by the Ehrlich colour reaction — 'first-step' was found to give no colour reaction, 'second-step' material gave a rapidly developing pink colour, becoming blue after 3—4 days, whereas an equal weight of the material issuing from the filter after the rather ill-defined third step (like the original tyrocidine) gave an initial slightly less strong pink colour darkening in 24—48 h to a considerably deeper blue-purple. Further analytical data on the fractions obtained by front analysis are given below, together with those on fractions obtained by elution and displacement development.

In order to test whether the fractions might be artefacts formed from originally homogeneous material by some chemical reaction mediated by the charcoal itself, all the material that had issued from the filter during a front analysis was collected, evaporated to dryness and subjected to front analysis in a filter of the same size packed with fresh charcoal. The resulting front analysis diagram had the same form as previously, except that, as expected, the relative heights of the first and second steps were somewhat increased. It is difficult to imagine how this result could have been obtained if the different fractions were due to alteration of an originally homogeneous material by the charcoal itself, unless such alteration occurred rapidly as soon as the tyrocidine came into contact with the charcoal.

The effect on the form of the front analysis diagram of adding increasing proportions of water to the solvent was investigated. Fig. 2 shows a family

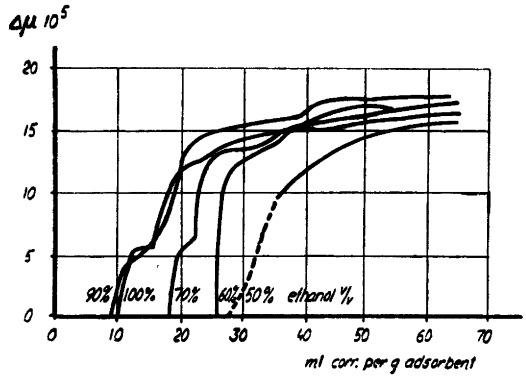

Fig. 2. Front analysis of tyrocidine hydrochloride (0.1 % w/v) on Carbo Activ III in different ethanol-water mixtures.

of plots for solvents of different water content. While the retention volumes increase with increasing water content, the selectivity of the adsorbent for the different components diminishes. It should be noted that tyrocidine hydrochloride crystals are more readily soluble in aqueous than in absolute alcohol, and that their speed of solution appears, for the range of concentrations studied here, to increase with the water content of the ethanol.

Tyrothricin. Fig. 3 shows a typical front analysis of tyrothricin on Carbo activ III. The diagram is consistent with what might be expected for a mixture of (say) 85 % of tyrocidine hydrochloride and 15 % of gramicidin; the step (at about 55 ml) that should correspond with gramicidin is not very sharp or

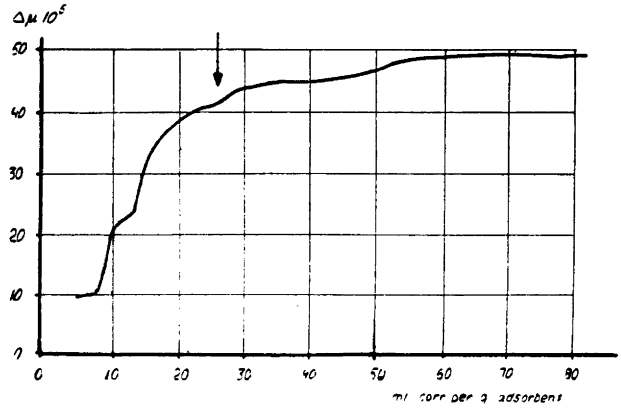

Fig 3. Front analysis of tyrothricin (0.23 % w/v) on Carbo Activ III in ethanol.

large, but this is a usual phenomenon with the later components on front analysis diagrams.

The material issuing from the filter before and after the cutting point (26 ml) indicated by the arrow on the diagram was evaporated to dryness, hydrolysed, and examined for amino-acids by two-dimensional partition chromatography. As expected, the amino-acids peculiar to gramicidin (glycine and alanine) were detected in the second but not in the first fraction. All the known constituent amino-acids of tyrocidine (valine, leucine, phenylalanine, tyrosine, proline, ornithine, glutamic acid, aspartic acid and tryptophan (colour reaction only) were recognised in both fractions, but it is of interest that no indication was obtained of the presence of other amino-acids. This suggests that any peptides other than gramicidin and tyrocidine that are present in tyrothricin must have a somewhat similar amino-acid composition.

Work with elution and displacement development

Experiments were carried out with a view to isolating the more strongly adsorbed components of tyrocidine in a pure state — since front analysis could only be used for isolating the least adsorbed component (I) that gave no colour with Ehrlich's reagent.

Recognition of the components. For this work the colour reaction with Ehrlich's reagent proved particularly valuable, since it soon became evident that, consistent with the data obtained by study of the fractions resulting from front analysis (see above), the component of tyrocidine corresponding to the second step (II) gave rapidly a bright pink colour with the reagent. Thus fractions rich in this component showed, in the early stages of the reaction, a *pink* colour about twice as intense as that obtained with an equal weight of unfractionated tyrocidine. This pink colour changed slowly to blue but only after 3—4 days. On the other hand fractions rich in the still more strongly adsorbed component(s) III gave very little colour during the first few hours of the reaction: with these a *pure blue* colour developed rather slowly, reaching after one day an apparent intensity slightly greater than that of the purple-blue colour given by an equal weight of unfractionated tyrocidine.

In view of the continual intensification of the colour, quantitative comparisons were unreliable, but it can be stated that fractions rich in II which were submitted to further study gave at least as much pink colour in the early stages of the reaction as double the weight of unfractionated tyrocidine, while fractions rich in III gave only as much pink colour in the early stages of the reac-

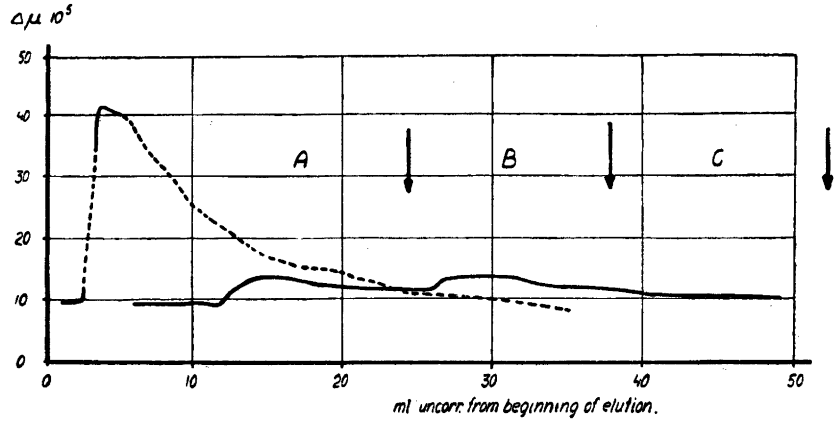

Fig. 4. Elution development of tyrocidine hydrochloride (ethanol: Carbo Activ III).
——— *Filter contains 2.12 g charcoal; pressed in 15 mg tyrocidine hydrochloride in 7 ml ethanol.*
- - - - - - - - *Filter contains 1.91 g charcoal; pressed in 52 mg tyrocidine hydrochloride in 3.3 ml ethanol.*

tion as would be obtained with 1/10 the weight of fractions rich in II. It seems likely that contamination of the components II and III in the corresponding fractions isolated did not exceed 20 %. There was of course no difficulty in preparing fractions embodying component I quite free from components II and III.

Fractionating procedures. For concentrating component II simple elution development with ethanol proved satisfactory, provided the chromatograms were not overloaded. Fig. 4 shows the optical diagram obtained in such an experiment. Cut A (containing 18 % of the total N of the tyrocidine) proved to be component I, nearly free from component II, which became apparent in cut B (16 % of the N); cut C (12 % of the N) appeared to consist of highly concentrated component II with little or no III (pure pink colour after 24 h).

If the chromatogram was more heavily loaded a single peak was obtained in the diagram (dotted line in Fig. 4), and this material was found on analysis to consist of components I and II very poorly resolved. There was no indication that component III was being eluted. It was thus evident that some displacing agent would be required for removing component III from the charcoal (*cf.* Tiselius [22]). Various possible agents were tried, namely picric acid, gramicidin, acetyl-DL-tryptophan and, at the suggestion of Dr. S. Claesson, stearic acid. All of these substances are weight for weight more

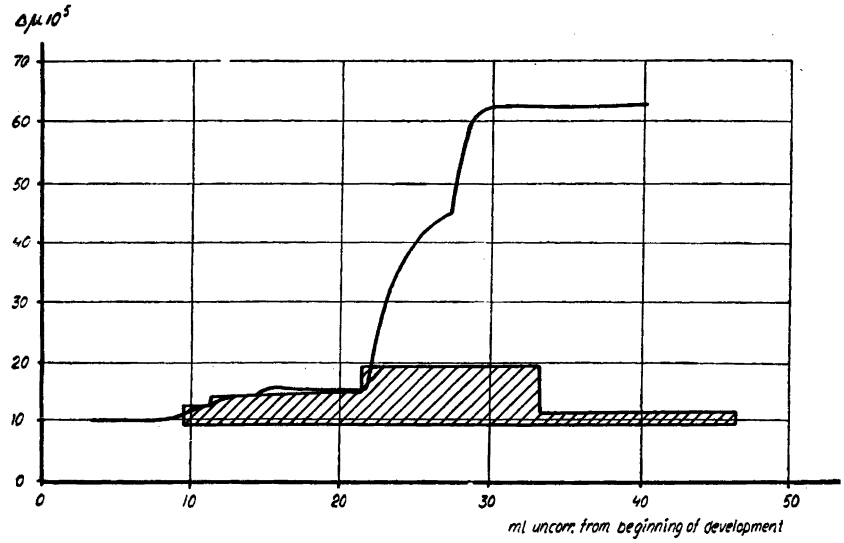

Fig. 5. Displacement development of 14 mg tyrocidine hydrochloride with 0.5 % w/v stearic acid in ethanol (see text). (The hatched area shows the content of tyrocidine-N in the various cuts).

strongly adsorbed on charcoal from ethanol solution than any of the components of tyrocidine.

14 mg tyrocidine HCl (in 7 ml ethanol) was pressed into a 1250 π mm^3 filter packed with 2.0—2.2 g Carbo activ III; development was then effected, under optical control, with pure ethanol or solutions in ethanol of stearic acid (0.5 % *w/v*: retention volume 13 ml/g), picric acid (0.5 % *w/v*: retention volume 14 ml/g), acetyl-DL-tryptophan (0.3 % *w/v*:retention volume 16 ml/g) or gramicidin (0.25 % *w/v*: retention volume 17 ml/g). The optical data showed clearly that only with stearic acid was any considerable displacement occurring. The amount of tyrocidine was determined in successive portions of the effluent during development, up to a total of 45 ml. In the runs with pure ethanol and with stearic acid, this was done by N (Kjeldahl) determination. With gramicidin and acetyltryptophan development, the tyrocidine in the effluent was determined by virtue of its amide-N content, the NH_3 being liberated by hydrolysis of the evaporated fractions in 1 : 1 *w/v* acetic acid: 10 *N* HCl in sealed evacuated tubes for 10 days at 38° (under these conditions gramicidin and acetyltryptophan liberate no NH_3).

The results showed that in the first 45 ml of effluent during development by simple elution, gramicidin displacement or acetyltryptophan displacement, only 30—40 % of the tyrocidine was recovered. On the other hand, 73 %

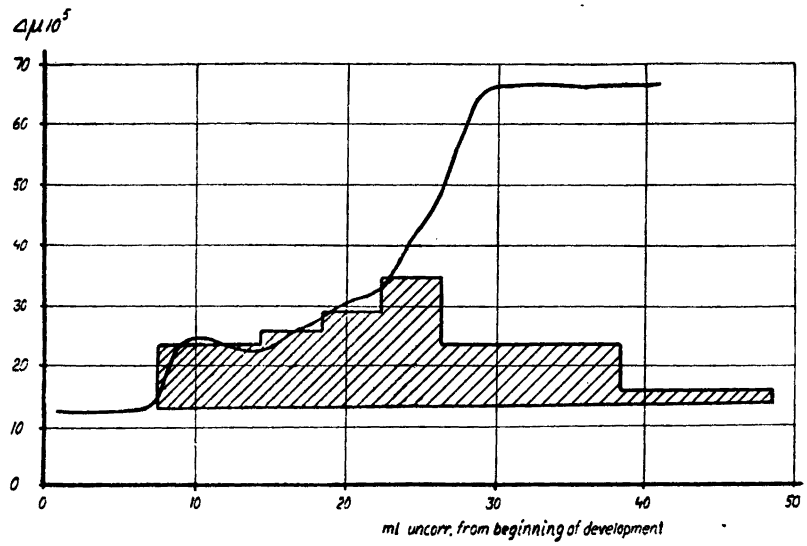

Fig. 6. Same experiment as illustrated in Fig. 5, but with 43 mg tyrocidine hydrochloride (see text).

was recovered in the stearic acid displacement. Fig. 5 shows the optical curve plotted together with the quantities of tyrocidine based on the N determinations. Fig. 6 shows a similar plot for an experiment in which 43 mg of tyrocidine hydrochloride was displaced in the same way. Here 79 % of the tyrocidine issued in the first 45 ml. In each case the initial flat portion of the curve was found to be due to component I, while components II and III were isolated in a state of mutual contamination from fractions in the region of the stearic acid front. Material issuing later, with the stearic acid, appeared to be almost purely component III, but the quantity was small. For preparative purposes it was therefore better to elute as much as possible of components I and II with pure solvent, and then to displace component III from the column with stearic acid solution. It proved easy to separate the mixture of peptide and stearic acid resulting from evaporation of the effluent by extracting this residue with boiling light petroleum (40—60°), dissolving the insoluble material in 50 % *w/v* aqueous ethanol, and filtering off traces of stearic acid which remained undissolved. On evaporating the filtrate, the peptide was obtained free from stearic acid and apparently almost free from component II.

In a typical preparation, 52 mg tyrocidine hydrochloride (1 % *w/v* in ethanol) was pressed into a filter packed with 2.0 g Carbo activ III. Elution was effected with 50 ml ethanol, and the effluent discarded. 60 ml 0.5 % *w/v*

stearic acid in ethanol was then passed, and the effluent was collected. On working it up as above, the yield was 10 mg.

Characterization of the components of tyrocidine

Apart from their colour reaction with Ehrlich's reagent and differing fluorescence, which have been noted above, no striking difference could be detected in the properties of the components.

In the preliminary stages of the work fractions obtained by front analysis were characterized with the results shown in Table 1.

Table 1. Characterization of fractions from front analysis of tyrocidine.

Fraction from front analysis	Components	$[\alpha]_D^{18°}$ (95 % *w/v*) aqueous ethanol, c = 1.1)	as % of total N of fraction	
			Tyrosine — N*	Amide — N*
'First step'	I	— 93°	6.6	13.9
'Second step'	I + II	— 104°	6.7	13.9
'Final' (same as unfractionated tyrocidine)	I + II + III	— 103°	7.7	14.6

Subsequently fractions were available in which components I, II and III had been substantially separated from one another. Like the original tyrocidine, these fractions showed little tendency to crystallise when concentrated in ethanol solution, amorphous gums resulting. Samples rich in each of the components were separately hydrolysed with acid, and the constituent amino-acids were examined by two-dimensional partition chromatography. Tryptophan appeared to be destroyed (the hydrolysates of fractions rich in tryptophan had more humin) but the other 8 amino-acids were all present in each hydrolysate, nor did their relative proportions seem to be greatly different in the different fractions. Diffusion constants were also determined for the different fractions, with the results shown in a following paper (Pedersen and Synge [19]).

DISCUSSION

The experiments described here permit one to hope that adsorption chromatography may be of value in work with other peptides having molecular

* Determined after hydrolysis in 1 : 4 *w/v* acetic acid — 6 *N* HCl in sealed evacuated tubes for 24 h at 110°.

weights of the order of a few thousand both for testing homogeneity and effecting isolations. The method of front analysis gave no indication of heterogeneity in gramicidin S, a peptide probably having a molecular weight of 1100—1200 (Pedersen and Synge [19]) for which there exists some evidence of homogeneity obtained by crystallographic and structural chemical studies (Crowfoot and Schmidt [20]; Consden, Gordon, Martin and Synge [6]). On the other hand with tyrocidine (molecular weight perhaps 2,500) front analysis revealed heterogeneity, proving in this case to be a more sensitive criterion than the commonly accepted one of constancy of properties on recrystallization. None of the other available information on tyrocidine is inconsistent with its being a mixture of components, and the evidence now presented may help to explain the controversy as to its tryptophan content (reviewed by Hotchkiss [1]). It was further possible, by elution development and displacement development to separate the different components of tyrocidine from one another. As concerns gramicidin (mol. wt. perhaps 4,000) neither the front analysis data nor the other information available suggest heterogeneity, although a mixture of peptides similar as to content of tryptophan, but differing in respect of the other amino-acids might well give a single step in front analysis.

If the peptides here studied are arranged in order of increasing adsorption on charcoal (gramicidin S, the 3 components of tyrocidine, gramicidin) it is seen that the extent of adsorption is correlated with the content of aromatic amino-acid residues, and particularly of tryptophan. This is in agreement with adsorption data for free amino-acids (Tiselius [13]; Tiselius, Drake and Hagdahl [21]). Neither the front analysis nor the displacement development results with acetyltryptophan and gramicidin suggest that there are sufficiently strong mutual displacement effects between the different peptides to give useful separations by the procedure employed in the carbohydrate series (Tiselius [22, 23]; Tiselius and Hahn [24]). The rather strong displacing effect of stearic acid may prove useful in other work involving the adsorption of peptides on charcoal.

When the front analysis first revealed the existence of an apparently tryptophan-free component (I) of tyrocidine which was similar in adsorption behaviour to gramicidin S, it was tempting to suppose that this substance might actually be gramicidin S, and that tyrocidine was simply a mixture of gramicidin S with other peptides crystallizing together in stoichiometric proportions. However, the amino-acid composition, optical rotation, diffusion constant and poor crystallizing power of component I all rule out this possibility, and it is evident that the relationship between gramicidin S and tyrocidine is a more subtle one. The amino-acid composition and the diffusion constants

of the different tyrocidine components reveal no striking differences between them, except as concerns the content and reactivity of the supposed tryptophan residues. It is perhaps most satisfactory to regard the different components as being the same peptide structure containing one or more loci at which may exist a tryptophan residue or else its precursors or successors in some biosynthetic process. However, before such a view could be substantiated or any clear answer given to the problem of the tryptophan of tyrocidine, it would be necessary to study the different components of tyrocidine by methods less equivocal than those based on colorimetry, fluorimetry or microbiological assay.

SUMMARY

1. The adsorption behaviour of gramicidin, tyrocidine, tyrothricin and gramicidin S has been studied by front analysis, mostly on charcoal chromatograms using ethanol as solvent.

2. Tyrocidine, which proved heterogeneous, has been further fractionated by elution and displacement development.

3. The resulting fractions have been characterized; the different components of tyrocidine appear to differ chiefly in their content of tryptophan.

4. The data are considered in relation to the information they yield both as to the peptides investigated and as to the possibilities of applying adsorption methods to other peptides having molecular weights of the order of a few thousand.

One of us (R. L. M. S.) took part in this work while on study leave from the Lister Institute of Preventive Medicine, London, England.

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