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Purification of Bacitracin and Some Properties of the Purified Bacitracin

JERKER PORATH

Institutes of Biochemistry and Physical Chemistry, University of Uppsala, Uppsala, Sweden

Bacitracin is a polypeptide which in its chemical and physical properties is especially suitable for studies of possible separation methods applicable to peptides. The aim of this investigation has been to purify the crude bacitracin as far as possible, to determine its approximate molecular weight and, finally, to estimate its amino acid composition.

The purification was performed by means of carrier displacement analysis [1]; the molecular weight determination by means of ultracentrifugation (according to the calculation method suggested by Archibald [2]) and the estimation of the amino acid composition mainly by means of the usual paper chromatographic methods.

Williams, Hagdahl and Tiselius have recently shown that certain difficulties are involved in the use of carrier displacement analysis on carbon (in press). These difficulties also appear in connection with the separation of polypeptides. It is to be expected, however, that better separation results could be obtained, if there were a large excess of one component the intention being to purify this main component. By means of the method described below a far-reaching purification is possible. A specimen of bacitracin partly purified by counter current distribution according to Craig [3] proved to be an excellent starting material.

THE CARRIER DISPLACEMENT METHOD

For the displacement analysis of compounds *e.g.* A_1, A_2 and A_3 (A_3 "the displacer") with increasing adsorption affinity a diagram such as that shown in Fig. 1 is obtained.

If small amounts of substances with intermediary adsorption affinity are introduced, these ought to appear at the boundaries v_1, v_2, and v_3. Suppose

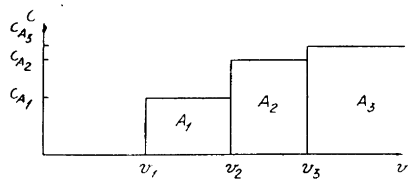

Fig. 1. Displacement analysis diagram for three components, concentration in effluent against effluent volume.

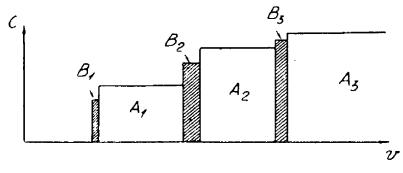

Fig. 2. Ideal carrier displacement analysis diagram. The components of a mixture (B_1, B_2 and B_3), separated by three carriers (A_1, A_2 and A_3).

B_1, B_2 and B_3 which are substances having such adsorption properties are introduced simultaneously with A_1 and A_2 into a column. If the isotherms of the system A_1, A_2, A_3 and the system B_1, B_2, B_3 are of the same shape, a diagram shown in Fig. 2 is obtained. The advantage of this method, the carrier displacement method, lies in the fact that the components of the mixture can be separated in the column by large volumes of interposed substances of a different nature [1]. These latter substances should be chosen in such a way as to make it possible to remove them easily from the required compounds.

If the isotherms of the carriers and those of the components in the mixture cross each other, some complications arise. If, alcohols for example are used as carriers for peptides and the column is loaded with large amounts of peptides, high elution peaks will be seen in the diagram (Fig. 3). There is more or less tailing in the regions behind the zone boundaries as indicated in the diagram. In this case it is even more important to separate the zone boundaries by large volumes of the carriers.

If the carriers are pressed into the column as in an ordinary displacement analysis (Fig. 1) the volumes of v_1, (v_2-v_1) and (v_3-v_2) will be determined by the retention volume v_3 of the displacer. The retention volume v_3 can be increased either by lowering the concentration C_1 of the displacer or by increasing the filter volume.

A much simpler way to vary at will the distances between the zone boundaries is to introduce the carrier solutions at equilibrium concentration. This will give a diagram shown in Fig. 4. This diagram differs from Fig. 1 in one respect.

The volumes $(v_2'-v_1')$ and$(v_3'-v_2')$ can be chosen arbitrarily at the fixed concentrations C_{A_1}, C_{A_2} and C_{A_3}.

This method offers several advantages. A small filter can be utilized for comparatively large amounts of substance and the distances between the zone boundaries can be conveniently chosen at will. A great number of carriers

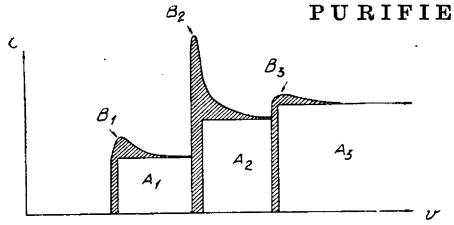

Fig. 3. Carrier displacement analysis diagram obtained for systems with crossing isotherms.

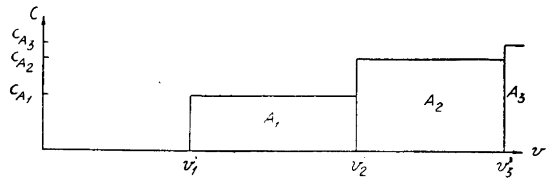

Fig. 4. Displacement analysis diagram with elongated steps.

can thus be used in a single experiment. It is also possible to get long steps over a greater range of concentrations of the carriers, a fact which seems to be of certain importance.

EXPERIMENTAL

Starting material: The preparation used had been purified by means of counter current distribution at the Research laboratories of Merck and Co. Inc., Rahway, N. J., U.S.A. ("Bacitracin 50 R 4996"). Its original activity was about 50 units/mg, but at the time of this investigation the activity had declined to 36—37 units/mg. The lyophilized product was faintly yellowish but the concentrated water solution was of a very deep red-brown colour. Paper chromatograms developed with acetic acid : butanol : water (1 : 4 : 5) indicated that the substance was a mixture consisting of at least five components. These components could be detected after treatment with ninhydrin by examination in visible and ultraviolet light.

Apparatus: A sectioned column of plastic filters each surrounded by a steel support of the type described by Hagdahl and Porath [5] was used. The progress of the analysis was followed by means of the Tiselius-Claesson interferometer [6].

Procedure: 50 % ethanolic solution in 0.1 *N* HCl, was used throughout as a solvent for the carriers. Quite as good a separation was obtained in neutral solution but complete inactivation occurred. Using this solvent, solutions of 0.5, 1.0 and 2.5 % *n*-heptanol-1 of *n*-octanol-1 and *n*-nonanol-1 respectively were prepared (volume percentages.) An ordinary displacement analysis was performed. From this experiment the equilibrium concentrations corresponding to 2.5 % nonanol were obtained: 0.43 % heptanol and 1.70 % octanol.

The filters (2, 4, 8 and 14 ml) were packed with a suspension of carboraffin supra charcoal and celite (1 : 3 parts by weight) in water. After washing with 0.1 *N* HCl/50 % ethanol solution, 50 mg bacitracin, dissolved in 3 ml of this solvent, was pressed into the column. This solution was followed successively by 20 ml 0.43 % heptanol solution, 25 ml 1.70 % octanol solution and finally by 2.5 % nonanol solution. The eluate was collected in graduated test tubes. Higher alcohols were removed from the fractions by extraction twice with an equal volume of hexane each time. The solvent was evaporated *in vacuo* over NaOH-pellets.

Fig. 5 shows a diagram of a typical experiment. Several experiments have given curves of the same shape. The nitrogen recovery varied from about 50 to 72 %. Most of the substance (about 60 % of the amount obtained in the fractions) was found in fraction 10 at the boundary between the heptanol and the octanol. The large amount of material in this fraction is indicated by the high elution peak shown in the diagram. There is also a free elution peak in fraction 3—5. As will be seen below, these fractions contain lower peptides.

The distribution of the nitrogen in the fractions of one experiment is shown in the table below.

Table 1. Nitrogen distribution.

Fraction	Mg N	Percent of the total amount of N
1— 4	0.144	2.0
5— 8	0.216	3.0
9	0.312	4.4
10	2.918	40.8
11	0.810	11.3
12	0.250	3.5
13—17	0.464	6.5
	5.11	71.5

INVESTIGATION OF THE FRACTIONS

1. Qualitative bacteriological tests. Corynebacterium xerosis* was used in order to determine qualitatively the distribution of the bacteriostatic activity over the fractions [4].

* A quantitative determination of the material in fraction 10 made at the Research Laboratories of Merck and Co. Inc., Rahway, N.J., U.S.A., indicated a decrease in the activity to about 27 units/mg (calculated on the free base). The inactivation may have occurred in the strongly acid solution or during shipment.

It might be considered that the comparatively low activity yield indicates that some still more active compound has not been displaced from the carbon. This is, however, probably not the case. 50 % propanol, a very powerful eluting agent, does not elute any higher peptide [7].

A completely inactivated material cannot be distinguished from the active substance neither by means of adsorption analysis nor by any other method, here used, except biological assay. Therefore it seems reasonable to assume that the inactivation must be due to some intramolecular rearrangement or at any rate a change which does not alter considerably the molecular weight. In order to study this it is necessary to follow more carefully the inactivation at the different steps during purification. Such controls have not been possible at these Institutes.

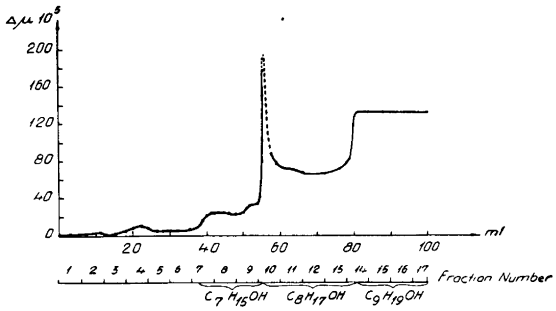

Fig. 5. Carrier displacement analysis diagram for a crude bacitracin preparation. Carriers: n-heptanol-1, n-octanol-1 and n-nonanol-1. Refractive index increment of effluent plotted against effluent volume.

The dried fractions were dissolved in small volumes of water. Each solution was sucked up into a circular filter paper of 10 millimeters' radius. Only about 0.5 mg of fractions 9 and 10 was taken, while about half the material of the other fractions was used. The papers were placed on blood agar plates containing 24 hours' old beef broth for 10 minutes at 37° C. The papers were removed and the blood agar on each plate were inoculated with one loopful of culture. After incubating 24 hours at 37° C the inhibition zones were measured.

The results are compiled in Table 2.

Table 2. Activity distribution.

Fraction no.	Activity *	Fraction no.	Activity *
1	0	8	0
2	0	9	++
3	0	10	+++
4	0	11	+++
5	0	12	++
6	0	13	+
7	0	14	+
		15	(+)

2. Paper chromatography. Small amounts of the material of the evaporated fractions were transferred to paper (Whatman no. 4). One-dimensional chromatograms were developed in the usual manner with acetic acid : *n*-butanol : water (1: 4 : 5). After treatment with 0.1 % ninhydrin dissolved in *n*-butanol the components were localized by examination in visible and ultraviolet light.

Fig. 6 is a map of the chromatograms showing the distribution of the peptides in the fractions obtained by the carrier displacement experiment.

* +++ indicates very strong act.
++ » » »
+ indicates very low act.
(+) » » » »
0 » no »

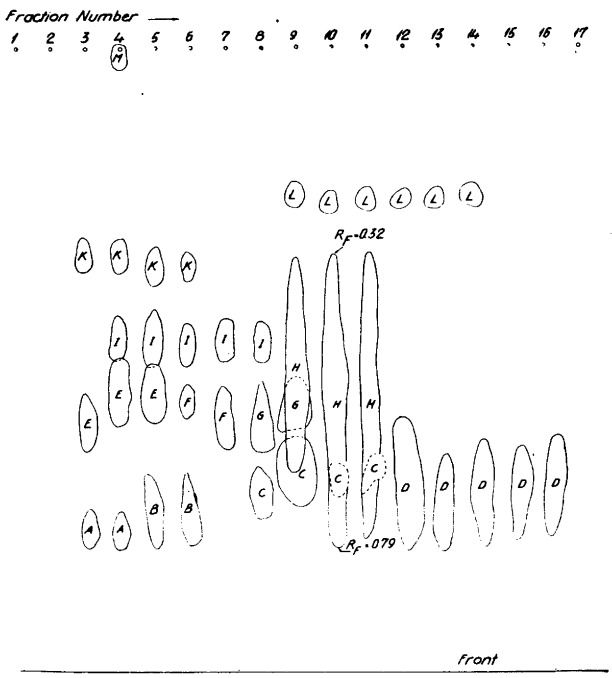

Fig. 6. Map of an one-dimensional paper chromatogram of the fractions obtained from the carrier displacement analysis of crude bacitracin (see Fig. 5).

The colours of the spots are given in Table 3. Fig. 7 shows a photography of chromatogram of the combined fractions 1—9 and fraction 10.

The most active fractions (10 and 11) contain at least three components corresponding to the spots H, C and L in the diagram. From the first nine fractions only small amounts of substance were recovered. Therefore, as the colours of the spots E and I were very intense, it seems reasonable to assume that the material of these fractions consists of lower peptides and, possibly, of amino acids. Since these compounds are inactive no further examination was made.

To establish that the lower peptides did not originate from the active peptide (as a results of a breakdown of the peptide in the acid medium influenced by the active carbon) a column was loaded with fraction 10 and another run was performed in exactly the same manner as before. Investigation of the new fractions shows that cleavage of the peptide chains does not occur. There was no detectable material in the new fractions 1—8 and only a very small amount in fraction 9. The chief material came in fraction 10 as was expected and a slight amount was also found in fractions 11 and 12. The paper chromatograms of fractions 10—12 show exactly the same spots as in the original experiment, but C and L were extremely weakly coloured. From elution

Fig. 7. Photography of chromatograms of the combined fractions 1—9 and fraction 10.

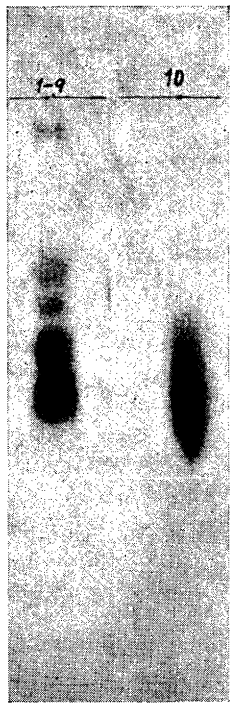

Table 3.

Spot	Colour in visible light	ultraviolet light
A	pink	dark blue
B	violet	yellow
C	— — —	light green
D	green	dark green
E	dark violet	blue green
F	pink	— — —
G	faint violet	light blue
H	greenish violet	dark green
I	faint violet	— — —
K	pink	blue *
L	pink	yellow
M	pink	blue *

experiments it has become clear that the original preparation only contains traces of the component L.

From the very faint colour of spot L in fraction 10 it was clear that it must contain an extremely small amount of material. In order to estimate the amount of material which causes the strong ultraviolet fluorescence of spot C, a series of chromatograms containing 50 mg total of crude bacitracin was developed. The system used was acetic acid: diethyl carbinol: water (1 : 4 : 5). All the material except the fluorescent matter remained in the original spot. A yellow spot (coloured before the ninhydrin treatment) at R_F 0.22 exhibited a very strong ultraviolet fluorescence, thus proving the identity with the material of spot C in fraction 10. Since the ninhydrin test was negative the material in spot C was probably not a peptide. This substance was eluted from the paper and the eluate was lyophilized. A very small amount (about 0.5 mg) of a bright yellow powder was obtained. The bacteriological test indicated that this pigment was completely inactive.

* Sometimes these spots appeared yellow.

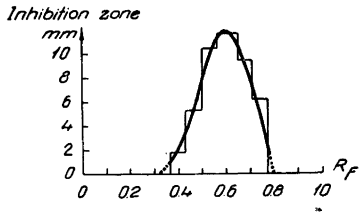

Fig. 8. Activity curve showing the distribution of activity for the fraction 10 from the carrier displacement analysis in a one-dimensional paper chromatogram (see Fig. 6).

In order to establish which component (or components) is responsible for the activity a test of fraction 10 was made as described below.

About 1 mg of dried substance from fraction 10 was transferred to a 2.5 cm broad paper strip (Whatman no. 4). A chromatogram was developed by use of acetic acid : *n*-butanol : water (1 : 4 : 5). The front was marked on the moist paper and after that the paper was washed with ether and dried. The paper was cut into 1 cm broad strips perpendicular to the direction of the development. The paper strips were placed on plates containing blood agar and were inoculated as described above. After 24 hours the inhibition zones were measured, inhibition zone defined as

$$\frac{\text{(width of the culture-free region)} - \text{(width of the paperstrip)}}{2}$$

Fig. 8 shows the activity curve obtained by plotting the width of the inhibition zones *versus* the R_F-values of the paper strips. The position of the activity coincides (as far as can be read from the diagrams) with that of spot H in Fig. 6, as also the maxima of intensity of both activity and colour.

SOME PROPERTIES OF THE PURIFIED BACITRACIN (FRACTION 10)

1. Check of the purity by means of electrophoresis on paper. Electrophoresis on paper was performed according to Kunkel and Tiselius [8], using the apparatus constructed by them.

About 1 mg of the substance dissolved in buffer solution was put on to a paper (Munktell no. 20) previously soaked in the same buffer. The paper was placed between two glass plates coated with silicone grease and the run was made by using 8 volt/cm for 5 hours. Acetate buffer, pH 4.8, μ 0.1, and veronal buffer, pH 8.6, μ 0.1, were used. The position of the peptide was determined by treatment of the paper with ninhydrin solution. On heating the paper from the electrophoresis runs only one spot could be detected in both cases (see Fig. 9). One paper strip from a run at pH 4.8 was cut into pieces and tested on biological activity in the manner described before.

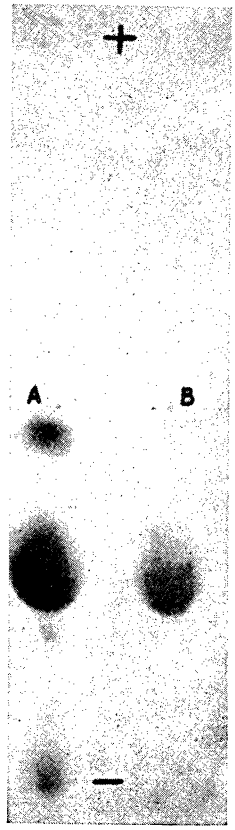

Fig. 9. Paper electrophoresis diagrams of crude bacitracin (A) and purified bacitracin (B). The run was made at pH 4.8 $\mu = 0.1$ using a field strength of 8 volt/cm during 5 hours.

The activity curve showed a maximum at a distance from the origin corresponding to that of the ninhydrin spot. A low activity was however observed throughout the whole region between the maximum and the origin. This indicates that there is some adsorption of the active peptide on the paper. In any event the experiments show that the bacitracin preparation is electrophoretically homogeneous as far as the sensibility of the ninhydrin reaction is concerned.

2. *Determination of the molecular weight.* Archibald has suggested a method of calculation which can also be used for determination of the molecular weight of comparatively small molecules by means of the ultracentrifuge [2]. The principle of this method is briefly described.

Consider that we are dealing with a monodisperse solution. The material flux Φ through the surface at a distance x form the axis of revolution will then be determined by the equation,

$$\Phi = x\,\Theta\,h\left(\omega^2 x\,n\,s - \mathrm{D}\frac{\delta n}{\delta x}\right) \tag{1}$$

where n is the concentration, ω the angular velocity of the rotor, s the sedimentation constant, D the diffusion constant, (x_a, x_b, Θ and h determine, as is shown in Figure 10, the shape and size of the cell).

If equation 1 is written,

$$\Phi = D\omega^2 x^2 n\,\Theta\,h\left(\frac{s}{D} - \frac{1}{\omega^2 x\,n}\frac{\delta n}{\delta x}\right) \tag{2}$$

it is easily seen that Φ will vanish for $\frac{1}{\omega^2 x\,n}\frac{\delta n}{\delta x} = \frac{s}{D}$. This is apparently the case

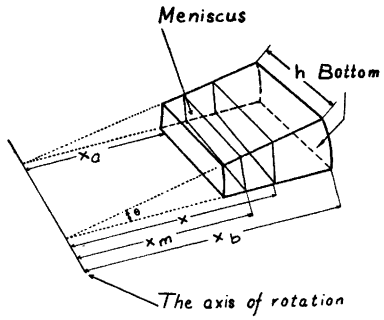

Fig. 10. Schematical drawing of the sedimentation cell of the ultracentrifuge.

when equilibrium is reached. At equilibrium this is valid for all points between x_a and x_b. It is possible, however, to determine $\frac{s}{D}$ at earlier stages of centrifugation. At the meniscus $x = x_m$ and at the bottom $x = x_b$ the material flow must evidently be zero and independent of the time from the start of the centrifugation. As $\frac{1}{\omega^2 x\, n} \cdot \frac{\delta n}{\delta x}$ can be calculated from the variation of refractive index (this is made in the same manner as described by Svedberg and Pedersen [9]) $\frac{s}{D}$ can be found by extrapolating the values of $\frac{1}{\omega^2 x\, n} \frac{\delta n}{\delta x}$ to the meniscus or the bottom. This determination is of importance, as the diffusion constant for low molecular compounds cannot easily be determined. The molecular weight can then be calculated from Svedberg's formula,

$$M = \frac{s}{D} \frac{RT}{1 - V\varrho} \tag{3}$$

Bacitracin hydrochloride (13.3 mg of preparation 10) was dissolved in 0.8889 ml 0.1 *M* potassium dihydrogen phosphate solution. The run was performed by means of an oil-driven ultracentrifuge at the speed of 400 r.p.s. At certain intervals exposures were taken. The calculated values of $\frac{1}{\omega^2 x\, n} \cdot \frac{\delta n}{\delta x}$ plotted against x gave a set of curves, some of which are shown in Fig. 11. From each curve two values of $\frac{s}{D}$ were obtained. The results are compiled in Table 4.

As sufficient amounts of the pure substance were not available, the partial specific volume of the unpurified preparation was used for the calculation of

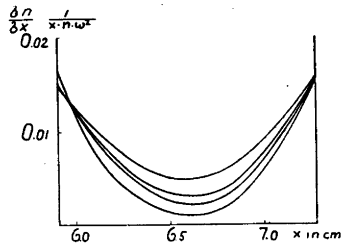

Fig. 11. Four curves showing the variation in the function $\frac{\delta n}{\delta x} \cdot \frac{1}{x\, n\, \omega^2}$ *with the distance from the axis of rotation (x) at four different times. The upper curve is obtained 840 minutes after the start and the lowest curve at 420 minutes after the start (see formula 2).*

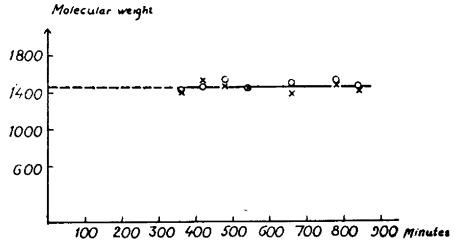

Fig. 12. Molecular weight of bacitracin as calculated from the values obtained for $\frac{s}{D}$ *at the meniscus (×) and at the bottom of the cell (○), for different times after the start of the run.*

the molecular weight. $V_{20} = 0.734$, the value used, is the mean value of two determinations according to Drucker [10] (0.732 and 0.736).

Fig. 12 shows the calculated molecular weights as a function of time. It is clearly seen that the molecular weight of the material at the bottom as well as at the meniscus is independent of time. This indicates that the preparation only contains molecules of the same or nearly the same weight. The average value of the molecular weight of these 14 determinations was found to be 1 460.

Table 4. s/D and M at different times after the start of centrifugation.

Time in minutes	s/D		M	
	Meniscus	Bottom	Meniscus	Bottom
360	0.0150	0.0152	1 410	1 430
420	0.0163	0.0157	1 540	1 480
480	0.0156	0.0164	1 470	1 550
540	0.0154	0.0155	1 450	1 460
660	0.0147	0.0160	1 390	1 510
780	0.0157	0.0162	1 480	1 530
840	0.0149	0.0157	1 400	1 480
			Average: 1 460	

In this actual experimental series the maximum deviation from the average is somewhat more than $\pm$ 6 % *.

3. *Some preliminary analytical data.* Purified bacitracin hydrochloride was hydrolyzed in a sealed tube with 6 *N* hydrochloric acid at 110° for 30 hours.

* A more detailed description will appear elsewhere.

From paper electrophoresis and paper chromatograms the following number of residues in the bacitracin molecule seem to be the most probable: aspartic acid 2, glutamic acid 1, leucine 1, isoleucine 2 or 3, phenylalanine 1, cysteine 1, histidine 1, lysine 1 and ornithine 1. A peptide so composed should have a molecular weight in excellent agreement with the value found by the ultracentrifugation. The amino acid analysis is however only to be considered as an estimation.

The bacitracin hydrochloride was found to contain 9.83 % Cl, 13.53 % N and 1.95 % S. The sulphur content, which was determined by a method recently developed by Paulson [11] corresponds to an equivalent weight of 1496, calculated on the free peptide.

SUMMARY

The investigation described above has shown how it is possible to reveal analytically the complexity of a preparation, previously highly purified by means of counter current distribution, and to further purify the main component, to give a monodisperse, electrophoretically homogeneous product.

By ultracentrifugation the molecular weight of bacitracin has been found to be *about 1460*.

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