Ayfin and Bacitracin: Resolution of Crude Products into Similar Series of Peptides

BY G. G. F. NEWTON AND E. P. ABRAHAM

The Sir William Dunn School of Pathology, University of Oxford

(Received 13 February 1950)

The production of an antibiotic named ayfin by a strain of *Bacillus licheniformis* was described by Arriagada, Savage, Abraham, Heatley & Sharp (1949) and by Hills, Belton & Blatchley (1949). Ayfin was extracted and partly purified by Sharp, Arriagada, Newton & Abraham (1949), who concluded that it consisted of several similar polypeptides and that it resembled bacitracin in its physical and chemical properties. Arriagada, Florey, Jennings & Wallmark (1949) found that the biological properties of ayfin also resembled those of bacitracin. The antibiotic bacitracin is formed by an organism which was first described as a member of the *B. subtilis* group (Johnson, Anker & Meleney, 1945), but which has now been classified by Dr K. Burdon as a strain of *B. licheniformis* (Meleney & Johnson, 1949).

Further investigations of ayfin were undertaken with several aims in view. Earlier experiments had indicated that the crude product might be resolved into inactive products and three or more active peptides by Craig's method of counter-current distribution under appropriate conditions. It appeared worth while to try to obtain the active constituents in a pure form, so that both their chemical and biological properties could be compared. Arriagada, Florey, Jennings & Wallmark (1949) showed that crude ayfin was able to cure mice of systemic infections with streptococci or staphylococci, but that it caused serious damage to the kidneys. Whether the nephrotoxicity of the material was due to an impurity, or was a property of one or all of the active constituents, was uncertain. It remained possible that one of the constituents of ayfin would prove to be a more valuable chemotherapeutic agent than the crude product.

The fact that bacitracin and ayfin were obtained from the culture fluids of similar aerobic spore-forming organisms, and that they resembled each other in many properties, suggested that some, at least, of the peptides present in the two products might be identical. The purity of commercial bacitracin was studied by Craig, Gregory & Barry (1949) using the method of counter-current distribution in a system composed of *sec.*-butanol and aqueous acetic acid. The material behaved as though it were largely composed of a single active peptide. But

since there was very poor resolution of the components of crude ayfin on counter-current distribution in this kind of system (Sharp *et al.* 1949), the question arose whether bacitracin might, nevertheless, contain more than one active constituent. At any rate, it seemed desirable to compare bacitracin and ayfin in a system that was favourable for separating peptides present in the latter.

The present paper is concerned with the resolution of crude ayfin by counter-current distribution in the system used for preliminary experiments by Sharp *et al.* (1949), and with the comparative behaviour of ayfin and bacitracin in this system. The acquisition of an apparatus similar to the 54-chamber apparatus described by Craig & Post (1949) made it possible to carry out experiments involving relatively large numbers of distributions.

METHODS

TECHNIQUE OF FRACTIONATIONS

The method of counter-current distribution between solvents, developed by Craig and his colleagues, has already proved valuable for resolving mixtures of polypeptides whose components are not easily isolated by other procedures (Gregory & Craig, 1948). Technical difficulties limit the number of distributions, and thus the resolution that can be obtained under given conditions in a single experiment, but this disadvantage is partly offset by the fact that a satisfactory interpretation of the results is often possible even when the separation of the constituents of a mixture is far from complete. The interpretation can be verified by further experiment, which can often lead to a reliable picture of the composition of the material under investigation. Once a reliable analysis of the mixture is available, short cuts may be devised which help to obviate the practical difficulties that would be encountered in carrying out many distributions on a preparative scale.

The solvent system. It is clearly advantageous to use a system of solvents in which there is as large a difference as possible between the partition coefficients of the various constituents of the mixture under investigation.

The distribution of a polypeptide between water and an organic solvent will depend greatly on the hydrophobic side chains, and the hydrophilic ionizable groups (if such groups are present), in the molecule. The effect of the latter groups on the partition coefficient will vary with their degree of ionization and hence with the pH of the system. In the case of crude ayfinin the overall partition coefficient defined as [total activity in organic solvent phase]/[total activity in water phase] rose sharply to a maximum in the region of pH 7, which appeared to be close to the isoelectric point of the major active constituent of the material (Sharp *et al.* 1949). Until the components of a mixture of such polypeptides have been obtained in relatively pure form, and their relative solubilities determined, it is not possible to predict the pH at which they will be most easily resolved. The curves representing the change of partition coefficient with pH may coincide or cross at some points and diverge widely at others.

Nevertheless, it appeared not unlikely that the greatest divergence in the partition coefficients of the constituents of crude ayfinin would be found in the isoelectric region, where the relative solubility of some, at least, of the constituents changed rapidly with pH. A pH close to 7 was therefore chosen for trial distributions. A solvent system consisting of amyl alcohol-*n*-butanol (4:1, by vol.) and 0.05M-potassium phosphate buffer, which appeared promising in preliminary work (Sharp *et al.* 1949), was used for most of the experiments described in the present paper. This is referred to as system I. The partition coefficient of the main active constituent of ayfinin in this system was close to unity, and the overall partition coefficient of the active substances in the crude material was shown not to vary, within the accuracy of the biological assay, over the range of concentrations encountered in the distributions.

A few experiments were carried out in a system composed of *sec.*-butanol and dilute acetic acid, similar to that used by Craig *et al.* (1949) in the study of bacitracin. In this system the pH was about 3 and the partition coefficient of the main active constituent of ayfinin was about 0.28. It is referred to as system II.

From a technical point of view system II had advantages over system I. Crude ayfinin was very soluble in system II, but had a solubility of less than 20 mg./ml. in system I, and with system II nothing was added to the preparation that was not removable by evaporation. Moreover, in some circumstances ayfinin was less stable at pH 7 than at pH 3 (Sharp *et al.* 1949).

Stability in the solvent system. During counter-current distributions with ayfinin, special attention had to be paid to the stability of the active material. If breakdown occurred to a significant extent during the distribution, or during the manipulations

between one distribution and another, the process would be less efficient and the results more difficult to interpret. Some inactivation took place in the experiments of Sharp *et al.* (1949) with system I, and although this was much too small for the inactivation products to account for the great resolving power of system I compared with that of system II, it was important that it should be eliminated.

The factors which govern the stability of ayfinin are not yet completely understood, but there is evidence that impurities present in aqueous and alcoholic solvents can be an important cause of loss of activity. A systematic investigation of this question might have been very laborious, and it was decided to make an empirical attempt to avoid inactivation by careful purification of the components of system I and by taking special precautions in concentrating the fractions obtained from the distributions. In some experiments a small quantity of 8-hydroxyquinoline was added to the system to minimize the possible deleterious effects of traces of heavy metals.

In the purified system I, 1 unit/ml. (about 0.03 mg./ml.) of a preparation of ayfinin containing more than 90 % of the major active constituent lost no detectable activity when shaken for 3 hr. at 37°. The information provided by activity measurements, however, is limited by the accuracy of the biological assay, and it would be incapable of detecting a change of one peptide into another showing similar activity. The most reliable evidence about the stability of ayfinin under the conditions used was obtained from the shape of the distribution curves and from the extent to which the results of successive series of distributions formed a consistent pattern. The nature of this evidence will appear in the description of the results.

Estimation of material in different fractions. In general, the best method of finding the amount of material in the various fractions from a counter-current distribution is by direct determinations of dry weight (Craig, Hogeboom, Carpenter & du Vigneaud, 1947). The use of this method would have imposed severe limitations on the counter-current distributions because of the low solubility of some of the components of crude ayfinin in system I. Estimations were therefore carried out by hydrolysing the peptides from samples of the various fractions and measuring the colour given by the resulting amino-acids when treated with ninhydrin in the manner described by Moore & Stein (1948). The colour was compared with that given by hydrolysates of standard amounts of the starting material.

The majority of amino-acids give similar colour yields (Moore & Stein, 1948) and it might be expected that this procedure would be satisfactory in the case of a mixture of peptides having similar amino-acid compositions and molecular weights. In

fact, the colour yields from crude ayfivin and from two of its purified active constituents were found to be almost identical, and 10–150 $\mu\text{g.}$ amounts of the peptides could be estimated with an accuracy of about $\pm 2 \mu\text{g.}$ Using this method, analytical counter-current distributions of a hundred theoretical plates could be carried out with little more than 150 mg. of partially purified material.

The activity of the various fractions was measured by the cylinder-plate method (Heatley, 1944), using *Corynebacterium xerosis* as a test organism (Arrigada, Savage, Abraham, Heatley & Sharp, 1949), and expressed in the units employed by these authors.

Procedure. In order that a series of experiments could be made with the same starting material, a number of batches of crude ayfivin hydrochloride (Sharp *et al.* 1949) were pooled and lyophilized. The resulting material, which contained 20 units/mg., is referred to as crude ayfivin hydrochloride. Unlike some batches of material, it appeared to lose no activity on storage at 4° for several months.

The resolution of crude ayfivin was carried out in two or more stages. An initial separation was obtained from a relatively small number of transfers, and the resulting fractions were then subjected to a larger number of transfers in the 54-chamber apparatus designed by Craig & Post (1949).

Initial distributions of several grams of material were carried out in eleven separating funnels (numbered 0–10) using system I. In one type of experiment, designed for the isolation of the main active peptide, the phosphate phase was mobile and transfers were continued until most of the active material had been withdrawn from the last funnel (10). Much labour was saved, and some additional purification obtained, by removing funnels from the zero end of the series as soon as they contained less than 1% of the active substance initially taken. In a second type of experiment, designed to isolate an active constituent less soluble than the main one in the alcohol phase, the latter phase was mobile. At the end of the distribution most of the substance concerned remained in the funnels, while nearly all the other active material had been withdrawn from funnel 10.

The amount of material in the fractions from the initial distribution was determined and those fractions chosen for further study were concentrated to a volume suitable for introduction into the 54-chamber apparatus. The equations derived by Williamson & Craig (1947), which relate the amounts of material in consecutive fractions to partition coefficients and the total number of transfers, were extended to allow a rapid interpretation of the results of estimations of the content of withdrawn fractions. Subsequent 100-plate distributions showed these interpretations to be essentially correct.

An initial distribution involving a limited number of transfers, followed by a theoretical interpretation and the reconcentration of selected fractions, was preferable for several reasons to a procedure beginning with a long fundamental run on the crude material. The approximate composition of the selected fractions was known and their behaviour on further distribution could be predicted with reasonable accuracy, thus enabling the maximum advantage to be obtained from the use of the 54-chamber apparatus. The greater purity of the selected fractions made it possible to introduce larger amounts of active peptides into this apparatus without exceeding the limits of solubility.

Analysis of distribution curves. Williamson & Craig (1947) showed that during a fundamental run of n transfers the fraction of material in successive tubes was given by the terms of a binomial expansion. The fraction $T_{n,r}$ in tube r for a substance of partition coefficient K could be calculated from the expression

$$T_{n,r} = \frac{n!}{r!(n-r)!} \left[\frac{1}{K+1} \right]^n (K)^r.$$

From this it followed that

$$\frac{T_r}{T_{r-1}} = FK, \quad \text{where } F = \frac{(n+1-r)}{r},$$

$$\text{and } \frac{T_r}{T_{r+1}} = F' \left[\frac{1}{K} \right], \quad \text{where } F' = \frac{(r+1)}{(n-r)}.$$

Hence K could be calculated from the ratio of the amount of material in successive fractions.

Suppose that R is the number of the tube from which the upper phase is being withdrawn, that w is the number of the fraction in the withdrawn series (the first withdrawal being numbered 1), and that T_w is the proportion of substance in the withdrawn fraction w . Then

$$\begin{aligned} T_w &= (\text{proportion of substance in the } R\text{th tube of a} \\ &\quad \text{fundamental run of } R+w-1 \text{ transfers}) \\ &\quad \times \frac{K}{K+1} \\ &= \frac{(R+w-1)!}{R!(w-1)!} \left[\frac{1}{K+1} \right]^{R+w-1} (K)^R \times \frac{K}{K+1}. \end{aligned}$$

It follows that

$$\frac{T_w}{T_{w+1}} = F'_w (K+1), \quad \text{where } F'_w = \frac{w}{R+w}, \quad (1)$$

and

$$\frac{T_w}{T_{w-1}} = \frac{F_w}{(K+1)}, \quad \text{where } F_w = \frac{R+w-1}{w-1}. \quad (2)$$

Using (1) and (2), values for K can rapidly be calculated from the amounts of material in adjacent tubes of the withdrawn series.

If the number of distributions is large and if there is a peak in the vicinity of fraction W of the withdrawn series, $\frac{T_w}{T_{w+1}} \rightarrow 1$ and the position of the peak is given approximately by the expression $W = R/K$.

RESULTS OF FRACTIONATIONS

Crude ayfvin was finally resolved into a number of peptides, of which at least three (referred to as A , B and C) had antibacterial activity. In system I the partition coefficients ($K = [\text{solute in alcoholic phase}]/[\text{solute in aqueous phase}]$) of the active peptides were such that $K_B < K_A < K_C$. A was present in considerably larger quantities than B or C .

The specific activity of A was similar to that of C and about four times as great as that of B .

The results of various counter-current distributions are given here in more or less chronological order.

Composition of crude ayfvin. Crude ayfvin was purified by an 8-transfer distribution in system I as described by Sharp *et al.* (1949). The material in tubes 2–7, which contained almost all the activity, was then subjected to fifty-three transfers in system I in the Craig-Post apparatus. The results, shown in

Further examination of fractions from an initial distribution. An initial purification in system I was carried out by twenty-seven transfers through eight

Fig. 1. 53-Transfer distribution of crude ayfvin in system I (amyl alcohol-*n*-butanol (4:1): 0.1M-potassium phosphate buffer, pH 7.1). For details see Experimental section (p. 265). O—O—O, percentage total dry weight estimated colorimetrically; ---, approximate theoretical interpretation.

separating funnels, the buffer phase being mobile. Fractions 11, 12 and 13 from the withdrawn series were pooled and lyophilized. The resulting material

Fig. 2. 100-Transfer distribution in system I (amyl alcohol-*n*-butanol (4:1): 0.05M-potassium phosphate buffer, pH 6.9) of fractions 7, 8 and 9, from an initial purification of crude ayfvin. For details see Experimental section (p. 265). O—O—O, percentage total dry weight estimated colorimetrically; ●...●, calculated curves.

Fig. 1, indicated that the mixture was complex and that at least 100 transfers would be required to resolve the main components. On the basis of these results it was decided to carry out a more extensive initial purification and to select fractions over a more limited range for further study.

was analysed by 100 transfers in system I in the Craig-Post machine and found to contain about 5% B (mixed with another compound), 72% A and 10% C . Material from fractions 7, 8 and 9 of the same withdrawn series was treated in a similar fashion. The distribution curve, shown in Fig. 2,

indicated that it contained about 21% *B*, 50% *A* and 8% *C*.

Comparison of systems I and II. Another portion of the material from fractions 7, 8 and 9 was subjected to 100 transfers in system II. The curve given in Fig. 3 shows that the material behaved as though it were practically homogeneous, and that in this system there was no significant resolution of *A*, *B* and *C* such as is shown in Fig. 2.

Fig. 3. 100-Transfer distribution in system II (*sec.*-butanol: 3% (w/v) acetic acid) of fractions 7, 8 and 9, from an initial purification of crude ayfivin. For details see Experimental section (p. 265). ○—○—○, percentage total dry weight estimated colorimetrically; ●...●...●, calculated curve.

Redistribution of fractions from a 100-transfer experiment. In order to obtain clear evidence about the stability of *A* under the conditions of the experiments, fractions which appeared to contain nearly pure *A* were redistributed in system I. Fractions 46–59 from the distribution shown in Fig. 2 and similar fractions from analogous experiments were pooled and concentrated. To obtain enough material for redistribution it was necessary to include fractions further from the peaks than was desired, but it was estimated at the time that the combined fractions contained about 95% *A*. The results of a further 100 transfers of this material are illustrated by the curve in Fig. 4, which indicated that it

contained 87% *A* and 12% *C*. Substance *B* was absent.

Fig. 4. 100-Transfer distribution of reconstituted ayfivin *A* in system I (amyl alcohol-*n*-butanol (4:1): 0.05M-potassium phosphate buffer, pH 6.9). For details see Experimental section (p. 265). ○—○—○, percentage total dry weight estimated colorimetrically; ●...●...●, calculated curve.

Fig. 5. Twenty-nine transfers, through eleven separating funnels, of crude ayfivin hydrochloride in system I (amyl alcohol-*n*-butanol (4:1): 0.0033M-potassium phosphate buffer, pH 7.0). Lower layer mobile. About 30% of the material has been withdrawn from the left side and does not appear in the figure. For details see Experimental section (p. 264). ○—○—○, dry weight of 0.1 ml. sample estimated colorimetrically; - - -, approximate theoretical interpretation.

Improved initial purification. The preceding results confirmed the original interpretation of the

distribution curve obtained from the initial purification, but showed that a still more extensive initial purification was desirable to obtain fractions containing nearly pure *A*. This was made by increasing the number of separating funnels and by withdrawing from the zero end somewhat earlier. Fig. 5 shows the results of such an experiment in system I. It indicated that the ratio of *A* to *B* in crude ayfvin was about 2.5 to 1. The discarded fractions which do not appear in the figure contained about 30 % of the starting materials.

Fig. 6. Twenty-eight transfers through nine separating funnels of crude ayfvin (enriched in *B*), in system I (amyl alcohol-*n*-butanol (4:1): 0.0033 M-potassium phosphate buffer, pH 6.9). Upper phase mobile. About 10 % of the material has been withdrawn from the left side and does not appear in the figure. For details, see Experimental section (p. 266). ○—○—○, dry weight of 0.04 ml. sample estimated colorimetrically; ---, approximate theoretical interpretation.

In an initial purification designed to concentrate *B*, the alcohol phase was mobile. Material containing a high proportion of *B* remained in the separating funnels (Fig. 6). The discarded fractions which do not appear in the figure contained about 10 % of the starting material.

Comparison of ayfvin and bacitracin. A distribution similar to that shown in Fig. 5 was carried out with a sample of commercial bacitracin (Commercial Solvents Corporation, Terre Haute, Indiana). The resulting curve resembled that in Fig. 5, and indicated that the ratio of *A* to *B* was 4:1. The contents of fractions 12, 13 and 14 of the withdrawn series from both the ayfvin and bacitracin distributions were concentrated, and equivalent amounts (80 mg.) of the two materials were mixed. The mixture was subjected to 100 transfers in system I and the distribution curve showed that it contained 93 % *A* and

7 % *C* (Fig. 7). There was no indication that the peak representing *A* could be resolved into two substances.

In an earlier experiment a different sample of commercial bacitracin (Ben Venue Laboratories, Bedford, Ohio: batch no. B124) was subjected directly to seventy-three transfers in the Craig-Post apparatus. The two major peaks of the distribution curve showed that the ratio of *A* to *B* in the sample

Fig. 7. 100-Transfer distribution of a mixture of purified ayfvin *A* and bacitracin *A* (equal amounts) in system I (amyl alcohol-*n*-butanol (4:1): 0.05 M-potassium phosphate buffer, pH 6.9). For details see Experimental section (p. 266). ○—○—○, percentage total dry weight estimated colorimetrically; ●—●—●, calculated curve.

was 3:1. The ratio of the partition coefficients, β , calculated from the position of these peaks (Barry, Sato & Craig, 1948) was the same as that calculated from the distribution of a similar fraction of ayfvin.

DISCUSSION

The results of the various counter-current distributions show that crude ayfvin was not purified to any appreciable extent in system II, but that it was resolved, or partly resolved, into at least seven components in system I. Five of these components appear in the distribution described by Fig. 1 and at least two more were present in the discarded fractions 0 and 8 of the distribution that preceded it.

The astonishing difference in the amount of resolution obtained in the two systems made it necessary to consider whether some of the products revealed in

system I might be formed by changes in the material during experiments with this system. Since the specific activity rose from 20 units/mg. for crude ayfivin to about 40 units/mg. for the material in the main peak, it was evident at an early stage that the distribution curves obtained in system I could not be largely accounted for by changes leading simply to loss of activity. Nevertheless, in some of the first experiments computation of the specific activity to be expected from the amount of impurity discarded indicated that either the specific activity was really more than 40 units/mg., or that about 25 % loss of activity had taken place. It now appears that up to 25 % inactivation did take place in early experiments (e.g. Fig. 1), but that this occurred during the re-concentration between distributions and that its amount depended on the manipulations at this stage. The fact that a satisfactory theoretical interpretation of the distributions was always possible indicated that the material was relatively stable in system I itself.

After purification of the solvents and modification of the method used for concentrating solutions, successive experiments could be made in which very little change, at least of substance *A*, appeared to take place. In one distribution of material containing a high percentage of *A*, substance *B* did not appear at all (Fig. 4). It was clear that neither *B* nor substances with smaller partition coefficients than *B* were being formed from *A* during the experiment, and that these substances were already present in crude ayfivin. The evidence with regard to *C* is less satisfying, but Fig. 4 indicates that less than 10 % of *A* could have been transformed to *C*, and Fig. 5 that very little, if any, transformation occurred. It is possible, of course, that such transformations took place in the culture fluid or during the preparation of crude ayfivin.

On the basis of the present results there is no reason to suppose that *A* is not a single compound. If it should be a mixture, distribution in other systems or a different method of purification will be necessary to resolve it. The status of *B* and *C* is less certain. They may well be single compounds, but it is still possible that further experiments with system I will show them to be heterogeneous.

The fact that no separation of bacitracin *A* and ayfivin *A* could be detected after 100 transfers in system I, and that the chemical properties of the two products are known to be very similar, justifies the provisional conclusion that they are in fact identical. The other components of commercial bacitracin have not been closely investigated, but they have partition coefficients very similar to some of those of crude ayfivin. In view of these findings it has been decided to abandon the name ayfivin and to refer to the active components of crude ayfivin as bacitracin *A*, *B* and *C*.

EXPERIMENTAL

Purification of reagents

Amyl alcohol and n-butanol. A modification of the method of Rowe & Phelps (1924) using *m*-phenylenediamine was found to be satisfactory for the removal of aldehydes.

The alcohols were treated in 5 l. batches with 1 g./l. of *m*-phenylenediamine hydrochloride, sufficient water being added to saturate the alcohol and leave a lower layer of about 100 ml./l. The mixture was allowed to stand in the dark at least 3 days, and was shaken occasionally. The lower aqueous layer was then run off and the alcohol layer washed with 100 ml./l. of water.

To remove 'peroxide' another 100 ml./l. of distilled water was then added together with a handful of ferrous sulphate crystals and a few ml. of 2*N*-H₂SO₄. The mixture was again kept at least 3 days in the dark with occasional shaking. The water layer was removed and the alcohol washed with batches of 100 ml./l. of water until the washings no longer gave a coloration with potassium ferricyanide. The alcohol was then distilled slowly in an atmosphere of hydrogen in an all-glass apparatus, using a lagged column 60 cm. high. The cylinder hydrogen was purified by washing with *N*-H₂SO₄ saturated with KMnO₄ followed by saturated aqueous AgNO₃. The fractions of *n*-butanol, b.p. 116–118°, and amyl alcohol, b.p. 128–132°, were used in the experiments described later. These were stored in the dark until required for use.

No reduction occurred when 1 ml. of the alcohol was shaken with 1 ml. of a strong solution of ammoniacal AgNO₃, and the mixture stored in the dark overnight. No red colour developed when the alcohol was added to a freshly prepared solution of pure KCNS and FeSO₄·(NH₄)₂SO₄·6H₂O. This method of testing for peroxide was suggested by Dr J. C. Smith.

Distilled water. To remove traces of heavy metals, laboratory-distilled water was treated with 8-hydroxyquinoline extracted several times with amyl alcohol and redistilled in an all-glass apparatus.

Estimation of dry weight by a standard photometric ninhydrin method

Construction of a standard curve. Two 5 mg. samples of crude ayfivin hydrochloride were weighed out in glass tubes (3 × $\frac{3}{8}$ in.) and 6*N*-HCl (0.4 ml.) was added to these and to two blank tubes. The tubes were sealed and heated at 110° for 18 hr. They were then opened and the HCl and water removed *in vacuo* over P₂O₅ and damp NaOH pellets. The residues were dissolved in water (or phosphate-buffer solution) to give solutions whose concentration in terms of ayfivin hydrochloride varied from 1500 to 25 µg./ml. Duplicate samples of 0.1 ml., containing the equivalent of from 150 to 2.5 µg. ayfivin hydrochloride, were taken from each solution and placed in 8 ml. colorimeter tubes (Evans Electroselenium Ltd., Harlow, Essex (E.E.L.) portable colorimeter). Ninhydrin solution (0.5 ml.), prepared according to the method of Moore & Stein (1948), was then added. Aluminium caps were placed on the tubes, which were heated 20 min. on a vigorously boiling water bath, after which 7.5 ml. of an *n*-propanol-water mixture (1:1, v/v) were added to each tube, using a standard automatic pipette. The colour density was read in the colorimeter using a no. 205 E.E.L. filter. The mean readings for the

standard solutions minus the blank reading were plotted against the weight of ayfivin hydrochloride known to be in the samples. A fresh standard curve was made for each new batch of solid ninhydrin used.

Estimation of unknown samples. A sample containing between 25 and 700 μg . of peptide was taken from the samples to be analysed by means of a syringe-pipette made by Mr S. Bush (Dryer, Ward, McIntosh & Fildes, 1923) and placed in a tube for hydrolysis. Two samples from a standard solution of ayfivin hydrochloride made up in 20% (v/v) aqueous ethanol, and of the plain solvent in which the sample was dissolved, were also taken.

The samples, frequently amyl alcohol-*n*-butanol or amyl alcohol-*n*-butanol-buffer mixtures, were evaporated to dryness in a brisk stream of air (18–22°), the tubes being maintained at 100° in a deep metal block. A compact apparatus, capable of drying eight tubes at a time, designed by Dr N. G. Heatley and made by Mr A. Broadway, was used for this purpose. 6*N*-HCl (0.4 ml.) was added to the dried samples, which were then hydrolysed in the standard manner.

After hydrolysis and drying of the hydrolysate, 0.5 ml. distilled water was added to each sample by means of an automatic pipette. Samples (0.1 ml.) were taken from these solutions and the colour densities measured in the standard manner. Any variation from one experiment to another was compensated by adjusting the standard curve to fit three values (corresponding to 25, 50 and 100 μg . ayfivin hydrochloride from the standard solution) which were redetermined in each case.

Readings were reproducible to ± 1 scale division (i.e. approx. 2 μg . ayfivin hydrochloride over the range 10 to 150 μg .).

Measurement of antibacterial activity

Test organism. The cylinder-plate method (Heatley, 1944) was used throughout. The plates were surface seeded with a suspension of an overnight culture of *Corynebacterium xerosis* matched to Burroughs Wellcome standard opacity tube no. 1 (Brown & Kirwan, 1915) and then diluted three times.

Diluting solution. Dilutions were made with a solution of 0.05*M*-potassium phosphate buffer (pH 6.8), which was also made 0.1*M* in respect of glycine. This solution was autoclaved to prevent the growth of contaminants. Dilute solutions of ayfivin, known to be unstable in the presence of certain impurities, were stable in this solution (Sharp *et al.* 1949).

Measurement of the activity in counter-current distribution fractions. A sample was taken from the upper phase of the fraction and placed in a graduated tube. An appropriate volume of diluting fluid, at least three times that of the sample, was then added to it. The two phases were then equilibrated and five times the volume of the sample of *n*-hexane was added and the mixture again equilibrated. After the hexane-alcohol layer had been withdrawn and discarded, a sample from the lower layer of the fraction was added and the combined solutions extracted again with *n*-hexane to remove any alcohol left in the aqueous solution. The hexane was discarded and the aqueous solution, which contained at least 95% of the active material, was made up to volumes containing approximately 1 and 0.5 unit/ml. The zones of inhibition given by these solutions were compared with those produced by a series of standards. The same pro-

cedure, slightly modified, was suitable when the fraction to be analysed was in a single phase. Units of activity were those defined by Arriagada, Savage, Abraham, Heatley & Sharp (1949).

Concentration of solutions

Ayfivin had occasionally been found to lose activity when recovered from dilute aqueous solution by lyophilization. Craig *et al.* (1949) reported that bacitracin had lost a certain amount of activity after isolation in a similar way. The effect of lyophilization on ayfivin has not proved to be consistently reproducible, but in view of the possibility of inactivation the method was not generally used in the present series of experiments. The fractions were concentrated in the following manner. Up to 500 ml. of the fractions to be concentrated (usually in two phases) were placed in a 1 l. flask connected through a splash-head to a trap cooled in solid CO₂ and ethanol. The flask was immersed in a water bath. When buffer solution only was to be concentrated, 10 ml. of purified amyl alcohol/100 ml. of buffer were added to prevent frothing; and with alcohol phase only 1.5 vol. of pure water were added initially.

The air was pumped out of the system while a stream of hydrogen (purified by bubbling through KMnO₄ and AgNO₃ solutions) was passed through the apparatus by means of a capillary reaching to the bottom of the flask. The pump was switched off before the distillation began. The hydrogen flow was then adjusted to be just sufficient to prevent bumping and the pump again started. As soon as distillation began, the bath, which was initially at room temperature, was allowed to heat up to 30°. The contents of the flask rapidly cooled to below 0°. The rate of distillation, which was 150–200 ml./hr., was mainly controlled by switching the pump on and off, and was adjusted so as just to prevent freezing.

Water or amyl alcohol were added occasionally so that two phases were present until the aqueous solution had reached its required concentration. The alcohol phase was not allowed to become anhydrous as inactivation of bacitracin has been noted under such conditions (Anker, Johnson, Goldberg & Meleney, 1948). The distillation was continued in a 500 ml. flask when the volume had dropped below 100 ml. The rate of heating was cut down as the volume of the concentrate decreased. The bath was at 10–15° during the final stages.

Counter-current distributions

(I) Initial distribution of 6 g. crude ayfivin hydrochloride in system I

Solvents. 4 l. 0.0033*M*-potassium phosphate buffer (pH 7.0) were shaken with a mixture of 600 ml. wet *n*-butanol: 300 ml. wet amyl alcohol. The upper layer was drawn off and discarded. The lower layer was equilibrated with a mixture of 1440 ml. amyl alcohol, 360 ml. *n*-butanol and 20 mg. 8-hydroxyquinoline, which had previously been just saturated with 0.0033*M* buffer solution.

Distribution procedure. Eleven separating funnels of 600 ml. capacity were used. These were fixed to a wooden frame with Terry clips so that they could all be shaken at once. 150 ml. portions of upper layer were placed in funnels 1–10. Crude ayfivin hydrochloride (6 g.) was shaken with 145 ml. upper and 120 ml. lower layer, and *m*-NaOH (13.8 ml.) was added to bring the lower layer to pH 7.0

(glass electrode). The dark, viscous oil which came out of solution as the pH was raised was spun down and the two clear layers transferred to funnel '0' where both layers were made up to the 150 ml. volume marks.

Seven fundamental transfers were made. The contents of funnel 0 were then discarded, no fresh lower layer having been added. After the ninth transfer upper '1' was discarded. On the eleventh transfer lower '10' was removed, and became the first member of the main withdrawn series. Upper '2' was also removed at this stage. The process was continued until nineteen fractions had been collected in the withdrawn series. Further discards of the slower moving components were made after the thirteenth, seventeenth, twenty-first, twenty-fourth and twenty-sixth transfers had been completed. Only three funnels of the fundamental series remained at the end of the experiment. The results are shown in Fig. 5.

(II) *Fifty-three transfers in system I on a partly purified sample of ayfivin*

Solvents. 800 ml. amyl alcohol, 200 ml. *n*-butanol and 800 ml. 0.1M-potassium phosphate buffer were shaken together; the pH at equilibrium was 7.1 (glass electrode). The solvents used in this experiment had not been specially purified.

Distribution procedure. Fractions 2-7 from an 8-transfer distribution, lower layer mobile (Sharp *et al.* 1949), were combined. 2N-Acetic acid was added until the pH of the lower phase dropped to 3.7 and then 1.5 vol. ether were added to reduce the solubility of ayfivin in the organic-solvent phase. Four further extractions of the latter with 10 ml. 2N-acetic acid were made and the combined extracts were lyophilized. Ayfivin acetate (126 mg.) was obtained, having an activity of 23 units/mg.

The ayfivin acetate was shaken with 10 ml. of each layer of solvent. A few mg. which did not dissolve were spun down and the clear solutions transferred to tube 0 of the Craig-Post 54-chamber apparatus. A fundamental run of fifty-three transfers was then carried out. The results are shown in Fig. 1.

(III) *One hundred transfers in system I on fractions 7, 8 and 9 from an initial purification of ayfivin*

Initial purification. Crude ayfivin hydrochloride (2 g.) was partially purified by a method very similar to that described in Exp. I. Nine funnels were used and twenty-seven lower layers of system I (0.0033M-phosphate) were passed through them, giving a withdrawn series of nineteen fractions. Fractions 7, 8 and 9 of the withdrawn series were lyophilized, giving 300 mg. of a mixture of ayfivin and phosphate.

One hundred transfers in the Craig-Post apparatus. 1000 ml. amyl alcohol and 250 ml. *n*-butanol were just saturated with pure water, and then mixed with 800 ml. 0.05M-potassium phosphate buffer. The pH at equilibrium was 6.9 (glass electrode). The dried sample was dissolved in 10 ml. upper and 9 ml. lower layer, and the pH adjusted to 6.9 with 0.05 ml. N-NaOH. All the material dissolved. The volumes were adjusted, and the solution transferred to tube 0 of the machine. A fundamental run of fifty-two transfers followed by forty-eight single withdrawals of the upper phase from empty tube 53 was then carried out (Craig & Post, 1949). The results are shown in Fig. 2.

(IV) *One hundred transfers in system II on fractions 7, 8 and 9 from an initial purification of ayfivin*

Fractions 7, 8 and 9 from the withdrawn series of an initial distribution identical with that described in Exp. III, were concentrated to 8 ml. *sec*.-Butanol was purified by a method similar to that used for *n*-butanol. Wet *sec*.-butanol was obtained on distillation. The acetic acid was purified by the method of Hutchinson & Chandler (1931). Wet *sec*.-butanol (900 ml.) was shaken with 500 ml. 4% (v/v) aqueous acetic acid (i.e. 1 vol. dry *sec*.-butanol to 1 vol. 3% acetic acid) giving 850 ml. upper phase and 550 ml. lower phase, pH 2.8 (glass electrode).

Distribution procedure. The concentrate of fractions 7, 8 and 9 in 8 ml. 0.05M-potassium phosphate buffer was equilibrated with 10 ml. upper layer (system II). The pH of the lower layer was adjusted to 2.8 with acetic acid and the volumes of each phase made up to 10 ml.

A preliminary distribution in system II (Sharp *et al.* 1949) had shown that volume changes occurred as the distribution proceeded when solutions of 20 mg./ml. of crude ayfivin hydrochloride were used. Therefore the first six transfers were carried out in stoppered tubes having volume marks on them. After two transfers, the upper layer being mobile, lower '0' and upper '1' had increased by 0.2 ml. while no changes were noted in '2'. These differences did not increase during the next four transfers. The contents of the tubes were then transferred to the appropriate tube of the Craig-Post apparatus, which had been turned so that upper '0' was over lower '6'. A further forty-six fundamental transfers followed by forty-eight single withdrawals from empty tube 53 were carried out. The results are shown in Fig. 3.

(V) *One hundred transfers in system I on nearly homogeneous ayfivin A reconcentrated from previous 100-transfer distributions*

Reconcentration. The contents of tubes 46-59 of Exp. III (Fig. 2), and of tubes containing material of similar purity from a 100-transfer distribution on fractions 11, 12 and 13 from the same initial purification, were reconcentrated. The combined fractions had an initial volume of 160 ml. upper and 120 ml. lower layers. These were concentrated to 5 ml. upper and 15 ml. lower layers by the method described above. The amyl alcohol was removed by adding an excess of anaesthetic ether, the lower layer was then drawn off and the ethereal solution shaken with 5 ml. 0.01N-HCl, which was then added to the first buffer extract. The combined aqueous solutions (20 ml.) were adjusted to pH 7.0 and extracted first with 20 ml. and then with 10 ml. *n*-butanol. The dry weight of a sample, which was removed at this stage, indicated that the total weight of material was about 110 mg. The combined *n*-butanol solutions were then extracted with 24 ml. 0.025M-KH₂PO₄ acidified to pH 3.2, followed by 8 ml. of the same solution at pH 2.3. The combined buffer solutions were brought to pH 6.3 and their volume reduced to 8 ml.

Solvents. 800 ml. amyl alcohol and 200 ml. *n*-butanol were shaken with 800 ml. 0.05M-potassium phosphate, which had previously been just saturated with *n*-butanol and amyl alcohol. The buffer phase was pH 6.9 (glass electrode) after equilibration.

Distribution procedure. 10 ml. of the organic phase and 1 ml. of pure water were added to the 8 ml. of reconcen-

trated ayfivin *A* solution. After the pH had been adjusted to 6.9, both layers were made up to 10 ml., and then transferred to tube 0 of the Craig-Post machine.

A fundamental run of fifty-two transfers followed by thirteen single withdrawals from empty tube 53 was first carried out; both layers were then removed from tubes 0-15 inclusive and the latter refilled with fresh upper and lower layers. The fundamental run was then continued until upper '13' was over lower '15' for the second time (eighty-two transfers). Withdrawal was then started again from empty tube 16 until 100 transfers had been completed. Thus material in tubes 17-53 and 0-15 had been through 100 fundamental transfers. The results are shown in Fig. 4.

(VI) *One hundred transfers on a mixture of ayfivin A and bacitracin A*

Initial purification. Ayfivin was initially purified as described in Exp. I. Fractions 12, 13 and 14 of the withdrawn series were pooled, extracted twice with ether to remove any oxine and then concentrated to 100 ml. Bacitracin (Commercial Solvents, batch no. B 480623) was initially purified in an identical manner to the ayfivin, followed by concentration of fractions 12, 13 and 14. About 60 mg. of ayfivin *A* and bacitracin *A* were mixed, and the combined solution further concentrated to 15 ml. when it was extracted with 9 ml. of the upper layer of system I before concentrating to 8 ml. The 9 ml. of upper layer was then added to the buffer concentrate and the pH of the latter adjusted to 6.9. The volume of each layer was then made up to 10 ml.

Distribution procedure. The solvents were prepared in the same way as described for Exp. V. Distribution was carried out in the same way as in Exp. V, until upper '13' was over lower '15' for the second time. Then both layers were removed from '16' to '25' inclusive, and replaced with fresh upper and lower phases. The fundamental run was then continued for another ten transfers, ninety-two in all, after which eight single withdrawals were made from empty tube 26. The material in tubes 27-53 and 0-25 had then been through 100 fundamental transfers. The results are shown in Fig. 7.

(VII) *Initial purification for the preparation of ayfivin B from crude hydrochloride in system I*

Solvents. 1200 ml. amyl alcohol, 300 ml. *n*-butanol and 20 mg. 8-hydroxyquinoline were shaken with just sufficient water to saturate the upper phase. This was then shaken with 700 ml. of 0.0033 M-potassium phosphate, which had previously been saturated with amyl alcohol and *n*-butanol. The pH of the buffer phase at equilibrium was 6.9 (glass electrode).

Distribution procedure. Crude ayfivin hydrochloride (1.6 g.) already partially enriched in *B* was dissolved in 50 ml. upper phase and 40 ml. lower phase. The pH of the latter was then adjusted to 6.9. The dark, viscous oil which came out of solution was spun down and both layers made

up to 50 ml. Nine separating funnels were used for the counter-current distribution; numbers 1-8 were filled with 50 ml. lower layer. The clear solution of crude ayfivin was transferred to funnel 0 and eight fundamental transfers carried out, the upper phase being mobile. The contents of funnel 0 were then withdrawn.

Twenty further transfers were then carried out, the contents of upper funnel 8 being withdrawn each time, forming a series of twenty fractions. Withdrawals from the trailing edge of ayfivin *B* were made after the eleventh and twenty-fourth transfers. Six funnels, 3-8, remained at the end of the experiment. The results are shown in Fig. 6.

SUMMARY

1. The antibiotic ayfivin, produced by a strain of *Bacillus licheniformis*, has been studied by the method of counter-current distribution between solvents, and its behaviour compared with that of bacitracin.

2. By distribution in a solvent system composed of amyl alcohol-*n*-butanol and 0.05 M-phosphate buffer at pH 6.8-7 (system I), crude ayfivin was resolved, or partly resolved, into at least seven components. Three of the components, described as *A*, *B* and *C*, were polypeptides showing antibacterial activity. The major constituent, *A*, showed an activity similar to that of *C* and about four times that of *B*.

Very poor resolution of the components of ayfivin was obtained on distribution in a system composed of *sec*.-butanol and dilute acetic acid.

3. The distribution curve of commercial bacitracin in system I was similar to that of partially purified ayfivin. There was no resolution of a mixture of ayfivin *A* and the corresponding component of bacitracin on distribution in this system. Ayfivin *B* and *C* behaved similarly to two other components of bacitracin.

4. It has been concluded from these and other results that the main active constituents of ayfivin and bacitracin are probably identical. The two products are so closely related that it is now proposed to abandon the name ayfivin and to refer to the active components of the latter as bacitracin *A*, *B* and *C*.

We are much indebted to Miss Ann Pill and Mr O. Boys for technical assistance.

The bacitracin used in this work was kindly sent by Dr F. L. Meleney to Prof. Sir Howard Florey, F.R.S. The work has been supported by a grant from the Medical Research Council for technical assistance and supply of materials.

REFERENCES

- Anker, H. S., Johnson, B. A., Goldberg, J. & Meleney, F. L. (1948). *J. Bact.* **55**, 249.
- Arriagada, A., Florey, H. W., Jennings, M. A. & Wallmark, I. G. (1949). *Brit. J. exp. Path.* **30**, 458.
- Arriagada, A., Savage, M. C., Abraham, E. P., Heatley, N. G. & Sharp, A. E. (1949). *Brit. J. exp. Path.* **30**, 425.
- Barry, G. T., Sato, Y. & Craig, L. C. (1948). *J. biol. Chem.* **174**, 221.
- Brown, H. C. & Kirwan, E. W. O'G. (1915). *Indian J. med. Res.* **2**, 763.
- Craig, L. C., Gregory, J. D. & Barry, G. T. (1949). *J. clin. Invest.* **24**, 1014.
- Craig, L. C., Hogeboom, G. H., Carpenter, F. H. & du Vigneaud, V. (1947). *J. biol. Chem.* **168**, 665.
- Craig, L. C. & Post, O. (1949). *Anal. Chem.* **21**, 500.
- Dryer, G., Ward, H. K., McIntosh, J. & Fildes, P. (1923). *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 78, p. 91.
- Gregory, J. D. & Craig, L. C. (1948). *J. biol. Chem.* **172**, 839.
- Heatley, N. G. (1944). *Biochem. J.* **38**, 61.
- Hills, G. M., Belton, F. C. & Blatchley, E. D. (1949). *Brit. J. exp. Path.* **30**, 427.
- Hutchinson, A. W. & Chandler, G. C. (1931). *J. Amer. chem. Soc.* **53**, 2881.
- Johnson, B. A., Anker, H. & Meleney, F. L. (1945). *Science*, **102**, 376.
- Meleney, F. L. & Johnson, B. A. (1949). *Amer. J. Med.* **7**, 794.
- Moore, S. & Stein, W. H. (1948). *J. biol. Chem.* **176**, 367.
- Rowe, A. W. & Phelps, E. P. (1924). *J. Amer. chem. Soc.* **46**, 2078.
- Sharp, V. E., Arriagada, A., Newton, G. G. F. & Abraham, E. P. (1949). *Brit. J. exp. Path.* **30**, 444.
- Williamson, B. & Craig, L. C. (1947). *J. biol. Chem.* **168**, 687.

The Biochemistry of Bacterial Toxins

4. THE LECITHINASE ACTIVITY OF *CLOSTRIDIUM HAEMOLYTICUM* TOXIN

By MARJORIE G. MACFARLANE

The Lister Institute, Elstree, Herts.

(Received 27 March 1950)

Oakley, Warrack & Clark (1947) observed that culture filtrates of *Clostridium haemolyticum* contained a haemolytic factor producing opalescence in egg-yolk emulsion; this lecithovitellin (L.V.) reaction could be neutralized by *Cl. oedematiens* type B antitoxic sera containing β -antitoxin. These facts indicated that *Cl. haemolyticum* toxin contained a lecithinase similar in biochemical action to those of *Cl. welchii* and *Cl. oedematiens* toxins, and closely related antigenically to *Cl. oedematiens* type B lecithinase, the β -toxin (Oakley *et al.* 1947; Macfarlane, 1948*a*). The presence of such a lecithinase has been verified by isolation of the products formed from lecithin by the action of the toxin, and by the examination of the inhibitory power of appropriate antitoxic sera upon the enzymic activity. A preliminary note on these findings has been published (Macfarlane, 1948*b*).

EXPERIMENTAL

Methods. Lecithinase activity was determined by the rate of liberation of acid-soluble P from lecithin under standard conditions, one enzyme unit (e.u.) liberating 100 μ g. P in 15 min. at 37°. The antilecithinase activity of sera was measured by their inhibitory action on P liberation by a test dose of toxin. These and other methods were similar to

those used previously (Macfarlane & Knight, 1941; Macfarlane, 1948*a*).

Toxins and antitoxins. Dry toxins prepared at the Wellcome Laboratories from a strain of *Cl. haemolyticum* originally isolated by Dr Vawter were used. These toxins, and samples of *Cl. haemolyticum* and *Cl. oedematiens* antitoxic sera, were given by Dr Oakley; the titres of the sera in β - and γ -antitoxin units by the L.V. test are referred to arbitrary standards (Oakley *et al.* 1947). The *Cl. welchii* antitoxin used was the Elstree no. 1 standard, containing 95 i.u./ml.

Hydrolysis of lecithin by Clostridium haemolyticum toxin

Isolation of products. Lecithin (0.65 mmol.) prepared from egg yolk was emulsified in 20 ml. 0.01 M-calcium chloride, pH 7.4, and incubated at 37° with 10 mg. toxin, further quantities of toxin (10 mg.) being added after 3 and 24 hr. incubation, and the mixture titrated back to pH 7.4 at intervals with NaOH. In 3 hr. 0.43 mmol. and in 19 hr. 0.56 mmol. acid were produced, equivalent to 67 and 86 % hydrolysis of the lecithin if only one linkage were attacked. Production of acid continued very slowly to a total of 0.79 mmol. at 42 hr. when the experiment was stopped. The mixture was separated, by extraction with ether several times, into a water-soluble fraction which contained 95 % of the original