AYFIVIN: EXTRACTION, PURIFICATION, AND CHEMICAL PROPERTIES.

V. E. SHARP, A. ARRIAGADA., G. G. F. NEWTON, AND E. P. ABRAHAM.

From the Sir William Dunn School of Pathology, Oxford.

Received for publication August 10, 1949.

THE production of ayfivin by the strain A–5 of *B. licheniformis* has been described by Arriagada, Savage, Abraham, Heatley and Sharp (1949), who grew the organism in potato-dextrose broth, and by Hills, Belton and Blatchley (1949), who later devised a more convenient synthetic medium. A method by which preparations of ayfivin with high antibacterial activity have been obtained from the culture fluids of the *B. licheniformis* is given in this paper. The chemical properties of the preparations indicate that ayfivin is a polypeptide.

EXPERIMENTAL.

Extraction.

Preliminary experiments showed that ayfivin was not extracted from the culture fluid by common organic solvents other than butanol or phenol. Adsorption on to charcoal was therefore used as the first step in its isolation. Elution was accomplished by the use of a two-phase mixture of butanol and dilute hydrochloric acid. The ayfivin in the eluate was transferred from water to butanol, and back to water, by appropriate adjustment of the pH of the solutions, and was then precipitated as a picrate.

In the course of these experiments the potato-dextrose medium was replaced by the synthetic medium of Hills *et al.* (1949). No significant difference was noticed in the behaviour of the active material from the two types of culture fluid during the course of purification.

Adsorption on to charcoal.

The culture fluid was clarified in a Sharples centrifuge and the resulting fluid was stirred for 10 minutes with about 0·75 per cent (w/v) of active charcoal (Farnell Grade 14 neutral or Grade LS neutral). The charcoal was then separated in a Sharples centrifuge or on a large filter. Different charcoals varied greatly in their suitability; with certain acid charcoals no active material could subsequently be eluted. The exact amount of charcoal used depended on the culture fluid. The best results were obtained when the amount was sufficient to adsorb from 90 to 95 per cent of the activity. If larger quantities were used, elution became more difficult and the final yield was reduced, presumably because a larger proportion of the antibiotic was then held on the most strongly adsorbing centres.

Elution.

Ayfivin was eluted by stirring the charcoal corresponding to each 100 l. of culture fluid with a mixture of 1 l. of *n*-butanol and 4 l. of 0·2 N aqueous hydrochloric acid. Butanol was adsorbed preferentially by the charcoal from the two-phase mixture. After filtration the eluate consisted almost entirely of water saturated with butanol. The yield of ayfivin in the eluate was from 40 to 50 per cent of that present in the culture fluid.

The partition coefficient [ayfivin in 0·2 N HCl]/[ayfivin in *n*-butanol] is about 10. In consequence, ayfivin eluted by the butanol is largely displaced into the aqueous phase. Here it is excluded from direct contact with the charcoal since the latter is covered by a layer of butanol. The use of the two liquid phases thus greatly favours elution.

A method of eluting substances from charcoal with a mixture of water and an organic solvent immiscible with water, such as benzene, was described by Steenberg (1944). The procedure differed from that used with ayfivin in that the substance to be desorbed was insoluble in the organic solvent by which it was displaced from the charcoal. Ayfivin, which is insoluble in ether and in benzene, was only eluted from charcoal in very poor yield by a mixture of water and benzene, or water and ether.

Little ayfivin was eluted by a solution of 0·2 N HCl saturated with butanol, or by butanol saturated with aqueous 0·2 N HCl. Acid ethanol was at one time used, but loss of activity occurred during concentration of the relatively large volume of solvent required for elution.

Transfer between water and butanol.

The eluate was adjusted to pH 7, saturated with sodium chloride, and shaken with one-fifth of its volume of butanol, which extracted the ayfivin. One fifth of its volume of water and two volumes of ether were mixed with the extract, and the pH of the aqueous phase was adjusted to 2 with hydrochloric acid. The aqueous phase, which then contained the ayfivin, was freed from dissolved butanol by extraction with ether, and its pH adjusted to 5.

Precipitation of crude ayfivin picrate.

The aqueous solution was cooled in ice, and a saturated aqueous solution of picric acid was added until there was no further precipitate of ayfivin picrate. The picrate was filtered and dried *in vacuo*.

Crude ayfivin hydrochloride.

The picrate was decomposed by dissolving it in 0·2 N ethanolic hydrochloric acid. Ayfivin hydrochloride formed a white flocculent precipitate on adding dry ether to the solution. It contained 20 to 30 units/mg. The unit of ayfivin is described by Arriagada, Savage, Abraham, Heatley and Sharp (1949).

Further Purification. Counter-current Distribution Between Solvents.

A number of methods were investigated for the further purification of crude ayfivin. They included precipitation from aqueous solution by salts, chromatography under various conditions, ionophoresis in silica gel (Consden, Gordon and Martin, 1946), and counter-current distribution between solvents. The method

of counter-current distribution between solvents, developed in recent years by Craig and his colleagues (Craig, 1944), was the most promising. Some preliminary results with this method are described here.

Ayfivin appeared to have an isoelectric point at about pH 7·1, and measurements of antibacterial activity showed that its partition coefficient between water and butanol varied rapidly in this region (see below). It was therefore thought possible that the components of crude ayfivin might be separated most efficiently in a system with a pH near to 7. Subsequent experiments showed a striking difference between the amount of resolution obtained in neutral and in acid solution.

Distribution between an amyl alcohol-n-*butanol mixture and water or phosphate buffer at pH* 7.

The amounts of activity found in the two phases when crude ayfivin (from 0·5 mg./ml. to 10 mg./ml.) was distributed between water or $\frac{m}{10}$ phosphate buffer, at pH 7, and a mixture of amyl alcohol and *n*-butanol (4 : 1) indicated that the overall partition coefficient of the active material was close to unity. The activity of crude ayfivin could be raised by eight-tube counter-current distributions in this system. Different batches of ayfivin contained varying amounts of material of low specific activity which congregated at both ends of the series. In general, the most active fractions (of about 30 to 40 units/mg.) were found in Tubes 3, 4, and 5. The accuracy of the method of assay was too low to make an exact analysis of the results on the basis of activity. Nevertheless, comparison of the experimental distribution curve for the activity with theoretical curves for a single substance indicated that the material contained significant amounts of three active substances. A major active constituent with a partition coefficient close to 1 showed a maximum near the centre of the series, while minor constituents appeared nearer the ends of the series. Different batches appeared to contain somewhat different amounts of the minor active constituents. The results of experiments with two batches are described below.

(1) Using separating funnels to hold the solvents, several grammes of crude ayfivin could be purified in one experiment. For preparative purposes the material in each funnel was transferred to the water phase at the end of the distribution, by adjusting the pH to 2 with hydrochloric acid and adding three volumes of ether. The pH of the aqueous solutions was then brought to 5 with sodium hydroxide, and the material from the different funnels precipitated in the form of the relatively insoluble picrate.

Fig. 1 illustrates the distribution of activity and of total material when one batch of ayfivin (Batch A) was subjected to eight transfers in a system composed of equal volumes of mutually saturated amyl alcohol—*n*-butanol (4 : 1) and water. The distribution was begun with 2 g. of ayfivin (22 units/mg.) and the aqueous phase (50 ml.) adjusted to pH 7 ; the alcohol phase was mobile. No change in pH was detected during the distribution. The material appeared to contain three active constituents, which contributed about 15 per cent, 80 per cent and 5 per cent, to the total activity and concentrated in the tubes near the beginning, the centre and the end of the series respectively. The specific activity of the major constituent in Tubes 3, 4, and 5 was about 40 units/mg. Calculations of partition

coefficients from the relative amounts of total material in adjacent funnels (Williamson and Craig, 1947) did not indicate that any of the material was homogeneous.

(2) The results of an eight-tube distribution on a smaller scale and with a different sample of ayfivin (Batch B) are shown in Fig. 2. The crude material, which had an activity of 20 units/mg., was part of a large batch that has been reserved for more extensive distributions.

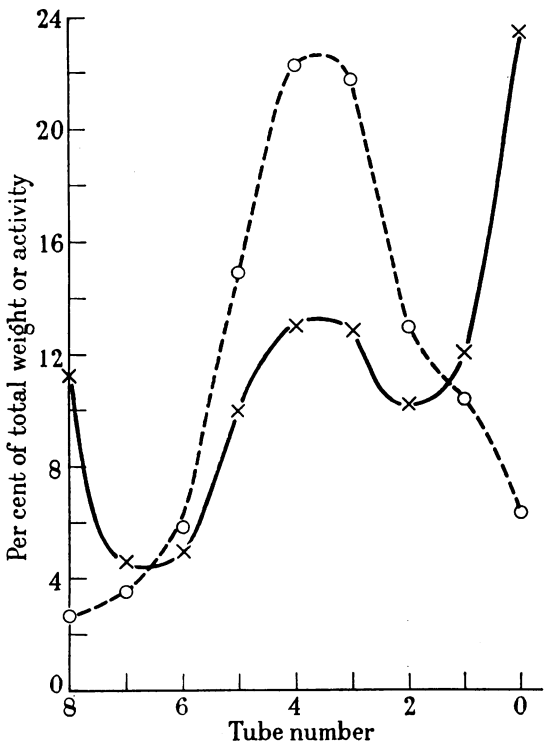

FIG. 1.—Eight-tube counter-current distribution of crude ayfivin (Batch A) between amyl alcohol-*n*-butanol (4 : 1) and water (pH 7). Upper phase mobile.

—×————×— Dry weight.
-○ - - - - - - - ○- Activity.

The experiment was carried out in stoppered test-tubes. The two phases, each of 8-ml., were amyl alcohol-*n*-butanol (4 : 1) and M/10 phosphate buffer at pH 6·8. The crude ayfivin hydrochloride (100 mg.) was dissolved in a mixture containing rather more than 8 ml. of each phase, and the pH was adjusted to 7 with N NaOH. The volume of each phase was made up to 9 ml., and a small amount of insoluble material was removed by centrifuging. Tube 0 was then filled with 8 ml. of each clear layer, and the remaining 2 ml. of the mixture was used for control determinations of dry weight and activity. The phosphate phase was mobile. At the end of the distribution 0·1 ml. amounts of both phases were removed from each tube and added to water (2 to 4 ml.) for determinations of

activity. The alcohols were extracted from the solutions by *n*-hexane before assay. The remaining alcohol phases were then removed, and the aqueous layers were extracted first with 3/5 volumes and then with 2/5 volumes of *n*-butanol. Each extract was mixed with the original alcohol phase from the corresponding tube. The weight of material in the extracts from each tube was determined by evaporating 4 ml. quantities of the solutions at 100° C. in a stream of air. The weight of phosphate extracted (< 0·05 mg./ml.) was negligible compared with the weight of peptide.

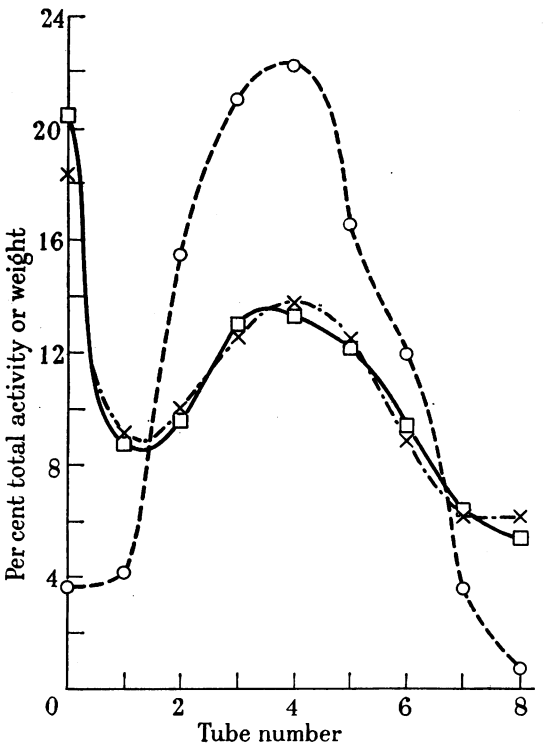

FIG. 2.—Eight-tube counter-current distribution of crude ayfivin (Batch B) between amyl alcohol-*n*-butanol (4 : 1) and m/10 phosphate buffer (pH 6·8). Lower phase mobile.

□————□ Dry weight. ○ - - - - - ○ Activity.
×—·—·—·—× Dry weight estimated by ninhydrin reaction.

The amounts of material in the different tubes were also estimated by using the ninhydrin reaction in the manner described by Moore and Stein (1948). Aliquots corresponding to from 20 to 150 μg. of material were evaporated to dryness, and the residues hydrolyzed with 0·3 ml. amounts of 20 per cent hydrochloric acid in sealed tubes placed in an oven at 110° C. for 18 hours. After removal of the hydrochloric acid the ninhydrin colours given by the amino acid mixtures were measured in a photoelectric colorimeter, and compared with those obtained from standard amounts of ayfivin. The method has proved convenient for the estimation of small amounts of material, and has given curves rather

close to those obtained by weighing. It will not be reliable if various peptides in a mixture give ninhydrin colours after hydrolysis which differ significantly in intensity.

In this distribution the material with the highest specific activity (about 30 units/mg.) was found in Tubes 3 and 4. The calculated partition coefficients of the material in the different tubes varied continuously, and no tube appeared to contain a homogeneous substance.

Distribution between sec. *butanol and* 0·5 N *acetic acid.*

The system already described had practical disadvantages. Its pH was that at which ayfivin showed a minimum solubility, and several manipulations were necessary to isolate the material in the different fractions, or to obtain it in a form suitable for the determination of dry weight. An alternative system was therefore investigated in which the partition coefficient of the activity had a convenient value. The two phases were prepared by mutually saturating equal volumes of *sec.* butyl alcohol and 0·5 N acetic acid. The pH of the aqueous phase was about 2·8. The system resembles that used by Barry, Gregory and Craig (1948) for the purification of commercial bacitracin.

The partition coefficient of ayfivin in this system expressed as $\frac{\text{activity per ml. in } sec. \text{ butanol}}{\text{activity per ml. in 0·5 N acetic acid}}$ was about 0·5. Fig. 3 shows the results obtained from an eight-tube distribution in which the volume of the butanol (4 ml.) was made twice that of the acetic acid (2 ml.) in order to bring the maximum activity near the centre of the series. The material used (80 mg.) was part of Batch B, whose distribution curve in the system at pH 7 is shown in Fig. 2. At the end of the experiment the amount of material in each tube was determined by evaporation of aliquots from the two phases. Activity was measured by the cylinder-plate method after adding 0·1 ml. of the alcohol layer, and 0·05 ml. of the lower layer, to an appropriate volume of water, and extracting the resulting aqueous solution with ether. An alternative method, in which all the material was transferred to the aqueous phase by the addition of 12 ml. of ether to each tube, gave almost identical results.

Fig. 3 shows that the distribution in this system, in contrast to the distribution in the system at pH 7, did not differ greatly from that to be expected if the preparation of ayfivin had consisted of a single substance. The fact that the curve rose somewhat more sharply to a maximum than the theoretical curve for homogeneous material could be attributed to small changes in the relative volumes of the phases, which occurred during the transfers. Except for Tube 8, which contained a small amount of inactive material, the activity-curve was roughly superimposable on the weight-curve. The specific activity of the material isolated from Tubes 3, 4 and 5 was no greater than that of the starting material. Very little resolution had therefore occurred under these conditions.

Further observations.

Although it has been established that the specific activity of crude ayfivin may be raised considerably by eight-tube counter-current distributions at pH 7, the units of activity in the separate fractions have usually appeared to total not

more than 80 per cent of those in the original material. It is thought probable that the loss of activity is partly caused by small amounts of impurities in the solvents used. Inactivation caused in this way becomes more serious the purer the ayfivin and the more dilute the solutions, and has complicated work on the further purification of the material.

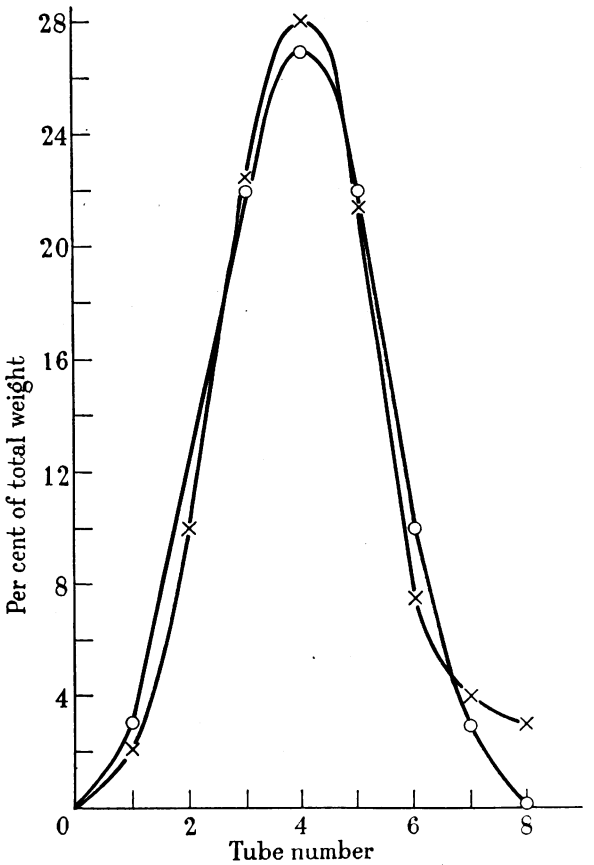

FIG. 3.—Eight-tube counter-current distribution of crude ayfivin (Batch B) between *sec* butyl alcohol and 0·5 N acetic acid. Lower phase mobile.

×———× Dry weight.
○———○ Theoretical curve for a single substance.

Physical and Chemical Properties.

The physical and chemical properties described here refer to preparations of ayfivin with an activity of about 40 units/mg., which were obtained from Fractions 3, 4, and 5 of eight-tube counter-current distributions at pH 7. These fractions contain the main active constituent of the crude material, but there is evidence that they are not homogeneous. Some of the properties will therefore need re-investigation if pure ayfivin becomes available.

Ayfivin hydrochloride gave the following results on elementary analysis:

C, 48·8 ; H, 7·3 ; N, 14·6 ; S, 1·5 ; Cl, 7·9. It contained 2·2 per cent of amino nitrogen according to the van Slyke method of determination. It showed no strong absorption bands in ultraviolet light, but had a weak blue fluorescence. It was very soluble in water or ethanol, sparingly soluble in acetone, and insoluble in ether. It dialyzed readily through a cellophane membrane.

Free ayfivin was much less soluble in water than the hydrochloride.

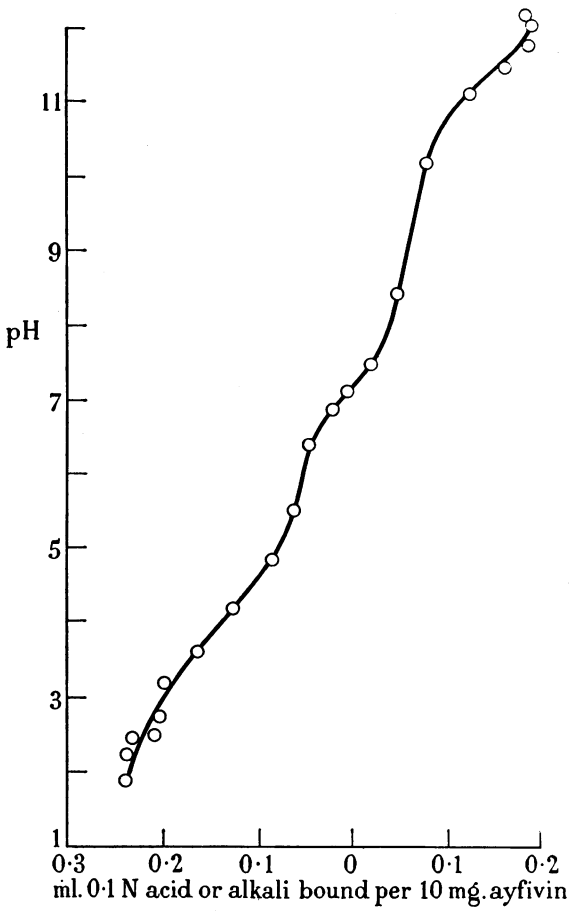

FIG. 4.—Titration curve of ayfivin.

Ionizable groups

Ayfivin behaved as a weak base. An aqueous solution of its hydrochloride had a pH of about 2·5. Electrometric titration, using a hydrogen electrode, showed that it contained groups ionizing between pH 2 and 5, pH 5·5 and 8·5, and pH 10 and 12 (Fig. 4). Changes in the partition coefficient of the activity between water and *n*-butanol when the pH was varied from 5·5 to 8·5 (see below) showed that ionization in this region was a property of the active material itself. The titration curve was compatible with the assumption that ayfivin consisted of

polypeptide. Buffering in the pH range 2–5 could be attributed to the free carboxyl groups of dicarboxylic amino acids; buffering in the pH range 5·5 to 8·5 could be due to the iminazole group of histidine and an α-amino group; and buffering in the pH range 10 to 12 could be due to free amino groups of diamino monocarboxylic acids. The " isoelectric point " of the material was at pH 7·1.

Solubility.

Ayfivin was extracted almost completely from aqueous solution between pH 2 and pH 10 by shaking with an equal volume of phenol saturated with water. It was not extracted significantly from water at any pH by ether, chloroform, amyl acetate, or benzene.

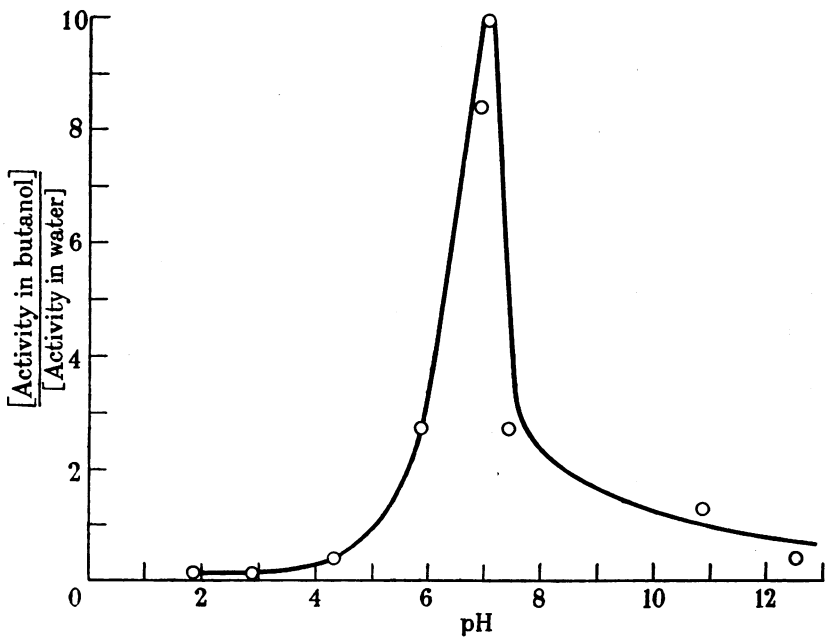

FIG. 5.—Variation with pH of the partition coefficient of ayfivin between *n*-butanol and water.

The partition coefficient of ayfivin (2 mg./ml.) between water and *n*-butanol varied greatly with the pH of the aqueous phase. Fig. 5 shows the results obtained by measuring the activity in the two phases. The relative solubility in butanol reached a maximum at the isoelectric point (pH 7·1), and fell sharply on either side of this value. The partition coefficient between water and amyl alcohol varied similarly with pH, though ayfivin was considerably less soluble in amyl alcohol than in butyl alcohol.

Precipitation and colour reactions.

Aqueous solutions of ayfivin gave amorphous precipitates with picric acid, picrolonic acid, Reinecke salt, mercuric chloride, and trichloracetic acid, and also on the addition of saturated ammonium sulphate or sodium chloride. They formed no precipitate with saturated aqueous solutions of benzoic or salicylic acid.

When benzoic acid or salicylic acid was precipitated in aqueous solutions of ayfivin very little activity was carried down on the precipitate.

Ayfivin gave a positive ninhydrin reaction. It gave no coloration with ferric chloride, or with alkaline nitroprusside in the presence or absence of cyanide. The Sakaguchi reaction for arginine and the Adamkiewicz reaction for tryptophane were negative. Knoop's reaction for histidine was positive.

Stability.

Ayfivin was stable for 10 minutes when heated at 100° C. in aqueous solution (1 mg./ml.) at pH 2, 7, or 10. It was stable in 2 N acid for 5 minutes at room

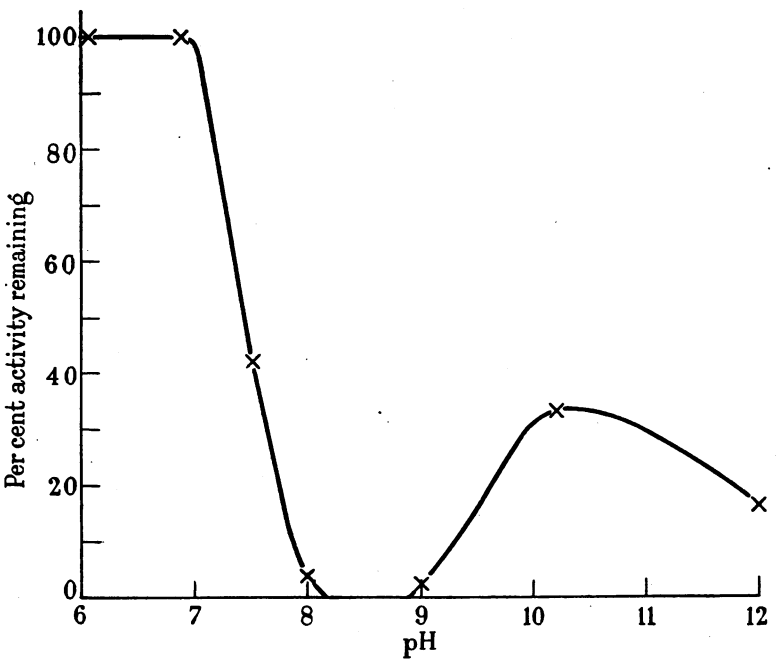

FIG. 6.—Inactivation of ayfivin in an aqueous solution (0·1 mg./ml.) at different pH values after 4 hours at 37° C.

temperature, but was inactivated when heated in 2 N acid at 100° C. for 5 minutes. It was largely inactivated when kept in N/10 sodium hydroxide for 4 hours at room temperature, or heated in N/100 sodium hydroxide for 15 minutes at 100° C. It was not inactivated by incubation with pepsin at pH 3, or with trypsin at pH 8.

Inactivation by copper ions.

In early experiments with purified ayfivin it was found that material kept at 37° C. for 4 hours in dilute aqueous solution (0·1 mg./ml.) showed a maximum instability between pH 8 and pH 9 (Fig. 6). Between pH 3 and pH 7 no activity was lost, and at pH 10–11 about 70 per cent of the activity was lost, while at pH 8–9 the loss of activity was almost complete. When dilute solutions of ayfivin at pH 8–9 were heated to 100° C. there was almost total loss of activity in 2 minutes

In contrast to the behaviour of purified material, the original culture fluid which had been adjusted to pH 8–9 showed no detectable loss of activity when kept for 4 hours at 37° C., or for 2 minutes at 100° C. The culture fluid therefore contained sustances that protected ayfivin from inactivation. It was found that the inactivation of purified ayfivin at pH 8–9 was largely or completely prevented by the following additions to the solution: commercial trypsin or pepsin (1 mg./ml.); horse serum (1 per cent); heart or lemco broth; glutamic acid, cysteine, or glycine (1 mg./ml.). Gelatine (1 mg./ml.) exerted some protective action, but was less effective than the other substances.

When the concentration of ayfivin was increased from 0·1 mg./ml. to 1 mg./ml. very little loss of activity was detected on keeping the solution at pH 8–9 for 4 hours at 37° C. The change in apparent stability with concentration suggested that the inactivation was caused by traces of an impurity in the solvent (laboratory distilled water). If the amount of ayfivin inactivated depended largely on the amount of the impurity, and thus on the volume of the solvent, the *proportion* of ayfivin inactivated would diminish as its concentration was increased.

It is now considered that a reaction of ayfivin with copper ions was responsible for this phenomenon. When the early experiments were later repeated no inactivation was found to occur. In the interval a new apparatus had been installed to supply distilled water. Inactivation occurred at pH 8–9 in the new distilled water, but not at pH 7 or below, when small amounts of copper sulphate were added to the solution. Table I shows the results obtained when ayfivin (0·1 mg./ml.) was incubated for 4 hours at pH 8·4 with varying amounts of cupric ions.

An amount of Cu^{++} as small as 0·25 μg./ml. caused substantial inactivation. Little, if any, inactivation was caused under similar conditions by a number of other heavy metal ions in concentrations approximately 10 times as large. The ions tested were Pb^{++}, Fe^{+++}, Zn^{++}, Co^{++}, Mn^{++}, Al^{+++}, Sn^{++}, and Hg^{++}. The loss of activity, which occurred in the presence of 2·5 μg. Cu^{++} per ml., was prevented by the addition of glutamic acid (1 mg./ml.) to the solution.

Attempts to restore the activity of ayfivin inactivated in the presence of cupric ions have not so far been successful.

TABLE I.

Cu++ μg./ml.	Per cent of activity remaining.
25·0	0
2·5	0
0·25	10
0·025	90
0·0025	100
0·00	100

Amino acids formed on acid hydrolysis.

When ayfivin was hydrolyzed for 24 hours with boiling 20 per cent hydrochloric acid almost all of its nitrogen appeared as α-amino nitrogen. The product showed a much stronger ninhydrin reaction than the original substance. When the ninhydrin reaction was carried out in the manner described by Moore and Stein (1948) the intensity of the colour shown by the hydrolysate from a given

weight of ayfivin hydrochloride was very close to that shown by the same weight of leucine. The hydrolysate gave a positive test for SH with nitroprusside in the presence, but not in the absence, of sodium cyanide.

Paper chromatography.

Using the method introduced by Consden, Gordon and Martin (1944), one-dimensional chromatograms on Whatman No. 1 paper were obtained from the ayfivin hydrolysate with the phenol-water system in the presence of HCN, with the phenol-water system in the presence of ammonia, and with the benzyl alcohol-water system. Chromatograms were also obtained from the acidic and from the basic plus neutral fractions of the hydrolysate which had been separated on Amberlite IR-4 by the method of Consden, Gordon and Martin (1948). The positions of the spots after development with ninhydrin indicated that the following amino acids were present: aspartic acid, glutamic acid, lysine, ornithine, histidine, cystine, phenylalanine, leucine and/or isoleucine. The amount of the leucines appeared to be larger than that of any other single amino acid.

Oxidation with D-amino acid oxidase.

The oxygen absorbed on treating ayfivin hydrolysate with a preparation of D-amino acid oxidase (Krebs, 1935) at 37° C. was measured in the Warburg manometric apparatus. The activity of the enzyme system was tested with DL-alanine as a substrate: the reaction was complete in 1 hour, and the oxygen taken up corresponded to the theoretical amount for the oxidation of the D-component. With ayfivin hydrolysate as a substrate the reaction appeared to be complete in 2 hours, and the amount of oxygen absorbed corresponded to 14 c.mm. per mg. of ayfivin hydrochloride. On this basis about 14 per cent of the nitrogen of the ayfivin hydrolysate was in the form of D-α-amino nitrogen.

DISCUSSION.

There can be little doubt that the main active constituent of ayfivin is a highly polar polypeptide. Although there is evidence that the most active preparations described in this paper are not homogeneous, their impurities appear to have physico-chemical properties and to contain amino acid radicals similar to those of the antibiotic itself. Even in the case of the crude product, the solubilities of the impurities were such that very little resolution occurred during an eight-tube counter-current distribution under acid conditions, and the material behaved as though it were largely a single compound. The fact that this material was quickly resolved into several components in a system at pH 7, and that some of the resulting fractions showed a considerable increase in specific activity, indicates that conclusions about the homogeneity of a polypeptide which are based on results from a restricted number of distributions in one system can easily be too optimistic. Craig, Mighton, Titus and Columbic (1948) have pointed out that the results of counter-current distributions, like those of all other methods, can only provide an answer to the question of the purity of a substance in terms of probability. With relatively large molecules several different criteria are desirable before the probability of a substance being homogeneous is considered to be very high.

The variation in the partition coefficient of ayfivin between butanol and water with changes in pH indicates that the active substance contains groups

that ionize over a wide range of hydrogen ion concentrations. The presence of such groups in the purified material is shown by the results of electrometric titration and of qualitative amino acid analyses. Anionic centres are provided by aspartic acid and glutamic acid, and cationic centres by histidine, ornithine and lysin. The hydrophobic side-chains of leucine and phenylalanine, which appear to be the main neutral amino-acids, would account in part for the high solubility of ayfivin in moist *n*-butanol at the isoelectric point. At this point the solubility may be increased by a displacement of equilibrium from the polypolar towards the non-charged form.

The electrometric titration of the purest preparation reveals a group which titrates in the region immediately on the alkaline side of the isoelectric point. A group in ayfivin, which ionizes in this region, may be concerned with the inactivation of the antibiotic that occurs under certain conditions in the presence of copper ions. The amount of inactivation is negligible at pH 7, but rapidly reaches a maximum at pH 8·5. A copper co-ordination complex is possibly involved in the reaction. The fact that there is a decrease in the amount of inactivation when the pH is raised from 8·5 to 10 would be understandable if a group inessential for antibacterial activity, either in ayfivin itself or in impurities, competed for copper at the higher pH. Competition by added amino acids and proteins has been shown to prevent inactivation.

An antibiotic named licheniformin, which is produced by a strain of *B. licheniformis*, has been studied by Callow, Glover and Hart (1947), and by Callow, Glover, Hart and Hills (1947). When grown under appropriate conditions, the strain A-5 of *B. licheniformis* also produces a licheniformin-like antibiotic (Hills *et al.*, 1949). Ayfivin and licheniformin differ markedly in chemical properties. Ayfivin, however, shows similarities to the antibiotic bacitracin, first described by Johnson, Anker and Meleney (1945). When the present work began little published information on the chemical properties of bacitracin was available, and it was doubtful whether the substance was a peptide (Anker, Johnson, Goldberg and Meleney, 1948). Both ayfivin and bacitracin could be extracted from culture fluid with *n*-butanol, but it was thought unlikely that they were identical because bacitracin was reported by Johnson, Anker, Scudi and Goldberg (1947) to be precipitated by benzoic acid and salicylic acid, and these reagents did not remove ayfivin from solution. A sample of commercial bacitracin, however, has now been found to behave like ayfivin towards benzoic and salicylic acid. The work of Barry, Gregory and Craig (1948) has established that purified preparations of bacitracin consist of polypeptide, and the amino acids they are reported to contain are very similar to the amino acids present in the most active preparations of ayfivin. Further investigations are required to establish the exact relationship between the two antibiotics.

Since this paper was written it has been shown that the inactivation in the presence of copper ions is accompanied by an uptake of oxygen and that it probably involves the oxidation of a suphur atom.

SUMMARY.

1. Preparations of ayfivin with high antibacterial activity were obtained from the culture fluid of the strain A-5 of *B. licheniformis*. The process of extraction involved adsorption of the active material on to charcoal, elution with a mixture

of *n*-butanol and dilute hydrochloric acid, transfer between butanol and water, and precipitation with picric acid. The crude product was further purified by 8-tube counter-current distributions in a system composed of amyl alcohol, *n*-butanol, and water or phosphate buffer at pH 7. No significant purification was obtained by 8-tube distributions in a system containing acetic acid at about pH 2·8.

2. The most active material behaved as a polypeptide with an isoelectric point at pH 7·1. It contained groups which ionized in the pH region 2–5, 5·5–8·5, and 10–12. On acid hydrolysis it yielded aspartic acid, glutamic acid, histidine, ornithine, lysine, cystine, phenylalanine, leucine and/or isoleucine.

3. The partition coefficient (ayfivin in *n*-butanol)/(ayfivin in water) varied rapidly with the pH in region of neutrality and was a maximum at the isoelectric point. On the alkaline side of the isoelectric point ayfivin was inactivated in the presence of copper ions, the amount of inactivation reaching a maximum at pH 8–9.

4. Ayfivin is quite different from licheniformin in its physical and chemical properties, but shows a similarity to bacitracin.

We are greatly indebted to Dr. D. W. Henderson and Mr. G. M. Hills of the Microbiological Research Department, Experimental Station, Porton, who supplied us with large amounts of culture fluid. Our thanks are also due to Miss R. Callow and Miss A. Pill for expert technical assistance.

This investigation has been aided by a grant to one of us (V.E.S.) from the Albert and Mary Lasker Foundation, and by the Medical Research Council, who have contibuted to technical and other expenses.

REFERENCES.

ANKER, H. S., JOHNSON, B. A., GOLDBERG, J., AND MELENEY, F. L.—(1948) *J. Bact.*, **55**, 249.

ARRIAGADA, A., SAVAGE, M. C., ABRAHAM, E. P., HEATLEY, N. G., AND SHARP, A. E. —(1949) *Brit. J. exp. Path.*, **30**, 425.

BARRY, G. T., GREGORY, J. D., AND CRAIG, L. C.—(1948) *J. biol. Chem.*, **175**, 485.

CALLOW, R. K., GLOVER, R. E., AND HART, P. D'A.—(1947) *Biochem. J.*, **41**, xxvii.

Iidem AND HILLS, G. M.—(1947) *Brit. J. exp. Path.*, **28**, 418.

CONSDEN, R., GORDON, A. H., AND MARTIN, A. J. P.—(1944) *Biochem. J.*, **38**, 224.—(1946) *Ibid.*, **40**, 33.—(1948) *Ibid.*, **42**, 443.

CRAIG, L. C.—(1944) *J. biol. Chem.*, **155**, 519.

Idem, MIGHTON, H., TITUS, E., AND GOLUMBIC, C.—(1948) *Anal. Chem.*, **20**, 134.

HILLS, G. M., BELTON, F. C., AND BLATCHLEY, E. D.—(1949) *Brit. J. exp. Path.*, **30**, 427.

JOHNSON, B. A., ANKER, H., AND MELENEY, F. L.—(1945) *Science*, **102**, 376.

JOHNSON, B. A., ANKER, H., SCUDI, J. V., AND GOLDBERG, J.—(1947) Paper presented at the Conference on Antibiotic Research held at Washington, D.C., Jan. 31–Feb. 1, under the auspices of the National Institute of Health.

KREBS, H. A.—(1935) *Biochem. J.*, **29**, 1620.

MOORE, S., AND STEIN, W. H.—(1948) *J. biol. Chem.*, **176**, 363.

STEENBERG, B.—(1944) 'Absorption and Exchange of Ions on Activated Charcoal.' Uppsala (Almquist and Wiksell).

WILLIAMSON, B., AND CRAIG, L. C.—(1947) *J. biol. Chem.*, **168**, 687.