The Preparation and Some Properties of Purified Micrococcin

By N. G. HEATLEY and HAZEL M. DOERY*
The Sir William Dunn School of Pathology, University of Oxford

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The formation of the antibiotic micrococcin by a species of *Micrococcus*, since given the number 7218 in the National Collection of Type Cultures, and its extraction from active broth, were described by Su (1948). The properties of his purest preparation, a colourless, amorphous powder, now believed to have been about 85% pure, were also given. The material is almost insoluble in water, but large doses can be given intravenously in finely divided form, the particles being rapidly taken up by the cells of the reticulo-endothelial system and persisting in the body for weeks or months. It can also be solubilized by certain detergents, and the results of animal experiments with particulate and solubilized micrococcin are described elsewhere (Markham, Heatley, Sanders & Florey, 1951; Markham, Wells, Heatley & Florey, 1951; Sanders, Florey & Wells, 1951; Heatley, Gowans & Florey, 1951).

Experience gained during the working up of some of the large batches of material needed for the animal experiments is described below, together with some chemical and physical properties of micrococcin.

EXPERIMENTAL

Methods

Source of crude culture fluid

Some batches of about 110 l. each were set up at Oxford in Lemco broth, as described by Su (1948), in aerated 5–10 l. bottles. In December 1948 two batches of 81 and 84 gal. were prepared in the Clevedon laboratories of the Distillers Co. Ltd.; the crude broth was acidified and after settling, the slurry was sent to Oxford for working up. Further batches were prepared at Clevedon under the control of the Medical Research Council. Later, the whole of the extraction, up to the preparation of a fairly pure amorphous solid, was done at Clevedon. During this work a cheaper medium giving a much enhanced yield of micrococcin was developed, and some observations were made on the biochemistry of the fermentation (Kelly, Miller & Hale, 1951).

Assay

Methods for the assay of micrococcin are reported elsewhere (Heatley, Kelly & Smith, 1951).

Extraction and isolation

Of the three processes described by Su (1948), the precipitation process was the most convenient and was used in the present work, but the following practical points may be worth mention.

General procedure. The crude culture fluid was adjusted to pH 2 and, after settling, the clear supernatant was discarded and the slurry centrifuged. The solid was extracted with ethanol two or three times and the extracts concentrated by vacuum distillation until all ethanol had been driven off; if necessary, water was added as the distillation neared completion. At this stage, depending on the nature and amount of anti-foam agent used in the fermentation, an extraction with ether was sometimes done, the ether being discarded. The watery suspension of pasty solid was rinsed from the flask with $CHCl_3$ into a separating funnel and well shaken with 5–6 successive portions of $CHCl_3$. The $CHCl_3$ extracts were passed through a column of Brockmann type alumina, starting with the last $CHCl_3$ extract. (Alternatively the weakest extracts were kept and used for the first extractions in the next batch.) The column was well washed with fresh $CHCl_3$, the purple fluorescent band was cut out, dried in air, and eluted with ethanol. The band containing the micrococcin was sharpened but not appreciably developed by $CHCl_3$, and each ml. of alumina in this band adsorbed 5–15 mg. of micrococcin. (The fact that the micrococcin was not washed through the column by large volumes of $CHCl_3$ suggested the possibility of an alumina trap as part of a continuous cyclical extraction procedure, but this was not followed up.)

Occasionally the fluorescent band was more diffuse and passed down the column more or less readily. This was traced to the presence of a small amount of ethanol in the $CHCl_3$. Care was therefore taken to remove all ethanol from the ethanolic extracts and from the $CHCl_3$.

Technique of ethanolic extraction. It was difficult to disperse the clay-like centrifuged acid precipitate of the crude culture fluid evenly in the ethanol except on a very small scale, and the extraction was correspondingly incomplete or tedious. A satisfactory solution, which might be of general use, was to allow the original acid precipitate to settle by gravity, then gradually to mix the uncentrifuged creamy slurry with a large volume of ethanol. The extraction was efficient, but a larger volume of extract had to be dealt with, and as the percentage of water was higher it is possible that more or different impurities were extracted.

The most convenient method was the following. The delivery tube of a centrifugal pump was connected to a nozzle of about 4 mm. internal diameter arranged within a large funnel as shown in Fig. 1 A. The stem of the funnel was connected to the intake of the pump, and enough ethanol was added to half fill the funnel and automatically prime the pump. The acid-precipitated solid, whether packed in the bowl of a continuous centrifuge of the Sharples type, or in separate centrifuge bottles, was dislodged by the stream of ethanol from the jet and fell into the funnel, from which it was re-circulated through the pump. After all the precipitate had been dislodged, the suspension was circulated for some minutes, at the end of which time a smooth finely divided suspension containing the minimum amount of water was obtained. After centrifuging, the sediment was

* Present address: Commonwealth Serum Laboratories, Melbourne, Australia.

readily re-suspended in fresh ethanol (for a second extraction) by shaking by hand. For handling larger amounts of precipitate the modification shown in Fig. 1 B was used.

Elution from alumina. The air-dried purple fluorescent zone of the alumina column was poured into another column and percolated with ethanol. The first few ml. of percolate were very concentrated—probably saturated, or nearly so—but became less so as elution proceeded. Although each drop of eluate was always perfectly clear a pad of precipitated amorphous material often formed in the first few ml. of eluate, although if this were collected in two

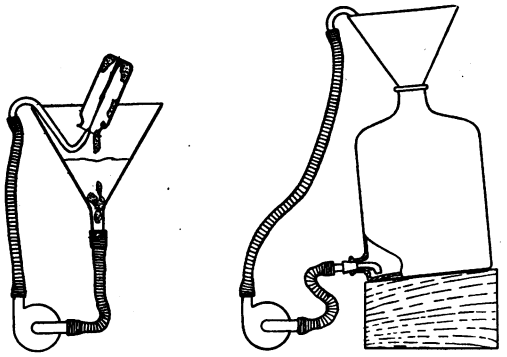

Fig. 1. Methods for suspending initial precipitate in ethanol. For explanation see text.

or three fractions each remained perfectly clear. Micrococcin is more soluble in 90% than in 100% ethanol (see below). When the almost water-free ethanol is passed through the column it probably removes some water from the air-dried alumina and thus acquires an increased solvent power for micrococcin. The water content of the successive eluates falls and the combined total eluate becomes supersaturated with micrococcin, which is precipitated.

Crystallization of micrococcin

Prolonged attempts to crystallize micrococcin were unsuccessful—Su (1948) reported the same—but very small crystals appeared spontaneously in an ethanolic concentrate which had been standing at room temperature for 2 or 3 weeks. In spite of this piece of good fortune it still proved impossible to obtain crystals from some concentrates of high activity and it was uncertain whether there were one or more active substances. Chromatography and an 8-funnel counter-current distribution by the method of Craig, Golumbic, Mighton & Titus (1945) in a mixture of water, $CHCl_3$, carbon tetrachloride, glacial acetic acid and ethanol gave no evidence for the presence of more than one active substance, and it was known that recrystallized material could come out of solution in the form of a gel. In some cases seeding of such a gel with crystals would lead to the gradual and complete conversion of the whole gel to crystalline form, either at room temperature or at 4°. On the other hand, certain gels would be only partly converted to crystals when seeded, no matter at what temperature or for how long they were left.

Another observation suggesting the occurrence of more than one active substance was the apparent variation in solubility of the material from different batches. Thus, it had been known that solutions containing 10 mg. of micrococcin per ml. were supersaturated, for seeding crystals increased in size; in certain other solutions, however, containing double the concentration of substance in terms of dry weight, activity, and fluorescence, similar seeding crystals not only failed to increase in size but actually disappeared. This at first appeared to be almost overwhelming evidence for the occurrence of more than one active substance, but was later explained by the presence of a little more water in the ethanol in the second experiment than in the first. (This could easily happen through the use of different batches of recovered ethanol, as was the practice at the time.)

The difficulty of crystallizing micrococcin seems to be due first to the rather exacting conditions required, and secondly to the presence, at any rate in some preparations, of an impurity which inhibits crystallization. Evidence for such an impurity is the fact that a gel which refuses to crystallize, if filtered as completely as possible from its mother liquor, then redissolved in fresh ethanol, will furnish at any rate a partial crop of crystals. Also, recrystallization of material which has already been crystallized never presents difficulty.

With more experience it has proved possible to obtain crystals regularly, the procedure being as follows:

The crude dry solid is dissolved in sufficient warm azeotropic ethanol to give a solution containing not more than about 20 mg./ml. This is filtered, then placed at 37°. Occasionally spontaneous crystallization occurs, but usually the solution remains supersaturated for many hours. It is seeded with a suspension of crystals in ethanol—this is important, for when dried the crystals quickly lose their solvent of crystallization and become amorphous. After some hours the suspension is gradually cooled, a convenient way of accomplishing this in the absence of a slowly cooling thermostat being to transfer the container (well wrapped, or preferably in a Dewar jar) to the incubator at 24°, to room temperature, and then to the refrigerator, at least 2 days being allowed for the transition.

Crystals can equally well be obtained from ethanolic solutions containing more water, but the initial concentration of micrococcin must then be appropriately increased.

Occasionally an amorphous gel is obtained in spite of slow cooling of a slightly supersaturated solution. In such a case the gel, which has positive thixotropic properties, is filtered off from the mother liquor as completely as possible and redissolved in ethanol, a crop of crystals being almost always obtained.

The mother liquor from which crystals had been removed still contained active material, but little or none of this was obtained in crystalline form in spite of further chromatography and repeated deposition from slowly cooled, seeded ethanolic solution. It separated as a compact brown granular solid which tended to adhere firmly to the walls of the container. In antibacterial activity, fluorescence, solubility in ethanol-water mixtures and in other solvents, melting point behaviour, and inactivity against a staphylococcus made resistant to micrococcin, it behaved like nearly pure micrococcin. The possibility that it is not identical with micrococcin cannot be ruled out, and it may or may not be significant that the chromatogram was not quite typical of that obtained in the first stage of purification, in that the purple fluorescent zone was less marked, and more of the activity appeared to occur in the top, less fluorescent, brown, band.

Chemical properties

Elementary composition

A finely divided, twice recrystallized, well mixed sample, pure white in colour, was found to contain C, H, N and S; halogens and P were absent. Some of the material was submitted to Drs Weiler and Strauss for C, H, N, S, *C*-Me and OMe determinations, with the following results: N, 13·6; S, 15·9; OMe, nil; *C*-Me, 6·9; C, 49·0; H, 4·6; C and H residue, 2·4 %.

In view of the residue, a sulphated ash determination of the same sample was asked for, a value of 3·77 being obtained. Attempts were made by the authors to obtain ash for further examination, but after much work and several consultations with, and further analyses by, Dr F. B. Strauss, the conclusion was reached that no true ash, other than traces of alumina and a trace of volatile ash, was present, but that during combustion an unusually stable organic residue might be left. This remained even after a glow had passed through the solid in an atmosphere of O_2; it was black in colour, and contained C and H. Dr Strauss kindly examined the emission spectrum of the same sample of micrococcin with Cu electrodes, and reported that there was no indication of the presence of any metal other than alumina.

Two more analyses by Drs Weiler and Strauss on the same sample of micrococcin gave the following values: (1) C, 49·5; H, 4·6; residue, 1·5; (2) C, 49·3; H, 4·6; no residue. $C_{25}H_{23}O_6N_6S_3$ requires: C, 49·7; H, 4·6; N, 13·9; S, 15·9 %; but owing to the special difficulty of combustion the analytical figures are probably subject to errors greater than usual.

Molecular weight

By Barger's method in 80 % ethanol + 20 % water with azobenzene as reference compound, the molecular weight appeared to be slightly greater than 2170 and definitely less than 2720. However, the solutions were of the order of 0·01-M and thus near the lower limit of workability of the method.

Behaviour to heat

Melting point. The material darkens and sinters at 222–228°, but does not have a sharp melting point.

On heating alone the powder softens to a yellowish syrup which darkens and bubbles, emitting a distinct but indefinable odour. On further heating yellow fumes are evolved and a tar condenses on the cooler parts of the tube, while the odour of ammonia can just be detected in the alkaline vapours. As the tar is driven off the black residue becomes hard and brittle.

On heating with lime ammonia is evolved.

On treating with a drop of sulphuric acid micrococcin chars at once, and on gently warming the black treacly mixture swells up and bubbles, as would a carbohydrate. There is a strong smell of acetic acid at first, which is later replaced by an equally strong smell of acetamide.

Tests for reactive groups

As shown above, methoxyl groups were absent, and the material contained 6·9 % of *C*-Me.

Ethanolic ferric chloride gave no appreciable colour with an ethanolic solution of micrococcin, and the nitrous acid test for phenols (Feigl, 1946) was also negative. Sodium nitrite in contact with a glacial acetic acid solution of micrococcin for 30 min. at room temperature before treatment with urea and neutralization caused no change in either fluorescence or antibacterial activity and no gas was evolved. Negative reactions were obtained with Schiff's and Millon's reagents and with ninhydrin. A hydrolysate prepared by digesting a 1 % solution of micrococcin in equal parts of concentrated hydrochloric acid and glacial acetic acid for 21 hr. in a sealed tube at 100–110° gave a clear solution while hot, but when cold a small amount of creamy coloured flocculent solid appeared. A ninhydrin-reacting substance was found in the hydrolysate, but this was identified as ammonia by the formation of ammonium picrate in the Conway diffusion apparatus. Ammonia present in the hydrolysate accounted for 24·2 % of the total nitrogen in micrococcin.

When solid micrococcin was added to a mixture of iodine and sodium azide (Feigl, 1946; test for —SH and =S) there was no trace of evolution of gas, but the particles of micrococcin stained brown. On adding an ethanolic solution of micrococcin to the reagent some of the micrococcin was precipitated, but there was still no evolution of gas.

Bromine was only slowly decolorized by dilute solutions of micrococcin, but both fluorescence and antibacterial activity were reduced. When micrococcin in solution in aqueous ethanol was treated with zinc and hydrochloric acid there was a pronounced smell of hydrogen sulphide and after 0·5 hr. both fluorescence and antibacterial activity had fallen to about 1 % of their original value.

Physical properties

Crystalline form

The crystals consist of fine long silky needles—sometimes extremely long in relation to their thickness—nearly always aggregated into fans, sheaves, or little balls. They are birefringent, showing crossed extinction, and in the dry state lose solvent of crystallization and birefringence and become pseudomorphs. On the other hand, in bulk the ethanol of crystallization is lost only slowly, even over calcium chloride in a vacuum desiccator, and partially dried crystalline material can apparently reabsorb ethanol from the vapour phase.

Solubility

In water. Although the microbiological assay of a saturated watery solution presented no difficulty the preparation of a just saturated solution was by no means easy. At room temperature values of less than 0·2 to more than 1·5 μg./ml. were obtained, and it was impossible to decide whether with the lower values the solution was not saturated, or whether with the higher values some particulate matter was present. None of the methods of preparing a saturated solution which were tried was satisfactory, but the solubility of micrococcin in water at room temperature is believed to be about 1 μg./ml.

In aqueous ethanol. The solubility was found to depend very markedly on the proportions of water and ethanol in the solvent, reaching a maximum at

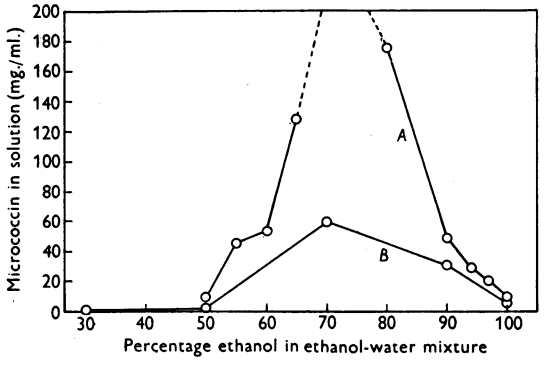

Fig. 2. Solubility of micrococcin in ethanol-water mixtures at 25±1° (curve *A*) and 2·8±0·1° (curve *B*).

about 75% ethanol, and falling steeply on either side. The actual values given in Fig. 2, which summarizes a series of experiments, are subject to several minor errors:

The process of saturation was carried out in constant temperature rooms where the suspension could be centrifuged and a sample of supernatant taken without materially changing the temperature, but the temperature in the rooms was seldom exactly the same at successive samplings. With the more dilute solutions it was found that 2 hr. or more might be required to reach equilibrium and with the stronger solutions, whose viscosity rose until at about 200 mg./ml. the fluid would hardly flow when the tube was inverted, very much longer might be required. Occasionally, with the stronger solutions, the whole might set to a stiff gel (due, perhaps, to a slight fall in temperature) which could not be centrifuged down. Then again, each time a sample was withdrawn, the composition of the solvent might change slightly by evaporation of ethanol and absorption of water, and owing to the steepness of the curve cause a significant change in solubility actually found.

In chloroform. In this solvent, whether carefully dried, or wet, solid micrococcin dissolved very rapidly. The excess solid became gelatinous and swelled until substantially the whole volume of the solution was occupied by a gel which was more or less rigid depending on the amount of solid taken. This interfered with sampling and it was not possible to obtain reproducible results, but at 20° the solubility in either dry or wet chloroform appeared to be about 8 mg./ml.

Ultraviolet fluorescence

Dry micrococcin had a definite but not especially powerful purple fluorescence in ultraviolet light. In solution it had a powerful purple fluorescence in all solvents tested, except in phenol. (This was due to

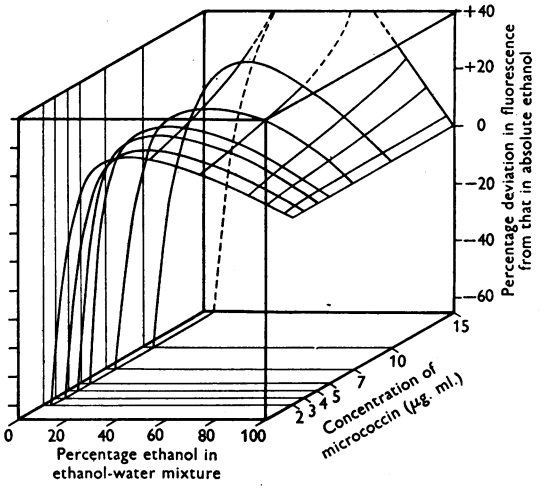

Fig. 3. The effect of the composition of the solvent on the expected fluorescence of micrococcin solutions, for different concentrations of micrococcin. (From Heatley, Kelly & Smith, 1951.)

quenching of fluorescence, not destruction of micrococcin, for removal of the phenol with ether restored the fluorescence.) The quantitative observations of fluorescence were made in the Beckman model DU spectrophotometer fitted with fluorimeter attachment, and were expressed as apparent concentration of micrococcin, as read from calibration curves (Heatley, Kelly & Smith, 1951).

Effect of temperature. There was no difference by eye between the fluorescence of ethanolic solutions at +2° and +20°, but at boiling point the fluorescence was slightly diminished.

Effect of dissolved oxygen. The fluorescence (measured by the Beckman apparatus) remained unchanged after H_2 had been bubbled through an ethanolic solution of micrococcin for 40 min. It was also unchanged on shaking with air or on adding H_2O_2.

Effect of water. The intensity of fluorescence increased as the proportion of water to ethanol was increased, reaching a maximum at about 60% of water and thereafter falling almost to zero. The magnitude of the effect depended on the concentration of micrococcin, and Fig. 3 shows the relation

between the three quantities—composition of solvent, concentration of micrococcin, and difference in fluorescence from that in pure ethanol.

Effect of acid. Hydrochloric acid, up to 0·2N, did not affect the fluorescence of an ethanolic solution.

Effect of alkali. Alkali (0·03N, final concentration) was observed to reduce the fluorescence of ethanolic solutions almost to zero, but on neutralizing and removing the precipitated NaCl it was completely restored, even after having been kept alkaline for several hours. The antibacterial activity was also unaffected by such treatment. When water was present the effect of the alkali was less marked, and in experiments in 98·5, 80 and 50% ethanol the fluorescence was reduced to 14, 33, and 51% of its original value, respectively. After neutralization over 90% of the original fluorescence was restored and no diminution of antibacterial action was detected.

When the concentration of alkali was altered the diminution of fluorescence changed also. In one experiment (in 98% ethanol) the following results were found:

Final concentration of alkali (N)	Fluorescence (% of original value)
0	100
0·01	29
0·02	17
0·03	11
0·05	7·5
0·1	4
0·2	2

No attempt was made to determine the pH of these ethanolic solutions.

Effect of sunlight. When a drop of micrococcin solution was placed on a filter paper and allowed to dry, the spot was invisible by daylight, but had a pure purple fluorescence in ultraviolet light. When the paper was exposed to sunlight or to ultraviolet light the spot became yellowish and the purple fluorescence in ultraviolet light was replaced by a less bright, pale-yellow fluorescence. The purple fluorescence disappeared completely after 5 min. in strong sunlight, but on adding a drop of ethanol to the filter paper the solvent immediately acquired a brilliant purple fluorescence, suggesting that only the surface of each micrococcin particle was initially affected.

The change appeared to be a light-activated oxidation, for no yellowing occurred even after several hours' exposure to sunlight if the paper were sealed in an evacuated tube. Similarly, the appearance and fluorescence of the spot were unchanged for weeks if kept in air in the dark. On extending the exposure to ultraviolet light or sunlight the yellowish fluorescence very gradually became stronger, while the intensity of the purple fluorescence washed out of the paper by ethanol became less, in parallel with the antibacterial activity. The yellow spot was not washed out of the filter paper by ethanol, water, or dilute acid; its fluorescence was reversibly quenched by alkali. The same change took place, though much more slowly, when an ethanolic solution of micrococcin was exposed to light. There was no evidence that, once started, the reaction would proceed in the absence of light.

Ultraviolet absorption

The absorption spectrum of twice crystallized micrococcin at 20 μg./ml. in ethanol is shown as curve *B* of Fig. 4. That of Su's (1948) purest preparation at the same concentration has been superimposed as curve *A*. The curves are closely similar, and it can be deduced that Su's preparation was about 86% pure, in terms of the crystalline material.

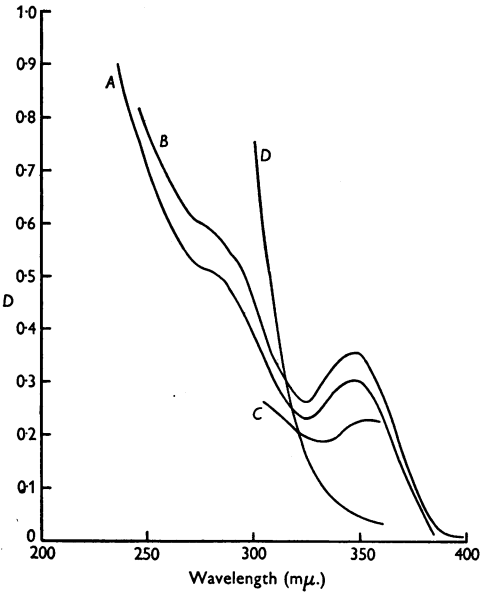

Fig. 4. Ultraviolet absorption spectra of micrococcin, at 20 μg./ml. in ethanol. Curve *A*, as reported by Su (1948) for his purest preparation. Curve *B*, twice recrystallized micrococcin. Curve *D*, copper, 1 in 10^4 (as sulphate) in ethanol. Curve *C*, twice recrystallized micrococcin, 20 μg./ml., plus copper, 1 in 10^4, after subtraction of curve *D*.

At a wavelength of 345 mμ. Beer's Law was found to be obeyed between concentrations of 5 and 60 μg./ml. in ethanol. The effect of traces of copper on the absorption are discussed below.

Infrared absorption

This was kindly examined by Dr L. N. Short. The presence of strong bands at 1657 and 1536 $cm.^{-1}$ in the spectrum of the solid suggested that the compound contains an amide group. In dilute solution in chloroform a band was given at 3384 $cm.^{-1}$, as would be expected for a mono-substituted amide. Many bands were given in the region 700–1400 $cm.^{-1}$, but no conclusions could be drawn from these regarding the structure of the compound.

Optical rotation

In 90% ethanol a twice-crystallized and carefully dried preparation, at a concentration of 50 mg./ml., had $[\alpha]_D^{21°} = +116° \pm 1°$.

The effect of heavy metals

Su (1947, unpublished) incubated small pieces of metallic copper, iron, and zinc for 24 hr. in an active crude culture fluid of the micrococcus and found no diminution of antibacterial activity. However, it has been found that traces of copper salts profoundly depress the fluorescence of ethanolic solutions of micrococcin, while tin, ferrous, and ferric salts have a definite though less marked effect. Attempts to find a stoicheiometric relation between copper and micrococcin showed that the effect was complicated: from several experiments the following points emerged:

(1) The main diminution of fluorescence on adding Cu occurred in less than 1 min.—possibly instantaneously. There was then a further small decrease during the next few hours. (2) Cu (as sulphate) at 0·1 p.p.m. gave an observable diminution of fluorescence; at 1 p.p.m. over 50% of the fluorescence of an ethanolic solution containing 20 μg. of micrococcin per ml. was abolished at once; but the fluorescence was not abolished completely, even with a copper concentration of 100 p.p.m., the highest practicable. (3) H_2O_2 had no effect on the fluorescence of ethanolic micrococcin alone. When added to micrococcin + copper some of the lost fluorescence was occasionally restored. Shaking a micrococcin-copper solution with air caused small variable changes in fluorescence. Prolonged bubbling of hydrogen through the solution had no effect. (4) The addition of BAL (2:3-dimercaptopropanol) to a copper-micrococcin solution restored some but not all of the fluorescence. This restoration was at first rapid—perhaps instantaneous—and there was sometimes a further slight increase in fluorescence during the next few minutes. Additional BAL had no effect. Copper mixed with BAL before adding to micrococcin solution had no effect on the fluorescence. (5) The effects of oxine or thioglycollate could not be followed because their coloured complexes with Cu interfered with the fluorescence of micrococcin. (6) The ultraviolet absorption of micrococcin was also modified by copper, as shown in Figs. 4 and 5, the change being in degree rather than in character. This change was also partly reversed by BAL, but owing to its smallness and to the strong absorption of mixtures of BAL and Cu, quantitative deductions were subject to a somewhat large error. However, it appeared that when BAL was added to a micrococcin-copper mixture the percentage restoration of fluorescence and of absorption (at 345 mμ.) was the same.

We are indebted to Dr W. S. Metcalf for suggestions and advice on this part of the work. He considered that the evidence available favoured the supposition that micrococcin formed a compound containing Cu.

The effect of copper on the antibacterial action of micrococcin. Some preliminary experiments were carried out to test whether the antibacterial action of micrococcin was modified by copper, as were its fluorescence and absorption spectrum. The conflicting results led to the reflexion that as the effect of copper was reversible, its effect on antibacterial action would be difficult to study, for the following reasons: (1) The fluorescence and absorption are measured in ethanol which must, for antibacterial assay, be largely evaporated. This would change the concentration of the volatile BAL, if present, and would probably cause precipitation of copper-micrococcin and copper-BAL complexes which might not redissolve in watery media. (2) The constituents of the medium would be expected to bind copper and alter its effective concentration. (3) The copper might itself inhibit the growth of the test organism. For these reasons further investigation of the effect of copper on the antibacterial effect of micrococcin was abandoned.

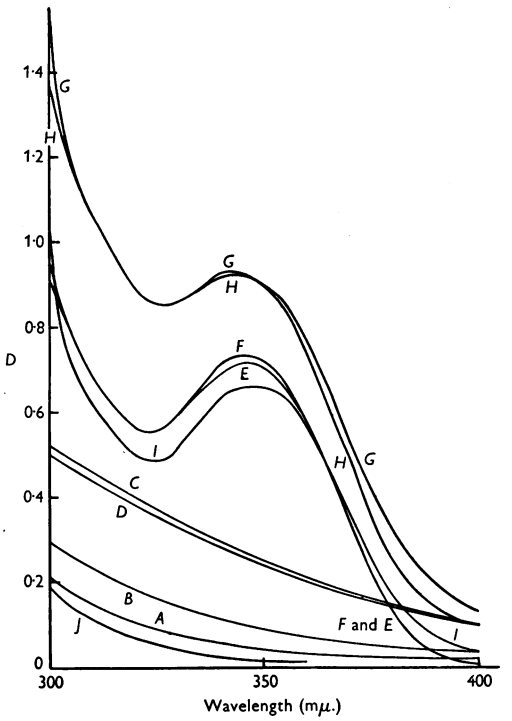

Fig. 5. Ultraviolet absorption spectra of micrococcin, copper sulphate, and BAL, and of mixtures of them, in ethanol. Copper (1 in 10^5) alone (curve *J*) and with 0·2, 0·5, 1 and 5 equivalents of BAL (curves *A–D*). Curves *E* and *F*, micrococcin 40 μg./ml. plus about 8 equivalents of BAL. Curves *G* and *H*, micrococcin, 40 μg./ml., plus copper, 1 in 10^5, plus 1 equivalent (curve *G*), and 5 equivalents (curve *H*) of BAL. (Approx. molar ratio of micrococcin:copper:BAL is 1:8:8 in curve *G* and 1:8:40 in curve *H*). Curve *I*, curve *G* minus curve *C*.

SUMMARY

1. The antibiotic micrococcin, extracted by Su (1948) from *Micrococcus* sp. (N.C.T.C. no. 6571), has been crystallized. The mother liquor may contain another antibiotic closely similar to micrococcin.

2. Micrococcin is almost insoluble in water. In ethanol-water mixtures its solubility rises to > 150 mg./ml. at 70–80 % ethanol, then falls to less than 10 mg./ml. in absolute ethanol.

3. It decomposes at 222–228°. $[\alpha]_D^{21°} = +116°$. It has a broad absorption in the ultraviolet and many bands in the infrared. An ethanolic solution has a strong purple fluorescence in near-visible ultraviolet light.

4. Both fluorescence and ultraviolet absorption are reduced by traces of copper and partly restored by BAL.

5. The molecular weight appears to be somewhat over 2000, and elementary analysis gave C, 49–49·5; H, 4·6; N, 13·9; S, 15·9 %. It gives a positive ninhydrin reaction only after acid hydrolysis, but this seems to be due to ammonia, not amino-acids. Nothing is known of its chemical nature.

We are indebted to Miss E. Page and Miss S. Long for valuable technical assistance throughout this work; to Mr B. K. Kelly, Director of the Medical Research Council Antibiotics Research Station, Clevedon, and his staff, for most of the micrococcin; and to Drs Ella M. Barnes and Kathleen Crawford and their assistants Miss C. King and Mr D. Jackson for some of the early fermentations. One of us (H.M.D.) is indebted to the Australian National Health and Medical Research Council for a personal grant.

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Metabolic Effects of Electrical Stimulation of Mammalian Tissues *in vitro*

By C. C. KRATZING (Australian National University Scholar)

[*Biochemical Laboratories, Institute of Psychiatry* (*British Postgraduate Medical Federation, University of London*), *Maudsley Hospital, S.E.* 5

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It has been found that electrical stimulation of slices of guinea pig brain cortex *in vitro* gives rise to large increases in the rate of oxygen consumption and aerobic glycolysis (McIlwain, 1951; McIlwain, Anguiano & Cheshire, 1951).

In order to test the scope of the new method, as to whether the response to electrical stimulation is confined to nerve tissue or is a property of other tissues, the technique has now been applied to rat diaphragm, and guinea pig lung and kidney slices.

Isolated whole frog muscle responds to electrical impulses by increased oxygen consumption which is several times that of the resting muscle (Meyerhof & Schulz, 1927; Fenn, 1927; Gemmill, 1934). Also Millikan (1937) has shown that *in vivo* cat muscle, during active contraction, can respire at a rate nearly twenty times that of the muscle at rest. Little work has been done to see what response is made to electrical impulses *in vitro* by other mammalian tissues.

METHODS

Guinea pig kidney cortex and lung slices, 0·35 mm. thick, were cut by means of a Stadie-Rigg's cutter. The thickness of slices was less than the maximum limit stated by Warburg (1930) and Field (1948) as being allowable for full oxygenation. Guinea pig-brain cortex slices were cut as described by McIlwain & Grinyer (1950). The diaphragms of young rats of about 100 g. were dissected out and the peripheral muscle portions used. According to Cohen (1949*a*) the diaphragms from animals of this size have a thickness of about 0·3 mm. The procedure in weighing tissues was that described by McIlwain (1951).

Krebs-Ringer phosphate solution, as described by Cohen (1949*b*), was used with the concentration of calcium reduced by half to prevent the formation of a precipitate. Glucose was added to give a concentration of 0·1 % (0·0056 M). Media were oxygenated at 37°.

The manometer flasks, of conical shape, were of about 18 ml. capacity; to some of them lead-in wires were inserted (type *A*, McIlwain, 1951). The tissues were fixed in small plastic and silver gauze tissue-holding electrodes (type *B*,