193. *Chemistry of Micrococcin* P. *Part II.*[1]

By (Mrs.) M. P. V. Mijović and James Walker.

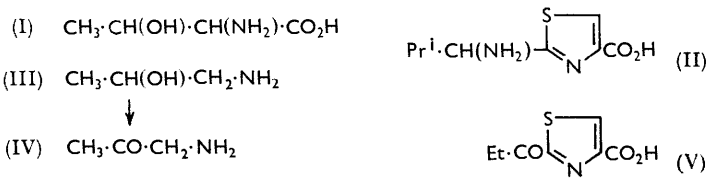
Two further components of the " acid-soluble fraction " from the acid hydrolysis of the antibiotic micrococcin *P* have been identified as aminoacetone and 2-aminopropan-1-ol (alaninol) respectively. The latter is configurationally related to D-alanine.

Quantitative determinations by the ninhydrin method have indicated the presence of two, four, and two molecules respectively of threonine, 2-aminopropan-1-ol, and 2-(1-amino-2-methylpropyl)thiazole-4-carboxylic acid per molecule of the antibiotic, and have given values of 2290, 2272, and 2296 for the molecular weight of micrococcin *P*.

The order in which various products are liberated on acid hydrolysis of micrococcin *P* has been studied; the carboxyl groups of the threonine residues appear to be free in the antibiotic.

In Part I [1] the products formed on acid hydrolysis of the antibiotic micrococcin *P* were separated into three groups, an " acid-insoluble fraction," an " ether-soluble fraction," and an " acid-soluble fraction." The present communication describes further progress in the study of the " acid-soluble fraction."

Paper chromatography had shown the presence in the " acid-soluble fraction " of four substances chromogenic towards ninhydrin, three of them giving typical purple spots and the fourth giving a bright yellow spot that slowly became purple. Two of these four components were readily purified by ion-exchange chromatography, and were shown to



be L-threonine (I) and 2-(1-amino-2-methylpropyl)thiazole-4-carboxylic acid (II) respectively, both of which gave purple spots on paper with ninhydrin. The substances responsible for the remaining purple spot and the atypical yellow spot ran closely together on paper, and were not well resolved by ion-exchange chromatography. They were, however, sufficiently well separated on paper to permit identification.

The mixture of the two components, after separation from threonine and 2-(1-amino-2-methylpropyl)thiazole-4-carboxylic acid (II) by ion-exchange chromatography, was partially purified by chromatography on a column of cellulose, and the resulting mixture was run on paper in butanol–acetic acid with an appropriate marker technique. (i) The substance giving the yellow spot was eluted and found to exhibit selective ultraviolet absorption, while the substance responsible for the purple ninhydrin reaction showed no selective absorption. The two substances were then converted into 2,4-dinitrophenyl derivatives by treatment with fluorodinitrobenzene, and, as the 2,4-dinitrophenyl derivatives of both substances passed into ether from alkaline solution, the parent compounds could not have been amino-acids. At this point we were aided by the observation of Cooley, Ellis, and Petrow [2] that 1-aminopropan-2-ol (III), a hydrolysis product from vitamin B_{12}, afforded on oxidation with acid permanganate " an unidentified product " giving with ninhydrin a yellow spot on paper slowly becoming purple at room temperature. It appeared to us that this " unidentified product " might be aminoacetone (IV), and

[1] Part I, Brookes, Fuller, and Walker, *J.*, 1957, 689.
[2] Cooley, Ellis, and Petrow, *J. Pharm. Pharmacol.*, 1950, **2**, 128, 535.

direct comparison with aminoacetone hydrochloride, obtained by hydrolysis of phthalimidoacetone, showed that the substance obtained on hydrolysis of micrococcin *P* and giving a yellow colour with ninhydrin on paper was indeed aminoacetone, which is, as we have also shown, formed from 1-aminopropan-2-ol (III) on oxidation with acid permanganate. In addition to its characteristic behaviour with ninhydrin, aminoacetone is readily detected on paper chromatograms by the red colour given with triphenyltetrazolium chloride solution. (ii) The substance responsible for the purple spot ran much faster than glucosamine and slightly faster than ethanolamine in butanol–acetic acid on paper. Like the latter amine, and unlike 3-aminopropan-1-ol, it reacted rapidly on paper with the periodate–permanganate spraying reagent.[3] The substance was, in fact, shown to be 2-aminopropan-1-ol (alaninol) by paper chromatography in two solvent systems, identical behaviour towards a range of spraying reagents, chromatography of the 2,4-dinitrophenyl derivatives, paper electrophoresis, and acid permanganate oxidation [4] to alanine. In contrast with previous reports [2] it was observed that 1-aminopropan-2-ol and 2-aminopropan-1-ol could be separated satisfactorily on paper chromatograms even although the difference in R_F values was quite small (0·05). There can be no doubt that the two previously unidentified substances reactive towards ninhydrin formed in the hydrolysis of micrococcin *P* are indeed aminoacetone and 2-aminopropan-1-ol, and a synthetic mixture of the two showed identical behaviour with that of the mixed unidentified compounds. The ultraviolet absorption spectrum of synthetic aminoacetone hydrochloride showed good agreement with that of the product from micrococcin *P*. The mixed aminoacetone–2-aminopropan-1-ol fraction from the hydrolysis of the antibiotic was lævorotatory, and, as L-2-aminopropan-1-ol is dextrorotatory, the 2-aminopropan-1-ol in micrococcin *P* is related configurationally to D-alanine, and is therefore D(−)-2-aminopropan-1-ol. A reference specimen of L(+)-2-aminopropan-1-ol was prepared from L-alanine.

The recognition of a volatile base other than ammonia in the hydrolysis products from micrococcin *P* caused us to re-examine the basic steam-volatile material previously considered [1,5] to be ammonia, and it was found to contain, in addition to ammonia, 2-aminopropan-1-ol and, surprisingly, aminoacetone; it was not expected that aminoacetone would survive in, and steam-distil from, an alkaline solution, but its characteristic behaviour on paper chromatography was readily observed.

Aminoacetone has only recently been detected in Nature as a metabolite of threonine produced in washed suspensions of staphylococci,[6] and its occurrence in micrococcin *P* together with threonine may indicate a common route of biosynthesis. It could arise from threonine by oxidation at the secondary alcohol group and decarboxylation of the resulting α-aminoacetoacetic acid.* Alternatively it could arise, *via* α-aminoacetoacetic acid, from acetylcoenzyme A and glycine, as there is evidence that acetate (or pyruvate) can inhibit the utilisation of glycine for the biosynthesis of δ-aminolævulic acid, and thence of porphyrins, by competing with succinate for glycine.[7]

Heatley and Doery [5] estimated the molecular weight of the original micrococcin, produced by a micrococcus, to be slightly greater than 2170 and definitely less than 2720 by Barger's method, but they were working near the limit of applicability of the method, and we have previously,[1] on the basis of the amounts of the various hydrolysis products isolated, placed the molecular weight of micrococcin *P* at about 2200. Quantitative

* It may also be noted that the amino-alcohol of vitamin B_{12}, D$_G$-1-aminopropan-2-ol, is formed by decarboxylation of L-threonine or of a closely related derivative (Krasna, Rosenblum, and Sprinson, *J. Biol Chem.*, 1957, **225**, 745).

[3] Lemieux and Bauer, *Analyt. Chem.*, 1954, **26**, 920.
[4] Billman, Parker, and Smith, *J. Biol. Chem.*, 1949, **180**, 29.
[5] Cf. Heatley and Doery, *Biochem. J.*, 1951, **50**, 249.
[6] Elliott, *Biochim. Biophys. Acta*, 1959, **29**, 446; *Nature*, 1959, **183**, 1051.
[7] Shemin, in " A Symposium on Amino Acid Metabolism " (ed. McElroy and Glass), The Johns Hopkins Press, Baltimore, 1955, pp. 738—739; cf. Nemeth, Russell, and Shemin, *J. Biol. Chem.*, 1957, **229**, 154; Gibson, Laver, and Neuberger, *Biochem. J.*, 1958, **70**, 77.

ninhydrin assays of the threonine, 2-aminopropan-1-ol, and 2-(1-amino-2-methylpropyl)-thiazole-4-carboxylic acid produced in the hydrolysis of micrococcin *P* have now given values of 2290, 2272, and 2296 respectively for the molecular weight of micrococcin *P*, and indicate the incorporation of two molecules of threonine, four molecules of 2-aminopropan-1-ol, and two molecules of 2-(1-amino-2-methylpropyl)thiazole-4-carboxylic acid per molecule of the antibiotic. The incorporation of two molecules each of threonine and the thiazole acid per molecule of micrococcin *P* is in accord with our previous conclusions.[1] The excellent agreement between the three values indicates that little or no destruction [8] of threonine could have occurred under the conditions used for the acid hydrolysis of micrococcin *P*. Aminoacetone was estimated by means of triphenyltetrazolium chloride, but it was obvious that the instability of aminoacetone precluded its quantitative recovery as the results indicated the presence of only 0·51 mol. Application of the conditions of the standard " acetyl group " determination to micrococcin *P* indicated that two molecules of volatile acid (propionic acid) are present per molecule of the antibiotic, rather than one as stated previously,[1] and similar treatment of the acetylated antibiotic indicated that there are probably four acetylatable groups per molecule of micrococcin *P*.

It appears that the carboxyl group of threonine is exposed in micrococcin *P*, as application of the Dakin–West [9] procedure to the antibiotic, followed by hydrolysis of the product and paper chromatography, showed the threonine to have been lost. In a control experiment *N*-benzoylallothreonine ethyl ester was submitted to the same procedure, and allothreonine was shown to be present in the subsequent hydrolysis mixture, in marked contrast to the behaviour expected when the carboxyl group of threonine (or of allothreonine) is exposed.[10]

The order in which various products are liberated in the acid hydrolysis of micrococcin *P* has been found to be: 2-aminopropan-1-ol, aminoacetone, threonine, 2-(1-amino-2-methylpropyl)thiazole-4-carboxylic acid, 2-propionylthiazole-4-carboxylic acid (V), and the dicarboxylic acid $C_{22}H_{19}O_5N_5S_4$, which affords the ester $C_{24}H_{23}O_5N_5S_4$ on treatment with methanol and sulphuric acid.[1,11]

Experimental

Butanol–acetic acid refers to the mixture butan-1-ol–acetic acid–water (63 : 10 : 27).

Partial Separation of the Components of the " Acid-soluble Fraction."—The " acid-soluble fraction " (2·8 g.) from the hydrolysis [1] of micrococcin *P* was dissolved in water (50 c.c.), filtered, and applied to a column (35 × 2·5 cm.) of " Amberlite IR-120(H)." The aqueous solution was washed through the column with distilled water until the effluent was neutral, and elution was then effected with N-hydrochloric acid. Fractions (each 20 c.c.) were collected automatically, fractions 22—44 containing the unidentified substances responsible respectively for yellow and purple ninhydrin-reacting spots on paper chromatograms. The brown oil (100 mg.) obtained on evaporation of these fractions was chromatographed on " Solka-floc " (30 g.) in butanol–acetic acid, and fractions (each 20 c.c.) were collected. Fractions 6—8 contained a trace of 2-(1-amino-2-methylpropyl)thiazole-4-carboxylic acid; fractions 7—14 contained the unidentified substance giving a purple spot with ninhydrin on separate paper chromatography in the same solvent mixture; and fractions 8—15 contained the unidentified substance giving the yellow spot. There was thus no significant separation of the two unknown substances and evaporation of the solvent from each fraction left a brown oil with an odour resembling that of meat extract. The material from fractions 9—14 was used for the subsequent experiments. The behaviour of the two substances towards a range of spraying reagents is shown in the following Table.

[8] Cf. Brockmann, Bohnsack, and Gröne, *Naturwiss.*, 1953, **40**, 223; Brockmann, Gröne, and Timm, *ibid.*, 1955, **42**, 125.

[9] Dakin and West, *J. Biol. Chem.*, 1928, **78**, 91, 745.

[10] Cf. Carter, Handler, and Melville, *ibid.*, 1939, **129**, 359; Carter and Stevens, *ibid.*, 1940, **133**, 117; Carter and Rissen, *ibid.*, 1941, **139**, 255.

[11] Brookes, Clark, Fuller, Mijović, and Walker, following paper.

Differential behaviour of unknown substances towards spraying reagents (Whatman No. 1 paper; butanol–acetic acid).

Reagent	Spot R_F 0·24	R_F 0·37
Ninhydrin	Yellow, with concave front, slowly becoming purple	Purple
Ammoniacal $AgNO_3$	White spot on brown background	Grey
$NaIO_4$–$KMnO_4$ [3]	No reaction	Fast reaction
Aniline phthalate	Brown	No reaction
p-Anisidine hydrochloride	Brown	No reaction
Folin–Marenzi reagent [12]	Blue (slowly)	No reaction
Triphenyltetrazolium chloride [13]	Red	No reaction

Ultraviolet-light Absorption by Unknown Compounds.—Fraction 12 from the previous experiment was dissolved in a little ethanol and applied as a streak to a paper chromatogram, which was then developed in the usual way with butanol–acetic acid. Side strips were cut off and sprayed with ninhydrin, thus revealing the positions of the main bands which were cut out. The organic material was eluted with methanol (25 c.c.), and the ultraviolet absorption was measured. The material giving the yellow spot with ninhydrin showed selective absorption with $\lambda_{max.}$ 276 mμ; that giving the purple ninhydrin reaction showed negligible absorption. Aminoacetone hydrochloride was subsequently shown to absorb maximally at the same wavelength.

2,4-Dinitrophenyl Derivatives.[14]—The solutions which had been used for the ultraviolet absorption measurements were evaporated to dryness, treated separately with ethanol (3 c.c.), trimethylamine (1·5 c.c. of a 1% aqueous solution), and 1-fluoro-2,4-dinitrobenzene (0·4 c.c. of a 7% ethanolic solution), and kept at room temperature for 4 hr. The ethanol was removed under reduced pressure, a drop of trimethylamine solution was added to each, and the mixtures were extracted with ether, giving extracts Y_I and P_I. After acidification the aqueous layers were extracted with ethyl acetate, giving extracts Y_{II} and P_{II}. For paper chromatography of the 2,4-dinitrophenyl derivatives,[15] Whatman No. 4 paper which had been impregnated with phthalate buffer (pH 6·0) was used with 30% propanol–light petroleum (b. p. 100—120°) (saturated with buffer) as the solvent system. Papers were always allowed to equilibrate for 4 hr. before the running, and the chromatography tank was covered with black paper. After the running, the papers were treated with hydrogen chloride gas to remove artefact spots. Y_I showed two spots of R_F 0·55 and 0·85, and P_I showed one main spot of R_F 0·52 and a faint one of R_F 0·85. Y_{II} and P_{II} gave no spots, showing that the two original ninhydrin-reacting substances were not amino-acids.

Paper Electrophoresis of Unknown Compounds.—Strips of Whatman No. 4 paper were loaded with material in the usual way, sprayed with a suitable buffer, and used with the central portion dipping into a bath of carbon tetrachloride to prevent drying. In this and all subsequent paper electrophoresis experiments a potential gradient of 20 v/cm. was applied for 1 hr. after which the papers were dried and developed with ninhydrin. Results are tabulated.

Buffer	pH	Distance moved towards cathode: Yellow spot	Purple spot
Phosphate, 0·125M	8·0	13·5 cm. (No separation)	
Phosphate, 0·125M	6·7	15 cm.	14 cm.
Acetate, 0·125M	4·0	28·5 cm.	26 cm.
Formic acid (10%)	1·0	30·4 cm.	28 cm.

Comparison of the Unknown Substances with Aminoacetone and 2-Aminopropan-1-ol.—(a) *Paper chromatography.* A mixture of aminoacetone, obtained as hydrochloride by hydrolysis of phthalimidoacetone, and 2-aminopropan-1-ol showed the same behaviour in all respects as the mixture of unknown substances in butanol–acetic acid on Whatman No. 1 paper (R_F 0·24 and 0·37 respectively) and on Whatman No. 3 paper (R_F 0·16 and 0·27). The R_F values of the components of the synthetic and the unknown mixtures in acetone–ethyl methyl ketone–water–formic acid (1 : 3 : 1 : 0·1) [16] on Whatman No. 1 paper were 0·38 (aminoacetone) and 0·46 (2-aminopropan-1-ol).

[12] Folin and Marenzi, *J. Biol. Chem.*, 1929, **83**, 109.
[13] Trevelyan, Proctor, and Harrison, *Nature*, 1950, **166**, 444.
[14] Cf. Sanger and Thompson, *Biochem. J.*, 1953, **53**, 353.
[15] Cf. Blackburn and Lowther, *Biochem. J.*, 1951, **48**, 126.
[16] Högström, *Acta Chem. Scand.*, 1957, **11**, 743.

(b) 2,4-*Dinitrophenyl derivatives.* The 2,4-dinitrophenyl derivatives obtained from aminoacetone and from the substance obtained from micrococcin *P* both showed two spots of R_F 0·55 and 0·85; in addition the synthetic material showed an extra spot of R_F 0·43. The 2,4-dinitrophenyl derivative of 2-aminopropan-1-ol moved at the same speed as that of the substance obtained from micrococcin *P* (R_F 0·52).

(c) *Paper electrophoresis.* In 0·125M-phosphate buffer (pH 6·7) the synthetic mixture of aminoacetone and 2-aminopropan-1-ol and the mixture of unknown compounds from micrococcin *P* showed identical behaviour. In 0·1M-sodium hydrogen sulphite aminoacetone moved 5 cm. (diffuse) and 2-aminopropan-1-ol moved 17·4 cm. in 40 min.; the mixture of unknown substances separated similarly.

Acid Permanganate Oxidation[4] *of Unknown Substances.*—Fraction 8 from the cellulose column, containing both the unknown substances, was dissolved in dilute sulphuric acid (0·125 c.c. containing 2 mmoles/c.c.) in a centrifuge tube, and aqueous potassium permanganate (0·5 c.c. containing 0·25 mmole/c.c.) was added. The permanganate colour had disappeared after 2 hr. at 50°, the manganese dioxide was spun down, and the supernatant solution was removed. The manganese dioxide was washed once with water, and the washings were added to the main solution. Aqueous barium chloride was added until no further precipitation took place, and the barium sulphate was spun down; the precipitate was also washed once with water. The solution and washings were evaporated to dryness under reduced pressure, and the residue was extracted with ethanol. The extract was taken to dryness and the residue was again extracted with ethanol; the resulting solution was used for paper chromatography and paper electrophoresis.

Chromatography on Whatman No. 3 paper in butanol–acetic acid revealed a spot which ran at the same rate as a standard alanine spot. This was not well separated from the aminoacetone spot, however, and the identity of the products in the oxidation mixture was proved by paper electrophoresis in 0·125M-phosphate buffer (pH 6·7) for 40 min. The oxidation products moved the same distances as did a standard mixture of alanine (4·6 cm.) and aminoacetone (11·8 cm.).

Steam-volatile Bases produced in the Hydrolysis of Micrococcin P.—The "acid-soluble fraction" from the hydrolysis of micrococcin *P* (1·48 g.) was basified with 2N-sodium hydroxide and steam-distilled until the distillate was neutral. Aliquot parts of the mixed total distillate (500 c.c.) were titrated against 0·1N-hydrochloric acid (phenolphthalein). With a molecular weight of 2290 for micrococcin *P*, the results indicated the formation of 4·64 mol. of steam-volatile base. The neutralised distillate was taken to dryness and paper chromatography in butanol–acetic acid on Whatman No. 1 paper, with 0·1% ninhydrin in butanol as developing spray, revealed the presence of aminoacetone and 2-aminopropan-1-ol. Repetition of the chromatography with, as developing spray, a solution[17] containing 0·1% of ninhydrin and 0·05% of ascorbic acid revealed, in addition, the presence of ammonia.

Optical Rotation of 2-Aminopropan-1-ol from Micrococcin P.—The optical rotation of the combined fractions containing aminoacetone and 2-aminopropan-1-ol, purified as described above, was measured. An aqueous solution (2 c.c.) containing the available material (180 mg.; consisting of a mixture of aminoacetone hydrochloride, 2-aminopropan-1-ol hydrochloride and, ammonium chloride) showed α_D −0·094°. The neutralised steam-distillate from the previous experiment was also found to be lævorotatory.

L-2-*Benzyloxycarbonylaminopropan-1-ol.*—(i) *N*-Benzyloxycarbonyl-L-alanine[18] (9·0 g.) was dissolved in ether (100 c.c.) and treated with diazomethane (*ca.* 2·5 g.) in ether (140 c.c.). After the reaction was complete, excess of diazomethane was destroyed with acetic acid, and the solvent was removed by distillation. Recrystallisation of the solid residue from light petroleum (b. p. 60—80°; 150 c.c.) afforded colourless needles (8·9 g., 93%) of the methyl ester, m. p. 43—44°, $[\alpha]_D^{23}$ −35·0° (*c* 2·5 in MeOH).

(ii) Lithium borohydride (1·3 g. of solid containing 68% of borohydride) was dissolved in purified tetrahydrofuran (100 c.c.), and the above methyl ester (6·5 g.) was added. After 3½ days at room temperature, water and dilute hydrochloric acid were added to the mixture to decompose excess of borohydride. The tetrahydrofuran was removed under reduced pressure and the aqueous mixture was extracted with ethyl acetate. Evaporation of the dried extract gave an oil (5·47 g.), which partly crystallised. This was chromatographed on alumina (150 g.,

[17] Keller-Schierlein, Mihailović, and Prelog, *Helv. Chim. Acta*, 1959, **42**, 315.
[18] *Org. Synth.*, 1943, **23**, 13.

activity I/II), ether eluting an oil (500 mg.) and ether–methanol eluting the required product as a crystalline solid (4·31 g., 75%). Recrystallisation from cyclohexane (400 c.c.) afforded L-2-*benzyloxycarbonylaminopropan*-1-*ol* as long needles, m. p. 75—78°, $[\alpha]_D^{22}$ −4·9° (*c* 2·5 in MeOH) (Found: C, 63·6; H, 7·4; N, 6·8. $C_{11}H_{15}O_3N$ requires C, 63·2; H, 7·2; N, 6·7%).

L-2-*Aminopropan*-1-*ol* (L-*Alaninol*).—Palladium black (390 mg.) was added to a solution of the above compound (3·9 g.) in methanol (100 c.c.), and hydrogen was bubbled through the stirred mixture until the effluent gases gave no turbidity with aqueous barium hydroxide (4 hr.). Acidification of the filtered solution with concentrated hydrochloric acid and evaporation to dryness gave a brown oil (2·35 g.), which was taken to dryness several times with methanol to remove excess of hydrochloric acid. The oil was finally applied in methanol (40 c.c.) to a column (22 × 2·5 cm.) of " Amberlite IR-400(OH) " resin, the free base being eluted with a further 200 c.c. of methanol. The methanol was distilled off through an efficient fractionating column, and the residue was distilled in a bulb-tube at 85—90° (bath-temp.)/12 mm.; L-2-aminopropan-1-ol was collected as a colourless oil (1·07 g., 77%), which rapidly became yellow, $[\alpha]_D^{22}$ +21·7° (*c* 3·06 in EtOH), $[\alpha]_D^{22}$ +26·1° (*c* 2·79 in N-HCl). Karrer, Portmann, and Suter [19] record b. p. 78—80°/12 mm. and $[\alpha]_D^{17}$ +20·1° (in EtOH).

Quantitative Ninhydrin Estimations of Threonine, 2-Aminopropan-1-*ol, and* 2-(1-*Amino*-2-*methylpropyl*)*thiazole*-4-*carboxylic Acid. Molecular Weight of Micrococcin* P.—Micrococcin *P* (100 mg.) was hydrolysed in boiling 20% hydrochloric acid (10 c.c.) for 6 hr., and the resulting suspension was diluted with water (20 c.c.) and filtered. Filtrate and washings (mixed) were continuously extracted with ether overnight and the aqueous phase was then evaporated to dryness under reduced pressure. The residue was taken up in an accurately measured volume (5 c.c.) of 3N-hydrochloric acid, and this solution, centrifuged to remove traces of insoluble yellow material, was used for the quantitative ninhydrin estimations. The paper-chromatographic technique of Pernis and Wunderly [20] was followed, and, as the first ninhydrin spray must be very fine, sparse, and even, immersion in 0·05% ninhydrin solution in acetone was adopted as described by Sheehan, Zachau, and Lawson.[21] Optical densities at 570 mμ were measured on a Unicam S.P.400 D.G. absorptiometer, and, owing to the weaker colours given by 2-aminopropan-1-ol and 2-(1-amino-2-methylpropyl)thiazole-4-carboxylic acid, only half (5 c.c.) the recommended volume of 50% propanol was used in the final dilution with these substances. To avoid differences between sheets of chromatography paper, the standard and the unknown solution were always run (butanol–acetic acid) on the same sheet.

One hundred parts by weight of micrococcin *P* were found to yield 10·40 parts of threonine, 13·20 parts of 2-aminopropan-1-ol, and 17·45 parts of 2-(1-amino-2-methylpropyl)thiazole-4-carboxylic acid from which it follows that molecular proportions of threonine, 2-aminopropan-1-ol, and 2-(1-amino-2-methylpropyl)thiazole-4-carboxylic acid are present respectively in 1145, 568, and 1148 parts of micrococcin *P*. As collateral evidence places the molecular weight of micrococcin *P* in the region of 2200, these figures give values of 2290, 2272, and 2296 respectively for the molecular weight of micrococcin *P*, and indicate the incorporation of 2 molecules of threonine, 4 molecules of 2-aminopropan-1-ol, and 2 molecules of 2-(1-amino-2-methylpropyl)-thiazole-4-carboxylic acid per molecule of micrococcin *P*.

Estimation of Aminoacetone.—The method used was based on that described by Fischer and Dörfel [22] for the quantitative estimation of reducing sugars on paper chromatograms. Aminoacetone hydrochloride used for the standard solution was purified by applying an ethanolic solution to a column of " Amberlite C.G. 120(H)," washing with ethanol until the effluent was neutral, and eluting the aminoacetone with 2N-hydrochloric acid in 80% ethanol. The eluate containing the aminoacetone hydrochloride was then treated with charcoal, filtered, and evaporated to dryness, affording the hydrochloride as a yellow gum which slowly crystallised. A standard solution (10 μg./0·01 c.c.) was prepared in 3N-hydrochloric acid. Known volumes (0·02—0·05 c.c., measured with an Agla syringe) were placed in small test-tubes and to each was added triphenyltetrazolium chloride (0·1 c.c. of a 4% solution in methanol) followed by 4N-aqueous sodium hydroxide (0·1 c.c.). Formation of the red formazan colour was instantaneous. Each sample was diluted with methanol–acetic acid (3·0 c.c. of a 10 : 1 mixture), and optical densities were measured at 482 mμ. Blank values were obtained by carrying out

[19] Karrer, Portmann, and Suter, *Helv. Chim. Acta*, 1948, **31**, 1617.
[20] Pernis and Wunderly, *Biochim. Biophys. Acta*, 1953, **11**, 209.
[21] Sheehan, Zachau, and Lawson, *J. Amer. Chem. Soc.*, 1958, **80**, 3352.
[22] Fischer and Dörfel, *Z. physiol. Chem.*, 1954, **297**, 164.

the procedure but omitting the aminoacetone. A similar series of determinations was carried out with known volumes of the preceding hydrolysate from micrococcin *P*, and the results were plotted graphically in the usual way [Found: 0·51 mol. of aminoacetone per mol. of micrococcin *P* (based on a molecular weight of 2290 for micrococcin *P*)].

Estimation of Propionic Acid formed in the Hydrolysis of Micrococcin P. *Acetylation of Micrococcin* P.—Micrococcin *P* (200 mg.) was dissolved in acetic anhydride (2 c.c.) containing 60% perchloric acid (1 drop) to give a yellow solution. Next morning the solution was poured into water, and the yellowish precipitate was extracted into chloroform. The chloroform extract was washed with aqueous sodium hydrogen carbonate, dried, and evaporated, affording a yellow residue. This was taken up in acetone (charcoal), and hexane was added to the warm filtered solution to incipient turbidity. The granular solid which separated was collected and dried at 20°/2 mm. for 4 hr.; it had m. p. 185—220° (decomp.) (Found: C, 49·8; H, 4·8; N, 12·1%); the infrared spectrum showed an additional carbonyl stretching band, but otherwise resembled that of micrococcin *P* fairly closely.

The standard "acetyl" determination procedure was applied by Dr. F. B. Strauss to micrococcin *P* and the result showed that 2·05 moles of volatile acid (propionic acid) were produced per mole of micrococcin *P* (based on a molecular weight of 2290 for micrococcin *P*). The same procedure was then applied to the above acetylated micrococcin *P*, and the total volatile acids (propionic and acetic acid) were determined. The results, corrected for the propionic acid present in the antibiotic initially, showed good agreement for a tetra-acetyl derivative (*M*, 2458), and indicated the presence of 4·16 moles of acetic acid per mole of acetylated micrococcin *P* (based on *M* 2290 for micrococcin *P*).

Dakin–West Degradation [23] *of Micrococcin* P.—Micrococcin *P* (20 mg.) was heated in pyridine (1 c.c.) and acetic anhydride (2·5 c.c.) at 135° for 6 hr. Solvents were removed under reduced pressure and the tarry residue was hydrolysed for 6 hr. with boiling 20% hydrochloric acid (25 c.c.). Paper chromatography in butanol–acetic acid and development with ninhydrin showed the presence of aminoacetone, 2-aminopropan-1-ol, and 2-(1-amino-2-methylpropyl)-thiazole-4-carboxylic acid in the hydrolysate, and the absence of threonine.

After the same treatment of *N*-benzoylallothreonine ethyl ester (20 mg., kindly supplied by Dr. D. F. Elliott), allothreonine was readily shown to be present in the hydrolysate.

Sequence of Liberation of Hydrolytic Fragments from Micrococcin P.—Micrococcin *P* (*ca.* 5 mg.) was hydrolysed at 100° with hydrochloric acid (3 c.c.) of the desired strength, and samples were withdrawn for paper chromatography every 10 min. (see Table). Each sample was placed on a small concave Polythene disc and dried in a vacuum-desiccator. The residue was taken

Time (in min.) after which hydrolysis product was present.

Product	Strength of hydrochloric acid N	3N	6N
2-Aminopropan-1-ol	70	30	10
Aminoacetone	80	50	15
Threonine	—	90	20
2-(1-Amino-2-methylpropyl)thiazole-4-carboxylic acid	—	160	30
2-Propionylthiazole-4-carboxylic acid	—	—	100
Acid $C_{22}H_{19}O_5N_5S_4$	—	—	140

up in absolute alcohol and spotted on to a paper chromatogram which was then run in butanol–acetic acid. Direct spotting of the hydrolysis mixture on to the paper without the intermediate drying and alcohol-extraction gave unsatisfactory chromatograms. The amino-compounds were detected by using ninhydrin in the usual way, and 2-propionylthiazole-4-carboxylic acid (V) was detected by its fluorescence in filtered ultraviolet light and by the violet colour it gave on immersion in ethanolic alkali followed by immersion in ethanolic *m*-dinitrobenzene.[24] The *dicarboxylic acid* $C_{22}H_{19}O_5N_5S_4$ separated after 90 min. from a hydrolysis in boiling 6N-hydrochloric acid as a white crystalline precipitate, from which the boiling supernatant liquid was removed through a filter stick; the solid was washed with water, methanol, and ether, and dried, but did not recrystallise satisfactorily; it had m. p. 271° (decomp.) (Found: C, 47·2; H, 2·8; N, 12·2; S, 23·5. $C_{22}H_{19}O_5N_5S_4$ requires C, 47·1; H, 3·4; N, 12·5; S, 22·8%). The substance

[23] Cf. Bullock and Johnson, *J.*, 1957, 3280.
[24] Cf. Savard, *J. Biol. Chem.*, 1953, **202**, 457.

(40 mg.) slowly dissolved on treatment with methanol (3 c.c.) and concentrated sulphuric acid (0·1 c.c.) on the water-bath (4 hr.), and a crystalline product separated; this was shown by its infrared absorption spectrum to be the dimethyl ester $C_{24}H_{23}O_5N_5S_4$ previously described.[1,11]

No substances reactive towards ninhydrin were liberated from micrococcin *P* by 6N-hydrochloric acid at 20° during 48 hr.

NATIONAL INSTITUTE FOR MEDICAL RESEARCH,
THE RIDGEWAY, MILL HILL, LONDON, N.W.7.

[*Received, August 18th,* 1959.]