575. *Actinomycin. Part I. Amino-acid Content.*

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Acid hydrolysis of actinomycin (Lehr and Berger, *Arch. Biochem.*, 1949, **23**, 503) yields, in addition to other as yet unidentified products, five amino-acids which have been identified as L-threonine, sarcosine, D-valine, L-methylvaline, and L-proline. Each of these has been isolated and identified by the preparation of derivatives. Comparisons with the amino-acid content of other actinomycins reported in the literature have been made.

SINCE the first isolation of the antibiotic actinomycin from *Actinomyces antibioticus* by Waksman and Tishler (*J. Biol. Chem.*, 1942, **142**, 519), a number of very similar compounds have been obtained from other actinomycetes (Waksman, Geiger, and Reynolds, *Proc. Nat. Acad. Sci.*, 1946, **32**, 117; Kocholaty, Junowicz-Kocholaty, and Kelner, *Arch. Biochem.*, 1948, **17**, 191; Trussell and Richardson, *Canadian J. Res.*, 1948, **26**, *C*, 27; Lehr and Berger, *Arch. Biochem.*, 1949, **23**, 503; Brockmann and Grubhofer, *Naturwiss.*, 1949, **36**, 376). The original work of Waksman and Tishler established that actinomycin which crystallised as orange-red platelets had a molecular weight of 770—1000 and an empirical formula approximating to $C_{41}H_{56}O_{11}N_8$. It was non-acidic and was precipitated from solutions in 10% hydrochloric acid on dilution, indicating weak basic properties. The presence of a quinone grouping and of hydroxyl groups was inferred from the results of acetylation and reductive acetylation experiments. Actinomycin, although a powerful antibiotic, is highly toxic to animals and thus has little or no therapeutic value.

The isolation of the material which we have used in the present work has been described by Lehr and Berger (*loc. cit.*) who prepared it from an unidentified strain of *Actinomyces* and we are very grateful to Dr. J. A. Aeschlimann of Hoffmann-La Roche Inc., Nutley, New Jersey, who made it possible for us to examine the compound which was supplied under the name of "Antibiotic X-45." In a previous note (*Nature*, 1949, **164**, 830), two of us (C. E. D. and

A. R. T.) have described a preliminary examination of this material and have compared the physical properties and empirical formula with those recorded for actinomycin by Waksman and Tishler (*loc. cit.*). It was concluded that the two antibiotics were almost certainly identical although a final decision on this point can only come from a knowledge of the detailed chemical structures. Complete acid hydrolysis with subsequent paper chromatographic analysis revealed the presence of five ninhydrin-reacting compounds, four of which were identified as D-valine, L-proline, threonine, and *N*-methylvaline, and the fifth compound was also believed to be an amino-acid. In these respects also, antibiotic X-45 and a sample of Waksman's actinomycin were identical. Somewhat similar conclusions concerning the nature of the acid hydrolysis products were later reached by Brockmann and Grubhofer (*loc. cit.*) who isolated three different actinomycins and reported preliminary chemical degradations of one of these which they named actinomycin C. They showed that this material had an approximate molecular weight of 800—900 and that the average value of several analyses indicated the formula $C_{40}H_{57}O_{11}N_7$. From an acid hydrolysis there were obtained L-threonine, L-proline, D-*alloiso*leucine (or D-*iso*leucine), *N*-methylvaline, and another *N*-methylamino-acid, m. p. 160°.

In view of the difference between the analytical results earlier reported by us for actinomycin and the figures quoted by Waksman and Tishler (*loc. cit.*), we have re-examined the analysis of " antibiotic X-45 " and have found that the air-dried material approximates to a pentahydrate and that the analysis of the anhydrous product corresponds to $C_{41}H_{58}O_{11}N_8$ rather than $C_{41}H_{54}O_{12}N_8$ as given in our original note (*loc. cit.*). The anhydrous material is very hygroscopic and suitable precautions must be taken during the preparation of the analytical samples. This revision of the molecular formula removes the major point of difference between Waksman and Tishler's actinomycin and " antibiotic X-45." We have further examined the hydrolysis of " antibiotic X-45 " and have isolated all of the five amino-acids in a crystalline state. The fifth amino-acid has been identified as sarcosine, having previously been shown to be an α-*N*-methylamino-acid by paper chromatographic techniques. Sarcosine has not previously been reported in an acid hydrolysate of a naturally occurring compound, although it occurs in the free state in the testicles of *Astropecten* (Kossel and Edlbacher, *Z. physiol. Chem.*, 1915, **94**, 264).

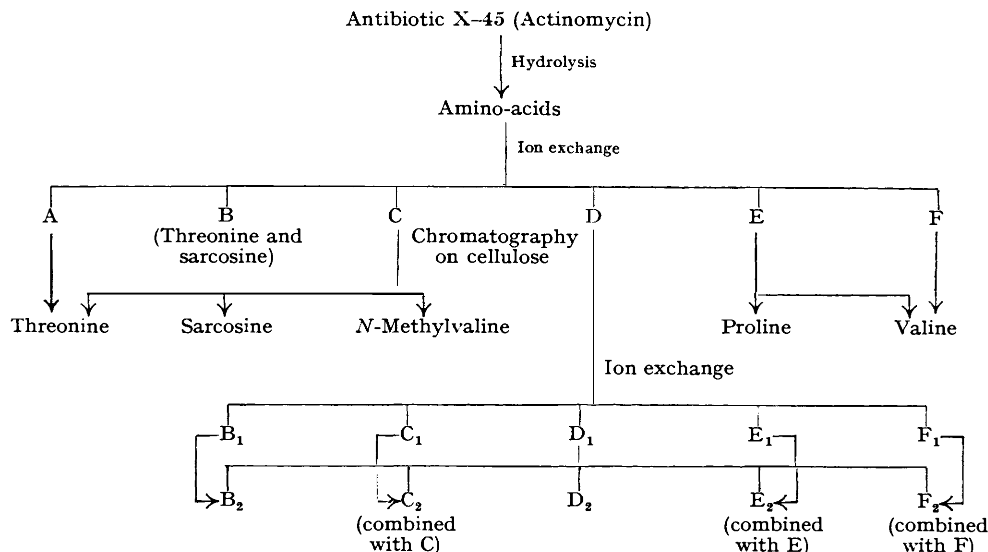
In order to settle the question of the identity or otherwise of our antinomycin with the actinomycin C of Brockmann and Grubhofer, we have exchanged samples with them and the two antibiotics have been compared in Göttingen and in Cambridge by *X*-ray and infra-red methods as well as by parallel hydrolysis and examination of the products by paper chromatography. These comparisons leave no doubt but that the two substances are different; in amino-acid content they appear to be the same, except that actinomycin C contains D-*iso*leucine or D-*alloiso*-leucine in place of the D-valine which occurs in actinomycin. Whether this represents the sole difference between them cannot yet be stated with certainty. Acid hydrolysis of actinomycin yields in addition to the above mentioned amino-acids other unidentified products, some obviously derived from the unknown chromophoric group in the molecule; until the nature of the remainder of the molecule has been determined the possibility that there are further points of difference must remain open, although from the general behaviour of the two actinomycins we incline to the view that only the amino-acid content differs. We should like to record our appreciation of the friendly and helpful contact which we have enjoyed with Professor Brockmann in our work on actinomycin.

The scheme which we have employed for the separation of the individual amino-acids depends on the preferential liberation of the compounds with dilute aqueous ammonia from a cation-exchange resin (cf. Partridge, *Biochem. J.*, 1949, **44**, 521) and may be represented diagrammatically (slightly simplified) as on p. 2948.

The nature of the amino-acids was such that they were not completely separated from each other on the ion-exchange column, and chromatography on cellulose columns was employed for the final separation of the threonine–sarcosine–*N*-methylvaline mixture. The latter method was not selected for the original separation of the amino-acid mixture because of the relatively small amounts of material which can be treated in each operation; with this particular mixture, however, cellulose chromatography undoubtedly gave a more efficient separation and it is recommended that it should be employed more extensively if the isolation of the amino-acids from the actinomycin hydrolysate should be repeated.

The stereochemical configurations of the valine (D-) and proline (L-) were deduced from the action of D-amino-acid oxidase on the mixed amino-acids. Threonine (Klein and Handler, *J. Biol. Chem.*, 1941, **139**, 103) and *N*-methylvaline do not react sufficiently quickly in the presence of this enzyme system for the results to be significant. Unfortunately the experimental

conditions employed in the large-scale hydrolysis of actinomycin were sufficiently vigorous largely to racemise the amino-acids so that the compounds isolated were optically inactive. However, examination of the various mother-liquors from the amino-acid crystallisations revealed that small amounts of the optically active forms were still present, and it was thus established that both the threonine and *N*-methylvaline were present in the L-form and confirmed that the proline was L-proline and the valine D-valine.



Only preliminary experiments have so far been carried out on the mode of attachment of the amino-acids within the actinomycin molecule. It seemed probable that some at least of the five might be present as a peptide and milder conditions of hydrolysis were examined in an attempt to isolate peptide fragments. Two such compounds were detected by paper chromatography, the first being identified as threonylproline and the second as valine attached to a non-ninhydrin reacting molecule. Further experiments along these lines are in progress but it is of interest that actinomycin gives a value of only one-third of a primary amino-group per molecule in the Van Slyke determination, on the assumption that the molecular weight is *ca.* 800. This may well be due to side reactions; Waksman and Tishler (*loc. cit.*) reported that there were no primary amino-groups in actinomycin. Thus, if the amino-acids we have isolated are linked in a peptide, then either the latter is further linked through the free amino-group (*e.g.*, in a cyclic peptide), or the terminal amino-acid is sarcosine or *N*-methylvaline.

EXPERIMENTAL.

The labelling of spots on the various chromatograms as A, B, C, etc., refers to individual compounds, the identity of which was inferred by direct comparison; *e.g.*, the spot due to proline has been referred to as B throughout, regardless of the nature of the solvent used for development of the chromatogram.

Actinomycin: "*Antibiotic* X-45."—The antibiotic formed bright red prisms, m. p. 255° (corr.), $[\alpha]_D^{16}$ $-367°$ (*c*, 0·25 in ethanol), from ethanol, methanol, or ethyl acetate–ligroin [Found, in an air-dried sample: C, 53·7; H, 7·3; N, 12·2; loss on drying at 120°/10^{-4} mm., 9·5. Calc. for $C_{41}H_{58}O_{11}N_8,5H_2O$: C, 53·0; H, 7·4; N, 12·1; H_2O, 9·7%. Found, in a sample dried at 120°/10^{-4} mm. over phosphoric oxide: C, 58·8; H, 7·1; N, 13·4; *C*-Me, 6·8; *N*-Me, 6·3; active H (Zerewitinoff), 0·39. Calc. for $C_{41}H_{58}O_{11}N_8$: C, 58·7; H, 7·0; N, 13·4; 2*C*-Me, 6·4; 2*N*-Me, 6·9%). The absorption spectrum showed maxima at 2370—2400 and 4420—4440 A.; $E_{1\,cm.}^{1\%}$ 238 and 161 respectively. Actinomycin is very soluble in acetone, benzene, chloroform, or acetic acid, moderately soluble in ethyl acetate, ethanol, methanol, or butanol, slightly soluble in ether or water, and insoluble in light petroleum. It is soluble in dilute mineral acid but insoluble in aqueous alkalis. Ethanolic potassium hydroxide gives a dark purple solution but the colour rapidly fades.

Acid Hydrolysis. Detection and Characterisation of Amino-acids produced. Single-dimensional Paper Chromatography.—Actinomycin (5 mg.) in 6N-hydrochloric acid (3 c.c.) was heated in a sealed tube at 100° for 48 hours and the product, after separation of the black insoluble material, was taken to dryness under reduced pressure and redissolved in a small volume of water (0·2 c.c.). One drop of this solution was subjected to paper chromatography, after neutralisation on the paper with ammonia vapour, using the upper layer of an *n*-butanol–acetic acid–water mixture (4 : 1 : 5) as solvent (henceforward

described as the butanol–acetic acid mixture). The chromatogram, after drying, was sprayed with a butanol solution of ninhydrin (0·1%) and heated at 100°, the following four spots being obtained (R_F values in parentheses; unless otherwise stated the colour of the spots was purple): A (0·19); B, yellow (0·28); C (0·43), and D (0·52). The last spot, D, differed from the others in that it did not develop at 70°. On the same chromatogram the following standard amino-acids were also present: alanine (0·23); valine (0·44); serine (0·13); glutamic acid (0·16); proline, yellow (0·27), and threonine (0·20). On this basis, A was tentatively identified as threonine, B as proline, and C as valine. The presence of a 1 : 2-hydroxyamino-compound was confirmed by spraying another similar paper chromatogram of the hydrolysate with the Nessler's periodic acid reagent (Consden, Gordon, and Martin, *Biochem. J.*, 1946, **40**, 33), a brown spot being obtained, of R_F 0·23, corresponding to a threonine standard. Chromatograms of other portions of the hydrolysate were carried out in various solvents: (i) Aqueous *s*-collidine: A (0·17); B, yellow (0·20); C (0·29), and D (0·35). Standards: glutamic acid (0·04); valine (0·29); threonine (0·19); alanine (0·17); and proline, yellow (0·20). (ii) Aqueous phenol: A (0·52, 0·54); B, yellow (0·88, 0·87); C (0·77, 0·77); and D (0·90, 0·90). Standards: threonine (0·53); proline, yellow (0·88); and valine (0·78). (iii) *n*-Butanol saturated with water, ascending chromatogram (Williams and Kirby, *Science*, 1948, **107**, 481): A and B unresolved (0·06—0·11); C (0·22); and D (0·38). Standards: threonine (0·06); proline, yellow (0·11); valine (0·23); and phenylalanine, green (0·32).

Two-dimensional Paper Chromatography.—Actinomycin (13·2 mg.) was hydrolysed with 6N-hydrochloric acid at 100° for 13 hours in a sealed tube. The product was treated as in the previous experiment and a portion of the aqueous extract of the hydrolysate was subjected to two-dimensional paper chromatography by the ascending technique (i) in aqueous phenol and (ii) in the butanol–acetic acid mixture. Five spots were obtained (R_F values in phenol given first): A (0·42, 0·19); B, yellow (0·72, 0·26); C (0·65, 0·40); D (0·74, 0·48); E (0·65, 0·17). The remainder of this hydrolysate was used for the characterisation of amino-acids D and E (below). Spot A of the single-dimensional chromatogram carried out in the butanol–acetic acid mixture is thus due to a mixture of threonine and amino-acid E, and spot C in the single-dimensional chromatogram is due to a mixture of valine and amino-acid E.

Deamination of Acid Hydrolysate.—An actinomycin hydrolysate (5 mg. of actinomycin treated with 1 c.c. of 9N-hydrochloric acid at 100° for 24 hours in a sealed tube, and the product clarified as before) was divided into two equal portions, and one of these was treated with nitrous fumes (from sodium nitrite and dilute hydrochloric acid) for 30 minutes. After addition of concentrated hydrochloric acid (0·1 c.c.), the solution was boiled for 5 minutes and then evaporated to dryness over potassium hydroxide in a desiccator, and the residue dissolved in water (1 c.c.), taken to dryness, and re-dissolved in water (0·5 c.c.). Examination of this solution on a paper chromatogram with the butanol–acetic acid mixture as solvent showed the presence of three spots, *viz.*, E (R_F 0·23), B, yellow (0·30), and D (0·55). Standards: threonine (0·24); proline, yellow (0·29); valine (0·47); and *N*-methylvaline (0·54). The untreated hydrolysate showed an additional spot C (0·47). In parallel experiments it was shown that standard threonine and valine were deaminated under these conditions and hence neither amino-acid D nor E contains a primary amino-group.

Chromatography on Paper pretreated with Copper Carbonate (cf. Crumpler and Dent, *Nature*, 1949, **164**, 441).—A drop (0·03 c.c.) of the above actinomycin hydrolysate was chromatographed on paper which had been lightly dusted with basic copper carbonate. The chromatogram was developed with water-saturated butanol and after drying was treated with ninhydrin solution and heated in the usual way. No spots were obtained from the hydrolysate, from standard threonine, proline, valine, or *N*-methylvaline under similar conditions. On the other hand blue spots were obtained from D-glucosamine and γ-aminobutyric acid. Amino-acids D and E were therefore both α-amino-acids.

Characterisation of Amino-acid D as N-*Methylvaline by Direct Comparison.*—Portions of the actinomycin hydrolysate, as used in the two-dimensional paper chromatography (above), were compared directly with various α-*N*-methylamino-acids on paper chromatograms with a variety of solvents and development with ninhydrin solution at 100°. The following was the behaviour of amino-acid D in the actinomycin hydrolysate: (i) Butanol–acetic acid mixture as solvent: D (0·52); *N*-methyl*iso*leucine standard (0·64); *iso*leucine standard (0·56). (ii) As (i): D (0·52); *N*-methylvaline standard (0·52). (iii) *s*-Collidine solvent: D (0·39); *N*-methylvaline (0·38). (iv) Aqueous phenol solvent: D (0·92); *N*-methylvaline (0·92). (v) A two-dimensional paper chromatogram of a mixture of threonine, valine, proline, and *N*-methylvaline was run first in aqueous phenol and then in the butanol–acetic acid mixture as solvents, the following 4 spots being obtained (R_F values in phenol first): threonine (0·48, 0·19); proline, yellow (0·86, 0·28); valine (0·74, 0·47); *N*-methylvaline (0·91, 0·58). This pattern agrees with that of the amino-acids A, B, C, and D obtained in a similar chromatogram of the actinomycin hydrolysate.

Characterisation of Amino-acid E as Sarcosine by Direct Comparison.—Further quantities of an actinomycin hydrolysate were used for direct comparisons of the properties of amino-acid E with those of sarcosine, by subjecting each to paper chromatography with development by ninhydrin at 100° as before: (i) Aqueous phenol, ascending chromatogram: E (R_F 0·77); sarcosine (0·77); Plattner and Nager (*Helv. Chim. Acta*, 1948, **31**, 2203) give sarcosine R_F = 0·80 in this solvent. (ii) Aqueous collidine: E (0·26); Plattner and Nager (*loc. cit.*) quote R_F = 0·21 for sarcosine. (iii) A two-dimensional chromatogram of a mixture of amino-acid E and sarcosine was run first in aqueous phenol and then in the butanol–acetic acid mixture as solvents, a single spot being obtained after development: R_F = 0·75 (aqueous phenol) and 0·24 (butanol–acetic acid).

Acid Hydrolysis of Waksman's Actinomycin.—Actinomycin (1 mg.; Waksman and Tishler, *loc. cit.*; sample kindly provided by Dr. S. Waksman) was hydrolysed with 9N-hydrochloric acid (1 c.c.) in a sealed tube at 110° for 19 hours and clarified as in the previous experiments. The hydrolysate was subjected to two-dimensional paper chromatography with aqueous phenol and the butanol–acetic acid mixture as solvents. Five spots were obtained after development with ninhydrin (R_F values in phenol first), the

position of which corresponded to those from the hydrolysate of the present sample of actinomycin (" antibiotic X-45 ") : A (0·51, 0·19); B, yellow (0·88, 0·28); C (0·76, 0·47); D (0·94, 0·57); E (0·76, 0·20).

Reaction of D-*Amino-acid Oxidase on the Acid Hydrolysate.*—The mixed amino-acids (21·9 mg.) from an acid hydrolysate of actinomycin were dissolved in water (1 c.c.) and neutralised to pH 8·2 with sodium hydroxide solution (0·31 c.c.; 0·414N). A portion of this solution (0·2 c.c.) was added to the enzyme system, comprising a D-amino-acid oxidase–flavine adenine dinucleotide mixture (1·5 c.c.), a catalase–ethanol mixture (0·2 c.c.) to remove hydrogen peroxide, and a 0·1N-pyrophosphate buffer (0·8 c.c.) to bring the pH to 8·3. The mixture was incubated at 38° for 20 hours in a Warburg manometer tube, after which the uptake of oxygen (144 μl.) was complete. A similar experiment using the neutralised hydrolysate (0·2 c.c.) with the addition of D-valine (0·39 mg.) in water (0·1 c.c.) was carried out simultaneously (uptake of oxygen, 230 μl.; and hence the uptake due to the added D-valine, 86 μl.; Calc. 85 μl.) and also a blank determination on the enzyme system (uptake of oxygen, 16·5 μl.). A sample of the solutions from each of the above experiments was subjected to two-dimensional paper chromatography with aqueous phenol and the butanol–acetic acid mixture as solvents, and the resulting chromatograms showed the complete disappearance of the valine spot in the first two cases. No amino-acid spots were obtained in the chromatogram of the enzyme system alone. Hence the valine is D-valine and the proline L-proline.

Milder Acid Hydrolysis.—Actinomycin (0·287 g.) was dissolved in 9N-hydrochloric acid (20 c.c.) and heated under reflux for 23 hours. The dark insoluble residue was separated and washed and the combined aqueous filtrate and washings, after thorough extraction with ether, were taken to dryness over potassium hydroxide in a desiccator. The residue was dissolved in water and subjected to paper chromatography, using the ascending technique and the butanol–acetic acid mixture as solvent; six spots were obtained after development with ninhydrin: threonine–sarcosine (0·19); proline, yellow (0·28); valine (0·43); *N*-methylvaline (0·52); F, yellow (0·56); and G (0·70). F and G were shown to be peptides by the procedure of Consden, Gordon, and Martin (*Biochem. J.*, 1947, **41**, 590). In a further chromatogram the portions of the paper containing the peptides were cut out and extracted separately with boiling water. The solvent was removed from the extracts in a vacuum and each of the peptide residues hydrolysed with 6N-hydrochloric acid in sealed tubes for 22 hours at 110°, the products were taken to dryness, and the residues examined by paper chromatography as before. Peptide F gave rise to two spots: F_1 (0·22) and F_2, yellow (0·28); standard threonine (0·22); standard proline, yellow (0·28). Peptide G gave only one main spot, of R_F 0·46; standard valine (0·46). Faint spots due to threonine (or sarcosine) and proline were also observed in this chromatogram, probably because of contamination with peptide F.

The experiment was repeated and the extracted peptides were deaminated by treatment with nitrous fumes, and then hydrolysed and chromatographed as before, whereafter peptide F gave a single orange spot with R_F 0·23; standard proline (0·23); and peptide G gave no spot. Peptide F was therefore threonylproline, this being the only peptide combination of proline with threonine or sarcosine which contains a primary amino-group. Peptide G must contain valine attached to a non-ninhydrin reacting fragment. These results were confirmed by chromatograms using *s*-collidine and aqueous phenol as solvents.

High-temperature Acid Hydrolysis.—Actinomycin (0·103 g.) was dissolved in N-hydrochloric acid (15 c.c.) and heated at 180° in a sealed tube for 1½ hours. The brown residue was separated and the aqueous filtrate after extraction with ether was examined by paper chromatography, using the butanol–acetic acid mixture as solvent and development with ninhydrin as usual. Five spots were obtained: threonine–sarcosine (0·18); proline, yellow (0·25); valine (0·42); *N*-methylvaline (0·51); and a new spot (0·12). The last was at first thought to be a peptide, but the compound was unchanged in its position on the paper chromatogram after extraction and hydrolysis with 4N-hydrochloric acid in a sealed tube for 19 hours at 100°. Two-dimensional paper chromatography of the original N-hydrochloric acid hydrolysate with aqueous phenol and the butanol–acetic acid mixture as solvents showed that the new spot had replaced threonine (R_F values in phenol first): sarcosine (0·76, 0·21); proline, yellow (0·91, 0·26); valine (0·80, 0·44); *N*-methylvaline (0·94, 0·52); new spot (0·14, 0·10).

Isolation of Amino-acids produced from Acid Hydrolysis.—Actinomycin (5 g.) was dissolved in 9N-hydrochloric acid (60 c.c.) and heated in a sealed tube at 110° for 40 hours. After cooling, the black residue (1·15 g.) was separated and the brownish filtrate continuously extracted with ether until a sample of the extract left no residue on evaporation. Removal of the solvent from the aqueous layer gave a brown gum which was redissolved in water and re-evaporated in order to remove all excess of hydrochloric acid. The gummy residue was then dissolved in water (250 c.c.), and a small quantity of insoluble material which had been precipitated was removed. The dark yellow filtrate was clarified by adsorption on a column of pre-treated cation-exchange resin, Zeocarb 215 (40 g.; 40—60 mesh size; 15 × 2·6 cm.; cf. Partridge and Westall, *Biochem. J.*, 1949, **44**, 418). The column was washed with water (50 c.c.) until the eluate was no longer coloured, and the amino-acids were then eluted with 0·15N-ammonia. The eluate was collected in 20-c.c. fractions, of which 1—14 were almost colourless, 15—17 contained ammonia and were brown, and further fractions were colourless and gave no colour with ninhydrin. Each of the fractions 1—17 was examined on a paper chromatogram using the butanol–acetic acid mixture as solvent and development with ninhydrin at 100°, whereby it was found that only fractions 9—15 inclusive contained amino-acids. Four spots corresponding to threonine–sarcosine, proline, valine, and *N*-methylvaline were obtained from each of these fractions although the threonine–sarcosine and *N*-methylvaline spots were stronger in the earlier fractions and the valine and proline spots were stronger in the later fractions. Fractions 9—14 inclusive were combined for the subsequent separation of the amino-acids, but as fraction 15 was coloured and also contained ammonia it was not included.

Meanwhile a column of Zeocarb 215 ion-exchange resin was constructed in three sections. The top section (25 g.; 40—60 mesh size; 18 × 1·9 cm.) was made up so that the amino-acids would be adsorbed on the upper two-thirds of the column (cf. Partridge and Westall, *loc. cit.*), and the middle (5 g.; 60—80 mesh size; 4 × 1·3 cm.) and lower sections (4 g.; 100—120 mesh size; 4 × 1 cm.) were used solely to correct irregularities on the amino-acid boundaries. Each section was fitted with a paraffin-disc float to deflect the incoming drops. A rough check was kept on the nature of the eluate by the use of a conductivity cell.

A solution of the amino-acids (fractions 9—14 above) was brought on to the column and washed with water until the eluate was colourless. Elution with 0·15N-ammonia was then commenced (14 c.c. per hour), and, when the amino-acids first appeared in it (22 hours), the eluate was collected in 10-c.c. fractions of which 1—26 inclusive were colourless, 27 was brown, and 28—30 colourless. Each of these was examined on a paper chromatogram using the butanol–acetic acid mixture as solvent. Fractions 1—18 inclusive which showed the threonine–sarcosine spot were also chromatographed on paper using aqueous phenol as solvent in order to differentiate between threonine and sarcosine.

Fraction 1 contained threonine and two new spots. Evaporation to dryness gave a pale brown waxy solid which rapidly became black in air and was not further examined.

Fraction 2 contained threonine and a little sarcosine. This fraction, (A), was used for the isolation of threonine.

Fractions 3 and 4 (B) contained threonine and sarcosine and were used in an unsuccessful attempt to isolate $\alpha\beta$-dihydroxybutyric acid after deamination before the isolation of threonine itself.

Fractions 5—7 (C) contained threonine, sarcosine, and *N*-methylvaline, all of which were separated by chromatography on cellulose (see below).

Fractions 8—18 (D) contained all five amino-acids.

Fractions 19—26 (E) contained valine and proline and were used for the isolation of these amino-acids.

Fraction 27 was brown and contained ammonia as well as valine.

Fraction 28 (F) also contained ammonia and valine, and was used for the isolation of valine.

Fractions 29 and 30 contained no amino-acids.

Fraction D was re-fractionated on the ion-exchange column and the various fractions examined by paper chromatography as before. Fractions corresponding to B, C, D, E, and F, *viz.*, B_1, C_1, D_1, E_1, and F_1 were obtained and a similar refractionation of D_1 yielded B_2, C_2, D_2 (1·04 g.), E_2, and F_2. B_1, B_2, C_1, C_2, E_1, E_2, F_1, and F_2 were used to supplement the corresponding fractions of the earlier separation.

Isolation of Threonine.—This was obtained partly from fraction C (including C_1 and C_2) and partly from fraction A. A column of powdered cellulose (65 g.; 10 × 4 cm.) was prepared using the butanol–acetic acid mixture as solvent, and metallic ions were removed by adding a solution of 8-hydroxyquinoline (100 mg.) in the same solvent (2 c.c.). The coloured band was washed right through the column and the washing continued until the eluate was colourless. Fraction C from the ion-exchange column was taken to dryness, redissolved in the butanol–acetic acid mixture, and brought on to the column. The chromatogram was developed with the same solvent, and the eluate collected in 10-c.c. fractions (1—31), the column being washed finally with water (100 c.c.), and the aqueous washings were concentrated (to 10 c.c.; fraction 32). Each of these fractions was examined for its amino-acid content by paper chromatography as before, using aqueous phenol as the solvent.

Fractions 2—4 inclusive contained *N*-methylvaline only and were used for the isolation of this amino-acid (see below).

Fractions 7—20 inclusive contained sarcosine only and were used for its isolation (see below).

Fractions 25—32 inclusive contained threonine only and were taken to dryness in a vacuum, giving a white amorphous solid (87·1 mg.). In addition, fraction A (29 mg.) from the original ion-exchange column was also added and the solid $[\alpha]_D^{19}$ +2·4 (*c*, 3·28 in water) was recrystallised from aqueous ethanol, to give colourless needles, m. p. 229—230° raised to 235—236° on further crystallisation and not depressed in admixture with an authentic specimen of DL-threonine, m. p. 236° (Found, in a sample dried at 90°/10^{-4} mm. for 12 hours: C, 39·7; H, 7·6; N, 12·0. Calc. for $C_4H_9O_3N$: C, 40·3; H, 7·6; N, 11·8%). The 2 : 4-dinitrophenyl derivative was prepared by adding a solution of the amino-acid (22 mg.) and sodium carbonate (46 mg.) in water (0·5 c.c.) to 1 : 2 : 4-fluorodinitrobenzene (45 mg.) in ethanol (1 c.c.). The mixture was shaken for 2 hours, the ethanol was removed, and the yellow aqueous solution remaining diluted to 5 c.c. and extracted with ether (5 × 1 c.c.) to remove excess of fluorodinitrobenzene. Acidification with 5N-hydrochloric acid (1 c.c.) precipitated a yellow oil which crystallised after some hours. Recrystallisation from ether–light petroleum (b. p. 40—60°) gave the derivative as yellow needles, m. p. 147°, undepressed on admixture with an authentic specimen of the threonine derivative (m. p. 147°) (Found: C, 42·3; H, 4·2; N, 15·1. Calc. for $C_{10}H_{11}O_7N_3$: C, 42·1; H, 3·9; N, 14·7%). Sanger and Porter (*Biochem. J.*, 1948, **42**, 287) give m. p. 152° for *N*-(2 : 4-dinitrophenyl)-DL-threonine.

Isolation of N-*Methylvaline.*—The *N*-methylvaline fraction from the chromatographic separation in the previous experiment was evaporated to dryness under reduced pressure at 60°. The residue was re-dissolved in water and again evaporated to dryness, to give a white amorphous solid (62 mg.). Similar treatment of other *N*-methylvaline-containing fractions yielded further quantities (122 mg.). The crude *N*-methylvaline fraction was purified by sublimation at 150°/0·01 mm. and the sublimate $[\alpha]_D^{17}$ 0·73 (c, 4·12 in water) crystallised from aqueous acetone as colourless plates, m. p. 255° (decomp.; in a sealed tube) undepressed by an authentic specimen of DL-*N*-methylvaline, m. p. 255° (Found, in a sample dried at 90°/10^{-4} mm. for 12 hours: C, 55·3; H, 10·2; N, 10·6. Calc. for $C_6H_{13}O_2N$: C, 54·9; H, 10·0; N, 10·7%). The mother-liquors from the crystallisation were evaporated to dryness, and the residue (24 mg.) was dissolved in 6N-hydrochloric acid (1·12 c.c.). The solution had $[\alpha]_D^{17}$ −26·2° (c, 2·14). The

2 : 4-dinitrophenyl derivative recrystallised from ether–light petroleum (b. p. 40—60°) had m. p. 173° alone or mixed with an authentic specimen, m. p. 173° (Found : C, 48·8; H, 5·0; N, 14·5. Calc. for $C_{12}H_{15}O_6N_3$: C, 48·5; H, 5·1; N, 14·1%). Plattner and Nager, *Helv. Chim. Acta*, 1948, **31**, 665, give m. p. 181·5—182° for *N*-(2 : 4-dinitrophenyl)-L-valine.

Isolation of Sarcosine.—The sarcosine fraction from the above chromatographic separation was evaporated to dryness, and the residue redissolved in water and again evaporated, to give a grey waxy solid (42 mg.; a further 49 mg. were obtained from other sarcosine-containing fractions), m. p. 205° (decomp. after darkening). Crystallisation from methanol–acetone gave colourless needles, m. p. 209° (decomp.) not depressed on admixture with an authentic specimen of sarcosine, m. p. 210° (Found : C, 40·7; H, 8·2; N, 15·6. Calc. for $C_3H_7O_2N$: C, 40·5; H, 7·9; N, 15·7%). A mixture of the product and authentic sarcosine could not be separated in a two-dimensional chromatogram using aqueous phenol and the butanol–acetic acid mixture as solvents. The 2 : 4-dinitrophenyl derivative formed yellow prisms (from aqueous methanol), m. p. 178° alone and mixed with an authentic specimen, m. p. 178° (Found : C, 42·3; H, 4·0; N, 16·2. $C_9H_9O_6N_3$ requires C, 42·4; H, 3·6; N, 16·5%).

Isolation of Valine.—Fraction E from the original ion-exchange separation was taken to dryness at 60° under reduced pressure, an amorphous pale brown solid (800 mg.) being obtained. This was dissolved in the minimum amount of hot water, two volumes of hot ethanol were added, and the mixture was kept at 0° for 12 hours, whereby valine was obtained as colourless plates (300 mg.), m. p. 274° raised to 289° after two recrystallisations from aqueous ethanol (Found : C, 51·7; H, 9·3; N, 12·1. Calc. for $C_5H_{11}O_2N$: C, 51·3; H, 9·5; N, 12·0%). No depression in m. p. was observed on admixture with authentic valine (m. p. 290°). The mother-liquors from the valine crystallisation were used for the isolation of proline. Further quantities of valine (34 mg.), $[\alpha]_D^{19}$ −3·8° (*c*, 2·63 in 6N-hydrochloric acid), were obtained similarly from fraction F which contained no proline. The 2 : 4-dinitrophenyl derivative formed yellow plates, m. p. 185° [from ether–light petroleum (b. p. 40—60°) (Abderhalden and Blumberg, *Z. physiol. Chem.*, 1910, **65**, 318, give m. p. 185° for *N*-(2 : 4-dinitrophenyl)-DL-valine] not depressed by an authentic specimen, m. p. 185°, prepared from DL-valine (Found : C, 46·8; H, 4·5; N, 15·3. Calc. for $C_{11}H_{13}O_6N_3$: C, 46·7; H, 4·6; N, 14·8%).

Isolation of Proline.—The mother-liquors from the valine preparation were evaporated to dryness under reduced pressure, redissolved in hydrochloric acid (20 c.c. of 1%), and mixed with a solution of ammonium rhodanilate (1·5 g.) in methanol (20 c.c.). The reddish-purple needles of proline rhodanilate which formed were separated, dried (540 mg.), and recrystallised from methanol–1% hydrochloric acid, then having m. p. 120°. A further crop (660 mg.) was obtained by concentration of the first mother-liquors and after recrystallisation as before was mixed with the first batch. The proline rhodanilate was shaken with 10% aqueous pyridine (11 c.c.) for 5 minutes, and after 12 hours the pyridine rhodanilate was separated, and the pale red filtrate treated with acetic acid (0·2 c.c.) and kept overnight. The colourless supernatant liquid was decanted from the small quantity of red precipitate and evaporated to dryness under reduced pressure, and the residue was dissolved and re-evaporated twice with water and finally with ethanol to remove pyridine and acetic acid. Recrystallisation from ether–ethanol gave colourless prisms of DL-proline (118 mg.), m. p. 204—204·5° not depressed on admixture with an authentic specimen, m. p. 205° (Found : C, 52·5; H, 7·7; N, 12·5. Calc. for $C_5H_9O_2N$: C, 52·2; H, 7·9; N, 12·2%). A sample of proline isolated from the mother-liquors from the above crystallisation had $[\alpha]_D^{19}$ −8·4° (*c*, 1·67 in water). The 2 : 4-*dinitrophenyl* derivative of DL-proline formed yellow needles, m. p. 189° alone and mixed with a synthetic specimen, m. p. 189° [Sanger and Porter, *loc. cit.*, give m. p. 137° for *N*-(2 : 4-dinitrophenyl)-L-proline] (Found : C, 47·4; H, 4·0; N, 15·2. $C_{11}H_{11}O_6N_3$ requires C, 47·0; H, 3·9; N, 14·9%).

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