Antibiotic Peptides from *Bacillus licheniformis*. Licheniformins A, B and C

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From the spore-forming aerobe *Bacillus licheniformis* Weigmann *emend.* Gibson, grown on an ammonium and sodium lactate medium, an antibiotic material, licheniformin, was prepared (Callow, Glover, Hart & Hills, 1947). It was obtained as the hydrochloride of basic material containing only carbon, hydrogen and nitrogen. It had bacteriostatic activity *in vitro* against numerous organisms, notably *Mycobacterium tuberculosis*, and protective effects against infections, including tuberculous infection, in mice. It was, however, toxic, causing damage to the kidneys after prolonged administration.

Further purification and chemical investigation was of importance from the practical point of view, to see whether the toxicity was inherent or could be separated from the antibiotic activity by fractionation. From the theoretical point of view, the constitution of a substance able to suppress experimental tuberculous infection was of the greatest interest. The crude active material for long resisted attempts at fractionation, but this was at last accomplished after it was found that separation into components could be effected by paper chromatography. This separation was used as a guide in devising a suitable scheme of countercurrent fractionation on a preparative scale.

The separation of three components, designated licheniformins A, B and C, is now described. They have proved to be peptides of very similar molecular weights and amino-acid composition, all having both antibacterial activity and toxicity, though to somewhat varying degrees.

Taken in conjunction with results obtained at the same time at two other centres of research, the Microbiological Research Department, Porton, and the Sir William Dunn Institute of Pathology, University of Oxford, the present results show that a single species of bacterium can produce at least six antibiotic polypeptides, one set of three, the licheniformins, on a medium with lactate and a high nitrogen/carbon ratio, and another set of three, the bacitracins, on a medium with glucose and a low nitrogen/carbon ratio. The late G. M. Hills collaborated not only with ourselves but also with the Oxford laboratories in devising suitable media for bulk production of antibiotic peptides from *B. licheniformis* and recognized the decisive influence of the medium on the type of antibiotic produced (Callow *et al.* 1947; Hart & Hills, 1947; Belton, Hills & Powell, 1949; Hills, Belton & Blatchley, 1949; Arriagada, Savage, Abraham, Heatley & Sharp, 1949).

EXPERIMENTAL

Materials and general methods

Organisms and culture conditions

We are indebted to the Microbiological Research Department, Porton, for culture fluids from the original strains of *B. licheniformis*, National Collection of Type Cultures (N.C.T.C.) no. 7072, and from the Oxford 'A5', and to Boots Pure Drug Co. Ltd. for culture fluids from N.C.T.C. nos. 5399 and 6816, and from 'Plate 2C', another strain isolated by Dr P. D'Arcy Hart. Culture was on the medium previously described, or modifications of this (Callow *et al.* 1947; Hart & Hills, 1947; Belton *et al.* 1949).

Extraction of licheniformin

The processing of early batches was carried out as described previously (Callow *et al.* 1947). Later, however, after experiments with different adsorbents, it was found that Decalso F (Permutit Co. Ltd., London), a sodium aluminosilicate, acted as an adsorbent of high capacity from which licheniformin was readily eluted by NaCl solution. The procedure described below is more rapid and convenient than the active-charcoal process.

The supernatant from the autoclaved and centrifuged culture was brought to pH 5–6 and further clarified, if necessary, by filtration through a layer of Hyflo Supercel (Johns-Manville Co. Ltd., London) and then run on to a dry column containing 5 g. of Decalso F for each litre of liquid. A convenient column was provided by an inverted Winchester-quart bottle with the bottom removed and a bung and tap fitted in the neck. Fluid was fed from a large aspirator bottle, closed at the top and with a wide-bore tap and bent delivery tube from the lower tubulure dipping into the liquid above the column, so forming a constant-level device. The rate of flow was 0·3–1·0 l./hr. When the fluid level finally fell to the level of the top of the adsorbent 10% (w/v) NaCl solution was run on as eluent. The rate of flow was decreased to about 2 drops/sec. and the effluent was tested at intervals by adding 1 drop to 0·5 ml. of saturated aqueous solution of picric acid. When a definite precipitate was formed, collection of the eluate was begun and continued until the picric acid test became very weak. About 50 ml. of 10% NaCl was sufficient to elute the licheniformin

present in 1 l. of culture fluid of normal potency (500–1000 units/ml.). A slight excess of picric acid solution was added to the eluate, the precipitate was allowed to settle and collected on a sintered-glass filter (porosity 3). The picrate was decomposed on the filter by grinding with conc. HCl, the solution sucked through and run into a large excess of dry acetone with stirring. Crude licheniformin hydrochloride was precipitated. It was collected and dried *in vacuo* and further purified by solution in methanol (4 ml./g.), filtration, and reprecipitation by pouring into 25 vol. of acetone. The usual yield was 40–50 mg. of material with an activity of between 5 and 10×10^6 units/g. from 1 l. of culture fluid. With the rather inaccurate bio-assay method used, no significant loss of activity was detected during isolation.

Paper chromatography

Amino-acid separations were carried out as described by Dent (1948). The special solvent mixture for separation of licheniformin is described below.

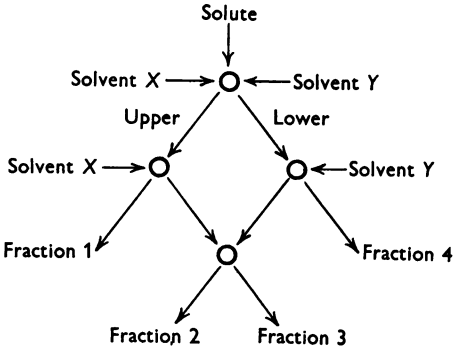
Antibacterial activity

This was estimated by the serial dilution method previously described (Callow *et al.* 1947) using *Mycobacterium phlei* as the test organism. We are indebted to Dr P. D'Arcy Hart for the numerous assays made in the course of this work.

Separation of licheniformins

Counter-current distribution between immiscible solvents

The system of fractionation described by Bush & Densen (1948) was used. In this n funnels, with final complete separation of the phases, yield $2n$ fractions. The system may be simply explained by means of a diagram showing the use of two funnels:



Bush & Densen (1948) give an equation which can be used to calculate the composition of the fractions in terms of the number of funnels used and the proportions of a substance in the upper and lower layers, p and q, respectively, where $p+q=1$. For a 12-funnel separation giving 24 fractions, these have the compositions: p^{12}, $12p^{12}q$, $78p^{12}q^2$, $364p^{12}q^3$, $1365p^{12}q^4$, $4368p^{12}q^5$, $12376p^{12}q^6$, $31824p^{12}q^7$, $75582p^{12}q^8$, $167960p^{12}q^9$, $352716p^{12}q^{10}$, $705432p^{12}q^{11}$, $705432p^{11}q^{12}$, $352716p^{10}q^{12}$, $167960p^9q^{12}$, $75582p^8q^{12}$, $31824p^7q^{12}$, $12376p^6q^{12}$, $4368p^5q^{12}$, $1365p^4q^{12}$, $364p^3q^{12}$, $78p^2q^{12}$, $12pq^{12}$, q^{12}. Using selected values of p and q a family of curves was drawn. By comparison of experimental graphs of weights of fractions against number in the series 1–24 it was then possible to estimate roughly the relative amounts and partition coefficients of major components of mixtures, by comparison of the heights and positions of the experimental maxima with the theoretical curves. The goodness of fit of a theoretical curve to the experimental curve for what appeared to be a single substance enabled one to judge its homogeneity with respect to partition coefficient. Bush & Densen also give an expression for the optimal proportions of upper and lower layers for separation of substances of known different partition coefficients. Thus, application of the procedure to separations on a preparative scale was simple over a range of partition coefficients, with appropriate alteration of the relative volumes of the two layers. At first ordinary separating funnels were used; later an automatic all-glass separating apparatus, as described by Craig & Post (1949), was constructed. This had 12 tubes each with a capacity of 92 ml. for the lower layer, and a maximum capacity of about the same for the top layer.

Paper chromatography

A large number of solvent mixtures were tried on one-dimensional paper partition chromatograms with descending flow. The only one effective in fractionating crude licheniformin was a mixture of collidine, lutidine and aqueous 2N-ammonia (1:1:2 by vol.). After vigorous shaking and separation, the upper phase was used in the trough and the lower used to saturate the atmosphere of the tank in which the chromatograms were run. Whatman no. 4 paper was used throughout. Spots were detected with ninhydrin spray in the way usual for amino-acids.

The procedure described below demonstrated the presence of three chromatographically distinct antibiotic components.

Licheniformin hydrochloride (1 mg.) was dissolved in water and transferred to four equally spaced spots at the top of a sheet of paper (length 50 cm.). The paper was irrigated with the upper layer of the solvent mixture. The solvent reached the end of the paper in 24 hr. and was allowed to run off the end of the sheet for another 24 hr. The paper was dried, washed thoroughly with ether until free of the odour of collidine and cut into four strips, each with one of the original spots at the top. Two of the strips were treated with ninhydrin and each was found to show several distinct zones of colour (Fig. 1). The four strips were laid side by side and the probable positions of the ninhydrin-positive spots marked in pencil on the untreated strips. The two untreated strips were now laid on the surface of two large agar plates (30×20 cm.) which had been previously poured with a uniformly distributed inoculum of licheniformin-sensitive bacteria, *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) or *Mycobact. phlei* (cf. Goodall & Levi, 1946). The plates were left at 0° for about 12 hr. and then incubated at 37° for 6 hr. (*Staph. aureus*) or 24 hr. (*Mycobact. phlei*). At the end of this time the paper was stripped from the surface of the agar and the plate examined for zones of inhibition (clear zones). The type of result which was obtained is shown in Fig. 1. The three clear zones on each agar plate corresponded with three coloured zones on the papers sprayed with ninhydrin. The three antibiotics separated by this procedure were named licheniformins A, B and C in order of increasing R_F values.

Unsuccessful attempts at fractionation

With paper-chromatographic analysis as a guide, various methods of larger-scale fractionation were tried. Fractional precipitation from aqueous solution by $(NH_4)_2SO_4$ or by methyl orange did not result in any separation. Activity was adsorbed by acid-washed alumina from methanolic solution, and material could then be eluted by aqueous methanol of increasing water content, but paper chromatography of the eluates showed that separation was far from complete. Elution of licheniformin adsorbed on Decalso F by dilute NaCl also gave only partial separation. Chromatography on a starch column in a mixture of collidine, lutidine and ammonia gave no evidence of fractionation. Ionophoresis in silica jelly (Consden, Gordon & Martin, 1946) promised interesting results, for under certain conditions three opalescent bands moved towards the cathode. It was, however, impossible completely to separate these bands, which tailed and became diffuse as they travelled, and little antibiotic activity could be recovered.

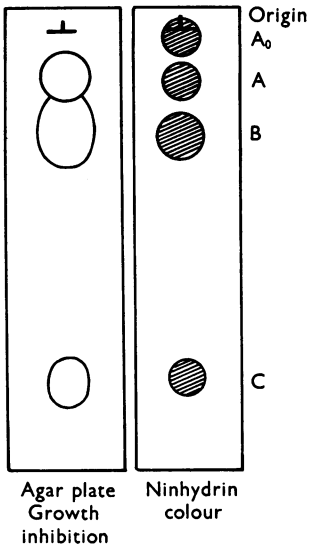


Fig. 1. Separation of three antibiotics by paper chromatography of crude licheniformin (for method see text). Zones of inhibition of bacterial growth are indicated by unshaded ovals. Zones coloured after spraying with ninhydrin are hatched.

Application of counter-current distribution

Several solvent systems were tried for the fractionation method outlined above. Licheniformin, as the hydrochloride, is stable but extremely soluble in water, and insoluble in the common organic solvents. Alkali metal hydroxides decompose licheniformin, but decomposition was not rapid in Sørensen borate-NaOH buffer at pH 12·38, and *n*-butanol extracted some activity from such a solution. *n*-Butanol and 8·5 N-NH_3 solution, *sec.*-butanol and 4·25 N-NH_3 solution and *n*-butanol, water, and piperidine (2:2:1 by vol.) all gave two liquid phases with licheniformin in each and were potentially useful for fractionation by partition, but they were unpleasant. Ultimately it was found that, in a mixture of phenol and water, a specimen of crude licheniformin had a partition coefficient (K) of 2·27 (ratio of concn. in upper water layer to concn. in lower layer); emulsification was largely prevented by addition of a little HCl. This system was used successfully.

The phenol-water mixture was prepared by melting the phenol ('detached crystals') and adding the appropriate amount of warm water. HCl (5 drops, about 0·25 ml., of 12 N/l.) was added, and the mixture equilibrated at room temperature. The crude licheniformin hydrochloride was dissolved in upper layer and the solution made up to the required volume and then added to the funnel containing lower layer; this constituted the 'first funnel' of the counter-current extraction. The final fractions were 12 water-rich upper layers and 12 phenol-rich lower layers. Recovery of material from these was at first done by way of the picrates, but this step was unnecessary and the following procedure was adopted, e.g. with 25 ml. upper layer and 75 ml. lower layer. Phenol was extracted from the upper layer with 80 ml. ether which was back-extracted with 2 × 20 ml. water. The combined water extracts were evaporated to dryness under reduced pressure; the residue was taken up in a little methanol and the solution poured into excess of acetone. The precipitated material was collected by centrifugation in a weighed 15 ml. tube, dried, and weighed. The lower layer was diluted with 20 ml. water and extracted with 200 ml. ether, which was back-extracted with 2 × 20 ml. water. The combined water extracts were then evaporated and material precipitated as before. Recovery was usually about 90% of the initial weight. Direct precipitation by pouring the layers into acetone was possible, but used much acetone.

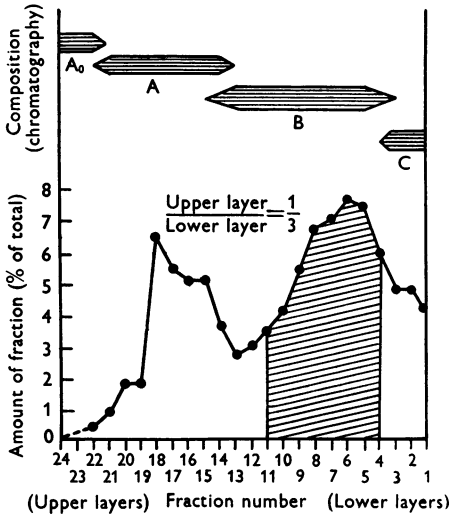


Fig. 2. Counter-current fractionation diagram for crude licheniformin hydrochloride partitioned between water (containing HCl) and phenol (for quantities see text). ▨ indicates material selected for refractionation to separate licheniformin B. The contents of each tube were also analysed by paper chromatography and the qualitative composition of the product is indicated diagrammatically above the curve.

The weighed fractions were examined by paper chromatography and a combined diagram could then be constructed in which weights and paper-chromatographic analyses were plotted against fraction numbers, as in Fig. 2, which gives results obtained with 2·14 g. of crude licheniformin hydrochloride prepared by the Decalso process from N.C.T.C. no. 5399 and partitioned between 12·5 ml. upper and 37·5 ml. lower layers (abbreviated as water/PhOH = 1/3).

The next step in the isolation of one of the constituents was to select fractions which were chromatographically homogeneous, or nearly so, with respect to the desired constituent. These were united and again submitted to counter-current fractionation. In doing this the ratio of upper and lower layers was altered so that the separation of the desired constituent from an adjacent one was more complete, e.g. alteration of the ratio water/PhOH to 1/0·75 brought constituent *B* into the upper layers with a maximum weight in fraction 18 and *C* was largely confined to the phenol layers. This is illustrated by curve *a* in Fig. 3, giving the results of taking fractions 4–11, wt. 0·92 g., from the previously illustrated fractionation (Fig. 2) and partitioning between 40 ml. upper and 30 ml. lower layer. A theoretical

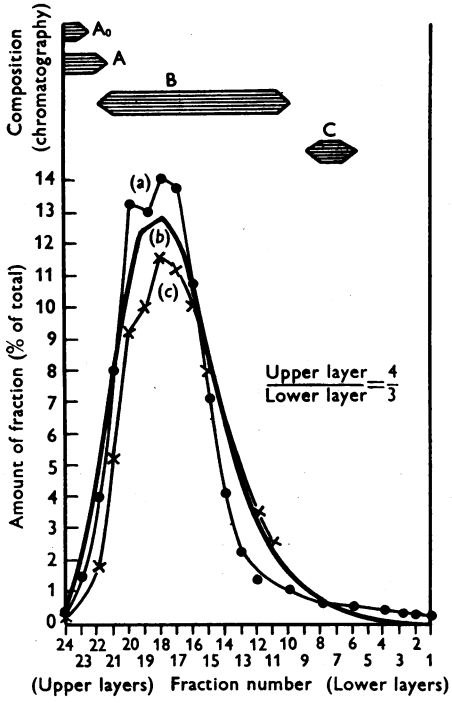


Fig. 3. (*a*) Counter-current fractionation diagram for partially purified licheniformin B hydrochloride partitioned between water (containing HCl) and phenol. The theoretical curve for $K = 1{\cdot}33$, $p = 0{\cdot}64$ with upper layer/lower layer = 4/3 is shown as a thick line (*b*). The qualitative compositions (paper chromatography) of the fractions are indicated diagrammatically above the curves. Curve *c* was obtained after two further counter-current fractionations of material represented by curve *a* (for details see text).

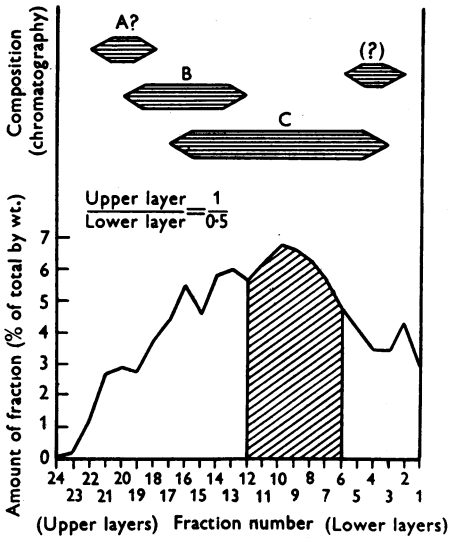


Fig. 4. Counter-current fractionation diagram for crude licheniformin C hydrochloride partitioned between water (containing HCl) and phenol. ▨ indicates material selected for refractionation. The qualitative compositions of the fractions (paper chromatography) are indicated diagrammatically above the curve.

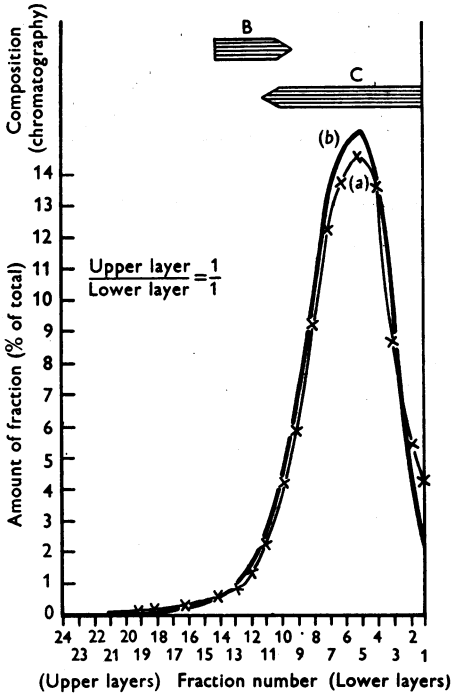


Fig. 5. Counter-current fractionation diagram for licheniformin C hydrochloride (*a*) partitioned between water (containing HCl) and phenol. A theoretical distribution curve (*b*) for $K = 0{\cdot}43$, $p = 0{\cdot}3$, is also shown. The qualitative compositions of the fractions are indicated diagrammatically above the curves.

curve, *b*, calculated on the assumption that $K = 1{\cdot}33$, is shown for comparison.

Generally, the procedure adopted was to select fractions rich in *B* from a number of different fractionations, unite them, fractionate with a ratio water/PhOH = 1/3 to remove the more water-soluble constituents, including *A*, to the upper layers, and then to unite the new fractions rich in *B* and conclude with a fractionation with a ratio water/PhOH = 4/3. The end result of one such fractionation is shown in curve *c* of Fig. 3.

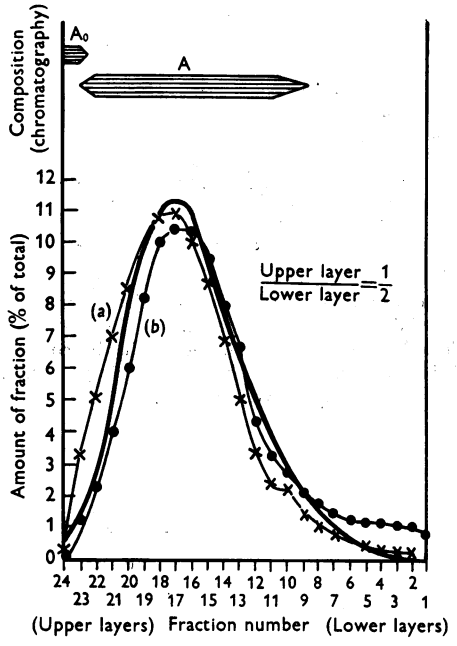


Fig. 6. Counter-current fractionation diagrams for two concentrates of licheniformin A hydrochloride partitioned between water (containing HCl) and phenol. A theoretical distribution curve for $K = 3{\cdot}0$, $p = 0{\cdot}6$ is also drawn (heavy line). The qualitative composition of the fractions (paper chromatography) is indicated diagrammatically above the curves.

Constituent *C* was isolated from a collection of *C*-rich fractions (wt. 3·8 g.) from various sources which, fractionated with a ratio water/PhOH = 1/0·5, gave the curve in Fig. 4. Fractions 6–12 from this (wt. 1·4 g.) were re-united and refractionated with a ratio water/PhOH = 1/1 with the result shown in Fig. 5, curve *a*. A theoretical curve *b* for $K = 0{\cdot}43$ has been drawn for comparison.

The same principles were applied to the separation of the constituent *A*. There was other material, A_0, more soluble in water, and remaining at or very near to the origin of the paper chromatograms, from which *A* had to be separated. A_0 was a brownish, deliquescent material and there was no evidence of segregation into a fraction of uniform partition coefficient. The counter-current fractionation diagrams of two concentrates of constituent *A* are shown in curves *a* and *b* of Fig. 6, together with a theoretical curve for material of $K = 3{\cdot}0$.

Licheniformin mixtures from different sources

By comparison of the counter-current fractionation diagrams of crude licheniformin from the five different strains of *B. licheniformis* worked up by active charcoal or Decalso, no evidence was obtained that there was any considerable difference in the proportions of the three main constituents. Charcoal preparations contained less *A* and *B* and more material highly soluble in phenol in comparison with Decalso preparations. Another constituent appeared on one occasion. In fractions 4–9 of a counter-current distribution at water/PhOH = 1/3 of a crude product from A 5, there was a constituent which moved slowly (at about the same rate as *a*) on a paper chromatogram. The amount of material available was not enough to encourage any attempt at isolation.

Some light was thrown on the composition of the material, often considerable in amount, at the extreme phenol-soluble end of the counter-current fractions by an experiment in which part of a lot of crude licheniformin was treated in 1% solution at pH 8–8·5 for 67 hr. at 37° with an equal weight of trypsin. The antibacterial potency was unaffected, and the only significant change in the counter-current diagram was a diminution of fraction 1 from 14% in the untreated material to 10% in the digested material. No effect on licheniformins A, B, or C was detectable.

Physical and chemical properties of licheniformins A, B and C

Physical properties

All three constituents in the form of hydrochlorides were white, amorphous, slightly hygroscopic powders, melting with decomposition at indefinite temperatures. Each behaved as a single substance when analysed by paper chromatography using the collidine/lutidine/ammonia solvent mixture.

Optical rotation. Crude mixtures had $[\alpha]_D$ − 36° to − 37·5° and $[\alpha]_{5461}$ − 42° to − 47° (*c*, 1) in water. Purified licheniformin A hydrochloride had $[\alpha]_D^{20°}$ − 37·4°; $[\alpha]_{5461}^{20°}$ − 45·2° in water (*c*, 0·19; *l*, 2 dm.). Licheniformin B hydrochloride had $[\alpha]_D^{20°}$ − 37·7°; $[\alpha]_{5461}^{20°}$ − 48·2° in water (*c*, 0·84; *l*, 4 dm.). Licheniformin C hydrochloride had $[\alpha]_D^{20°}$ − 36·8°; $[\alpha]_{5461}^{20°}$ − 44·9° in water (*c*, 1·18; *l*, 4 dm.).

Absorption spectra. The ultraviolet absorption spectra, measured on a Beckman spectrophotometer in aqueous solution, showed a rapidly increasing general absorption at decreasing wavelength below 2500 A. Bands corresponding to phenylalanine or inflexions in this region were observed with preparations of all three constituents, most clearly with licheniformin B, least clearly with licheniformin C, which had the higher general absorption. Selected curves are given in Fig. 7. No firm conclusions could be based on these absorption spectra, for, apart from generally absorbing impurities, there was some evidence in one fractionation of a material with a partition coefficient between those of licheniformins B and C and not visible on paper chromatograms and which had an intense band at 2480 A.

Molecular weight. We are indebted to Dr A. G. Ogston for determinations of molecular weights from sedimentation constants on the ultracentrifuge and diffusion constants. The values obtained were as follows: licheniformin A,

4400; licheniformin, B, 3800; licheniformin C, 4800. Full details are given in an Addendum to this paper (Addendum II).

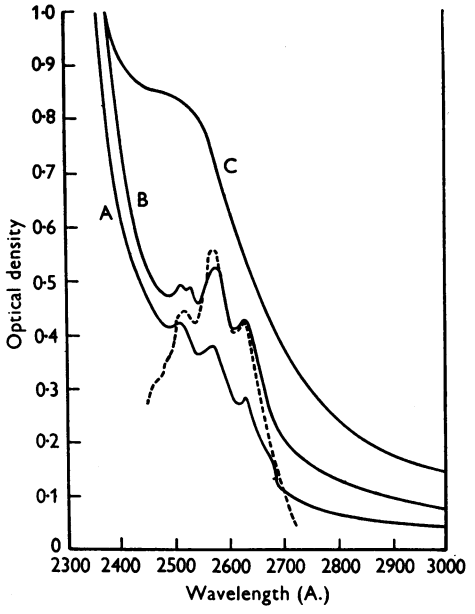


Fig. 7. Ultraviolet absorption spectra of licheniformins A, B and C and of phenylalanine (broken line). A and B were 0·2% (w/v) of hydrochloride in water, C was 0·1% (w/v) hydrochloride in water, and phenylalanine was 0·05% (w/v) in water.

Elementary analysis

The figures below are for material dried over P_2O_5 for 3–6 days.

Licheniformin	C (%)	H (%)	N (Dumas) (%)	Cl (%)
A	44·4	7·0	18·1	11·9
B (i)	44·7	7·2	18·4	10·4
B (ii)	46·2	7·1	17·7	11·3
C (i)	47·5	8·1	17·8	11·5
C (ii)	48·0	7·1	17·1, 16·4	—
C (iii)	47·5	7·7	18·1	—
C (iv)	45·2	7·0	17·0	10·5

(Analyses by Weiler & Strauss, Oxford, and by the Institute micro-analytical laboratory.)

Qualitative observation of peptide constitution

All three materials on acid hydrolysis gave amino-acids, evidently derived from an original peptide molecule. A sample of each antibiotic (250 μg.) was hydrolysed for 18 hr. with 6N-HCl at 105° in a sealed tube and after removal of acid the product was in each case analysed by two-dimensional paper partition chromatography. Licheniformins A and B gave chromatograms which indicated the presence of aspartic acid, serine, glycine, arginine, lysine, proline, valine and phenylalanine. Licheniformin C gave in addition a spot which was indistinguishable from glutamic acid.

Quantitative estimation of amino-acids in licheniformin: chemical method

Serine, glycine, proline, arginine and aspartic acid were estimated colorimetrically as their dinitrophenyl derivatives. A sample (40·3 mg.) of licheniformin A hydrochloride previously dried to constant weight at 100° was hydrolysed for 16 hr. in a sealed evacuated tube with excess 6N-HCl at 105°. The acid was removed and the residue dissolved in water and diluted to 4 ml. A sample of this solution (1·5 ml.) was allowed to react with an excess of fluorodinitrobenzene (FDNB) (0·15 ml.) in aqueous methanolic bicarbonate (6 ml. 5% (w/v) $NaHCO_3$, 6 ml. methanol) (Sanger, 1945) for 90 min. and the solution then acidified (10 ml. 3N-HCl).

From this point until all estimations were complete, operations were conducted entirely in a dimly lit room so as to avoid photodecomposition. The acid solution was extracted five times with 20 ml. peroxide-free ether, each extract being washed with 3 ml. water and the washings returned to the aqueous phase. Ether was removed from the aqueous phase under reduced pressure and the volume then adjusted to 50 ml. and 50 ml. of N-HCl added. Colour intensity was measured at once in a Hilger Spekker absorptiometer using a 1 cm. cell, tungsten lamp, violet filter (601) and heat filter (H 503). The amount of dinitrophenyl-(DNP-) arginine was determined by reference to a standard curve. The results are given in Table 1. Licheniformin B was treated in the same way. The combined ether extracts from the arginine determination were concentrated and the residue dried in a desiccator over KOH.

Use of Celite columns. Separation of the DNP-amino-acids was conducted on buffered Celite columns (Perrone, 1951). The Celite (Johns-Manville Co., London, S.W. 1), grade 545 (25 g.), was mixed with buffer (16 ml.) and peroxide-free wet ether (100 ml.) and stirred with a high-speed mechanical stirrer for 30 min. The suspension was poured into a suitable glass tube and the Celite packed with the packing tool described by Howard & Martin (1950). The diameter of the glass tube was chosen to give a final column about 15 cm. high. It was possible to separate from a single sample of 1·5 ml. of hydrolysed licheniformin hydrochloride (10 mg./ml.) the pure DNP derivatives of aspartic acid, serine, glycine, proline, valine and phenylalanine, but to do so it was necessary to use four successive columns of differing pH and the losses thus became serious. For quantitative estimation it was found that reproducible results could not be obtained unless the estimation of any one amino-acid was completed on a single column and within the compass of a single working day. The amino-acid which was being estimated was required to form a well defined band, separated from any other band, and the method of collection of the effluent was standardized both in this procedure and in the preparation of reference curves. When the band was 1 cm. from the bottom of the column the receiver was replaced by a measuring cylinder and when the trailing edge of the band left the column the volume of solvent in the cylinder was noted and a further 15 ml. of solvent collected before the receiver was changed. The solvent was evaporated as rapidly as possible *in vacuo*, the residue was dissolved in 1% $NaHCO_3$, the volume adjusted to 100 ml., and the colour intensity measured at once in an absorptiometer. By observing these precautions the variation between successive determinations of the same amino-acid was never greater than $\pm 5\%$.

Aspartic acid and serine. The Celite column was prepared in ether using buffer *A* of Blackburn (1949) and the ether then displaced by washed $CHCl_3$. The ether-soluble DNP-amino-acids from 1·5 ml. of licheniformin solution (10 mg. licheniformin HCl/ml.) were dissolved in the minimum volume of washed $CHCl_3$ and transferred to the column. The column was developed with $CHCl_3$ until the two slow bands (aspartic acid and serine) were well separated; the fast bands were discarded. In order to speed development the $CHCl_3$ was replaced by $CHCl_3$-butanol (99:1, v/v) and this solvent by $CHCl_3$-butanol (98:2, v/v). The serine band was collected first and the aspartic band then pushed through with $CHCl_3$-butanol (95:5, v/v). Estimations were made as described above. The results are given in Table 1.

Table 1. *Amino-acids in licheniformins* A *and* B

(Arginine, glycine, serine, proline and aspartic acid were estimated colorimetrically after conversion to their DNP derivatives and separation of the mixture on Celite. Phenylalanine, valine and lysine were estimated microbiologically.)

	Amino-acid (mg./100 mg. licheniformin)		N as % total N licheniformin	
	A	B	A	B
Aspartic acid	2·2	2·2	1·28	1·26
Glycine	11·9	11·8	12·3	11·9
Serine	6·5	6·9	4·8	5·0
Proline	5·5	5·5	3·7	3·6
Arginine	21·0	21·0	37·3	36·6
Phenylalanine	8·3	9·0	3·9	4·2
Valine	4·8	5·5	3·2	3·6
Lysine	36·6	38·6	38·6	40·0
Total	—	—	105·08	106·16

Glycine and proline. The procedure was the same as that used for aspartic acid and serine except that ether was used as solvent and the column was buffered with a mixture of 0·5 M-KH_2PO_4 (975 ml.) and 0·5 M-Na_2HPO_4 (25 ml.). The fast bands ($R \rightarrow 1$) were discarded and the next two bands (proline and glycine) collected. The results are given in Table 1.

Valine, lysine and phenylalanine. No column could be devised which would separate these three amino-acids as their DNP derivatives in a manner which eliminated all danger of error from overlapping bands and we accordingly abandoned the method in favour of microbiological assay.

Synthetic mixture. A mixture of glycine (25 mg.), serine (25 mg.), valine (25 mg.), phenylalanine (25 mg.), aspartic acid (25 mg.), arginine (75 mg.), and lysine (150 mg. monohydrochloride, $2H_2O$) in water was diluted to 15 ml. and samples of 0·5, 1 and 2 ml. used for preparation of the DNP derivatives. Three successive determinations were made of colour intensity for arginine, aspartic acid, serine, glycine and proline at each of these levels, the procedure outlined above being followed in each case. The results were highly reproducible, e.g. scale reading on absorptiometer: 0·475, 0·475, 0·485 for DNP-arginine from 2 ml. solution, and 0·25, 0·27, 0·26 for DNP-arginine from 1 ml. solution; 0·56, 0·58, 0·57 for proline from 2 ml. solution, and 0·38, 0·38, 0·39 for proline from 1 ml. solution.

Quantitative amino-acid analyses: microbiological methods (Miss K. R. de Bouk)

An account of these is given in an Addendum to this paper (p. 567).

D-*Amino-acids in licheniformins* A *and* B

A preparation of D-amino-acid oxidase was made from sheep kidney by the method of Negelein & Brömel (1939). Purification was carried only to the stage of the first $(NH_4)_2SO_4$ precipitation. Samples of the peptides were

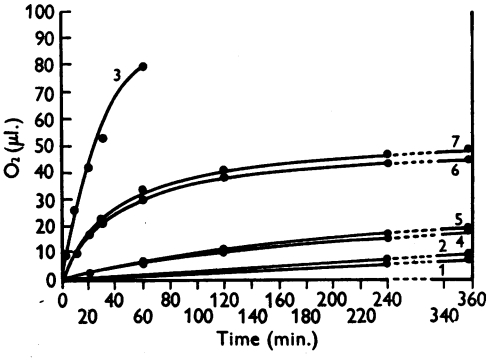


Fig. 8. Oxygen uptake (in μl. at 37°) during oxidation of hydrolysed licheniformin by D-amino-acid oxidase (for conditions see text). Curve 1, no substrate; curve 2, L-phenylalanine; curve 3, D-phenylalanine; curve 4, hydrolysed horse serum globulin; curve 5, hydrolysed casein; curve 6, licheniformin A; curve 7, licheniformin B.

hydrolysed for 8 and 16 hr. at 100° in 6 N-HCl in sealed, evacuated tubes. As controls, samples of casein and horse serum globulin were hydrolysed in the same way. The acid was removed from each sample by leaving in a desiccator over KOH for several days and the residue dissolved in water. The pH was adjusted to 8·0 by addition of 5% $NaHCO_3$ and the solution diluted to a concentration equivalent to 10 mg. peptide or protein/ml. The amount of D-amino-acid in each preparation was estimated by following O_2 uptake in Warburg manometers. Each flask contained 0·5 ml. hydrolysed peptide, 1·8 ml. sodium pyrophosphate buffer (pH 8·3, 0·067 M) and, in the centre cup, 0·2 ml. KOH (20%, w/v). The side arm of each Warburg flask contained 0·2 ml. enzyme. As controls three flasks were prepared, one with no substrate, one with D-phenylalanine (0·5 ml. ≡ 2·5 mg. amino-acid) and one with L-phenylalanine (0·5 ml. ≡ 2·5 mg. amino-acid). The result of a typical experiment is shown in Fig. 8.

Three successive runs were performed on separate samples of each peptide hydrolysed for 8 hr. and one on each peptide hydrolysed for 16 hr. The results were the same within the limits of error of the method. The two proteins hydrolysed for 8 hr. and for 16 hr. showed a slightly greater O_2 uptake than the control or the L-phenylalanine (10 μl. in 360 min.). After subtraction of the control value the corrected O_2-uptake figures for licheniformin A were 40, 37, 35, and 45 μl. O_2/5 mg. peptide hydrochloride, and for licheniformin B 45, 39, 35, and 45 μl. O_2/5 mg. peptide hydrochloride. These figures indicate that each peptide contained the same proportion of amino-acid in the D form. In the hope that the

D-amino-acid could be identified, the material from each Warburg flask was freed from enzyme (heat coagulation) and a portion was used for two-dimensional paper partition chromatography. Both peptides had initially given eight different amino-acids on hydrolysis and the same eight amino-acids were still present after oxidation with D-amino-acid oxidase. The conclusion is that in each peptide at least one species of amino-acid is present in both optical forms.

End amino groups in licheniformins A *and* B

The method of Sanger (1945) for the estimation of end groups using fluorodinitrobenzene was applied to each of the peptides. Each peptide (25 mg.) was treated with FDNB in the usual way and, after hydrolysis for 24 hr. at 105° with 10N-HCl (sealed tube), the product was separated into a water-soluble and an ethyl acetate-soluble fraction. The ethyl acetate-soluble fraction was passed through a column of silica gel and the very faint yellow band collected. Measurement of the colour intensity of this band in each case showed that it represented less than 6% of the theoretical value required for one end group. A sample of each DNP-peptide was also hydrolysed for 6 hr. with 6N-HCl, but no end group was found. Confirmation that both peptides had failed to react with FDNB was obtained by paper chromatography of the water-soluble fraction from the acid hydrolysis. This fraction contained ε-dinitrophenyllysine, and in addition, all the other amino-acids present in a simple acid hydrolysate of the peptide. The DNP-lysine was put on a column of silica with methyl ethyl ketone-ether (Sanger, 1945) as solvent. A single band indistinguishable from synthetic ε-DNP-lysine was obtained.

Biological tests

Antibacterial activity. The three antibiotics were tested *in vitro* against *Mycobact. phlei* and the greatest dilution capable of inhibiting surface growth in Hartley digest broth was recorded. Licheniformin A (hydrochloride) inhibited at 1 in 5×10^6; licheniformin B at 1 in 10×10^6; and licheniformin C at 1 in $0{\cdot}32 \times 10^6$. The figures quoted are averages for three successive tests.

Chronic toxicity (mouse). Table 2 shows the results of chronic toxicity tests for which we are indebted to Dr Janet Niven. The antibiotics were made up in sterile saline at about 5 mg./ml. and injected subcutaneously in mice. A sample of unfractionated licheniformin corresponding in properties with the licheniformin hydrochloride used in previous toxicity tests (Callow *et al.* 1947) was used for comparison and a sample of bacitracin was also tested. The assessment of kidney damage is subjective, but was always made by the same person and indicates the degree of micro-anatomical alteration in the kidneys of the experimental animals.

Mouse protection test against tuberculous infection. As licheniformin A was found by the chronic toxicity test to be much less toxic than B or C, it was tested further for its ability to control tuberculous infection in mice. The tests were made by the procedure outlined in an earlier paper (Callow *et al.* 1947) and streptomycin was simultaneously tested as a control. We are indebted to Dr P. D'Arcy Hart and Dr R. J. W. Rees for carrying out the tests. Licheniformin A was tested at two dose levels, 3 mg./day and 6 mg./day. The lower dose caused no obvious toxic symptoms but failed to control the infection; the higher dose controlled the infection to a lesser degree than streptomycin at the level of 0·3 mg./day and caused some kidney damage.

Table 2. *Chronic toxicity for mice of licheniformins* A, B *and* C, *compared with standard preparation and with bacitracin*

(Licheniformin A hydrochloride 5 mg./ml., licheniformin B hydrochloride 5 mg./ml., licheniformin C hydrochloride 7 mg./ml., bacitracin* 5 mg./ml., licheniformin standard (S 785) 5 mg./ml. Dose schedule 0·2 ml. morning, 0·4 ml. evening, and 0·4 ml. Saturday for 14 days (no dose given on Sundays). Each test made on three animals. The degree of kidney damage is indicated; +, slight; + + + +, severe.)

Licheniformin					
A	B	C	Bacitracin	S 785	Control (saline)
+	+ + + +	+ + +	+ +	+ + +	—
+	+ + +	+ + + +	+ + +	+ + +	—
+	+ + + +	+ + + +	+ + + + (died)	+ + +	—

* A specimen of commercially manufactured product provided by Dr F. L. Meleney.

DISCUSSION

A large number of antibiotics have been reported in the culture filtrates from aerobic spore-forming bacilli (Baron, 1950). The nature of the substance responsible for antibiotic action is not known in every case, but where a pure or nearly pure product has been isolated, activity has frequently been found in a peptide fraction (Gilliver, Holmes & Abraham, 1949; Sharp, Arriagada, Newton & Abraham, 1949; Howell, 1950; Catch, Jones & Wilkinson, 1949; Barry, Gregory & Craig, 1948; Consden, Gordon, Martin & Synge, 1947; Hotchkiss, 1944). In some instances an antibiotic peptide, which was at first thought to be homogeneous, has later been resolved into several distinct substances (Gregory & Craig, 1948).

In the present investigation, numerous attempts at purification of licheniformin hydrochloride by precipitation, salt formation and adsorption chromatography gave little or no evidence of heterogeneity, but paper partition chromatography showed that the best preparation of licheniformin previously obtained (Callow *et al.* 1947) was a mixture of at least three antibiotics. Counter-current distribution methods (Bush & Densen, 1948; Craig & Post, 1949) were successful in separating these three antibiotics, but nevertheless the purity of the final products, the hydrochlorides of licheniformins A, B and C, cannot be regarded as absolutely established. Each substance gave a single zone of colour with ninhydrin after paper chromatography and this zone coincided entirely with the zone of antibiotic activity. Each substance gave a distribution curve in a 24-stage counter-current fractionation which followed closely the theoretical curve for a pure compound. The materials are, therefore, each homogeneous with respect to partition coefficient between two pairs of solvents, but no crystalline derivative could be obtained.

We have been informed that Dr L. H. Kent, Dr B. T. Tozer and Dr J. H. R. Slade at the Microbiological Research Department have obtained very similar results with products from the A 5 strain on ammonium lactate medium. Like ourselves, they would put forward only with the greatest reserve the statement that any fractions are chemically homogeneous.

Biological activity

Crude licheniformin hydrochloride (Callow *et al.* 1947) had a wide antibacterial spectrum, but its main interest lay in its considerable activity against acid-fast organisms and in its potentialities as an antitubercular drug. The limitation to the use of the crude antibiotic was that it caused severe damage to the kidney. Purified licheniformin C was less active against acid-fast organisms than the original crude preparation and caused more extensive kidney damage. Licheniformin B was rather more active *in vitro* than the original preparation, but it also caused extensive kidney damage. Licheniformin A was much less toxic than either of the other fractions, but still caused some slight kidney damage and was less effective than streptomycin in controlling tuberculosis in mice (p. 565). It would be tempting to suppose that renal toxicity is not an inherent property of licheniformin A and that the small degree of kidney damage evident in our tests was due to contamination of this substance with another peptide but, since the criteria of purity were fairly exacting, we do not ourselves favour this view. Renal toxicity has been reported also with several other antibiotic peptides obtained from related bacteria, and attempts to separate this toxicity from the antibacterial activity have not been entirely successful (Miller, McDonald & Shock, 1950; Smith, Schultz, Ott & Payne, 1949; Abraham & Newton, 1950).

Chemical structure

The difficulty experienced in separating the three compounds indicated some similarity of composition, but we did not expect such close similarity as was actually found. Licheniformin C was readily distinguishable from the other two peptides in that it gave, on acid hydrolysis, glutamic acid in addition to the eight amino-acids found in licheniformins A and B. In other respects the three compounds showed remarkable similarity. Elementary analyses did not distinguish between them; all three had the same optical activity and about the same molecular weight; all three, after reaction with fluorodinitrobenzene, failed to give any 'end group' (Sanger, 1945) on acid hydrolysis.

Licheniformins A and B, being the more active antibiotics, were examined in greater detail than C. As can be seen from Table 1, the amounts of amino-acids produced on hydrolysis are strikingly similar: they account quite well for the whole of the original peptides and it seems unlikely that the difference in toxicity between A and B could be due to some unrecognized non-peptide component. The difference between the molecular weights of licheniformins A and B (4400 and 3800) is scarcely significant, having regard to the errors of the method, and in Table 3 the number of amino-acid residues/molecule of peptide is calculated on the assumption that the mean of these figures is the molecular weight of both peptides. There are three major sources of error in this calculation: (i) molecular weight, (ii) percentage composition as determined by micro-analyses and (iii) estimation of individual amino-acids by colorimetric or microbiological methods. Tristram (1950) has pointed out that in order to estimate the stoicheiometric ratio of amino-acids in a protein or peptide the percentage error in the analysis of any one amino-acid must not exceed $\pm 0{\cdot}4/R \times 100$, where R is the number of residues of that amino-acid. We have not had sufficient material to permit repeated quantitative analyses on our antibiotics, but we consider that the methods used cannot be relied upon to be more accurate than $\pm 5\%$, thus it may well be that the error approaches 10% with a few of our amino-acid estimations.

Table 3. *Amino-acid residues in licheniformins* A *and* B

(Results are calculated assuming a molecular weight of 4100 for each peptide. Figures are given to nearest whole number but where there is doubt, the calculated analytical figure is given in brackets. For definition of permissible error, see text.)

	Licheniformin A	Licheniformin B	Permissible error (%)
Aspartic acid	1	1	40
Glycine	7 (7·4)	7 (7·2)	5
Serine	3	3	13
Proline	2	2	20
Arginine	6 (5·65)	6 (5·5)	6·7
Phenylalanine	2 (2·4)	2 (2·5)	13
Valine	2	2	20
Lysine	12	12	2·8

The last column of Table 3 gives the percentage accuracy required to calculate, with certainty, the number of residues of each amino-acid present in the unit of molecular weight 4100. It is apparent from these figures that our failure to find any significant difference in residue numbers between the two peptides does not necessarily prove that they have the same quantitative composition.

With these reservations in mind the close chemical resemblance between licheniformins A and B is

nevertheless quite striking and the difference in toxicity is difficult to explain. A difference in optical form of the constituent amino-acids of these two peptides is not attractive as an explanation, since each peptide, after hydrolysis, gave an amino-acid mixture in which the same proportion of amino-acid was present in the D form; however, the species of D-amino-acid was not established in either case. We are more inclined to suggest that the difference in toxicity between licheniformins A and B may be attributable to a difference in amino-acid sequence within the two peptides. Some attempts were made to confirm this idea by partial hydrolysis experiments, but both compounds gave mixtures of basic peptides which we could not fractionate.

The suggestion has already been made (Work, 1949) that certain antibiotics may act by interference with protein synthesis. It was pointed out that protein synthesis is a species-specific process and that inhibition of protein synthesis by metabolite analogues might be expected to be species-specific also. We have now obtained some evidence (Campbell & Work, 1951) that peptides are normal intermediates in protein synthesis and it is therefore not surprising that amino-acid sequence in an antibiotic peptide may influence toxicity. We have, as yet, no clue to the cause of the common nephrotoxic action of antibiotic peptides.

SUMMARY

1. The licheniformin of Callow *et al.* (1947) has been separated by the use of counter-current distribution into three antibiotic peptides, licheniformins A, B and C.

2. Licheniformins A and B are both hydrolysed by acid to give glycine, serine, proline, valine, phenylalanine, lysine, arginine and aspartic acid. Licheniformin C gives these amino-acids and, in addition, glutamic acid.

3. All three peptides have similar molecular weights (3800–4800), optical rotations and elementary compositions.

4. Licheniformins A and B are more active against *Mycobacterium phlei* than licheniformin C, and are less toxic to mice than licheniformin C.

5. Licheniformin A, the least toxic peptide, was less effective than streptomycin in controlling a tubercular infection in mice.

6. Quantitative amino-acid analyses were made on licheniformins A and B and the stoicheiometric ratio of the amino-acids calculated for each peptide.

7. Licheniformins A and B are both devoid of reactive end amino groups (fluorodinitrobenzene) and are presumably cyclic.

8. The difference in biological activity between licheniformins A and B is regarded as due to a difference in amino-acid sequence between the two peptides.

ADDENDUM I

Microbiological Estimation of Lysine, Valine and Phenylalanine in Licheniformin

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Although the estimation of lysine, valine and phenylalanine by microbiological methods is a standard procedure, some difficulty was experienced in using, unmodified, the media of earlier authors. After testing the media of Dunn, Shankman, Camien, Frankl & Rockland (1944), Barton-Wright (1946), Sauberlich & Baumann (1946) and Steele, Sauberlich, Reynolds & Baumann (1949), some modifications were made to the last two, and good acid production with linearity over a substantial part of the curve was attained. A brief account of these modifications is given. The results obtained are given in Table 1 (p. 564).

MATERIALS AND METHODS

Cultures. Lactobacillus arabinosus 17–5 was used for the determination of valine and phenylalanine; *Leuconostoc mesenteroides* P–60 was used for the determination of lysine.

Stock stab cultures were carried in a Nymon & Gortner (1946) medium, modified as follows. For 100 ml. of medium: Difco bacto-peptone, tryptone and yeast extract, 0·5 g. each; liver extract, 10 ml.; glucose 1·0 g.; sodium acetate (hydrated), 1·0 g.; 0·5 ml. each of salt solutions *A* and *B* (see basal medium); agar, 1·5 g. This medium maintained vigorous growth for all the organisms commonly used for amino-acid assays (*Lb. arabinosus* 17–5, *Lb. mesenteroides* P–60, *Streptococcus faecalis* R, *Lactobacillus fermenti* 36

Leuconostoc citrovorum, *Lactobacillus brevis*), and was an improvement on published media on which cultures tended to die out. Transfers were made monthly. This practice was discontinued, however, in the case of *Lb. arabinosus* owing to a gradual departure in linearity of response and the latter was maintained in the dried state. Before use in assays the organisms were grown in broth of the same composition as the above, with the agar omitted.

Basal medium. Medium I of Sauberlich & Baumann (1946) was used with *Lb. arabinosus* 17–5 with the following modifications. Serine, glycine and proline were omitted from the medium as unnecessary; sodium acetate (hydrated) was increased from 20 to 33 g./500 ml. of double-strength medium; 200 mg. of L-asparagine was replaced by 640 mg. of DL-aspartic acid; riboflavin was reduced from 0·5 to 0·25 mg. (see below).

Medium VI of Steele *et al.* (1949) used with *L. mesenteroides* P–60 was modified by increasing the vitamins. 500 ml. of double-strength medium contained: aneurin 1·0 mg.; pyridoxin, 1·6 mg.; calcium D-pantothenate, 1·0 mg.; nicotinic acid, 2·0 mg.; *p*-aminobenzoic acid, 0·1 mg.; biotin, 0·005 mg.; folic acid, 0·01 mg.; riboflavin, 0·25 mg. The smaller quantity of riboflavin used in both media I and VI served to diminish the colour of the medium and hence to facilitate titration, and in no way affected the assays.

The inorganic salt solutions used to make up 500 ml. of media (double strength) were as follows: Solution *A*, 500 mg. of KH_2PO_4 and 500 mg. of K_2HPO_4 in 5 ml. Solution *B*, 200 mg. of $MgSO_4.7H_2O$, 10 mg. of $FeSO_4.7H_2O$, 20 mg. of $MnSO_4.4H_2O$, and 10 mg. of NaCl in 5 ml.

Standard curve. The synthetic DL-acid was used, after confirming that the D-form was not available with the media employed; however, calculations were based on the L-isomers only. The range (per tube) for the construction of the linear portion of the curve was as follows: L-lysine HCl, 0–50 μg., L-valine and L-phenylalanine, 0–25 μg.

Preparation of samples for assay. Licheniformin hydrochloride (15 mg.) was hydrolysed in 6N-HCl for 16 hr. at 105° in a sealed evacuated tube. The HCl was removed over KOH and the residue made up to 10 ml. in water. Portions taken were adjusted to pH 6·8.

Assay procedure. The sample was added to 5 ml. quantities of double-strength medium and the tubes sterilized at 10 lb./in.^{2} for 5 min. This gave less darkening of the medium than with the longer periods and higher pressures commonly used. The lactic acid was titrated to pH 6·8 with 0·1N-NaOH using Smith's (1930) indicator suitably altered so as to produce the colour change of orange-grey to pure grey at pH 6·8 instead of 6·9. This modified indicator, containing 0·09 g. of bromothymol blue and 0·10 g. of alizarin red sulphonate dissolved in 100 ml. of 30% (v/v) aqueous ethanol, gives a much sharper end point than the bromothymol blue always recommended in the literature for microbiological assay titrations.

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ADDENDUM II

Sedimentation and Diffusion of Licheniformins A, B and C

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Sedimentation. This was observed in a Svedberg oil-turbine ultracentrifuge by the method of Cecil & Ogston (1948). The hydrochlorides were dissolved in 0·2 M-NaCl to a concentration of 1 g./100 ml. Owing to the relatively small molecular weights and high diffusion coefficients, clear sedimentation boundaries were not obtained; the sedimentation constants were therefore measured by the method of Gutfreund & Ogston (1949). These results are probably not more accurate than ±5%.

Diffusion. This was measured in the Gouy diffusiometer (Coulson, Cox, Ogston & Philpot, 1948). The same solutions were used as for sedimentation. Since an equilibrium diffusate could not be prepared by dialysis, the solvent into which diffusion took place was NaCl of a concentration calculated to equal that of diffusate on the basis of the Gibbs-Donnan equilibrium. The observed diffusion coefficients are then comparable with the sedimentation constants. Both sedimentation and diffusion constants are corrected to their values in water at 20° (Table 1). In calculating the molecular weights, a value of 0·75 has been assumed for the partial specific volume.

Table 1. *Sedimentation and diffusion constants*

Licheniformin	S_{20} (corr.) × 10^{13}	D_{20} (corr.) × 10^{7}	Mol.wt.
A	0·96	21·0	4400
B	0·86	22·4	3800
C	0·95	19·4	4800

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A Solubility Analysis of Crystalline Ox-Liver Catalase

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Loew (1901) first demonstrated that the decomposition of hydrogen peroxide by plant and animal tissues was due to an enzyme which he named catalase. Other workers proceeded to isolate and purify this enzyme and eventually Zeile & Hellström (1930) established that the enzyme was an iron-porphyrin protein. This was confirmed by Keilin & Hartree (1936) and Stern (1935), who also proved that the iron-porphyrin was protohaematin. Sumner & Dounce (1937) crystallized ox-liver catalase soon afterwards; since then many other liver and erythrocyte catalases and a bacterial catalase have also been prepared in a crystalline state.

Catalase has been found to be present in all aerobic cells so far examined and is present in high concentrations of from 10^{-6} to 10^{-7} M in the liver, kidneys and erythrocytes of mammals. Dounce (1943) has stated that the catalase of liver is confined to the cytoplasm of liver cells, but has since found (1951) that this observation was due to the method of preparation of the liver-cell nuclei and that nuclei prepared by an improved method do contain catalase.

Stern & Wyckoff (1938), using an ultracentrifuge method, found the molecular weight of ox- and horse-liver catalases to be about 250 000, and from the iron content of their preparations calculated that the catalase molecule contained four atoms of iron. Sumner & Dounce (1937) observed that only about half the iron of their crystalline ox-liver catalase preparations was bound as haematin, and Lemberg, Norrie & Legge (1939) later demonstrated