Antibiotics produced by *Bacillus licheniformis*

1. A Practical Chemically Defined Medium for Production of Licheniformin

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SUMMARY: The production of licheniformin-like antibacterial activity in culture by a single strain of *Bacillus licheniformis* required neutral or alkaline conditions, conveniently attained by the use of lactate rather than glucose as a source of carbon in a chemically defined medium.

When the medium contained initially about 0.04–0.10% nitrogen, supplied as asparagine or as ammonium lactate, and the cultures, harvested after 4–9 days at 37°, were sterilized by bringing to pH 2.5 and autoclaving, the inhibitory dilution against a test strain of *Mycobacterium phlei* was 1/160 or greater.

Fluid from cultures in a chemically defined medium, containing 0.06 M ammonium lactate and 0.05 M sodium lactate, inhibited the test organism at a dilution of 1/200–1200 (geometric mean, 530) in 44 consecutive batches of 100–200 l. culture fluid produced by incubation of cultures in shallow layers. The pH value of the harvested fluid was about 9 and the antibiotic material was partly bound by the cells. It was largely freed by adjustment to pH 2.5.

When amino-acids were added to the medium either as a mixture of known amino-acids, or as a casein hydrolysate, the maximum titre was attained earlier, but with no significant change in its value. A similar result was obtained with yeast extract added alone or with casein hydrolysate.

Licheniformin is an antibiotic whose production by a strain of *Bacillus licheniformis* was first reported by Callow & Hart (1946). The isolation of highly concentrated material and its chemical and biological properties were described briefly by Callow, Glover & Hart (1947), and in more detail by Callow, Glover, Hart & Hills (1947). Cultures with similar biological and chemical properties were obtained only from those spore-bearing aerobic organisms (11 of 81 cultures examined) which were identified as *B. licheniformis* by the criteria of Gibson (1944).

The present communication describes the development of a chemically defined medium for the production of active culture fluids. A preliminary communication indicated the reasons for adopting the formula now in use (Hart & Hills, 1947) which has lactate as main source of carbon and ammonia as source of nitrogen. The efficacy of such simple nutrients was not foreseen at the beginning of the work, since at that time aerobic spore-bearers were reported either as failing completely to produce antibiotic from such sources, as in subtilin production (Jansen & Hirschmann, 1944) or as producing relatively low yields in comparison with those from more complex media, as in tyrothricin production (Lewis, Dimick & Feustel, 1945). Hence, in spite of the desirability of using a chemically defined medium to facilitate isolation of the active material free from impurities introduced in the medium, it was considered expedient to examine antibiotic production in a complex medium of controllable

composition. A suitable medium for this purpose was the CCY medium of Gladstone & Fildes (1940) in which the nitrogen is supplied as the amino-acids of an acid hydrolysate of casein, supplemented with a tryptic digest of the same protein to supply tryptophan. Other water-soluble growth factors are provided by a boiled yeast extract.

METHODS

The techniques were in general those of Callow *et al.* (1947), modified as described below.

Cultures. The organism used throughout was the Hampstead strain of *B. licheniformis*, now N.C.T.C. 7072, maintained on glycerol asparagine agar. Experimental media were inoculated from a liquid glucose asparagine culture as described by Callow *et al.* (1947). In addition, spores from the original culture, as received from Hampstead, were stored in the dried state by washing off in distilled water, heating for 90 min. at 60° to destroy vegetative forms, and drying in 0.05 ml. amounts in 10 × 45 mm. tubes over P₂O₅ at a pressure of 0.05 mm. Hg. The tubes of dried spores were evacuated again in test-tubes, which were sealed and stored at 2°.

For convenience, spore suspensions were used in the inoculation of the medium finally adopted for routine production. They were prepared from the tubes of dried spores, which were incubated at 37° for 6 hr. in tryptic beef broth; 0.5 ml. was then seeded into asparagine glucose liquid medium without Mn (Callow *et al.* 1947), and incubated overnight at 37°. One and a half ml. of this culture was seeded on to 30 ml. quantities of solid maintenance medium (Callow *et al.* 1947) in 8 oz. medical flats. Complete sporulation occurred in 7 days at 37°. The spore suspensions were standardized by the method of Callow *et al.* (1947).

Culture media. The following media were tested for production of antibiotic: the CCY medium of Gladstone & Fildes (1940), the glucose + asparagine medium of Callow & Hart (1946), the two 'growth' media of Callow *et al.* (1947), and modifications of these.

Conditions of incubation. All cultures were incubated for varying periods at 37°. Most of the work was done with stagnant cultures of 15 ml. of medium in 50 ml. conical flasks, 50 ml. in 250 ml. conical flasks or 1 l. in rectangular enamelled-steel trays, approx. 25 × 40 cm. In each case the average depth of medium was about 1 cm. Separate flasks or trays, or a pool of replicates were used for each determination, since it was found that disturbance of the pellicle by sampling led to irregular results on continued incubation. In a few tests 15 ml. cultures were shaken in 50 ml. flasks at 120 cycles/min., the amplitude being 2.5 cm.

Precision of the method of assay. Experience with the method as described by Callow *et al.* (1947) suggested that even two-fold differences between single assays could not be regarded as significant. This was confirmed by statistical analysis of the results for 20 consecutive 150 l. batches of culture. Assays were carried out in duplicate, or sometimes triplicate, on samples of culture which had been subjected to three different treatments. Bartlett's test showed no

significant heterogeneity of variance of the log potency between the three treatments ($P > 0.25$) and there was no significant correlation ($r = +0.085$; $P > 0.1$) between the log potency and its standard error within the observed range of end-points (dilutions of 1/300–1/1500). A pooled estimate of the variance, based on the 71 degrees of freedom available from the 60 samples, corresponded to a standard error of 0.11 for the log potency and an insignificant difference ($P = 0.95$) for observations in the ratio less than 2.0. Since this ratio was only diminished to 1.5 for the difference between the means of triplicates, it was usually expedient to save labour on replication of assays in favour of confirmation of results by total repetition of experiments. Crucial tests were, however, planned in a form providing internal evidence of accuracy. Geometric rather than arithmetic means were used in pooling the results of assays, in view of the uniformity of variance of the log potency.

RESULTS

Media with casein hydrolysates as source of nitrogen

Since the original medium of Callow & Hart (1946), with asparagine as source of N, contained 3 % glucose, modifications of CCY medium with this concentration of sugar were compared with normal CCY medium containing 0.65 % sodium lactate (58 mm), and a modification without either lactate or glucose. Table 1 shows that, although it was possible to get moderate titres at 6 days

Table 1. *The effect of carbon source on licheniformin production in CCY medium compared with glucose asparagine medium*

Medium	Licheniformin units/ml. at (days)			pH at (days)		
	2	4	6	2	4	6
CCY, no lactate	101	40	20	8.5	9.1	9.3
CCY + 58 mm lactate	80	40	20	8.7	9.3	9.4
CCY + 150 mm glucose	20	20	20	5.7	7.5	8.3
Asparagine + 150 mm glucose	23	80	101	6.9	8.0	7.2

with the medium of Callow & Hart (1946), CCY medium with the same glucose concentration produced no antibiotic within this period, whereas in the absence of glucose moderate titres were reached within 2 days, declining on further incubation. It appeared from the pH changes that a slightly alkaline reaction was an essential but not sufficient condition for antibiotic formation. Excessive alkalinity, on the other hand, was to be avoided.

In other experiments titres as high as 320 units/ml. (i.e. cultures inhibiting at a dilution of 1/320) were reached as early as 18 hr. and were sometimes maintained for as long as 4 days after inoculation. Initial acidity delayed or prevented the attainment of a high titre even when good growth occurred. In media with and without lactate, the omission of glycerophosphate buffer or its replacement by an equivalent amount of phosphate had no effect (Table 2). In all cases a pH value of about 9 was reached by the third day and subsequently the titre fell considerably.

Although moderate titres were reached in CCY medium, this was never used for routine production of antibiotic, since observations on the conditions of antibiotic formation in that medium led to modifications of chemically defined media that gave consistently higher titres. Hence, further modification of CCY medium was not undertaken.

Table 2. *The effect of buffer on licheniformin production in CCY media*

Buffer (33 mm)	Lactate concentration (mm)	Licheniformin units/ml. at (days)	
		3	6
None	0	225	112
	58	225	80
Phosphate	0	190	80
	58	112	80
Glycerophosphate	0	160	66
	58	225	55

Media with asparagine as a source of nitrogen

The glucose asparagine medium of Callow & Hart (1946) had a number of disadvantages due to the glucose. Thus the glucose concentration was critical and dependent on the type of vessel used for incubation. With 2 % glucose, 50 ml. of medium in 250 ml. conical flasks gave the optimum titre of 320 units/ml., but 250 ml. medium in cylindrical flasks of about 18 cm. diam. gave more variable and, on the whole, lower titres, although the average depth of medium in both cases was about 1 cm. Glucose also gave highly viscous culture fluids and tough pellicles, to which the active material was bound, and from which it was difficult to isolate.

Table 3. *The effect of replacing glucose by lactate in media with asparagine as source of nitrogen*

Source of carbon	Concentration (mm)	Licheniformin units/ml. at (days)				pH at (days)			
		2	3	4	6	2	3	4	6
Lactate	16.7	225	225	160	160	8.8	9.0	9.0	9.0
Lactate	33.3	160	80	320	320	8.8	9.0	9.0	9.0
Lactate	67	160	160	160	450	8.8	9.0	9.0	9.0
Lactate	133	80	160	160	320	8.8	9.0	9.2	9.0
Lactate	250	14	—	20	14	9.0	—	9.5	9.3
Glucose	110	20	160	160	225	6.5	7.0	7.4	8.6

Since (i) the maximum titre was not reached in this medium until after the initial acid fermentation had ceased and the medium had either regained neutrality or had even become alkaline, and (ii) the maximum titre was reached earlier in those modifications of the CCY medium which never became acid, the effect of replacing glucose by lactate was tested (Table 3). On the basis of this, and similar experiments, it appeared that the maximum titre was reached

earlier with low concentrations of lactate but the best titres were reached in about 6 days with about 0.75 % sodium lactate (67 mM). The lactate concentration, however, was not critical and only in high concentrations, roughly equivalent in total carbon to the glucose previously used, were poor titres observed. Similar results were observed with 15, 50 and 1000 ml. volumes of medium provided the average depth was about the same; variations in this depth from 5 to 15 mm. merely delayed the appearance of maximum activity for periods up to 24 hr.

Media with ammonia as source of nitrogen

Although replacing the unsatisfactory media containing high glucose concentrations, the asparagine lactate medium was still not suitable for large-scale production because of difficulty in obtaining adequate supplies of asparagine. Replacement of asparagine by a mixture of ammonium chloride and sulphate, as used by Fildes (1938), gave low titres: 40–80 units/ml. after

Table 4. *Licheniformin production and pH changes with ammonia as source of nitrogen and lactate as source of carbon*

Lactate (mM)	Ammonia* (mM)	Licheniformin units/ml. at (days)			pH at (days)		
		1	2	4	1	2	4
33	17	40	80	80	8.0	8.0	9.2
67	33	40	80	160	8.2	8.8	9.4
133	67	20	80	160	8.2	8.8	9.2
267	133	0	0	40	7.6	8.4	8.6

* The ammonia was added as an equinormal mixture of thrice recrystallized ammonium chloride and sulphate.

2–6 days with 25–50 mM lactate. The nitrogen content (17 mM), however, was less than 25 % that of the asparagine medium. When increasing the concentration of ammonium salts it was necessary to increase the concentration of metabolizable anions (as in penicillin production on chemically defined media; Jarvis & Johnson, 1947), in order to prevent the development of acidity which could not be adequately controlled by an increased buffer capacity. Titres were thus increased to 160 units/ml. at 4 days with 33–67 mM ammonia and 67–133 mM lactate (Table 4). There was little activity with 133 mM ammonia and 267 mM lactate, but this was not due to adverse pH changes, since development of alkalinity was little delayed compared with that in cultures in less concentrated media. Similar titres were reached 24–48 hr. earlier in shaken cultures and even higher titres, up to 640–1280 units/ml., when the ammonium salts were replaced by a commercially available solution of ammonium lactate. The medium could also be made up with lactic acid (British Pharmacopoeia standard, approx. 12 N) neutralized partly with NaOH and partly with ammonia; but this method was inconvenient, owing to the need for boiling the medium at an alkaline pH to hydrolyse lactide, and a variable decrease in pH value which occurred on autoclaving. This method was therefore abandoned

owing to the uncertain loss of ammonia and the need for a final adjustment of pH under aseptic conditions. It served, however, to show, using 1 l. quantities of medium in trays, that the amount of ammonia (sp. gr. 0.880) could be 2, 3 or 4 ml. giving 30, 45 or 60 mm NH_4^+ , and the initial pH could be 6.4, 7.0 or 7.5, with little effect on the titre at 7 days (Table 5).

Table 5. *The effect of initial pH and ammonia content of media*

All media contained 10 ml. lactic acid (B.P.)/l.

Ammonia (sp.gr. 0.880) (ml./l.)	Initial pH	Licheniformin units/ml. at (days)		
		6	7	11
2	6.4	600	300	200
	7.0	250	400	200
	7.5	50	500	400
3	6.4	500	500	600
	7.0	300	400	500
	7.5	20	300	1000
4	6.4	400	600	600
	7.0	400	500	600
	7.5	40	800	400

This medium was made up most conveniently with commercial 50 % ammonium lactate and 50 % sodium lactate, using 10 ml./l. The initial pH 7.0–7.2 fell to 6.5–6.7 after autoclaving 15 min. at 15 lb./sq. in. Analysis showed about 110 mm lactate (by the method of Friedemann & Graesser, 1933) and 60 mm ammonia, after autoclaving. In one experiment triplicate daily assays at 4–9 days showed a maximum titre of 1100 units/ml. at 5 days, but this hardly differed significantly ($P \approx 0.95$) from the lowest assays of 680 units/ml. observed at 4 and 9 days. The standard error of the log potency was 0.108, identical with the pooled estimate observed later on 20 consecutive batches of culture fluid.

Effects of amino-acids and other growth factors

The inclusion of 0.15 % acid-hydrolysate or tryptic digest of casein in the basal ammonium sodium lactate medium stimulated growth and gave a markedly raised titre during the first 2–4 days. The maximum titre was usually attained during this period (Table 6), but was not significantly different from that in the control flasks at 6–7 days. This effect was not due merely to increased available nitrogen, because the substitution of an equivalent amount of nitrogen as ammonium lactate did not give an early high titre. A known mixture of amino-acids behaved similarly to the casein hydrolysate. The addition of yeast extract, alone, or with tryptic casein also stimulated growth and gave high titres in 24–48 hr.

The addition of purines and pyrimidines gave a maximum titre in 4 days. A mixture of certain growth factors was inhibitory, viz. biotin, 0.005 μM ; Ca pantothenate, riboflavin and aneurin, 1 μM ; oleic acid and pyridoxin, 2 μM ; nicotinamide, 5 μM ; haemin, 10 μM ; choline chloride, 20 μM ; and inositol,

100 μM . The inhibition was traced to haemin, which was present at 10 μM . In the absence of the haemin a stimulation of growth was observed with the other substances. Van Heyningen (1948) reported inhibition of growth of *B. licheni-*

Table 6. *Effects of amino-acids and other stimulants of growth*

In all experiments duplicate flasks were assayed for each treatment. The titres quoted are the geometric means for the number of experiments indicated in parentheses.

Supplement	Licheniformin, units/ml. (no. exp.) at (days)						
	1	2	3	4	5	6	7
Nil	0 (3)	30 (3)	80 (3)	160 (3)	300 (3)	700 (2)	1300 (1)
Ammonium lactate (17 mM)	—	20 (1)	—	400 (1)	—	600 (1)	300 (1)
Yeast extract ($\equiv$ 10 % fresh yeast)	900 (1)	450 (4)	600 (3)	500 (4)	400 (3)	500 (3)	250 (2)
Tryptic digest of casein ($\equiv$ 0.15 % casein)	600 (1)	600 (4)	800 (3)	600 (4)	800 (3)	800 (3)	400 (2)
Yeast extract + tryptic digest of casein	1300 (1)	1300 (3)	1000 (3)	700 (3)	500 (3)	300 (1)	—
Acid hydrolysate of casein ($\equiv$ 0.15 % casein)	—	400 (1)	—	600 (1)	—	600 (1)	150 (1)
Amino-acid mixture*	—	100 (1)	1300 (1)	1300 (1)	2500 (1)	500 (1)	—
Purines + pyrimidines†	—	0 (1)	40 (1)	900 (1)	450 (1)	300 (1)	—

* Glycine, alanine, serine and glutamate, 1000 μM ; leucine, *isoleucine*, *norleucine*, proline, L-hydroxyproline, phenylalanine, tyrosine, aspartate, L-arginine and lysine, 500 μM ; valine, threonine, L-cystine, methionine and L-histidine, 200 μM ; tryptophan, 100 μM . Concentrations, except where shown otherwise, are of the L-form in DL-mixture. Glutamine, 500 μM , was also present.

† Adenine, guanine, xanthine, cytosine, thymine and uracil, 50 μM each.

formis and of a number of other aerobic sporing bacilli by haematin in agar at ten times the concentration we used. The haemin effect in our experiments was rather erratic. In a few cultures, growth was observed after a lag of 3–4 days, whereas replicates remained clear for several weeks. This difference was not associated with variations in the initial pH of the medium.

Routine production

The simple ammonia + lactate medium was used without supplements of yeast extracts or casein hydrolysates. One litre volumes were incubated for 6 days in enamelled steel trays with loose-fitting lids. The pH rose to 8.00–9.75. The cultures were acidified to pH 2.5 with about 8 ml. 12 N-HCl/l. and boiled for 20 min., since Callow & Hart (1946), using glucose asparagine medium, had found that some such treatment was needed to free the antibiotic from the cells. After cooling the liquid rapidly in a 120 gal./hr. milk cooler, the cellular debris was removed in a continuous centrifuge.

In 44 consecutive batches, the potencies of the whole culture lay within a range of 270–1200 units/ml., with a geometric mean of 530. Results as low as 270 units/ml. for some early batches were believed to be due to over-heating of the hot-room, when freshly autoclaved vessels of medium for a succeeding

batch were allowed to cool there, preparatory to inoculation at 37°. When the flasks of medium were allowed to cool before transfer to the hot-room, low results were observed only irregularly and were almost certainly due to variability in the assay. In spite of such irregularities, the potencies of the culture fluids were significantly correlated with those of the whole culture ($r = +0.675$; $P > 0.999$) over the complete series (Fig. 1) though shorter series were not always adequate to show it (Table 7).

Fig. 1. The recovery of licheniformin in culture fluids. The fluid was removed after boiling 20 min. at pH 2.5; series I, single observations $\square$, two identical observations $\square$; series II, cultures also supplying data on removal of fluid at pH 9 and at pH 2.5 without boiling (Table 7) $\triangle$; series III, cultures also supplying data on removal of fluid at pH 2.5 without boiling (Table 7), single observation $\diamond$, two identical observations $\diamond$, three identical observations $\diamond$.

It was found that under these conditions the active material was no longer firmly bound to the cells and over 50 % could be recovered from the supernatant on centrifuging the whole culture at the pH reached during incubation (Table 7). The average loss, however, was highly significant ($P > 0.999$) in the 12 unselected batches on which the determination was carried out. The loss was diminished, but was probably still significant ($P = 0.98$), on centrifuging at pH 2–3 and 36 batches were needed to show significance at this level. Finally, the loss was decreased to 7 %, and was then insignificant ($P = 0.92$) after boiling at this pH and centrifuging either at the same pH or at pH 5. The latter value, pH 5, was frequently convenient, since it was also used for the subsequent adsorption of the active material on charcoal.

DISCUSSION

The routine medium described is produced cheaply from chemicals available commercially in bulk. Since highly purified chemicals and minimal inocula were not used, we have not established the minimal nutritional requirements of *B. licheniformis*. The mineral requirements, in particular, were not examined,

Table 7. *The recovery of active material in culture fluids*

Conditions of removal of cells	No. of batches treated	Correlation coefficient of log potencies of whole culture and supernatant <i>r</i>	Probability of <i>r</i> due to chance <i>P</i>	Percentage recovery		
				Mean	Fiducial* limits of mean <i>P</i> =0.95	Probability of loss* due to chance <i>P</i>
At final pH of culture (8.00–9.75)	12	0.47	0.1	56	45–69	0.001
At pH 2.05–3.17	36	0.54	0.001	88	79–99	0.021
After boiling 20 min. at pH 2.05–3.17	44	0.68	0.001	93	83–103	0.08

* Since Bartlett's test showed no significant heterogeneity of the variance of the log recovery (*P*=0.26), a pooled estimate was used in calculating the fiducial limits and probability of loss due to chance. This is half the probability of an equal chance deviation without regard to sign.

so that although Mn^{++} was supplied at $1.3 \times 10^{-5}M$ as for subtilin production (Jansen & Hirschmann, 1944), the optimal requirement is unknown. Similarly it is not known whether other trace elements are required, such as Zn which is essential for subtilin production (Feeney, Lightbody & Garibaldi, 1947; Feeney & Garibaldi, 1948), and which may have been adequately supplied as an impurity in the chemicals used.

The absence of any increase in the peak titre when the media were supplemented with amino-acids or growth factors, either as pure chemicals or in the form of casein hydrolysates or yeast extracts, contrasts with tyrothricin production (Lewis *et al.* 1945) in which a supplement of tryptone supplying less than one-sixth of the N supplied as ammonium sulphate increased the yield five- to eight-fold when citrate or better, malate, was present. It is, however, to be emphasized that ammonia is likely to serve as an adequate source of N both for growth and antibiotic production by other members of the *Bacillus* group, since it usually supports some growth, which may be improved by 'training' (Knight, 1936). Previous failures have in certain cases been due to the development of adverse conditions, such as pH, during incubation. Thus it is now recognized that ammonia suffices for subtilin production (Feeney, Garibaldi & Humphreys, 1948) in spite of earlier reports to the contrary (Jansen & Hirschmann, 1944). Here, as well as with tyrothricin (Lewis *et al.* 1945), it is essential to avoid acidity by supplying also a metabolizable anion such as citrate. A more elaborate example of pH control using this principle

to maintain, successively, optima both for growth and antibiotic production, is given by the work of Jarvis & Johnson (1947) on penicillin. With bacitracin production (Anker, Johnson, Goldberg & Meleney, 1948) utilization of ammonia does not appear to have been tested; with amino-acids, however, L-glutamate was not consistently replaceable by D-glutamate, asparagine or glycine. The fact that replacement did sometimes occur suggests the possibility that it depended on the initiation of enough growth to cause breakdown of the amino-acids followed by utilization of ammonia.

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