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Isolation from Liver of New Factors Essential for the Growth of *Lactobacillus helveticus* and *Streptococcus lactis* R.

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Snell & Peterson (1940), Stokstad (1941), Mitchell, Snell & Williams (1941), and O'Dell & Hogan (1943) have described the isolation, from various sources, of substances which stimulate the growth of *Lactobacillus helveticus* (*casei* ϵ) and *Streptococcus lactis* (*faecalis*) R. All of these workers used adsorption on norite and elution with ammoniacal solutions as an essential step in the process of isolation. The factor thus isolated by Mitchell *et al.* (1941) from spinach leaves was named folic acid.

Attempts have been made in the present work to prepare by similar methods a concentrate of folic acid, but using as starting material a precipitate obtained by the addition of ethanol to a concentration of 70% (v/v) during the manufacture of a commercial liver extract ('Plexan', made by Glaxo Laboratories Ltd.) by the method of Laland & Klem (1936). This precipitate was dissolved in water and the solution treated with norite. The adsorbate was eluted by ethanolic ammonia, following the procedure of Snell & Peterson (1940). The resulting eluate stimulated the growth of *L. helveticus* when, added to the medium of Landy & Dicken (1942) used for testing for folic acid.

The eluate was next adsorbed on Decalco (an ion-exchange adsorbent made by the Permutit Co. Ltd.), and the adsorbate eluted with hot 10% aqueous sodium chloride solution. Both eluate and filtrate stimulated growth of the test organism. It was also found that at least part of the active material was present in the precipitate formed when silver nitrate was added to the eluate at pH 7. By combining these three methods, there was prepared a concentrate of which 0.5 ml. gave good growth with *L. helveticus*; this concentrate contained under 100 μ g. total solids/ml.

Attempts to dissolve the active fraction in chloro-

form, preparatory to attempting partition chromatography (Martin & Synge, 1941), led to the discovery that most of the activity was extracted by chloroform from both acid (pH 3) and alkaline (pH 11) solutions, although folic acid is said to be insoluble in chloroform. Both the chloroform-soluble and chloroform-insoluble fractions were capable of stimulating the growth of *L. helveticus* and *S. lactis*, and the responsible factors are therefore provisionally designated factor HL1 and factor HL2 respectively. The latter may be identical with folic acid.

Extraction of a more refined liver extract ('Examen', made by Glaxo Laboratories Ltd.) by chloroform also gave an active fraction; this, on treatment with Decalco, gave an active filtrate and an eluate that was only slightly active. Similarly, when Examen was run through a column of Decalco and the filtrate and the hot 10% sodium chloride eluate were extracted with chloroform, the extract from the filtrate markedly stimulated the growth of *L. helveticus*, whilst the extract from the eluate gave only partial stimulation. The chloroform-soluble factors present in the Decalco eluate and filtrate are designated factors HL3 and HL4 respectively. When the two fractions were combined, growth equal to that given by the chloroform extract from Examen was obtained. It is possible that factor HL1 is a mixture of factors HL3 and HL4.

With one exception, factors HL1, HL3 and HL4 were not affected by acetylation, benzoylation, treatment with nitrous acid or boiling with water, dilute acid or dilute alkali. The activity of HL4 in the growth of *L. helveticus*, however, was largely destroyed by nitrous acid, but not the activity for *S. lactis*. Its synergistic action with factor HL3 towards either organism was not decreased.

EXPERIMENTAL

Microbiological procedure

The organisms used in this investigation were *Lactobacillus helveticus* (casei ϵ) strain no. 7469 and *Streptococcus lactis* (faecalis) Rogers, which were obtained from the American Type Culture Collection, Georgetown University, Washington, D.C., and *Streptococcus lactis* (National Collection of Type Cultures 8043) obtained from The Lister Institute of Preventive Medicine.

Preparation of inoculum. A transfer was made from an agar stab culture to a tube of the Snell & Strong riboflavin test medium (cf. Barton-Wright & Booth, 1943), to which had been added 0.25 μ g. riboflavin/10 ml. medium. The time of incubation was 16–18 hr., at 37° for *L. helveticus* and at 30° for *S. lactis* R. After incubation the cultures were centrifuged aseptically, the supernatant medium poured off and 10 ml. of sterile 0.9% saline solution added, followed by aseptic centrifugation. The supernatant saline solution was poured off and the cells were suspended in 10 ml. of 0.9% saline. 1 ml. of this suspension was then added to 100 ml. of sterile saline solution and this diluted suspension was used for inoculation.

The basal medium employed was a slight modification of that described by Landy & Dicken (1942):

Component	Wt./ml. of basal medium
Acid-hydrolyzed casein	5.0 mg.
<i>dl</i> -Tryptophan	0.1 mg.
<i>l</i> -Cystine	0.1 mg.
Glucose	10.0 mg.
Sodium acetate	6.0 mg.
<i>d</i> -Calcium pantothenate	0.1 μ g.
Pyridoxin	0.1 μ g.
Riboflavin	0.1 μ g.
Nicotinic acid	0.1 μ g.
<i>p</i> -Aminobenzoic acid	0.1 μ g.
Biotin	0.0002 μ g.
Adenine	0.01 mg.
Guanine	0.01 mg.
Uracil	0.01 mg.
Xanthine	0.01 mg.
Sodium chloride	5.0 mg.

Inorganic salts: solution A and solution B (see below) 0.5 ml. of each/100 ml. of completed medium

Estimation of amount of growth. The fermentations were carried out in ordinary bacteriological or chemical tubes (18 $\times$ 150 mm.), the acid produced being titrated against 0.1N-NaOH with *L. helveticus* and 0.05N-NaOH with *S. lactis* R (Barton-Wright & Booth, 1943). It is necessary to use the lower concentration of standard NaOH for *S. lactis* R, as this organism produces less acid than *L. helveticus*. When the buffer in the medium (sodium acetate) and the glucose are increased to 2%, the titres will be more than doubled. Cultures inoculated with *L. helveticus* were incubated at 37° and those inoculated with *S. lactis* R at 30°, each for 72 hr.

The acid-hydrolyzed casein was prepared from commercial casein and purified by treatment with lead acetate and adsorption on charcoal (10%). *Inorganic salt solution A*

was composed of 25 g. K_2HPO_4 , 25 g. KH_2PO_4 in 250 ml. of water. *Inorganic salt solution B* was modified to contain 10 g. $MgSO_4 \cdot 7H_2O$, 0.5 g. $MnSO_4 \cdot 4H_2O$, 0.1 g. $FeCl_3$ in 250 ml. of water; five drops of conc. HCl were added to this solution.

Separation of active fractions

The 70% (v/v) ethanol-insoluble precipitate from Plexan was fractionated by adsorption on charcoal and elution with aqueous ethanolic ammonia (Snell & Peterson, 1940), precipitation with silver nitrate solution and adsorption on Decalso. The details of the method are recorded in Fig. 1. Solution A, the charcoal eluate, contained 2.9 mg. total solids/ml., and stimulated the growth of *L. helveticus* when added to the folic acid test medium of Landy & Dicken (1942). Solution B, the filtrate after adsorption by Decalso, had only slight growth-promoting activity, whereas solution C, the Decalso eluate, containing only 0.1 mg. total solids/ml., was very potent.

Solution C was further fractionated by extraction with chloroform after adjusting one part to pH 3 and another to pH 11 (details in Fig. 2). The materials obtained from both chloroform extracts were more active than the residual aqueous solutions. This was a surprising observation since folic acid and related factors have been stated to be insoluble in chloroform (Mitchell *et al.* 1941; O'Dell & Hogan, 1943). Further extraction with chloroform failed to remove any more active material. Since the chloroform-soluble and chloroform-insoluble factors stimulated the growth of *L. helveticus* and *S. lactis*, they are provisionally designated factor HL1 and factor HL2 respectively; the latter may be identical with folic acid.

Typical results, indicating the relative activities of the different fractions in stimulating the growth of *L. helveticus*, are given in Table 1.

In view of the separation of a chloroform-soluble growth factor from the 70% ethanol precipitate, it was decided to extract a more refined liver extract (Examen) with chloroform, with the object of obtaining a larger quantity of this factor. The chloroform extract thus obtained was treated with Decalso. The reverse procedure, that is, adsorption of Examen on Decalso followed by chloroform extraction, was also tried. Details of the fractionation are given in Fig. 3, and the relative activities of the fractions in stimulating the growth of *L. helveticus* in Table 2.

The chloroform extract proved to be extremely potent and, on treatment with Decalso, most of the activity passed into the filtrate. A mixture of the Decalso eluate and Decalso filtrate, however, gave enhanced growth equal to that given by the original chloroform extract. When Examen was treated with Decalso, most of the activity was present in the filtrate, though the eluate was more potent than

70% ethanol-insoluble precipitate ($\equiv$ 50 kg. liver) dissolved in water (6 l.), adjusted to pH 3 and filtered through charcoal (40 g.)

Charcoal washed with 50% ethanol (2×400 ml.) and eluted by stirring with $N-NH_4OH$ in 50% ethanol (2×1 l.) at 60–70°

Eluate evaporated to dryness, residue dissolved in water (400 ml.), and solution adjusted to pH 3

Precipitate discarded and solution filtered through charcoal (5 g.)

Charcoal washed with 50% ethanol (2×200 ml.) and eluted by stirring with $N-NH_4OH$ in 50% ethanol (2×500 ml.) at 60–70°

Eluate evaporated to dryness and residue dissolved in water (500 ml.) = *Solution A*

Solution A (250 ml.) treated with 5% $AgNO_3$ solution (40 ml.) and neutralized to pH 7 with NaOH

Precipitate washed with water and decomposed with $N-HCl$ (20 ml.)

Solution adjusted to pH 3 and filtered through charcoal (4 g.)

Charcoal eluted with $N-NH_4OH$ in 50% ethanol (2×200 ml.)

Eluate evaporated to dryness and residue dissolved in 200 ml. of water

Solution (150 ml.) adjusted to pH 4.5 and filtered through Decalco (100 g.; washed with 1% acetic acid); Decalco washed with hot water

Decalco eluted with hot 10% NaCl solution (100 ml.) Filtrate and washings (150 ml.) = *Solution B*

Eluate extracted with phenol, extract diluted with 5 vol. of ether and extracted with water = *Solution C* (50 ml.)

Fig. 1. Fractionation of the 70% ethanol-insoluble precipitate from Plexan.

Charcoal-Decalco eluate (*Solution C* (Fig. 1); 30 ml.)

<p style="text-align: center;">adjusted to pH 3 and extracted with chloroform (3×50 ml.)</p> <p>chloroform extract washed with water and extracted with 5% $NaHCO_3$ solution (3×20 ml.)</p> <p>aqueous extract adjusted to pH 3 and extracted with phenol. Extract diluted with ether and extracted with water = <i>Solution D</i> (30 ml.)</p>	<p style="text-align: center;">adjusted to pH 11 and extracted with chloroform (3×50 ml.)</p> <p>chloroform extract washed with water and extracted with $N-HCl$ (3×20 ml.)</p> <p>aqueous extract adjusted to pH 3 and extracted with phenol. Extract diluted with ether and extracted with water = <i>Solution F</i> (30 ml.)</p>
aqueous solution = <i>Solution E</i>	aqueous solution = <i>Solution G</i>

Fig. 2. Fractionation by chloroform extraction of the charcoal-Decalco eluate from the 'Plexan' precipitate.

Table 1. *Relative potencies of fractions obtained from 70% (v/v) ethanol-insoluble precipitate from Plexan in stimulating the growth of L. helveticus*

	Volume (ml.) of fraction added to 10 ml. of medium				
	0.0	0.1	0.5	1.0	2.0
	Volume (ml.) of 0.1 N-NaOH required to neutralize the acid formed in 10 ml. medium				
Solution A, Charcoal eluate	0.3	8.0	—	—	—
" B, Decalco filtrate	0.3	0.5	1.3	—	—
" C, Decalco eluate	0.3	3.1	6.3	—	—
" D, Chloroform extract (pH 3)	1.0	4.7	9.8	9.8	9.8
" E, Chloroform insoluble (pH 3)	0.7	0.8	1.4	2.6	4.4
" F, Chloroform extract (pH 11)	1.0	—	8.7	9.5	9.7
" G, Chloroform insoluble (pH 11)	0.7	0.7	1.5	2.4	6.8

Fig. 3. Fractionation of Examen by adsorption on Decalso and extraction with chloroform.

Table 2. Relative potencies of fractions obtained from Examen in stimulating growth of *L. helveticus* (measured by acid-production)

	Volume (ml.) of fraction added to 10 ml. of medium			
	0.0	0.5	1.0	2.0
	Volume (ml.) of 0.1 N-NaOH required to neutralize the acid formed in 10 ml. medium			
Solution H, Chloroform extract	0.8	6.3	7.3	9.7
" I, Decalso eluate of chloroform extract	0.1	0.7	1.0	1.4
" J, Decalso filtrate of chloroform extract	0.1	2.6	3.9	6.1
Solutions I + J	0.1	5.7	6.8	9.9
Solution K, Decalso eluate of Examen	0.8	3.0	3.1	4.3
" L, Decalso filtrate of Examen	0.8	6.3	8.5	9.9
" M, Chloroform extract of Decalso eluate	0.8	3.0	3.1	4.2
" N, Chloroform extract of Decalso filtrate	3.8	6.2	7.4	8.9

the eluate from the chloroform extract. The filtrate and eluate were then extracted with chloroform, and in both instances the extracts were active. These results appear to establish the presence of two different chloroform-soluble factors, one termed factor HL3 being present in the Decalso eluate, and the other, factor HL4, in the Decalso filtrate. They obviously differ from factor HL1 which, though soluble in chloroform and adsorbable on Decalso, is a more potent growth stimulant, and from factor HL2, which is chloroform-insoluble. It is possible, however, that factor HL1 is a mixture of factors HL3 and HL4.

Action of reagents on the various growth factors

Acetylation. The chloroform extract from Examen and the Decalso eluate and filtrate therefrom (solutions H, I and J) were acetylated, by adding to 15 ml. of each of the solutions, 2 ml. of 2N-NaOH and 0.2 ml. of acetic anhydride, repeating the addition after an interval of 2-3 min. The mixture was

then left at room temperature for 15 min., neutralized and concentrated *in vacuo* to 15 ml. The activities of the solutions on *L. helveticus* and *S. lactis* R were unaffected by this treatment. The Decalso eluate and filtrate from Examen itself (solutions K and L) were also acetylated; again the activity was unaffected.

Benzoylation. Samples of solutions H, I and J (30 ml.) were shaken with benzoyl chloride (100 mg.) and 4N-NaOH (1.0 ml.) and left at room temperature for 1 hr. The mixture was acidified, extracted with ether to remove benzoic acid, and adjusted to pH 6. No loss of activity occurred. It is therefore concluded that factors HL3 and HL4 are not inactivated by benzoylation.

Nitrous acid. Samples (30 ml.) of solutions H, I and J were treated with NaNO_2 (100 mg.) and 2N-HCl (1.0 ml.); after standing at room temperature for 2 hr. each solution was treated with sulphamic acid (150 mg.) to destroy excess nitrous acid. After 10 min., the solutions were adjusted to pH 6. The activity on *L. helveticus* of the Decalso filtrate

from the chloroform extract (solution J) containing factor HL4 was markedly decreased, though not its activity for *S. lactis* R; however, its effect in presence of factor HL3 decreased for either organism.

Effect of heat, acid and alkali. The stability of factors HL3 and HL4 was tested under the following conditions: (a) refluxing solutions I and J for 3 hr.; (b) boiling for 3 hr. with 2N-NaOH; (c) boiling for 3 hr. with 2N-HCl. Neither of the factors was appreciably affected, whereas, according to Mitchell *et al.* (1941), folic acid is unstable under these conditions.

The effect of a mixture of factors HL3 and HL4 on *L. helveticus* was also tested on a medium in which the casein hydrolysate was replaced by a mixture of 16 amino-acids. Considerably less growth was obtained with this medium than with the original medium, suggesting that some additional factor may have been present in the casein hydrolysate (Table 3).

Table 3. *Effect of factors HL3 + HL4 from Examen on the growth of L. helveticus, using medium containing casein hydrolysate or 16 amino-acids*

	Volume (ml.) of solution added to 10 ml. of medium					
	0.0	0.1	0.5	1.0	2.0	4.0
	Volume (ml.) of 0.1N-NaOH required to neutralize acid produced					
Casein hydrolysate medium	0.2	2.7	4.3	6.4	8.2	—
Amino-acid medium	0.2	—	0.9	1.3	3.0	5.5

An aqueous solution of the chloroform-soluble factors did not exhibit any characteristic absorption bands, but an aqueous solution of the chloroform-insoluble fraction from Examen gave an absorp-

tion spectrum similar to that obtained by Mitchell (1944) for folic acid, with a maximum at 267m μ and a minimum at 249m μ . These were displaced at pH 11 to 282 and 271m μ respectively.

DISCUSSION

Assuming that factor HL2 is folic acid, the evidence now presented suggests the existence of two chloroform-soluble factors, each of which can replace folic acid as an essential growth factor for *L. helveticus* and *S. lactis*. The two factors can be separated by treatment with Decalso. The factor not adsorbed on Decalso (HL4) appeared to be more unstable to HNO₃ than the eluate factor (HL3).

SUMMARY

1. Attempts have been made to prepare a concentrate of folic acid from by-products obtained in the manufacture of a commercial liver extract. In addition to a fraction that could not be extracted from aqueous solution by chloroform, other fractions soluble in chloroform and capable of stimulating the growth of *Lactobacillus helveticus* and *Streptococcus lactis* were obtained.

2. These fractions are believed to contain three or possibly four different growth factors. The chloroform-insoluble factor, designated factor HL2, is probably identical with folic acid. Factors HL1 and HL3 are soluble in chloroform and adsorbed on Decalso, but whereas HL1 is a potent growth-stimulant, HL3 is only slightly active. Factor HL4 is also soluble in chloroform and is a potent growth-stimulant, but it is not adsorbed on Decalso. Factor HL1 may be a mixture of factors HL3 and HL4. Factors HL3 and HL4 exert a synergistic action on one another.

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