CCXXIII. GROWTH FACTORS FOR BACTERIA

VI. FRACTIONATION AND PROPERTIES OF AN ACCESSORY FACTOR FOR LACTIC ACID BACTERIA[1]

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In recent years there has been considerable interest in the nature, distribution and function of accessory nutritive factors required by certain bacteria in addition to suitable nitrogen, carbon and mineral sources. The excellent reviews by Knight [1936] and by Burrows [1936] make it unnecessary to summarize here any but the most recent developments in this field.

Knight [1937] has reported that the basic factor necessary for the growth of *Staphylococcus aureus* in acid-hydrolysed media can be replaced by mixtures of vitamin B_1 and nicotinic acid or its amide. Richardson [1936] reports that for anaerobic growth this organism requires uracil and pyruvic acid in addition to the above basic factor.

Lwoff & Lwoff [1937] have discovered that factor V, which is necessary for growth of certain haemophilic organisms, can be completely replaced by very small amounts of Warburg coenzyme or cozymase. Mueller [1937, 1, 2] has identified pimelic acid as an accessory substance needed for growth of a strain of the diphtheria bacillus.

Orla-Jensen *et al.* [1936, 1, 2] have shown that riboflavin is required by certain lactic acid bacteria and state that one or more other accessory substances are necessary for proper growth of these organisms. Wood, Anderson & Werkman [1937] have confirmed the above work with respect to riboflavin, which they find is also required by the propionic acid bacteria in addition to the ether-soluble factor previously reported [Wood, Tatum, and Peterson, 1937]. Tatum *et al.* [1936] believe that vitamin B_1 is also required by some of the propionic acid bacteria.

In the third paper of this series [Snell *et al.* 1937] it was demonstrated that the addition of an aqueous potato extract to a peptone-glucose-mineral salts medium markedly increased its ability to support growth of *Lactobacillus delbrückii*. A similar medium prepared with acid-hydrolysed peptone or casein plus tryptophan proved to be inadequate even in the presence of the potato extract, and it was concluded that the intact peptone contained a second substance essential for the normal nutrition of this organism. Preliminary examination of the "potato factor" and the "peptone factor" indicated that the former was an acidic substance whilst the latter was basic in character. Further, it was

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found that an aqueous liver extract was capable of replacing both factors, inasmuch as the addition of such material allowed good growth and fermentation in a medium composed of tryptophan, acid-hydrolysed casein, glucose and mineral salts.

In this paper are presented the results of a further study of the properties of one of the active substances from liver and of attempts to effect its isolation. Inasmuch as previous experience had suggested that the "peptone factor" might prove to be less dispensable than the "potato factor" for lactic acid bacteria, the former has been made the object of the present investigation.

The selection of a medium suitable for assaying preparations of the "peptone factor" presented some difficulties, the chief problems being to find a nitrogen source free from the substance in question, and to avoid misinterpretation of results arising from multiple deficiencies. Media containing peptone or casein from which the active substance had been removed either by acid hydrolysis or treatment with alkali supported somewhat erratic growth, when sources of the "peptone factor" were added. The certainty of response to active material was found to be greatly improved by the inclusion of sodium acetate in the medium. The sodium acetate presumably exerts a buffer action, but may also have a specific function concerned with the initiation of growth. Following the suggestion of Orla-Jensen *et al.* [1936, 1] the effect of riboflavin was investigated and proved to be decidedly beneficial, the flavin apparently having been previously present in suboptimum quantities. As expected, it was found necessary to include tryptophan in media containing acid-hydrolysed peptone as the source of nitrogen. The addition of cystine to such media also seemed to improve the growth response.

With the aid of a basal medium built along the lines indicated above a search for potent sources of the "peptone factor" was made, and considerable fractionation of a suitable material (liver extract) carried out. The active material has been found to be an acidic ether-extractable organic substance and appears to be essential for growth and fermentation of a variety of lactic acid bacteria. It exhibits detectable stimulatory action on *L. casei* in dilutions as high as 1 : 300,000,000, and thus seems definitely to act as a growth accessory substance in the sense in which this term is used by Burrows [1936].

Experimental

Cultures

Pure cultures of 13 species of lactic acid bacteria were selected on the basis of their fermentation characteristics as representative of the various types of bacteria in this group. They were as follows: *Lactobacillus delbrückii* 3; *L. casei* 1; *L. helveticus*; *L. bulgaricus*; *L. mannitopoeus* 26; *L. arabinosus* 17–5; *L. pentosus* 124–2; *L. pentoaceticus* 41–11; *L. gayoni* 36; *Bacillus brassicae* 6–26; *B. lactis acidi* B1 1; *Leuconostoc mesenterioides* P–60; and *Streptococcus lactis* R. Cultures were made in yeast-water glucose agar. For inoculum the cells from 10 ml. of a 24 hr. culture in yeast-water glucose medium were centrifuged down and resuspended in 10 ml. of 0·9 % saline solution. 1 % inoculum was used. For purposes of routine testing *L. delbrückii* was usually employed. *L. casei* proved to have some advantages and was used in part of the later work.

Technique of biological testing

Two media, both deficient in the accessory factor, were developed. Medium A had the following % composition: acid-hydrolysed peptone 0·5, glucose 1,

sodium acetate 0·6, tryptophan 0·01, cystine 0·01; inorganic salts;[1] riboflavin 0·01 mg./100 g. The acid-hydrolysed peptone was prepared by autoclaving a 35 % solution of Bacto peptone in 20 % sulphuric acid (by volume) overnight. The sulphuric acid was exactly removed with barium hydroxide. Medium B consisted of NaOH-treated peptone 0·5, glucose 1, sodium acetate 0·6, cystine 0·01 %; inorganic salts; riboflavin 0·01 mg./100 g. The sodium hydroxide treatment consisted in allowing an 8 % solution of Bacto peptone in *N* NaOH to stand 23 hr. at room temperature; the alkali was neutralized with glacial acetic acid.

The media were first made up in 10 times the above concentrations, and the fractions to be tested were incorporated during the subsequent dilution. The fermentations were carried out in test-tubes containing a total volume of 10 ml. of medium. The tubes were autoclaved at 15 lb. pressure for 15 min., cooled, and inoculated. *L. delbrückii*, *L. casei*, *L. helveticus*, *L. bulgaricus*, and *B. lactis acidi* were incubated at 37°, all other cultures at 28°. Fermentation was followed by direct titration of the acid produced during 5–6 days' growth. The close correlation between growth and acid production previously noted [Snell *et al.* 1937] was also observed throughout the present work. Growth of the test organisms on either of the unsupplemented media was usually too slight to be detected visually.

Sources of the growth factor

An alcohol-soluble liver fraction[2] proved to be a rich source of the active substance. The potency of this material as compared with that of Difco yeast

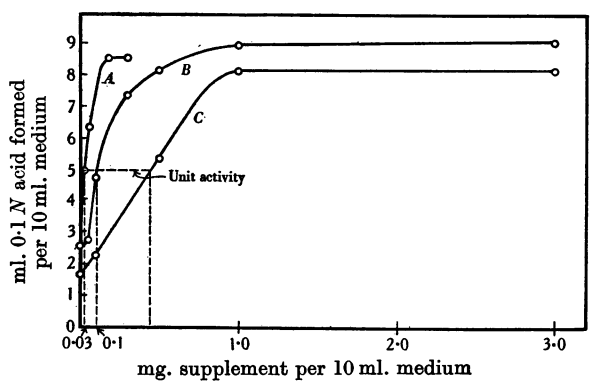

Fig. 1. Effect of various supplements on acid production by *L. delbrückii* in medium A. *A*, alcoholic extract of liver (Wilson & Co.). *B*, dried pork liver. *C*, yeast extract (Difco).

extract and of dried, ground pork liver is indicated in Fig. 1. The alcohol-soluble liver fraction is roughly 10 times as active as the yeast extract and 3 times as active as the dried liver per unit of weight. Similar data have shown it to be 2–3 times as active as a water extract of liver, and about 20 times as active

[1] The inorganic salts were used in the following concentrations: K_2HPO_4, 0·50 g.; KH_2PO_4, 0·5 g.; $MgSO_4$, $7H_2O$, 0·2 g.; NaCl, 0·01 g.; $FeSO_4$,$7H_2O$, 0·01 g.; $MnSO_4$, $3H_2O$, 0·01 g.; in 1000 ml. of medium.

[2] A by-product of the commercial preparation of the pernicious anaemia factor, consisting of that portion of an aqueous extract of beef liver which remains soluble in 92 % alcohol. We wish to thank Dr David Klein of the Wilson Laboratories, Chicago, for supplying the liver fractions used in this work.

as a commercial, lactose-free, whey concentrate. In view of its high activity and ready availability the alcohol-soluble liver fraction has been used as the starting material in the following fractionation procedure.

Apparently uncontrollable variations in growth on identical media at different times rendered it difficult to base a unit of activity directly on the fermentation. Kögl's scheme [1936] of correcting such inherent variability by reference to a suitable standard preparation was therefore adopted. A quantity of the alcohol-soluble liver fraction was set aside as a standard. One unit of activity was defined as that activity residing in 0·03 mg. of the standard preparation. The results of a large number of assays of this preparation indicate that the unit so defined is a satisfactorily constant value, and represents a conversion of approximately one-half the available glucose into lactic acid.

Fractionation procedure

The procedure finally adopted for preparation of active concentrates is illustrated by the following run. Details of the assay results are given in Table I.

(1) *Lead precipitation.* 75 g. of the alcohol-soluble liver fraction were dissolved in 400 ml. of water, and filtered. The insoluble portion was washed well with water, and to the combined filtrate and washings was added a solution of 105 g. lead subacetate in 300 ml. of water. The heavy yellow precipitate was filtered off, resuspended once in water, filtered and the washings added to the main filtrate. Concentrated ammonia was then added until the mixture was just alkaline to litmus. The second yellow precipitate was at once filtered off, the filtrate being collected in a flask containing an excess of 5% acetic acid. The precipitate was resuspended once in water, filtered and the washings added to the main filtrate. Excess lead was removed with H_2S, the precipitated PbS boiled up twice with water and the deep yellow washings added to the main filtrate:

(*a*) Starting material: 75 g. = 2,500,000 units (1 unit in 0·03 mg.).

(*b*) Lead filtrate solids: 90 g. = 2,250,000 units (1 unit in 0·04 mg.).

Although no apparent concentration of the factor was accomplished by this treatment, it was found necessary to make the subsequent step successful.

(2) *Charcoal adsorption.* The lead filtrate, which had a *p*H of 3·6–4·0, was diluted to 6 litres and stirred 1 hr. at room temperature with 130 g. of active charcoal (Pfanstiehl, "Norite A"). The charcoal was filtered off, washed well with water, stirred twice in succession with 500 ml. of 50% alcohol at 70–80° for 45 min. each time, and then twice with 500 ml. of a mixture of pyridine-methyl alcohol-water (1 : 2 : 1) at room temperature. The deep yellow eluates were combined and evaporated to dryness at reduced pressure below 60°. The dry material was a deep brown, hygroscopic syrup:

(*a*) Charcoal eluate: 9·2 g. = 2,300,000 units (1 unit in 4γ).

(*b*) Charcoal filtrate: inactive.

(3) *First ether extraction.* The charcoal eluate was dissolved in 100 ml. of water, the solution adjusted to *p*H 6·8–7·0 with NaOH and extracted continuously with ether for 12 hr. The *p*H of the aqueous residue was brought to 1·0 with H_2SO_4 and the extraction continued for 24 hr. longer, the extracts being removed at the end of 5, 12, and 24 hr. respectively. The acid ether extracts were combined and the solvent removed from all fractions *in vacuo*:

(*a*) Neutral ether extract: 2·0 g., inactive.

(*b*) Acid ether extract: 1·9 g. = 1,900,000 units (1 unit in 1γ).

(*c*) Aqueous residue: inactive.

The acid ether extract consisted of a viscous, yellow-brown syrup, from which a mass of colourless, needle-shaped crystals usually separated. When the mixture was rubbed up with a small amount of chloroform, the syrupy part dissolved and the crystals could be filtered off. After recrystallization from ethyl acetate the substance proved to be inactive, and was shown by neutralization equivalent, M.P., mixed M.P. and preparation of the *p*-phenylphenacyl derivative to be identical with succinic acid.

(4) *Second ether extraction.* It was found that the active substance in the acid ether extract above could be enriched by re-extracting with ether. A solution of 2·4 g. of such material in 25 ml. of water was adjusted to *p*H 1·0 with H_2SO_4 and continuously extracted with ether. The extract was collected in three portions after 3, 15, and 20·5 hr. of extraction:

(*a*) Starting material: 2·4 g. = 2,420,000 units (1 unit in 1γ).
(*b*) First fraction: 1·357 g. = 452,000 units (1 unit in 3γ).
(*c*) Second fraction: 0·503 g. = 1,006,000 units (1 unit in 0·5γ).
(*d*) Third fraction: 0·154 g. = 308,000 units (1 unit in 0·5γ).
(*e*) Aqueous residue: 200,000 units.

The second fraction above represents the most active concentrate so far obtained, and for brevity is referred to in subsequent discussions as Fraction L.

Table I. *Activity of liver fractions*

	Activity							
	Concentration in γ per 10 ml.				ml. 0·1 *N* acid formed per 10 ml. medium			
Supplement to medium A*	(*a*)	(*b*)	(*c*)	(*d*)	(*a*)	(*b*)	(*c*)	(*d*)
None	—	—	—	—	1·9	1·8	—	—
Alcohol-soluble fraction of liver	30	60	180	300	6·0	8·4	9·2	9·2
Lead filtrate	30	60	180	300	4·9	8·1	9·3	9·2
Norite filtrate	30	60	180	300	1·8	1·8	2·0	3·9
Norite eluate	2	4	12	20	5·0	8·0	9·3	9·3
Neutral ether extract	1	3	5	10	1·9	1·9	2·0	2·2
1st acid ether extract	0·5	1	3	5	3·5	6·2	9·1	9·3
2nd acid ether extract:								
(*a*) 1st fraction	0·5	1	3	5	2·3	2·7	4·2	5·7
(*b*) 2nd fraction	0·5	1	3	5	5·6	7·1	8·9	8·9
(*c*) 3rd fraction	0·5	1	3	5	6·2	7·9	8·9	8·9

* Assay organism: *L. delbrückii*.

Properties of the active substance

(1) *General.* Fraction L consisted of a yellow-white, sticky solid, free from sulphur, halogens and phosphorus, but containing 4·4 % N (micro-Kjeldahl). It dissolved easily in water, absolute alcohol, acetone, ethyl acetate and glacial acetic acid at room temperature, sparingly in ether or chloroform and hardly at all in benzene or light petroleum. The ash from a potent preparation showed no activity.

(2) *Stability.* Data showing the activity of potent preparations after being treated in various ways are given in Table II.

The active substance was quickly destroyed at 150°, but appeared to be stable at 80–90°. Autoclaving in neutral solution had an adverse effect only after several hours. The substance was apparently much more resistant to acid than to alkali, but was readily destroyed by *N* acid at 100°. Information obtained incidentally in connexion with the fractionation procedure and other operations described below make it appear probable that the factor is stable to 10 % Na_2CO_3 and to NH_4OH for at least $\frac{1}{2}$ hr. at room temperature. A neutral

Table II. *Stability of the active material to acid and alkali*

Supplement to medium A*	Treatment	Activity: Concentration per 10 ml.		Activity: ml. 0·1 *N* acid formed per 10 ml. medium	
None	—	—	—	1·8	1·8
Alcohol-soluble fraction of liver	None	0·5	1·0 mg.	9·6	9·6
,, ,,	In *N* NaOH 24 hr. at room temperature	0·5	1·0 mg.	1·7	2·0
,, ,,	In *N* NaOH 1 hr. at 100°	0·5	1·0 mg.	1·8	1·8
,, ,,	In *N* HCl 1 hr. at 100°	0·5	1·0 mg.	2·3	2·0
,, ,,	In *N* HCl 24 hr. at room temperature	0·5	1·0 mg.	9·7	9·5
Acid ether extract	Not autoclaved	1	3γ	5·0	8·8
,,	Autoclaved at 15 lb.: *p*H 6·9, 2 hr.	1	3γ	3·7	8·0
,,	*p*H 6·9, 5 hr.	1	3γ	1·5	2·8
,,	Heated, 150°, 30 min.	1	3γ	1·5	1·5
Fraction L	Unheated	0·3	1·0γ	4·8	8·2
,,	80–90°, 3 hr.	0·3	1·0γ	4·8	7·1

* Assay organism: *L. delbrückii*.

solution of an active preparation showed no loss of activity after 5 months' storage in diffuse daylight.

(3) *Effect of various reagents.* (*a*) *Diazomethane.* A chloroform solution of an ether extract (1 unit in 3γ) was treated with an excess of diazomethane in ether, allowed to stand overnight and then evaporated to dryness under reduced pressure. The remaining waxy solid was completely inactive.

(*b*) *Acetylation* of an ether extract (1 unit in 1γ) with acetic anhydride in pyridine solution at 100° resulted in complete loss of activity. A control solution treated in the same manner but with glacial acetic acid in place of acetic anhydride showed no loss of activity.

A repetition of the experiment using 3:5-*dinitrobenzoyl chloride* in place of acetic anhydride led to a considerable decrease in activity (1 unit in 5γ), although shaking the same material with 3:5-dinitrobenzoyl chloride and 10% Na_2CO_3 at room temperature had no noticeable effect.

(*c*) *Hydrogen peroxide.* A 2·7% solution of a charcoal eluate (1 unit in 3γ) heated to 100° for 1·5 hr. in 3% hydrogen peroxide showed only a slight diminution in activity (1 unit in 3·4γ), although the solution was somewhat bleached by the treatment.

(*d*) *Nitrous acid.* The activity was decreased somewhat less than one-half by allowing an active fraction (1 unit in 0·75γ) to stand overnight at 8° in the presence of excess nitrous acid generated from sodium nitrite plus dilute acetic acid (product: 1 unit in 1·25γ).

(*e*) *Bromine.* Exposure of a potent concentrate (1 unit in 0·75γ) to the action of excess bromine in glacial acetic acid at room temperature for 30 min. also resulted in destruction of approximately one-half the activity (product: 1 unit in 1·25γ).

(4) *Precipitation reactions.* Attempts to secure fractions of increased activity by the use of precipitation reactions have met with no success. The active material was not precipitated by Neuberg's reagent, $HgCl_2$ in alcohol, phosphotungstic acid in sulphuric acid solution, lead subacetate, lead acetate or lead acetate and ammonia. In all but the lead precipitations the activity of the

unprecipitated material was decreased rather than increased by the procedure, while the precipitated portions were inactive or showed only slight activity. No enhanced activity was observed in any case by combining the filtrate and precipitate. The bulk of the material invariably remained in the soluble portion.

Barium salts prepared from an active ether extract were largely soluble in 95 % alcohol. The insoluble barium salts exhibited no activity when freed from barium and assayed. The alcohol-soluble salts were active, but in no lower concentration than the starting material (1 unit in 1γ).

No concentration was effected by precipitating with ammoniacal silver oxide.

(5) *Adsorption.* Lloyd's reagent, aluminium oxide and the sulphides of lead, silver, and mercury all failed to adsorb the active substance from water solution. It was adsorbed by various activated charcoals, but complete adsorption occurred only when relatively large quantities of adsorbent were used.

Table III*a*. *Effect of growth factor on other species of lactic acid bacteria*

		Supplement to medium A		Activity: ml. 0·1 *N* acid formed per 10 ml.		Activity: Visible growth*	
Organism	Fraction	γ per 10 ml.					
L. arabinosus	None	—	—	6·1	6·1	+ + +	+ + +
	Alcohol-soluble liver	60	300	9·4	9·0	+ + + +	+ + + +
	Acid ether extract	0·8	4·0	9·4	9·4	+ + + +	+ + + +
L. bulgaricus	None	—	—	1·2	1·2	–	–
	Alcohol-soluble liver	60	300	3·8	5·1	+ +	+ + +
	Acid ether extract	0·8	4·0	2·6	3·3	+ +	+ +
L. casei	None	—	—	1·1	1·0	–	–
	Alcohol-soluble liver	60	300	7·1	8·7	+ + +	+ + + +
	Acid ether extract	0·8	4·0	5·5	8·1	+ + +	+ + + +
L. helveticus	None	—	—	1·5	1·8	±	±
	Alcohol-soluble liver	60	300	5·0	8·5	+ + +	+ + + +
	Acid ether extract	0·8	4·0	2·1	6·2	+	+ + +
L. pentosus	None	—	—	3·1	3·0	+ +	+ +
	Alcohol-soluble liver	60	300	7·4	9·4	+ + + +	+ + + +
	Acid ether extract	0·8	4·0	4·8	9·4	+ + + +	+ + + +
L. gayoni	None	—	—	0·8	0·8	±	±
	Alcohol-soluble liver	60	300	3·8	4·7	+ + + +	+ + + +
	Acid ether extract	0·8	4·0	2·1	4·2	+ + +	+ + + +
L. mannitopoeus	None	—	—	0·9	0·9	+	+
	Alcohol-soluble liver	60	300	4·5	4·8	+ + + +	+ + + +
	Acid ether extract	0·8	4·0	2·3	4·7	+ + +	+ + + +
L. pentoaceticus	None	—	—	0·8	0·8	±	±
	Alcohol-soluble liver	60	300	3·8	4·7	+ + + +	+ + + +
	Acid ether extract	0·8	4·0	2·1	4·2	+ + +	+ + + +
B. brassicae	None	—	—	6·0	6·0	+ +	+ +
	Alcohol-soluble liver	60	300	9·3	9·0	+ + + +	+ + + +
	Acid ether extract	0·8	4·0	9·2	9·3	+ + + +	+ + + +
B. lactis acidi	None	—	—	2·0	2·0	+	+
	Alcohol-soluble liver	60	300	5·8	9·4	+ + +	+ + + +
	Acid ether extract	0·8	4·0	5·5	9·3	+ + +	+ + + +
L. mesenterioides	None	—	—	1·9	1·9	+	+
	Alcohol-soluble liver	60	300	5·7	8·6	+ + + +	+ + + +
	Acid ether extract	0·8	4·0	4·4	9·0	+ + + +	+ + + +
S. lactis	None	—	—	2·2	2·2	+	+
	Alcohol-soluble liver	60	300	3·9	4·1	+ + +	+ + +
	Acid ether extract	0·8	4·0	3·7	4·6	+ + +	+ + +

* – indicates no visible growth; ± questionable; + slight growth; + + fair growth; + + + heavy growth; + + + + very heavy growth (with sediment).

Effect of the growth factor on other species of lactic acid bacteria

The effect of the active substance on other members of the group of lactic acid bacteria has been investigated. The results of the experiments are given in Table III*a*. In those cases where fairly good growth occurred in the basal medium alone, subcultures were made from the control tubes after 24 hr. into a second set of tubes with and without the accessory substance added. The results are given in Table III*b*. The growth factor appears to be essential in

Table III*b*. *Effect of growth factor on other species of lactic acid bacteria*

Organism	Supplement to medium A: Fraction	γ per 10 ml.		Activity: ml. 0·1 *N* acid produced per 10 ml. medium		Visible growth*	
L. arabinosus	None	—	—	1·4	1·5	±	±
	Alcohol-soluble liver	60	300	9·2	9·2	+ + + +	+ + + +
	Acid ether extract	0·8	4·0	9·3	9·4	+ + + +	+ + + +
L. pentosus	None	—	—	0·3	0·4	−	−
	Alcohol-soluble liver	60	300	7·3	9·3	+ + + +	+ + + +
	Acid ether extract	0·8	4·0	5·8	8·5	+ + + +	+ + + +
B. brassicae	None	—	—	1·3	1·3	−	−
	Alcohol-soluble liver	60	300	8·8	8·2	+ + + +	+ + + +
	Acid ether extract	0·8	4·0	9·3	9·1	+ + + +	+ + + +
B. lactis acidi	None	—	—	1·8	1·9	±	±
	Alcohol-soluble liver	60	300	6·1	9·3	+ + +	+ + + +
	Acid ether extract	0·8	4·0	4·4	9·3	+ + +	+ + + +
L. mesenterioides	None	—	—	0·9	0·9	−	−
	Alcohol-soluble liver	60	300	3·6	4·5	+ + + +	+ + + +
	Acid ether extract	0·8	4·0	3·3	3·6	+ + + +	+ + + +
S. lactis	None	—	—	0·8	0·8	−	−
	Alcohol-soluble liver	60	300	2·9	3·1	+ + +	+ + +
	Acid ether extract	0·8	4·0	2·5	3·0	+ + +	+ + +

* As in Table III*a*.

every one of these cases. Enough of the essential substance is apparently contained in the washed cells of the original inoculum to permit the amount of growth indicated in Table III*a*.

Visual observations of the amount of growth are given as an aid in interpreting the effect of the stimulatory substance on the bacteria. Thus the rather moderate acidity produced by *S. lactis* really corresponds to heavy growth, since this organism rarely produces more than 0·5% lactic acid in a culture, whereas organisms such as *L. casei* will produce three times as much. Similarly such organisms as *L. pentoaceticus* or *L. gayoni* produced low titratable acidities not because growth was not heavy but because non-acidic products such as ethyl alcohol are prominent end-products in the fermentations which they bring about [Stiles *et al.* 1925; Peterson & Fred, 1920; Pederson, 1929].

Failure of known compounds to replace the active substance

Several of the compounds reported in recent years to be active stimulants for bacteria or plants have been assayed for their effect on the test organism used in this work. The results are given in Table IV. None of the compounds tested was at all effective in replacing the acid ether extract from liver.

Table IV. *Effects of various physiologically active compounds on acid production**

Material tested	Concentration: Range per 10 ml.	Concentration: Optimum γ per 10 ml.	Activity of optimum concentration ml. 0·1 *N* acid per 10 ml.
None	—	—	1·9
Acid ether extract	1 –10γ	3	9·4
Auxin-*a*†	0·5–10γ	5	1·9
Sodium salts from urine (pimelic acid)†	0·5–100γ	100	1·9
β-Indolylacetic acid	1γ–10 mg.	10	2·0
Nicotinamide	0·5–5γ	5	1·9
Vitamin B_1	0·5–5γ	5	1·9
Uracil	0·5–5γ	5	2·0
Nicotinamide plus vitamin B_1	0·5–5γ + 0·5–5γ	5 + 5	2·0
Pyruvic acid	0·5–5γ	5	1·8

* Medium A. Test organism: *L. delbrückii*.

† The authors wish to thank Prof. F. Kögl for the sample of auxin-*a* and Prof. J. H. Mueller for a sample of sodium salts containing pimelic acid.

Comparison of the active substance with tryptophan and riboflavin

It was of interest to compare the weights of tryptophan, riboflavin, and Fraction L required to produce a growth response. The medium used in each case was deficient in the particular substance being tested, but was provided with an abundance of the other two. Results of suitable experiments are quoted in Table V.

Table V. *Comparison of the effects of tryptophan, riboflavin and the acidic ether-soluble factor on growth of* L. casei

Medium	Supplement	Activity: γ supplement per 10 ml. medium				Activity: ml. 0·1 *N* acid produced per 10 ml. medium			
A	Tryptophan	0	1	3	5	1·5	2·8	4·8	5·4
		10	30	50	100	6·1	6·6	7·2	7·2
B	Riboflavin	0·0	0·05	0·1	0·3	1·4	3·3	5·3	8·3
		0·5	1·0	3·0	5·0	9·7	9·7	9·7	9·7
B	Acid ether extract L (1 unit = 0·5γ)	0·0	0·01	0·03	0·05	1·1	2·0	2·0	4·5
		0·1	0·3	0·5	1·0	5·0	5·7	7·0	8·8
		3·0	5·0	—	—	9·4	9·4	—	—

The effect of tryptophan becomes apparent at a concentration of 0·1γ per ml. of medium, and reaches a maximum at slightly over 3γ per ml. Riboflavin is detectable in a concentration of less than 0·005γ per ml., and reaches its maximum effect at 0·05γ per ml. Fraction L can be readily detected in concentrations of 0·001–0·003γ per ml., the maximum effect being attained at 0·1–0·3γ. The active substance in Fraction L thus appears to act as a true accessory factor, comparable with riboflavin.

Discussion

The fact that the active substance is extracted by ether from acid but not from neutral solutions seems to indicate that it is an acid. This behaviour taken together with its non-precipitability by the various reagents tried shows quite definitely that it has no basic properties. The precipitation tests also make it probable that the substance does not belong to any of the common classes of

nitrogenous compounds brought down by these reagents, i.e. amino-acids, N bases, purines, pyrimidines etc. although such deductions must necessarily be of a tentative character. The information obtained concerning the stability of the active substance under various conditions and the effect of certain reagents on it must be regarded at present more as a guide to further attempts at purification than as a basis for extensive speculations regarding its possible chemical nature.

The relationship of the accessory growth substance described in the present paper to the "potato factor" and the "peptone factor" previously reported [Snell, *et al.* 1937] is not entirely clear. The basal media used in the present work were presumably free from both the latter factors and yet supported excellent growth in the presence of the active substance from liver. Probably the former results were complicated by multiple deficiencies. Thus the "potato factor" has been found stimulatory but non-essential when the medium contained $CaCO_3$ or especially sodium acetate, and the medium previously used for assaying the "peptone factor" appears in the light of present knowledge to have been deficient in riboflavin. It seems probable that the present factor is the active substance other than riboflavin, which was contained in preparations of the "peptone factor".

It will be recalled that the "peptone factor" appeared to be a basic substance, whereas the active substance from liver is quite obviously an acid. This discrepancy is perhaps explicable on the basis that the latter exists partially in a combined form in natural materials. Only a fraction of the total activity of fresh liver is contained in a water extract thereof, and of that portion which does go into solution only a part is soluble in 92% alcohol. Similarly crude peptone contains considerable amounts of the active substance, but none of it is extractable by ether under acid conditions.

Fraction L may of course contain active substances corresponding to both the "potato factor" and the "peptone factor", but no indications of any such heterogeneity have ever been observed, although combinations of the fractions resulting from the various operations have frequently been assayed.

A possible relationship to the ether-extractable substance reported by Wood, Tatum and Peterson [1937] to be essential for the propionic acid bacteria is by no means excluded. Experiments designed to test this point have yielded conflicting results and are being continued.

The active substance from liver differs from most other acidic factors reported necessary for bacteria. The "sporogenes vitamin" [Knight & Fildes, 1933] is much more stable to alkali than is Fraction L. The plant auxins do not replace it nor does nicotinic acid, either alone or in combination with vitamin B_1. While there seems to be a rough similarity between the properties of the active substance and those of pantothenic acid [Williams *et al.* 1933], the available evidence is too limited to warrant discussion of the relationship of the two factors. The factor reported by Orla-Jensen *et al.* [1936, 1] as essential for lactic acid bacteria in addition to riboflavin was stable to alkali. If it is required by the organisms used in this work, it must be present in the basal medium.

Summary

A procedure is described for obtaining from an alcohol-soluble liver extract an acidic, ether-extractable substance, which is essential for the normal growth of 14 species of lactic acid bacteria. The effect of the concentrate is evident in amounts as low as 0·003γ per ml. of medium.

The active substance is rather labile to heat, acid and especially to alkali and is somewhat labile to mild treatment with bromine and nitrous acid. It is not affected by a number of common precipitating agents or by hydrogen peroxide.

Comparison of properties and direct replacement tests seem to indicate that the active substance differs significantly from other bacterial growth factors.

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