GROWTH FACTORS FOR BACTERIA

III. SOME NUTRITIVE REQUIREMENTS OF LACTOBACILLUS DELBRÜCKII[1]

E. E. SNELL, E. L. TATUM AND W. H. PETERSON

Departments of Agricultural Chemistry and Agricultural Bacteriology, University of Wisconsin

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The existence of substances which stimulate the growth of microörganisms when added to culture media in small amounts has been known for many years. Since the pioneer work of Wildiers (1901) with yeast, and of Bertrand (1904) with bacteria, a great many microörganisms have been reported to require for growth certain "stimulants" present in extracts of plant and animal tissue. An adequate review of the earlier literature on this subject is given by Peskett (1933), while most of the later literature has been reviewed by Sayhun and collaborators (1936).

In a previous paper Wood, Tatum and Peterson (1936) reported two factors necessary for vigorous growth of certain propionic acid bacteria in a synthetic medium. Both factors are present in yeast extract. One was prepared from a water extract of potato, the other was obtained from corn. Since the propionic acid bacteria are generally considered to be closely related to the *Lactobacillus* group, an investigation of the requirements of certain of the latter organisms was begun. This paper deals with sources and properties of certain factors essential for vigorous growth of a representative organism of the group.

A water extract of potato was very effective in stimulating growth of *Lactobacillus delbrückii* in a peptone medium; peptone itself contained a factor necessary for growth in acid hydrolyzed media; and both factors could be obtained from a water extract

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of liver. These substances have been partially purified, and some of their properties determined.

EXPERIMENTAL

Cultures and media. Pure cultures of the following microorganisms were used in this investigation: *Lactobacillus delbrückii*, culture 3; *Streptococcus lactis*, culture R; and *Lactobacillus pentoaceticus*, culture 41-11. Cultures of *L. delbrückii* were incubated at 37°C.; the other cultures at 28°C.

In all cases the basal medium contained 1 per cent glucose, mineral salts,[2] and a suitable source of nitrogen, peptone or hydrolyzed casein. The final concentration of all components of the medium, other than those whose effect on bacterial growth it was desired to determine, was the same in all tubes of any one experiment. One per cent of inoculum from a 48-hour culture of the organisms in malt sprouts medium was used in all cases. The fermentations were carried out in test tubes $\frac{5}{8}$ inch in diameter, containing 10 cc. of medium.

Analytical methods. The amount of acid produced was determined by titration with 0.1 N sodium hydroxide using bromthymol-blue as the indicator. Acidity was expressed in cubic centimeters of 0.1 N acid produced per 10 cc. of medium. When the weight of acid produced was desired, this was calculated as lactic acid. Reducing sugar was determined by the micro-method of Stiles, Peterson and Fred (1926). Nitrogen in various extracts was determined by the micro-Kjeldahl method described by Pregl (1930), using copper selenite as the catalyst.

Selection of organism and medium. The crude potato extract used in these experiments was prepared as described by Tatum and co-workers (1934). Whole Wisconsin potatoes were washed and ground. The juice was expressed, autoclaved at 15 pounds pressure for one-half hour, and the coagulated protein filtered off. The filtrate was then concentrated and stored under toluene. For use, it was diluted so that 1 cc. was equivalent to approximately 1 gram of raw potato.

[2] The mineral salts were used in the following concentrations: K_2HPO_4, 0.50 gram; KH_2PO_4, 0.5 gram; $MgSO_4 \cdot 7H_2O$, 0.2 gram; NaCl, 0.01 gram; $FeSO_4 \cdot 7H_2O$, 0.01 gram; $MnSO_4 \cdot 3H_2O$, 0.01 gram; H_2O, 1000 cc.

The effect of this extract on certain of the lactic bacteria was determined by adding it in amounts not exceeding 3 cc. per 10 cc. of medium to media containing either 0.5 per cent peptone, 0.5 per cent malt sprouts, or 1.0 per cent corn. Tubes of these media were then inoculated with cultures of *S. lactis*, *L. delbrückii* and *L. pentoaceticus*. The amount of acid produced was determined after 8 days. The results are given in table 1. The crude extract brought about a definite increase in acid production in

TABLE 1

Effect of potato extract on production of lactic acid in various media

ORGANISM	CRUDE POTATO EXTRACT ADDED TO MEDIUM*	0.1 N ACID PRODUCED IN 8 DAYS PER 100 CC. MEDIUM		
		Malt sprouts medium	Peptone medium	Corn mash medium
	cc.	cc.	cc.	cc.
S. lactis, No. R	0	0.73	1.77	0.24
	1	1.38	2.45	1.10
	2	1.75	2.24	1.44
	3	1.88	2.74	1.28
L. delbrückii, No. 3	0	2.02	0.75	1.72
	1	4.17	3.86	3.13
	2	4.52	7.10	3.96
	3	5.58	9.25	3.95
L. pentoaceticus, No. 41-11	0	1.36	0.47	0.15
	1	2.25	0.53	1.00
	2	2.40	0.54	1.25
	3	2.51	0.67	1.10

* One cubic centimeter potato extract represented approximately 1.0 gram raw potato and contained 11.9 mgm. solids.

every case. However, the stimulation was especially striking in the case of *L. delbrückii* on the peptone medium. This organism and this medium were therefore employed in attempts at further purification of the active principle in the potato extract.

Rôle of potato extract in glucose fermentation by L. delbrückii. More than one explanation for the increased acid production noticed might be suggested. A plant extract might bring about increased acid production (1) by supplying some sugar more easily fermented than glucose; (2) by increasing the fermentation

rate of the individual cells; (3) by increasing the number of cells, each of which ferments sugar at approximately the same rate as a cell in the absence of the "stimulant;" (4) by changing the course of the fermentation, resulting in production of a greater proportion of acidic substances per unit of glucose destroyed; (5) by buffering the medium so that acid production would not slow down the rate of fermentation; or (6) by a combination of the

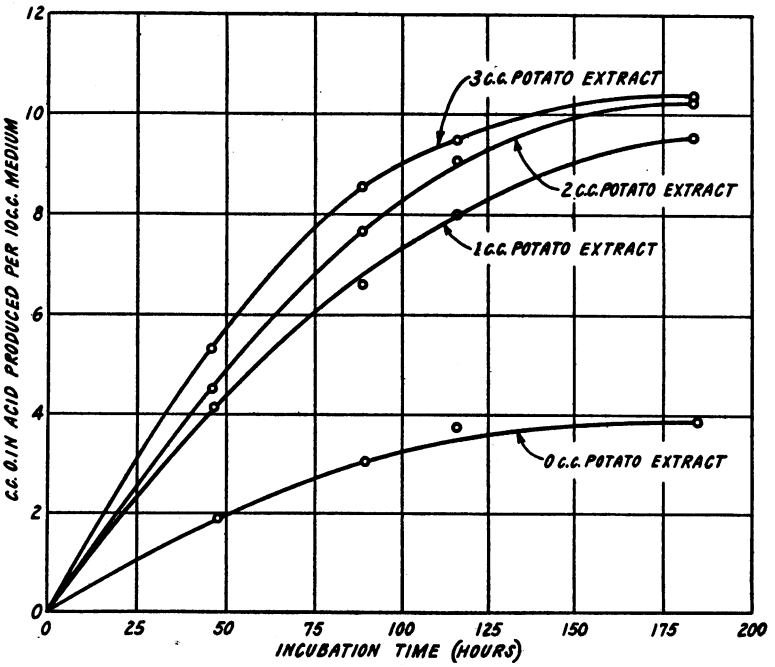

FIG. 1. STIMULATING EFFECT OF POTATO EXTRACT ON ACID PRODUCTION IN PEPTONE MEDIUM

above effects. Data indicating which of these effects are significant are given below.

A series of tubes were prepared containing different concentrations of the potato extract. Duplicate tubes were titrated at intervals from 0 to 185 hours. The results are given in figure 1. The potato extract caused an increased rate of acid production from the beginning of the fermentation. Furthermore, this

increase was not proportional to the amount of extract added, as might be expected, were it due to addition of small amounts of a substance easily fermented with production of acid.

The increased acid production was not due to an increase in the ratio of acid produced to the glucose destroyed (table 2). Assuming that only lactic acid was produced, almost perfect equivalence was obtained between acid production and glucose fermentation. This indicates that potato extract did not change the course of the normal fermentation, and that the increased production of acid was caused by increased glucose fermentation rather than by utilization of substances in the added potato extract.

TABLE 2

Dissimilation of glucose to lactic acid by L. delbrückii

POTATO EXTRACT IN 10 CC. OF MEDIUM	AFTER 48 HOURS		AFTER 96 HOURS		AFTER 144 HOURS	
	Lactic acid produced	Glucose destroyed	Lactic acid produced	Glucose destroyed	Lactic acid produced	Glucose destroyed
cc.	*mgm. per 10 cc.*	*mgm. per 10 cc.*	*mgm. per 10 cc.*	*mgm. per 10 cc.*	*mgm. per 10 cc.*	*mgm. per 10 cc.*
0	14.3	15.1	18.9	19.7	23.4	23.7
2	63.2	66.6	74.8	76.6	84.5	82.9*
3	64.5	67.2	77.6	79.4	85.2	83.1*

Basal medium: 0.5 per cent peptone, 1 per cent cerelose, mineral salts.

* Ten cubic centimeters of medium (without potato extract) contained 84.9 mgm. glucose.

It was observed that in all tubes increased acid production was correlated with greatly increased visible growth. Direct plate counts were made to check this observation. Platings were made after a 48-hour fermentation in order to avoid loss of viability from exposure of the organisms to a high acidity. Later work has shown that these counts are minimum figures since the organisms occurred in chains which were extremely difficult to break up into the constituent cells. Results (table 3) show a 100-fold increase in bacterial numbers for a 4-fold increase in acid, a 200-fold increase for a 5-fold increase in acid. The most pronounced effect of the potato extract, therefore, was to increase bacterial numbers. Increased acid production followed as a necessary consequence.

To determine whether the potato extract was equally stimulating in a medium containing $CaCO_3$, fermentations were carried out in 8-ounce bottles containing 100 cc. of 3-per-cent glucose-peptone medium, to which an excess of sterilized $CaCO_3$ was added aseptically. Samples for analysis were removed at intervals with sterile pipettes. The data are given in table 4. The rate of fermentation was markedly accelerated by the extract

TABLE 3

Correlation between numbers of bacteria and acid production

POTATO EXTRACT ADDED	AVERAGE NUMBER PER CUBIC CENTIMETER	0.1 N ACID PER 10 CC. MEDIUM
cc.		*cc.*
0	182,000	0.43
1	18,875,000	1.78
2	34,500,000	2.00
3	46,570,000	2.22

Basal medium: 0.5 per cent peptone, 1 per cent cerelose, mineral salts.

TABLE 4

Effect of potato extract on glucose fermentation by L. delbrückii in presence of calcium carbonate

POTATO EXTRACT PER 10 CC. MEDIUM	GLUCOSE FERMENTED* AFTER						
	24 hours	48 hours	72 hours	96 hours	142 hours	190 hours	238 hours
cc.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
0	27.9	76.5	124.8	145.5	175.5	204.9	229.0
1	29.7	128.4	192.1	206.4	235.6	248.6	263.3
2	33.5	147.2	208.3	229.9	243.4	259.8	270.9

Basal medium: 0.5 per cent peptone, 3.0 per cent glucose, mineral salts.

* Original medium contained 275.9 mgm. reducing sugar per 10 cc.

during the first half of the fermentation. Later, a depleted sugar supply became a limiting factor so that eventually destruction of sugar in the controls nearly reached that in the supplemented cultures. The data show clearly that the potato extract acts as a stimulant rather than as a buffer.

The initial oxidation reduction potential of a medium is often a factor in determining whether or not bacteria can initiate growth. However, potentiometric data showed that the action of the

potato extract was not due to its effect upon the oxidation-reduction potential of the medium. Although the extract lowered the oxidation potential, quantities of cysteine, cystine, or thioglycollic acid which lowered the potential in like amount were without effect upon growth of the bacteria.

In early experiments, considerable variation in growth was observed in similar experiments conducted at different times. An investigation of factors causing these variations showed that the final amount of growth may differ widely with the amount of inoculum, even though the smallest inoculum used was capable of initiating growth in the medium. Sterilization near neutral reaction often produced acidic substances, which stimulated growth of the organism to some extent. Therefore, size of inoculum and conditions of sterilization were carefully controlled in subsequent experiments.

Rôle of peptone in the growth of L. delbrückii. In an effort to develop a basal medium more nearly synthetic in character it was found that although the test organism grew well in a peptone or sodium caseinate (nutrose) medium in the presence of potato extract, acid hydrolyzates of peptone or casein were ineffective under the same conditions. The inclusion of tryptophane (ordinarily destroyed by acid hydrolysis) in these hydrolyzed media made initiation of growth possible, but in no case did growth in such media approach that in the unhydrolyzed media (see table 6). The disparity was not remedied by addition of cystine or cysteine to the medium. Evidently some substance other than tryptophane, and like tryptophane, essential for proper growth of the organism, was destroyed by the acid hydrolysis. It appears that *L. delbrückii* requires for vigorous growth unknown factors contained in potato extract and in peptone, in addition to known amino acids and a fermentable carbohydrate.

PREPARATION AND ACTIVITY OF POTATO FRACTIONS

In determining the effect of the various preparations on the growth of the test organism, the fractions were added in amounts equivalent to the original crude potato extract used in their preparation. A 0.5-per-cent peptone, 1-per-cent glucose, mineral

salts medium was used as a base. The results are presented in table 5. The fractions containing the greatest proportion of the stimulating substance are compared in figure 2. Details regarding the various treatments and conclusions therefrom follow:

1. Hydrolysis. 5.5 cc. concentrated H_2SO_4 were added to 50 cc. potato extract. Hydrolysis was carried out over night in the autoclave at 15 pounds pressure. Sulphuric acid was removed

TABLE 5

Biological activity of potato extract fractions

Cubic centimeters 0.1 N acid produced per 10 cc. medium

SUPPLEMENT TO BASAL MEDIUM	SUPPLEMENT ADDED PER 10 CC. MEDIUM*			
	0	1 cc.	2 cc.	3 cc.
Crude potato extract	1.61	6.73	8.05	8.74
Hydrolyzed potato extract	1.45	1.91	1.52	1.91
Ether extract of potato extract	1.40	3.90	5.10	6.03
Ether extract of potato extract plus sodium acetate			7.30	8.20
Residue from ether extraction	1.45	4.48	4.94	4.83
Ether extract plus residue		6.25	8.10	8.40
Ether-alcohol filtrate of potato extract		6.40	7.50	8.20
Ether-alcohol precipitate of potato extract		1.80	4.10	4.75
Lead acetate-ammonia filtrate from potato extract		4.40	5.80	6.32
Neuberg precipitate of potato extract		3.21	3.55	4.02
Neuberg filtrate of potato extract		6.30	7.92	8.60
Neuberg fractions combined		6.78	7.90	9.08

Basal medium: 0.5 per cent peptone, 1 per cent glucose and mineral salts.

* All fractions were added so as to be equivalent to the original crude potato extract.

with barium hydroxide. The stimulating substance was completely destroyed by this treatment.

2. Ether extraction. Crude potato extract was concentrated to small volume, acidified with sulphuric acid until acid to Congo red. Anhydrous calcium sulphate was mixed with the concentrated extract, and the whole allowed to dry. The resulting product was extracted continuously with ether for several days. Ether was removed from the extract, the residue was taken up in water, neutralized with sodium hydroxide, and diluted to volume. The residue from the ether extraction was boiled up with water,

filtered, neutralized, and concentrated to volume. The stimulatory substance was extracted by ether. The residue was less active than the extract.

3. Alcohol-ether precipitation. This procedure, as used for liver extract, has been described by Koehn and Elvehjem (1936). One

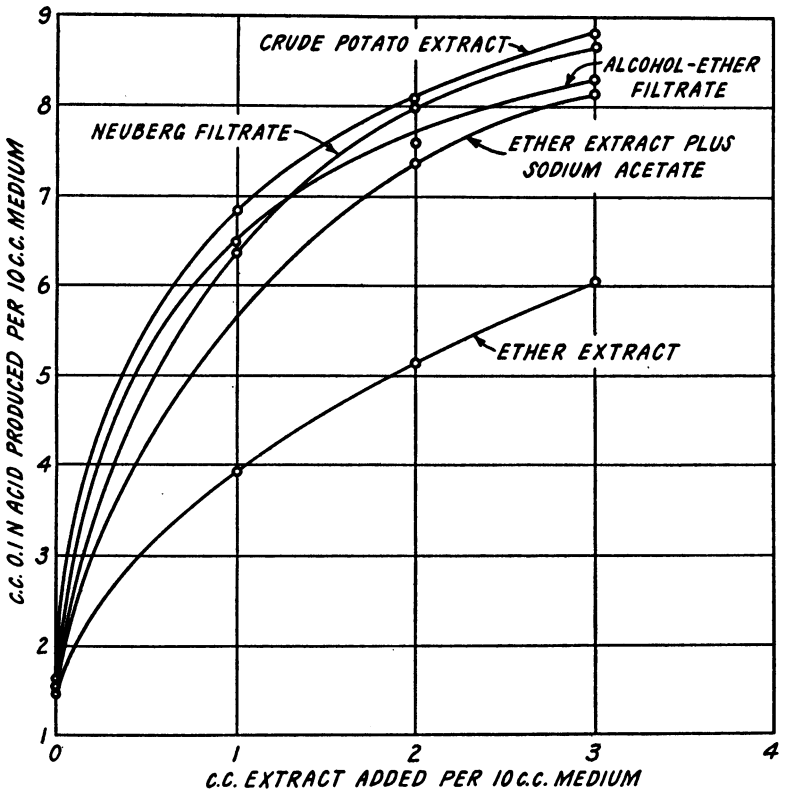

FIG. 2. STIMULATING EFFECT OF POTATO EXTRACT FRACTIONS ON ACID PRODUCTION

volume of concentrated potato extract was treated with 5 volumes of ethyl alcohol, then 6 volumes of ethyl ether were added with constant stirring. The precipitate was removed by centrifuging, washed with alcohol-ether, and dissolved in distilled water. The alcohol-ether solution was concentrated until all the organic solvents had been driven off, then diluted to volume with dis-

tilled water. The major portion of the dark-colored constituents of the extract were obtained in the insoluble fraction by this procedure. The stimulatory substance was soluble in the neutral alcohol-ether mixture.

4. Treatment with lead acetate and ammonia. Proteins, starch, sugar, and substances hydrolyzable to glucose were removed from the extract by precipitation with lead acetate in the presence of an excess of ammonia (Tatum, Peterson and Fred (1934)). The excess ammonia was boiled off from the filtrate, and the lead removed with hydrogen sulfide. Excess hydrogen sulfide was removed by boiling. The stimulatory substance was not precipitated by this treatment, and is therefore probably not related to the above substances.

5. Treatment with Neuberg's reagent. Crude potato extract was made alkaline with sodium carbonate and precipitated with mercuric acetate as described by Neuberg and Kerb (1912). The mercury was removed from filtrate and precipitate with hydrogen sulfide, and the excess hydrogen sulfide was removed by boiling. Although all proteins, peptides, and amino acids in the extract should be precipitated by this reagent, the filtrate was practically as active as the untreated extract.

Extraction of the crude potato extract with butyl alcohol by the method of Dakin (1918) was tried, but did not separate essential from non-essential substances.

When an amount of sodium acetate approximately equal to that occurring in the Neuberg filtrate was added to the ether extract, the effect of the latter became equal to that of the Neuberg filtrate, probably because of the increased buffer capacity.

The following figures show that the nitrogen content of the fractions is not a measure of their effect in stimulating growth:

Extract	Dry weight of 1 cc. mgm.	Nitrogen content of 1 cc. mgm.
Crude potato	11.98	0.775
Alcohol-ether filtrate	7.9	0.532
Alcohol-ether precipitate	3.5	0.239
Ether extract		0.049
Neuberg filtrate		0.118

From the above results, certain properties of the potato stimulant may be deduced. It is stable to autoclaving; soluble in water, ethyl alcohol, and ether. It is probably not a protein, peptide, amino acid, or a carbohydrate yielding glucose on hydrolysis. Since it is extracted by ether under acid conditions, it is not basic in character, and may be an acid of fairly simple nature. It is destroyed by prolonged acid hydrolysis.

TABLE 6

Activity of peptone fractions

Cubic centimeters 0.1 N acid produced per 10 cc. medium

SUPPLEMENT TO MEDIUM	PEPTONE EQUIVALENT OF ADDED FRACTIONS	
	0.4 per cent	0.8 per cent
None	0.75	
Hydrolyzed peptone	1.2	1.8
Peptone	7.05	8.4
Butyl alcohol extract of peptone	1.25	1.65
Residue from butyl alcohol extraction	3.63	4.05
Butyl alcohol extract plus residue	4.50	7.05
Residue from ethyl alcohol extraction of peptone	4.05	4.9
Ethyl alcohol extract of peptone	6.15	8.4

Basal medium: 0.9 per cent hydrolyzed casein, 0.01 per cent tryptophane, 1 per cent glucose, mineral salts, 0.5 cc. Neuberg filtrate of potato extract (per 10 cc. of medium).

PREPARATION AND ACTIVITY OF PEPTONE FRACTIONS

The medium used in determining which components of the peptone were necessary for growth contained, besides glucose and mineral salts, hydrolyzed casein (0.9 per cent), tryptophane, and the Neuberg filtrate of potato extract. Growth in this medium in the absence of peptone was very scant. The peptone fractions were added to this medium in amounts representing 0.4 or 0.8 per cent of the original peptone.

Results are summarized in table 6. The difference in effect of peptone and hydrolyzed peptone should be especially noted. Hydrolysis was carried out in the same manner as with potato extract.

As with potato extract, butyl alcohol extraction effected no

separation of essential constituents and seemed to destroy some of the activity. Peptone was entirely insoluble in ethyl ether so no separation could be effected with this solvent.

Ethyl alcohol extract. Extraction was made by the procedure described by Sayhun and co-workers (1936). Fifty grams of peptone were dissolved in hot water (about 35 cc.), and 250 cc. of hot 95-per-cent ethyl alcohol were added. The mixture was shaken frequently during a period of 24 hours, then allowed to stand for 24 hours longer. The clear supernatant liquid was decanted, and the extraction repeated twice, with 250-cc.-portions of 80 per cent alcohol. The alcoholic extracts were combined, acidified with 1 N sulphuric acid to pH 5.4. After standing a few hours the slight precipitate was filtered off. Almost all of the activity of the original peptone remained in the alcohol-soluble fraction. The extent of the concentration is shown by the following dry weight determinations:

	mgm. per cc. of 4 per cent equivalent
Original peptone	40.0
Alcoholic extract of peptone	15.4
Residue from alcoholic extraction	20.3

Since almost all of the activity was concentrated in this extract, it was used as a starting point in the preparation of all fractions other than those mentioned above. The various treatments were as follows with fermentation data given in table 7.

1. Solubility in acetone. Five cubic centimeters of the alcohol extract were treated with 25 cc. of acetone. The white flocculent precipitate was centrifuged off, washed with acetone once, and dissolved in water. The material was only slightly soluble in approximately 80 per cent acetone.

2. Adsorption and dialysis. The active factor was adsorbed by filtering through Norite under acid, neutral, or alkaline conditions, but only a slight loss in activity was suffered on filtering through Supercel. It was readily dialyzable through a collodion membrane.

3. Treatment with lead acetate-ammonia and Neuberg's reagent. These precipitations were carried out as described above for

potato extract. The active material was precipitated almost completely by lead acetate and ammonia and by Neuberg's reagent. In the case of the latter, the slight effect of the filtrate may be ascribed to its salt content. Addition of sodium acetate and sodium chloride gave about the same effect as addition of Neuberg filtrate.

TABLE 7

Treatment of ethyl alcohol extract of peptone and activity of fractions

Cubic centimeters of 0.1 N acid produced per 10 cc. medium

SUPPLEMENT TO MEDIUM	PEPTONE EQUIVALENT OF ADDED FRACTIONS	
	0.4 per cent	0.8 per cent
Original extract	6.15	8.4
Acetone soluble	1.95	2.8
Acetone insoluble	3.8	5.2
Norite filtrate		
pH 9.0	1.45	2.40
pH 7.0	1.70	2.35
pH 6.0	1.50	2.45
Supercel filtrate pH 6.0	5.55	8.65
Collodion dialysate	6.50	7.5
Phosphotungstic acid precipitate	3.1	4.4
Phosphotungstic acid filtrate	1.5	1.8
Phosphotungstic acid fractions combined	6.25	6.9
Barium hydroxide filtrate	3.42	4.85
Lead acetate-ammonia filtrate	1.65	1.9
Lead acetate-ammonia precipitate	2.85	4.0
Neuberg filtrate	1.1	1.5
Neuberg precipitate	4.41	5.2
Neuberg fractions combined	4.3	5.4

Basal medium: 0.9 per cent hydrolyzed casein, 0.01 per cent tryptophane, 1 per cent glucose, mineral salts, 0.5 cc. Neuberg filtrate of potato extract (per 10 cc. medium).

4. Precipitation with barium hydroxide. To the neutral alcohol extract a saturated solution of barium hydroxide was added until no further precipitation took place. The solution was filtered and the excess barium hydroxide removed from the filtrate with sulphuric acid. The active material was not precipitated.

5. Phosphotungstic acid precipitation. This precipitation was

carried out in the usual manner with 30 per cent phosphotungstic acid dissolved in 5 per cent sulphuric acid. Excess sulphuric and phosphotungstic acids were removed from filtrate and precipitated with barium hydroxide. The excess of the latter was removed with sulphuric acid. Phosphotungstic acid would precipitate basic organic substances, such as any basic amino acids or peptides occurring in the peptone. The active substance was largely precipitated by this reagent.

The above properties suggest that the active substance is either a basic amino acid or a compound with properties similar to such acids.

LIVER AS A SOURCE OF ESSENTIAL GROWTH FACTORS FOR L. DELBRÜCKII

Liver and extracts of liver have long been used as sources of biological substances stimulatory to growth. Various preparations from liver were therefore obtained in order to determine whether this material could be used as a convenient source of the factors necessary for the growth of *L. delbrückii*.

Preparation of extracts. The water extract of liver used in these experiments was obtained from the Wilson Laboratories of Chicago. It is essentially a dried water extract of fresh liver from which proteins have been removed by coagulation.

The ether extract of the water soluble constituents of liver was made in the same manner as described for potato extract.

The alcohol-ether precipitate, vitamin B_2 and hepatoflavin fractions were prepared by the method of Koehn and Elvehjem (1936).[3] The preparation of the alcohol ether precipitate has already been described. The hepatoflavin fraction was removed from the alcohol-ether soluble portion of the liver extract by adsorption upon fuller's earth and subsequent elution with pyridine and methyl alcohol. The portion not adsorbed is the vitamin B_2 fraction.[4] Since the pure hepatoflavin is unstable to light, special precautions were taken with this extract to prevent its decomposition.

[3] These extracts were prepared by Dr. C. J. Koehn for his vitamin investigations and portions of them kindly supplied to the authors.

[4] The terminology used in this paper is that of Koehn and Elvehjem (1936).

Activity of liver fractions. All extracts were added to the basal medium on the basis of the dry weight of the liver extract from which they had been prepared. The effect of the crude water-extract of liver on growth in various media is shown in table 8. Although neither potato extract alone nor extracts of peptone alone are effective in producing good growth on hydrolyzed casein medium containing tryptophane, the liver extract does support such growth and must therefore contain both factors, or substances capable of replacing them. This is confirmed by the fact that the presence of potato extract in the hydrolyzed casein medium with liver extract does not increase acid production over

TABLE 8

Stimulation of growth and acid production by a water extract of liver

Cubic centimeters of 0.1 N acid produced per 10 cc. of medium

MEDIUM*	AMOUNT OF LIVER EXTRACT ADDED TO MEDIUM			
	0	0.1 mgm.	1 mgm.	10 mgm.
0.5 per cent peptone	0.25	0.25	2.30	6.25
0.015 per cent tryptophane	0	0	0.65	2.80
0.9 per cent hydrolyzed casein + 0.015 per cent tryptophane	0.25	0.30	4.15	9.00
0.9 per cent hydrolyzed casein + 0.015 per cent tryptophane + 1 cc. potato extract	0.75	1.30	3.00	8.75

* Also contained 1 per cent glucose and mineral salts.

that with liver extract alone except when the latter is present in very small amounts.

The distribution of the substance capable of replacing the potato extract factor was further investigated. For this purpose the effect of the different fractions of liver extract on growth of the test organism in peptone medium was determined. The results are given in table 9. The active substance was concentrated in the alcohol-ether precipitate; the vitamin B_2 fraction contained it in much smaller amounts, while the flavin fraction was entirely inactive. The activity of the vitamin B_2 fraction was much increased, however, by the addition of the flavin fraction.

The acid-ether extract of liver extract showed some stimulating properties. The substance extracted may be the same as that obtained from potato extract. This is not the main factor present, as is shown by the much greater activity of the residue from ether extraction. In the case of potato extract, the residue was less active than the ether extract. Stimulatory substances in the potato extract were also concentrated in the filtrate from the alcohol-ether precipitation, whereas in the case of liver extract they are concentrated in the precipitate. These facts indicate that factors other than those contained in potato extract and

TABLE 9

Activity of liver fractions

Cubic centimeters of 0.1 N acid produced per 10 cc. of medium

NUMBER	SUPPLEMENT TO PEPTONE MEDIUM	WEIGHT OF LIVER EXTRACT REPRESENTED BY SUPPLEMENT			
		0	5 mgm.	10 mgm.	30 mgm.
1	Crude liver extract	0.35	5.1		
2	Acid-ether extract of liver extract	0.4	2.3	2.7	3.1
3	Residue from ether extraction	0.4	6.35	7.1	
4	Alcohol-ether precipitate fraction	0.35	5.3	6.7	7.85
	Alcohol-ether dialysate		5.25	7.55	
5	Heptoflavin fraction	0.35	0.35	0.35	0.35
6	Vitamin B_2 fraction	0.35	0.45	0.45	5.55
7	Hepatoflavin + vitamin B_2 fractions		2.85	5.6	5.4
8	Hepatoflavin + alcohol-ether precipitate		5.32	6.82	8.2
9	Vitamin B_2 + alcohol-ether precipitate		5.9	7.5	8.25
10	Vitamin B_2 + hepatoflavin + alcohol-ether precipitate		6.5	7.55	8.2

Basal Medium: 0.5 per cent peptone, 1 per cent glucose, mineral salts.

present in liver extract are capable of replacing the potato factor. An alternative explanation would be to assume that both factors are identical, but that in the case of liver extract it is in combination with a substance precipitated by alcohol-ether, from which it is not released under the acid conditions of ether extraction employed. The stimulatory substances contained in the alcohol-ether precipitate of liver extract were completely dialyzable.

DISCUSSION

The known properties of the three factors are summarized in table 10. The peptone and potato factors are evidently different. While all of the diagnostic tests have not been applied to the liver factor, it appears to contain two factors, one of which is identical with the potato factor, and another which can replace this factor. Constituents in the liver extract are also able to replace the peptone factor, which is necessary in addition to the potato factor for luxuriant growth on acid hydrolyzed media.

TABLE 10

Comparison of peptone, potato, and liver factors

PROPERTY	FACTOR		
	Peptone factor	Liver factor	Potato factor
Heat stable (pH 7.0)	+	+	+
Dialyzable	+	+	+
Solubility			
(1) Water	+	+	+
(2) 80 per cent ethyl alcohol	+	+	+
(3) Ethyl ether	−	Partially	+
Precipitated by			
(1) Alcohol-ether	+	+	−
(2) Neuberg's reagent	+		−
(3) Lead acetate-ammonia	+		−
(4) Phosphotungstic acid	+		
Adsorption on norite	+		
Destroyed by acid hydrolysis	+		+

Properties of the potato factor indicate that it is similar to the factor reported by Wood, Tatum and Peterson (1936) for the growth of certain propionic acid bacteria. Its effect on *L. delbrückii* is not correlated with its nitrogen content, and since it was extracted by ether from acid solutions, it seems to be an acid.

Kayser in 1894 showed that peptone is a very good source of nitrogen for lactic acid bacteria, especially in the presence of juice from vegetables such as onions. ZoBell and Meyer (1932), in attempting to prepare a synthetic medium which would support growth of *Brucella*, observed that in no case did known simple carbon sources in the presence of a nitrogen source support

growth nearly so well as peptone. They state "the accelerated multiplication noted from the use of peptone . . . may be due to other factors than its nitrogen content." Sayhun and co-workers (1936) obtained a preparation from the alcoholic extract of peptone which was very active in stimulating the growth of *Escherichia coli* in a synthetic medium. The factor was precipitated by phosphotungstic acid, and was destroyed by prolonged boiling with acid. Our factor is probably closely related to this factor. They reported the separation of their peptone factor into two fractions with butyl alcohol. In our case, the butyl alcohol soluble constituents of the peptone are practically without effect on the growth of *L. delbrückii*. Solubility and precipitation reactions of the factor indicate that it is a basic, nitrogenous compound of fairly low molecular weight.

The activity of liver extract in replacing potato extract was not correlated with the presence of vitamin B_2, since an extract of liver containing vitamin B_2 was comparatively inactive in stimulating growth of the test organism in a peptone medium.

SUMMARY

A water extract of potato, when added to simple media stimulated growth and acid production of a number of lactic acid bacteria. This action was most effective with *Lactobacillus delbrückii* on peptone-glucose-mineral-salts medium. The stimulating action is evident also in media buffered with $CaCO_3$, and results primarily in an increase in the number of cells.

Tryptophane is an amino acid essential for the growth of this organism. For luxuriant growth in the presence of hydrolyzed casein and tryptophane two unknown factors are necessary. One of these occurs in the Neuberg filtrate or acid-ether extract of a water extract of potato, and may be an acid of fairly low molecular weight. The other factor is basic in character, and occurs in peptone. Both are destroyed by prolonged acid hydrolysis.

Liver extract contains both of the necessary factors or factors capable of replacing them. Vitamin B_2 does not seem to play a rôle in the stimulation by liver preparations.

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