CCLXVII. GROWTH FACTORS FOR BACTERIA.[1]
V. VITAMIN B_1, A GROWTH STIMULANT FOR PROPIONIC ACID BACTERIA.

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IN a previous paper [Wood *et al.* 1936] it was shown that for growth and fermentation the propionic acid bacteria require an ether-extractable acid found in aqueous extracts of yeast, potatoes and liver and in corn steep water. This stimulant was found to be essential for growth on a synthetic medium containing ammonium sulphate as the source of nitrogen. Although fairly good growth was obtained with this combination, continued transfer gave inconsistent results. The organisms generally failed to grow after the 3rd or 4th transfer. However, the addition of amino-acids in the form of hydrolysed caseinogen greatly increased cell growth and fermentation and made repeated transfer possible with consistent results.

In the present investigation a second growth factor for the propionic acid bacteria has been found which stimulates growth particularly in the presence of amino-acids. This paper deals with the sources, preparation and properties of this accessory factor, which has been found to be identical with vitamin B_1.

EXPERIMENTAL.

Cultures. Pure cultures of six strains of propionic acid bacteria were used in this study. These were selected from those studied by Hitchner [1934] and on the basis of his classification are representative cultures. The following were used: *Propionibacterium pentosaceum* 11; *P. freudenreichii* 33; *P. jensenii* 54; *P. zeae* 56; *P. pentosaceum* 57; and *P. arabinosum* 61. All cultures were carried on Difco yeast extract medium and were transferred at least twice on ammonium sulphate medium before inoculating in order to deplete the inoculum of any growth factors contained in the original medium. *P. pentosaceum* 11 was used as a test organism for most of the investigations, as in previous work, but the findings were checked against the other cultures.

Medium and analytical methods. The supplemented synthetic medium developed in the previous work [Wood *et al.* 1936] was used throughout this investigation. This has the following composition: glucose 1%; sodium acetate, 0·6%; ammonium sulphate, 0·3%; inorganic salts; ether extract of 3 g. yeast extract per 100 ml. Fermentation was followed by direct titration of the acid produced.

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Effect of protein hydrolysates on fermentation. It has been shown in the previous paper that the basal medium with the addition of hydrolysed caseinogen gave a fermentation practically equivalent to crude yeast extract medium. As this was thought to be due to the amino-acids added, it seemed that two factors were necessary for good growth; viz. the ether-soluble acidic factor and some necessary or beneficial amino-acids. However, when the amount of hydrolysed caseinogen which was necessary for maximum fermentation was compared with the amount of crude yeast extract required, it was apparent that the yeast was much more potent. Furthermore, the optimum concentration of hydrolysed caseinogen was greatly in excess of the probable amino-acid requirements, judged by the amounts needed by other bacteria [Fildes & Richardson, 1935; Mueller, 1935]. Two explanations of this seemed possible; yeast extract may contain some particularly beneficial amino-acids which are present in caseinogen in only small amounts, or yeast extract may contain an active ether-insoluble factor other than amino-acids which is present in small amounts in caseinogen hydrolysate.

If amino-acids were concerned, it seemed probable that hydrolysates of proteins other than caseinogen might have very different effects owing to their differences in amino-acid content. Therefore sulphuric acid hydrolysates of gelatin, egg albumin and a sample of purified caseinogen (reprecipitated and washed until vitamin-free) were prepared according to standard procedures. These were added to the basal medium and their activities compared with that of the crude caseinogen hydrolysate. A sample of crude yeast extract was also hydrolysed in the same way and tested. The results (Fig. 1) show that one of the

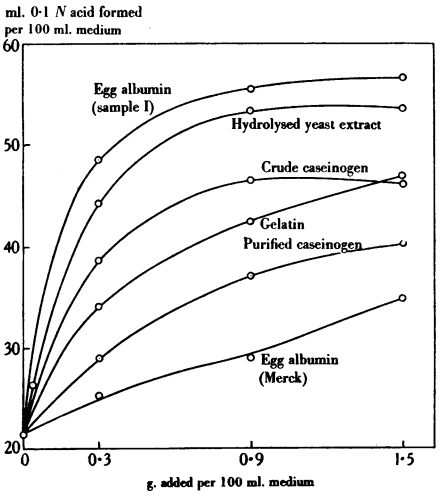

Fig. 1.

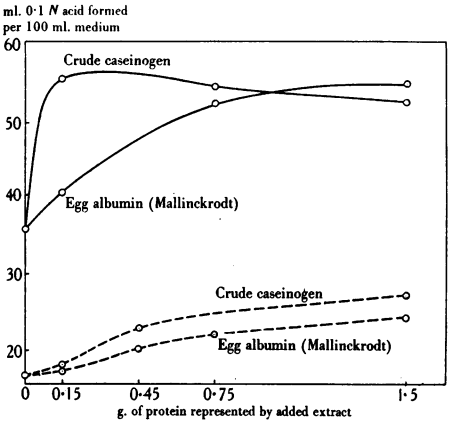

Fig. 2.

Fig. 1. Effect of hydrolysed materials on acid production.

Fig. 2. Stimulating effect of alcohol extracts of crude caseinogen and egg albumin on acid production.

—— 0·45 % hydrolysed purified caseinogen + 0·3 % $(NH_4)_2SO_4$. --- 0·3 % $(NH_4)_2SO_4$.

hydrolysed egg albumins (sample 1) and hydrolysed yeast extract were much more effective than the crude caseinogen hydrolysate. Gelatin was almost as effective as the caseinogen. However, purified caseinogen was much inferior to the crude caseinogen, and a second sample of egg albumin (Merck) was still less

effective. These results indicated that some factor other than amino-acids was primarily responsible for the superiority of yeast extract. In the first place there was no correlation between the known amino-acid contents of the different proteins and their observed activities; and in the second place there should have been no variation in the amino-acid composition of the two samples of caseinogen and the two egg albumin samples. It seemed probable that some factor in the hydrolysed proteins which was not a constituent of the protein molecule was primarily responsible for their activity. It has been found that 95 % alcohol and 85 % acetone extracts of crude yeast extract contain all the factors necessary for vigorous growth and fermentation. Therefore extracts of unhydrolysed crude caseinogen and egg albumin (impalpably powdered, Mallinckrodt) were made by continuous extraction with 95 % alcohol. The solvents were removed and aqueous solutions of these extracts were added in varying concentrations to the basal synthetic medium with and without hydrolysed purified caseinogen. The results (Fig. 2) show that these extracts contained some factor which was only slightly stimulating in the basal ammonium sulphate medium, but which had a very decided effect in the presence of hydrolysed purified caseinogen.

These results indicated quite conclusively that the stimulating factor was neither an amino-acid nor a constituent of the protein molecule, although its activity seemed to be associated with the utilization of amino-acids. It seemed possible that the original materials from which the proteins were derived might be better sources of the factor than the crude proteins. Therefore milk powder and dried egg white were extracted with alcohol and acetone. The extractions were carried out by covering the materials with the solvent, allowing to remain for 1 or 2 days with frequent shaking, and finally filtering off the insoluble residues. The solvents were removed and aqueous solutions of the extracted material were tested by adding them to the basal medium in the presence of hydrolysed purified caseinogen. It was found that the extracts of egg white were fairly active, whilst the milk powder extracts were even more active than the continuous alcohol extract of caseinogen. These extracts were just as potent in a basal medium containing 0·15% of hydrolysed purified caseinogen as in one containing 0·45 %. The data in Fig. 3 show the effects of the three most active extracts on the production of acid in the presence of 0·15 % hydrolysed purified caseinogen. The maximum stimulation was reached with a very low concentration of these extracts. The units chosen were those amounts which produced maximum stimulation when added to 100 ml. of medium containing hydrolysed purified caseinogen. The dry weights of the various extracts were as follows:

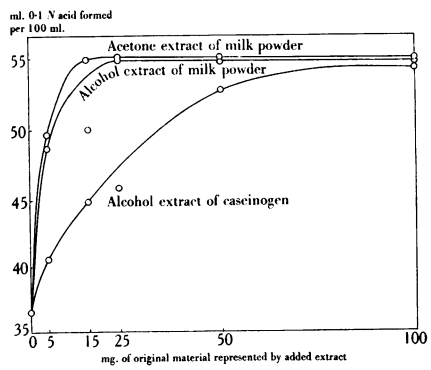

Fig. 3. Stimulating effects of extracts of milk powder and crude caseinogen on acid production.

Extract	Weight of one unit γ	Weight of original material represented by one unit of extract mg.
Alcohol extract of caseinogen	882	100
Alcohol extract of milk powder	555	25
Acetone extract of milk powder	15	15

It should be pointed out that recovery of the factor from the milk powder was probably much lower than from caseinogen, since the latter was extracted continuously whilst the milk powder was merely shaken with the solvents. Milk powder seemed to be the richest and best source of the stimulant, and the acetone extract was by far the most active per unit of dry weight.

Maximum stimulation in 100 ml. of medium was obtained with 0·9 mg. of alcohol extract of caseinogen (the extract of only 0·1 g. of crude caseinogen) in the presence of only 0·15 g. hydrolysed purified caseinogen. In contrast to this the optimum concentration of hydrolysed crude caseinogen was 0·9 g. (Fig. 1). This high optimum concentration was probably due to partial destruction of the stimulant during the acid hydrolysis. Enough active stimulant still remained, however, to cause the superiority of hydrolysed crude caseinogen over hydrolysed purified caseinogen.

Response of other cultures to the stimulant.

In order to find out whether cultures of propionic acid bacteria other than *P. pentosaceum* 11 require or are benefited by the factor, five additional cultures were tested on the basal medium with hydrolysed caseinogen (0·15 %) with and without the stimulant. Table I gives the results of this experiment, in which

Table I. *Response of different cultures to the stimulant.**

Addition to basal hydrolysed caseinogen medium	Culture No.					
	11	33	54	56	57	61
	ml. 0·1 *N* acid formed per 100 ml. medium					
None	37	53	20	29	32	33
Acetone extract milk powder, 36γ/100 ml.	55	54	21	37	45	62

* Inoculated from second transfer in $(NH_4)_2SO_4$ medium.

culture 11 is included for comparison. From these results it appears that different strains of propionic acid bacteria vary in their requirements for this alcohol-soluble factor. Cultures 11 and 61 respond very definitely. Cultures 56 and 57 respond to a less degree. Culture 54, a rather weak fermenter, apparently was not affected, whilst culture 33, a vigorous fermenter, grew just as well without the factor. Culture 33 either does not require the stimulant for its growth or, if it does, it can synthesize it under the cultural conditions used.

Relation of the stimulant to growth stimulants for other organisms.

In order to determine whether our factor was similar to other stimulants described in the literature, as many of these factors as were available were tested for their ability to replace it. The concentration of each substance tested was varied over a wide range on each side of the optimum values reported for other organisms. The figures in Table II give the maximum acid productions with these materials[1] in the basal medium containing 0·15 % hydrolysed purified caseinogen. Inositol, indoleacetic acid, ascorbic acid and nicotinic acid amide had no effect. Pantothenic acid was slightly active in the highest concentration, probably because of impurities present, since 1·25 mg. had less effect than did 15γ of acetone extract of milk powder. The sample was a 50 % pure preparation of the calcium salt of pantothenic acid which according to Dr R. J. Williams stimulated yeast growth in a concentration of 2·9γ per 100 ml.

[1] The authors wish to thank Dr R. J. Williams for the sample of pantothenic acid and Dr C. J. Koehn for the nicotinic acid amide.

Table II. *Effects of various materials on acid production.*

Material tested	Concentration range per 100 ml.	Activity: Concentration* per 100 ml.	Activity: Acid formed per 100 ml. ml. 0·1 *N*
None	—	—	38
Acetone extract of milk powder	0·7 to 70 γ	15·0 γ	55
dl-Inositol	5 to 500 mg.	500 mg.	39
Indoleacetic acid	0·01 to 100 mg.	0·01 mg.	31
Ascorbic acid	0·5 to 100 mg.	50 mg.	41
Nicotinic amide	0·1 to 100 mg.	0·1 mg.	31
Pantothenic acid	0·25 γ to 1·25 mg.	1·25 mg.	47

* Most active or least toxic concentration.

The fractions of liver extract[1] which were tested for stimulation of *L. delbrückii* [Snell *et al.* 1936] were also tested for ability to replace the propionic stimulant. The hepatoflavin fraction was inactive, the vitamin B_2 fraction had some activity, but the ether-alcohol precipitate fraction was quite active. This fraction was also most effective for *L. delbrückii*.

Properties of the stimulant.

In order to obtain some information regarding the chemical nature of the stimulant, various physical properties were investigated. Approximately 5 units of the stimulant were used for each of the various treatments and the preparations and fractions obtained were tested for activity by adding them in varying concentrations to 10 ml. of the basal medium containing 0·15 % purified hydrolysed caseinogen. The results for one concentration (1 unit per 100 ml.) are given in Table III. It should be mentioned that high acidity was accompanied in every case by increased visible cell growth.

Table III. *Physical properties of stimulant.*

Treatment or fraction tested	Acid formed per 100 ml. medium ml. 0·1 *N*	Conclusion
No stimulant	38	—
Untreated stimulant	55	—
Dried 60°, 12 hours	56	Stable
Heated 1 hour in *N* H_2SO_4 126°	55	Stable
Heated 1 hour in *N* NaOH 126°	45	Partially destroyed
Steam distillate, *p*H 4	32	Non-volatile
Steam distillate, *p*H 9	34	Non-volatile
Vacuum distillate, 2 mm. Hg, 120°	39	Non-volatile
Vacuum residue	53	Still active
Acetone extract, *p*H 5·0	53	Extracted
Acetone extract, *p*H 8·0 (NaOH)	57	Extracted
Acetone extract, *p*H 8·0 ($Ba(OH)_2$)	55	Extracted
Chloroform extract, *p*H 4·0	45	Partially extracted
Chloroform extract, *p*H 8·0 ($Ba(OH)_2$)	41	Partially extracted
Ether extract, *p*H 4·0	35	Not extracted
Ether extract, *p*H 8·0	37	Not extracted
Treated $BaSO_4$, *p*H 6·8	56	Not adsorbed
Treated norite, *p*H 6·0	40	Adsorbed
Treated norite, *p*H 8·0	37	Adsorbed
Treated norite, *p*H 7·0	32	Adsorbed

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

The factor is stable to dry heat at a temperature of 60° for 12 hours. It is stable to autoclaving at neutrality and in *N* H_2SO_4 solution but is largely destroyed by *N* NaOH under the same conditions. The factor is not volatile with steam either at *p*H 4 or 9, nor is it volatile at 120° and 2 mm. pressure.

It was hoped that some indication of whether the stimulant was acidic or basic in nature could be obtained by extraction from acid and alkali. Aqueous solutions of the factor were adjusted to *p*H 4 and 8 with H_2SO_4 and NaOH respectively, dried on anhydrous $CaSO_4$ and extracted continuously with solvents for 24 hours. Acetone extracted the stimulant completely under all conditions, the chloroform extractions were incomplete at both *p*H levels and the stimulant was not ether-soluble. It seemed possible that the factor might be a neutral compound as there was no difference between recovery from alkaline and from acid solutions.

From the results given in this table it can only be concluded that the factor is stable to acid and labile to alkali, and that it is non-volatile, soluble in acetone, slightly soluble in chloroform, insoluble in ether and adsorbed on norite.

Replacement by vitamin B_1.

The solubilities and stability of the factor suggested that it might be similar to vitamin B_1. Samples of this vitamin were therefore tested for their activity in replacing the stimulant in the basal medium containing 0·15% hydrolysed purified caseinogen. Fig. 4 shows the effect of these samples as compared with

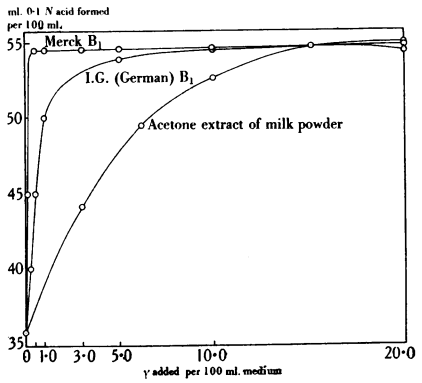

Fig. 4. Effects of vitamin B_1 and acetone extract of milk powder on acid production.

that of acetone extract of milk powder. Both vitamin B_1 samples replaced the stimulant completely. The optimum concentration of acetone extract of milk per 100 ml. was 15γ, that of the I.G. (German) sample of B_1 was 5·0γ and the sample of crystalline B_1 (Merck) was active at a concentration of 0·5γ.

Discussion.

The complete replacement of our stimulant by vitamin B_1 in very low concentrations indicates that the two factors are identical. This supposition is supported by the similarities of the properties of the stimulant and of vitamin B_1. The solubility of the factor in water, alcohol and acetone, the insolubility in ether; the stability to acid and destruction by alkali; the adsorption on norite; and the non-volatility are all characteristic properties of vitamin B_1.

The materials in which our stimulant has been found, yeast, caseinogen and milk, are known to contain this vitamin. Egg albumin and egg white were not as rich in our factor as the other materials and are known to contain little vitamin B_1. The facts that our stimulant is not a part of the protein molecule and that the washed vitamin-free caseinogen was not active further support the theory of its identity with vitamin B_1. It is probable that the activity of vitamin B_1 would not be completely destroyed even by hydrolysis in 20 % H_2SO_4; hence the observed activity of the protein hydrolysates would not contradict the theory that the stimulant is the same as vitamin B_1.

Vitamin B_1 has been reported to be stimulatory or essential for other micro-organisms. Schopfer [1935] showed that *Phycomyces blakesleeanus* is stimulated by vitamin B_1 and suggested the use of this organism in vitamin B_1 assay. It has also been reported [Sunderlin & Werkman, 1928] that many bacteria are able to synthesize vitamin B_1. The more vigorous growth of propionic acid bacteria obtained by Sherman & Shaw [1923] in association with certain other bacteria might possibly have been due to synthesis of vitamin B_1 by the associated organisms. *P. freudenreichii* No. 33 may itself synthesize this vitamin, since it grows with equal vigour with or without its addition to the medium.

The function of vitamin B_1 in the metabolism of propionic acid bacteria is not definitely known. Its most important role may be in stimulating growth and multiplication since its most striking effect is the greatly increased cell growth induced. It is of great interest that vitamin B_1, a specialized substance essential for higher animals, is also required by single-celled microorganisms such as the propionic acid bacteria.

SUMMARY.

The stimulating action of protein hydrolysates on the acid production by certain propionic acid bacteria has been found to be due in part to a factor which is neither an amino-acid nor a part of the protein molecule.

The stimulant was obtained from unhydrolysed caseinogen, egg albumin, yeast extract and milk powder by extraction with alcohol or acetone. The acetone extract of milk powder was most effective, since it was active in a concentration of 15γ per 100 ml.

The effect of the stimulant was most pronounced in the presence of amino-acids and it stimulated four of six cultures tested.

Inositol, pantothenic acid, ascorbic acid, hepatoflavin, nicotinic acid amide and indoleacetic acid could not replace the stimulant.

The properties of the stimulant were similar to those of vitamin B_1, and crystalline vitamin B_1 (Merck) completely replaced it in a concentration of $0{\cdot}5\gamma$ per 100 ml. of medium.

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