190. GROWTH FACTOR AND OTHER NUTRITIONAL REQUIREMENTS OF THE ACETONE BUTANOL ORGANISM, *CL. ACETO BUTYLICUM*¹

BY A. E. OXFORD,² J. O. LAMPEN AND W. H. PETERSON

*From the Department of Biochemistry, College of Agriculture, University
of Wisconsin, Madison, Wisconsin, U.S.A.*

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THIS investigation has been mainly concerned with the following fundamental problem: What is the simplest possible medium in which normal growth of *Cl. acetobutylicum* will take place? The recent observations of Woolley *et al.* [1939] and of Snell & Williams [1939] have shown that the related organism, *Cl. butylicum*, will grow normally on a synthetic medium containing glucose, in which asparagine is the sole source of nitrogen and to which exceedingly minute amounts of pure biotin have been added. Weizmann & Rosenfeld [1939] have already reported that biotin is a growth factor for *Cl. acetobutylicum*, which differs from *Cl. butylicum* mainly in being proteolytic and in yielding acetone as a fermentation product in place of isopropyl alcohol. These authors, however, did not use pure biotin, but concentrates rich in that growth factor obtained from egg yolk (by following the fractionation procedure of Kögl & Tönnis [1936] up to a certain point) and from various plant materials.

Thanks to the great generosity of Prof. Kögl of Utrecht, we have had at our disposal a small specimen of nearly pure biotin and have been able to confirm the general conclusion reached by Weizmann & Rosenfeld [1939], but not the particular one they claimed to have established, which was, to quote their own words, that "the second factor, biotin, together with the first factor, asparagine, brings the specific nutritive level up to 70 % as compared with maize". In our experience, *Cl. acetobutylicum* will show practically no growth in a synthetic medium containing asparagine and biotin and no other growth factors. Another factor, which we propose to call the BY factor, not identical with any known factor for micro-organisms, is absolutely essential for growth, while asparagine is not essential. In a medium with glucose as the sole source of carbon and the ammonium ion (supplied as ammonium phosphate) as the sole source of nitrogen, normal growth will take place provided that adequate amounts of both biotin and the BY factor are supplied. Neither factor will support growth in the complete absence of the other.

Yeast extract is particularly rich in the BY factor which has been obtained in a very concentrated form, but not yet in a pure state, from this material. Our best concentrate will enable marked growth of the organism to take place, in presence of excess of biotin, when supplied at the low concentration of 0.025 µg./ml. The biotin requirement for a similar result in presence of excess of the BY factor is even smaller and is of the same order as the known requirement

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² International Fellow in the Natural Sciences of the Rockefeller Foundation.

by *Cl. butylicum*. The chemical properties of the BY factor are on the whole very similar to those of biotin; it is a weak acid, very stable to the action of acids and alkalis and is destroyed by nitrous acid.

As already stated, the nitrogen requirements of the organism in presence of the growth factors are very simple. Ammonium phosphate is in fact more suitable than asparagine, or asparagine and ammonium salts, in that growth begins after 28–30 hr., whereas on the asparagine basal medium growth is usually delayed until the third day. This long lag period can be considerably shortened in a number of ways:

(a) by addition of hydrolysed casein, or an artificial amino-acid mixture simulating hydrolysed casein;

(b) by addition of suitable mixtures of a few amino-acids, valine plus methionine being especially good in this respect;

(c) by addition of cysteine or Na_2S .

Finally, some preliminary work has been carried out on the fermentation of glucose in the simplest possible medium containing ammonium phosphate as the sole source of nitrogen and minute but adequate amounts of the two highly purified growth factors. Although the production of solvents does occur, the yield is not as high as in a "normal" fermentation, and the titratable acidity at the end is correspondingly higher. An impure biotin preparation obtained from cane molasses is better in this respect than pure biotin. The reason for this is at present unknown. Most of the common mono- and di-saccharides, as well as starch and mannitol, can also be utilized for growth and solvent production on the above virtually synthetic medium. We have also satisfied ourselves that *Cl. acetobutylicum* can be propagated indefinitely on such a medium; in other words, no unsuspected growth factor was carried over in the initial inoculum. We cannot at the moment offer any explanation of the claim of Brown *et al.* [1939] that the organism can be propagated indefinitely on a glucose-amino-acid-ammonium sulphate-salts medium, save that their amino-acid mixture, which was not exclusively composed of synthetic acids, must have contained both biotin and the BY factor.

EXPERIMENTAL

Cultures and medium. Unless otherwise stated *Cl. acetobutylicum* Strain 5 was used throughout this investigation. This is a strain that has been used in the commercial production of acetone and butyl alcohol. The basal medium most commonly employed has the following composition: 2% glucose (C.P.); 0.1% asparagine; 0.1% $(\text{NH}_4)_2\text{SO}_4$; 0.11% Speakman's salt mixture (KH_2PO_4 ; K_2HPO_4 ; Mg, Mn and ferrous sulphates); reduced iron (at the bottom of the tube). Each tube contained 35 ml. of medium and was incubated at 37° in a closed jar made anaerobic by moistened oats. The standard inoculum was prepared from a 2-day culture which had been inoculated with a spore suspension on soil and heated at 100° for 2 min. before incubation. The medium we used for growing the inoculum was similar to the above, but in it the $(\text{NH}_4)_2\text{SO}_4$ was replaced by 0.52% peptone. A portion of the culture was centrifuged, the supernatant liquid poured off and the deposit resuspended in an equal volume of sterile distilled water. 0.3–0.4 ml. of this suspension was used as the inoculum. After 3 days' incubation the gas in the tubes was removed by suction and the turbidity read in the Evelyn colorimeter. The reading is inversely proportional to the turbidity or amount of growth. A reading of 100 denotes no growth. "Pure biotin" refers to the crystalline preparation received from Prof. Kögl, which he designated as about 75% pure biotin methyl ester.

Biotin as growth factor for Cl. acetobutylicum. No growth of the organism took place in a 2% glucose–0.1% asparagine–Speakman's salts–basal medium containing pure biotin in a concentration much higher than necessary for optimum growth of the related organism, *Cl. butylicum*, Strain 21 [cf. Peterson *et al.* 1940]. The addition of a mixture of growth factors known to be required by certain micro-organisms (riboflavin, pantothenic acid, nicotinic acid, vitamin B₆, inositol, pimelic acid, uracil, glutathione, vitamin B₁, β -alanine and biotin) gave a similar negative result, as did a mixture of two pyrimidines (uracil and cytosine) and four purines (guanine, adenine, xanthine and hypoxanthine). A biotin-free preparation (*Cl. butylicum* assay) was, however, obtained from yeast extract which alone did not support the growth of *Cl. acetobutylicum*, but when added in conjunction with pure biotin enabled the organism to grow normally as shown by turbidity measurements in the colorimeter.

This biotin-free preparation (referred to subsequently as yeast extract preparation) was obtained as a by-product in the concentration of the second BY factor described later. Difco yeast extract was extracted for 30 hr. with absolute alcohol in a Soxhlet apparatus. The cold extract was filtered, evaporated to dryness *in vacuo* and taken up with cold water. The small amount of residual insoluble gum was extracted with 1000 parts of boiling water, and the filtered extract, after evaporation to dryness, was again taken up in hot water and filtered. The clear neutral solution so obtained contained approximately 50 μ g. of dissolved matter per ml. and was essentially free from biotin (see Table 1). Table 1 further shows the response of *Cl. acetobutylicum* to an excess of added biotin in presence of an excess of the yeast extract preparation after incubation at 37° for 3 days.

Table 1. *Biotin as growth factor for butyl organisms*

Organisms and supplement	Supplement μ g./ml.		Colorimeter readings	
	Tube 1	Tube 2	Tube 1	Tube 2
I. <i>Cl. butylicum</i> :				
(1) + pure biotin	0	0.00023	91.5	22
(2) + yeast extract preparation	5	7	85.7	89
II. <i>Cl. acetobutylicum</i> :				
(1) + pure biotin	0	0.00023	92	98
(2) + yeast extract preparation	5	7	85	83.5
(3) + pure biotin and yeast extract preparation	0.00023 5	—	22	—

Table 2. *Response of Cl. acetobutylicum to varying amounts of biotin*

Yeast extract preparation added in excess (5 μ g./ml.)		
Pure biotin added μ g./ml.	Reading	Gas production
0.0000055	66.5	Very slight
0.000011	65	Slight
0.000028	53	Moderate
0.00011	32.5	Good
0.00023	31	Very good

Table 3. *Response of Cl. acetobutylicum to varying amounts of the BY factor*

Each tube contained 1 mg. of molasses concentrate as source of biotin

Yeast extract preparation μ g./ml.	Reading
0	96
0.7	80
2.8	37.5
5.6	29

Table 2 shows the response of the latter organism to varying amounts of biotin in presence of an excess of the yeast extract preparation, while Table 3 shows the converse, viz. the growth response to varying amounts of the yeast extract preparation in presence of an excess of biotin, supplied in this instance as a concentrate obtained from cane molasses by extraction with cold ethyl acetate, evaporation of the extract to small volume and subsequent aqueous extraction of the filtered extract. The biotin preparation, which was virtually free from the BY factor, was the brown gum left on evaporation of this aqueous extract and is subsequently referred to as "molasses concentrate". By assay against *Cl. butylicum* it was estimated to contain 0.035 $\mu\text{g.}$ of biotin per mg. In order to conserve the supply of pure biotin, the above molasses concentrate was used as a source of biotin wherever possible.

Evidence that biotin and the BY factor are the only growth factors required

The following experiments showed that the yeast extract preparation did indeed contain all the other necessary factors and that there was no carry-over of growth factors from the inoculum. A standard inoculum was made in the following medium: 2 % glucose, 0.1 % asparagine, 0.1 % $(\text{NH}_4)_2\text{SO}_4$, Speakman's nutrient salts, synthetic *dl*-valine (9 $\mu\text{g./ml.}$), yeast extract preparation (5 $\mu\text{g./ml.}$), pure biotin (0.00023 $\mu\text{g./ml.}$). After 42 hr. the culture showed a good turbidity; it was centrifuged, the supernatant was decanted off completely and the hard deposit of cells was resuspended in an equal volume of sterile distilled water. 0.4 ml. of this suspension was then used as an inoculum for a second tube of medium (35 ml.) of identical composition. This culture showed good growth after 2 days. After another such transfer two more were made into a similar medium but without valine. A sixth transfer was finally made into three separate tubes of the same basal medium without valine:

- (a) with biotin but with no yeast extract preparation,
- (b) without biotin but with yeast extract preparation,
- (c) with both biotin and yeast extract preparation.

Tube (c) showed good growth after 36 hr. and gave a reading of 21 after 3 days, whereas the corresponding final readings with (a) and (b) were 100 and 83.5 respectively. There can be little doubt therefore that the yeast extract preparation contains all the factors other than biotin which are necessary for growth. Three other strains of *Cl. acetobutylicum*, viz. American Type Culture Strains 862 (Kluyver's *Cl. acetonigenus*) and 824 (Rettger's strain), and Weizmann's original strain, responded exactly as did the strain 5. In every case there was no growth on the medium containing only biotin, minimal growth on the medium containing only yeast extract preparation and good growth and gas production in 3 days on the medium containing both growth factors.

Purification and properties of the BY factor

No evidence has yet been secured that the active biotin-free preparations obtained from yeast extract contain more than one growth factor for *Cl. acetobutylicum*. A considerable concentration of this factor has been achieved by following the scheme outlined below, which, in essence, consists of a preliminary concentration of active material by alcohol extraction of Difco yeast extract, followed by a separation from amino-acids by ether extraction at pH 1 and then a separation from the stronger organic acids by ether extraction at pH 7. Our unit for BY factor activity is that amount of material, which, dis-

solved in 1 ml. of the glucose-asparagine- $(\text{NH}_4)_2\text{SO}_4$ basal medium containing also 29 $\mu\text{g.}$ of molasses concentrate, will give a reading of 60 after 3 days' anaerobic incubation at 37° . This somewhat arbitrarily chosen unit is really more satisfactory than that corresponding to half-maximum growth, owing to the difficulty often experienced in making an even suspension from the ropy bacterial deposit in a culture showing maximum growth.

No claim is made that the values given for the activities of the various fractions are more than rough approximations. There is evidence that the amino-acids present in the cruder fractions, although not growth factors or growth inhibitors in the strict sense, nevertheless have a considerable effect on the growth of the organism. The extent of the purification achieved may be summarized thus: Difco yeast extract has an activity of 1 unit in 20 $\mu\text{g.}$, is rich in amino-acids and also contains biotin; the "ether-soluble pH 7 extract" finally obtained has an activity of 1 unit in 0.05 $\mu\text{g.}$, is free from amino-acids and also essentially free from biotin. The purification procedure is given in more detail in Fig. 1.

Fig. 1.

A fair proportion of relatively inactive material can be removed from this preparation by extraction with an excess of boiling light petroleum, but a more promising mode of purification is to remove coloured impurities by precipitation with phosphotungstic acid and to extract the colourless material recovered from the phosphotungstic acid filtrate with boiling alcohol. Before the alcohol treatment the product contained 1 unit in 0.03 $\mu\text{g.}$ Further work is in progress on the purification of the factor. A brief survey was made of various sources of the factor. Difco yeast extract was the best source, while bakers' yeast, brewers' yeast, alcoholic extracts of corn and bran, distillers' solubles and liver were also quite active. Casein, dried whey, skim milk powder, meat scrap meal and molasses contained little activity.

The factor is adsorbed on norite at pH 4 and may be eluted by acetone-ammonia, or alcohol-ammonia. It is not eluted by 50% alcohol. It is not adsorbed on permutit, Lloyd's reagent, or "filtercel". The factor is stable to

2N NaOH or 2N H₂SO₄ for 10 min. at 100° and to 30 min. autoclaving at neutrality at 15 lb. pressure. Bromination in chloroform at 25° inactivates it; biotin is not destroyed by this treatment. The factor is destroyed by cold alkaline KMnO₄ and by HNO₂. At least 90 % of it is destroyed by 10 min. boiling with excess H₂O₂. It may be noted here that we have been unable to obtain active extracts from hydrolysed casein with unpurified ether, but extracts made with peroxide-free solvent have shown definite, though weak, activity. The BY factor, in contrast to biotin, is completely extracted from yeast by autoclaving with 50 volumes of water. Autolysis gives no increase. The factor is apparently not precipitated by neutral lead acetate, but it has been difficult to recover more than 50 % of the original activity in the filtrate.

*Suitability of various simple nitrogenous nutrients in presence
of both growth factors*

In the experiments described below, the source of biotin was "pure biotin", and the source of the BY factor was the material obtained by a boiling chloroform extraction of the ether-soluble pH 7 extract which had previously been extracted with boiling light petroleum and with boiling benzene. It contained 1 unit in 0.05 µg. and was free from biotin.

The basal medium for *Cl. acetobutylicum* previously employed contained both asparagine and (NH₄)₂SO₄ as sources of nitrogen. The latter was added because in its presence the maximum growth attained with both growth factors in excess seemed to be a little heavier than in the basal medium containing asparagine as sole source of nitrogen. In neither case however did growth begin until the end of the 2nd day, often not until the 3rd day. A basal medium containing (NH₄)₂SO₄ as sole source of nitrogen was found to be even less suitable, but a remarkable improvement was effected by using ammonium phosphate (0.1 %) as the sole source of nitrogen, the initial pH of the medium being 6.3–6.5. In this medium growth invariably began after 28–30 hr., sometimes sooner, and the maximum growth attained, in the presence of excess of the growth factors, was practically the same as in the asparagine–(NH₄)₂SO₄ basal medium. Table 4 gives the results of one such comparison made between the two basal media. All the tubes were incubated in the same anaerobic jar and each tube contained 1.1 µg. of the BY factor per ml. The ammonium phosphate medium contained (NH₄)₂HPO₄ (0.1 %) adjusted to pH 6.5 by addition of the requisite amount of H₃PO₄.

Table 4. *Comparison of nitrogen sources*

Biotin added µg./ml.	NH ₄ phosphate basal			Asparagine–(NH ₄) ₂ SO ₄ basal		
	40 hr.	64 hr.	Reading after 5 days	40 hr.	64 hr.	Reading after 5 days
0	–	–	90	–	–	93
0.00006	±	+	80	–	±	73
0.00012	±	+	78	–	±	65
0.0007	++	+++	61	–	±	39
0.0014	++	+++	30	–	++	38

Although the final response to amounts of biotin insufficient for maximum growth is poorer on the ammonium phosphate medium, it is equally good when excess of biotin is present, and in all instances growth begins about a day earlier on this medium. It is a singular fact that an asparagine–ammonium phosphate

medium shows but little improvement over the customary asparagine-ammonium sulphate basal medium in this respect. It is therefore justifiable to conclude that although *Cl. acetobutylicum* is undoubtedly a proteolytic organism it can make, and perhaps prefers to make, all the amino-acids it requires from the glucose and the ammonium ion, in this respect resembling other proteolytic organisms which are not strict anaerobes, e.g. *Ps. fluorescens* and *Pr. vulgaris*. It is also noteworthy that filtrates from these last-named organisms, grown in a purely synthetic glucose-NH₄Cl medium, undoubtedly contained relatively large amounts of both biotin and the BY factor. On the other hand, *Cl. acetobutylicum*, when grown in a medium containing an excess of both growth factors, seems to remove them from solution almost entirely.

It may be mentioned that when biotin is supplied as molasses concentrate, the superiority of the ammonium phosphate basal medium is much less marked, since growth begins much earlier in the asparagine-(NH₄)₂SO₄ tubes than when biotin is supplied in the form of pure biotin.

Effect of various sulphur-containing and iron-containing compounds on the growth of the organism in simple media

The ensuing remarks refer only to growth in the asparagine-(NH₄)₂SO₄ basal medium containing excess of pure biotin and the BY factor as on page 1590. It has been found that the addition of small amounts of certain reducing agents, or the oxidized form of these substances, enables growth to begin much sooner than normally. The following is a list of the active substances so far encountered and the concentrations at which they have been found to be active:

Cysteine	1-3.5 mg. per tube (but not 10 mg. per tube)
Cystine	Ditto
Na ₂ S, 9H ₂ O	1-4 mg. per tube (but not 6 mg. per tube)
Na hydrosulphite	1 mg. per tube
K ferricyanide	2 mg. per tube

The following have no such effect in speeding up growth: Na₂SO₃, Na thiol-acetate, catechol quinol. NaCN and K ferrocyanide inhibit growth altogether. Of the active substances, cysteine, cystine and Na hydrosulphite seem to be the most effective. The extent to which these compounds shorten the lag phase is evident from Table 5.

Table 5. *Effect of S- and Fe-containing substances on growth*

Addition μg./ml.	Growth after				Final reading after 3.5 days
	37 hr.	42 hr.	47 hr.	61 hr.	
None	-	-	-	±	26
Cysteine HCl: 29	+	++	+++	+++	22.5
Cysteine HCl: 58	+	++	+++	+++	24.5
Cysteine HCl: 101	++	+++	+++	+++	22
Cystine: 29	+	++	+++	+++	30
Na ₂ S, 9H ₂ O: 29	±	.	++	.	41.5
Na ₂ S, 9H ₂ O: 58	+	.	+++	.	39
Na ₂ S, 9H ₂ O: 87	+	++	+++	+++	43
Na ₂ S, 9H ₂ O: 116	±	±	++	+++	22
Na hydrosulphite: 29	+	.	+++	.	28
K ₃ Fe(CN) ₆ : 58	-	±	++	+++	23

The addition of cysteine hydrochloride (29 μg./ml.) and of Na₂S, 9H₂O (87 μg./ml.) also hastened growth by a few hours in the ammonium phosphate

basal medium. In fact, tubes containing these additions behaved exactly as a tube containing molasses concentrate (43 $\mu\text{g./ml.}$) as source of biotin, but no added cysteine or Na_2S . The molasses concentrate gave a negative ninhydrin reaction, but may of course contain sulphur compounds other than amino-acids.

Methionine has a similar, but much weaker, effect than cysteine and cystine. Since it cannot act as a redox agent, it is considered with other amino-acids in the next paragraph.

Effect of amino-acids on the growth of the organism in simple media

It has to be admitted that, even in the ammonium phosphate medium, growth does not begin as soon as when a small amount of ether-extracted hydrolysed casein (equivalent to 500 $\mu\text{g.}$ of casein per ml.) is added in addition to pure biotin and the BY factor concentrate.

A few preliminary observations on the effect of single amino-acids and artificial mixtures of amino-acids on the growth of *Cl. acetobutylicum* in the asparagine- $(\text{NH}_4)_2\text{SO}_4$ basal medium are here recorded. It has become abundantly evident that the problem is one of great complexity, not only because certain amino-acids seem to be helpful and others antagonistic to growth, but because their effects are markedly different according to whether the growth factors are present in excess or in amounts barely sufficient to support good growth. There is further a surprising lack of parallelism between growth and the utilization of glucose when mixtures of amino-acids are present.

In the first place, an artificial mixture of 19 amino-acids simulating hydrolysed casein [Hutchings & Woolley, 1939] did have much the same effect as ether-extracted hydrolysed casein. When 1 mg. of this mixture was added per tube, growth was evident after only 25 hr. incubation. The mixture contained 8 amino-acids of which optically inactive synthetic specimens were available, and the effects of these acids, viz. glycine, *dl*-alanine, *dl*-valine, *dl*-leucine, *dl*-serine, *dl*-phenylalanine, *dl*-threonine and *dl*-methionine, were next studied. Of these, valine and methionine (particularly when added together) were definitely helpful, and leucine definitely inhibitory to growth. Table 6 brings out these findings very clearly.

Table 6. *Effect of amino-acids on the growth of Cl. acetobutylicum*

Basal medium: 2% glucose-asparagine- $(\text{NH}_4)_2\text{SO}_4$ -Speakman's salts.

Pure biotin = 0.00023 $\mu\text{g./ml.}$

BY factor = 5 $\mu\text{g.}$ yeast extract preparation per ml.

Addition $\mu\text{g./ml.}$	Growth after			Reading after 4 days
	37 hr.	41 hr.	59 hr.	
None	-	-	++	31
<i>dl</i> -Valine: 9.2	$\pm$	+	+++	26
<i>dl</i> -Methionine: 4.6	$\pm$	+	+++	27
Valine and methionine (as above)	++	+++	+++	29
<i>dl</i> -Leucine: 5.7	-	-	$\pm$	33
8 amino-acids: total concentration = 29	++	+++	+++	30

It should be pointed out, however, that the residual glucose after 4 days' incubation was highest in the valine plus methionine tube (0.60%) and lowest in the valine tube (0.08%). Growth and fermentation are two quite distinct activities of the organism, as is shown by the results of the concluding sections.

Fermentation of glucose in the ammonium phosphate basal medium containing the growth factors

Although detailed analyses for individual fermentation products have not been made, the yield of solvents, when biotin is supplied as pure biotin, and the BY factor as a highly active concentrate, approximates to, but does not quite equal, the normal. If biotin is supplied as molasses concentrate the titratable acidity at the end of the fermentation is invariably lower than when pure biotin, in amounts equal to or greater than those present in the molasses concentrate, is supplied in its stead (see Table 7). The yield of acetone is usually 4-6% of the sugar fermented, and, given sufficient biotin, the organism will completely or almost completely ferment 2% glucose in the medium. It has to be remembered that the BY factor is supplied in an impure form, and the possibility certainly exists that a third "accessory" factor may be required for normal fermentation as distinct from normal growth. Further work on this aspect of the problem is in progress. The ash obtained on ignition of the molasses concentrate has no activity as an accessory factor.

Table 7. *Fermentation of glucose on a virtually synthetic medium*

BY factor supplied as 1.1 µg. of concentrate per ml.

No. of exp. and period of incubation days	Biotin, source and amount µg./ml.	Initial conc. glucose %	Final conc. glucose %	Titrateable acidity, ml. 0.1 N NaOH per 10 ml. of medium
I. 3-5	Pure biotin: 0.003	2.0	Nil	6.4
	Molasses concentrate: 29	2.0	Nil	3.1
II. 2-5	Pure biotin: 0.006	2.0	0.5	3.7
	Molasses concentrate: 29	2.0	0.5	2.7
III. 3-5	Pure biotin: 0.006	2.0	0.1	3.0
	Molasses concentrate: 29	2.0	0.1	2.4
IV. 5-5	Pure biotin: 0.006	3.0	0.8	4.0
	Molasses concentrate: 29	3.0	0.4	2.5

Sugars fermented by Cl. acetobutylicum on a virtually synthetic medium containing ammonium phosphate as the only source of nitrogen

In the presence of pure biotin and a highly active concentrate of the BY factor, the following were fermented: glucose, galactose, mannose, fructose, xylose, *l*-arabinose, sucrose, lactose, cellobiose, maltose, mannitol, starch, salicin. The lag period, the yield of acetone and the final acidity showed considerable variations from sugar to sugar. No growth took place on the following: glycerol, dulcitol, sorbitol, sorbose, *d*-arabinose, rhamnose, trehalose, heptose, inulin, lactic acid and pyruvic acid. Further work along these lines is in progress.

SUMMARY

Two growth factors are required by *Cl. acetobutylicum*, viz. biotin and another factor, present in yeast extract, which is not identical with any known growth factor for micro-organisms, and which has been obtained in a highly concentrated form. In presence of these two factors the organism will grow normally and also produce solvents from glucose with ammonium phosphate as

the sole source of nitrogen. There is no strict correlation between growth and fermentation, and the presence of other factors may be necessary in order that normal solvent production may take place concurrently with normal growth. Certain other sugars are also fermented in the simple ammonium phosphate medium containing the two growth factors.

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