CLXVIII. THE SPECIFIC NUTRITIVE REQUIREMENTS OF *CLOSTRIDIUM ACETOBUTYLICUM* (WEIZMANN). II

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IN our first communication [Weizmann & Rosenfeld, 1937] we have shown that *Cl. acetobutylicum* (W.) requires for a normal fermentation of sucrose in a synthetic medium the presence of asparagine and of an unknown compound which has been termed "activator". Thus in accordance with Knight's [1936] classification of micro-organisms by their specific nutritive requirements, *Cl. acetobutylicum* (W.) has a very restricted synthetic power, its nutritional level being comparable with that of numerous pathogenic bacteria.

The unknown part of the nutritive requirements of *Cl. acetobutylicum* hitherto referred to as "activator", has been the subject of further investigation with the ultimate aim of elucidating the chemical constituent or constituents which are responsible for its biological activity. Prior to a further purification and isolation of the "activator" in a chemically pure state it was thought advisable to ascertain whether those chemically defined substances whose growth-promoting properties have already been described in the literature, might also prove to be active in the case of *Cl. acetobutylicum* (W.). In this way it was also hoped to obtain some information about the number of factors involved with the "activator".

I. *The action of compounds of known chemical constitution*

In connexion with the oxido-reduction potential of the medium as a limiting factor for the development of micro-organisms [Kanel, 1937] we investigated the combined influence of cysteine and ascorbic acid. Although we observed a conspicuously beneficial influence on the development of the bacteria in the presence of ascorbic acid, no essential increase of fermentation intensity could be detected. Likewise a negative influence on the fermentation intensity has been found in the case of uracil, nicotinic acid and all possible combinations of this latter substance with aneurin, β -alanine and asparagine.

II. *The action of biotin*

The possibility that a biotin-like substance might be required for the growth and metabolism of *Cl. acetobutylicum* (W.) has already been discussed in our first paper. We are now able to present experimental evidence that biotin, as described by Kögl and co-workers, is one of the essential extraneous factors required by *Cl. acetobutylicum* (W.) for a normal fermentation of carbohydrates to butanol and acetone. The difficulty of preparing chemically pure biotin did not permit a direct test, yet the consistent results obtained by three independent procedures justified the assumption that the experimental responses observed can be ascribed to biotin.

By following the procedure given by Kögl & Toennis [1936] for the extraction and purification of biotin from egg yolk and testing the action of the individual

fractions on the fermentation intensity with *Cl. acetobutylicum* (W.) a clear-cut parallelism was observed between the growth-promoting properties as in the case of yeast found by Kögl, and the stimulation of the butanol-acetone fermentation.

An activator extract from maize which had been subjected to practically the same purification procedure as the above egg yolk extract, showed that the active principle in maize has the same properties as the one from egg yolk, particularly the quantitative adsorbability on charcoal and the eluability therefrom with an ammonia-acetone mixture. For the third independent proof we made use of the different biotin content of seeds which had been quantitatively measured by Kögl & Haagen-Smit [1936]. Parsley and cress seeds being relatively rich in biotin (4900 and 5150 saccharomyces units per g.) have been compared with maize, the latter being relatively poor in biotin. We have found that the ratio of the numbers of activator units of parsley and maize is the same in the case of a normal fermentation with *Cl. acetobutylicum* as that found by Kögl in the case of yeast.

III. The action of the "third factor".

In the experiments where purified egg yolk extract or maize extract were tested for their biotin content and activating power, it was observed that the fermentation velocity failed to increase after the steep initial rise in contradistinction to the corresponding curves obtained with maize or yeast autolysate (see Fig. 1). A similar observation was made both with yeast autolysate treated with acetone and separated from the insoluble part, and with a cress extract rich in biotin. This deviation from the normal behaviour, as in a maize mash, is understood if one assumes that biotin + asparagine account quantitatively only for the initial steep rise of the fermentation velocity curve. The subsequent gradual increase of the curve is caused by a "third factor" which is present in maize or in the dialysate of yeast autolysate, and absent from chemically pure biotin or from purified yeast autolysate.

Fig. 1. $t = 36.5^\circ$. I (x), dialysate of yeast autolysate; II (□), maize mash; III (o), yeast autolysate, acetone-treated (part soluble in 50% acetone).

An experimental proof of the necessity of a "third factor" in order to obtain a normal fermentation velocity curve as illustrated in Fig. 1, curve I or II,

was given by comparing the fermentation velocity of a system consisting of a solution rich in biotin but poor in the "third factor" with that of a solution devoid of biotin but rich in the "third factor", e.g. a mixture of parsley extract and yeast autolysate (dialysate) which on storage (2 years) had lost its biotin activity. A considerable increase of the fermentation velocity was observed, indicating the existence of the "third factor". This factor is presumably of low molecular weight. It is more stable on storage than biotin and—according to the gradual increase of the fermentation velocity curve which it causes—has a lower biological activity per g. than biotin. For the time being no further assumption is made regarding its chemical nature.

In summarizing the present state of research about the specific nutritive requirements of *Cl. acetobutylicum* (W.) we come to the following conclusion.

If the level of the specific nutritive requirements in maize is taken as 100, then the corresponding level in the presence of asparagine (first factor) amounts roughly to 25 %. Owing to the difference in the biotin content of the inoculant used this value shows fluctuations between 4 and 8 ml. gas per hr., the asparagine factor remaining constant.

The second factor, biotin, together with the first factor, asparagine, brings the specific nutritive level up to 70 % as compared with maize.

The remaining 30 % are due to the "third factor".

EXPERIMENTAL

The general experimental procedure used in this work has already been described in detail in our first paper [1937]. The fermentation intensity as a quantitative measure for the amount of the activating factors has likewise been obtained by graphical evaluation of the fermentation curve and expressed as ml. of gas formed per hr. The fermentation intensity of a 2.5 % maize mash served as a standard. In accordance with our earlier experiments the activator content of maize was found to be 1.30 activator units (A.U.) per g. The curve obtained by plotting activator units against fermentation intensity (ml./hr.) from a series of fermentations with varying amounts of maize mash in the presence of 30 mg. asparagine (Fig. 1, curve I) enables the activator content of other solutions or sources to be read, provided that the values for the fermentation intensity are plotted on the same scale.

I. The action of compounds of known chemical constitution

(a) *The influence of ascorbic acid with and without additional cysteine and asparagine.* In a series of 50 ml. round flasks containing 0.60 g. sucrose, 1.0 ml. of 0.80 *M* phosphate buffer pH 6.8 and 0.15 g. filter pulp, the additions quoted in Table I were made. The sterilized flasks were inoculated with 1 ml. of a culture of *Cl. acetobutylicum* (W.) in 5 % maize mash, and incubated.

Table I

t = 33°, total volume 38 ml. tap water.

mg. asparagine	30	30	30	30	None	30	None
mg. ascorbic acid	None	2	4	10	10	2	2
mg. cysteine hydrochloride	None	None	None	None	None	15.8	15.8
Fermentation velocity ml./hr.	3.0; 3.6	4.6	4.6	4.4	0.8	4.5	0.6

The experiments with ascorbic acid show a slight increase of the fermentation velocity above the value observed with asparagine alone. The result of the

experiment without asparagine in the presence of cysteine and ascorbic acid (last column) shows that asparagine cannot be replaced by cysteine.

(b) *The influence of uracil.* Uracil has been found to be an essential nutritive factor for the anaerobic growth of *Staphylococcus aureus* [Richardson, 1936]. The fermentation velocity observed in the presence of asparagine and 20 mg. uracil was 2.7 ml./hr. compared with 3.0 ml. for asparagine alone. The experimental conditions were the same as in (a).

(c) *Nicotinic acid, aneurin and β -alanine.* The combinations of nicotinamide or nicotinic acid with aneurin have been proved to be important growth factors for *Staph. aureus* [Knight, 1937]. It was of interest to test these compounds with *Cl. acetobutylicum* (W.). Although negative results with β -alanine have already been reported in our previous communication, the influence of this substance in combination with the others was retested. The amounts per standard experiment in presence of 30 mg. asparagine were:

1.2 mg. nicotinic acid;
2.0 mg. β -alanine;
0.12 mg. aneurin.

All possible combinations have been tried in duplicate. Compared with the fermentation velocity in the presence of asparagine the actions of all compounds and their combinations were invariably negative.

II. The action of biotin

The most convenient starting material for the preparation of chemically pure biotin proved to be egg yolk [Kögl & Toennis, 1936]. If biotin were required for the propagation of *Cl. acetobutylicum* (W.) both crude egg yolk and the extracts prepared therefrom should produce an increase of the fermentation velocity in our standard fermentation test. 1.378 kg. egg yolk (51 % dry matter) prepared by boiling 100 eggs for 15 min. and then separating the egg white, served as stock for the subsequent experiments.

A. *Activating effect of egg yolk.* 6.4 g. of moist egg yolk were suspended in tap water and the suspension made up to 100 ml. Varying amounts of this suspension were added to 50 ml. round flasks containing 0.60 g. sucrose, 30 mg. asparagine, 1.0 ml. 0.08 M phosphate pH 6.8 and 0.15 g. filter pulp (standard conditions). The volume was made up with tap water to 38 ml. and the sterilized flasks inoculated as usual. Table II gives the fermentation velocities for the corresponding amounts of egg yolk.

Table II

$t=34^{\circ}$.

6.4 % egg yolk (mg. suspension moist egg yolk)	None	19.2	38.4	76.8	160	320	640	770 mg. maize as 5 % mash
Fermentation velocity, ml./hr.	5.1	15.6	15.4	15.9	15.3	15.5	15.3	16.0

Although it was to be expected from the figures given by Kögl & Toennis [1936] and Kögl & Haagen-Smit [1936] that egg yolk (3700 s.e./g.) contains about six times as much biotin as maize (600 s.e./g.) which under our experimental conditions would correspond to 130 mg. moist egg yolk, maximum speed was already observed with 19.2 mg. i.e. 1/7 of the calculated amount. It may be that egg yolk contains appreciably more biotin than can be extracted by boiling

water, and that this can be made available from insoluble precursors of biotin by the bacterial enzymes. Further experimental evidence is required for the elucidation of this discrepancy.

1. *Activating effect of egg yolk extract.* 1.35 kg. of the same moist egg yolk tested in (A) were boiled for 1 hr. with approximately 2 l. distilled water and filtered hot. The residue was again heated with 1 l. distilled water and filtered. The joint turbid filtrates (1870 ml.) were treated with acetone (935 ml.), filtered, and the filtrate evaporated *in vacuo* to dryness. The residue was dissolved in 410 ml. water and treated with 1720 ml. 96 % alcohol. After cooling for 1 hr. the solution was filtered and again evaporated *in vacuo*.

The residue was dissolved in 200 ml. water and tested with *Cl. acetobutylicum* (W.) under standard conditions, 1 ml. of the ten-times diluted test solution (=1) corresponded to 0.68 g. egg yolk. The influence of added inositol was also tested. Table III shows the results.

Table III

ml. acetone- and alcohol- treated egg-yolk ex- tract	0.3	0.3	1.0	1.0	3.0	3.0	10.0	10.0
Corresponding amount of moist egg yolk in mg.	202	202	675	675	2020	2020	6750	6750
mg. inositol	None	11	None	11	None	11	None	11
Fermentation velocity, ml./hr.	9.4	10.0	8.6	11.7	8.9	8.6	10.6	11.1

The maximum speed is already attained with 0.3 ml. of the test solution (0.6 mg. dry matter). Inositol exhibits a small but conspicuous influence, yet it is not able to bring the maximum speed to that of maize mash.

2. *Lead acetate treatment of solution (1).* 193 ml. of solution (1) were treated with 20 ml. saturated lead acetate solution, centrifuged and the clear yellow liquid, which gave no further precipitate upon addition of more lead acetate, treated with H_2S for the removal of Pb. The filtered solution including the washings was evaporated *in vacuo* to dryness. A 0.17 % solution (2) in water was prepared from the dry residue and tested under standard conditions with and without additional inositol. The results resemble, in principle, those given in Table III with the exception that this time inositol was without any influence: average fermentation velocity without inositol, 9.7 ml./hr.; in the presence of inositol 9.8 ml./hr.

3. *Phosphotungstic acid treatment.* To 234 g. of solution (2), 12.5 g. conc. sulphuric acid were added and the mixture was precipitated with 100 ml. 50 % phosphotungstic acid solution in water. After filtration the precipitate was washed with 100 ml. distilled water which had been acidified with 7 ml. 10 % HCl. After decomposition of the precipitate with 2.5 l. 2 % $Ba(OH)_2$, and removal of the excess Ba^{++} with H_2SO_4 the solution was concentrated *in vacuo* to 197 ml. (solution (3P)). The filtrate, including the washings of the phosphotungstic acid precipitate, was freed from excess of phosphotungstic acid with 150 g. $Ba(OH)_2 \cdot 8H_2O$. Neutralization, removal of Ba^{++} , filtration and concentration gave solution 3S (485 ml.). The activating effects of both solutions 3P and 3S were tested under standard conditions, the results being given in Table IV.

Both the precipitate (3P) and the solution (3S) show activity, yet comparison of the results given in columns 1 and 6 indicates that the precipitate contains at least four times as much of the activating factor as the solution. It

Table IV

 $t=35^{\circ}$. 1 ml. 3P = 2.5 ml. 3S.

No. of exp.	1	2	3	4	5	6	7	8	9
ml. solution 3 P	0.2	0.5	1.0	1.0	—	—	—	0.5	1.0
ml. solution 3 S	—	—	—	—	1.0	2.0	20.0	1.3	2.5
Fermentation velocity, ml./hr.	13.7	15.3	15.5	1.0	9.5	13.4	11.9	10.7	12.4
				No					
				asparagine					

seems quite possible that the solution 3S owes its activity to the washing with acidified water in which the active compound is partially soluble.

4. *Adsorption on charcoal and elution.* 150 ml. of solution (3P) were shaken for 1 hr. with 15 g. charcoal (Merck, med.) filtered and washed with 200 ml. distilled water; filtrate and washings were evaporated *in vacuo* giving solution 4a, 85 ml. The charcoal was again shaken with 300 ml. 50 % alcohol for 2 hr. The yellow alcoholic filtrate was evaporated to dryness *in vacuo* and dissolved in water, giving solution 4b, 89 ml.

For eluation the charcoal was shaken three times for 2 hr. with 500 ml. of a mixture composed of 220 ml. 17 % NH_3 + 900 g. acetone made up to 1.5 l. with distilled water (60 % acetone, 2.5 % NH_3). The joint eluates were evaporated *in vacuo* to dryness and redissolved in 167.5 ml. water. The solution 4c showed an orange colour. Solutions 4a, 4b and 4c were tested for their activity under standard conditions. Solution 4c (eluate) was diluted ten times for the test. The results are given in Tables V and VI.

Table V

 $t=34^{\circ}$.

ml. solution 4c eluate 1 : 10	0.0	0.2	0.5	1.0	2.0	5.0	10.0	20.0
Fermentation velocity, ml./hr.	4.4	6.7	6.2	5.9	7.4	10.4	10.3	10.4

Table VI

ml. 4a	1.0	1.0	—	—	1.0	1.0
ml. 4b	—	—	1.0	1.0	1.0	1.0
ml. 4c eluate, orig.	—	0.5	—	0.5	—	0.5
Fermentation velocity, ml./hr.	4.4	7.6	9.1	10.4	9.7	10.4

The results in Tables V and VI obtained with *Cl. acetobutylicum* (W.) confirm the results obtained by Kögl & Toennis [1936] with yeast, viz.:

1. The adsorbed biotin is not eluted by water (Table VI, col. 1).
2. 50 % alcohol removes some of the adsorbed biotin from charcoal (Table VI, col. 3).
3. Biotin is eluted by a mixture containing 60 % acetone and 2.5 % NH_3 .

The solutions obtained from each step of purification show equal responses both when tested for their growth-promoting properties towards yeast and their stimulating influence upon the velocity of a butanol-acetone fermentation of sucrose in a strict synthetic medium. Both effects may therefore be ascribed to the same chemical substance, viz. biotin.

B. *Biotin in maize extract.* If biotin from egg yolk is identical with the activating principle in the standard medium for *Cl. acetobutylicum* (W.), viz. in maize mash it should be possible to obtain analogous results from maize extract using the same purifying procedure as for egg yolk extract.

The maize extract was prepared by stirring 600 g. ground maize with 3 l. $N/10$ HCl for 4 hr. at room temperature (24°) in the presence of toluene, which procedure, as we knew from previous experiments [Weizmann & Rosenfeld, 1937; Weizmann & Davies, 1937] brings all the activator into solution. The treatment at low temperature has the advantage that the starch can be readily separated afterwards by centrifuging or filtering. The milky filtrate including the washings was exactly neutralized to pH 7.0 with $2N$ NaOH whereupon the colloidal suspension flocculated out. The solution was again filtered and the filtrate concentrated *in vacuo* to 87.1 g. Analysis showed the N distribution to be as follows:

600 g. maize (1.70 % N) with 10.20 g. N gave $\left\{ \begin{array}{l} 87.1 \text{ g. conc. extract} = 1.15 \text{ g. N} \\ 10.32 \text{ g. precipitate} \\ \text{upon neutralization} = 0.30 \text{ g. N.} \end{array} \right.$

14.2 % of the total N was dissolved during the extraction, 11.2 % is contained in the active concentrate.

For the activator test the concentrated extract was diluted ten times. 1.45 ml. of the diluted extract corresponded to 1.0 g. maize. One series was carried out under standard conditions, in a parallel series the addition of asparagine was omitted. Fig. 2 shows the fermentation velocity observed for a given amount of extract.

Fig. 2. Maize extract. $t=35.5^\circ$. o, in the presence of asparagine; x, without asparagine.

The curve without asparagine shows a very low activity indicating the insufficient asparagine content of the test solution. 8 ml. extract (70 mg. protein) are necessary for some asparagine-like action.

Lead acetate treatment of maize extract. To 50 g. extract from the preceding experiment 50 ml. of saturated lead acetate solution were added. After vigorous shaking the precipitate was filtered by suction and the clear yellow filtrate delead with H_2S . The lead-free solution was concentrated *in vacuo* and made up to 48 g. (solution B_2). 5 g. of solution B_2 contained 10.5 mg. N, i.e. 21.6 % of the corresponding amount before lead acetate treatment (48.7 mg. N).

For the test the concentrate was diluted ten times. 1 ml. of the dilute solution corresponded to 0.51 g. maize. Besides the standard test, a parallel series without asparagine was carried out. Table VII shows the results.

Table VII

$t=35^\circ$.

ml. B_2 (1 : 10)	0.1	0.2	0.5	1.0	2.0	4.0
Fermentation velocity in ml./hr. with asparagine	5.2	9.2	10.9	12.9	17.0	18.5
Without asparagine	0.9	0.6	0.6	0.6	1.2	0.9

As in the case of egg yolk extract there is no removal of the activating factor by the treatment with lead acetate. The inactivity of the "activator" solution in the absence of asparagine demonstrates clearly the dependence of the biotin activity on the presence of asparagine.

Adsorption on charcoal of biotin-containing maize extract. 5 g. of solution B₂ (preceding exp.) were shaken with 1 g. charcoal. After centrifuging and washing the charcoal three times on the centrifuge (the last two washings contained colloidal charcoal) the residual (colourless) liquid including the washings was evaporated *in vacuo*, made up to 50 ml., filtered from traces of charcoal and kept for test.

Elution. The charcoal adsorbate was stirred three times with 20 ml. of an acetone-NH₃-water mixture as described in biotin-charcoal adsorbate from egg yolk. A clear yellow eluate was obtained. The combined eluates were evaporated *in vacuo* for the removal of NH₃ and acetone and made up to 50 ml. The test was carried out as usual. The results are given in Table VIII. 1 ml. eluate corresponds to 0.51 g. maize.

Table VIII

t = 35.0°.

ml. eluate	0.0	0.5	1.0	2.0	5.0	10.0	15.0	0.0	5.0
ml. washing	0.0	0.0	0.0	0.0	0.0	0.0	0.0	15.0	5.0
Fermentation velocity, ml./hr.	4.2	8.9	9.6	12.8	11.4	13.1	13.0	4.2	11.3

The eluate is active but the maximum speed obtained is only 70 % of that observed in the preceding purification steps, where the maximum speed was equal to that of a maize mash. Apparently a factor required for a normal fermentation velocity has been eliminated in the last purification (third factor). In case this factor has not been adsorbed by charcoal it should be possible to reconstruct the activating system as it was before adsorption by combining the eluate with the washings. Yet the experimental test of such a combination showed a negative response, as may be seen from the last column in Table VIII. One is therefore led to assume that the eliminated factor has also been adsorbed like biotin on charcoal, but could not be eluted with acetone-NH₃ mixture. Further experimental evidence on the adsorption of the "third factor" on charcoal is given in the third section of the experimental part. No difference in the behaviour of biotin from egg yolk and from maize has been observed.

C. *The ratio of biotin contents in maize, parsley or cress seeds.* For the preparation of the seed extracts care was taken that the conditions for the extraction were identical in all three cases. The extraction was carried out at 40° with *N*/100 HCl. In every case during the extraction the ratio of HCl to seed was chosen so as to keep the acidity at pH 5. This procedure differs from that recommended by Kögl & Haagen-Smit [1936], viz. heating the ground seed with water for 30 min. at 100° and separating the biotin solution from the seeds by ultra-filtration. Firstly, owing to evaporation during ultra-filtration by suction it has seemed to us difficult to obtain a reliable aliquot part from a given amount of liquid; secondly, the treatment of maize at 100° causes swelling of the starch which cannot then be separated by simple centrifuging or filtration; finally, as was already pointed out, the recovery of biotin by our procedure is quantitative.

Maize. 200 g. ground maize were stirred with 1000 ml. *N*/100 HCl for 3½ hr. at 40°. The pH during the extraction was 4.7. An aliquot part of the centrifuged extract was neutralized to pH 7.0 with *N* NaOH. After filtration from the

flocculent precipitate an aliquot part of the filtrate was concentrated *in vacuo* to 323 g. which corresponded to 117.5 g. maize. (1 g. maize = 2.75 ml. extract.) The activity of the extract was tested in the customary manner. Table IX gives the corresponding results.

Table IX

	$t = 36.5^\circ$.			
ml. extract	0.0	0.2	0.4	0.8
Fermentation velocity, ml./hr.	5.1	13.0	15.7	18.3
Activator units	(50 mg. maize)	(123 mg. maize)	(200 mg. maize)	(350 mg. maize)
	0.065	0.16	0.26	0.40

Parsley. 87 g. milled parsley (light petroleum-extracted) were stirred with 1000 ml. *N*/100 HCl for $3\frac{1}{2}$ hr. at 40° , *pH* during extraction 5.2. An aliquot part of the centrifuged extract was neutralized with NaOH to *pH* 7.0. After filtration from the precipitate formed, an aliquot part of the filtrate was concentrated *in vacuo* to 240 g. which corresponded to 50.8 g. parsley (defatted). 1 g. parsley = 4.72 ml. extract.

Cress seeds. 52 g. milled and defatted garden cress seeds were stirred for $3\frac{1}{2}$ hr. at 40° with 780 ml. *N*/100 HCl. The *pH* during the extraction was 4.9. The separation of the extract from the insoluble residue was rendered difficult by the swelling of constituents of this type of seed. After neutralization, filtration and evaporation 134 g. concentrated extract were obtained which corresponded to 21.05 g. defatted seeds. For the activity test the solution was diluted ten times.

The results of the activity test together with those obtained from the parsley extract are given in Table X.

Table X

ml. parsley extract	0.1 21 mg. parsley	0.2 42 mg. parsley	None	None	None	None
ml. cress extract	None	None	None	0.2 12.7 mg. cress	0.4 25.4 mg. cress	0.8 51 mg. cress
Fermentation velocity, ml./hr.	14.6	20.4	5.7	12.3	13.7	12.8

Evaluation of the results obtained with maize, parsley and cress extracts

The speed values obtained from the fermentation experiments with our reference media, viz. maize extract (in the presence of asparagine) are plotted in

Fig. 3. A = 50 mg. maize; B = 12.7 mg. cress; C = 123 mg. maize; D = 21 mg. parsley; E = 25.4 mg. cress; F = 200 mg. maize; G = 350 mg. maize; H = 42 mg. parsley.

Fig. 3 as activator units (A.U.) against fermentation velocity (ml./hr.). Within the limit of experimental error the speed values observed with maize extract are the same as those obtained with the corresponding amount of maize mash under the same conditions. The activator content of the HCl extract deriving from 1 g. maize was therefore taken as 1.30 A.U. The speed values obtained from the fermentations with parsley and cress extracts were plotted on the curve obtained from maize extract. The corresponding activator contents were read on the abscissae.

The A.U. values thus obtained from Fig. 3 are:

Maize extract	Parsley extract	Cress extract
Corresponding to 123 mg. maize = 0.16 A.U.	42 mg. parsley = 0.60 A.U. 21 mg. parsley = 0.21 A.U.	12.7 mg. cress = 0.15 A.U. 25.4 mg. cress = 0.23 A.U.
i.e. 1.3 A.U. per g. maize	i.e. mean 12.2 A.U. per g. parsley (defatted)	i.e. mean 10.5 A.U. per g. cress seed (defatted)
	9.8 A.U. per g. parsley (orig.)	8.1 A.U. per g. cress seed (orig.)

For comparison of our results with the biotin contents of maize, parsley and cress as obtained by Kögl & Haagen-Smit [1936] we transform Kögl's saccharomyces units into the corresponding activator units. Accordingly maize contains 600 saccharomyces units (S.E.) or 1.3 activator units (A.U.) per g. parsley contains 4900 S.E. or 10.6 A.U. per g. and cress contains 5150 S.E. or 11.2 A.U. per g. Despite the difference in the test methods used the agreement of our values with those of Kögl is satisfactory.

III. *Experimental evidence for the existence of the "third factor"*

We tried to find a convenient laboratory method for the preparation of activator from yeast autolysate which avoids both the difficult separation of the active solution from the colloidal constituents of the yeast cell, or the tedious process of collection and evaporation of the dialysates of yeast autolysate. We overcame these difficulties by treating the yeast autolysate with an equal volume of acetone and separating the active solution from the insoluble parts by centrifuging. By evaporating the acetone solution *in vacuo* a dry orange coloured hygroscopic product was obtained. Table XI shows the results of the activity test with this product.

Table XI

mg. dry autolysate	0.0	1.45	2.9	5.8	11.5	23.0	46.0
Fermentation velocity, ml./hr.	5.2	7.6	12.1	11.5	12.7	12.9	13.9
A.U.	0.050	0.065	0.13	—	—	1.04	2.07
Without added asparagine							
mg. dry autolysate	92.5	92.5	108	145	250	500	
Fermentation velocity, ml./hr.	17.9	20.1	18.4	22.0	26.0	27.6	
A.U.	4	4	—	—	10	20	

In the presence of 92.5 mg. dry autolysate the addition of asparagine has no stimulating effect upon the fermentation velocity, the optimum amount is already contained in the amount of autolysate used. Therefore an addition of asparagine at higher amounts of autolysate has been omitted.

The fermentation velocities have been plotted as curve III in Fig. 1. While the corresponding test with the dialysate of yeast autolysate (prepared according to the procedure given in our previous paper), curve II, Fig. 1 gives a fermentation velocity curve practically identical with the one obtained with maize mash

(curve I, Fig. 1), the acetone-treated yeast autolysate (curve III) follows the other curves only in the initial part up to 0.13 A.U. After that point the fermentation velocity remains fairly constant to 2 A.U. Up to this point both the yield of solvents and the titration acidity remain normal. According to the activating properties the autolysate obtained by separating the insoluble matter (yeast cells together with some protein) with acetone is different from those autolysates which have been obtained either by filtration or by dialysis. The acetone treatment removed a factor (we term it "third factor") from the autolysate which in maize mash, yeast autolysate or dialysate of yeast autolysate causes a further increase of the fermentation velocity curve (I and II, Fig. 1) from 0.13 to 1.0 A.U. A deficiency of the same character, viz. the failure to attain maximum speed observed in the case of a maize mash, was also met with in the charcoal eluates of biotin from purified egg yolk and maize extracts (Tables V and VIII) and in cress extract (Table X).

The increase of the fermentation velocity above 2 A.U. (Table XI) has a different biochemical significance, the yields of alcohol and acetone decreasing, and the titration acidity increasing. At 20 A.U. practically no butanol and acetone are formed, the fermentation products being only butyric and acetic acids. The same observation was made also with parsley mash. At first sight it seemed that overdoses of biotin change the butanol-acetone fermentation into a butyric acid fermentation. Yet one has to take into consideration that the buffering capacity of the media is also increased by the addition of large amounts of yeast autolysate or parsley mash. (Parsley mash required for the same change of pH three times as much acid as maize mash.) In this case the media would acquire the properties of maize mash plus CaCO_3 . A final answer concerning the biochemical significance of this interesting observation must be postponed until the experiment has been repeated with chemically pure biotin.

An experimental proof of the existence of the "third factor" was given by combining a solution rich in biotin but poor in the "third factor" with a solution poor in biotin but rich in the "third factor". Parsley extract is an example of the "biotin" solution. As a solution of the "third factor" we used a dialysate of yeast autolysate which had entirely lost its biotin activity after 2 years' storage (dialysates D_1 and D_2 in our earlier paper). The results of the subsequent experiments are mean values from three parallel runs; the mean deviation from the stated fermentation velocity is also given.

The charcoal treatment with D_1 and D_2 (15 ml. D_1 or D_2 + 3 g. Merck's charcoal were shaken for 5 min. and filtered) was carried out in order to remove traces of biotin eventually present in the stored dialysates.

A. Solutions rich in biotin

	Fermentation velocity ml./hr.
Parsley extract { 0.1 ml.	11.9 $\pm$ 0.41
{ 0.5 ml.	13.4 $\pm$ 1.16
Yeast autolysate 1.7 ml.	13.5 $\pm$ 0.46

B. Solutions containing the "third factor"

	Fermentation velocity ml./hr.
Dialysate of yeast autolysate D_1 (1936) { 0.1 ml.	6.03 $\pm$ 1.73
{ 0.2 ml.	7.07 $\pm$ 0.18
Dialysate of yeast autolysate D_2 (1936) { 0.6 ml.	2.7 $\pm$ 0.22
{ 1.2 ml.	2.1 $\pm$ 0.0

Combinations of A and B

		Fermentation velocity ml./hr.			Fermentation velocity ml./hr.
1. Parsley extract	{0.1 ml. }	16.7 ± 0.76	Parsley extract	{0.5 ml. }	16.2 ± 0.6
D ₁	{0.12 ml. }		D ₁	{0.12 ml. }	
2. Parsley extract	{0.1 ml. }	13.9 ± 0.28	Parsley extract	{0.5 ml. }	18.2 ± 0.42
D ₁ (charcoal-treated)	{0.13 ml. }		D ₁ (charcoal-treated)	{0.13 ml. }	
3. Yeast autolysate	{1.7 ml. }	17.4 ± 1.9			
D ₂	{0.6 ml. }				
4. Parsley extract	{0.1 ml. }	12.1 ± 0.49	Parsley extract	{0.5 ml. }	14.0 ± 0.0
D ₂ (charcoal-treated)	{0.75 ml. }		D ₂ (charcoal-treated)	{0.75 ml. }	

Results

Combination 1 (untreated D₁) shows a positive activation of the biotin solution in the form of parsley extract.

Combination 2 (D₁ charcoal-treated) shows a positive but weak activation of the biotin effect obtained by parsley extract alone. With more parsley extract full activation was obtained.

Combination 3 (D₂ as source of the "third factor" with a freshly prepared dialysate as a source of biotin) shows together a positive activation.

Combination 4 (D₂ charcoal-treated with parsley extract as a source for biotin) showed no activation; in distinction to Combination 2 an increased amount of parsley extract gave likewise a negative response. The "third factor" in dialysate D₁ seems to be present in a higher concentration than in dialysate D₂. The "third factor" is to some extent adsorbed by charcoal; this has already been indicated by the results quoted in Table VIII.

The presence of the "third factor" in D₁, i.e. the first fractions obtained by dialysis of yeast autolysate, points to the fact that the factor in question is substance of low mol. wt.

DISCUSSION

The results of our experiments have revealed that one of the hitherto unknown nutritive factors which are required by *Cl. acetobutylicum* (W.) for a normal butanol-acetone fermentation is identical with biotin, a substance which, according to Kögl, promotes the growth of yeast as one of the bios factors. These two actions of biotin, viz. the stimulation of yeast growth and the stimulation of the fermentation intensity of a butanol-acetone fermentation can be correlated with one another by the intelligible assumption that the increased fermentation velocity (ml. gas/hr.) due to the presence of biotin is proportional to the concentration of the bacterial metabolic enzymes which in turn are proportional to the bacterial population. Biotin may, therefore, also promote the growth of *Cl. acetobutylicum* (W.). This assumption still requires direct experimental confirmation, yet it has acquired a high degree of probability since Kögl & Fries [1937] and Kögl & v. Wagtendonk [1938] have shown that the growth-promoting action of biotin is not restricted to a particular strain of yeast, but is also required by some fungi and by *Staph. aureus* in addition to aneurin and nicotinamide [Knight, 1937]. It is quite possible that biotin might prove to be an active growth stimulant with other bacteria, where it has not yet been suspected, e.g. with *Bact. anthracis* [Landy, 1939].

The main reason why we did not confine our measuring procedure to the estimation of the influence of the medium upon the growth of the bacterium was based on the following fact. The media which stimulate the growth of *Cl. acetobutylicum* (W.) do not necessarily produce a fermentation in which the yields

of alcohol and acetone, the final titration acidity and the fermentation velocity are identical with the butanol-acetone fermentation of a maize mash, to which we always refer as a normal fermentation. Maize contains all the specific nutritive requirements for *Cl. acetobutylicum* (W.) in a particular harmonic combination. We endeavoured to look at the fermentation as a whole; we did not accept, for example, any fermentation velocity as a true measure for the activator content unless the yields of products and the titration acidity were normal. In many instances we tested microscopically the appearance of clostridia and spores as a sign that the bacteria had completed their normal life cycle.

For the comparison of experiments which had been carried out at temperatures different from 37°, or at a different total volume, use was made both of the temperature coefficient of the fermentation velocity which, between 33 and 38°, was found to be 2₁₀, and of a simple relationship which exists between the fermentation velocity and the volume of the medium, viz. fermentation velocity/volume = K. In the case of a normal medium e.g. maize mash K_{37°} was found empirically equal to 0.6. For example 40 ml. of a 2.5% maize mash showed a fermentation velocity of 24.0 ml./hr. at 37° and 38 ml. of a 2.5% maize mash showed a fermentation velocity of 16.0 ml./hr. at 34°. The calculated value was 15.95 ml./hr.

The above-mentioned normal value for K, viz. 0.6 was found to be much higher with a 2.5% parsley-mash-sugar medium, viz. K_{37°} = 1.2 (35.0 ml. gas/hr., 34°, total volume 38 ml.). Yet in this particular case the fermentation cannot be called a normal one as no alcohol or acetone has been formed but only butyric and acetic acid. Despite the complexity of the activating system of *Cl. acetobutylicum* (W.) the activator unit (A.U.) as defined in our previous paper has been adhered to for practical reasons. Owing to the "third factor" the deviations from the normal shape of the fermentation velocity curve above 0.15 A.U. had to be taken into account. In case of a deficiency in the "third factor" the activator content should be measured in the range of 0.05–0.15 A.U. of the fermentation velocity curve.

From curve III, Fig. 1, which described the influence of biotin in the presence of asparagine (as far as such a curve can be abstracted from experiments if only a small amount of the "third factor" was present) it can be seen that a further increase of the biotin concentration above 0.15 A.U. has no essential influence upon the fermentation velocity. 0.15 A.U. seems to be the optimum amount of biotin required by the bacteria under the given experimental conditions. The multiplication of the bacteria at this stage amounts to 4000% (unpublished experiments); the approximate amount of A.U. corresponding to a 100% multiplication, as in Kögl's test, would be of the order of $0.15 \times 1/40 = 0.0038$ A.U., and as 1.0 A.U. = 540 saccharomyces units (Kögl), 0.0038 A.U. = 2.0 s.e./40 ml. and therefore 0.0038 A.U. = 1.3 s.e./25 ml.

This calculation is naturally only approximate, as the multiplication of the bacteria is not strictly proportional to the biotin concentration (Kögl). The figures obtained have therefore only the qualitative significance that the biological action of one saccharomyces unit (2.5×10^{-4} µg. biotin) is probably of the same order both in the case of *Saccharomyces cerevisiae* and *Cl. acetobutylicum* (Weizmann).

SUMMARY

1. *Clostridium acetobutylicum* (Weizmann) belongs to those heterotrophic bacteria whose synthetic power is very restricted; it cannot synthesize the factors required for growth.

2. Even in the presence of asparagine, combinations of aneurin, nicotinic acid and β -alanine, or uracil cannot replace the growth factors present in maize or yeast.

3. It has been demonstrated that one of the active growth factors required for *Cl. acetobutylicum* (Weizmann) is biotin.

4. In order to bring the nutritive level of a synthetic medium up to 70 % of the nutritional level represented by a maize mash both asparagine and biotin must be present.

5. The missing 30 % of the full nutritional requirements is due to a "third factor", for the existence of which experimental evidence has been brought forward.

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