LXXXV. THE ACTIVATION OF THE BUTANOL-ACETONE FERMENTATION OF CARBOHYDRATES BY *CLOSTRIDIUM ACETOBUTYLICUM* (WEIZMANN)

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For the elucidation of the mechanism of the fermentation it seemed to us essential that the medium in which the fermentation takes place should be synthetic, in order to eliminate any unknown influence. Up to now no one has effected a normal butanol-acetone fermentation in a strictly synthetic medium. Weyer & Rettger [1927] have already shown that *Clos. acetobutylicum* grows only in synthetic media which contain a specific nitrogen supply like peptone or egg albumin. Simple amino-acids or ammonium salts could not be substituted for the highly complex compounds. Yet the fermentation was still far from being a normal one, the yield of butanol being only a small fraction of that obtainable by fermenting maize. Later on Weinstein & Rettger [1933] found that the addition of zein to a semisynthetic (peptone-containing) medium gave a yield of solvents corresponding to about 21 % calculated on the sugar employed. Prolamins from other cereals were able to replace zein; other high-molecular proteins gave low yields of butanol. The authors concluded therefore that the prolamins are a necessary constituent for a normal yield of butanol.

Further evidence regarding the difficulty of effecting a normal butanol-acetone fermentation in a semisynthetic medium (yeast-water, glucose) with *Clos. acetobutylicum* was given by Langlykke *et al.* [1935]; they obtained a very low yield of butanol and acetone combined with a high final titration acidity.

I. Theoretical

In order to proceed to a normal butanol-acetone fermentation in a strictly synthetic medium our first task was to obtain a more thorough knowledge concerning the constituent in maize which is necessary for a normal fermentation. A fermentation has hitherto been termed "normal" when the yield of solvents, i.e. butanol and acetone, was identical with that obtained by fermenting a maize mash of a corresponding carbohydrate content. We consider the yield to be only one of the factors determining a so-called normal fermentation, and that the definition should also take into account the fermentation velocity. We have studied the influence of the constituents affecting the fermentation velocity in respect of a normal yield of butanol and have obtained information not only about the "activator" of the butanol-acetone fermentation—as we term the active constituent—but also about the fermentation itself.

Since the conception of bios raised by Wildier has been confirmed by Kögl [1936; Kögl & Toennis, 1936] and many others, there has been a marked trend to solve analogous problems in the vast field of bacterial metabolism, viz. to investigate the necessary specific requirements.

It is to be expected that related species of bacteria would have similar specific requirements for their metabolism, and conversely if similar specific requirements are observed the existence of a genetic relationship may be suggested which hitherto has not been recognized.

By comparing the experimental results concerning the specific requirements of *Clos. acetobutylicum* (Weizmann) [Weizmann, 1915; 1919] with those of butyric acid bacteria described by Tatum *et al.* [1933], and of propionic acid bacteria [Tatum *et al.* 1936; Fromageot & Laroux, 1936] we have come to the conclusion that they have the following features in common.

These bacteria require for their metabolism the presence of at least two compounds, which separately do not exhibit any action. The one is asparagine which may be replaced by aspartic acid or other compounds of related structure. Both the American and the French investigators have brought forward experimental evidence, especially for the propionic acid bacteria, that this compound plays a role in the nitrogen metabolism of the bacteria. The second compound, the description of which will be the main subject of this paper, is a low-molecular substance of still unknown constitution and widespread occurrence. This compound which stimulates, in combination with the first compound, viz. asparagine, the fermentation of sucrose with *Clos. acetobutylicum* has also been recognized, both by Fromageot & Laroux and Tatum as the second necessary requirement for the glucose fermentation in the case of propionic acid bacteria.

According to the experimental conditions (maize mash media) it is highly probable that the butyric acid bacteria of Tatum also need the second compound, in spite of his statement that asparagine is the only factor responsible for the stimulation of the production of butanol by butyric acid bacteria. If the necessary specific requirements for the fermentation are prepared from natural sources the dualism cannot often be immediately recognized in the crude extracts from yeast, potatoes, green plants etc. [see Tatum *et al.* 1936, p. 160], unless the asparagine is separated, as e.g. by treatment with mercuric acetate and sodium carbonate [Tatum *et al.* 1936]. This dualism of activating principles made it necessary in our experiments to carry out the quantitative measurements of the second compound—the activator—in the presence of asparagine in order to exclude possible errors derived from the accidental presence or absence of this compound.

The activating principle of still unknown constitution is, in our opinion, a coenzyme-like substance. We are not yet advanced enough in our knowledge about the activator to be able to describe the mechanism of the activation, i.e. the action of the activator as growth-promoter like biotin [Kögl & Toennis, 1936] or its function as hydrogen and phosphate carrier like cozymase [Euler *et al.* 1936; Vestin, 1936], or Warburg & Christian's [1936] coenzyme from red blood cells. Yet the experimental fact—often observed in this laboratory—that insufficiently anaerobic conditions or lack of activator or both lead to the formation of acids (mainly butyric) which inhibit the fermentation, seems to point to the fact that the activator may play an essential role as hydrogen carrier in the enzyme system of the bacteria in bringing about the unusual hydrogenation of butyric acid or β-hydroxybutyric acid to butanol, besides the obvious favourable effect upon the growth of the bacteria.

II. Experimental

1. *Experimental procedure* (*General*)

Measurement of the gas formed during the fermentation was adopted as the most sensitive, reliable and convenient procedure for kinetic studies. After

several trials with long test-tubes as fermentation vessels we decided to use 50-ml. long-necked flasks containing always 40 ml. of medium so as not to leave too much air space above the surface of the liquid, thus ensuring the easy production of anaerobic conditions. The addition of pulped filter-paper [Weyer & Rettger, 1927] or asbestos fibre proved to be essential to media poor in solid matter, in order to obtain reproducible results.

The gas was collected over water under slightly reduced pressure in graduated glass cylinders of about 500 ml. capacity and capable of being read to an accuracy of 1 ml.

A series of these graduated cylinders was kept in an incubator room at 37°.

Inoculant. The inoculant used in all cases was a 24-hour culture of *Clos. acetobutylicum* (Weizmann) in 5 % maize mash, inoculated always from spores in order to ensure constant vigorous fermentation. It was prepared by inoculating about 10 ml. 5 % sterile maize mash with 1 ml. of spore suspension from a 10-day old fermented maize tube. After subsequent pasteurization for 3 min. in boiling water and immediate cooling the tubes were incubated at 37°.

Sterilization. It is necessary to sterilize or resterilize the media immediately before inoculation in order to provide anaerobic conditions for the bacteria. The maize mash was sterilized at 25 lb./sq. in. for 2 hours, the sugar and other solutions were only steam sterilized.

2. *Velocity of a maize mash fermentation*

The fermentation of a maize mash, to which we always refer as standard or normal fermentation, was carried out by inoculating 40 ml. of resterilized 2·5 % maize mash with 1 ml. of a vigorous 24-hour culture of *Clos. acetobutylicum* strain D in 5 % maize mash as inoculant.

A parallel experiment was carried out by using strain H as inoculant. This strain was obtained from strain D by a gradual increase of the time of pasteurization from 3 up to 13 min. The result is given in Fig. 1. Strain H is definitely weakened by this treatment in comparison with strain D.

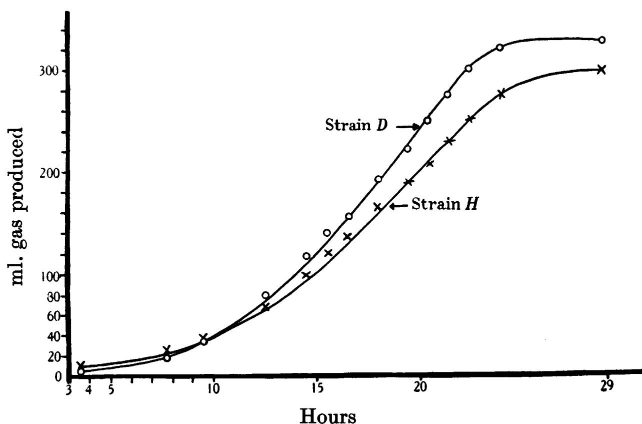

Fig. 1. Fermentation of maize mash.

The curves consist principally of three parts: (1) the initial part with increasing fermentation velocity, (2) the main part, with constant and maximum fermentation velocity and (3) the final part of the fermentation curve with decreasing

velocity of gas evolution. The curves given as examples show that about 60% of the total gas formed is within the linear part of the curves; 21 curves which we have examined in this connexion showed that on an average 66% of the total gas given off was formed within the linear part of the curve. We therefore felt justified in assuming that the steepness of the linear part of the fermentation curve is a characteristic of the fermentation intensity. As will be shown later, the fermentation intensity depends largely on the activator content of the medium. A quantitative measure of the activator concentration is therefore given by the steepness of the fermentation curve. The maximum fermentation velocity is independent of the amount of bacteria in the inoculant, or of any other influence on the initial growth period.

3. *Comparison of fermentation velocities of maize samples used*

The maize used for the preceding experiments was freshly harvested (October 1935) Palestine maize. The maximum fermentation velocity seen from the curves in Fig. 2 amounted to 24·5 ml. per hour for strain D. After 8 months practically the same value (25·2 ml. per hour) was observed using the same maize and the 14th generation of the same strain.

Several experiments were carried out with another sample of maize of unknown origin which had been kept in stock for more than a year. We will always refer to this sample as "old maize". In order to render possible the comparison of the results obtained by the use of the different samples we give the corresponding values of the fermentation velocities in Table I. Both strain H and strain D were tried as inoculant.

Table I. *Comparison of fermentation velocity of a 2·5% maize mash prepared from "old maize" with that of one from maize "1935".*

	Maize "1935"		Maize "old"	
	Strain H	Strain D	Strain H	Strain D
Fermentation velocity, ml. per hour	19·6	24·5	16·3	20·7
Ratio strain H/strain D	0·80		0·79	

The fermentation velocity of "old" maize is only 80% of that of the maize "1935". The lower fermentation velocity may possibly be due to decrease of activator content during storage but as a corresponding decrease in the case of maize "1935" has not been observed after a year's storage, it is possible that the low value observed with "old" maize was peculiar to that sample. Both strains tested showed equal responses to the two different samples of maize, the ratio of velocities observed remained constant.

4. *Distribution of activator in maize*

During the search for a suitable method for the isolation and preparation of the activator, it was of interest to get some information about the distribution of the activator in maize between the bran and the starch. If a 2·5% maize mash, prepared in the usual way, is allowed to stand overnight the bran as well as the husks settle down fairly well, and a crude separation of the supernatant fluid from the bran can be effected by careful siphoning.

(1) 40 g. of the supernatant fluid representing an opalescent starch solution without solid particles were filled into a fermentation flask, resterilized, inoculated with 1 ml. maize mash culture of *Clos. acetobutylicum* strain H and incubated at 37·5°.

(2) 40 g. of the settled suspension of bran and husks were filled into a fermentation flask, resterilized, inoculated and incubated as in (1). We may regard it as a starch suspension which is relatively rich in bran compared with ordinary maize mash.

(3) 40 g. of ordinary 2·5 % well mixed maize mash were fermented simultaneously with (1) and (2). The maize used was "old"; the experiments were carried out in duplicate. The results are given in Table II.

Table II. *Distribution of activator in maize*

	(1) Starch suspension free from bran	(2) Ordinary mash	(3) Starch suspension rich in bran
Fermentation velocity, ml. per hour	6·1, 6·1	16·2, 16·4	18·8, 18·8

Though the ratio of the observed velocities may not be absolutely true, owing to the crude method of separating and to the fact that it is not exactly known what amount of activator has been rendered soluble by the intense treatment which maize mash undergoes during the sterilizing process, it is quite obvious from the above results that the bulk of the activator is held in the bran of the maize. Some maize mash was malted with malt extract at 58° for 16 hours in order to saccharify the starch. The bran preparation prepared by this method was found to have a low activating effect probably owing to the activator being rendered soluble by this method. The fermentation velocity observed was 6 ml. gas per hour using the optimum amount of bran (grains) which corresponded approximately to 1·5 g. of maize "old" per 40 ml. of 1·5 % sucrose.

(5) *Extraction of the activating principle from maize*

Extraction of maize with 85 % alcohol. Weinstein and Rettger conclude from the results of their experiments that only the prolamin, i.e. the zein which they obtained by extracting maize with 80 % alcohol, is able to activate a semisynthetic medium so that a normal yield of butanol is obtained, while the extracted maize loses its ability to ferment normally. Yet a comparison of the quantitative relationship between the minimum amount of zein required for a normal fermentation in a semisynthetic medium which they found to be 4 % and the zein content of a 5 % maize mash which is 0·25 %, makes it probable that maize contains 16 times as much activator as is contained in zein. We found it therefore desirable to check this calculation by determining experimentally the exact ratio of fermentation velocity between normal maize and maize from which the zein has been quantitatively extracted.

100 g. maize meal ("old") were suspended in 500 ml. 85 % alcohol and shaken on the machine for 5 hours; the maize meal was separated from the alcoholic extract and washed with 100 ml. 85 % alcohol. The whole extraction procedure was repeated three times in exactly the same manner. The last (fourth) extract gave only a very faint turbidity when diluted with cold water. The extracted maize was dried on filter-paper (91 g.). From this sample a 5 % mash was prepared; 20 g. of this mash were filled into each of 3 fermentation flasks and made up to 40 mg. with tap water. Three experiments with the corresponding amount of ordinary maize (20 g., 5·25 % mash) were carried out. After re-sterilization the flasks were inoculated as usual with 1 ml. of a 24-hour culture in maize mash (strain H) and incubated at 37° (Table III).

A 5 % maize mash contains according to Tatum as much activating constituents as a medium containing 4 % zein. In a 5 % maize mash (0·25 % zein)

Table III

	Normal maize ("old")	Maize ("old") zein-free
Fermentation velocity, ml./hour	16·2, 16·6, 15·9	13·9, 15·0, 14·0
	Mean value 16·2	Mean value, 14·3

the distribution of activator should be accordingly $\frac{0 \cdot 25}{4 \cdot 0} = \frac{1}{16}$ in the zein and $\frac{15}{16}$ in the residue, i.e. 93·7 %. Experimentally we have found $\frac{14 \cdot 3 \times 100}{16 \cdot 2} = 88 \cdot 3$ %.

Extraction of maize with 0·1 N *HCl.* Dilute hydrochloric acid proved to be more effective for extracting the activator from maize. Maize (zein-free) which was shaken for 4 hours with hydrochloric acid (*p*H of the maize meal suspension: 2·1) centrifuged, neutralized and made up into a 5 % sterile mash exhibited a very sluggish and incomplete fermentation, thus confirming the results of earlier experiments of Weizmann & Davies [1937].

The activator content of fermented maize mash. The strong amylolytic and proteolytic activities of *Clos. acetobutylicum* (Weizmann) make it highly probable that the insoluble part of the activator which is present in the bran of the maize will become soluble during the fermentation process. If the activator is not used up in the metabolism of the bacteria, as would be the case if the activator were a specific nutritive factor, it should be possible to demonstrate the presence of the activator in the "spent wash".

(*a*) *Preparation of the "spent wash".* The fermented liquid from 8 litres 5 % maize mash was evaporated in a Faust-Heim hot air evaporator to 1·3 litres. 6·7 g. sodium phosphate (Na_3PO_4, $12H_2O$) and 33·2 ml. of 2 *N* NaOH were required to adjust the acidity of the concentrated spent wash to *p*H 6·2.

(*b*) *Fermentation test with "spent wash" as activator.* (1) To 0·5 g. of sucrose in long test-tubes were added 3·0 ml. of conc. "spent wash". Total volume made to 12 ml. with tap water. Inoculant: 1 ml. culture in 5 % maize mash. Temperature 37°. (2) A parallel experiment was made exactly the same as (1) except that the spent wash used (3·0 ml.) was separated from solid particles by centrifuging. All experiments were carried out in duplicate. The fermentation velocities found were: 1·3 ml./hr. for (1) and 3·3 ml./hr. for (2). By centrifuging the spent wash a constituent was separated which had an unfavourable effect upon the growth of the bacteria. A microscopical examination showed degeneration in the fermentation where the original spent wash was used (1), while (2) showed normal and healthy forms of the bacteria.

Under the experimental conditions with which we worked in this experiment, the values of the fermentation velocity observed can only be compared with speed values obtained under similar experimental conditions. The theoretical activator content of 3·0 ml. spent wash is approximately one activator unit (1 A.U.). (For definition of activator unit see p. 628.) Supposing that under the experimental conditions used, 1 A.U. corresponds to 5–6 ml. per hour, we may guess the yield of activator in the spent wash as being roughly 1/2 to 2/3 of the theoretical. This figure has of course only qualitative significance.

We did not endeavour to obtain an activator extract from maize, as yeast proved to be a very convenient source for this purpose.

6. *Preparation of activator extracts from yeast*

In spite of the unsatisfactory results obtained by Langlykke *et al.* [1935] who fermented a glucose-yeast water medium with *Clos. acetobutylicum* (Weizmann) it seemed possible that the procedure hitherto employed for the extraction of the

activator from yeast could be improved by adopting Willstätter's [1928] method of controlled autolysis.

Assuming that the activating substance might be associated with cellular protein as "symplex" in the sense of Willstätter [Willstätter & Rohdewald, 1934] we chose conditions favourable for the liberation of the yeast proteases. Therefore the prescription given by Grassmann & Haag [1927] was used with little alteration.

The results of the following experiments make it highly probable that the liberation of the activator is considerably helped by the action of the yeast proteases. We compared the activating effects of two different autolysates. (1) Yeast autolysate separated from the cell residue 1 hour after plasmolysis with ethyl acetate which did not produce any proteolytic activity according to the findings of Grassmann. (2) Yeast autolysate separated from the cell residue after the incubation of the autolysate at *p*H 6·5 for 48 hours.

It was shown by Grassmann and we were able to confirm it in the case of the proteolysis of both gelatin and soya proteins, that yeast autolysate prepared in this way exhibits optimum proteolytic activity.

(1) 200 g. of baker's yeast were plasmolysed with 10 ml. ethyl acetate, the mass being kneaded until complete liquefaction took place (5 min.). After standing at room temperature for 1 hour the mass was centrifuged and filtered. The filtrate was kept under a layer of toluene. Measured portions of the autolysate carefully freed from toluene were added to 10 ml. of a 6 % cane-sugar solution in tap water, the total volume (including 0·5 ml. inoculant) made up with tap water was 15 ml. Before inoculation the medium was sterilized twice on successive days for 1 hour at 100°. The fermentation was carried out in long pyrex test-tubes (16 × 220 mm.) at 37°.

Table IV

ml. inoculant	0·5	0·5	0·5
ml. yeast autolysate as described	None	0·5	1·0
Corresponding amount of fresh yeast, g.	None	0·5	1·0
Rate observed, ml. of gas per hour	0·34	0·78	0·95

(2) 100 g. of baker's yeast were plasmolysed with 5 ml. ethyl acetate. After the yeast had become liquid 100 ml. distilled water were added and the autolysate was kept neutral by gradual addition of 25 ml. 5 % $Na_3PO_4,12H_2O$. A layer of toluene prevented putrefaction during the subsequent autolysis at 37° for 48 hours. After incubation the autolysate was made up to 400 ml., filtered, freed from toluene and sterilized after readjustment of weight. The fermentation was carried out as described in the preceding experiment.

Table V

Total volume, 15 ml.; $t=37°$.

ml. inoculant	0·5	4·0	0·5	0·5	0·5	0·5	0·5
ml. yeast autolysate as described	None	None	0·2	0·5	1·0	2·0	5·0
Corresponding amount of fresh yeast, g.	None	None	0·05	0·125	0·25	0·5	1·25
Rate observed, ml. per hour	0·22	3·33	1·50	2·54	3·92	4·48	5·16

The results given in Tables IV and V show that the extract obtained by short autolysis is practically free from activator. The parallelism between the activator

content and protease content of the two autolysates is obvious. The absolute values of the fermentation velocity observed in these experiments are low compared with the values in other fermentations which are described later in this paper. The relative values given are comparable with one another as is evident to some extent from the rate obtained under the same conditions with 25 % maize mash inoculant (Table V, col. 2) and is still more evident from the following two sets of experiments. The activator solution used for both experiments was prepared by dialysing yeast autolysate against distilled water and concentrating the joined dialysates; 1 ml. of the concentrated dialysate corresponded to 1 g. of fresh yeast.

Exp. 1 (Table VI). 8 long test-tubes, each containing 0·6 g. cane-sugar, varying amounts of dialysate as shown in the table and made up to 15 ml. total volume with tap water (including 1 ml. inoculant) were connected with graduated collecting tubes and incubated at 37°.

Table VI

ml. activator solution	0·05	0·10	0·20	0·50	1·0	2·0	5·0	10·0
Fermentation velocity ml./hr.	1·6	0·5	4·4	0·7	2·3	2·3	2·1	Stopped after 53 ml. gas evolved

The experiments of Table VII were carried out in 50-ml. flasks containing 0·6 g. of cane-sugar in a volume of 40 ml. together with 0·5 g. of sterilized filter pulp. The same and varying amounts of activator solution were used as in the preceding experiment. The individual experiments were carried out in duplicate.

Table VII.

1 ml. inoculant strain D; $t=37{\cdot}5°$.

ml. dialysate of autolysed yeast	None	0·1	0·2	0·5	1·0	2·0
Corresponding amount of fresh yeast	None	0·1	0·2	0·5	1·0	2·0
Rate observed, ml. per hr.	1·2	3·8	8·6	20·0	21·7	19·6
% yield of solvents calculated on the sugar employed	Not estimated	Not estimated	Not estimated	30·7 Mean value	35·6 Single value	Not estimated

It is surprising to see the striking effect of small alterations in the experimental conditions on the fermentation intensity. Special experiments have proved that the filter pulp cannot be omitted without diminishing the fermentation velocity. In this experiment we succeeded for the first time in obtaining artificially a "normal" fermentation, comparable both in velocity and in yield of solvents with a normal maize mash fermentation (see Fig. 1). With the exception of the activator every constituent of the medium is chemically defined. The yield of solvents was estimated by distilling off one-third of the fermented liquid and by counting the number of drops of the distillate given by a 5 ml. stalagmometer which had been checked with known mixtures of fermentation solvents with water. The results obtained by this method were quite satisfactory for control purposes, and they agreed with the results which were obtained when the fermentation was carried out on a larger scale.

A medium consisting of 14 ml. D_1 (=dialysate of yeast autolysate 1st fraction) as activator, 2·1 g. asparagine and 42 g. of sucrose and 2 g. asbestos pulp made up to 2800 ml. with tap water was inoculated with 80 ml. inoculant

(24 hours old) of exactly the same relative composition. A parallel experiment was carried out simultaneously but instead of the dialysate D_1 28 ml. of the second fraction, D_2, of a dialysate from yeast autolysate was used as activator. (The preparation of D_1 and D_2 is described later.) Result: 13·6 g. (D_1), 14·0 g. (D_2) solvents were collected over saturated K_2CO_3 solution, i.e. 31·5 (D_1), 32·4 (D_2) % on the weight of sugar (43·2 g.) including the amount of sugar in the inoculant used.

The acetone content of the solvents obtained was 21·8 % for D_1 and 23·0 % for D_2. The fermentation was in both cases complete in 40 hours at 36°, 21·3 (D_1 and D_2) litres of gas being formed.

7. *Activator in yeast-water*

The yeast-water was prepared by heating one part of yeast in the presence of egg white with 5 parts of water under slight pressure [van der Lek, 1930]. The clear yellow filtrate was used.

To 6 flasks (50 ml.) containing 0·6 g. sucrose and 0·5 g. filter-pulp, increasing amounts of yeast-water were added as given in Table VIII; the volume was made up to 40 ml. with tap water.

Table VIII

1 ml. inoculant strain D; $t=38·5°$.

ml. yeast-water	10, 10	20, 20	40, 40
Corresponding amount fresh yeast, g.	1·67	3·33	6·67
Max. rate observed	18, 18·0	15·4, 16·4	18·4, 28·9
Main rate, ml. per hour	4·6, 5·6	5·5, 7·2	7·3, 6·5

The fermentation carried out with yeast-water as source of activator behaves abnormally. With the exception of one experiment with 40 ml. yeast-water, which showed a gas evolution of 28·9 ml. per hour, the fermentation velocity decreased to the low values which have been specified as "main rate" in Table VIII, as the principal part of the fermentation was going on at the low rate. The higher temperature prevailing during this experiment is probably not the reason, as other fermentations carried out simultaneously behaved normally. The best of these fermentations with yeast-water was that in which 40 ml. yeast-water were used. If we compare the amounts of yeast required for the preparation of that amount of activator which produces an optimum fermentation, we find the following: 0·5 g. baker's yeast for the dialysate from yeast autolysate (see Table VII), 6·67 g. for yeast-water (Table VIII). The method of preparing activator solutions from yeast by controlled autolysis is obviously superior to the procedure involved in preparing yeast-water, both in yield and in the general influence on the fermentation.

8. *Influence of the activator concentration on the fermentation velocity*

For the quantitative estimation of the activator from kinetic results an intimate knowledge of the influence of the activator concentration on the fermentation velocity was of importance. We therefore completed the results given in Table VII by estimating the fermentation velocity using 0·3, 0·4 and 0·5 ml. of the same dialysate from autolysed yeast. The total volume (40 ml.), amount of filter-pulp (0·5 g.), sucrose (0·6 g.) and inoculant corresponded to the standard conditions adopted in the experiment of Table VII. The results obtained are shown in Table IX.

Table IX

ml. dialysate	0·30	0·40	0·50
Number of experiments	7	5	10
Fermentation velocity, ml./hr. (mean value)	13·2	17·7	21·1

From these data and those given in Table VII the curve in Fig. 2 was constructed by plotting the values of fermentation velocity against the corresponding amount of activator solution. Each velocity value was taken from an individual

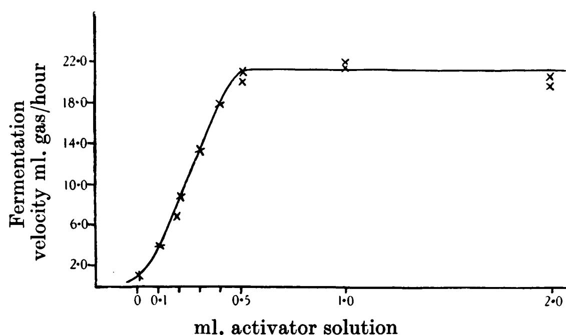

Fig. 2.

fermentation curve as shown in Fig. 1. This graph shows that between the limits of 0·1 and 0·5 ml. of activator solution the fermentation velocity is proportional to the activator concentration. At lower activator concentrations the curve bends towards the ordinate owing to the activator content of the inoculant used. Higher activator concentrations than 0·5 ml. of this activator solution are without influence on the fermentation velocity.

9. *Definition of the activator unit* (A.U.)

One activator unit (A.U.) is defined as that amount of substance which is just sufficient to produce maximum velocity in a butanol-acetone fermentation of a solution containing 0·6 g. sucrose and 0·03 g. asparagine (0·2 millimol) in a total volume of 40 ml. at 37°.

The customary requirements for bacterial growth such as Ca, Mg, PO_4 etc., and also the requirement of filter-pulp for liquid media are omitted from this definition.

The dialysate of yeast autolysate contains for example 2·0 A.U. in 1·0 ml., as can be seen from Fig. 3, or, as the dialysate contained 7·4 % dry matter, the relative concentration of this activator solution may be given as 27 A.U. per g. (dry).

10. *Fractional dialysis of yeast autolysate*

For preparative purposes it was of interest to obtain some information regarding the activator content of the different fractions obtained during dialysis. We have omitted to give experimental details for the preparation of the dialysate from yeast autolysate which we used in the preceding experiments. The main difference between the earlier procedure and that now to be described was that we did not unite the fractions obtained but analysed them separately.

2 kg. of baker's yeast (Polish) were plasmolysed with 200 ml. of ethyl acetate. After liquefaction (2 min. after the addition of ethyl acetate) the mass was

mechanically stirred and kept neutral (to litmus) by the gradual addition of 500 ml. 10 % sodium phosphate solution ($Na_3PO_4, 12H_2O$) during 80 min. (the room temperature was 26°); 160 ml. toluene were then stirred into the autolysate. After incubation at 37° for 24 hours another 130 ml. 10 % sodium phosphate solution were added and also 100 ml. of toluene to replace the loss by evaporation. The autolysate was incubated for another 36 hours.

Fractional dialysis. The yeast autolysate (2925 g. including yeast cells) was poured into the parchment container of a rotating Gutbier dialyser and dialysed against 6 l. distilled water at room temperature (25°). A mixture of 200 ml. xylene and 150 ml. chloroform was added to the distilled water. After 16 hours' dialysis the clear yellow dialysate was evaporated at 37 mm. Hg. The dialysis was continued with another 6 l. of distilled water and after 16 hours the dialysate was evaporated *in vacuo* together with the portion obtained previously to about 500 ml., the formation of a precipitate being then observed. The concentrate was kept in the refrigerator overnight, the solution was then filtered and the residue washed twice with 25 ml. ice-cold water. Washings and filtrate were united and steam sterilized. The dialysate which had become dark weighed 557 g. and was called D 1. The residue was dried in a desiccator (3·7 g.).

The second fraction D 2 was obtained by further dialysis (16 hours) of the autolysate against 6 l. of distilled water, followed by evaporation and separation of the insoluble precipitate formed. 289 g. of concentrate (D 2) and 1·20 g. (dry) of insoluble residue were obtained.

Before the dialysis was continued the content of the parchment cell was evaporated to approximately 1·5 l. The united concentrates of the three dialysates obtained on three successive days gave the third fraction, D 3 (451 g.).

The insoluble residue which separated out during evaporation weighed dry 3·20 g. D 3 represents the concentrate of two other successive dialysates (486 g.). No precipitate was obtained during evaporation. The final fraction D 5 consists as in D 4 of the united concentrates of two successive dialysates; in the sterilized fraction D 5 weighed 523 g.

The contents of the parchment cell were quantitatively removed and evaporated *in vacuo* to 1273 g. (for analytical data of the other fractions see Table XI) (total N 2·585 g., amino-N as percentage of total N, 25·0 %).

The precipitates obtained during the evaporation of D 1, D 2, D 3 were powdered and mixed (8·1 g.); this residue is called P.D.

Tyrosine content of P.D. 0·5 g. substance was dissolved in 30 ml. *N*/10 HCl and filtered from insoluble constituents (0·040 g.) and 10 g. of potassium acetate were dissolved in the clear yellow filtrate; tyrosine crystallized out in fine needles. On recrystallization from water 0·17 g. of pure tyrosine was obtained. The total tyrosine content of P.D. was therefore 2·45 g. Deducting 2·45 g. tyrosine (0·190 g. N) from 8·10 g. total P.D. (1·165 g. N) there remain 5·65 g. of residue with 0·975 g. N, i.e. 17·3 % N. Both the high nitrogen content and the formation of a silver salt insoluble in dilute ammonia suggest the presence of a purine compound. With regard to uropterin, a characteristic fluorescent dye isolated from the purine fraction of human urine by Koschara [1936] it may be of interest to describe the fluorescence observed in the purine fraction of P.D.

In 7 *N* HCl	... ...	violet (faint)
0·1 *N* HCl	... ...	no fluorescence
*p*H 6	... ...	yellow-green
*p*H 9–10	... ...	blue-green (intense)

The fluorescent principle was adsorbed by frankonite from neutral solution and was redissolved with pyridine and hydrochloric acid. It seems to us probable therefore that uropterin is also present in yeast.

Further analytical data referring to the fractions D 1 to D 5 are given in Table XI. In making the nitrogen balance of the dialysis of yeast autolysate, we find that out of 34·8 g. N in the original autolysate 29·3 g., i.e. 84 %, have passed the parchment membrane. The total amount of N recovered was 31·9 g., i.e. 91·7 %. The main loss of nitrogen is probably due to loss of ammonia during evaporation.

11. *Activator content of the different dialysates obtained by the dialysis of yeast autolysate*

Influence of added asparagine. For the quantitative estimation of the activator content in the different fractions D 1, D 2 etc. the standard conditions as laid down for the definition of the activator units were used. In this connexion we studied for the first time the influence of additional asparagine. The amount of asparagine was chosen with the object of maintaining a constant nitrogen level for the bacterial nitrogen requirements. The quantities of asparagine used varied accordingly, but they remained always in the optimum range, viz. 30–150 mg. per 40 ml.

The results of the experiments showing the influence of increasing activator concentration on the fermentation velocity with and without additional asparagine are given in Table X.

Table X

a, without asparagine; *b*, with additional asparagine.

Fermentation velocity of:	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	ml. containing 1 A.U.
D 1	(0·04)		(0·08)		(0·16)		(0·32)		(0·64)		0·08
	2·5	18·2	4·8	24·4	12·4	24·2	26·4	—	24·6	—	
D 2	(0·04)		(0·08)		(0·16)		(0·32)		(0·64)		0·58
	1·6	8·7	2·6	12·7	4·6	14·8	11·0	18·6	24·0	25·2	
D 3	(0·06)		(0·16)		(0·30)		(0·60)		(1·20)		0·80
	2·2	5·0	3·8	9·1	11·6	15·8	22·3	22·1	25·0	26·0	
D 4	(0·30)		(0·80)		(1·5)		(3·00)		(5·00)		3·00
	1·1	5·4	7·6	12·8	15·8	20·0	24·8	24·8	25·8	27·1	
D 5	(2·0)		(4·5)		(8·0)		(16·0)		(32·0)		16·0
	3·2	6·0	6·8	6·9	12·8	11·8	16·7	19·2	13·7	15·8	
Solid residue from D 1 to D 3	(1·3 mg.)		(2·6 mg.)		(20·8 mg.)		(39 mg.)		(48 mg.)		—
	1·8	3·7	1·9	10·1	4·6	10·5	7·4	17·5	10·3	20·7	
5 % maize mash	(1·0)		(3·0)		(6·0)		(11·0)		(21·0)		15·4
	1·2	2·2	5·8	13·7	10·4	15·8	17·0	16·9	22·4	25·2	

The figures in brackets indicate the ml. of activator solution used in the corresponding experiment.

From the figures in the last column in Table X representing the amount of ml. which contain one activator unit (1 A.U.) we calculated, having taken into consideration the dry matter content (see Table XI), both the relative purity which we express as A.U. per g. dry matter and the total activator content of the individual fractions also expressed in activator units (Table XI).

We are now able to compare the relative activator contents of maize and yeast. In this comparison we have not taken into account the activator content of the non-dialysable part. 1 g. fresh yeast contains 9 A.U., 1 g. dry yeast 36 A.U.

Table XI

	D 1	D 2	D 3	D 4	D 5	Insoluble residue from D 1 to D 3
Total N, g.	15·00	3·94	6·67	2·01	0·52	1·165
Amino-N % of total N	61·3	68·3	66·3	62·0	52·8	40·2
Wt. of dry matter, g. per 100 ml.	33·15	15·07	16·07	4·53	1·32	—
Total amount of A.U.	6950	5000	5650	162	237	187
A.U. units per g. dialysate (dry)	37·7	115·0	78·1	7·3	4·7	23·6

has about 26 times as much activator as 1 g. of maize which contains according to Table X only 1·3 A.U.

All dialysates combined contain 17,795 A.U. per 329·7 g. of dry matter, i.e. 54·0 A.U. average activator content per g. compared with 27 A.U. per g. in the dialysate of yeast autolysate which we prepared before.

The fractions D 1, D 2 and D 3 contain 99 % of the activator which passed through the parchment membrane. The fraction D 2 (115·0 A.U. per g. of dry matter) represents relatively the purest solution of activator which we have prepared. With fraction D 5 we did not obtain the maximum fermentation velocity given by the other soluble fractions. On the contrary, we observed a decrease in the fermentation velocity with increasing amount of activator. The microscopical examination of the bacteria in this medium showed degeneration and we are led to assume the presence of an inhibiting substance in D 5 (Table X).

The results given in Tables X and XI show that for the quantitative recovery of the activator free from inhibiting constituents derived from the dialysate of yeast autolysate it is sufficient to collect only three fractions (which consist in themselves of six successive dialysates).

The influence of asparagine on the activating effect of the three main fractions D 1, D 2 and D 3 requires closer consideration. We have postulated in our definition of the activator unit that the real activator content appears only if the fermentation is carried out in the presence of asparagine. The experimental results in Table X show that there is a definite stimulating influence on the activator. It may also be seen that the influence of additional asparagine depends mainly on two factors: primarily on the kind of activator, since different solutions, D 1, D 2, D 3 and maize respond differently; in the second place, the stimulating effect is the greatest in activator concentrations between 0·2 and 0·8 A.U. It is small or even absent at very low and very high activator concentrations. However, the fact which really justifies the inclusion of asparagine in the definition of the activator unit, is that only in the presence of asparagine are we able to compare activator solutions of different compositions, or from different sources.

If the velocities obtained with D 1, D 2 and D 3 in the presence of asparagine (Table X) are plotted against the activator units, the graph shown in Fig. 3 is obtained.

In considering the graph we compare at first the curve given in Fig. 2, which shows the influence of increasing amounts of activator on the fermentation velocity, with the corresponding curves in Fig. 3. We see that they exhibit qualitatively the same character, but quantitatively the curve in Fig. 2 can only be compared with that obtained with D 2, the latter being a little less steep. The maximum fermentation rate is reached simultaneously at the same activator concentration as in the presence of asparagine. For this reason the A.U. values calculated for that dialysate are still correct.

While generally we have to expect different curves from different activator solutions, it may be seen from Fig. 3 that in the presence of asparagine we are able to describe with a single curve the influence of the activator concentration on the fermentation velocity. On the other hand we may conclude that the

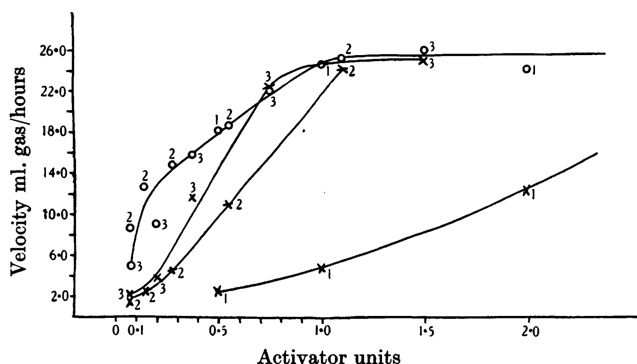

Fig. 3. The points o correspond to the experiments with asparagine. The points × belong to those without asparagine; the numbers indicate the fraction of dialysate used (e.g. $\times_2$ means experiment without asparagine, D2 as activator).

varying steepness of the curves obtained in the experiments without asparagine is caused by different contents of asparagine or some substance which acts like asparagine and can be replaced by it.

12. *Optimum concentration of asparagine*

It can be seen from the results of the following experiment (Table XII) that the range of the optimum concentration of asparagine is quite wide, viz. from 15 to 150 mg. per 40 ml. In this connexion it is of interest to mention that the optimum concentration of asparagine necessary for the butyric acid fermentation is of the same order, viz. 60 mg. per 100 ml. [Tatum *et al.* 1935].

Standard conditions as in previous experiments were employed, viz. 600 mg. sucrose, 500 mg. filter-pulp and 0·2 millimol of phosphate (pH 6·8); 1 ml. 5 % maize mash inoculant served as source of activator. To experiment No. 5 no filter-pulp was added.

Table XII

Total volume, 40 ml.; $t=37°$.

No. of experiment	1	2	3	4	5	6
Millimol asparagine	0	0·1	0·2	0·5	0·5	1·0
Velocity, ml./hr.	1·6	4·1	4·4	4·8	1·4	4·7

The apparent discrepancy between the fermentation velocity which we find in this experiment (4·8 ml./hr.) and that we have observed elsewhere, using the same experimental conditions, viz. 2·2 ml./hr. (Table X, last row, first column), is probably due to the fact that at low activator concentrations, small changes in concentration cause large changes in fermentation velocity.

13. *Other sources of activator; activator from green plants*

We investigated the activator contents of grass, lucerne and vetch. The leguminous plants were of special interest as it is known that they contain a relatively high amount of asparagine [Abderhalden, 1911].

1. *Grass.* Fresh grass (perennial) was finely cut, 2·0 g. were placed in 50 ml. round flasks containing 0·6 g. sucrose and the volume was made up to 40 ml. with tap water. After sterilizing, 1 ml. inoculant (strain H) was added.

2. *Lucerne.* (*a*) 2 g. and (*b*) 4 g. of the finely cut fresh material were put into 50-ml. round flasks, each containing 0·6 g. sucrose, total volume 40 ml. made up with tap water; inoculant 1 ml. 24-hour culture in 5 % maize mash (strain D).

3. *Vetch.* The fresh material was finely cut as above. (*a*) 2 g. and (*b*) 4 g. were put into two 50-ml. round flasks each containing 0·6 g. sucrose, total volume 40 ml., inoculant as in the lucerne experiment.

4. *Vetch extract.* 20 ml. of chloroform were poured on to 20 g. of fresh, finely cut vetch, 200 ml. distilled water added and the mixture incubated at 37°. After 48 hours' incubation the autolysate was filtered. The clear yellow filtrate including the washings (twice 50 ml. water) was evaporated on the water-bath and finally made up to 50 ml. (*a*) 2·5, (*b*) 5·0, (*c*) 10·0 ml. of the extract corresponding to 1, 2 and 4 g. of fresh vetch were filled into three 50-ml. round flasks each containing 0·600 g. sucrose and 0·5 g. filter-pulp, total volume 40 ml. made up with tap water, inoculant 1 ml. culture in 5 % maize mash strain D.

Table XIII shows the fermentation velocity observed in these experiments.

Table XIII

$t = 37·5°$.

	1. Grass	2. Lucerne		3. Vetch		4. Vetch extract		
No. of experiment		*a*	*b*	*a*	*b*	*a*	*b*	*c*
Fermentation velocity, ml./hr.	8·0	13·1	17·1	18·1	21·6	8·0	13·1	26·6

If we compare equal weights of the fresh materials we find lucerne to be superior to grass and vetch to be superior to lucerne in their activating effect. 4 g. of fresh vetch (3 *b*) and the vetch extract of 4 g. vetch (4*c*) have about the same activator content, thus showing that the extracting procedure employed was exhaustive.

14. *Influence of the initial sucrose concentration on the fermentation velocity*

From the shape of a normal fermentation curve it might be anticipated that the fermentation velocity would be independent of the initial carbohydrate concentration—at least for concentrations not exceeding 1·5 %. A series of experiments, the results of which are given in Table XIV, confirmed this conclusion at saccharose concentrations between 0·37 and 10·0 %.

Six 50-ml. flasks with varying amounts of sucrose and 0·4 ml. of dialysate from yeast autolysate as constant quantity of activator and 0·5 g. filter-pulp were inoculated with 1 ml. of a 24-hour culture of strain D in 5 % maize mash; total volume 40 ml., made up with tap water; *t*, 37°.

Table XIV

g. of sucrose	0·15	0·30	0·60	1·20	2·400	4·00
Velocity, ml./hr.	18·3	18·1	18·3	17·0	17·0	19·1
% yield of solvents calculated on the sugar	36·3	32·0	30·9	28·5	19·7*	12·1 *

* The fermentation was not completely finished.

In these fermentations with higher sugar concentrations than 1·5 % a decrease in the fermentation velocity was observed when more than 400 ml. gas had been

produced. This inhibition may be ascribed to the fermentation products, especially, according to Tschekan [1935], to the butanol formed. As 400 ml. gas produced correspond approximately to 0·3 % butanol, we may regard this concentration as the limiting concentration at which the inhibiting action of the fermentation products begins. By this observation we got independent evidence of the suitability of the standard sugar concentration, viz. 1·5 %, used in this investigation.

15. *Adsorption of the activator. Influence of acidity on its stability*

Previous to the actual adsorption experiments it was desirable to know the stability of the activator in acid and alkaline media.

(*a*) A mixture of 25·4 ml. of dialysate from yeast autolysate with 9·5 ml. *N* HCl brought the acidity to *p*H 3·45. (*b*) Mixture of 24·1 ml. of dialysate with 5·6 ml. 2*N* NaOH gave *p*H 9·32 measured with the glass electrode. (*a*) and (*b*) were kept for 3 hours in the steamer, neutralized and the activator contents estimated from the rate of a standard fermentation. The amount of activator calculated for 1 A.U. was used. For comparison one experiment was made with untreated dialysate. The experiments were carried out in duplicate. No influence on the activator could be noticed by this treatment, the fermentation rate observed for the untreated was 25·5 ml./hr., for acid-treated (*p*H 3·45) 22·3 ml./hr., for the alkali-treated (*p*H 9·32) 24·8 ml./hr.

At *p*H 12·4 the activator in maize mash is damaged to an appreciable extent when incubated at 37°. The deteriorating effect of the alkali was increased when the alkaline maize mash had been further heated for half-an-hour in boiling water (Table XV).

Table XV

Strain H.

	1. Normal mash ("old" maize)	2. Maize mash 2 days *p*H 12·4 at 37°	3. Like 2 but additional heating
Velocity, ml./hr. mean value of two experiments	16·2	9·2	7·1

The *p*H was adjusted to 12·4 by the addition of 8 ml. 2*N* NaOH to 150 ml. 5 % maize mash; for the readjustment to neutrality an equivalent amount of *N* HCl was used.

Adsorption experiments. As adsorbents of the activator we tried an aqueous suspension of $Al(OH)_3$ (type B according to Willstätter & Kraut [1922]), kaolin, fuller's earth and frankonite. With none of these adsorbents have we been able to effect a complete adsorption of the activator either from the original or from the diluted solution.

Frankonite proved to be the best adsorbing agent in slightly acid solution as may be seen from the following experiment.

To (1) 20 ml. $Al(OH)_3$ suspension, (2) 2 g. of kaolin, (3) 2 g. of fuller's earth, (4) 2 g. of frankonite, in stoppered flasks, 16·7 ml. of an acidified mixture (*p*H 4·0) of dialysate (60 ml.) from yeast autolysate with 6 ml. *N* HCl were added and the volume made up to 30 ml. with water. The flasks were shaken for 5 min., the contents were centrifuged and the residual liquid, after readjustment to *p*H 6·0, tested for activator content. The amount corresponding to 1 A.U. was taken. The rates observed were (1) 13·4 ml./hr; (2) 21·0 ml./hr; (3) 17·1 ml./hr; (4) 12·8 ml./hr; a blank experiment without absorbing agent showed a rate of 18·4 ml./hr.

Assuming that the activator is present in an unfavourable concentration for the adsorbent, a 3-step adsorption of the undiluted activator solution, which was

adjusted to *p*H 3·8, was carried out. 60 ml. dialysate from autolysed yeast were adjusted to *p*H 3·8 by the addition of 7 ml. *N* HCl. The initial activator content amounted to 120 A.U.

I. 5 ml. were taken for the test.

II. 62 ml. of the acid dialysate (111 A.U.) were shaken with 51 g. of frankonite and centrifuged.

III. 40 ml. of the residual liquid from II were shaken with 10 g. frankonite, centrifuged and 5 ml. kept for test.

IV. 23·7 ml. of the residual liquid from III were again shaken with 10 g. of frankonite and centrifuged. 5 ml. were taken for the test. The residual liquids destined for the test including the one without adsorbent were adjusted to the original *p*H (6·2). The amount corresponding to 1 A.U. was used for the test fermentation.

Result

		Dry weight of residual liquid in %
Fermentation velocity without adsorption (mean of 3 values)	14·9 ml./hr. = 0·74 A.U.	100
1st step adsorption (mean of 2 values)	11·6 ml./hr. = 0·55 A.U.	96·8
2nd step adsorption (mean of 2 values)	7·1 ml./hr. = 0·35 A.U.	80·7
3rd step adsorption (mean of 2 values)	7·0 ml./hr. = 0·34 A.U.	60·8

Although conditions as favourable as possible for the adsorption of the activator and a relatively high amount of adsorbent were used, only about 50 % of the activator was adsorbed after two successive adsorption steps. The third step did not increase this value. We tried also to separate the activator adsorbed from the frankonite, both in the first and in the second step of the adsorption. The residual liquid from the frankonite which still contains an appreciable amount of activator was removed by washing with acidified water in the centrifuge and the frankonite suspended in 0·2 % disodium phosphate solution at 37°. No activator however was found in the solution obtained; probably on account of the small affinity of the activator for the frankonite, it was lost during the washing process.

16. *Glycine, alanine and β-alanine as nitrogen supply in the fermentation of sucrose*

The question whether asparagine is a specific source of nitrogen which can be replaced only by closely related dicarboxylic acids, e.g. glutamic acid, still requires investigation before a conclusive answer can be given. In this section we restrict ourselves to the fundamental question, viz. if the action of asparagine is specific, or if simple amino-acids such as glycine or alanine are able to replace it. The influence of β-alanine was of special interest in this connexion as Williams & Rohrwald [1936] described this substance as an extremely effective growth stimulant for yeast.

Standard conditions were used, viz. total volume 40 ml. (tap water), substrate 0·6 g. sucrose, inoculant 1 ml. culture (strain D) in 5 % maize mash, 0·5 g. filter-pulp. For the experiments with glycine and alanine the inoculant added served also as a source of activator; in some experiments with β-alanine, additional activator was used in the form of the dialysate D 1, as shown in Table XVI; $t = 37°$.

As indicated in Table XVI none of these substances exhibits any effect comparable with that of asparagine (compare these results with those given in Tables X and XII and Fig. 4).

Table XVI. *Influence of glycine, alanine and β-alanine on the fermentation velocity*

Glycine millimol	1·0	—	—	—	—	—	—	—	—
Alanine millimol	—	1·0	—	—	—	—	—	—	—
β-Alanine millimol	—	—	1·0	1·0	—	1	—	1	—
Dialysate D 1, ml.	—	—	—	0·01	0·01	0·04	0·04	0·10	0·10
Fermentation velocity, ml./hr.	0·6	0·0	0·7	0·8	0·8	1·6	1·6	4·9	4·9

17. *Lactoflavin and cozymase as activator*

On account of the stability of the activator towards the action of dilute alkali (pH 9·3) and the difficulty of adsorbing the active principle on frankonite in neutral and slightly acid solution, we assumed that lactoflavin would be without any activating effect. Yet we observed occasionally the appearance of a yellow-green fluorescent dye (alkali-labile and therefore not identical with the fluorescent dye described on p. 629) in fermented maize or rice mash and also when we used a dialysate of yeast autolysate as activator which had been prepared from Vienna yeast. This fluorescence was not observed in the fermented liquid of "old" maize or in any of the dialysates obtained from Polish yeast. However, it was thought advisable to test the influence of pure lactoflavin on the fermentation. It was also of interest to learn if the hydrogen carrier in the alcoholic fermentation, viz. cozymase, is able to play a similar role in the butanol-acetone fermentation—alone or in combination with lactoflavin with and without additional asparagine. Each flask contained 0·6 g. sucrose, 1 ml. $M/50$ phosphate buffer pH 6·8 and 0·4 g. filter-pulp. In flasks Nos. 8 and 9 the sterile cozymase solution was added immediately before inoculating; inoculant 1 ml. of a 24-hour culture (strain D) in 5 % maize mash; total volume 40 ml. made up with tap water. The cozymase solution contained 100 γ per ml. in sterile water, the lactoflavin solution 60 γ per ml. and a 1·5 % asparagine solution was used. The amounts used are shown in Table XVII.

Table XVII

$t = 37°$.

No. of flask	1	2	3	4	5	6	7	8	9	10	11
ml. asparagine sol.	5	5	5	5	—	—	5	5	5	2	2
ml. cozymase sol.	—	0·5	1·0	2·0	2·0	2·0	2·0	2·0	2·0	—	—
ml. lactoflavin sol.	—	—	—	—	—	1·0	1·0	—	1·0	1·0	3·0
Fermentation velocity, ml./hr.	1·8	1·8	1·8	1·9	0·7	0·6	2·2	1·9	2·3	1·8	0·0

The inhibiting effect of lactoflavin in relatively large amounts, e.g. 180 γ/40 ml., has also been observed at concentrations of 300 and 600 γ/40 ml. in independent experiments. It seems to us that the flavin acts like oxygen in these concentrations and prevents the formation of the necessary anaerobic conditions. The results in Table XVII show clearly that under the conditions employed lactoflavin and cozymase have no activating effect upon the fermentation—either alone or combined, with or without the presence of asparagine.

III. Discussion

From the results of our experiments it has become evident that two main factors are necessary for a normal fermentation of carbohydrates by *Clos. acetobutylicum* (Weizmann): asparagine and a substance of unknown constitution to which we have always referred as "the activator". Both belong, generally speaking, to the organic catalysts, as in their absence the fermentation does not

proceed at all or at least very slowly. On account of their stability towards heat, dilute acid and alkali, their low molecular weight and their indifference towards the substrates of the fermentation which they activate they can be classified as coenzymes.

The coenzymes known are characterized as substances of definite structure with a specific action at extremely low concentrations. 1 mol. of coenzyme may catalyse the transformation of an infinite number of substrate molecules, but in practice it gradually becomes inactive owing to some side reaction.

A critical consideration reveals that the activator system concerned in the activation of the butanol-acetone fermentation does not completely correspond to this definition of a coenzyme, especially regarding the concentration of the compounds in question. Asparagine, e.g., displays its optimum action at a relatively high concentration, viz. 0·2 millimol per 40 ml., enough to supply the nitrogen requirements of the bacteria. This means that asparagine acts as a specific nitrogen supply if it is used up during the propagation of the bacteria. If however the organic residue resulting from its deamination (fumaric acid) remains unchanged and combines again enzymically with ammonia present in the medium to give asparagine or aspartic acid, then they behave like specific nitrogen carriers. Quastel & Woolf [1926] have brought forward experimental evidence that aspartic acid may function biologically as a nitrogen carrier. Although it has not yet been proved, we strongly believe that these compounds play a similar role in the metabolism of *Clos. acetobutylicum*. The amount of dry matter which is required from the "activator" in order to produce maximum fermentation velocity, viz. 8·7 mg. = 1 A.U. in the case of our preparation D 2, cannot yet be quoted as an argument against our assumption that the activator is a coenzyme, as the purification of the activator has not been carried very far. The fact that the activator is not used up during the fermentation but remains at a fairly high concentration in the fermented liquid (spent wash) supports strongly the assumption of the coenzymic nature of the activator.

We cannot decide from our experimental results whether the "activator" is a single substance or not. The sudden fall of the asparagine curve in Fig. 4, below 0·2 A.U. may possibly be explained by the assumption that the amount of an essential compound has been diminished below a certain critical concentration, at which the influence of this compound on the fermentation velocity is a different one, although we may assume with the same right that 0·2 A.U. is a "critical" activator concentration for the case of the activator being a single substance.

Furthermore we have shown in our experiments that it is possible to adsorb 26 % of a given amount of activator on frankonite at *p*H 3·8 while the bulk of dry matter (97 %) remained in solution. In our adsorption experiments, the difficulty of adsorbing a low-molecular substance was further increased by the fact that the dialysates which we used were lacking in high-molecular co-adsorbents, in the presence of which adsorption takes place much more easily.

The fermentation system does not necessarily require highly complex protein, such as peptone and zein, for nutritive and activating purposes as Weinstein & Rettger concluded from their experimental results. We found that the bacteria are able to synthesize their own protein from low-molecular sources in presence of the complete activator system which by itself is constituted of low-molecular substances. The power of the bacterial enzymes to synthesize protein is in fact just as remarkable as their known proteolytic activity. We found regularly a decrease of amino-N from 60 to 30 % of the total N, which may well be compared with the increase of the ratio amino-N to total N from a negligible value in maize to 31·5 % in spent wash. This ratio of amino-N/total N seems to be the

most suitable for the bacteria as it is reached independently of the nature of the nitrogenous starting material.

In connexion with the dual activator system, activator + asparagine, which, as we believe, exists in the propionic acid fermentation, in the butanol-acetone fermentation and also, though not definitely proved, in the fermentation of carbohydrates with butyric acid bacteria, we find it not out of place to emphasize that the optimum asparagine concentration is the same for all three types of fermentation. Glycine and alanine proved to be equally unable to replace asparagine.

Now the question arises: In what manner is the nitrogen carrier, viz. asparagine, linked with the activator? Let us consider two principal possibilities:

1. The activator acts like biotin, it stimulates the propagation of the bacteria. Asparagine promotes the propagation by providing an efficient nitrogen supply.

The concentration of enzymes available for the fermentation of carbohydrates increases proportionally to the number of bacteria thus causing a more vigorous fermentation than without the activating system.

2. The activator is an auxiliary substance for the enzyme system of the bacteria, it facilitates especially the hydrogen transfer either from the substrate or from the enzyme to butyric, or possibly to β-hydroxybutyric, acid.

The crude activator solutions contain also a growth-promoting factor which in combination with asparagine as nitrogen carrier limits the propagation of the bacteria and therewith the enzyme concentration available.

The first possibility based on a few natural suppositions does not explain the fact that, despite the satisfactory growth observed at low activator concentrations, the yield of solvents may be low, especially in the presence of chalk, while at higher activator concentration the trapping effect of added neutralizing agents does not influence the yield of solvents. Therefore the second possibility deserves to be considered although it is more complicated and involves more suppositions.

Concerning the influence of asparagine it may be added that the organic skeleton of this nitrogen carrier may also play a role in the oxidation-reduction system of the fermentation. According to the structural relationship the role played by asparagine may be similar to that of fumarate in the respiration of muscle and other tissues [Szent-Györgyi, 1935].

SUMMARY

1. Both the yield and the fermentation velocity of a 2·5% maize mash inoculated with *Clos. acetobutylicum* (Weizmann) form the basis of the definition of a normal butanol-acetone fermentation.

2. At least two compounds are required for a normal butanol-acetone fermentation in synthetic media, viz. asparagine and a substance of still unknown constitution. These two compounds are separately without any action.

3. The "activator" is a low-molecular thermostable substance, which occurs in green plants and seeds. Yeast is a rich source of activator, and the preparation of an activator solution therefrom by autolysis and dialysis has been described.

4. With an activator preparation from yeast we have been able for the first time to effect a normal butanol-acetone fermentation in a medium which, except for the activator, was strictly synthetic.

5. Concerning the distribution of the activator in maize we were unable to confirm the experimental results of Weinstein & Rettger [1933] who found that

the activator is contained in the zein of the maize. We have shown that zein contains only a small fraction of the total activator content in maize.

6. No highly complex proteins are required such as peptone or prolamins for a normal butanol-acetone fermentation. The bacteria are able to synthesize their own protein.

7. Lactoflavin and cozymase have no essential influence on the butanol-acetone fermentation either alone or in combination.

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