THE NUTRITION OF CLOSTRIDIUM KLUYVERI

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Received for publication November 3, 1947

When a culture medium containing ethanol, calcium carbonate, and the usual inorganic salts is inoculated with black mud and incubated anaerobically at 30 C, a spontaneous fermentation soon begins that often results in the conversion of much of the alcohol to a mixture of acetic, butyric, and caproic acids (Barker, 1937*b*). A considerable quantity of methane is also evolved. In such fermentations the dominant organisms are *Methanobacterium omelianskii* and *Clostridium kluyveri*. The former organism causes the oxidation of ethanol to acetic acid and the coupled reduction of carbon dioxide to methane, whereas the latter organism has been shown to be responsible for the formation of butyric and caproic acids (Barker and Taha, 1942). Until recently, however, it was not possible to obtain an adequate understanding of the chemical reactions catalyzed by *C. kluyveri* because pure cultures of this organism could not be grown except in very complex media, and even then growth was scanty and the yield of caproic acid was low. As a preliminary to further metabolic studies it was necessary therefore to investigate the nutrition of the organism in some detail. In the present paper it is shown that *C. kluyveri* has unusual nutritional requirements that can be satisfied by the use of a relatively simple, completely synthetic medium.

EXPERIMENTAL PROCEDURES AND RESULTS

Basic growth requirements. In previous studies appreciable growth of *C. kluyveri* could only be obtained in media containing inorganic salts, a reducing agent such as thioglycolate, ethanol, and an abnormally high concentration of yeast autolysate (Barker and Taha, 1942). An earlier attempt to replace the yeast autolysate by some other nutrient was unsuccessful, so the conclusion was reached that yeast autolysate contains one or more substances of special nutritive value for *C. kluyveri* that are not present in most other complex media. At the beginning of the present investigation it was therefore decided to fractionate yeast autolysate in order to identify its active constituents. The following experiments were done with strain K.1.

The first fractionation procedure was a separation of volatile acids by steam distillation. It was at once found that the distillate was highly active in stimulating growth in a basal medium containing ethanol and a small quantity (1 vol. per cent) of yeast autolysate, whereas the residue from the steam distillation was completely inactive. By the Duclaux distillation method the volatile acid fraction was shown to consist largely of acetic acid, and the growth-stimulating activity of the fraction could be entirely accounted for by the acetate it contained.

The addition of larger amounts of acetate to the basal medium improved the growth far beyond the highest level previously attained with maximal concen-

trations of yeast autolysate. Table 1 shows that growth increases from zero with no added acetate to a maximum when the acetate concentration is about 0.8 per cent.

When acetate is added in excess, the amount of growth is dependent upon the supply of ethanol. Table 2 illustrates this relation. With 1 per cent sodium acetate and 0.8 per cent ethanol, rapid and abundant growth is obtained in an otherwise adequate medium.

When adequate amounts of ethanol and acetate are provided only a small quantity of yeast autolysate or other similar material is required. For example,

TABLE 1

*The effect of acetate concentration on growth**

$CH_3COONa \cdot 3H_2O$ ADDED %	MAXIMAL TURBIDITY†
0.0	0
0.1	14
0.2	36
0.4	60
0.8	64

* The medium contained the inorganic salts of medium 1, 0.4 per cent ethanol, and 20 vol. per cent of acetate-free yeast autolysate.

† Measured with a Klett-Summerson photocolorimeter.

TABLE 2

*The effect of ethanol concentration on growth**

ETHANOL ADDED g/100 ml	MAXIMAL TURBIDITY† (2 − log G) × 1,000
0.0	0
0.1	27
0.2	51
0.4	70
0.8	104

* Medium 1 contained 0.075 per cent Difco yeast extract, 1.0 per cent sodium acetate, and the indicated amounts of ethanol.

† Measured with an Evelyn photocolorimeter.

50 mg of Difco yeast extract per 100 ml of medium are sufficient to allow maximal growth. Even this small amount of yeast extract can be entirely replaced by two growth factors, biotin and *para*-aminobenzoic acid, provided they are added at the rate of 0.3 μg and 5 μg, respectively, per 100 ml.

In common with many other microorganisms, *C. kluyveri* requires carbon dioxide as a nutrient. In our early experiments, which were carried out entirely in test tubes, no carbonate was added directly to the medium but some carbon dioxide was supplied by the pyrogallol-carbonate seal used to remove oxygen. When the organism is grown under such conditions in a liquid medium, it pro-

duces a uniform turbidity that reaches a maximum in 24 to 48 hours. The cells are actively motile and generally occur singly. Only after growth has ceased do they begin to settle and form a conspicuous sediment. Large cultures of 1 to 10 liters in the same medium behave quite differently even when provided with a small pyrogallol-carbonate seal. The bacteria develop much more slowly and occur mainly as large clumps of immotile cells resting on the bottom or adhering to the walls of the vessel. The bulk of the medium remains perfectly clear. Sometimes such cultures even fail to develop at all. These phenomena were found to be due to a deficiency of carbon dioxide. The same type of growth can be obtained in test tube cultures by replacing the carbonate in the anaerobic seal with sodium hydroxide or by using a solution of a chromous salt instead of pyrogallol as an oxygen absorbent.

The addition of a small amount of sodium carbonate to the medium entirely eliminates the above-mentioned signs of carbon dioxide deficiency in both large and small cultures. The quantity of sodium carbonate required for maximal growth is of the order of 0.1 to 1 mg per 100 ml. For routine cultures we have made a practice of adding 10 mg Na_2CO_3 per 100 ml medium.

On the basis of the foregoing results the following synthetic medium (medium 1), which supports excellent growth of *C. kluyveri*, was developed: ethanol, 0.8 g; sodium acetate hydrate, 0.8 g; M/1 pH 7.0 KH_2PO_4-Na_2HPO_4 buffer, 2.5 ml; $(NH_4)_2SO_4$, 50 mg; $MgSO_4 \cdot 7H_2O$, 20 mg; $CaSO_4 \cdot 2H_2O$, 1 mg; $FeSO_4 \cdot 7H_2O$, 0.5 mg; $MnSO_4 \cdot 4H_2O$, 0.25 mg; $NaMoO_4 \cdot 2H_2O$, 0.25 mg; biotin, 0.3 μg; *para*-aminobenzoic acid, 5 μg; sodium thioglycolate, 50 mg; Na_2CO_3, 10 mg; and glass-distilled water, 100 ml. The thioglycolate can be replaced by 20 mg $Na_2S \cdot 9H_2O$, which is best added after autoclaving. When a strictly synthetic medium is not required, the growth factors may be replaced by 50 to 100 mg Difco yeast extract. It is best to inoculate the medium very soon after it is autoclaved. Since *C. kluyveri* is an obligate anaerobe, the culture medium must be protected from oxygen by means of a pyrogallol-carbonate seal or some similar device. When an active inoculum is used, good growth is obtained in 24 hours at 35 C.

Utilization of organic substrates other than ethanol and acetate. After the fact that both ethanol and acetate are needed as macronutrients had been established, the possibility of replacing these substrates by structurally related compounds was explored.

In testing for acetate substitutes a basal medium containing an excess of ethanol (1 per cent) was used and the compound to be tested was added in a concentration of 0.1 to 0.4 per cent. In some experiments, when it was thought that substrates to be tested might be inhibitory in such concentrations, a small, limiting amount of acetate, usually 0.1 per cent, was added both to the experimental culture and to the control. Either growth stimulation or toxicity could then easily be detected. In all experiments growth was measured with either a Klett-Summerson or an Evelyn photocolorimeter.

The following compounds were tested: propionate, butyrate, valerate, caproate, lactate, pyruvate, glycine, *alpha*-alanine, *beta*-alanine, and *alpha*-aminobutyrate. Positive results were obtained in these experiments only with propionate and

butyrate, although evidence has been obtained from metabolic experiments (Bornstein and Barker, 1948) that valerate can also be utilized to a slight extent as a substitute for acetate. Typical results obtained with several fatty acids are given in table 3.

In concentrations below 0.5 per cent, propionate is almost as good a substrate as acetate. Growth occurs rapidly and is roughly proportional to the amount of propionate added. At concentrations above 0.5 per cent, propionate is somewhat inhibitory as compared with acetate and both the rate of growth and yield of cells are less. The bacteria can still multiply, however, in media containing as much as 2 per cent sodium propionate and 2 per cent ethanol. Two per cent acetate is also somewhat inhibitory, though reduced growth occurs even with 2.5 per cent acetate and ethanol. With high substrate concentrations a period of

TABLE 3

*The growth of Clostridium kluyveri on ethanol and higher homologues of acetic acid**

SODIUM SALT ADDED (g/100 ml)		MAXIMAL TURBIDITY†
Acetate	0.2	38
"	0.4	70
Propionate	0.1	16
"	0.4	78
"	0.1, acetate 0.2	53
Butyrate	0.2	20‡
"	0.1, acetate 0.2	42
Valerate	0.1	0
"	0.1, acetate 0.2	32
Caproate	0.1	0
"	0.1, acetate 0.2	32

* Medium 1 was used containing 0.65 per cent ethanol, 0.5 vol. per cent yeast autolysate, plus the indicated fatty acid salts.

† Measured with a Klett-Summerson photocolorimeter.

‡ Growth began after 35 days' incubation.

adaptation appears to be required; two or three transfers in the same medium are needed before maximal growth is attained.

Butyrate is not as effective a substrate as acetate or propionate. It is readily metabolized by *C. kluyveri* when a small amount of acetate is also provided, but in the absence of the latter a long lag period is often observed before the bacteria begin to develop normally. The best growth with butyrate is only about half that obtainable with an equimolar quantity of acetate. This is to be expected since the catabolism of acetate involves two steps, acetate to butyrate to caproate, whereas with butyrate as a substrate only the second step occurs (Bornstein and Barker, 1948; Barker, Kamen, and Bornstein, 1945).

In testing for ethanol substitutes essentially the same methods were used as in testing for acetate substitutes, except that the medium contained 1 per cent acetate and, in some experiments, a small amount of ethanol. The compounds tested were propanol, butanol, lactate, and pyruvate. Negative results were ob-

tained with all four substrates. Lactate and pyruvate were tested repeatedly under various conditions so that we feel that the inability of strain K.1 to use these compounds is conclusively established. Unfortunately the experiments with propanol and butanol were not done in the presence of ethanol, so that the negative results obtained thus far are not completely conclusive in view of the possibility that even 0.1 per cent solutions of these compounds may be inhibitory to the organism.

Utilization of carbon dioxide. After carbon dioxide was shown to be essential for the normal growth of *C. kluyveri*, it seemed worth while to look for an actual utilization of carbon dioxide in growing cultures. The possibility of detecting carbon dioxide utilization by chemical means was enhanced by the observation that no carbon dioxide appeared to be formed in the fermentation process.

A preliminary experiment showed that there is a small disappearance of carbon dioxide of the order of magnitude of 0.1 to 0.2 mM per 100 ml of medium 1 and that it is dependent upon the growth of the organism. There was no loss of carbon dioxide from cultures placed in a refrigerator immediately after being

TABLE 4

The dependence of carbon dioxide utilization on substrate concentration

SUBSTRATE CONCENTRATION*		INITIAL CO_2	FINAL CO_2	CO_2 UPTAKE
	%	*mM/100 ml*	*mM/100 ml*	*mM/100 ml*
Sodium acetate	0.1	0.28	0.20	0.08
" "	0.2	0.28	0.13	0.15
" "	0.4	0.28	0.08	0.20
Sodium propionate	0.1	0.22	0.17	0.05
" "	0.2	0.22	0.12	0.10
" "	0.4	0.22	0.07	0.15

* In medium 1 containing 0.4 per cent ethanol and 0.5 vol. per cent yeast autolysate.

inoculated, and when a culture was incubated for a short period until growth started and was then placed at 5 C, the loss was greatly reduced.

A second experiment was designed to demonstrate a possible correlation between the amount of carbon dioxide utilized and the quantity of cells formed. Medium 1 was used, containing 0.01 per cent Na_2CO_3 and varying concentrations of acetate or propionate. The tubes used as culture vessels were fitted with "oxsorbent" seals and were tightly closed with rubber stoppers during the incubation period. After growth had ceased, the cultures were analyzed for carbon dioxide. The initial carbon dioxide content of the media was obtained by analyzing a second set of inoculated tubes which had been kept at 5 C.

The results of this experiment are presented in table 4. It can be seen that in all cultures a small but significant amount of carbon dioxide disappeared. With either acetate or propionate the carbon dioxide uptake increased with substrate concentration and, consequently, with the yield of cells. By comparison of the data in tables 1 and 4 it may be concluded that there is an almost direct proportionality between cell yield and carbon dioxide uptake with acetate as a sub-

strate. By carrying out an ethanol-acetate fermentation in the presence of carbon dioxide labeled with C^{14}, it was found that at least 70 per cent of the carbon dioxide utilized could be recovered in the washed bacterial cells. Little or none of the labeled carbon from the carbon dioxide went into the fatty acids that are the main products of the fermentation (Bornstein and Barker, 1948).

Enrichment cultures. *C. kluyveri* was originally isolated from black mud by the use of an enrichment medium containing ethanol and calcium carbonate as the main ingredients. This medium consistently allows the enrichment of methane-producing bacteria but does not always yield good cultures of *C. kluyveri*.

Now that the unique nutritional requirements of *C. kluyveri* are known, its enrichment from natural sources can be achieved with greater certainty and rapidity by the use of medium 1, slightly modified. Biotin and *para*-aminobenzoic acid are used in preference to yeast extract since even small amounts of the latter stimulate the growth of a variety of amino-acid-fermenting bacteria that interfere with the isolation of *C. kluyveri*. In order to eliminate sulfate-reducing and methane-producing bacteria as far as possible, it is desirable to replace most of the sulfate in medium 1 by chloride and to reduce the carbonate to 1 mg per 100 ml. In this way a highly specific enrichment medium is obtained. The inoculum should be pasteurized to eliminate nonsporulating bacteria and, of course, anaerobic conditions must be maintained.

By the use of this enrichment method we have obtained active cultures of *C. kluyveri* in a few days from black mud. Such cultures contain relatively few bacteria of other types. The isolation of pure cultures from the enrichments is very easily accomplished by the shake culture technique.

DISCUSSION

The most notable result of the present investigation is the demonstration that an anaerobe belonging to the group of butyric acid bacteria can satisfy its energy requirements by metabolizing a mixture of ethanol and acetate. All other butyric acid bacteria require a more complex substrate such as a carbohydrate, lactate, or pyruvate (Bhat and Barker, 1947), or one of the amino acids (Barker, 1937*a*). Not only does *C. kluyveri* not require such compounds but it is unable to utilize them. In order to grow anaerobically it must have ethanol and acetate or one of its close homologues. These substrates are more or less quantitatively converted into a mixture of higher fatty acids (Barker, 1947; Barker, Kamen, and Bornstein, 1945; Bornstein and Barker, 1948).

So far no substrate has been found that can be substituted for ethanol. Even propyl alcohol does not appear to be utilized by *C. kluyveri*, though there is still a slight possibility that it can be metabolized under special conditions. Lactate and pyruvate are definitely excluded as ethanol substitutes.

Acetate can be replaced by only two compounds tested thus far, propionate and butyrate. Fatty acids with longer chains do not support growth, though there is evidence that valerate may be metabolized to a limited extent. According to present information any alteration of the acetic acid molecule, other than the substitution of a methyl or ethyl group for one of the hydrogens, destroys its usefulness for this organism.

There is nothing unusual about the fact that *C. kluyveri* requires carbon dioxide for growth, but its ability to cause a net disappearance of carbon dioxide is noteworthy. Only a relatively few heterotrophic bacteria can do this, since such organisms generally form more carbon dioxide than they consume. Most of the carbon dioxide fixed by heterotrophic anaerobes studied thus far is found in catabolic products. *C. kluyveri* is exceptional in this respect since most of the assimilated carbon is present in the bacterial cells.

The demonstration that *C. kluyveri* needs two simple organic compounds for its energy metabolism raises the question regarding the existence of other obligate anaerobes having similar nutritional requirements. A few such organisms are already known. Perhaps the first to be reported was *Clostridium sporogenes*, which grows on a mixture of amino acids, some of which are oxidized while others are reduced (Fildes and Richardson, 1935; Stickland, 1934). A similar type of energy metabolism has been established for *Clostridium botulinum* (Fildes, 1935; Clifton, 1940). Recently the growth of *Clostridium lacto-acetophilum* on lactate and glycerol was shown to be dependent upon the presence and simultaneous utilization of acetate (Bhat and Barker, 1947). The substrate requirements of this organism are very similar to those of *C. kluyveri*, differing only in the substitution of lactate for ethanol. Undoubtedly other anaerobic bacteria requiring more than one organic compound for their energy metabolism will be found in the future.

Certainly the best way to find such organisms is by the use of the enrichment culture method. It has been shown that this method can be applied very successfully in the isolation of *C. kluyveri* and *C. lacto-acetophilum* from natural sources. Since a two-substrate requirement results from the need for one compound as a reductant and another as an oxidant, enrichment media for new bacteria of this type should contain a pair of potentially oxidizable and reducible compounds as the main ingredients. As reductants, compounds such as simple primary and secondary alcohols, glycols, polyalcohols, fatty acids, amino acids, hydroxy acids, and hydrogen gas might be used. As oxidants, a variety of fatty acids, amino acids, unsaturated and keto acids, ketones, aldehydes, and even polyalcohols could be tested. The number of combinations of oxidants and reductants is almost unlimited. Sugars or other readily fermentable compounds should be avoided since they favor the development of the usual one-substrate organisms.

A study of two-substrate organisms may contribute a great deal to our understanding of anaerobic transformations of organic materials under natural conditions. Such processes nearly always involve a multiplicity of organisms and substrates. Winogradsky (1932) has pointed out certain limitations of the pure culture method when applied to complex problems of soil microbiology. For a complete understanding of microbial processes in nature the interaction of substrates as well as of organisms must be taken into consideration.

SUMMARY

It has been shown that *Clostridium kluyveri* grows very well in a synthetic medium containing inorganic salts, ethanol, acetate, biotin, and *para*-amino-

benzoic acid as the main ingredients. No compound has been found that can be substituted for ethanol. Acetate can be replaced by propionate and, less adequately, by butyrate. *C. kluyveri* does not attack glucose, pyruvate, or other common fermentation substrates. Carbon dioxide is consumed during growth, most of it being used for the synthesis of cell constituents. By the use of the synthetic medium the isolation of *C. kluyveri* from natural sources is greatly facilitated.

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