BACTERIAL METABOLISM

I. THE REDUCTION OF PROPIONALDEHYDE AND OF PROPIONIC ACID BY CLOSTRIDIUM ACETOBUTYLICUM

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Carbohydrates are fermented by Clostridium acetobutylicum and related microorganisms with the formation of acetic acid, n-butyric acid, acetone, ethanol, and n-butanol. It has been conclusively proved that the butanol arises by the reduction of the butyric acid (1, 2). As yet, however, there is no exact information as to the sequence of reactions by which butyric acid is formed from glucose by such microorganisms, although several investigators have advanced speculative schemes to account for such a transformation. The nature of these schemes has recently been described in a number of reviews (3–6) and need not be considered here, except to note that, with the exception of the hypothesis advanced by Neuberg and Arinstein (7), all of these schemes have followed the early suggestion of Bühner and Meisenheimer (8) that butyric acid is formed in some manner from aldol. The latter is assumed to arise from the condensation of 2 molecules of acetaldehyde formed in turn from the carbohydrate, although as yet neither of these substances has been detected in fermentation induced by butanol-forming microorganisms.

In some experiments made several years ago, the writers attempted to determine if such bacteria could utilize either aldol or its dehydration product, crotonic aldehyde. The latter was readily reduced to n-butanol by actively fermenting cultures of Clostridium acetobutylicum, while aldol proved to be of such great toxicity to the bacteria, that cessation of fermentation invariably followed its addition in even minute amounts to actively fermenting cultures. In consequence, experiments of this type were
abandoned. Recently a similar experience with aldol has been recorded by Johnson, Peterson, and Fred (9). The distinct toxicity displayed by aldol when added to cultures of microorganisms which supposedly form it in the course of their chemical activities cannot be taken as a negation of its possible importance in the formation of butyric acid, for it is quite conceivable that in the sequence of reactions occurring within the bacterial cell such a toxic intermediary substance may have but a transitory intracellular existence.

One might reasonably expect to obtain indirect evidence relative to the role of aldol in the formation of butyric acid, if it could be shown that the addition of acetaldehyde to actively fermenting cultures led to an enhanced yield of butyric acid and butanol. Such an experiment presents a number of technical difficulties, including the volatility of the aldehyde, the necessity of working with small quantities of the substance to avoid toxic interference with the normal course of the fermentation, and the difficulties attending the exact analytical determination of small increments in the yield of the various fermentation products. In order to circumvent these difficulties a similar type of experiment with the less volatile propionaldehyde in place of acetaldehyde has been conducted.

The former aldehyde of course is not a normal intermediary in the chain of chemical events induced by butanol-forming microorganisms. Theoretical justification for the employment of propionaldehyde in such an experiment is, however, to be found in the present day concept of the mechanism of enzyme action, according to which the specificity of enzyme action is largely determined by the structural nature of particular groupings of the enzyme and substrate molecules. Consequently one may reasonably expect the enzymes of a butyric acid-forming microorganism to form from propionaldehyde products analogous to those supposedly formed from acetaldehyde. If such were the case, on the basis of the aldol hypothesis one would anticipate the formation from propionaldehyde in fermenting cultures of one or more of the following aldols: \( \alpha \)-methyl-\( \beta \)-hydroxyvaleraldehyde, \( \beta \)-hydroxyvaleraldehyde, and \( \alpha \)-methyl-\( \beta \)-hydroxybutyraldehyde, according to whether the propionaldehyde underwent condensation respectively with itself or with acetaldehyde in either of the two theoretically possible modes. In subsequent reactions these re-
spective aldols should form \( \alpha \)-methylvaleric acid and 2-methylbutanol, \( n \)-valeric acid and \( n \)-pentanol, and \( \alpha \)-methylbutyric acid and 2-methylbutanol. The detection of any one of these products among the end-products of the fermentation of glucose by \textit{Clostridium acetobutylicum} in the presence of propionaldehyde would offer convincing proof of the validity of the aldol hypothesis.

Experimentally none of these anticipated end-products was found in cultures of \textit{Clostridium acetobutylicum} to which propionaldehyde was added, although under the conditions of the experiment their detection should have presented no difficulty. Aside from the normal products of the fermentation, the only other substance obtained was \( n \)-propanol, obviously formed by the reduction of the propionaldehyde. As a result it appears unlikely that the aldol condensation plays a significant rôle in the formation of butyric acid and \( n \)-butanol from glucose by \textit{Clostridium acetobutylicum}. In this connection it is of interest to note that Johnson, Peterson, and Fred (9) have recently shown that pyruvic acid, which is generally conceded to be the immediate precursor of acetaldehyde in fermentations, is readily fermented by \textit{Clostridium acetobutylicum} without any noteworthy increment in the yields of butyric acid and butanol.

In the fermentation under discussion butyric and acetic acids are reduced to the corresponding alcohols. According to Desborough \textit{et al.} (10) and Speakman (2), the latter acid is also converted to acetone. According to Kluyver and Donker (11), this ketone arises by the condensation of acetaldehyde with acetic acid to form acetoacetic acid which in turn is decarboxylated to acetone, as first suggested by Raistrick and Clark (12). One might expect propionaldehyde to behave analogously, forming methyl ethyl ketone, but this product could not be detected in the experiments outlined above.

When propionic acid was incorporated in the mash fermented by \textit{Clostridium acetobutylicum} it was reduced to propionaldehyde and \( n \)-propanol without the production of any ketones whose origin might be traced to this acid.

**EXPERIMENTAL**

**Materials**—The strain of \textit{Clostridium acetobutylicum} used in the present experiments was donated by Mr. Horace Hall, who kindly supplied us with a number of cultures. Previous experience with
this strain had shown it to be an exceedingly vigorous fermenter. As a result of some preliminary experiments made with this organism in media to which Eastman propionaldehyde was added, it was found that the commercial product was decidedly toxic even when added in minute amount to vigorously fermenting cultures. Upon fractionation the crude material was found to consist of a mixture of propionaldehyde and a higher boiling fraction, presumably the aldol, α-methyl-β-hydroxyvaleraldehyde. The high boiling material was found to be very much more toxic to the cultures than was pure propionaldehyde. The latter, boiling at 48.8–49°, was obtained in about 40 per cent yield by distillation of the commercial product through a 1 meter packed column. Only such purified material was used in the large scale experiment described below.

Reduction of Propionaldehyde—A seeding culture of Clostridium acetobutylicum was prepared by inoculating 400 cc. of sterile 5 per cent corn mash and incubating at 37° for 24 hours. This culture was then added to a sterile mixture composed of 8 gm. of dipotassium phosphate, 8 gm. of monopotassium phosphate, 70 gm. of Merck’s peptonc, and 900 gm. of invert sugar in 12 liters of tap water. After 24 hours incubation at 37°, by which time a vigorous fermentation was in progress, the addition of the propionaldehyde was begun. For this addition 36.3 gm. of the aldehyde were dissolved in 1100 cc. of sterile water. This solution was run into the fermenting mixture at a rate of 20 to 25 cc. per hour by means of a tube reaching to the bottom of the fermentation flask. At the expiration of 72 hours the fermentation had ceased. It is perhaps of interest to note that in similar experiments without the addition of propionaldehyde the period of active fermentation is of essentially the same duration.

The fermented mixture was made definitely alkaline with sodium hydroxide and distilled until a volume of distillate equal to 8/15 of the initial volume had been collected. The distillate was redistilled through a 150 × 2 cm. jacketed packed column of the total condensation variable take-off type, which was operated with a reflex ratio of 50:1. Three definite fractions boiling at 55°, 88°, and 92–93° were obtained. The latter, as is characteristic of the butanol-water azeotrope, separated into two layers. Distillation was continued until about 800 cc. of water had been
collected. Each of the above fractions, as well as the intermediary fractions, was saturated with anhydrous potassium carbonate and the non-aqueous layers separated. These were washed with a saturated solution of sodium bisulfite, dried over anhydrous copper sulfate, and refractionated through a 40 X 1 cm. column of beads, whereby there were obtained 112 gm. of n-butanol, 8 gm. of ethanol, and 27 gm. of n-propanol. Each of these alcohols was identified by conversion to the 3,5-dinitrobenzoate in the usual manner. From the bisulfite solution, 48 gm. of acetone were obtained.

The alkaline residue from the first distillation was extracted with ether. The latter upon evaporation yielded 3 gm. of a yellow oily material insoluble in water and sodium carbonate solution, but readily soluble in acetone. It was dissolved in a minimum quantity of ether, dried over anhydrous sodium sulfate, and, following the removal of the solvent, an attempt was made to distil it. Nothing passed over below 170°, at which temperature decomposition accompanied by a distinct odor of acrolein began.

The aqueous residue from the ether extraction was made acid to Congo red with sulfuric acid and steam-distilled. The distillate was neutralized with barium hydroxide and evaporated to dryness. The mixed barium salts were decomposed with dilute sulfuric acid and the liberated acids extracted with ether and transferred to 100 cc. of water. The resulting solution was subjected to a Duclaux distillation, whereby the mixture was found to consist of only acetic and butyric acids.

Reduction of Propionic Acid—83 gm. of redistilled propionic acid were converted to the calcium salt and were added to 18 liters of a medium of the composition used in the preceding experiment. This was inoculated with 900 cc. of a seeding culture in 5 per cent corn mash and incubated at 37°. The fermentation was over in 72 hours, when the culture was distilled without a column until 12 liters of distillate were obtained. This was again distilled and the 8 liters of condensate were fractionated through the large column used in the preceding experiment. The first few drops of condensate which came over at 50–51° had a definite odor of propionaldehyde and were identified as such by the skatole test described by Mulliken (13). As in the preceding experiment, the various fractions were treated successively with potassium carbonate, sodium...
bisulfite, and anhydrous copper sulfate. The dry products were then distilled through a Podbielniak precision column, whereby three fractions boiling at 78.5–80°, 97.4°, and 117° were obtained. These were identified respectively as n-propanol (46.7 gm.), ethanol (15.8 gm.), and n-butanol (110 gm.). The bisulfite solution yielded 59 gm. of acetone. The residue remaining after the distillation through the large column was saturated with potassium carbonate and extracted with ether. The other extract yielded nothing of interest.

SUMMARY

When added to actively fermenting cultures of Clostridium acetobutylicum, both propionaldehyde and propionic acid are reduced to the corresponding alcohol, n-propanol, without the accompanying formation of any other end-products which are not normally formed by this microorganism in the fermentation of carbohydrates.

As a result doubt is cast upon the commonly accepted hypothesis that aldol condensation constitutes an important intermediary step in the formation of butyric acid and n-butanol from carbohydrates by Clostridium acetobutylicum.

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