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The Stability of some Bacterial Enzymes toward Heat and Chemical Bactericides

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SUMMARY: The effect of heat, HgCl_2 and cetrimide on decarboxylase systems of some typical lactic acid bacteria and coliform organisms has been investigated. The enzymes appeared to be reversibly inactivated by heat, for the cultures in milk and in broth retained considerable decarboxylase activity after having been heated to 100° for 60 min. Furthermore, decarboxylation continued after sterilization of the cultures with HgCl_2 and with cetrimide.

It is well known that some enzymes even in an impure state can withstand temperatures considerably higher than that usually considered to cause irreversible denaturation of the protein moiety. Mellanby & Woolley (1913), Van Slyke & Cullen (1914), Stapp (1924), Bodansky (1924) and Northrop (1932) reported examples of stability of enzymes in the temperature range 80 – 100° . Miyake & Ito (1924) reported a diastase in *Aspergillus oryzae* which, although inactivated in a few seconds at 140° , required 130° for 15 min. and 120° for 60 min. for destruction. These appear to be the highest recorded temperatures at which any activity is retained by crude enzyme preparations. In spite of these early observations no one seems to have considered the possibility that bacterial enzymes might withstand the heat treatment commonly applied in pasteurization processes. The present work developed from the discovery of a heat-stable decarboxylase system in a strain of *Streptococcus diacetilactis*. The results show that bacterial enzyme systems may survive the killing of the organism by heat or bactericides and may continue under suitable conditions to produce characteristic end-products such as carbon dioxide and diacetyl.

MATERIALS AND METHODS

Test organisms. Four cultures of lactic streptococci were used. Two (DL and US) were strains of *Strep. diacetilactis*, a variant of *Strep. cremoris* with the unusual property of producing carbon dioxide, diacetyl and acetic acid in addition to the main-end product, lactic acid. This organism was first described by Matuzewski, Pijanowski & Supińska (1937), and characterized in more detail by Thiel (1940). The third culture was a strain of *Strep. cremoris* (HP), and the fourth (MX13) was a dairy 'starter' containing a mixed flora of strains of *Strep. lactis*, *Strep. cremoris* and betacocci. The stock cultures were maintained in daily subculture in sterilized skim milk at 22° . All these cultures clotted milk within 24 hr. at 22° and gave a maximum acid concentration of c. 0.8 % (calculated as lactic acid) within 36 hr.

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Four strains of coliform organisms were used. According to the classification proposed by Wilson (1935) they were culture no. 5, *Bacterium aerogenes*, Type I; no. 13, *Bacterium coli*, Type II; no. 53, Intermediate, Type II; no. 83, *Bact. aerogenes*, Type II. All four strains produced a considerable amount of CO₂ in milk and in broth. Strains, nos. 13, 53 and 83, continued to produce gas during incubation for 12 days at 37° and strain 5 for 20 days. All the strains except no. 53 clotted skim milk at 37° within 7 days. The stock cultures were maintained in daily subculture in sterilized skim milk at 37°.

Media. (i) Skim milk sterilized in the autoclave at 12 lb. for 20 min. (ii) A broth containing 2.0 % lactose, 0.2 % yeast extract (Oxoid), 1.0 % Lab. Lemco, 1.0 % peptone, 0.8 % Na₂HPO₄ and sodium citrate sterilized as in (i).

Estimation of carbon dioxide. The CO₂ produced during growth of a culture was estimated by absorption in 0.1 N-baryta which was subsequently back-titrated with 0.1 N-oxalic acid, the amount of CO₂ being expressed in ml. at N.T.P. The culture vessels were 150 ml. conical flasks containing 100 ml. of culture medium connected to absorption flasks also of 150 ml. capacity.

Estimation of diacetyl. The diacetyl produced was estimated colorimetrically by the method of Pien, Baisse & Martin (1936) as modified by Cox & Wiley (1939).

RESULTS

In preliminary work the practice was to inoculate twenty-one flasks of medium with the organism under test, connect the flasks to absorption vessels containing baryta, incubate at 22°, and estimate the residual baryta in three flasks on each of seven successive days. The average of the three values obtained on each day was used to calculate the aggregate amount of CO₂ formed by the culture up to that time. Later the procedure was simplified by detaching the absorption flasks each day for titration and replacing them immediately with flasks containing fresh baryta. In this way the CO₂ production of the same three or four cultures was followed for the whole experimental period, with the advantage that possible variations from one culture flask to another were greatly reduced. This variation in procedure did not affect total CO₂ production, although there was a tendency for the end-point to be reached a little earlier since the admission of oxygen to the system apparently influences slightly the rate of reaction but not the position of the end-point. Table 1 shows the cumulative volumes (at N.T.P.) of CO₂ produced by the four cultures of lactic streptococci over a 7-day period. Culture HP, a typical *Strep. cremoris*, produced a negligible amount of CO₂. The other cultures produced CO₂ continuously during the 7-day period, the total volume produced being similar for all three. All the cultures clotted the milk medium within the first 24 hr. and acid production was negligible from then on. The numbers of lactic acid bacteria in clotted milk cultures are known to be static and thus it is evident that continued CO₂ production after the first 24 hr. under these conditions must be due to the metabolic activity of non-proliferating (so-called 'resting') bacterial cells.

Table 1. *Carbon dioxide produced by 100 ml. cultures of lactic streptococci in milk at 22°*

Days ...	1	2	3	4	5	6	7
	CO ₂ produced (ml. N.T.P.)						
Culture							
US	11	21	29	34	38	40	43
DL	10	20	28	33	37	39	42
HP	1.5	2	3	4	4.5	5	5
MX 13	8	22	32	38	42	45	48

Controls of 100 ml. sterilized milk produced c. 2 ml. CO₂ during the 7-day period.

Carbon dioxide production after death of the bacteria

After the CO₂-producing characteristics of the cultures had been established, experiments were set up in which some of the cultures, after growth under the described experimental conditions for various times, were treated in the following ways: (i) heated to 80° for 2 min. and rapidly cooled; (ii) heated to 100° momentarily and rapidly cooled; (iii) treated with HgCl₂ at a concentration of 0.2 % w/v in the medium; (iv) treated with cetrimide 1 % w/v (cetyltrimethylammonium bromide). Incubation and daily estimation of CO₂ was continued with these flasks as with the controls for 7 days.

Table 2. *Carbon dioxide produced by 100 ml. milk cultures of Strep. diacetylactis in 7 days at 22°, the organisms being killed at various times from the start of the experiment.*

Killed after (days): ...	1	2	3	4
	CO ₂ produced (ml. N.T.P.)			
Method of killing				
Heated at 80°, 5 min.	44	41	43	40
Heated at 100° momentarily	42	43	42	44
HgCl ₂ , 0.2 % (w/v)	40	43	44	42
Cetrimide 1.0 % (w/v)	40	39	41	41

Controls (viable organisms) produced 42–43 ml. during the same period.

Table 2 gives some typical results with culture DL. It will be observed that the various treatments had no significant effect on the total volume of CO₂ produced in 7 days. Similar results were obtained with all four cultures of lactic streptococci. The 'killed' cultures were tested for viable organisms after subjection to the various treatments by subculture into sterilized skim milk, and where HgCl₂ had been used, Na₂S was added in slight excess to eliminate the effect of mercury ions transferred with the inoculum. All four treatments were found to have sterilized the cultures. It was thus clear that the killed lactic streptococci contained an enzyme system capable of producing CO₂ in volumes similar to those produced by the viable bacteria.

Thermal destruction point of the bacterial enzymes

Preliminary trials indicated that the CO₂-producing enzyme system of the lactic streptococci retained considerable activity after being heated at 100° for 1 hr. in milk but that it was less stable in broth. A more precise heating

technique permitted more detailed observations to be made. The flasks containing 100 ml. of culture and connected to CO₂-absorption flasks were plunged into a large water-bath maintained at about 2° above the desired temperature of treatment and agitated frequently. The time required for the culture fluid to reach the required temperature was checked by means of a thermometer in a control flask in the bath. After immersion for the desired time the flasks were removed from the bath and cooled in ice-water. In practice not more than 3 min. were required to raise the temperature to 100° and not more than 1 min. to cool to room temperature. These relatively short times could not significantly affect the results. Table 3 gives the volumes of

Table 3. *Carbon dioxide produced by 100 ml. cultures of Strep. diacetylactis during 5 days following the killing of the 2-day cultures by heat treatment*

Heated at 80° for (min.) ...	5	15	30	40	60	120
	CO ₂ produced (ml. N.T.P.)					
Milk culture	23	22	23	23	22	20
Broth culture	25	22	21	20	19	18
Heated at 100°						
Milk culture	22	18	13	12	11	3
Broth culture	18	15	4	3	3	2

Controls (viable organisms) in milk and in broth produced *c.* 23 and 26 ml. respectively during the 5-day period.

CO₂ produced in a subsequent 5-day period at 22° when cultures of strain DL after growth at 22° for 2 days in skim milk and in broth were heat-treated as indicated in the table. These figures clearly indicate that the enzyme(s) concerned in CO₂ production was remarkably heat-stable in milk culture. It retained 100 % of its activity after heating at 80° for 60 min. and 45 % at 100° for 60 min., whereas in broth the figures were *c.* 75 and 13 % respectively.

The possibility that prolonged heating might cause a significant chemical decarboxylation of the substrate in the medium was excluded by control experiments in which cultures were heated in the bath as described, and the gases evolved during heating were drawn off and bubbled through baryta solution. Estimation of the CO₂ evolved gave a result never exceeding 2 ml. It is a common practice in work on decarboxylation by micro-organisms to deduct from the amount of CO₂ produced by living cells the amount produced by boiled cell controls on the ground that the latter represents CO₂ due to spontaneous decarboxylation in the presence of boiled cell material. Depending on the precise heat treatment given, on the culture medium used and on the micro-organism under investigation this practice could result in appreciable error, since the results detailed above show that in some cases CO₂ production from boiled cells may arise from heat-stable enzyme systems.

Production of carbon dioxide by killed coliform bacteria

A few experiments on the same general lines as those described above were carried out with coliform bacteria in place of lactic streptococci. Cultures of all four strains after destruction by heat or disinfectants continued to produce

CO₂ as shown by the typical results for strain 13 given in Table 4. The preliminary growth period in sterilized milk was 3 days at 37° and the figures indicate that after the various treatments, which all sufficed to kill the organisms, CO₂ production at 37° in the subsequent 7 days was almost equal to that in the control. There was an indication that the decarboxylases present in coliform organisms were not quite as resistant to the treatments applied as were the decarboxylases of the lactic streptococci.

Table 4. *Carbon dioxide produced by 100 ml. milk cultures of a coliform organism (strain 13) during the 7 days following the killing of the 3-day cultures by various treatments.*

Method of killing	CO ₂ produced (ml. N.T.P.)
Heated at 80°, 5 min.	39
Heated at 100°, 1 min.	30
HgCl ₂ , 0.2 % (w/v)	34
Cetrimide, 1.0 % (w/v)	34
Control (viable organisms)	40

DISCUSSION

It has long been known that the velocity of enzyme-controlled reactions increases with rise in temperature to a maximum at about 50° and decreases thereafter as the enzyme is progressively denatured, until at about 80° most enzymes are irreversibly denatured. Bodansky (1924) believed that in some cases heat inactivation is reversible, but few investigations into the occurrence of such heat-stable enzymes appear to have been made. The present work indicates that among bacteria, heat-resistant enzyme systems may be quite widespread. From the fact that the activity of the decarboxylative enzymes of the lactic streptococci and coliform organisms ceased above 80° but recommenced on a return to low temperature it appears that the phenomenon is due to a true reversible inactivation of the type reported by Bodansky. A few experiments indicated that the lactic streptococci of *Strep. diacetylactis* type continued to form diacetyl as well as CO₂ after killing of the organisms.

This suggests that production of other relatively complex metabolic products by heat-stable enzyme systems in killed bacteria is likely.

The stability of the decarboxylase enzyme system towards a potent protein denaturant (HgCl₂) and a surface-active bactericide (cetrimide) is also remarkable, and there seems to be no other recorded instance of the phenomenon.

The present findings may have a wide significance especially in commercial processes where pasteurization procedures are used to destroy bacteria. In cheese manufacture, for instance, it is generally assumed that heat treatment of milk at 72° for 15 sec. not only kills over 90 % of the bacteria present as contaminants but that it also eliminates any further influence of these organisms on the final product. Since it appears that some of the bacterial enzyme systems can survive milk pasteurization this assumption is false. The heat-stable enzyme systems may have a significant influence on the cheese-ripening process. (The production of gas, for instance, has an important

bearing on the texture of cheese.) If this proves to be so the hygienic quality of the raw milk used after pasteurization for cheese manufacture is much more important than has generally been supposed because all enzymic reactions due to bacteria are not checked by the killing of the bacteria themselves.

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