DEGENERATIVE PROCESSES IN A STRAIN OF CLOSTRIDIUM BUTYLICUM

A. KUTZENOK AND M. ASCHNER

Department of Bacteriology, Hebrew University, Jerusalem, Israel

Received for publication April 16, 1952

The butyric acid forming bacteria, if continuously subcultured, undergo certain changes which affect morphological as well as physiological features. These changes were described as early as 1893 by Grimbert as degenerative changes of his cultures. Winogradsky (1902) found that his *Clostridium pasteurianum* ceased to form spores or clostridial forms in subcultures, and that growth was mainly in the form of long chains of vegetative cells. The rate of fermentation decreased, and the strain lost its ability to fix nitrogen and finally died out. Bredemann (1909) stated that none of his 30 strains could be maintained by direct subculture for any length of time. Continuous cultivation was possible only by the regular use of spores as inoculum. References to this process have continued to appear in the literature until recent years. However, in spite of the early recognition of this phenomenon and its obvious theoretical and practical importance, no detailed study of the degenerative process itself is found in literature.

It is our aim in this work to describe in detail the process of degeneration as it occurs in consecutive cultures; and, if possible, to determine the external and internal factors differentiating these degenerate cultures from their parent cells.

MATERIALS AND METHODS

Description and taxonomic position of our strain. A strain of *Clostridium butylicum*, isolated from soil in Jerusalem, was used in this study. The strain ferments vigorously various sugars but has only a weak diastatic action on starch. Fermentation of glycerol, formation of acetylmethylcarbinol, and liquefaction of gelatin do not occur. Fermentation of corn mash is sluggish and incomplete.

In addition to various amounts of butyric and acetic acid, the following products are formed during the fermentation of glucose: butanol 20 to 25 per cent of the sugar fermented, isopropyl alcohol 8 to 10 per cent, and small amounts of acetone. The most characteristic property of this organism is its ability to form considerable amounts of a slimy polysaccharide at the height of fermentation. This mass usually accumulates at the top of the fermenting medium or may be ejected from the fermentation flask by the pressure of the gas evolved.

Our strain appears to be identical with an organism isolated in Holland by Beijerinck (1893) and designated by him *Granulobacter butylicum*. This designation has also been used by Van der Lek (1930) who reinvestigated Beijerinck's original strain and described zooglea- and isopropyl alcohol formation. Langlykke,

Peterson, and McCoy (1935) applied the name *Clostridium butylicum* to strains which produce small amounts of acetone and large amounts of isopropyl alcohol in addition to butanol; but neither in this publication nor in another dealing with the same organism (Langlykke, Peterson, and Fred, 1937) is polysaccharide formation described. Koepsel and Johnson (1942), however, working with a strain of *C. butylicum*, again described polysaccharide formation.

In the 6th edition of *Bergey's Manual* (Breed, *et al.*, 1948), *C. butylicum* is not recognized as a species separate from *C. butyricum* and the name *C. butylicum* is considered to be a synonym of *C. butyricum*. However, since we find that the production of isopropyl alcohol and the formation of polysaccharide are constant and characteristic properties which clearly differentiate our strain from the other butyric acid formers, we use in the following Beijerinck's name, as amended by Donker (1926), as the designation for our organism until a more generally accepted nomenclature for this group of organisms is at hand.

The degenerative phenomena. The strain usually could be transferred 6 to 10 times by direct passage without any noticeable physiological change. Following this period certain phenomena began to appear; fermentation slowed down and remained incomplete. No polysaccharide appeared, and clostridial forms and spores were entirely absent. The bacteria at this stage were obviously of low vitality, and the culture, in spite of frequent subculture, usually failed to survive. The maximum number of passages which could be maintained before the strain died out was 15 to 18.

Actively growing subcultures and normal fermentations could be obtained constantly only by using the so-called "heat-shocking" of spore suspensions. This process is commonly used in the acetone-butanol fermentation industry as cited by Prescott and Dunn (1949).

Medium. The broth used consisted of: tap water, peptone (Difco) 0.5 per cent, "marmite" 1.0 per cent, glucose 3.0 per cent, in 1 liter Erlenmeyer flasks.

Inoculation was done by transferring 10 ml of a freshly prepared starter at the height of fermentation to 1 liter of medium in an Erlenmeyer flask. The flasks were filled to the neck with medium and were used immediately after they had been autoclaved and rapidly cooled. The starter was prepared in the following way. Sporulation was induced by adding a few drops of fermenting medium or polysaccharide to a dish of sterile soil. Spore suspensions were heated in boiling water for 10 to 40 seconds in a thin walled test tube of small diameter.

The morphology of bacterial colonies was examined in the following manner. The medium described above to which 2 per cent agar was added was poured into the central chamber of a Conway dish (Conway and Byrne, 1933). A mixture of pyrogallol and sodium bicarbonate was placed in the marginal canal which encircles the central chamber. After sowing, several drops of water were added to the pyrogallol-bicarbonate mixture and the entire Conway dish was covered by a watch glass attached to the dish by a layer of plasticine.

Analytical methods. Butanol and ethanol were determined by Johnson's method (1932), acetone by Goodwin's modification of Messinger's method (1920), isopropyl alcohol by the technique of Langlykke, Peterson, and McCoy (1935),

volatile acids by the method of Virtanen and Pulkki (1928), and residual sugar according to Stiles, Fred, and Peterson (1926).

Serological methods. The antigens of degenerated cells were prepared by centrifugation and washing of a culture which had undergone degeneration. The antigen of normal cells was prepared by mixing polysaccharide and fermenting broth of a culture, which had been started from a spore suspension, in a Waring blendor and washing this mixture 5 times in distilled water. Antigens were preserved by the addition of toluol.

Antisera were prepared by the intravenous inoculation of the above described antigens into rabbits.

In order to avoid the spontaneous sedimentation of clostridia present in the antigen, agglutinations were performed in a 15 per cent glycerin solution containing 0.2 per cent NaCl (Nachtigal, 1940).

EXPERIMENTAL RESULTS

Physiological differences between normal cells and cells undergoing degeneration. The following experiments illustrate how degenerative cells differ physiologically from their normal ancestors.

A fermentation from heat-shocked spores was started and was subcultured serially in 1 liter Erlenmeyer flasks by transferring 10 ml aliquots of the culture at the height of fermentation. The final products of every passage were determined and the results are summarized in figure 1.

It is clear that the cells undergoing degeneration differ from the normal ones: in lower use of sugar, in lower production of butanol and isopropyl alcohol, and in increased production of butyric acid.

Cultures undergoing degeneration produced no polysaccharide, and starch formation was not detected by iodine. The amount of sugar used and of solvents produced decreased gradually with a relative rise in the butyric acid produced until butyric acid and gas were the only metabolic products which could be demonstrated.

To demonstrate the biochemical reactions of degenerated cells in comparison to normal ones, resting cellular suspensions of degenerated cells were used. However, the degenerate cells turned out to be very sensitive, and their activity ceased during the cooling, centrifugation, and washing necessary for the preparation of the cellular suspension. Thus suspensions of degenerated resting cells could not be employed to measure biochemical activity.

Morphological differences between normal cells and cells undergoing degeneration. In normal fermentations bacillary forms are seen at the beginning and clostridial forms appear later in the fermentation. Microscopic examination revealed that beginning with the 6th passage, fewer and fewer clostridia were formed, in the 8th passage only very few were observed, and no clostridia at all were present in the 9th.

There was also a difference in the appearance of the bacillary forms in the fermentations after the 7th passage. In progressive passages bacillary forms could be seen with increasing frequency to contain granules which stained brown

with iodine. Usually 2 or 3 granules were present in each cell and they were distributed in a manner similar to the metachromatic granules in *Corynebacterium diphtheriae*. These granules could also be stained vitally with trypan blue. The number of the bacillary forms containing granules increases with each passage until these are practically the only type of cells present.

Both microscopic and chemical data definitely indicate a progressive process. From the microscopic appearance of the serial passages, the impression is gained that the degenerative physiological properties of the serial passages are not due to a corresponding change in the biochemical properties of all of

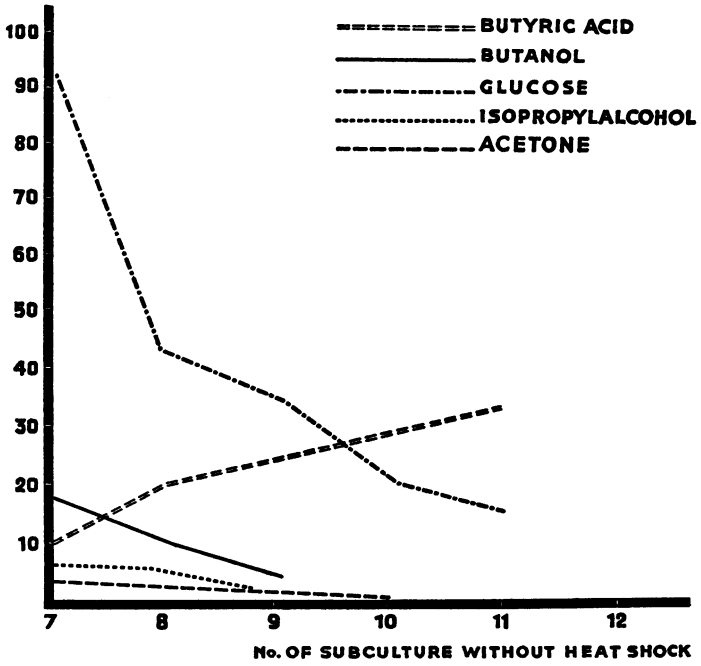


Figure 1. The progress of degeneration in successive subcultures as seen in the change of chemical activity of the culture.

Fermentation products are given in percentages of glucose utilized with the curve giving percentages of glucose consumption superimposed.

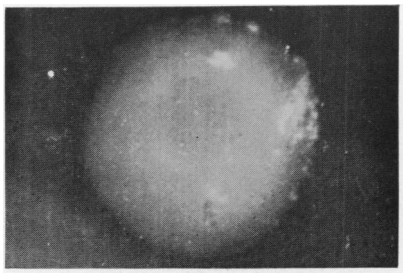
the cells in the fermentation. Instead they are due to the increase in number of a special morphological form which seems to be responsible for the peculiarities of the "degenerative" fermentations.

Differences in colony morphology between colonies grown from normal and from degenerated cells. When cells from a normal fermentation were plated on a solid medium under anaerobic conditions, large mucoid colonies were always obtained in which gas bubbles could sometimes be seen (figure 2). It made no difference whether the inoculum consisted of spores, bacillary forms from the beginning of the fermentations, or polysaccharide material containing chiefly clostridial forms. However, when degenerated cells were sown, morphologically variant colonies appeared. They were rather small and flat with no trace of mucoid

material or of gas bubbles. With higher magnification, chains of bacteria arranged in a wavy pattern could be seen at the edges (figure 3). In contrast to the mucoid colonies which developed regularly and could be subcultured without difficulty, it was very difficult to obtain growth of degenerated cultures, and usually not more than 2 or 3 of every 10 Conway dishes inoculated gave positive results. Strictly anaerobic conditions had to be maintained, and the material for inoculation had to be taken from an actively fermenting culture. This type of colony could not be subcultured.

The comparative appearances of the two kinds of colonies are similar to the S-R variation in other bacterial species. The S or M types correspond to our normal form, whereas the degenerated form is a typical R type. These results were also confirmed with the aid of a serological technique.

Serological results. The antigen prepared from normal fermentations was called "S" antigen, and that from the degenerated fermentations was called "R" antigen. The end titer of the antisera obtained after 2 months of immunization with these antigens was 1:400. Since we were interested only in the somatic



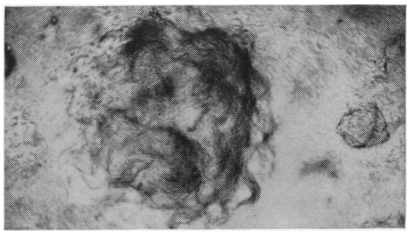


Figure 2. (left) Colony from normal cells of *Clostridium butylicum*
Figure 3. (right) Colony from degenerative cells of *Clostridium butylicum*

antigens, all agglutinations and adsorptions were carried out with cells boiled for 2 hr. These experiments are summarized in table 1. It will be seen from the adsorption experiments that in addition to a common antigen there is an additional antigen present only in S cells. This is indicated by the agglutinin remaining in anti-S sera after adsorption with the R antigen, which agglutinates only S cells.

Factors enhancing degeneration. Experiments were carried out in 20 ml test tubes, filled with 10 ml of semisolid medium which if not otherwise stated was the medium described above with the addition of 0.25 per cent agar. Subcultures were made by transferring 0.5 ml of culture. Cultures were examined microscopically for evidence of degeneration.

When cells were grown on a medium with ammonium sulfate as the source of nitrogen, degeneration started after 3 passages, and the culture usually died out in the 5th passage. Degeneration in the standard medium started only after the 10th passage. In the course of growth, the ammonium sulfate medium became acid (pH 4.3 to 4.5) since the cells utilized the ammonium ion.

The possibility that the lower pH was the enhancing factor in degeneration was considered. In order to test this hypothesis the following experiments were

carried out. An excess of $CaCO_3$ was added in one series of passages to the ammonium sulfate medium. While degeneration occurred in the 3rd passage of the medium without $CaCO_3$, it did not occur until the 10th passage in the series with $CaCO_3$.

Using standard semisolid medium acidified to pH 5.5 with H_2SO_4, HCl, and by the addition of citrate buffer, with and without $CaCO_3$ addition, it was shown that degeneration always occurred in the acid media in the first few passages, and could be prevented by the addition of $CaCO_3$.

Phenol, which according to Habs and Mohr (1935) induces R variation in *C. perfringens*, was also tested; it was found that 0.09 to 0.18 per cent induced degeneration in the 3rd passage. The effect of phenol could not be prevented by the addition of $CaCO_3$. When the organism was subcultured in liquid media,

TABLE 1

Agglutination of "S" and "R" cells of Clostridium butylicum with anti-"S" and anti-"R" sera

ANTISERUM	ANTIGEN	TITER OF AGGLUTINATION				
		1/10	1/50	1/100	1/200	1/400
S	S_{100} c	+	+	+	+	±
S	R_{100} c	+	+	+	+	±
R	R_{100} c	+	+	+	+	+
R	S_{100} c	+	±	−	−	−
$S/_{ad}$ S_{100} c	S_{100} c	±	−	−	−	−
$S/_{ad}$ S_{100} c	R_{100} c	±	−	−	−	−
$S/_{ad}$ R_{100} c	S_{100} c	+	+	+	+	−
$S/_{ad}$ R_{100} c	R_{100} c	±	−	−	−	−

the degenerative changes induced by phenol were the same as those occurring during spontaneous degeneration.

It was also found that 0.5 per cent NaCl also induced degeneration in the 4th to the 5th passage (controls 10 passages).

Maintenance of degenerated strains. It is very difficult to maintain a strain for any period after it has undergone degeneration. The only method found to be useful consisted of maintaining the organism on the semisolid medium described above containing an excess of $CaCO_3$. Transfers had to be made during active fermentation; they could not be made after the end of fermentations or in the absence of $CaCO_3$. Using this method it was possible to maintain strains for some weeks.

DISCUSSION

From these experiments it appears that the "degeneration phenomenon" described is a further example of the well known S → R variation. A similar

connection between metabolic changes and S → R variation apparent in our experiments has been described previously, notably by Tracy (1938) in the case of *Lactobacillus plantarum*.

The outstanding physiological difference between the S and R strains of *C. butylicum* was the inability of the latter to reduce butyric acid to butanol.

The following mechanism may be assumed to take place in a degenerating culture. One of the factors which enhances degeneration is an acid reaction. Acid formed at the commencement of fermentation will encourage the formation of R cells which in the next generation, by producing more acid, will set up an "autocatalytic" R enhancing effect. This effect is increased with subculture until S cells can no longer be detected and eventually the culture dies out due to overproduction of acid.

SUMMARY

Changes in the metabolism of a strain of *Clostridium butylicum* undergoing degeneration in subcultures without heat shock treatment have been investigated.

After degeneration started, the strain used up less sugar and produced progressively less butanol and isopropyl alcohol and more butyric acid. The culture usually died out after the 12 to 15th passage.

As degeneration proceeded clostridial forms decreased until they disappeared completely and no polysaccharide was formed. The vegetative cells showed a typical granulation. Degeneration was enhanced by acidifying the medium, by phenol, and by NaCl.

On solid media, normally fermenting cells produce "S" colonies, and cells from fermentations undergoing degeneration produce "R" colonies.

By agglutination and agglutinin adsorption tests it has been shown that cells from normal fermentations contain a somatic antigen in addition to those present in cells from fermentations undergoing degeneration. Degeneration in this species, therefore, appears to be similar to the well known S-R variation in other bacteria.

REFERENCES

BEIJERINCK, M. W. 1893 Ueber die Butylalkohol-Gaerung und das Butylferment. Verhandel. Koninkl. Ned. Akad. Wetenschap. 2e Sect. 1, No. 10.

BREDEMANN, G. 1909 *Bacillus amylobacter* A.M. et Bredemann in morphologischer, physiologischer und systematischer Beziehung. Zentr. Bakt. Parasitenk., Abt. I, **23**, 385–568.

BREED, R. S., MURRAY, E. G. D., AND HITCHENS, A. P. 1948 *Bergey's manual of determinative bacteriology*. Sixth edition. The Williams and Wilkins Co., Baltimore, Md.

CONWAY, E. J., AND BYRNE, A. 1933 An absorption apparatus for the microdetermination of certain volatile substances. I. The microdetermination of ammonia. Biochem. J., **27**, 419–429.

DONKER, H. J. L. 1926 Bijdrage tot de kennis der Boterzuur-Butylalcohol en Acetongistingen. Thesis, Delft.

GOODWIN, L. F. 1920 The analysis of acetone by Messinger's method. J. Am. Chem. Soc., **42**, 39–45.

GRIMBERT, L. 1893 Fermentation anaerobie produite par le *Bacillus orthobutylicus*. Ses variations sous certaines influences biologiques. Ann. inst. Pasteur, **7**, 353–402.

HABS, H., AND MOHR, W. 1935 Experimentelle Untersuchungen über Variationsformen des Fränkelschen Gasbrandbacillus. Zentr. Bakt. Parasitenk., Abt. I, **133**, 129–139.

JOHNSON, M. J. 1932 Determination of small amounts of ethyl and butyl alcohols. Ind. Eng. Chem., Anal. Ed., **4**, 20–22.

KOEPSEL, H. J., AND JOHNSON, M. J. 1942 Dissimilation of pyruvic acid by cell free preparations of *C. butylicum*. J. Biol. Chem., **145**, 379.

LANGLYKKE, A. F., PETERSON, W. H., AND FRED, E. B. 1937 Reductive processes of *C. butylicum* and the mechanism of formation of isopropyl alcohol. J. Bact., **34**, 443–453.

LANGLYKKE, A. F., PETERSON, W. H., AND MCCOY, E. 1935 Products from the fermentation of glucose and arabinose by butyric acid anaerobes. J. Bact., **29**, 333–347.

NACHTIGAL, D. 1940 *Personal communication.*

PRESCOTT, S. C., AND DUNN, C. G. 1949 *Industrial microbiology.* McGraw-Hill Book Co. New York.

STILES, H. R., FRED, E. B., AND PETERSON, W. H. 1926 A rapid method for determination of sugar in bacterial cultures. J. Bact., **12**, 427–439.

TRACY, R. L. 1938 Comparative metabolism of the R and S variants of *Lactobacillus plantarum* (Orla Jensen). J. Bact., **36**, 467–479.

VAN DER LEK, J. B. 1930 Ondersoekingen over de Butylalkoholgisting. Thesis, Delft.

VIRTANEN, A. J., AND PULKKI, L. 1928 The volatility with steam of water-soluble organic substances. J. Am. Chem. Soc., **50**, 3138–3157.

WINOGRADSKY, S. 1902 *Clostridium pasteurianum*, seine Morphologie und seine Eigenschaften als Butter-säureferment. Zentr. Bakt. Parasitenk., Abt. II, **9**, 43.