# DISSOCIATION AND LACTASE ACTIVITY IN SLOW LACTOSE-FERMENTING BACTERIA OF INTESTINAL ORIGIN

A. D. HERSHEY AND J. BRONFENBRENNER

*Department of Bacteriology and Immunology, Washington University School of Medicine, St. Louis, Missouri*

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In 1918 Bronfenbrenner and Davis observed that intestinal bacteria were frequently encountered which failed to show up as lactose fermenters in the routine diagnostic procedure, but which readily did so on subsequent cultivation in media containing lactose in higher concentrations. Studies of the conditions under which the fermentation of lactose by these organisms could be enhanced, with the purpose in view of improving methods for their detection, were reported at that time.

Bacteria exhibiting similar variability in fermentation were early described by Neisser (1906) and Massini (1907), under the name *Bacillus coli-mutabile*. They have been studied more recently by Kriebel (1934), Sandiford (1935), and, with special reference to bacterial variation, by Lewis (1934) and Hall (1935).

In the course of continued studies of the metabolism of slow lactose-fermenting bacteria of the *Escherichia coli* type, we have made some observations which we believe may contribute toward elucidation of metabolic mechanisms which determine lactose fermentation by these organisms.

The cultures used in the present study were isolated from unselected human fecal specimens by plating on lactose agar containing China blue-Rosolic Acid (C.R.) indicator (Bronfenbrenner, 1918). Of six samples examined, slow lactose-fermenting organisms were obtained from one, in which they were the predominating type. Several single colony strains of both the slow-fermenting and typical *E. coli* varieties were transferred

to plain agar slants, and maintained by monthly transplant for use as required.

In order to obtain variants of increased fermenting power from these slow lactose-fermenting cultures, they were subjected to daily serial transfer in a lactose medium as in the method previously used (Bronfenbrenner and Davis, 1918). Some elaborations of technic were introduced to facilitate the study of conditions affecting the change from slow to rapid fermentation, and particularly to determine the parts played by bacterial variation and selection respectively in this change. These will be described.

Duplicate serial transfers of five slow-fermenting strains were made daily in two series of 1 per cent lactose C-R peptone water cultures, one series consisting of open tubes plugged with cotton, the second of Dunham fermentation tubes sealed with paraffin. At the end of each 24 hours, one culture in each series was used to inoculate the subcultures, the duplicate being held in the incubator for daily observation. All sub-cultures from the closed tube series were made from the inner gas measuring tube. At frequent intervals, transfers from the open tube culture series were made into sealed fermentation tubes to observe any changes in fermentation reactions, which were thus always compared under identical conditions. At the same time these cultures were streaked on plates of lactose C-R agar for the purpose of estimating the relative numbers of fermenting variants. The same examination was made of cultures kept without transfer, corresponding to subculture I of each series (table 1). Observed increases in the ability of the cultures to form acid and gas in lactose subculture, were always concurrent with the finding of rapidly fermenting variants on the plates. No changes in fermenting power were seen until the twelfth day in standing cultures, or until the twelfth passage in the transfer series. As the experiment progressed, the fermenting power, and the proportion of fermenting variants present, increased to a maximum sometime about the twentieth day. These changes occurred with the same regularity, but were never as marked, in cultures of the open tube series. The proportion of fermenting colonies

(in per cent of the total colonies) on the plates made from subculture I at 1 day, 10 days, and 20 days, and from subculture XX after 24 hours, for each of the two series of cultures, are compared in table 1.

From the foregoing results it is evident that the change in fermenting power occurring after a prolonged cultivation in lactose medium is due to the appearance of variants and that this change is greater under conditions of partial anaerobiosis.

Furthermore, it appears that while variation occurs after about 12 days, the multiplication of actively fermenting variants thereafter goes on faster than that of non-fermenting variants.

TABLE 1

*Differential plate count showing per cent of fermenting colonies in lactose cultures after aging and after serial subcultures*

| STRAIN | SUBCULTURE I, 0–10 DAYS (PER CENT BLUE COLONIES) | | SUBCULTURE I, 20 DAYS (PER CENT BLUE COLONIES | | SUBCULTURE XX, 1 DAY (PER CENT BLUE COLONIES) | |
|---|---|---|---|---|---|---|
| | Open tubes | Closed tubes | Open tubes | Closed tubes | Open tubes | Closed tubes |
| a | 0 | 0 | 100 | 100 | 90 | 100 |
| b | 0 | 0 | 5 | 30 | 50 | 100 |
| c | 0 | 0 | 0 | 10 | 10 | 100 |
| d | 0 | 0 | 50 | 30 | 50 | 75 |
| e | 0 | 0 | 10 | 30 | 50 | 100 |

This appears even more clearly from the following experiment designed to define the growth conditions favoring selection of fermenting variants. Several media made up in fermentation tubes were inoculated in duplicate with pure cultures of fermenting and non-fermenting strains respectively. The media consisted of the following: (1) 1 per cent peptone, 0.5 per cent sodium chloride; (2) 1 per cent lactose added to a synthetic basal medium containing 0.5 per cent ammonium sulfate, 0.5 per cent sodium chloride, and M/20 phosphate buffer of pH 7.0; (3) 3 per cent lactose added to the same base; (4) 1 per cent sodium citrate added to the same base; (5) 1 per cent sodium succinate added to the same base; (6) 1 per cent lactose and 1 per cent sodium succinate combined in the same base; and (7) 1 per cent lactose, 1 per cent peptone and 0.5 per cent sodium chloride.

The relative amounts of growth appearing in 24 hours in the closed and open portions of the tubes for the two variant strains were separately recorded, as shown in table 2.

It is evident from this experiment that conditions of limited oxygen supply are favorable to the selection of fermenting variants, but only when the lactose is accompanied by an additional source of carbon.

The frequent observation of colonial dissociation in lactose-containing media, directed attention to the possibility that these changes might be in some way related to changes in lactose fermenting power. It was particularly of interest to look for

TABLE 2

*Relative growth intensity of fermenting and non-fermenting strains in various media as a function of oxygen supply*

| MEDIUM | OPEN TUBES | | CLOSED TUBES | |
|---|---|---|---|---|
| | Fermenting variant | Non-fermenting parent | Fermenting variant | Non-fermenting parent |
| (1) Peptone water | + | + | ± | ± |
| (2) Lactose 1 per cent (no additional carbon) | ++ | ++ | + | ± |
| (3) Lactose 3 per cent (no additional carbon) | + | ++ | ± | ± |
| (4) Citrate medium | + | + | − | − |
| (5) Succinate medium | + | + | − | − |
| (6) Lactose-succinate | ++ | ++ | + | − |
| (7) Lactose-peptone | ++ | ++ | + | − |

possible similarities between the rapidly fermenting variants and true *Escherichia coli.*

Accordingly, colonial variants were isolated from a number of the plates made during the course of the preceding experiments, and maintained in pure culture for subsequent examination. Grossly recognizable forms consisted of smooth and rough, large mucoid, small translucent, and occasional striated and stellate colonial types. Not all colonial variants were found in dissociated cultures of a single strain, although some colonial dissociation accompanied increase in fermenting power in all cases. Both fermenting and non-fermenting variants were obtained in the various colonial forms. Excepting differences in

colonial form, and rate of lactose fermentation, all dissociants appeared to be identical with one another, and with the parent organisms. They were motile, gram-negative rods, tending occasionally toward the coccoid form. No capsules were demonstrated by the method of Hiss, even in the mucoid cultures. They all grew in the citrate medium previously described, and failed to produce indol or $H_2S$, or to liquefy gelatin. Succinate and lactate were fermented slowly, and formate with explosive gas formation. Glucose, galactose, mannitol, and sucrose were fermented with acid and gas. These organisms, therefore, whether attacking lactose slowly or rapidly, and regardless of their colonial type, differ from typical *E. coli* in growing on citrate and succinate media, and in producing no indol, thus corresponding more closely to *Aerobacter aerogenes*. The failure to find apparent correlation between lactose-fermenting power and colonial form is in accord with the recent experience of Hall (1935).

Of critical importance in considering the relation between rapidly fermenting variants of the slow lactose fermenters and typical *E. coli* species, is the question of their mutability. Hall (1935) and Stewart (1928) as well as other investigators have failed to observe reversion from fermenting variant to non-fermenting parent types. As a result there is a tendency in the literature to refer to these organisms which have "regained" the capacity to ferment lactose actively, as differing in no essential respect from other lactose-fermenting species. On the contrary, in our experiments, by serial cultivation on the above described synthetic medium with sodium succinate as the only source of carbon, reversion was readily effected.

The fermenting variant used was first obtained in single cell culture by repeated plate isolation. A normal *E. coli* strain was used as control. Both cultures were inoculated into the succinate medium, and six successive subcultures were made at two- or three-day intervals. Lactose-fermenting power was measured at intervals by inoculating into lactose fermentation tubes. From the sixth transfer tube, both cultures were then carried through a series of 20 daily subcultures in lactose fer-

mentation tubes. Results are given in table 3. Relative lactose fermenting capacity is indicated by amount of gas measured in linear millimeters in the standard tubes, and presence or absence of acid.

These results show that variants possessing heightened capacity to ferment lactose are by no means stable in this respect as compared to *E. coli* itself. Deprived of lactose-fermenting power by serial cultivation on succinate medium, the culture slowly regains this power during subsequent serial cultivation on lactose, behaving in every respect like the original slow lactose-fermenting strain.

TABLE 3

*Loss of lactose-fermenting power on succinate and its recovery in lactose media*

| | NUMBER OF SERIAL SUB-CULTURE | AMOUNT OF GAS AND ACID IN 5 DAYS ON LACTOSE | | | |
|---|---|---|---|---|---|
| | | Fermenting variant | | *E. coli* control | |
| | | Gas | Acid | Gas | Acid |
| On succinate..... | I | 45 | + | 50 | + |
| | IV | 0 | − | 50 | + |
| | VI | 0 | − | 50 | + |
| Subsequently on lactose..... | I | 0 | − | 50 | + |
| | X | 10 | + | 50 | + |
| | XX | 35 | + | 50 | + |

It was of interest also to inquire to what extent mutability was an invariable characteristic of the slow lactose-fermenting forms. Hall (1935) has defined the criterion of lactose mutation as "repeated isolation of rapid lactose fermenters from a parent type which perpetuates both itself and the mutant indefinitely. . . .," and other investigators (Kriebel, 1934; Stewart, 1928) have concurred in the finding that slow fermenting individuals isolated from the dissociated culture retain their variability. In our experience most strains have done so, but a few single cell strains, after repeated plate-isolation, failed to show any tendency to change in their lactose-fermenting power, although still readily undergoing colonial dissociation like that observed in the parent strain. The strains used were obtained by culturing from a

large number of white colonies appearing on the lactose C-R plate streaked from a dissociated culture of the parent strain, and were purified by repeated plating, and culturing from single colonies. Their mutability was tested by daily serial subculture in lactose broth, and by aging in lactose broth for 20 days. The parent strain was subjected to similar treatment as a control. After the 20-day period, the control strain, the original single cell strains, and organisms from the two series of lactose cultures, were transferred to lactose fermentation tubes for comparison. At the same time, lactose C-R streak plates were made from the lactose cultures. Although colonial variants were recognizable on all of these plates, and both blue and white colonies appeared

TABLE 4

*Lactose fermentation stability of a selected strain during dissociation in lactose medium*

| TREATMENT OF CULTURE | AMOUNT OF GAS AND ACID (FORMED) IN 5 DAYS ON LACTOSE | | | |
|---|---|---|---|---|
| | Purified strain | | Parent strain control | |
| | Gas | Acid | Gas | Acid |
| Untreated original culture | 0 | − | 0 | − |
| 20 serial transplants in lactose | 0 | − | 18 | + |
| 20 days aging in lactose | 0 | − | 20 | + |

in most cases, a few strains like the one described in table 4, yielded only white colonies.

In this table are shown the results of fermentation tests with one of these strains, and the control strain, during the course of this experiment. The amount of gas formed in 5 days is given in linear millimeters, and presence or absence of acid as detected by china blue indicator.

From this experiment it is evident that colonial dissociation may occur without accompanying changes in lactose-fermenting power, and that daughter strains may be selected in which the capacity to ferment lactose is more radically and perhaps permanently depressed.

Two series of experiments were now carried out in an effort to determine in what particular the biochemical mechanisms of slow

lactose fermentation differ from those utilized by the actively fermenting strains. These experiments were necessarily concerned therefore with the question whether the lactose-fermenting capacity of the various strains depends exclusively on their ability to hydrolyze lactose to its fermentable monosaccharides, or whether some additional lactose-fermenting mechanism may be involved.

The preliminary experiments consisted of manometric measurements of the respiration of washed, non-proliferating cells in the presence of lactose, and of its constituent monosaccharides, respectively. It was first of all necessary to determine whether, as anticipated, an actual difference could be seen in the capacity of the cellular enzymes of the various strains to ferment lactose, and to exclude the possibility that "secreted" enzymes were primarily concerned. Further, it was reasoned that, if lactose fermentation by these organisms is a quantitative function merely of their lactase activity, then, supposing their monosaccharide-fermenting power to be roughly equal, the observed discrepancy between rates of oxidation of lactose and of monosaccharides respectively would give an inverse measure of lactase content. For the purposes of these measurements, unstandardized bacterial suspensions could be used. Oxygen uptake was measured in the Barcroft apparatus using washed cell suspensions in M/20 phosphate buffer of pH 7.2, containing M/20 lactose. The vessels were provided with side arms permitting the addition of sufficient M/1 solution of the monosaccharide mixture (glucose and galactose) to make the final concentration M/40 with respect to each. Readings of the monometer were made at three successive 10 minute intervals for each substrate, and are expressed directly in the linear values. These values are shown in table 5.

Thus, it was evident that differences in lactose-fermenting power could be attributed to the intracellular enzyme activities of the respective organisms.

Experiments of a second series were designed to give separate comparative measurements of lactose-hydrolyzing, and lactose-fermenting activity of cells of the various cultures. Use was made of the fact that toluol inhibits oxidation processes without destroying hydrolytic function (Karstnom, 1930).

Enzyme preparations consisting of washed cell suspensions of equal turbidity of *E. coli*, a slow lactose-fermenting strain, and a rapidly fermenting variant of the latter, respectively, were divided each into two parts. One of these was shaken up with toluol and left for 2 days, while the other was held as control for the same length of time previous to its use in the experiment.[1] In addition to the cell suspensions, equivalent amounts of supernatant fluid obtained by centrifugation of the fresh cell suspension, and of the same suspension after autolyzing two days under toluol, were tested for lactase activity. They will be referred to as supernatant and autolysate respectively. The test mixtures were made up to contain equal quantities of the

TABLE 5

*Stimulation of lactose oxidation by washed cells on addition of monosaccharide*

| SUBSTRATE | $O_2$ UPTAKE (CM./10 MIN.) | PER CENT STIMULATION BY MONOSACCHARIDES |
|---|---|---|
| For *E. coli:* | | |
| Lactose | 1.54; 1.60; 1.60 | 56 |
| Monosaccharides | 2.41; 2.50; 2.52 | |
| For slow-fermenting strain: | | |
| Lactose | 0.30; 0.31; 0.30 | 1,400 |
| Monosaccharides | 4.44; 4.57 | |

preparation to be tested for the enzyme, lactose in 0.5 per cent or glucose in 0.25 per cent concentration, and M/20 phosphate buffer of pH 7.2, when diluted with distilled water to a total volume of 10 cc. Incubation was made at room temperature, and the determinations were carried out after 24 and 48 hours. At the conclusion of each experiment, the mixtures were plated to show absence of contamination. Table 6 lists the mixtures prepared for the examination of each strain, and the changes which occurred during the period of incubation as measured by the Schaffer-Hartmann micro-method for determination of reducing sugar.

[1] Preliminary tests showed that it was necessary to treat the cell suspension with toluol for two days, after which no viable cells remained, and oxidase activity was destroyed.

From these values the amount of *lactose hydrolyzed* was calculated from the increase in glucose value for tube (1) over its control tube (3), multiplied by the lactose copper-reduction value 1.67.

The amount of *lactose fermented* in the absence of toluol in the same period of time was similarly calculated from the decrease in glucose value obtained by comparison of tubes (2) and (3).

Comparison of tubes (4) and (5) showed the amount of *glucose fermented under toluol,* and it was found in each case that toluol

TABLE 6

*Copper reduction value calculated as grams per cent of glucose after incubation of test mixture*

| | STRAINS | | | |
|---|---|---|---|---|
| | *E. coli* | | Slow fermenting strain | Fermenting variant strain |
| | 24 hours | 48 hours | 48 hours | 48 hours |
| 1. Lactose, cells, toluol | 0.37 | 0.41 | 0.26 | 0.30 |
| 2. Lactose, cells | 0.20 | 0.13 | 0.24 | 0.16 |
| 3. Lactose, boiled cells | 0.27 | 0.27 | 0.25 | 0.25 |
| 4. Glucose, cells, toluol | 0.23 | 0.22 | 0.18 | 0.18 |
| 5. Glucose, cells | 0 | | 0 | 0 |
| 6. Glucose, boiled cells | 0.20 | 0.22 | 0.20 | 0.20 |
| 7. Cells alone, toluol | 0 | | 0 | 0 |
| 8. Cells alone | 0 | | 0 | 0 |
| 9. Lactose, supernatant | 0.25 | 0.26 | 0.27 | 0.26 |
| 10. Lactose, autolysate | 0.27 | 0.27 | 0.27 | 0.28 |

successfully inhibited monose fermentation. From tubes (5) and (6), giving the amount of *glucose fermented* in the absence of toluol, it was evident that the preparation was capable of active fermentation under these conditions. Examination of tubes (7) and (8) proved that the bacterial suspensions themselves were free from reducing sugars. Tubes containing the autolysate and supernatant fluid, which were included to detect any soluble lactase present, remained unchanged. A summary of the calculated results is given in table 7.

Thus, it is seen that slow lactose-fermenting strains are deficient in the enzyme lactase, and that increase in fermenting

capacity is accompanied by corresponding increase in this enzyme. The lactase of *E. coli* is an intracellular enzyme which is not set free from the protoplasm to any appreciable extent even after the death and autolysis of the cells. There is no indication of a lactose-fermenting mechanism other than preliminary hydrolysis to the constituent monosaccharides.

It should be emphasized, in conclusion, that variations in lactase activity described throughout this paper are merely quantitative and that in no instance under our observation have variants shown any metabolic property which was wholly absent in the parent organism (Diehl, 1919). Thus, the phenomena suggesting specific elaboration of enzymes corresponding to a

TABLE 7

*Simultaneous lactose fermentation and lactase activity expressed in grams per cent of sugar changed*

| ENZYME PREPARATION | TIME | CONTROL (GLUCOSE FERMENTED UNDER TOLUOL) | CONTROL (GLUCOSE FERMENTED) | LACTOSE FERMENTED | LACTOSE HYDROLYZED |
|---|---|---|---|---|---|
| | *hours* | | | | |
| *E. coli* cells | 24 | 0 | 0.20 | 0.12 | 0.16 |
| *E. coli* cells | 48 | 0 | | 0.23 | 0.23 |
| Slow fermenting strain cells | 48 | 0 | 0.20 | 0.02 | 0.02 |
| Fermenting variant cells | 48 | 0 | 0.20 | 0.14 | 0.12 |
| Original amount of sugar present | | 0.20 | 0.20 | 0.45 | 0.45 |

defined substance supplied in the medium in our opinion indicate no more than the realization of a potential, perhaps latent, physiologic capacity of the bacterial species in question.

## SUMMARY

1. Slow lactose-fermenting intestinal bacteria of the *Bacillus coli-mutabile* type of Neisser and Massini manifest stable metabolic characters distinct from typical *Escherichia coli* in addition to their lactose mutability.

2. Colonial dissociation invariably occurs concomitantly with metabolic variation in the five strains studied, but no direct correlation between colonial type, and fermentative capacity was observed.

3. It was found possible to induce variation by suitable manipulation both from the slow to rapidly fermenting types, and *vice versa*. Occasional slow-fermenting daughter strains were obtained which appeared stable toward lactose.

4. The fermentation of lactose by the bacteria studied is the function of an intracellular lactase, and its variations are the variations in the activity of this enzyme.

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