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Damage to *Streptococcus Lactis* Resulting from Growth at Low *p*H

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ABSTRACT

HARVEY, R. J. (The Dairy Research Institute, Palmerston North, New Zealand). Damage to *Streptococcus lactis* resulting from growth at low *p*H. J. Bacteriol. **90**:1330–1336. 1965.—Growth of *Streptococcus lactis* below *p*H 5.0 results in damage to the cells. A period of growth below *p*H 5.0 is required before this damage is expressed, and a period of growth above *p*H 5.0 is required to correct the damage. Part of the damage consists of a reduction in the specific activities of a number of enzymes. At the same time, the differential rate of total protein synthesis is unchanged. During recovery from growth at low *p*H, the enzymes subject to damage are formed at a differential rate which is higher than normal until the normal specific activities are restored. The growth rate characteristic of the new *p*H is then resumed. The results are consistent with either of two mechanisms of damage. Growth at low *p*H could result either in direct inactivation of a number of enzymes, or in loss of control of the differential rates of synthesis of individual enzymes.

A number of enzymatic activities of *Escherichia coli* and *Micrococcus lysodeikticus* have been shown to vary with the *p*H of the medium during growth (Gale and Epps, 1942). It was considered that these changes tended either to counter the change in external *p*H or to compensate for the change by maintaining certain essential activities at a fixed level.

In the present work, similar changes in the levels of certain enzymes have been found to occur in *Streptococcus lactis* grown at *p*H values below 5.0. Since during growth of lactic streptococci the *p*H falls as a result of the formation of lactic acid from carbohydrate, these changes might be expected to have a function similar to that proposed by Gale and Epps (1942).

However, it is shown that metabolic damage occurs in cells of *S. lactis* during growth at low *p*H, and that the changes in enzyme levels account for at least part of this damage.

MATERIALS AND METHODS

Organism. The organism used was *S. lactis* ML_3. The culture was maintained by daily subculture in sterile skim milk.

Culture media. Unless otherwise stated, the media used in all experiments had the following composition: lactose, 0.25%; Casamino Acids (Difco), 0.25%; yeast extract (Difco), 0.25%; $MgSO_4$, 0.02%; phosphate buffer, 0.075 M. The desired *p*H was obtained by mixing medium containing K_2HPO_4 with medium containing KH_2PO_4. The concentration of phosphate in the medium was sufficient to prevent significant changes in *p*H during an experiment; changes were always less than 0.1 *p*H unit.

Growth conditions. Inocula were prepared from cultures grown overnight in medium with an initial *p*H of 6.5. The cells were harvested, either by centrifugation or by filtration on a membrane filter, and were suspended in fresh medium at the *p*H of the experimental medium. After incubation for 1.5 to 2 hr, this suspension was added to the experimental medium to give a concentration of 15 to 30 μg (dry weight) of cells per ml.

Experimental cultures were grown in stoppered test tubes (30 by 250 mm) containing ca. 100 ml of medium. The cultures were immersed in a water bath at 30 C.

Measurement of growth. Growth was followed by measurement of the optical density at 600 mμ of samples removed periodically from the culture; a Beckman model DU spectrophotometer with 1-cm absorption cells was used. The dry weight of cells per milliliter was calculated from the optical density. A cell concentration of 1 mg (dry weight) per ml corresponded to an optical density at 600 mμ of 1.942. The relationship between bacterial dry weight and optical density was independent of the *p*H of growth and the *p*H of measurement, and was linear to an optical density of 0.3. The specific growth rate, k, in hr^{-1}, was calcu-

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lated from:

$$k = \frac{2.303 (\log_{10} x_2 - \log_{10} x_1)}{t_2 - t_1}$$

in which x_1 and x_2 are the optical densities at times t_1 and t_2, respectively.

Shifts in pH. Rapid changes in the *p*H of the medium were accomplished by adding fresh medium, equilibrated at 30 C, containing either K_2HPO_4 or KH_2PO_4, depending upon the direction of the shift. Fresh medium at the final *p*H was also added to obtain the desired cell density. In performing a *p*H shift, the culture was diluted two to four times; the extent of dilution had no effect upon subsequent growth.

Measurements of pH. A glass electrode and a Radiometer 22 *p*H meter were used to measure *p*H. The electrode was standardized with 0.05 M potassium phthalate buffer and Beckman *p*H 7 standard buffer.

Radioactivity measurements. Radioactivity was measured at room temperature by use of a liquid scintillation system (NE8301; Nuclear Enterprises, Ltd., Edinburgh, Scotland). The scintillation fluid was 0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis-[2(5-phenyloxazolyl)] benzene in toluene. For measurement of radioactivity in bacterial cells, the cells were deposited on a 25-mm Millipore filter, washed, and dried under an infrared lamp. The filter was then placed flat on the bottom of the counting vial, cells down, and 2 ml of scintillation fluid were added. Radioactivity measurements on aqueous solutions were made by spotting the solution onto filter paper, and then drying the paper and placing it flat on the bottom of a vial containing 2 ml of scintillation fluid.

Toluene treatment. One drop of toluene was added to 1 ml of cell suspension, and the mixture was shaken for 60 min at room temperature.

Enzyme assays. Activity of the glycolytic pathway was estimated by electrometric titration of the lactic acid produced from glucose by washed-cell suspensions at *p*H 7.0. Reaction mixtures contained, in a total volume of 5 ml: D-glucose, 10^{-2} M; $MgSO_4$, 5×10^{-3} M; phosphate buffer (pH 7.0), 5×10^{-3} M; chloramphenicol, 100 μg/ml; washed cells, 1 to 2 mg/ml. Nitrogen was bubbled through the reaction mixture for 5 min before addition of glucose at zero-time. The *p*H was maintained at 7.0 by adding 0.1 N NaOH with an Agla micrometer syringe. The unit of activity was taken as the mass of cells (or protein) producing 2 meq of acid per min.

Hexokinase was assayed in toluene-treated cells by the method of Sherman (1963). Reaction mixtures contained, in a total volume of 0.5 ml: D-glucose-C^{14} (10^5 counts per min per μmole), 10^{-3} M; adenosine triphosphate (ATP), 1.5×10^{-3} M; tris(hydroxymethyl)aminomethane chloride buffer (*p*H 7.0), 0.10 M; $MgSO_4$, 5×10^{-3} M; and cells, 0.05 to 0.2 mg (dry weight). Samples (0.05 ml) were removed at intervals and absorbed onto strips (5 by 1.5 cm) of dry diethylaminoethyl (DEAE) cellulose paper. The strips were washed three times with 150 ml of deionized water in a Millipore filter apparatus and were dried under an infrared lamp. The area containing the original spot was cut out, and the radioactivity was determined as described above. Further conversion of glucose-6-phosphate by the toluene-treated cells would not have affected the assay, since all the intermediates in glycolysis are strongly anionic and would be retained on the DEAE paper and their activity counted. The unit of activity was taken as that mass of cells (or protein) phosphorylating 1 μmole of glucose per min under the conditions of assay.

Acetate kinase was assayed in toluene-treated cells by the method of Rose (1955). Aceto-coenzyme A (CoA)-kinase activity was not present. The unit of acetate kinase activity was taken as that mass of cells producing 1 μmole of acetyl phosphate per min.

Protein was estimated by the method of Lowry et al. (1951).

Chemicals. Scintillation chemicals were purchased from Nuclear Enterprises, Ltd.; uniformly labeled glucose and DL-valine-*1*-C^{14}, from the Radiochemical Centre, Amersham, England. Chloramphenicol was obtained from Parke, Davis & Co., Detroit, Mich., and adenosine-5′-triphosphate from L. Light & Co., Colnbrook, England. All other chemicals used were reagent grade.

Results

Effect of pH on specific growth rate. Figure 1 shows the effect of *p*H of the growth medium on the specific growth rate of *S. lactis* at 30 C over the range of *p*H 4 to 7. The maximal specific growth rate was at *p*H 6.3. At *p*H 4.0, the specific growth rate approached zero.

Effect of rapid changes of pH on growth. When a culture growing at *p*H 5.2 was rapidly transferred to *p*H 6.2, the specific growth rate normal for *p*H 6.2 was assumed almost immediately. A lag of 5 min at most may have occurred. The transfer from *p*H 5.5 to 6.3 gave the same results. Figure 2 and Table 1 show the results of representative experiments.

When the transfer was from an initial *p*H of 4.2 to *p*H 5.2 or to *p*H 6.2, the specific growth rate immediately after the shift was less than normal for the final *p*H (Fig. 3, Table 1). This growth rate was maintained for a short period, then gradually increased. The specific growth rate normal for the final *p*H was assumed after about 0.5 doubling of cell mass.

The results of shifts from high to low *p*H are shown in Fig. 4 and Table 1. When a culture growing at *p*H 6.3 was transferred to *p*H 5.5, the specific growth rate normal for *p*H 5.5 was assumed almost immediately. The specific growth

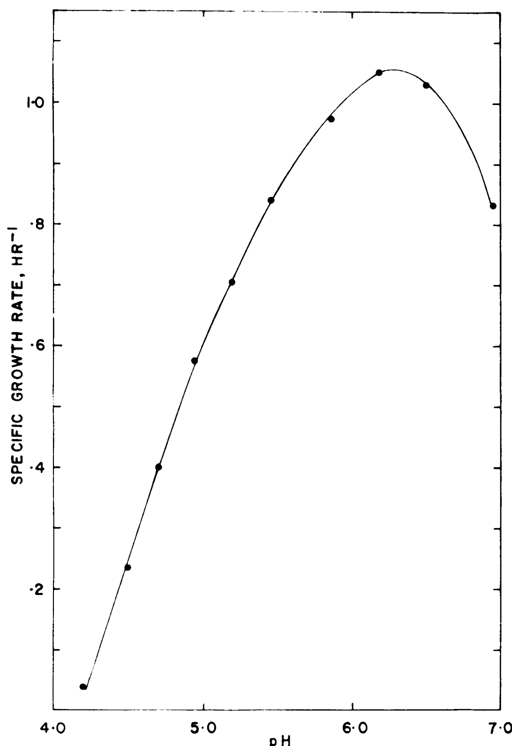


FIG. 1. *Effect of pH on the specific growth rate of Streptococcus lactis ML_3.*

rate may have been higher than normal during the first 5 min after transfer. When the shift was from *p*H 6.3 to *p*H 4.5, the initial specific growth rate was higher than is normal for *p*H 4.5. This transient growth rate was maintained for about 0.5 doubling, after which it decreased and the specific growth rate normal for *p*H 4.5 was attained.

A summary of the shift-up and shift-down experiments is presented in Table 1. A shift-up from an initial *p*H of 5.0 or above was followed by immediate assumption of the growth rate normal for the new *p*H. When the initial *p*H of growth was below 5.0, a shift-up resulted in a transient growth rate lower than normal for the final *p*H, and the ratio (transient growth rate to normal growth rate at final *p*H) decreased as the initial *p*H of growth was decreased.

The appearance of transient growth rates can be attributed neither to the magnitude of the *p*H change, nor to the associated change in ionic strength. The shift-up from *p*H 4.2 to *p*H 5.2 resulted in a transient growth rate, whereas that from *p*H 5.2 to *p*H 6.2 did not. When *S. lactis* was subjected to a rapid change in ionic strength at *p*H 6.3, equivalent to the change occurring in the shift from *p*H 4.5 to *p*H 6.3 in the presence of 0.075 M phosphate buffer (s = 0.076 to s = 0.118), the normal growth rate was immediately resumed.

If a fraction of the cells were killed by the sudden *p*H change, the initial growth rate after the shift, as measured by optical density, would appear to be lower than normal. However, the

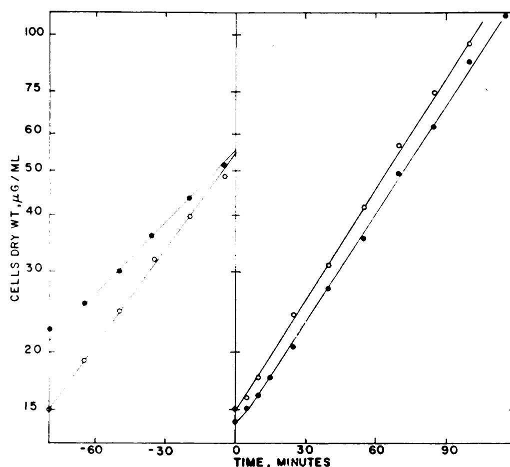


FIG. 2. *Effect of increase in pH on growth of Streptococcus lactis ML_3. At time-zero the pH was rapidly increased as described in Materials and Methods. Symbols: ●, increase from pH 5.2 to pH 6.2; ○, increase from pH 5.5 to pH 6.3.*

TABLE 1. *Summary of pH shift-up and shift-down experiments*

Expt	*p*H		Specific growth rate (k), hr^{-1}			
				At final *p*H		
	Initial	Final	At initial *p*H	Transient	Control*	Final
Shift-up	5.5	6.3	.980	None†	1.08	1.11
	5.2	6.2	.705	None	1.09	1.10
	5.0	6.3	.505	None	0.97	0.98
	4.7	6.3	.400	.840	1.08	1.05
	4.5	6.3	.144	.470	0.91	0.92
	4.4	5.8	.287	.490	1.08	1.04
	4.2	5.2	.041	.242	0.705	0.705
	4.2	6.2	.041	.368	1.09	1.06
Shift-down	6.3	5.5	.950	None	0.812	0.790
	6.3	4.5	.950	.591	0.235	0.230
	5.5	4.5	.812	.560	0.235	0.223

* Specific growth rate of a culture grown initially at the final *p*H of the shift.

† Final specific growth rates resumed without detectable transient growth rates.

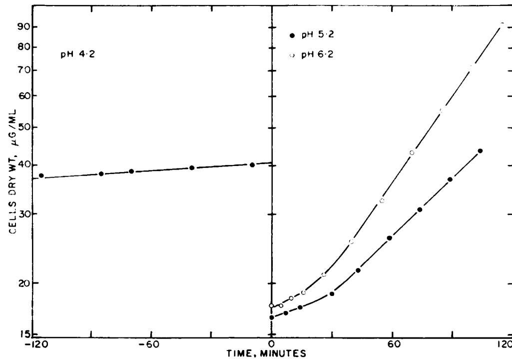


FIG. 3. *Effect of increase in pH on growth of Streptococcus lactis* ML_3. *Culture grown for 5 hr at pH 4.2, shifted to pH 5.2 and to pH 6.2 at time-zero.*

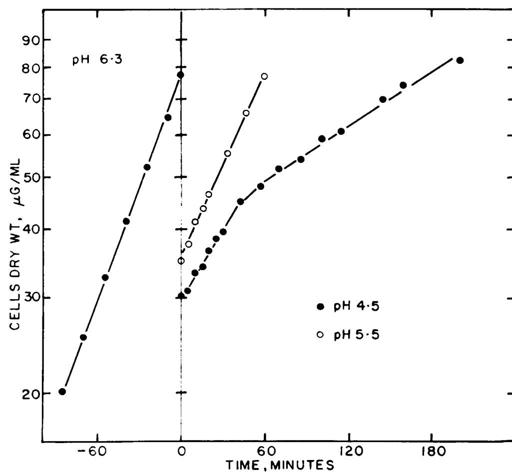
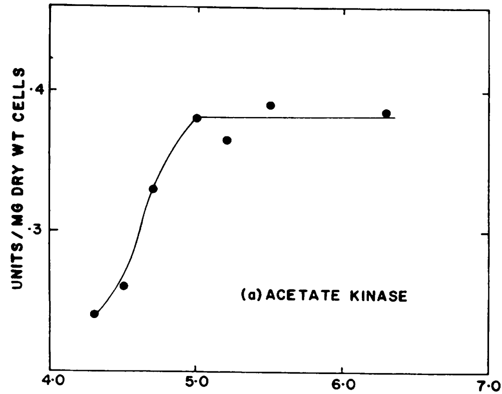
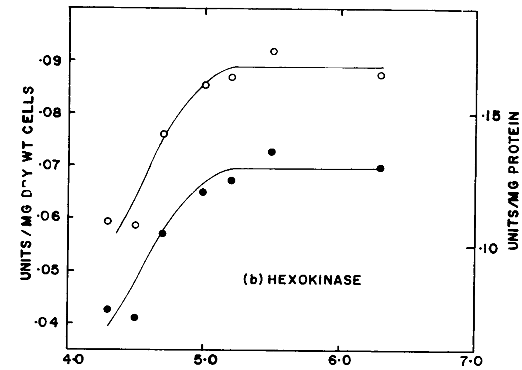


FIG. 4. *Effect of decrease in pH on growth of Streptococcus lactis* ML_3. *Culture grown initially at pH 6.3, shifted to pH 5.5 and to pH 4.5.*

normal growth rate would not then be assumed until at least two to three doublings have occurred; in the shift-up experiments described here the normal rate was assumed after only 0.5 doubling. Further, the growth rate immediately after a shift from high to low pH was higher than normal. Thus, it can be concluded that the transient growth rates are not a result of killing of a fraction of the population during the shift.

Effect of pH of growth on enzyme activities. The foregoing experiments indicate that growth of *S. lactis* at pH values below 5.0 alters or damages the cell in some manner which results in a decreased growth rate. This "damage" could consist of reduction in the specific activities of a number of enzymes, and consequent reduction of the activity of the pathways in which they operate. Acetate kinase, the initial enzyme in





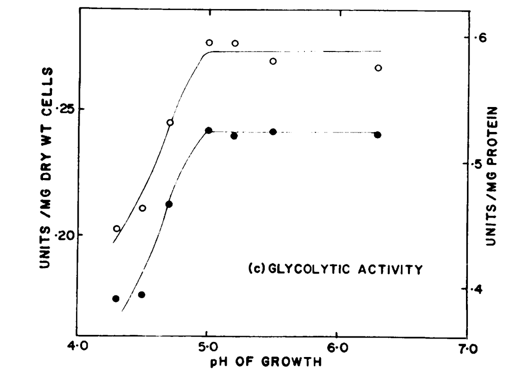


FIG. 5. *Effect of pH of growth on enzyme activities. Cultures were grown for one to two generations at the pH indicated. The cells were harvested on a membrane filter, washed three times with cold tris-(hydroxymethyl)aminomethane chloride buffer (0.01 M, pH 7.0), and suspended in the same buffer. Enzyme assays and measurement of bacterial dry weight and protein were made on these suspensions. (a) Acetate kinase activity. Medium contained 0.2% sodium acetate. (b) Hexokinase activity. Medium contained 0.25% glucose in place of lactose. (c) Activity of the glycolytic pathway. Medium same as in b. Symbols:* ○, *units per mg of protein;* ●, *units per mg (dry weight) of cells.*

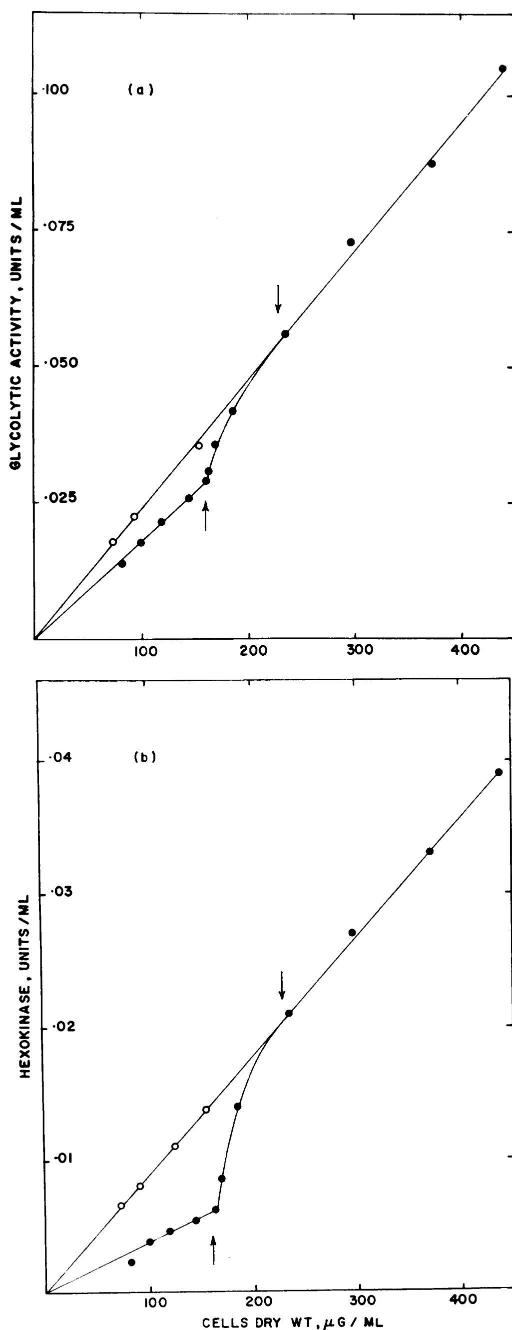


FIG. 6. *Recovery of activity of the glycolytic pathway and of hexokinase activity after growth at pH 4.3. (a) Differential rate of formation of glycolytic activity. (b) Differential rate of formation of hexokinase. Symbols:* ○, *culture grown at pH 6.3;* ●, *culture grown initially at pH 4.3, shifted to pH 6.3. Shift from pH 4.3 to 6.3 was carried out at the point indicated by the lower arrows. Growth rate normal for pH 6.3 assumed at the point indicated by the upper arrows. Results after the pH shift have been corrected for the dilution which occurred during the shift. Samples for enzyme assays were prepared as described in the legend of Fig. 5.*

TABLE 2. *Incorporation of* DL*-valine-1-C^{14} into cells of Streptococcus lactis ML_3 grown at pH 6.3 and pH 4.3**

*p*H	Differential rate of C^{14} incorporation†
4.3	46.74 ± 1.389‡
6.3	45.32 ± 0.894‡

* Medium contained: D-glucose, 0.25%; Casamino Acids, 0.050%; yeast extract, 0.050%; $MgSO_4$, 0.02%; DL-valine-*1-C^{14}*, 0.10 μc per ml. The specific activity of valine was the same in all media. Samples (2 ml) of culture were taken at intervals. The cells were deposited on 25-mm Millipore filters, washed once with water, twice with cold 5% trichloroacetic acid, once with 0.05% DL-valine, twice more with 5% trichloroacetic acid, and once more with water. After drying, the radioactivity was measured as described in Materials and Methods.

† Expressed as counts per minute per microgram (dry weight) of cells.

‡ Difference not significant at 10% level (t = 0.863)

fatty acid synthesis, hexokinase, the initial enzyme of the glycolytic pathway, and the overall activity of the glycolytic pathway were studied.

Figure 5 shows the specific activities of acetate kinase, hexokinase, and the glycolytic pathway in cells grown at various *p*H values in the range of *p*H 4.3 to 6.3. Above *p*H 5.0, the *p*H of growth had no effect on the specific activities. Below *p*H 5.0, the specific activities decreased rapidly with decreasing *p*H of growth in all cases. Similar results were obtained when the specific activities were expressed as units per milligram of protein.

Recovery of enzyme activity after growth at low pH. A culture of *S. lactis* was grown at *p*H 4.3 and shifted to *p*H 6.3. Samples for hexokinase and glycolysis assay were taken at intervals before and after the shift. Assays were also made on samples from a culture growing continuously at *p*H 6.3. Figures 6a and b are differential plots of glycolytic and hexokinase activity, respectively.

During growth at *p*H 4.3, the differential rates of formation of glycolytic activity and of hexokinase were 0.18 and 0.04 units per mg (dry weight) of cells, respectively, compared with 0.24 and 0.09 units per mg (dry weight) of cells during growth at *p*H 6.3. This result confirms that the lowered specific activities shown in

Fig. 5 represent lowered differential rates of enzyme formation.

Within 5 min after shifting the pH from 4.3 to 6.3, the differential rate of formation of both glycolytic activity and hexokinase increased to four to five times the rate normal for pH 6.3, then gradually decreased. When the specific activity normal to pH 6.3 had been restored, the normal differential rate of formation was assumed. At this point, the specific growth rate characteristic of pH 6.3 was resumed.

Effect of pH of growth on amino acid incorporation. Preliminary experiments established that more than 97% of DL-valine-*1-C*^{14} incorporated into cells of *S. lactis* could be recovered with the cell protein. Measurement of DL-valine-*1-C*^{14} incorporation could thus be used as a measure of total protein synthesis. The differential rates of incorporation of DL-valine-*1-C*^{14} into *S. lactis* growing at pH 4.3 and at pH 6.3 are shown in Table 2. There was no significant difference between the two rates. After completion of the incorporation measurements, the culture growing at pH 4.3 was shifted to pH 6.3. A typical transient growth rate was obtained, showing that the usual damage had occurred.

DISCUSSION

Changes in specific growth rate with changes in pH in the range of pH 5.0 to 6.3 are attributable only to effects of pH on the rate of the enzymatic reactions of the cell. A sudden change in pH within this range is followed by immediate assumption of the specific growth rate characteristic of the final pH.

Below pH 5.0, the effects of pH on enzyme kinetics must still occur. In addition, growth at such low pH alters or damages the cell in some manner, resulting in a decreased growth rate. A period of growth at high pH is required to correct this damage, and a period of growth at low pH is necessary before the damage is expressed.

In the streptococci, the catabolism of carbohydrate via the glycolytic pathway is the major source of metabolic energy. During growth at pH values below 5.0, the specific activities of the glycolytic pathway and of the initial enzyme of the pathway, hexokinase, are reduced. *S. lactis* is unable to synthesize most of the monomers required for the synthesis of protein and nucleic acids. The number of biosynthetic enzymes possessed by the organism is thus limited. Fatty acids are virtually the only monomers synthesized de novo in the complex medium required for growth. The specific activity of the initial enzyme in the pathway, acetate kinase, is reduced during growth below pH 5.0.

Thus, in the range of pH in which damage occurs, the specific activities of enzymes involved in the major metabolic pathways of the organism are reduced. Gale and Epps (1942) found a similar reduction in a number of the enzymatic activities of intact cells of *E. coli* grown at low pH. At the same time, the level of other enzymatic activities increased. It was proposed that, in general, a change in external pH is followed by an alteration in the enzymatic constitution of the cells such that an attempt is made to counter the external change, and to maintain certain essential activities at a constant level.

No such teleological interpretation can be placed upon the present results, which indicate that the changes in enzymatic activities constitute at least part of the damage to the cell. Two lines of evidence point to this conclusion. First, both cell damage and enzymatic changes begin to occur at the same pH, that is, at pH 5.0. Second, during recovery from growth at low pH, the time at which the normal specific activities of the glycolytic pathway and of hexokinase are restored coincides with the time at which the normal specific growth rate is resumed.

Whereas the differential rate of formation of a number of enzymes is reduced during growth at low pH, the differential rate of total protein synthesis, as measured by amino acid incorporation, is unchanged. Thus, the reduction in enzyme activity at low pH cannot be a result of nonspecific damage to the enzyme-forming systems. The high differential rate of synthesis of hexokinase observed immediately after a shift from low to high pH supports this conclusion. The possibility that the reduction in enzyme levels is due to an increased rate of protein turnover at low pH is also excluded.

Damage during growth at low pH must occur by either one of two mechanisms. (i) The differential rate of formation of individual enzyme proteins is unchanged at low pH but a certain fraction of the enzyme protein formed is inactive, either as a result of formation in an inactive state, or of inactivation after formation. (ii) During growth at low pH, the control of the differential rates of synthesis of individual proteins is lost, whereas the differential rate of total protein synthesis is unchanged. Thus, while the specific activities of some enzymes are reduced, those of others will be increased. Although it seems more likely that the first mechanism in fact operates, no further distinction can be made on the basis of the present results.

There remains the question of the nature of

the direct effects of external pH upon the cell, which lead to the observed changes in growth rate and to cell damage by either of the mechanisms described above. Two possibilities can be considered. (i) Changes in external pH are continuously reflected in changes in the intracellular ionic composition. Above pH 5.0, the intracellular ionic changes affect only the rates of enzymatic reactions; below pH 5.0 they result in damage to the cell. (ii) The cell can maintain a constant intracellular ionic composition as the external pH decreases to pH 5.0. The enzymatic reactions affected by the change in pH would then be those operating in contact with the external environment, such as specific transport systems. Below pH 5.0, the control of intracellular ionic composition is lost, and the resulting changes are the cause of cell damage.

Whatever the nature of the intracellular ionic changes produced by a change in external pH, it can be concluded that they occur relatively rapidly, since if the external pH is changed from pH 4.3 to pH 6.3 the differential rate of enzyme formation increases to four to five times normal within 5 min after the shift. Further, after a shift-up from an initial pH of growth above 5.0, the lag before the normal growth rate is assumed is not more than 5 min.

Literature Cited

GALE, E. F., AND H. M. R. EPPS. 1942. The effect of the pH of the medium during growth on the enzymic activities of bacteria (*Escherichia coli* and *Micrococcus lysodeikticus*) and the biological significance of the changes produced. Biochem J. **36**:600–618.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.

ROSE, I. A. 1955. Acetate kinase of bacteria (acetokinase), p. 591–595. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 1. Academic Press, Inc., New York.

SHERMAN, J. R. 1963. Rapid enzyme assay technique utilizing radioactive substrate, ion exchange paper, and liquid scintillation counting. Anal. Biochem. **5**:548–554.