THE MECHANISM OF THE HETEROLACTIC FERMENTATION: A NEW ROUTE OF ETHANOL FORMATION[1]

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The mechanism of ethanol production in bacterial fermentations has not been elucidated. Among the alcohol-producing bacterial systems available for study, the mixed ethanol-lactate fermentation characteristic of the heterofermentative lactic acid bacteria presents an interesting and apparently relatively simple case for experimentation. The major products of glucose fermentation by these organisms are lactic acid, ethanol, and carbon dioxide. Gayon and Dubourg (1901), Peterson and Fred (1920), and Nelson and Werkman (1935) have shown the occurrence of two fermentation patterns among the heterofermentative lactobacilli. With glucose as substrate, these are: (1) the production of lactate, ethanol, and CO_2, with occasional traces of acetate and (2) the formation of glycerol along with lactate, acetate, and CO_2. Among the heterofermentative cocci, genus *Leuconostoc*, Pederson (1929) found only the first type—glucose fermentation yielded equimolar quantities of lactate, ethanol, and CO_2. Friedemann (1939) confirmed this fermentation balance using *Leuconostoc dextranicum* as did Bäng (1945) for *Leuconostoc citrovorus*. With one culture of *Leuconostoc mesenteroides* grown at 37 C, Friedemann recovered lactate accounting for 80 per cent of the glucose fermented. This observation has not been amplified.

The relatively simple array of products of glucose fermentation by the genus *Leuconostoc* suggested this as a likely system for the study of the mechanism of bacterial ethanol formation. In addition, the apparent occurrence of products in a fixed ratio indicates the possibility that either the lactate or the ethanol plus CO_2 arises from a precursor other than pyruvate. If all three products arose from pyruvate via lactic dehydrogenase and carboxylase plus ethanol dehydrogenase, it should be possible, by altering the growth and fermentation conditions, to shift the ratio of the end products.

In the present work, the products of glucose fermentation by *Leuconostoc mesenteroides* were examined at various pH levels using resting cell suspensions as a source of enzymes. In agreement with the fermentation data for growing cultures, glucose yielded equimolar amounts of lactate, ethanol, and CO_2 at all hydrogen ion concentrations tested.

Enzyme experiments with cell extracts have demonstrated diphosphopyridine nucleotide-linked dehydrogenases for ethanol, lactate, triosephosphate, and 2,3-butylene glycol. The lactic dehydrogenase is specific for D(−)lactic acid, the

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optical isomer formed in the *Leuconostoc* fermentation. No evidence has been obtained for a carboxylase reaction. The presence of an active system which forms acetoin from pyruvate in either dried cells or cell-free extracts, as reported by Beckhorn (1948), interferes with the measurement of a carboxylase type system. Attempts to demonstrate aldolase and isomerase in extracts from *Leuconostoc* cells have been uniformly negative, suggesting that the glycolytic mechanism of the genus *Leuconostoc* may depart from the usual Embden-Meyerhof pattern of reactions.

METHODS

Bacteriological. Leuconostoc mesenteroides, strain 39, from the laboratory culture collection, was used throughout the metabolic experiments. The culture was maintained by transfer at 30-day intervals in a stock agar containing one per cent each of yeast extract and tryptone, 0.5 per cent K_2HPO_4, 0.3 per cent glucose, 10.0 per cent liver extract, and 1.5 per cent agar. To obtain cells for manometric experiments and for the preparation of extracts, the culture was transferred to medium AC1-B, which is prepared by adding aseptically 1 ml of a vitamin-salts mixture per 100 ml of AC1 medium. AC1 medium contains 1 per cent each of tryptone and yeast extract plus 0.5 per cent K_2HPO_4 and 1 per cent glucose. The vitamin-salt solution contains per 100 ml, thiamin hydrochloride, 100 mg; $MnSO_4 \cdot 4H_2O$, 2 gm; $MgSO_4 \cdot 7H_2O$, 4 gm; NaCl, 100 mg; $FeSO_4 \cdot 7H_2O$, 100 mg; and ascorbic acid, 500 mg—sterilized by filtration through a sintered glass filter.

For inoculum a transfer was made from stock agar to a tube of AC1 medium; the tube incubated 24 hours at 30 C, transferred to a second tube of AC1 medium which, after 12 to 15 hours at 30 C was used to inoculate AC1-B medium (0.1 per cent inoculum). Cells were collected by centrifugation from 12-hour cultures in AC1-B medium, washed with one-tenth the growth volume of a 1:20 dilution of vitamin-salt solution listed previously, and resuspended in the same diluted salt solution at levels appropriate for the experiments. Dried-cell preparations were obtained by suspending the washed cells in distilled water, approximately 1 g per 3 ml of water, and drying *in vacuo* over $CaCl_2$ or "drierite."

Enzyme extracts were prepared either from dried cells or from resting cell suspensions with a Raytheon 9 kc, 50 watt oscillator by suspending 100 mg of cells per ml in 0.02 M $NaHCO_3$ (or 0.03 M Na_2S) and subjecting them to treatment for 3 hours. Approximately 50 per cent of the cells were ruptured during this operation. The cellular debris was removed after oscillation by centrifugation at 9,000 rpm at 10 C in a Sorvall SS-1 centrifuge. The clear, straw-colored supernatant liquid obtained was stored at −20 C until used.

The growth yield (growth increment per unit of carbohydrate) was determined for several strains of lactic acid bacteria, namely: *Leuconostoc mesenteroides*, strain 39, *Streptococcus faecalis*, strain 10C1, and *Lactobacillus delbrueckii*. The cultures were grown in AC medium, the *Leuconostoc* at 30 C and the other two cultures at 37 C. The AC medium was supplemented with 0.05 ml of the sterile vitamin-salt mixture indicated before per 10 ml medium. After growth, the

turbidity of the cultures was measured with an Evelyn photoelectric colorimeter using a 660 mμ filter and 18 by 150 mm calibrated test tubes. The growth curves with each culture were followed until the maximum growth was reached—approximately 12 hours in all cases. To ensure anaerobic growth, the air space in the tubes was flushed with sterile nitrogen and the tube closed with a rubber stopper to prevent the incorporation of oxygen into the medium when the tubes were shaken before each turbidity measurement.

Chemical. Acid production was followed manometrically in 0.0084 M sodium bicarbonate in an atmosphere of 5 per cent CO_2 and 95 per cent nitrogen. Carbon dioxide formation was measured under nitrogen atmosphere at 28 C in various buffer solutions—with proper correction for retention (Umbreit *et al.*, 1949). Analyses for fermentation products were performed on Warburg cup contents after acidification with 0.1 volume of 3 N H_2SO_4. Where protein removal was necessary, one ml 10 per cent trichloracetic acid was added to an equal volume of cup contents. After centrifugation the deproteinized supernatant liquid was used for the determination of glucose according to the method of Horvath and Knehr (1941) and lactic acid according to the method of Barker and Summerson (1941). Ethanol was determined by the method of Friedemann and Klaas (1936) on the acidified, but not deproteinized, cup contents. Acetoin was determined by the method of Barritt (1936).

The occurrence of diphosphopyridine nucleotide-linked dehydrogenase in cell-free extracts was demonstrated by following the increase in optical density at 340 mμ with the Beckman spectrophotometer. The reversal of the dehydrogenase reactions was followed with reduced diphosphopyridine nucleotide, prepared according to the method of Ohlmeyer (1938), by measuring the decrease in optical density at 340 mμ. In each case conditions for the experiments are indicated in the protocols.

In tests for the occurrence of aldolase the formation of triosephosphates was followed either by measuring the appearance of alkali-labile phosphate according to Meyerhof and Lohmann (1934) or by the colorimetric technique of Sibley and Lehninger (1949). Sodium fructose-1,6-diphosphate was prepared from the barium salt purchased from Schwarz Laboratories. Fructose, in sodium fructose-1,6-diphosphate, was determined by the method of Roe (1934).

EXPERIMENTAL RESULTS

By using complete medium in which the quantity of growth is dependent upon the level of energy-yielding substrate, the growth yield of the heterolactic fermentation was compared with the homofermentative mechanism. In addition the growth yield of the heterolactic fermentation was measured on hexose and pentose. The measurements were similar to those of Monod (1942) who has shown that for *Escherichia coli* and *Bacillus subtilis*, maximum growth is a linear function of the concentration of the energy source. A comparison of the growth of three strains of lactic acid bacteria as a function of the substrate concentration is shown in table 1. Although these organisms grow measurably in the basal AC medium, without added carbohydrate, the growth increment as

measured turbidimetrically per unit of substrate is approximately linear over the range tabulated. As shown in the table, *Lactobacillus delbrueckii* and *Streptococcus faecalis*, the two homofermentative organisms used, gave equivalent growth yields per unit of substrate. Under similar conditions, however, *L. mesenteroides* gave somewhat less growth, i.e., 70 per cent of that shown for the two homofermentative organisms. From these limited data it appears that the heterofermentative mechanism yields less energy per unit of substrate than does the homofermentative mechanism.

TABLE 1

Growth of lactic acid bacteria as a function of carbohydrate concentration

Basal medium: Tryptone, yeast extract, phosphate, salts; see methods.

SUGAR ADDED	GROWTH: OPTICAL DENSITY × 10^{3}*			
	Streptococcus faecalis	*Lactobacillus delbrueckii*	*Leuconostoc mesenteroides*	
	glucose	glucose	glucose	arabinose
per cent				
0.05	176		39	47
0.10	240	161	77	80
0.15	275		91	114
0.20	316	212	123	144
9.25	355		146	170
0.30	390	321	182	213
0.35	395		210	226
0.40		362		
Increase in optical density × 10^{3} per 0.1 per cent (CH_2O)	75	73	53	63
Increase in optical density on molar basis†	75	73	53	52†
Relative growth glucose *Streptococcus faecalis* = 1	1	0.98	0.7	0.7

* Evelyn colorimeter, 660 mμ filter, 18 mm test tubes, basal medium, without inoculum = 0.

† Molecular weight pentose/hexose = 150/180 = × 0.833 for arabinose.

A comparison of the pentose, arabinose, with glucose as an energy source for *L. mesenteroides* gave approximately the same growth yield per mole of substrate, thus suggesting that the energy available to this organism from pentose and hexose fermentation is equivalent. The products of pentose fermentation by the genus *Leuconostoc* are equimolar quantities of lactate and acetate, instead of lactate, ethanol, and CO_2 as yielded by hexose (Pederson, 1929).

While experimental results with more strains and varied basal media would be desirable to ensure the general validity of these data, the present observations do suggest that the heterolactic fermentative mechanism yields less energy per

mole of hexose than does the homofermentative mechanism. Furthermore, in the heterolactic fermentation hexose and pentose appear to yield approximately the same energy per mole.

The products of glucose fermentation at two pH values with resting cell suspensions of *L. mesenteroides* are shown in table 2. Ten micromoles of glucose were allowed to ferment at each pH and the products determined. The yields of products in these experiments confirm the observations with growing cultures, namely the formation of one mole each of lactate, ethanol, and CO_2 per mole of glucose fermented (Pederson, 1929). Moreover, from these data it seems likely that the adherence to an equimolar ratio of products is an inherent property of this heterofermentative mechanism.

In determining the conditions for the fermentation of glucose by resting cells of *L. mesenteroides*, the rate of fermentation was observed to be markedly de-

TABLE 2

Products of glucose fermentation by cell suspensions of Leuconostoc mesenteroides

Per Warburg Cup: 0.3 ml 0.033 M phosphate buffer, pH indicated; 0.2 ml (10 μM) 0.05 M glucose; 0.3 ml (= 4 mg dry weight) cell suspension; water to 4.0 ml; atmosphere N_2, 28 C.

pH	4.5		7.0	
	μM	μM/100 μM C_3	μM	μM/100 μM C_3
Glucose used	10	50	9.35	50
Products formed:				
Lactate	10.2	51	8.3	44.4
Ethanol	11.3	56	8.95	47.8
CO_2	9.6	48	9.7	51.9
Carbon recovery per cent		104		93.6
O/R balance		0.86		1.08

pendent upon the pH and on the nature of the buffer employed. Data illustrating the influence of pH on the fermentation rate are shown in figure 1. As may be noted, using phosphate buffer, the rate of fermentation at pH 5 is markedly greater than at pH 7, even after proper adjustment for CO_2 retention. The nature of the buffer is also significant, as indicated by the fact that at pH 5 the fermentation rate in the presence of butyrate approximated the rate in phosphate buffer, whereas with acetate as buffer, the rate of fermentation as measured by CO_2 released from glucose is markedly stimulated. In the experiment shown, the fermentation rate with acetate is approximately twice the rate in phosphate or butyrate buffer. The effect of pH and of acetate on the fermentation rate is suggestive of the conditions which favor formation of ethyl or butyl alcohols in the clostridial fermentations for solvent production (Peterson and Fred, 1932) and the acetone-ethanol fermentation of *Bacillus acetoethylicus* (Arzberger, Peterson, and Fred, 1920). In the case of the genus *Clostridium*, the influence of pH has been attributed to the reduction of the free acids in the un-

dissociated form to the analogous alcohols. Such a hypothesis is compatible with the present observations and suggests the desirability of testing for the reduction of acetate to ethanol during the fermentation of glucose by cell suspensions. If this should prove to be the case, it may be that acetate or an acetate derivative

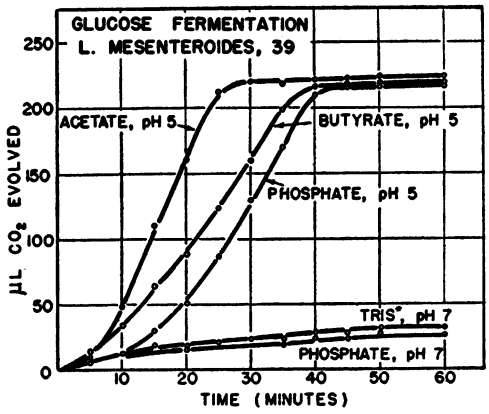

Figure 1. Effect of pH and buffer on rate of glucose fermentation (CO_2 release) by *Leuconostoc mesenteroides*.

Per Warburg Cup: 2.0 ml 0.03 M buffer, as indicated; 0.3 ml (3.7 mg dry weight) cells· 0.2 ml (10 μM) glucose; total volume: 3.0 ml; atmosphere N_2, 28 C.

*Tris buffer = tris(hydroxymethyl)-aminomethane (Gomori, 1946).

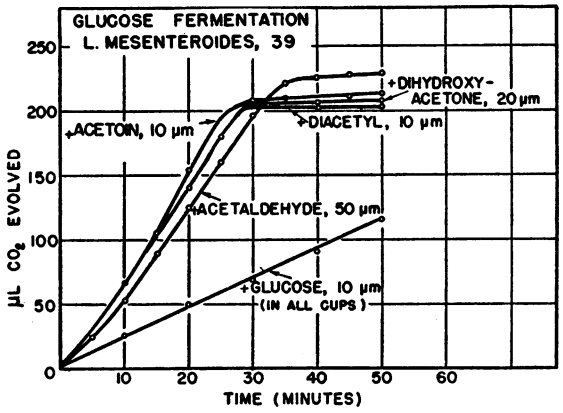

Figure 2. Acceleration of fermentation rate by hydrogen acceptors

Per Warburg Cup: 2.0 ml 0.03 M phosphate buffer, pH 7.0; 0.3 ml (4.5 mg dry weight) cells; 0.2 ml (10 μM) glucose; total volume: 3.0 ml; atmosphere N_2, 28 C.

is an intermediate in the formation of ethanol in the heterofermentative mechanism.

On the hypothesis that acetate stimulates the rate of fermentation through its action as a hydrogen acceptor, other possible hydrogen acceptors were added to cell suspensions fermenting glucose at neutral reaction. As shown in figure 2, acetate, acetaldehyde, acetoin, and dihydroxyacetone were equally effective in

stimulating the fermentation rate. From these observations it would seem that the rate limiting step in the heterofermentative mechanism is the formation of a suitable hydrogen acceptor at some stage of the fermentation pathway. The stimulatory effect of acetaldehyde may indicate that this is the immediate precursor of ethanol—although the aldehyde very probably does not arise by a carboxylase reaction. The stimulatory effect of acetoin suggests the presence of a butylene glycol dehydrogenase in the *Leuconostoc* cells. Beckhorn (1948) has previously shown the rapid conversion of pyruvate to acetoin by resting or dried cells of *L. mesenteroides*. He was, however, unable to demonstrate the presence of a carboxylase or to show the participation of acetaldehyde in acetoin formation.

On the basis of these experiments, the mechanism of ethanol formation in *L. mesenteroides* would appear to involve an oxidative step coupled with a hydrogen acceptor usually formed at a later stage in the pathway. The fermentation balances (fixed quantities of lactate, ethanol, and CO_2) and the sensitivity of the heterolactic fermentation rate to pH indicate that the glycolytic system of heterolactic organisms departs at least in some step from the classical Embden-Meyerhof scheme. It would seem that both lactate and ethanol cannot arise via phosphoglyceraldehyde, since a variation in end product ratio would be expected, and indeed the latter situation has been shown to occur for the homolactic fermentation (Gunsalus and Niven, 1942). In order to study further the mechanism of glycolysis in the heterofermentative cocci, cell-free extracts were prepared and examined for the occurrence of the enzymes involved in the usual glycolytic scheme.

Dehydrogenases. Using cell-free extracts, spectrophotometric measurements were made for both the oxidation and the reduction of diphosphopyridine nucleotide using a variety of substrates for which dehydrogenases were suspected on the basis of the Embden-Meyerhof scheme and of the products formed in the fermentation. The measurements for diphosphopyridine nucleotide reduction were run at alkaline reaction in order to shift the equilibrium in favor of the reduced form of diphosphopyridine nucleotide (Racker, 1950).

As shown in figure 3, active dehydrogenases for ethanol, lactate, and 2,3-butyleneglycol are present in extracts of *L. mesenteroides*. In the first portion of figure 3, diphosphopyridine nucleotide is shown to be reduced at alkaline pH in the presence of ethanol and reoxidized by the addition of an excess of acetaldehyde. Chemically-reduced diphosphopyridine nucleotide is also oxidized in the presence of acetaldehyde. The second portion of the figure includes data for the reduction of diphosphopyridine nucleotide with hexosediphosphate as hydrogen donor—an observation taken as the presumptive evidence for the occurrence of triosephosphate dehydrogenase since the triose would presumably arise from hexosediphosphate by aldolase action. Thus the presence of aldolase, though not necessarily isomerase, in the fermentation scheme of this organism, was expected. The mechanism of diphosphopyridine nucleotide reduction by hexosediphosphate is not defined by these measurements; however, further data concerning the aldolase reaction will be presented in a later portion of this paper.

In the presence of reduced diphosphopyridine nucleotide formed enzymatically with sodium fructose-1,6-diphosphate as reducing agent (figure 3, part 2), the addition of an excess of pyruvate resulted in immediate reoxidation of the diphosphopyridine nucleotide thus indicating the presence of a lactic dehydrogenase. However, the addition of lactate at alkaline reaction to a cell-free extract effected no reduction of the diphosphopyridine nucleotide present. The lactate used was a sample of L(+)lactate recovered from a *Clostridium per-*

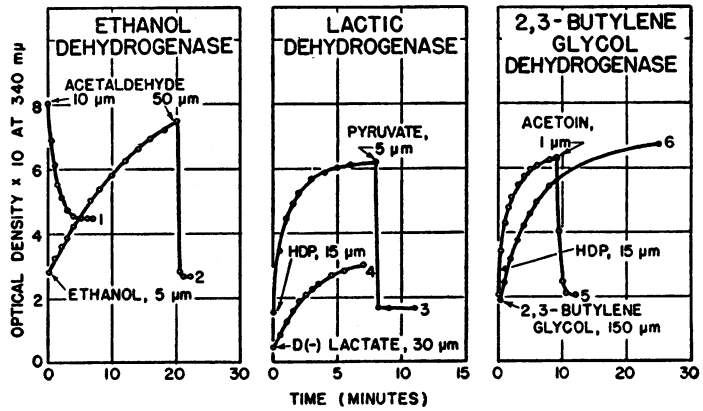

Figure 3. Evidence for diphosphopyridine nucleotide-linked dehydrogenases for ethanol, lactate, and butyleneglycol in cell-free extracts.

Beckman quartz-cuvettes with total volume of 3.0 ml, 0.2 to 0.4 μM diphosphopyridine nucleotide were used. For the oxidation of diphosphopyridine nucleotide H_2, 0.2 μM diphosphopyridine nucleotide H_2 was used. Where acetaldehyde or pyruvate was formed, 90 μM semicarbazide were added as a trapping agent. Further additions were as follows:

Ethanol dehydrogenase:	1. 0.3 M pyrophosphate buffer, pH 8.0 1.0 μM $MnCl_2$
	2. 0.2 M pyrophosphate buffer, pH 9.0 1.0 μM $MnCl_2$
Lactic dehydrogenase:	3. 1.5 ml veronal buffer, pH 7.0 (Michaelis, 1931) 52 μM Na_2HAsO_4 60 μM NaF
	4. 0.5 M pyrophosphate buffer, pH 9.0
2,3-butyleneglycol dehydrogenase:	5. 1.5 ml veronal buffer, pH 6.5 52 μM Na_2HAsO_4 60 μM NaF
	6. 2.0 ml veronal buffer, pH 6.5.

fringens fermentation by R. C. Bard. As shown in figure 4, L(+)lactate does not act as a hydrogen donor with these extracts, but D(−)lactic acid does serve as a reductant for diphosphopyridine nucleotide thus indicating that the lactic dehydrogenase present is specific for the D(−)isomer. Orla-Jensen (1942) and Pederson (1929) have previously shown that laevorotatory, D(−), lactic acid is produced by the majority of heterofermentative cocci.

The presence of a diphosphopyridine nucleotide-linked 2,3-butyleneglycol dehydrogenase in the cell-free extracts is shown by the oxidation of reduced

diphosphopyridine nucleotide in the presence of acetoin and the reduction of diphosphopyridine nucleotide with butyleneglycol as substrate at alkaline reaction (figure 3, part 3).

The presence of a triosephosphate dehydrogenase, presumably glyceraldehyde-1,3-diphosphate dehydrogenase in the extracts is suggested from the reduction of diphosphopyridine nucleotide in the presence of hexosediphosphate (figure 3). In order to obtain further evidence, 3-phosphoglyceraldehyde (obtained through the courtesy of Dr. E. Baer) was used as substrate. As shown in figure 5, diphosphopyridine nucleotide is reduced in the presence of cell-free extract, arsenate, and 3-phosphoglyceraldehyde. Furthermore 3-phosphoglyceric acid serves as an oxidant for reduced diphosphopyridine nucleotide in the presence

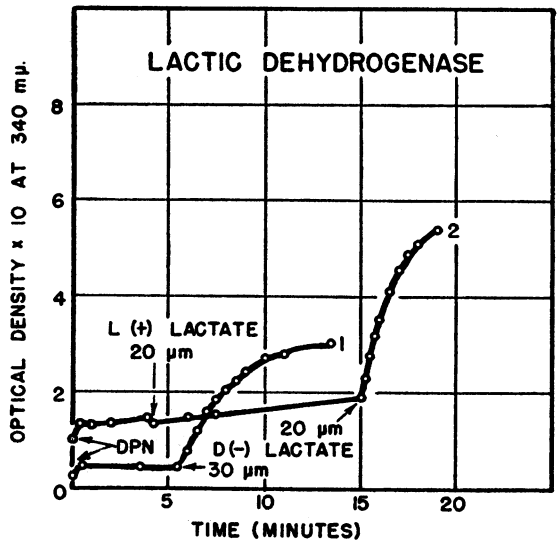

Figure 4. Specificity of *Leuconostoc mesenteroides* lactic dehydrogenase for D(−)lactic acid.

Protocol as figure 3, curve 4.

of adenosine triphosphate, but not in its absence. These observations indicate the presence of a typical triosephosphate dehydrogenase (Warburg and Christian, 1939) and as well, adenosine triphosphate-phosphoglycerate transphosphorylase.

From these experiments it is concluded that lactate and ethanol may well arise from their usual precursors, namely pyruvate and acetaldehyde. Furthermore, the triosephosphate dehydrogenase step from 3-phosphoglyceraldehyde operates in these organisms and is presumably functional in the glucose fermentation mechanism.

In order to determine if the triosephosphate arises from the normal aldolase split of sodium fructose-1,6-diphosphate to phosphoglyceraldehyde and dihydroxyacetone phosphate, and if phosphoglyceraldehyde is in equilibrium with dihydroxyacetone—isomerase reaction—the following experiments were run.

Aldolase-isomerase. Using dried cells and cell-free extracts, obtained by a

number of procedures, attempts to demonstrate the presence of aldolase, measured both by the accumulation of alkali-labile phosphate and by the formation of a chromogen with 2,4-dinitrophenylhydrazine by the method of Sibley and Lehninger (1949), were uniformly negative. Parallel experiments using extracts of *Clostridium perfringens*—shown by Bard and Gunsalus (1950) to contain active aldolase—are shown in table 3. As may be seen, the experiments with *C. perfringens* gave a positive test for aldolase whereas the experiments with *L. mesenteroides* were completely negative. One is unable to conclude with assurance that negative evidence concerning an enzyme is proof of its non-occurrence in a particular organism. However, from the variety of methods used to prepare extracts, and the stability of aldolase from other tissues and bacteria, it seems likely that aldolase does not function in the glycolytic scheme

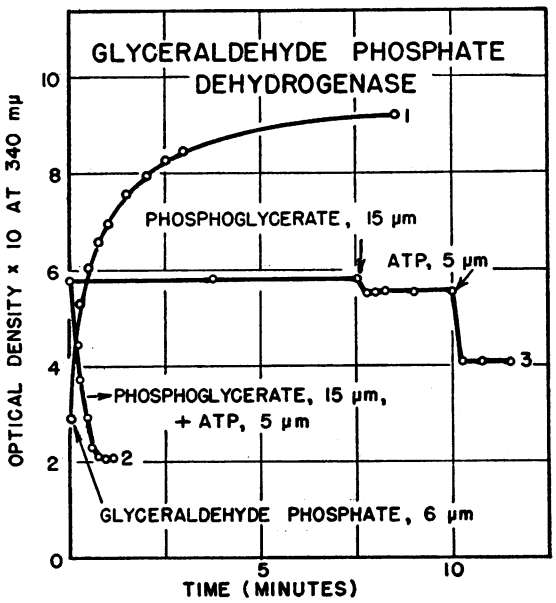

Figure 5. Evidence for *triosephosphate dehydrogenase.*

of the heterofermentative lactic acid bacteria, and thus that some departure from the usual Embden-Meyerhof scheme occurs in these bacteria. In the absence of aldolase, the reaction(s) by which sodium fructose-1,6-diphosphate acts as a reductant for diphosphopyridine nucleotide remains to be determined.

Without aldolase activity, it has not been possible to test for the presence of isomerase in the forward direction. However, since triosephosphate dehydrogenase was shown to be present in the extracts (figure 5), a test for isomerase was performed by reversing the reaction in the presence of adenosine triphosphate, 3-phosphoglyceric acid, and a suitable reductant (2,3-butyleneglycol) for diphosphopyridine nucleotide to catalyze the formation of 3-phosphoglyceraldehyde.

Evidence for the occurrence of the enzymes necessary for this determination has been presented in figures 3 and 5, the end product being 3-phosphoglyc-

eraldehyde. In the presence of isomerase, the latter substance is converted mainly to dihydroxyacetone phosphate. Both those esters can be measured together as alkali-labile phosphate and can be differentiated by the chromogen method of Sibley and Lehninger (1949) since dihydroxyacetone phosphate gives a very intense color by this method, whereas 3-phosphoglyceraldehyde gives practically no color.

As shown in the second half of table 3, alkali-labile phosphorus, but not equivalent chromogen formation, occurs in the presence of 3-phosphoglyceric acid and adenosine triphosphate, with 2,3-butyleneglycol as reductant. Both the adenosine triphosphate and the reductant are required for appreciable formation

TABLE 3

Tests for aldolase and isomerase

Protocols: Veronal buffer pH 7, 1.2 ml; cysteine, 0.1 ml (10 μM); cell extract, 0.2 ml; total volume, 2.5 ml.

For hexosediphosphate (HDP) add, $FeSO_4$, 0.25 μM; hydrazine, 200 μM; hexosediphosphate, 12.5 μM.

For 3-phosphoglyceric acid (PGlyc) add, diphosphopyridine nucleotide (DPN), 300 μg; 2,3-butanediol (2,3 Bg), 25 μM; 3-phosphoglyceric acid, 12.5 μM; ATP, 12.5 μM.

ADDITIONS	LEUCONOSTOC MESENTEROIDES EQUIVALENTS PHOSPHORUS		CLOSTRIDIUM PERFRINGENS EQUIVALENTS PHOSPHORUS	
	Chromogen*	Alkali labile†	Chromogen	Alkali labile
	μg		μg	
I				
HDP, 12.5 μM	0	23	198	159
HDP + hydrazine	0	28	218	246
II				
3-PGlyc	1	9		
3-PGlyc + ATP	0	17		
3-PGlyc + ATP + 2,3 Bg	2	120		

* Chromogen = Sibley and Lehninger colorimetric test for trioses, expressed as μg of alkali-labile phosphorus.

† *Ortho*-phosphate liberated by N NaOH 20 minutes, 25 C.

of alkali-labile phosphate. These data are interpreted as evidence for the reversal of the triosephosphate dehydrogenase, as shown in figure 5, and for the accumulation of 3-phosphoglyceraldehyde without formation of dihydroxyacetone phosphate. Thus the absence of triosephosphate isomerase in these extracts and presumably in the glycolytic scheme of *L. mesenteroides* is indicated. Under the conditions used in these experiments, the acetoin formed by the oxidation of 2,3-butyleneglycol does not interfere with the Sibley and Lehninger method.

SUMMARY

Growth experiments and fermentation balances with the heterofermentative coccus, *Leuconostoc mesenteroides*, strain 39, indicate a lower energy yield per mole of glucose fermented than in the homofermentative lactic acid bacteria.

Fermentation balances with cell suspensions indicate the fermentation of glucose to 1 mole each of lactate, ethanol, and CO_2. No evidence for departure from this ratio of products has been observed.

Diphosphopyridine nucleotide-linked dehydrogenases for ethanol, lactate, 2,3-butyleneglycol, and 3-phosphoglyceraldehyde have been demonstrated in cell-free extracts. Acetate, acetaldehyde, acetoin, and dihydroxyacetone stimulate the rate of fermentation by cell suspensions.

Aldolase and isomerase do not appear to be present in this organism.

A departure from the Embden-Meyerhof scheme and the presence of a new glycolytic mechanism in these organisms are indicated.

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