QUANTITATIVE STUDIES OF ENZYME SYSTEM OF HETEROLACTIC FERMENTATION

I. SOME ASPECTS OF CYTOPLASM OF *LEUCONOSTOC MESA- TEROIDES* AS THE SITE OF HETEROLACTIC FERMENTATION

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Studies on enzymes have been conducted so far mostly with isolated cell-free or purified preparations under artificial conditions, and the results of such studies have been used to infer rather vaguely the intracellular activity of the enzyme in question and its participation in a supposed metabolic pathway. The establishment of a metabolic pathway has been based, on the other hand, mainly upon the data of flow of radioactive isotopes of substrates and was supported enzymologically only by evidences that the activity of the corresponding enzymes was "qualitatively" found in the cells. It is, however, highly probable that the activity *in situ* of an enzyme differs considerably from what is estimated under the artificial conditions, since intracellular milieu in metabolizing cells is scarcely known and may differ possibly from the conditions used for cell-free system. The studies of this series were undertaken to evaluate quantitatively the activity *in situ* of enzymes involved in the heterolactic fermentation system of *Leuconostoc mesenteroides*. Total potential activity of enzymes as well as their enzymological characteristics were studied using cell-free or purified enzyme preparations, and the conditions inside the cells where these enzymes function were examined with fermenting cells. The informations thus obtained will make possible the calculation of activity of the enzymes when they function under the conditions prevailing in the fermenting cells. Theoretically, the activity evaluated should coincide with the rate of overall fermentation by the cells. It is expected that the studies will bring about more precise understandings of the functioning of enzymes *in vivo*. A study along the same line was also performed on the homolactic fermentation system of *Lactobacillus plantarum* (1-6).

As the first step of these studies the present paper deals with the examination of conditions prevailing in the soluble part of the cytoplasm of *Leuconostoc mesenteroides* with special reference to the factors which may affect the activity of the fermentation system.

MATERIALS AND METHODS

Organism. *Leuconostoc mesenteroides* BO7 used throughout this work

was isolated and identified in this laboratory. The strain is a typical heterolactic fermenting coccus producing D(-)-lactic acid from glucose and belongs to α -type(?), since it has an ability to form mannitol from fructose. The organism was maintained as a stab culture in malt extract-bouillon-agar containing CaCO_3 .

Preparation of Cells. The organism was grown at 30° in a medium of the following composition: 2% glucose, 1% peptone, 1% meat extract, 0.1% yeast extract, 0.5% sodium chloride and 2% anhydrous sodium acetate in tap water. The pH of the medium was adjusted to 7 before sterilization. Throughout the studies a fresh medium was inoculated with an overnight culture in the same medium and cells were harvested in a late logarithmic phase of growth when the turbidity reached to a definite value corresponding to a cell density of the dry weight of 425 mg per liter. The cells harvested were washed once with cold 0.85% saline.

Fermentation of Glucose by Whole Cells. The cells prepared as described above were suspended so as to give a cell density of 4-5 mg dry weight per ml in a fermentation medium of the following composition: 0.1 M potassium phosphate buffer (pH 6.6); 3×10^{-3} M MgCl_2 ; 3×10^{-5} M MnCl_2 . The suspension was preincubated at 30° and fermentation was started by adding glucose solution to a final concentration of 50 $\mu\text{moles/ml}$.

Determination of Fermentation Products. Samples were taken during the course of fermentation and diluted with 10-20 volumes of either 5% trichloroacetic acid or 2.55% perchloric acid to stop the reaction. After the cells were removed by centrifugation, the supernatant solution was analyzed for lactic acid, ethanol and glucose. Lactic acid was determined according to the method of BARKER and SUMMERSON (8), ethanol by the method of LUNDQUIST (9) and glucose by the method of SOMOGYI and NELSON (10, 11). In the experiments where CO_2 production was followed (Fig. 2, upper part), fermentation was carried out in several Warburg flasks. In individual flasks the reactions were stopped after different time lapses by tipping in 0.1 ml of 6 N sulfuric acid from the side arm. After CO_2 evolved was read on the manometer, the contents of each flask were analyzed for residual glucose.

Fractionation of cellular structures. Procedure for the fractionation of cellular structures is illustrated in Fig. 1. Throughout the process cells or cellular fractions were suspended in or washed with 0.02 M tris buffer, pH 7.5, containing 3×10^{-3} M MgCl_2 in order to prevent dissociation of membraneous or particulate structures. The short period of sonic treatment and its repetition after removal of broken fragments were intended to avoid overdestruction of cellular structures into finer fragments. Centrifugations were performed in the cold.

Volume determination of cellular fractions. Volume of whole cells or cellular structures obtained as described above was estimated by means of inulin space method (12). Cells or cellular fractions were packed by

Fig. 1. Fractionation of cellular structures. 0.02M tris buffer (containing 3×10^{-3} M MgCl_2) pH 7.5 was used as suspending medium throughout the experiment.

centrifugation for 10 min at $20,000 \times g$ and after removal of the supernatant, the packed material was mixed with a known volume (usually 2 ml) of 1% inulin solution. The mixture was kept for 30 min at 0° and centrifuged again for 15 min at $20,000 \times g$. Concentration of inulin before and after the mixing with the cellular material was determined by hydrolyzing the solution with 0.2N HCl for 10 min at 100° and estimating fructose by the method of SOMOGYI and NELSON (10, 11).

Volume of particulate fraction was estimated directly after centrifugation for 60 min at $100,000 \times g$ by marking the centrifuge tube, since the particles were believed to be packed so tightly that there was scarcely any

Fig. 2. Fermentation of glucose by intact cells under the standard conditions. Initial glucose concentration: 45 μ moles/ml.

○—○ glucose consumed, ●—● lactate formed, ■—■ ethanol formed, ×—× CO₂ evolved.

Cell density; Upper part 4.74 mg dry cells/ml: Lower part 2.72 mg dry cells/ml.

interparticulate space.

Determination of inorganic ions. Cells were allowed to ferment glucose under the conditions described above. 100 ml samples of fermenting cell suspension were withdrawn at intervals and cooled rapidly in an ice bath (approx. -10°) to stop fermentation. The cells were separated from the medium by centrifugation in the cold for 7 min at 20,000 $\times g$ and washed twice rapidly with 50 ml of cold water. Leakage of inorganic phosphate during the washing was found to be negligible compared with the amount subsequently extracted. The washed cells were suspended in about 10 ml of distilled water and to the cell suspension were added 50 ml of boiling distilled water and extracted at 100° for 3 min. Cells were removed by centrifugation, washed once with distilled water and the washing was

combined with the extract to make up 100 ml. Inorganic phosphate in this extract was determined by the colorimetric method of NAKAMURA (13). For determination of Na^+ , K^+ and Mg^{++} , the extract was further passed through a column of Amberlite IRA 410 (Cl^- form) in order to remove phosphate ion and other interfering anions. Na^+ and K^+ were estimated by flame-spectrophotometry using Lange Flammen Photometer Model IV. Mg^{++} was titrated with $1.0 \times 10^{-3} \text{ M}$ ethylenediaminetetraacetate using Dortite BT (Wako Chemical Co., Osaka, Japan) as an indicator in 0.1 M NH_4OH - NH_4Cl buffer (pH 10).

Determination of coenzymes. Nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP) and their reduced forms (NADH_2 and NADPH_2) were assayed of the cells fermenting glucose as described above. In order to effect an instantaneous stop of fermentation, 0.5 ml of the fermenting cell suspension was poured into 4.5 ml of acid- or alkali-extraction medium (14) which was placed in a boiling water bath. Extraction was continued for 2 min and subsequent determination of nicotinamide adenine dinucleotide coenzymes was performed according to a modification (15) of the method of BASSHAM *et al.* (14).

Thiamine pyrophosphate determination was made essentially according to the thiochrome method of FUJITA and MATSUI (16). Cells corresponding to 43.8 mg dry weight or cellular fractions derived therefrom were extracted with 1.1 ml of 0.1 N sulfuric acid for 5 min at 100° . The extract was neutralized to pH 4.7 with NaOH and treated with phosphatase (Sankyo and Co., purified from Takadiastase) for 3-8 hours at 45° . The hydrolysate was then treated successively with HCl - KCl and BrCN - NaOH , and thiochrome developed was assayed with Farrand Fluorospectrophotometer at $440 \text{ m}\mu$ using an exciter wave length of $360 \text{ m}\mu$. Treatment of the extract with permutit was omitted because the results were essentially the same with or without the treatment.

For the determination of coenzyme A (CoA) the cells corresponding to 455 mg dry weight were suspended in 7.1 ml of water and the pH of the suspension was adjusted to 4.0. The suspension was placed in a boiling water bath for 3 min and after removal of residue by centrifugation the supernatant was concentrated under reduced pressure to 1 ml. After acidification to pH 1.0 with perchloric acid, this sample was adsorbed to a column of a mixture of 200 mg of Norit SX-30 and 100 mg of Celite 520, and eluted with 25% ethanol containing 0.5% ammonia. The eluate (5 ml) was concentrated under reduced pressure to 1 ml and 0.1 ml was analyzed for CoA by the method of WAKIL *et al.* (17).

Determination of adenosine phosphates. Thirty ml of fermenting cell suspension were quickly mixed with 150 ml of boiling water and extracted for 3 min at 100° . After removal of cells by centrifugation the extract was concentrated under reduced pressure to about 1 ml and the pH was adjusted to 1 by the addition of 2.55% perchloric acid. This sample was

then passed through a column of a mixture of Norit SX-30 and Celite 520 (100 mg each) and the nucleotides were subsequently eluted with 25% ethanol containing 0.5% ammonia. The eluate was further concentrated under reduced pressure to 1.5 ml and analyzed for ADP and ATP by the enzymatic method of HOGHORST *et al.* (18).

Chemicals and Enzymes. NAD, NADP, NADH₂, NADPH₂, CoA, phosphoenolpyruvate, pyruvate kinase for the assay of ADP and hexokinase for the assay of ATP were purchased from Nutritional Biochemicals Co.. Thiamine pyrophosphate was obtained from Sankyo and Co., Tokyo. Glucose-6-phosphate dehydrogenase for NADP and ATP determination was prepared from brewer's yeast according to KORNBERG *et al.* (19). Alcohol dehydrogenase for NAD determination was prepared according to RACKER (20) from baker's yeast. Lactic dehydrogenase for ADP assay was purified from *Leuconostoc mesenteroides* by the method which is to appear in the following report (21).

RESULTS

Fermentation by whole cells

For the quantitative treatise on the enzyme system of heterolactic fermentation it was necessary to set up standardized conditions under which the cells of *Leuconostoc mesenteroides* ferment glucose at a definite and steady rate. After some preliminary experiments the conditions described in MATERIALS AND METHODS were adopted. Fig. 2 shows that the cells ferment glucose under the conditions according to the formula of heterolactic fermentation:

It was also shown that fermentation proceeds at a fairly constant rate of 5–7 μ moles per hour per cells of 1 mg dry weight. Fermentation was carried out throughout the studies under these conditions and the fermentation rate by the whole cells was used as a reference in the examination of activity *in vivo* of enzymes of this system.

Dimension of field of fermentation

In order to estimate the concentration of various substances at the site of heterolactic fermentation it was requisite to know the dimension of the site per unit mass of cells. Assuming that the system of heterolactic fermentation is localized in the soluble part of the cytoplasm, the dimension of this part was evaluated by volume determination of cellular fractions. Cells of *Leuconostoc mesenteroides* were fractionated into various structural fractions as illustrated in Fig. 1 and the volume of these fractions measured as mentioned above. Table 1 shows the results thus obtained. It was postulated that difference in quantity between crude cell walls and cell walls (Fig. 1) was due to the cytoplasmic membrane fraction (together

with attaching particles) which was digested by the treatment with lipase, ribonuclease and trypsin. Quantity of membrane-particulate fraction in Table 1 represents the sum of quantities of membrane fraction thus cal-

Table 1. Weight and Volume of Cellular Fractions per Cell Mass.

Cellular Fractions	Weight		Volume	
	g	%	cm ³	%
Whole cell	1.00	100	2.19	100
Cell wall	0.16	16.0	0.51	23.3
Membrane+particulate	0.18	18.0	0.30	13.7
Cytoplasm	0.66	66.0	1.38	63.0

culated and that of the particulate which was directly measured. Volume or weight of the soluble part of the cytoplasm was obtained by subtracting other fractions from whole cells. It was thus found that the soluble part of the cytoplasm comprises 63 per cent in volume and 66 per cent in weight of whole cells. The figure for cytoplasmic volume of 1.38 cm³ per unit cell mass (1 g dry weight) was used in all the calculations of intracellular solute concentration mentioned below.

Hydrogen ion concentration of cytoplasm

Hydrogen ion concentration of cytoplasm is undoubtedly one of the important factors which affect enzyme activity *in vivo*. There is, however, no reliable method by which to know the pH value inside the cells, not

Fig. 3. Change in pH of sonic lysate with dilution.

to speak of in actively metabolizing cells. The authors were, therefore, obliged to guess the pH of the cytoplasm by a rather indirect method (1) using glucose fermenting cells corresponding to 0.64 g dry weight (cell volume 1.4 cm³). As can be seen in Fig. 3, pH of the sonic lysate of the cells washed in cold water rose with dilution with distilled water. Since cytoplasm was already diluted in the original lysate with suspending and intercellular water, the pH of the cytoplasm was evaluated by extrapolating the curve of Fig. 3 to an undiluted state of the cytoplasm (volume of external water being given as volume of lysate minus volume of cells themselves). The pH value of 5.9 thus obtained was assumed to be the pH of the cytoplasm *in vivo*.

Intracellular concentration of some inorganic ions

Concentration of ions also is one of the fundamental factors in determining the intracellular milieu. Besides influencing nonspecifically the ionic strength of the cytoplasm, some ions act specifically on certain enzymes, as instanced by the activating effect of magnesium ions on NADP linked glucose-6-phosphate dehydrogenase. Furthermore, phosphate participates directly in some of the reaction steps of heterolactic fermentation (phosphoketolase, triosephosphate dehydrogenase, *etc.*). Experiments were performed, therefore, to study the intracellular concentration of some principal inorganic ions.

Fig. 4. Change in intracellular concentration of inorganic ions during fermentation.

Fig. 4 shows cytoplasmic concentration of Na^+ , K^+ , Mg^{++} and $\text{PO}_4^{=}$ in the fermenting cells. It was assumed that these ions extracted with hot water exist in the cytoplasm in a form of free solutes. The results in Fig. 4 show that the monovalent cations Na^+ and K^+ existed in very high levels (10^{-1} M) while the level of Mg^{++} was of the order of 10^{-3} M. Inorganic phosphate existed in a level of 10^{-2} M. Levels of K^+ and $\text{PO}_4^{=}$ rose slightly with the onset of fermentation, while Na^+ showed a tendency to decrease during fermentation. Mg^{++} inside the cells also decreased during the course of fermentation.

The amount of Cl^- of non-fermenting cells was roughly measured by the method of BOLZ (22), and was found to be 1.8×10^{-1} M. An attempt to determine the amount of Ca^{++} in the cells by flamephotometry failed to demonstrate a significant amount of this cation. From the sensitivity of the method employed, the level of Ca^{++} in these cells may be assumed to be lower than 10^{-5} M.

Intracellular concentration of coenzymes related to heterolactic fermentation. The pathway of heterolactic fermentation is generally considered to involve six dehydrogenases which require nicotinamide adenine nucleotide coenzymes, although the coenzyme specificity is not yet fully established for some of the dehydrogenases (23, 24). In this system, an enzyme phosphoketolase which cleaves xylulose-5-phosphate into glyceraldehyde-3-phosphate and acetylphosphate, requires thiamine pyrophosphate as a coenzyme (25). Further, acetylphosphate is believed to be reduced to acetaldehyde via acetyl-CoA (26). Intracellular concentration of these coenzyme in heterolactic fermentating cells was also estimated. Fig. 5

Fig. 5. Levels of nicotinamide adenine nucleotide coenzymes during fermentation.

●—● NAD, ○—○ NADH₂, ■—■ NADP, □—□ NADPH₂.

illustrates the change in the intracellular levels of NAD, NADP and their reduced forms during steady rate fermentation. As may be seen in the figure, nicotinamide adenine nucleotide coenzymes were shown to have considerably high levels (NAD+NADH₂, 2.3×10^{-3} M; NADP+NADPH₂, 0.6×10^{-3} M). It should be noticed that the levels of reduced forms of both coenzymes rose with the onset of fermentation and kept constant during steady rate fermentation. The level of NADPH₂ was higher than that of NADP, while only a minor part of the total NAD was in a reduced state in the fermenting cells. The intracellular levels of the coenzymes were calculated in this experiment assuming that they exist only in the cytoplasm and in free form. That this is actually the case was indicated by the following experiment.

Cells grown and harvested as described above were suspended in 0.05 M phosphate buffer containing 3×10^{-3} M MgCl₂ and disrupted completely by sonic treatment (10 kc, 50 min). The sonic lysate was then fractionated into soluble and cell wall particulate fractions by centrifugation for 90 min at $100,000 \times g$. The soluble cytoplasmic fraction thus obtained was further separated into high and low molecular weight fractions by means of a Sephadex G-50 column. All these treatments were performed in the buffer used for suspending the cells. The fractions thus prepared were analyzed for NAD and NADP by the method reported earlier (15), and the results are summarized in Table 2. Since these coenzymes exist in non-fermenting

Table 2. Distribution of Nicotinamide Adenine Nucleotide Coenzymes among Cellular Fractions.

Fractions	NAD	NADP
	$\mu\text{moles/g dry weight}^a$	
Whole cell	3.18	0.29
Cell wall+membran+particulate	0.05	0.02
Cytoplasm	3.39	0.39
Low molecular	3.36	0.40
High molecular	0.07	0.00

^a $\mu\text{moles/cells of 1 g dry weight or fraction derived therefrom}$

cells for the most part in oxidized form (15), NADH₂ and NADPH₂ were not assayed. The results shown in the Table indicate that nicotinamide adenine nucleotide coenzymes exist almost exclusively in the soluble cytoplasmic fraction. Taking in account the mild and rapid procedure used to separate the high and low molecular weight fractions of the cytoplasm, it may also be concluded that only a minor part of the coenzymes is bound to the enzyme proteins.

It should be mentioned that due to unknown reasons disrupted cells always showed a higher NADP content than whole cells.

Table 3. Distribution of Thiamine Pyrophosphate among Cellular Fractions.

Fractions	Thiamine Pyrophosphate $\mu\text{moles/g dry weight}^a$
Whole cell	0.26
Cell wall+membrane+particulate	0.03
Cytoplasm	0.17
Low molecular	0.24
High molecular	0.00

^a $\mu\text{moles/cells of 1 g dry weight or fraction derived therefrom}$

Owing to the lack of a sufficiently sensitive method of analysis, thiamine pyrophosphate and CoA in fermenting cells could not be measured. Table 3 shows the thiamine pyrophosphate content of cellular fractions prepared as just mentioned for nicotinamide adenine nucleotide coenzymes. Approximately 90 per cent of thiamine pyrophosphate was found in the soluble cytoplasmic fraction in free form. Cytoplasmic concentration of this coenzyme calculated from the mean of four determinations was $1.6 \times 10^{-4} \text{ M}$. It should also be mentioned that the nonphosphorylated form of thiamine appears to exist but in a very small amount, since no measurable thiochrome was detected when phosphatase digestion of samples was omitted.

Intracellular level of CoA was found to be much lower than that of other coenzymes. A concentrated extract from cells corresponding to 455 mg dry weight failed to give any measurable extinction at $300 \text{ m}\mu$ when assayed by the method of WAKIL *et al.* (17). The level of CoA in the cytoplasm was evaluated from the sensitivity of the method used and was found to be less than 10^{-5} M .

Intracellular level of ATP and ADP during fermentation

Heterolactic fermentation generates 1 mole of ATP per 1 mole of glucose fermented. It may be thus expected that intracellular level of ATP may rise considerably during fermentation. Furthermore, the level of ATP or ADP may affect directly the activity of some enzymes of this system of which these nucleotides are the substrates. Experiments were performed, there-

Fig. 6. Cytoplasmic levels of ATP and ADP during fermentation.

fore, to follow the change in the level of ATP and ADP during steady rate fermentation. As can be seen in Fig. 6 the levels of these nucleotides were, contrary to expectation, almost constant. This finding seems to indicate that ATP generation as the result of fermentation is constantly cancelled by the activity of some form of ATP consuming system under these conditions. Cytoplasmic level of ADP was much higher ($1.6 \times 10^{-3} \text{ M}$) than that of ATP ($0.5\text{--}0.7 \times 10^{-3} \text{ M}$).

DISCUSSION

The present study was undertaken to provide some basic aspects of the cytoplasm of *Leuconostoc mesenteroides* which is the site of heterolactic fermentation. Efforts were made to examine as many factors as possible with those cells fermenting glucose under the standardized conditions. When this was impossible because of technical limitations, experiments were performed with resting cells which were prepared under the same conditions as the cells used for fermentation experiments.

Volume determination of whole cells and cellular fractions revealed that unit mass (1g dry weight) of cells of this organism has a volume of 2.19 cm^3 , of which cell wall and membrane-particulate fractions occupy 23.3 and 13.7 per cent, respectively. Total cell number was counted for the same cell mass by means of PETROFF-HAUSER counting chamber and found to be 4.2×10^{12} . Assuming a perfectly spherical form, an average cell may be thus represented schematically as a sphere of 1.0μ diameter with an outermost cell wall layer of $58 \text{ m}\mu$ thickness. The soluble part of cytoplasm has a volume of 1.38 cm^3 per unit cell mass or 63 per cent of whole cell volume. It should be mentioned that this volume includes also the nuclear area of the cytoplasm. Therefore, some correction will be necessary for this figure, when the volume of the nuclear part can somehow be known. A very rough estimate of nuclear volume is possible from electronmicrographs of thin sections of bacteria, and it does not appear to exceed 15 per cent of the total cytoplasmic volume. Concentration of various molecules in the cytoplasm was tentatively calculated throughout the studies using this uncorrected figure.

Cytoplasm of *Leuconostoc mesenteroides* was shown to contain fairly large amounts of monovalent cations. These cations and magnesium ion are approximately balanced by considerably high levels of Cl^- and phosphate ion. Moreover, acidic intermediates and end products of fermentation may probably contribute somewhat to affect the pH of the cytoplasm which was presumed to be 5.9. A slight rise of intracellular level of potassium and phosphate at the beginning of fermentation is apparently due to active transport (of potassium) or passive diffusion (phosphate) of the ions from the fermentation medium. Sodium ion appears, on the other hand, to leak gradually into the medium which does not contain this ion. The drop of the level of magnesium ion can not be explained by leakage, since the

medium contains this cation at the same level as in the cytoplasm. It should be mentioned in this respect that soluble magnesium ion in the cytoplasm comprises only a small part of the total magnesium content of the cells. Comparison with the result of magnesium determination of perchloric acid digest of the cells showed that free magnesium ion in the cytoplasm (Fig. 4) represents only 1 per cent of the total magnesium in the cells. Thus, the intracellular level of free magnesium ion seems to be affected by more complex factors than monovalent cations which exist for the most part in a free form.

Experiments on the distribution of coenzymes within the cells revealed that nicotinamide adenine nucleotide coenzymes and thiamine pyrophosphate exist principally in the cytoplasm in a free form. Characteristically high levels of the former coenzymes in lactic acid bacteria have already been reported (15). Thus, NAD concentration in the cytoplasm of *Leuconostoc mesenteroides* is as high as $2 \times 10^{-3} \text{ M}$ (Fig. 5). This means that NAD dependent dehydrogenases in these cells are almost saturated with respect to this coenzymes, since affinity for NAD of the known dehydrogenases is considerably high (K_m of the order of 10^{-5} M). More than half of the total NADP was found to be in reduced form during fermentation, while in non-fermenting cells practically no NADPH_2 was detected (zero time in Fig. 5). This finding points to the participation of this coenzyme in heterolactic fermentation which has so far not been established.

In contrast to nicotinamide adenine nucleotide coenzymes, CoA appears to exist in a very low level. More exact estimation of this coenzyme is dependent on the development of a more sensitive method of analysis.

Of considerable interest was the result that the intracellular level of ATP was constant during fermentation. In heterolactic fermentation 1 mole of ATP is generated from each mole of glucose fermented. This means that, under the conditions used, 5-7 μmoles of ATP has to be formed in one hour in the cells of 1 mg dry weight, corresponding to a rate of increase in cytoplasmic ATP concentration of $4-5 \times 10^{-2} \text{ M}$ per min. Since no consumption of ATP for biosynthetic use is possible under these conditions, the observed constant level of ATP suggests the presence of some ATP utilizing system in fermenting cells.

The present study revealed that the milieu inside the cells differs in some respects considerably from those conditions under which cell-free enzyme experiments are performed. For instance, the ionic strength of the cytoplasm is much higher than that of buffers used for enzyme studies. NAD concentration of as high as 10^{-3} M is also scarcely used in spectrophotometric assay of dehydrogenases. Thus, it is possible that the activity *in vivo* of an enzyme is far from what is measured in a cell-free state. Cell-free assays can at most provide informations for enzyme content of the cells but not always for its activity in the cells. An example of this situation will be shown in a following report (21).

SUMMARY

As a preliminary step of quantitative studies of the heterolactic fermentation system, standard conditions for steady rate fermentation were set up. Some basic aspects of the cytoplasm of *Leuconostoc mesenteroides* were studied under the defined conditions. These included dimension of the cytoplasm, internal pH, and concentration of several inorganic ions. Distribution and levels in the cells of NAD, NADP, thiamine pyrophosphate and CoA were also studied. Cytoplasmic levels of ATP and ADP were followed and found to be constant during fermentation.

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