STUDIES ON THE DEHYDROGENASES OF BUTYRIC ACID BACTERIUM B_2

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By

Choseki FURUSAKA

Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University, Sendai, Japan.

(*Received December 1, 1949*)

There are many working hypotheses concerning the mechanism of butyric acid formation, the reduction of butyric acid to butyl alcohol and acetone to isopropyl alcohol by the bacteria of genus *Clostridium*. Decisive evidence explaining how so much energy is supplied for the reduction processes that change the carboxyl to the alcohol group has not yet been obtained. From the point of view that these reactions are mainly catalysed by the dehydrogenase systems of the bacteria, a report of some investigations that have been made on several kinds and textures of the dehydrogenases of the bacteria, using Thunberg's methylene blue reducing method, are offered in this paper. Stickland (1 and 2) or Kocholaty and Hoogerheide (3 and 4) have occasionally studied the dehydrogenases, chiefly of *Cl. sporogenes*, grown in a peptone medium without carbohydrates, but their studies were limited chiefly on dehydrogenases of amino acids.

Experiment

(1) *Organism.*

The organism, donated by Mr. Saji, a technician of the Japan Brewery Company, was named "strain B_2" temporarily. According to his experiments, when grown in corn mash, it was a rod with dimensions of 1.2μ to 1.5μ by 4.5μ to 6.0μ, with terminal spores whose dimensions were 1.2μ to 1.25μ by 1.8μ to 1.95μ, was motile, gram-positive and deeply stained with I-IK solution. When cultivated in either corn mash or a semisynthetic medium containing 2% glucose the organism produced, chiefly, butyric and acetic acid with small amounts of acetone, butyl alcohol, ethyl alcohol and acetoin.

Contribution from the Laboratory of Microbiology.

(2) *Preparation of Cell Suspensions.*

The organism was grown in 350 ml. SKH broth* with calcium carbonate which had been inoculated with 10 ml. of 24 hour subculture, was incubated for 20 to 24 hours at 37°C, then centrifuged, washed once with normal saline and resuspended in 15 to 25 ml. of saline, according to the quantities of cells. As soon as the suspension was prepared the air was removed by introducing N_2 gas and it was stored in ice water. In order to remove all the calcium carbonate from the washed suspension it was treated with acetic acid which stopped all dehydrogenation. Then the supernatant of the culture liquid was filtered through serveral pieces of gauze, centrifuged for one minute at low speed (500~1,000 r. p. m.), the calcium carbonate precipitated in the bottom and the cells remained in the supernatant. The supernatant was again centrifuged at a higher speed and the calcium carbonate free cells were collected. In measuring the dry weight of cells hydrochloric acid solution (ca. 10%) was added to a known volume of cell suspension to dissolve any remaining calcium carbonate. The whole was diluted with distilled water and centrifuged at high speed. The precipitates were collected, dried at 80°C and weighed.

(3) *Procedure and Methods.*

a. Measurement of the Dehydrogenase Activity.

The activities of the dehydrogenases were measured in Thunberg's tubes and noted with the time** or the velocity*** of methylene blue reduction. The hydrogen ion concentrations of the reaction systems were adjusted at pH 5.0, 7.0 and 7.5 each with M/17.5 phosphate buffer at room temperature. All experiments were conducted at 37°C and substrate concentrations prepared at 1/35 (M/L) unless otherwise stated. Acids as substrate were used after neutralization.

b. The Effect of Washing the Cell Suspension with Acid.

Washing the cell with 1% acetic acid stopped the activity of dehydrogenation with ethyl alcohol and diminished the activity of dehydrogenation with pyruvic acid. The effect was almost the same with 1% HCl solution

* SKH broth consists of: 2% commercial glucose, Koji extract (100 ml. per liter), yeast extract (100 ml. per liter), peptone 0.5% and Speakman's salts (K_2HPO_4-0.5 g., KH_2PO_4-0.5 g., $MgSO_4$-0.2 g., NaCl-0.01 g., $MnSO_4$-0.01 g. and $FeSO_4$-0.01g. per liter).

** The time necessary for the reduction of the dye from 6.25×10^{-5} Mol/L until 5.0×10^{-7} Mol/L or from 1.43×10^{-4} Mol/L until 2.86×10^{-5} Mol/L was noted.

*** The velocity, Q_{Mb}, was calculated from the time cited above, and was equivalent to the quantity of methylene blue (m-Mol) reduced by 1 mg. dried cells in 1 second, unless otherwise stated.

as acetic acid since the activity of dehydrogenation with both pyruvate and crotonate disappeared almost completely.

c. The Effect of Washing with Saline.

The cells washed once in saline then again suspended in saline were divided into two parts. One part was stored in ice water and the other was washed two more times in saline and again suspended in the same volume as the first sample. The dehydrogenase activities of both suspensions were tested with glucose. The effect of the washing was so serious that the activity of the second sample dropped to about one third of the first.

d. The Effect of Oxygen.

Washed cell suspension was divided into two test tubes provided with both gas inlet and outlet. One was aerated with air, the other with nitrogen gas. When the displacement was complete both were vigorously shaken for two minutes. The activities of both suspensions were tested immediately with glucose. When the substrate was added there was no remarkable difference in activity but when substrate was not added (decoloration with self substrates stored in the cells) the velocity of methylene blue reduction diminished markedly in the sample treated with air. After two hours storage in anaerobic conditions, activity decreased considerably but deterioration was much more serious when stored in contact with air. In the preparation of the cell suspension it was found better for the suspension to be shaken with air during the washing process and then centrifuged and stored in an anaerobic condition.

e. The Change in the Hydrogen Ion Concentration before and after the Dehydrogenation Reaction.

If the hydrogen ion concentration of the reactant changes before and after the reaction, it must be considered that the reaction velocity varies from time to time, otherwise, we should be led to misjudge the results.

However in my experiments, the changes in the hydrogen ion concentration were negligible, ranging between 0.07 to 0.02 in pH value in case when glucose, crotonate or β-oxybutyrate were used as the substrate. Therefore it is not necessary to consider changes in hydrogen ion concentration before and after the reaction.

(4) ***Results.***

a. The Relative Velocity of Dehydrogenation with Various Substrates.

The relative velocities of dehydrogenation with various substrates are summarised in Table I.

Table I. The velocity of dehydrogenation with various substrates.

Substrates \ Hydrogen ion con. ($Q_{Mb} \times 10^{-8}$)	pH 5.0	pH 7.0	pH 7.5
Glucose	0.4-9.0 2.9(6)	13-105 47.4(21)	14-87 40.1(6)
Mg-hexosediphosphate	5.7(1)	11-20 15.5(2)	14-39 26.5(2)
Glycerophosphate	2.4-13 8.1(5)	8.5-8.7 8.6(2)	0.4(1)
Acetaldehyde	Inhibition (2)	Inhibition (2)	Inhibition-0 Inhibition(2)
Formate	Inhibition-0.7 0(2)	0-10 4.5(4)	2.1-2.6 2.4(2)
Acetate	Inhibition (2)	Inhibition (2)	Inhibition(2)
Propionate	1.2(1)	8.0-2.0 5.0(2)	22(1)
Butyrate	4.7-6.7 5.7(2)	0.2-11 3.0(7)	0.4(1)
Citrate	0.5-0.9 0.7(2)	1.0-4.9 2.9(4)	0.6(1)
Lactate	0.9-4.5 2.3(3)	2.2-62 16.8(6)	12-14 13.0(2)
Malate	1.9-4.8 3.4(2)	0.3-6.0 3.2(2)	Inhibition-2.3 1.2(2)
Succinate	2.2(1)	2-15 8.5(2)	22(1)
Crotonate	2.6-1.9 2.3(3)	2.0-8.7 4.7(9)	4.4(1)
β-Oxybutyrate	1.4-17 5.7(2)	0.8-29 5.2(9)	4.9-10 7.5(2)
Pyruvate	21-97 54(4)	4-35 19.4(7)	12(1)
Methyl alcohol	Inhidition (1)	Inhibition (1)	4.3(1)
Ethyl alcohol	0.6(1)	4.7(1)	5.7(1)
n-Propyl alcohol	0(1)	25(1)	12(1)
i-Propyl alcohol	0(1)	—	3.2(1)
n-Butyl alcohol	0(1)	6.0(1)	5.5(1)

The values before and after the mark - indicate the maximum and the minimum values in several experiments. The numbers in the mark ( ) indicate the numbers of experiments dupplicated. The values defore the numbers of experiments indicate the mean velocity.

The velocity of dehydrogenation by the cell suspensions varied considerably from experiment to experiment. For example in the case of glucose the values of Q_{Mb} took 1.0, 1.3, 1.3, 1.9, 2.0, 2.8, 3.8, 3.8, 3.9, 3.9, 4.0, 4.1, 4.2, 4.6, 4.7, 5.0, 5.8, 8.5, 10.5×10^{-7}.

The order of the ralative velocities was found to be as follows: pyruvate, glucose (these two substrates dehydrogenated very rapidly)>magnesium-

hexosediphosphate, succinate, propionate, lactate, n-propyl alcohol (these dehydrogenated moderately) > scdium-glycerophosphate, β-oxybutyrate, butyrate, ethyl alcohol, crotonate, n-butyl alcohol(these were dehydrogenated slowly) > methyl alcohol, acetate, acetaldehyde (these were not dehydro genated or inhibition occurred).

The comparison of the values of the strain B_2 with *Cl. sporogenes*, obtained by Kocholaty and Hoogerheide (3), taking the activity with pyruvate as a standard, is shown in Table II. The activity with pyruvate, ethyl alcohol, formate, and citrate showed almost same values, but activity with glucose, lactate, propionate, methyl alcohol and β-oxybutyrate differed markedly.

Table II. Comparison of the dehydrogenases of *Cl. sporogenes* and of strain B_2.

Strains and experimentor / Relative velocity / Substrates		*Cl. sprogenes* (W. Kocholaty and I. C. Hoogerheide)	Strain B_2 (Auther)
Pyruvate (standard)		51	51
Ethyl alcohol		20	24
n-Butyl alcohol		20	23
Glucose		12	168
n-Propyl alcohol		12	13
Lactic acid		11	54
Formic acid		7	10
Butyric acid		4	1.2
Acetaldehyde		5	0
Citric acid		3	2
Propionic acid		0	92
Methyl alcohol		0	18
β-Oxybutyric acid		0	31
Coditions of culture	Medium	Neopepton 3%	Glucose 3% Pepton 0.5%
	Time	16–20hrs.	20–24hrs.
Hydrogen ion concentration		pH 9.4	pH 7.5
Dyes used		Brilliant cresyl blue	Methylene blue

However, Kocholaty and Hoogerheide observed that when the glucose was added in the culture medium, the activities with glucose and with ethyl alcohol increased markedly. I can not decide without further experiments whether these differences originated from differences of the strains, of

the culture conditions, of the dyes or of the other conditions under which the tests were made.

b. The Relationships between Hydrogen Ion Concentration and the Activities of Dehydrogenation with Various Kinds of Substrates.

The velocity of methylene blue reduction, from which the relative velocities of various kinds of substrates were calculated, varied from culture to culture, and even with the same culture the relative velocity varied from experiment to experiment, so it was not possible to derive an optimum hydrogen ion concentration of each dehydrogenation system directly from the values shown in Table I.

This was done by referring the values resulting from an experiment in which the relative reduction velocity at different pH values was measured simultaneously. The values shown in the table cited are mean values obtained from several series of the experiments. Glucose, succinate, magnesium-hexosediphosphate, lactate, formate and citrate were dehydrogenated at pH 7.5 most rapidly, which was taken to be the optimum condition, and under neutral conditions they were dehydrogenated at a moderate rate while at pH 5.0, glucose, succinate, formate and citrate were dehydrogenated very slowly. Alcohols such as ethyl, n-propyl, i-propyl and butyl alcohol showed the same texture and were dehydrogenated most rapidly at pH 7.5 and at pH 5.0 very slowly or not at all.

Pyruvate and sodium-glycerophosphate were dehydrogenated most rapidly at pH 5.0 and slowly at pH 7.5.

Butyrate, β-oxybutyrate, crotonate and malate were dehydrogenated at pH 5.0 and 7.0 at almost the same rate. This is very interesting in contrast with the fact that the optimum hydrogen ion concentration of the fermentation was on the acidic side.

c. On the Formic Hydrogenlyase and the Formic Hydrogenase.

Butyric acid bacteria produced much hydrogen and carbon dioxide gases. Donker and others postulated formate as the initial basis of these gases. To make clear the view cited above and to decide whether the hydrogen gas may be used as a hydrogen donator in the fermentation, I made a test for the presence of formic hydrogenlyase and formic hydrogenase. These enzyme acitivities could not be recognized in the cells of 24 hour old culture, but could be recognized in an 18 hour old culture.

The activity of the formic hydrogenlyase was shown by Q_{2H_2}* as 0.5 (Hydrogen production by the cells from formate continued for two hours linearly). The activity of the formic hydrogenase in 1 atm. of H_2 gas was

* Q_{2H_2} : $1/2\,H_2{}^{\mu l}$/ 1 mg. dried cells, 1 hr.

strong and its values were shown as follows: $Q_{Mb}=0.5\times10^{-7}$ at pH 7.5, $Q_{Mb}=2.4\times10^{-7}$ at pH 7.0, and obstructive at pH 5.0. In conclusion it can be said that hydrogen gas is formed from formate and that the hydrogen gas which is formed is activated by the formic hydrogenase.

It is hard to determine definitely the physiological state in which enzymes are really active and through which processes the fermentation really proceeds, without further study on the problems relating to substrate concentration, cultivation conditions, treatment during experiments and other variable conditions.

Therefore it can be seen that the results obtained were evaluated in order to give an outline of the enzyme systems possessed by butyric acid bacteria.

d. Michaelis Constants of Several Enzyme Systems.

Influences of the substrate concentration on the velocity of the enzymes such as pyruvic, crotonic, butyric, β-oxybutyric, glycerophosphoric and lactic dehydrogenase, were studied. These are active in an acid condition and are supposed to be important in the metabolism of carbohydrates (see

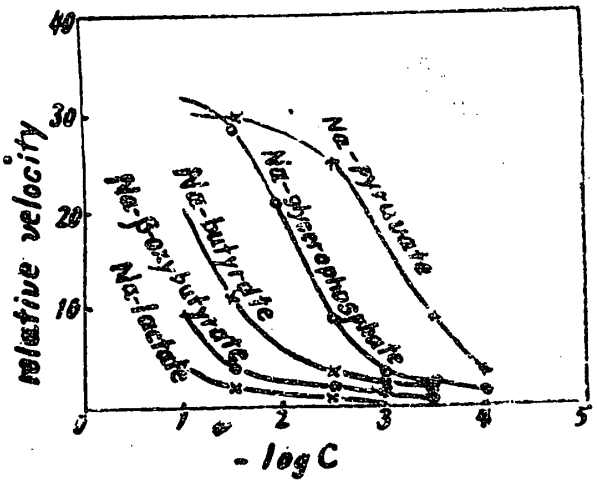

Fig. 1. Influence of substrate concentration on the relative velocity of dehydrogenation.

Fig. 1, 4 and 5). Fig. 1 shows the pS-curves of the various enzymes at pH 5, the half velocity substrate concentrations (the Michaelis constant) obtained from the figures are as follows: pyruvic dehydrogenase; 8.4×10^{-4} Mol*, glycerophosphoric dehydrogenase; 2.0×10^{-3} Mol**. For the sake of

* The K_m value obtained by W. Kocholaty and Hoogerheide (5) with *Cl. sporogenes* and Briliant cresyl blue was 8×10^{-3} Mol.

** The K_m value obtained by Green (1936. Biochem. J. 30, 629), with enzyme preparation isolated from muscle of rabbit at pH 7.2 using methy-lene blue was 1.1×10^{-3}

examining the values obtained from the curves and for calculation of the Michaelis constants of enzymes such as butyric, β-oxbutyric and lactic dehydrogenase, which had a low affinity to the substrate that I could not obtain the Michaelis constants from the curves, I tried to find the values with the following fomula of Micharlis-Menten;

$$v = V_{max} / (1 + K_m/[s]) \text{ or } 1/v = 1/V_{max} + K_m/V_{max} \times 1/[s]$$

In the case of pyruvic dehydrogenase values 1/v kept a linear value against 1/[s] and the value Km (8.2×10^{-4}) agreed well with the value from the curve. In the case of sodium-glycerophosphate, the Km value, showing some deviation from the former, was 2.5×10^{-3}. But in the case of enzymes of low affinity with the substrate the values 1/v did not keep linear values against 1/[s] and therefore a definite value could not be obtained.

All the experiments were done with considerable cares, the substrate concentrations were determined by alkalimetric titration, so that there should be no significant errors. But the acute Km values were not obtained, therefore it is supposed that some error might have been made in the method of measuring the velocities of decoloration.

Following data show the texture of decoloration against time.

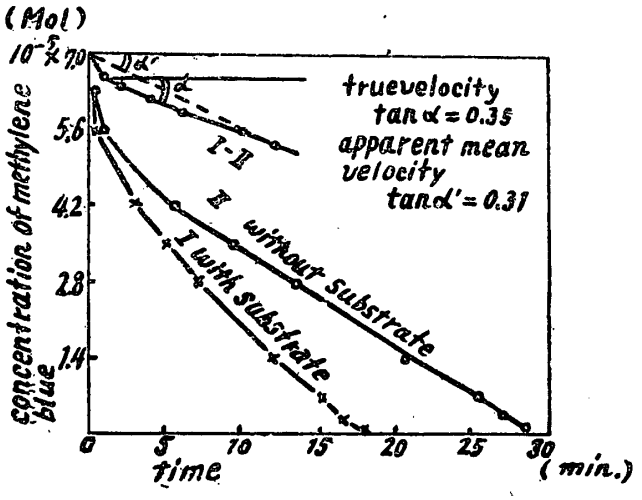

Fig 2. Rate of methylene blue reduction.

As can be seen from the figure, decoloration velocity was at first very rapid, then slower and then constant, showing the linearity of decoloration against time.

This indicates that in the initial stage some unknown substrates other than those supplied were consumed in the methylene blue reduction. It is supposed that the velocity of methylene blue reduction with a substrate is between the velocity referred to pyruvate and that of butyrate. In the

former experiments the cell suspensions in the Thunberg's tubes were incubated in a water bath for ten minutes at 37°C before mixing with substrate. When the time of incubation before mixing was shortened to one minute, the decoloration curve against time showed the linear values shown in Fig. 3.

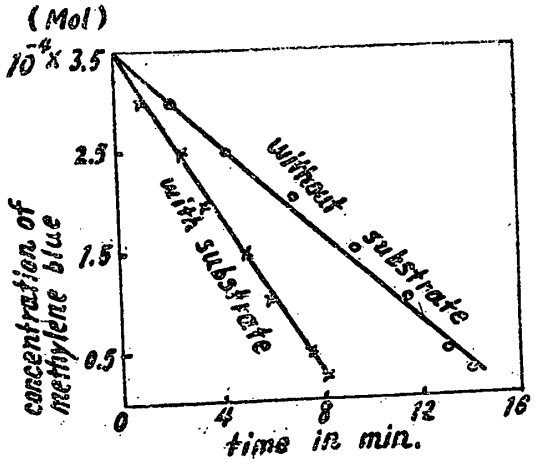

Fig. 3. Rate of methylene blue reduction.

Thus, the velocity values obtained from the former experiments indicate mean values and are not satisfactory for use in kinetic treatments. The experiments to obtain the Michaelis constants referred to β-oxybutyrate and to crotonate were tried again by an improved method. (see Figs. 4 & 5). The Michaelis constants of β-oxybutyric dehydrogenase were 2.1×10^{-2} at

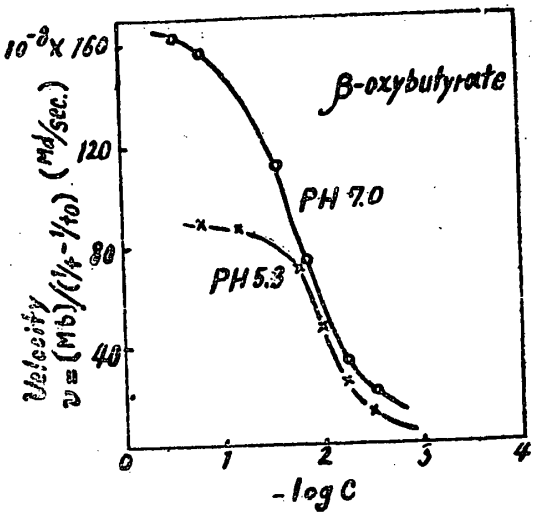

Fig. 4. Influence of substrate concentration on the relative velocity of dehydrog

pH 7.0 and 1.0×10^{-2} at pH 5.0*, when measured from the pS-curve. But in this case too, the values 1/v against 1/[s] deviated considerably from a straight line, and so the Km values thus obtained were not very accurate.

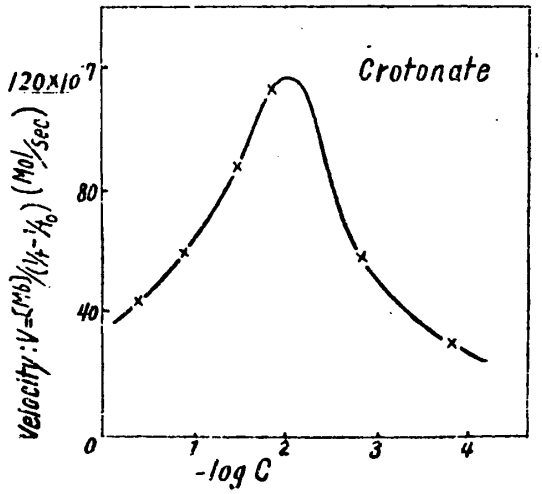

Fig. 5. Influence of substrate concentration on the relative velocity of dehydrogenation.

In the case of crotonate as shown in Fig. 5, substrate inhibition were noticeable at concentrations higher than M/70. It is clear that this was not inhibition by reaction products, because the methylene blue concentration was dilute enough and the methylene blue reduction against time proceeded in line values as shown in Fig. 3. The changes of hydrogen ion concentration caused by the addition of the substrate of higher concentration might not invite the inhibition, because the maximum deviation in hydrogen ion concentration caused by the addition of substrate was only pH 0.1 from the initial solution. The experiments with the substrates of low concentration, in which many difficulties were encountered, will be performed again using more accurate procedures.

e. Promotive Effect of Phosphate to Pyruvic Dehydrogenase.

If the pyruvate were decomposed by phosphoroclastic reaction as Koepsell and Lipmann investigated (5, 6), the dehydrogenation of the pyruvate would be promoted by the addition of phosphate. The following experiments proved this to be true. The dehydrogenation of pyruvate was noticeably rapid when the reaction was carried out in a phosphate buffer at pH 5.0, while in the citrate buffer even though phosphate was added no dehydrogenation could be recognized, as shown in Table III. This indicates the possibility that the citrate buffer may inhibit the dehydrogenation of pyruvate.

* The K_m values obtained by Green (1937. Biochem. J. 31, 934), with the enzyme preparation of *l*-β-oxybutyric acid dehydrogenase using oxygen as a hydrogen acceptor was 5×10^{-3} at pH 7.3.

Table III. Inhibiton of citrate buffer.

	A	B	C	D	E	F
Citrate buffer	1.0ml	1.0ml	1.0ml	1.0ml	—	—
Phosphate buffer	—	—	1.0	1.0	2.0ml	2.0ml
Distilled water	1.5	1.0	0.5	—	0.5	—
Pyruvate	—	0.5	—	0.5	—	0.5
Time of methylene blue reduction	>1hr.	>1hr.	>1hr.	>1hr.	795 〃	148
Q_{Mb}.	—	—	—	—	0	1.2×10^{-7}

Conditions of experiments: Cell suspensions: 0.5 ml (dried weight 8.7 mg).
Methylene blue solution: M/1,000. 0.2 ml
Total volume : 3.2 ml
pH : 5.0.
Temp.: 37°C.

To avoid the inhibitory effect of citrate buffer, the contents of the Thunberg's tubes were prepared as follows: before mixing, the cell suspension alone was placed in the hollow stopper and the other agents in the main tube, restricting the time of contact of the cells with citrate to the minimum. The results thus obtained are shown in Table IV. The dehydrogenation of pyruvate was markedly promoted by the phosphate buffer, while the other substrates were but little affected (see Table V).

Table IV. Influence of buffer.

	A′	B′	C′	D′
Citrate buffer	1.0ml	1.0ml	1.0ml	1.0ml
Phosphate buffer	—	—	1.0	1.0
Distilled water	1.5	1.0	0.5	—
Pyruvate	—	0.5	—	0.5
Time of methylene blue reduction	>100′	>100′	>100′	24′

Conditions of experiments: Cell suspensions: 0.5 ml.
Mb : M/1,000. 0.2 ml.
Total volume : 3.2 ml.
pH : 5.0.
Temp.: 37°C.

Table V. Promotive effect of phosphate buffer on the dehydrogenation with pyruvate.

	Citrate buffer only				With phosphate buffer			
Citrate buffer	1.0ml	1.0ml	1.0ml	1.0ml	1.0ml	1.0ml	1.0ml	1.0ml
Phosphate buffer	—	—	—	—	1.0	1.0	1.0	1.0
Distilled water	1.5	1.0	1.0	1.0	0.5	—	—	—
Substrate	—	lactate 0.5	pyruvate 0.5	glycerophosphate 0.5	—	lactate 0.5	pyruvate 0.5	glycerophosphate 0.5
Time of methylene-blue reduction	99′	45′	44′	137′	33′	23′	75″	29′
Q_{Mb}	—	7.2×10^{-9}	7.5×10^{-9}	inhibition	—	7.4×10^{-9}	4.6×10^{-7}	2.5×10^{-9}

Experimental conditions: Cell suspension: 0.5 ml (dried weight 5.4 mg).
Mb: M/1,000. 0.2 ml.
Total volume: 3.2 ml.
pH: 5.0.
Temp.: 37°C.

References

1. Stickland, L. H. 1934. Biochem. J. 28, 1746.
2. Stickland, L. H. 1935. Biochem. J. 29, 888.
3. Kocholaty, W. and J. C. Hoogerheide. 1938. Biochem. J. 32, 437.
4. Hoogeerheide, J. C. and W. Kocholaty. 1918. Biochem. J. 32. 949.
5. Koepsell H. J. and M. J. Johnson. 1942. J. Biol, Chem., 145, 379.
6. Lipmann. 1947. Advances in Enzymology vol. 6, 231.