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# 63. Takayuki Misawa, Hiroyasu Kaneshima, and Masuo Akagi : Studies on the Metabolism of Borate. Ⅳ.*1 Effect of Borate on Glyceraldehydephosphate Dehydrogenase.*2

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In the previous paper,*1 it was reported that the levels of fructose 1, 6-diphosphate (FDP) in the liver of the rats and guinea pigs revealed a remarkable tendency to rise within four hours after oral administration of borax and also that anaerobic glycolysis of glucose in the homogenates of liver and brain of the animals were considerably inhibited by borate.

In the present study it was found that also *in vitro* experiments with the homogenates of liver and brain of guinea pigs the levels of fructose 6-monophosphate and FDP in anaerobic glycolysis system markedly rised by the addition of borate.

It would be desirable to investigate the effects of borate on each enzymic step in the pathway from FDP to lactate, and find out which of the enzymes involved might be inhibited by borate.

Aldolase*5 was omitted from the investigations because boron is involved as borate buffer in the reaction mixture of the assay system of this enzyme by the method of Herbert, *et al.*[1] Therefore the effects of borate on glyceraldehydephosphate dehydrogenase (GAP dehydrogenase), the enzyme of the second step, was studied and it was found that this enzyme was obviously inhibited by borate.

Furthermore the effects of borate on lactate dehydrogenase*6, the enzyme of the last step of anaerobic glycolysis system in which nicotinamid adenine dinucleotide (NAD) was involved as coenzyme, were also studied. In this paper, the results of the above investigation will be described.

## Methods

**Purification of GAP Dehydrogenase**——The enzyme was isolated and purified in the crystalline form from baker's yeast through the method described by Krebs[2] based on the similar method of Kunitz and McDonald.[3]

**Assay of Yeast GAP Dehydrogenase**——Assay of yeast GAP dehydrogenase was carried out by measuring the rate of change of optical density at 340 mμ, the absorption maximum of NADH by the method of Krebs[2] which was essentially identical to that originally described by Warburg and Christian[4] for the dehydrogenase from yeast. Because of the fact established by Warburg and Christian that free D-glyceraldehyde (GA) also reacts with coenzyme in the presence of phosphate if 1000 times as much enzyme is used, and also of the fact confirmed by Meyerhof, *et al.*[5] that the same phenomenon is observable as in the reaction with GA 3-phosphate, the effects of borate on yeast enzyme in our research were tested with both GA 3-

*1 Part Ⅲ. M. Akagi, T. Misawa, H. Kaneshima : This Bulletin, **11**, 1461 (1963).
*2 D-glyceraldehyde-3-phosphate : NAD oxydoreductase (phosphorylating).
*3 Nishi-15-chome, Minami-2-jo, Sapporo (三沢隆行, 金島弘恭).
*4 Nishi-5-chome, Kita-12-jo, Sapporo (赤木満洲雄).
*5 Ketose-1-phosphate aldehyde-lyase.
*6 L-lactate : NAD oxydoreductase.

1) D. Herbert, *et al.* : Biochem. J., **34**, 1108 (1940).
2) E.G. Krebs : Methods in Enzymology, **I**, 407 (1955).
3) M. Kunitz, McDonald : J. Gen. Physiol., **29**, 393 (1946).
4) O. Warburg, W. Christian : Biochem. Z., **303**, 40 (1939).
5) O. Meyerhof, P. Oesper : J. Biol. Chem., **170**, 1 (1947).

phosphate and GA as substrates. As the same effect of borate was found in both cases, the most part of our study was carried out with GA, which was obtainable more easily from commercial source, as substrate instead of GA 3-phosphate. Specific activity was determined by the method of Krebs too.

**Assay of GAP Dehydrogenase in Liver Homogenate**——As the assay method described above was not applicable to homogenate, GAP dehydrogenase in liver homogenate was assayed as described below. In a 10 ml. glass stoppered test tube was prepared an incomplete reaction mixture consisted of 1.5 ml. of pyrophosphate-cysteine buffer (pH 8.5), 0.1 ml. of NAD, 0.1 ml. of sodium arsenate, 0.3 ml. of GA 3-phosphate. The concentrations of all the reagents were same as in the assay of yeast GAP dehydrogenase described above. Reaction was initiated by the addition of the final component, 1.0 ml. of 10% liver homogenate. The total volume of complete reaction mixture was 3.0 ml. After incubation for 20 min. at room temperature the reaction was stopped by the addition of 3.0 ml. of 10% trichloracetic acid. The mixture was centrifuged and the supernatant fluid was taken in a silica cuvette. Reading at 340 mμ was taken; it was corrected for any absorption at zero time.

**Assay of Rabbit Muscle Lactate Dehydrogenase**——The assay of lactate dehydrogenase*7 was carried out by the method of Kornberg.[6]

**Assay of Lactate Dehydrogenase in Liver Homogenate**——Assay of lactate dehydrogenase in liver homogenate was carried out as described below. In a 10 ml. glass stoppered test tube was prepared an incomplete reaction mixture consisted of 1.0 ml. of $KH_2PO_4$-$K_2HPO_4$ buffer (pH 7.4), 0.1 ml. of NADH, 0.1 ml. of sodium pyruvate, and 0.8 ml. of water. The concentrations of all the reagents were same as in the assay of rabbit muscle lactate dehydrogenase by Kornberg.[6] Reaction was started by the addition of the final component, 1.0 ml. of 10% liver homogenate prepared with 0.01*M* NaCl. After incubation for 20 min. at room temperature, the reaction was stopped by the addition of 3.0 ml. of 10% trichloracetic acid. The mixture was centrifuged and the supernatant fluid was taken in a silica cuvette. Reading at 340 mμ was taken; it was corrected for any absorption at zero time.

**Determination of Fructose 6-Monophosphate and FDP in Tissue Homogenates**——Fructose 6-monophosphate and FDP in tissue homogenates were determined spectrophotometrically by the method of Roe, *et al.*[7]

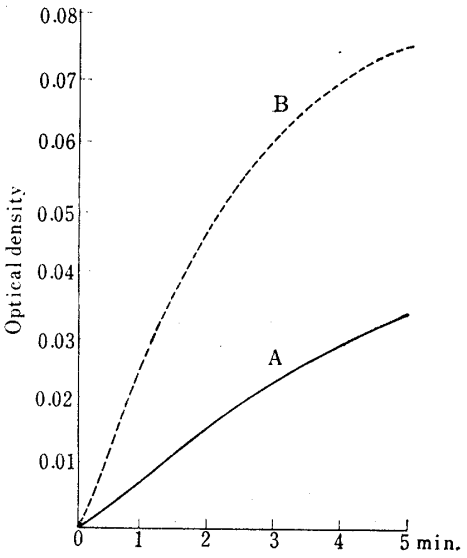


Fig. 1. Effect of Borate on Yeast Glyceraldehydephosphate Dehydrogenase with D-Glyceraldehyde 3-Phosphate as Substrate

Curve A, 0.003*M* borate; Curve B, control. 0.0025*M* GAP was used as substrate, and the enzyme activities were measured at pH 8.5, at room temperature by the method of Krebs.

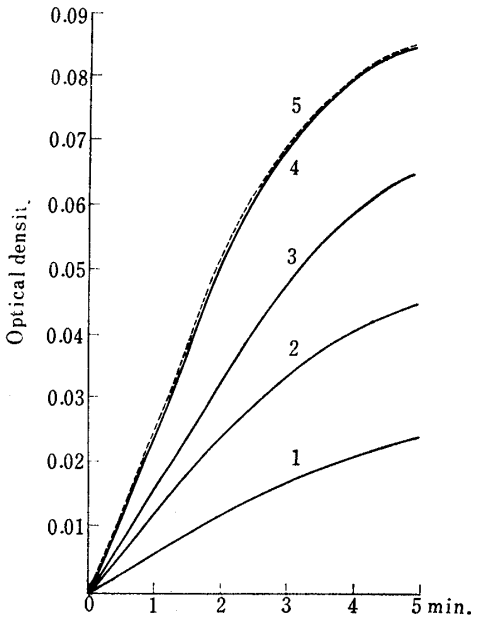


Fig. 2. Effect of Borate on Yeast Glyceraldehydephosphate Dehydrogenase with D-Glyceraldehyde in Relation to Various Borate Concentrations

Curve 1, 0.01*M* borate; Curve 2, 0.005*M* borate; Curve 3, 0.003*M* borate; Curve 4, 0.001*M* borate; Curve 5, control. 0.002*M* GA was used as substrate, and the enzyme activities were measured at pH 8.5, at room temperature.

*7 Lactate dehydrogenase was the preparation of Sigma Chemical Company.

6) A. Kornberg: Methods in Enzymology, **I**, 441 (1955).
7) J. H. Roe, *et al.*: J. Biol Chem., **210**, 703 (1954).

## Results

**Effects of Borate on Yeast GAP Dehydrogenase and on GAP Dehydrogenase in Liver Homogenate**——Effects of borate on yeast GAP dehydrogenase were investigated by addition of 0.1 ml. of 0.09 *M* borate to the reaction mixture. The yeast GAP dehydrogenase was found to be inhibited remarkably by 0.003 *M* borate (this term in every case refers to sodium tetraborate except otherwise noted) in both cases using GA 3-phosphate and GA as substrate, and the results are shown in Fig. 1 and 2, respectively. Fig. 2 showed, in addition, the degrees of inhibition by various concentrations of the borate.

Effects of Borate on GAP dehydrogenases in liver homogenates of rats and guinea pigs were investigated by addition of 0.2 ml. of 0.15 *M* borate (0.01 *M*, final concentration) to the reaction mixture. As indicated in Table I, also the GAP dehydrogenases in liver homogenates were evidently inhibited by borate.

Table I. Effect of Borate on Glyceraldehydephosphate Dehydrogenase in Liver Homogenate

| | Opt. density of NADH after 20 min. | | |
|---|---|---|---|
| | Borate[a] | Control | Ratio B/C |
| Rat liver homogenate | 0.010 | 0.026 | 1:2.6 |
| | 0.011 | 0.033 | 1:3.3 |
| | 0.010 | 0.028 | 1:2.8 |
| | | average | 1:2.9 |
| Guinea pig liver homogenate | 0.015 | 0.033 | 1:2.2 |
| | 0.010 | 0.030 | 1:3.0 |
| | 0.012 | 0.031 | 1:2.5 |
| | | average | 1:2.5 |

*a*) 0.01 *M* borate.

The enzyme reactions were performed at pH 8.5, at room temperature. GAP dehydrogenase activities in liver homogenate were measured as described in the section of Methods.

**Effects of Borate on Muscle Lactate Dehydrogenase and on Lactate Dehydrogenase in Liver Homogenate**——Effects of borate on lactate dehydrogenase from rabbit muscle was investigated by addition of 0.1 ml. of 0.15 *M* borate (0.005 *M*, final concentration) to the reaction mixture. Effect of borate on nonpurified lactate dehydrogenases in liver homogenates of guinea pigs was also tested by addition of 0.2 ml. of 0.15 *M* borate (0.01 *M*, final concentration) to the reaction mixture. In both cases lactate dehydrogenases were found not to be affected by borate at all as shown in Fig. 3 and Table II respectively.

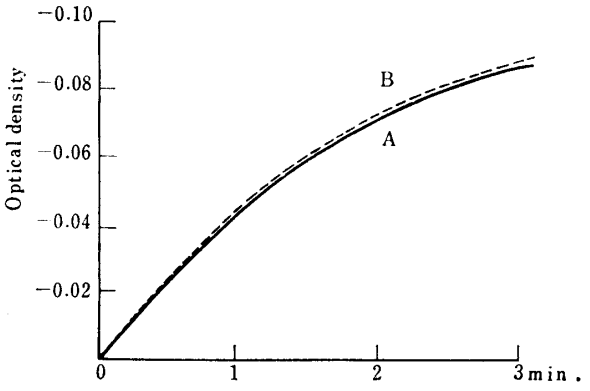


Fig. 3. Effect of Borate on Lactate Dehydrogenase from Rabbit Muscle

Curve A, 0.005*M* borate; Curve B, control. The enzyme activities were measured at pH 7.4, at room temperature by the method of Kornberg.

**Effects of Borate on the Levels of Fructose 6-monophosphate and FDP in Anaerobic Glycolysis of Homogenate**——With liver and brain homogenates of guinea pigs, glycolytic tests by the method of

LePage[8] were carried out after addition of 0.033 *M* boric acid-potassium bicarbonate buffer (0.01 *M*, final concentration) to the reaction mixture, and after 30 min. incubation at 38° levels of fructose 6-monophosphate and FDP were determined. As represented in Table Ⅲ, the both sugar phosphates levels were found to rise obviously by the addition of borate.

Table Ⅱ. Effect of Borate on Lactate Dehydrogenase in Liver Homogenate

| Opt. density of NADH after 20 min. | | Opt. density of NADH after 20 min. | |
|---|---|---|---|
| Borate[a] | Control | Borate[a] | Control |
| 0.025 | 0.025 | 0.024 | 0.024 |
| 0.019 | 0.020 | 0.024 | 0.022 |
| 0.031 | 0.030 | 0.020 | 0.020 |

*a*) 0.01*M* borate.
The enzyme reactions were performed at pH 7.4, at room temperature. Lactate dehydrogenase activities in liver homogenate were measured as described in the section of Methods.

Table Ⅲ. Effect of Borate on the Levels of Fructose 1,6-Diphosphate and Fructose 6-Monophosphate in Anaerobic Glycolysis of Tissue Homogenate

| | Experimental No. | Sugar phosphates levels in reaction mixture after 30 min. | | | | | |
|---|---|---|---|---|---|---|---|
| | | FDP | | | F6P | | |
| | | Control (μg./ml.) | Borate[a] (μg./ml.) | Rate of increase (%) | Control (μg./ml.) | Borate[a] (μg./ml.) | Rate of increase (%) |
| Rat liver homogenate | 1 | 9 | 27 | 200 | 5 | 22 | 330 |
| | 2 | 17 | 33 | 94 | 8 | 23 | 190 |
| | 3 | 10 | 22 | 120 | 6 | 18 | 200 |
| | 4 | 16 | 29 | 81 | 7 | 13 | 86 |
| | 5 | 14 | 31 | 120 | 8 | 21 | 160 |
| | | | average | 123±12 | | average | 195±41 |
| Guinea pig liver homogenate | 1 | 18 | 85 | 360 | 11 | 18 | 64 |
| | 2 | 10 | 52 | 420 | 8 | 20 | 150 |
| | 3 | 29 | 84 | 189 | 12 | 39 | 210 |
| | 4 | 31 | 101 | 220 | 4 | 16 | 300 |
| | 5 | 23 | 86 | 270 | 12 | 34 | 180 |
| | | | average | 291±43 | | average | 200±39 |

*a*) 0.01*M* borate.
The reactions were performed at pH 7.6, at 38°. The sugar phosphates were determined by the method of Roe, *et al.*

**Effects of Sugars or Polyhydroxy Compounds on Borate Inhibition of GAP Dehydrogenase**——As borate had been known to react with sugars or polyhydroxy compounds and form complexes with them, we tested whether the borate inhibition of GAP dehydrogenase is reversed by the addition of excess sugars or polyhydroxy compounds or not. Several compounds such as glucose, sorbose, ribose, mannitol and glycerol were tested, but none of these were appeared to reverse the inhibition by borate in the experimental condition as shown in Fig. 4 and 5.

**Effects of Dialyse on Borate Inhibition of GAP Dehydrogenase**——It was investigated whether or not the inhibition of GAP dehydrogenase by borate may be reversed by dialysis. Three volumes of 0.15 *M* borate were added to enzyme solution whose specific activity was $1.85\times10^3$, and so the specific activity reduced to $0.160\times10^3$. As control,

8) G. A. LePage : J. Biol. Chem., **176**, 1009 (1948).

enzyme solution added three volumes of distilled water was used. After both enzyme solutions were dialysed in 500 ml. of water for 24 hours and 48 hours in a cold room, the specific activities of the both were compared with each other. As is seen from the results shown in Table Ⅳ the specific activity of the enzyme solution added borate became equal to that of control enzyme solution. By this fact, the borate inhibition was found to be reversed by dialysis.

**Graphical Analysis of Borate Inhibition of GAP Dehydrogenase**——In Fig. 6, the reciprocals of the reaction velocities with 0.003 *M* borate and with no borate were

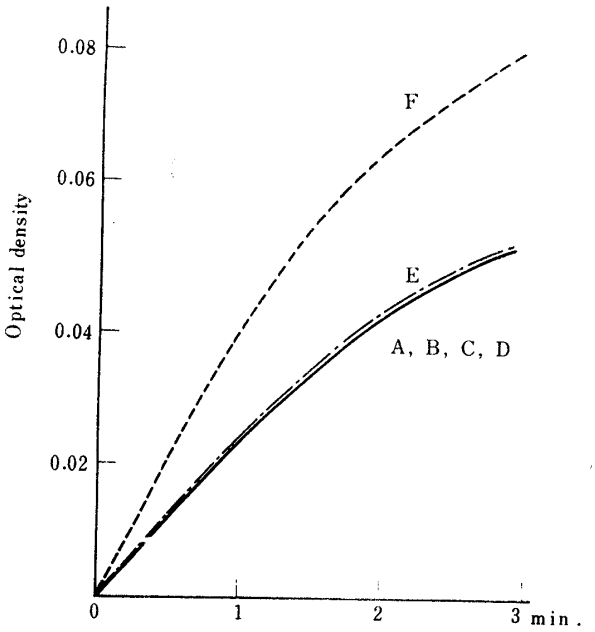


Fig. 4. Effect of Sugar or Sugar Alcohol on Borate Inhibition of Yeast Glyceraldehyde-phosphate Dehydrogenase

Curve A, 0.003*M* borate and 0.12*M* glucose; Curve B, 0.003*M* borate and 0.12*M* sorbose; Curve C, 0.003*M* borate and 0.033*M* ribose; Curve D, 0.003*M* borate and 0.033*M* mannitol; Curve E, 0.003*M* borate alone; Curve F, control (no borate).

In Curve A, B, C, and D, borate and sugar or sugar alcohol were added to the reaction mixture adjusted to pH 8.5 with 0.03*M* sodium pyrophosphate-0.003*M* cysteine buffer and in Curve E, borate alone was added to the same reaction mixture.

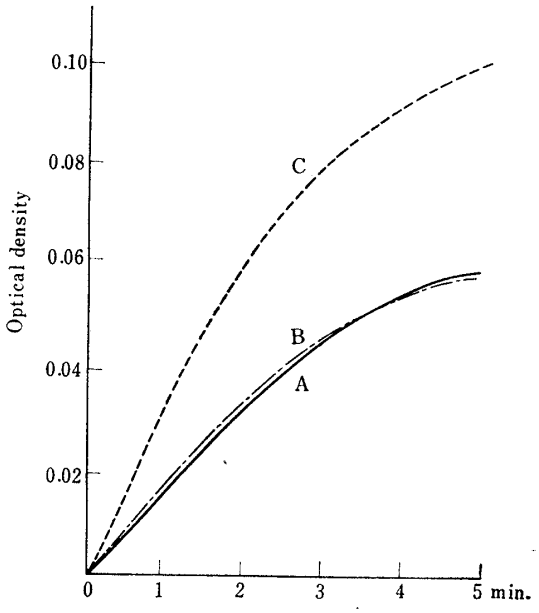


Fig. 5. Effect of Glycerol on Borate Inhibition of Yeast Glyceraldehyde-phosphate Dehydrogenase

Curve A, 0.003*M* borate and 0.04*M* glycerol; Curve B, 0.003*M* borate alone; Curve C, control (no borate).

In Curve A, borate and glycerol were added to the reaction mixture adjusted to pH 8.5 with 0.03*M* sodium pyrophosphate-0.003*M* cysteine buffer, and in Curve B, borate alone was added to the same reaction mixture.

Table Ⅳ. Effect of Dialyse on Borate Inhibition of Yeast Glyceraldehydephosphate Dehydrogenase

| Hours for dialysis (hr.) | Specific activity of enzyme | |
|---|---|---|
| | Control | Borate |
| 0 | 1.850×10 | 0.160×10 |
| 24 | 2.390×10 | 2.310×10 |
| 48 | 1.588×10 | 1.583×10 |

Dialyses were carried out in 500 ml. of water with cellophane tube for dialyse in a cold room at 3°. Specific activities were measured by the method of Krebs.

plotted respectively against the reciprocals of several concentrations of substrate (2.5 $\sim 32 \times 10^{-3}$ *M* GA) by the method of Lineweaver and Burk.[9] In Fig. 7, the reciprocals of the reaction velocities for two different concentrations of substrate (GA) were plotted respectively against several concentrations of inhibitor by the method of Dixon.[10] On the basis of the analysis of enzyme inhibition given by them, it was concluded that borate inhibition was of competitive to the substrate, and was greatest with low concentrations of substrate. From the Lineweaver-Burk plots in Fig. 6 the Michaelis constant (Km) and the value of the maximum velocity (Vm) with excess of substrate were calculated as $1.72 \times 10^{-2}$ *M* and $1.47 \times 10^{-1}$ (expressed in optical density of reduced NAD) respectively and from the Dixon's plots in Fig. 7 the enzyme-inhibitor dissociation constant (Ki) was calculated as $2.8 \times 10^{-3} M$ under the experiments.

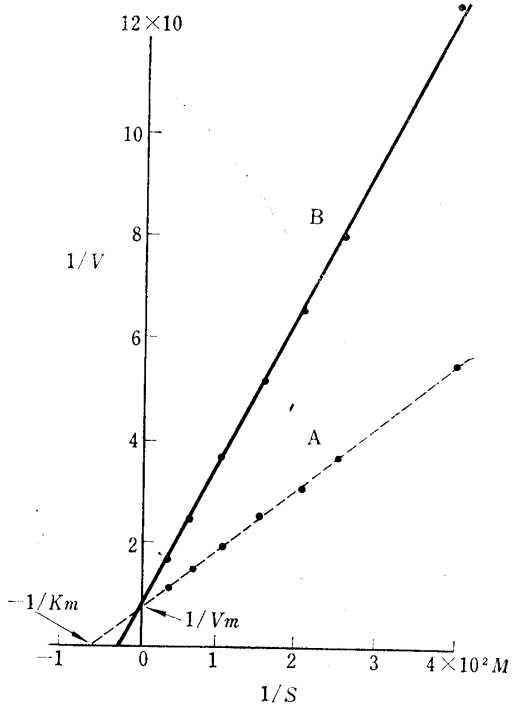


Fig. 6. Lineweaver-Burk Plots

*V*, initial velocity expressed in opt. density of NADH/min.; *S*, substrate (GA) concentration. Curve A, no borate; Curve B, 0.003*M* borate. The reactions were performed at pH 8.5, at room temperature. *Km* and *Vm* express the Michaelis constant and maximum velocity respectively.

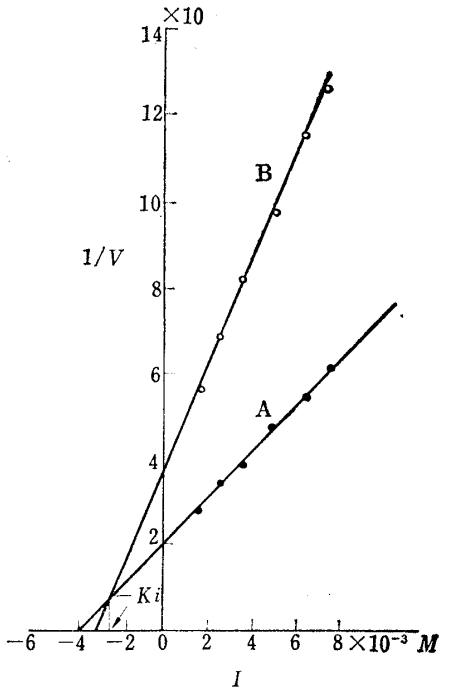


Fig. 7. Dixon's Plots

*V*, initial velocity expressed in opt. density of NADH/min.; *I*, inhibitor (borate) concentration. Curve A, 0.016*M* GA as substrate with the inhibitor; Curve B, 0.008*M* GA as substrate with the inhibitor. The reactions were performed at pH 8.5, at room temperature. *Ki* express the enzyme-inhibitor dissociation constant.

## Discussion

In the present study it was found that in anaerobic glycolysis of liver homogenates of rats and guinea pigs fructose 6-monophosphate and FDP were accumulated in the system by the addition of borate, and that GAP dehydrogenase was inhibited remarkably by borate, while lactate dehydrogenase was not affected by borate at all.

The nature of the inhibitory effect of borate on GAP dehydrogenase was found to be a competitive type from the Lineweaver-Burk plots or the Dixon's plots. The inhibitory effect of borate was reversed by dialysis but not reversed by the excess sugars or

9) H. Lineweaver, D. Burk : Am. Chem. Soc., **56**, 658 (1934).
10) M. Dixon : Biochem. J., **55**, 170 (1953).

polyhydroxy compounds at all up to the concentrations used. Thus the inhibition seemed not to be caused by the complex formation of the borate with the substrate.

Recently a formation of complex between borate and NADH has been reported,[11] in the present study, however, borate inhibited the enzymatic activity of GAP dehydrogenase but not that of lactate dehydrogenase at all.

The inhibition for alkaline phosphatase has been suspected by Zittle, *et al.*[12] to be caused by direct binding of borate anion with enzyme which could be competitively substituted by the substrate. As was discussed by Zittle, *et al.*,[12] it seems likely that borate inhibits GAP dehydrogenase by a similar mode to that for alkaline phosphatase, urease, arginase and pepsine as an anion, rather than it binds with coenzyme or substrate.

From the facts of borate inhibition of GAP dehydrogenase and accumulation by the effect of borate, of fructose 6-monophosphate and FDP in glycolysis system observed with liver homogenate of rats and guinea pigs, it would be explained that the accumulation of FDP in livers of the animals dosed borax which had been reported in the previous paper*1 should mainly be caused by the inhibition of glycolysis in the livers due to the inhibition of GAP dehydrogenase by borate. This disturbance of sugar metabolism may result in the fall of ATP production and the facts might account for one of the important factors of boron poisoning.

## Summary

1. It was found that fructose 1, 6-diphosphate and fructose 6-monophosphate were accumulated by borate in anaerobic glycolysis system of liver homogenates of guinea pigs.

2. Glyceraldehydephosphate dehydrogenase*2 (GAP dehydrogenase) from yeast and in liver homogenates of guinea pigs and rats were found to be inhibited by borate. The inhibition was observed in both cases in which free D-glyceraldehyde or D-glyceraldehyde 3-phosphate was used as substrate.

3. GAP dehydrogenase was inhibited competitively by borate, and the inhibition was reversed by dialysis, but not reversed by excess sugars or polyhydroxy compounds in the experimental condition.

4. Borate has no effect on lactate dehydrogenase*6 from rabbit muscle and also in liver homogenates of guinea pigs.

5. Toxicity of borate was discussed from biochemical point of view.

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11) P. Strittmatter : J. Biol. Chem., **239**, 3043 (1964).
12) C. A. Zittle, *et al.* : Advances in Enzymology, **12**, 493 (1951).