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Studies on the Metabolic Effects of Borate. VI.[1] Effects of Borate on the Reduction of Methemoglobin

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The spontaneous reduction of methemoglobin was markedly inhibited by borate and this inhibition was recovered by reduced nicotinamide adenine dinucleotide. Also the inhibition of glyceraldehydephosphate dehydrogenase activity in bovine blood by borate was observed. The toxicity of borate to blood was discussed.

In the previous paper[1] of this series, it was reported that the administered inorganic borate entered into red blood cell of guinea pig and induced remarkable accumulation of methemoglobin(MeHb). Furthermore it was also found that borate inhibited anaerobic glycolysis and depressed the level of lactate concentration in red blood cell of experimental animals.

From the view-point of potential toxicity of borate toward blood, these findings prompted us to investigate the interrelationship between the accumulation of MeHb and the inhibition of glycolysis in red blood cell induced by borate.

In the present study it was observed that, *in vitro* experiments with the whole blood of guinea pig, the spontaneous reduction of MeHb was markedly inhibited in the presence of borate. It was also found that this inhibition was completely recovered by the addition of sufficient amounts of reduced nicotinamide adenine dinucleotide (NADH). However, the effect of reduced nicotinamide adenine dinucleotide phosphate (NADPH) on the recovery of inhibition was found to be slight. To elucidate the different actions of these two coenzymes toward recovery of inhibition caused by borate, the effects of borate on the activities of glyceraldehydephosphate dehydrogenase[3] (GAP dehydrogenase), lactate dehydrogenase,[4] and glucose-6-phosphate dehydrogenase[5] (G6P dehydrogenase) in red blood cell were investigated.

Materials and Methods

Animals and Diet——Male guinea pigs (0.6—0.9 kg, body wt.) were used. The keeping and diet were the same as described in the previous paper.[6]

Bloods——Blood of guinea pig was withdrawn by heart puncture and was added 2 mg of potassium oxalate to per ml of blood. Bovine blood was collected from local slaughterhouse, Sapporo Chikusan Kosha, by cutting across carotide artery and kept with acid citrate dextrose (ACD). Human blood with ACD was obtained from Red Cross Blood Center, Sapporo.

Only fresh blood was aseptically used throughout these experiments.

Chemicals——NADH and NADPH were obtained from Sigma Chemical Co., U.S.A. All other chemicals were obtained from Wako Pure Chemical Ind., Ltd., Japan.

Nitrite Solution——Sodium nitrite was recrystallized and dissolved in 0.9% sodium chloride solution.

1) Part V: H. Kaneshima, T. Misawa, and M. Akagi, *J. Hyg. Chem. Japan*, **12**, 14 (1966).
2) Location: *a) Nishi-15-chome, Minami-2-jo, Sapporo. b) Nishi-7-chome, Kita-15-jo, Sapporo.*
3) D-Glyceraldehyde-3-phosphate: NAD oxidoreductase (phosphorylating)
4) L-Lactate: NAD oxidoreductase.
5) D-Glucose-6-phosphate: NADP oxidoreductase.
6) M. Akagi, T. Misawa, and H. Kaneshima, *Yakugaku Zasshi*, **82**, 934 (1962).

Borate Solution——Boric acid solution (0.82 M) was adjusted to pH 7.0 with potassium bicarbonate solution.

Enzyme Protein Fractions——Enzyme protein fractions of GAP dehydrogenase, lactate dehydrogenase, and G6P dehydrogenase activities were prepared from hemolysate (pH, 7.0) of cattle according to the method of Hennessey, *et al.*[7] The used concentration of protein were 1.0 to 2.5 mg per ml for the assays of enzymatic activities.

GAP Dehydrogenase——The assay of activities was carried out by measuring the rates of change of optical density at 340 mμ, the absorption maximum of NADH, by the method of Velick.[8]

The assay mixture contained in a total volume of 3 ml: 2 mM glyceraldehyde-3-phosphate, 1 mM NAD, 0.56 mM sodium arsenate, 26 mM nicotinamide, 2 mM cysteine, 20 mM phosphate buffer (pH, 8.5), 0.2 ml of borate solution, and 0.3 ml of enzyme protein fraction.

Lactate Dehydrogenase——The assay of this enzyme was carried out by the method reported by Kornberg.[9] The assay mixture contained the following components in a total volume of 3 ml: 0.33 mM pyruvate, 0.33 mM NADH, 0.1 mM magnesium chloride, 0.06M phosphate buffer (pH, 7.4), 0.2 ml of borate solution, and 0.5 ml of enzyme protein fraction.

G6P Dehydrogenase——The method for measuring of the activities of the enzyme was that of Kornberg and Horecker.[10] The assay mixture contained the following components in a total volume of 3 ml: 0.67 mM G6P, 0.5 mM NADP, 6.7 mM magnesium chloride, 14.8 mM glycylglycine buffer (pH, 7.5), 0.2 ml of borate solution, and 0.5 ml of enzyme protein fraction.

MeHb Determination——MeHb determinations were carried out by the Evelyn-Malloy method[11] adapted to Hitachi Perkin-Elmer spectrophotometer, Type 139.

Results

Effects of Borate Concentrations on the Rates of MeHb Reduction——To the mixtures of 2 ml of fresh blood of guinea pig and 0.04 ml of 1% sodium nitrite solution were added 0.025 ml, 0.05 ml, and 0.1 ml of borate solution, respectively (final concentrations; 0.01 M, 0.02 M, and 0.04 M, respectively).

The mixtures were incubated with occasional shaking at 30°. The levels of produced MeHb were determined at one-hour intervals and the amounts of MeHb were expressed as per cents of the total hemoglobin (Hb). As collected in Fig. 1, the amounts of MeHb established maxima after two hours in all cases, and thereafter the levels of MeHb decreased gradually with times.

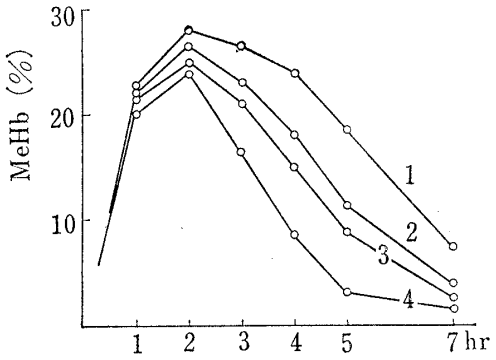

Fig. 1. Effects of Borate on the Reduction of MeHb in Guinea Pig Blood

curve 1, 0.04M borate; curve 2, 0.02M borate; curve 3, 0.01M borate; curve 4, control.
To 2 ml of blood of guinea pig were added 0.04 ml of 1% $NaNO_2$ solution (final concent.; 2.8 mmole) and borate solution. In the course of incubation at 30°, MeHb were determined by the method of Evelyn-Malloy.[11]

Effects of NADH and NADPH on the Rates of MeHb Reduction in the Presence of Borate——As described above, MeHb was produced by the treatment of guinea pig's blood with nitrite in the presence of borate (final concentration; 0.02 M) and two hours later (at maximum of MeHb levels) added NADH to the mixture to make its final concentration as 5 mM. Similarly, NADPH was added to the mixture as described for NADH.

Maxima and its rates of decrease were summarized in Table I.

7) M.A. Hennessey, A.M. Waltersdorph, F.M. Huennekens, and B.W. Gabrio, *J. Clin. Invest.*, **41**, 1257 (1962).
8) S.F. Velick, "Methods in Enzymology," Vol. 1, ed. by S. P. Colowick and N. O. Kaplan, Academic Press, Inc., Publishers, New York, 1955, 401.
9) A. Kornberg, *J. Clin. Invest.*, **1**, 441 (1955).
10) A. Kornberg and B.L. Horecker, *J. Clin. Invest.*, **1**, 323 (1955).
11) K.A. Evelyn and H.T. Malloy, *J. Biol. Chem.*, **126**, 655 (1938).

Table I. Effects of NADH and NADPH on the Reduction of MeHb in the Presence of Borate

Compounds	Final conc. (mM)	Maximum MeHb[a] (g/dl)	Decreasing rate from maximum MeHb 1 hr %	2 hr %	3 hr %	4 hr %	5 hr %
Control		4.00±0.18	71	47	25	18	11
Borate	20	4.29±0.10	88	69	46	37	26
NADH	5	4.08±0.16	57	35	13	8	2
NADH + Borate	5+20	4.22±0.07	73	48	25	15	7
NADPH	5	4.09±0.18	64	42	23	13	10
NADPH + Borate	5+20	4.23±0.09	83	62	42	34	22

a) Values are means±standard errors for five guinea pigs. MeHb determinations were performed as described in Fig. 1, after addition of NADH and NADPH respectively at 2 hours.

Reduction of MeHb was markedly improved by the addition of NADH and three hours later the levels of MeHb approached to that of control. On the other hand, the recovery of inhibition with NADPH was slight being compared with the case of NADH.

Effects of Borate on the Activities of Enzyme Protein Fraction from Bovine Red Blood Cell ——In the presence of borate, the activities of the following enzyme fractions were assayed: GAP dehydrogenase, lactate dehydrogenase, as NAD–NADH dependent, and G6P dehydrogenase sa NADP–NADPH dependent.

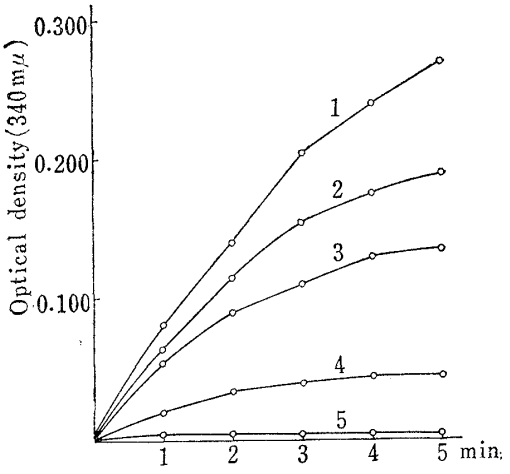

Fig. 2. Effects of Borate on Glyceraldehydephosphate Dehydrogenase

curve 1, control; curve 2, 0.001M borate; curve 3, 0.002M borate; curve 4, 0.01M borate; curve 5, 0.02M borate. 0.002M GAP was used as substrate, and the enzyme activities were measured at pH 8.5, at room temperature by the method of Velick[8]

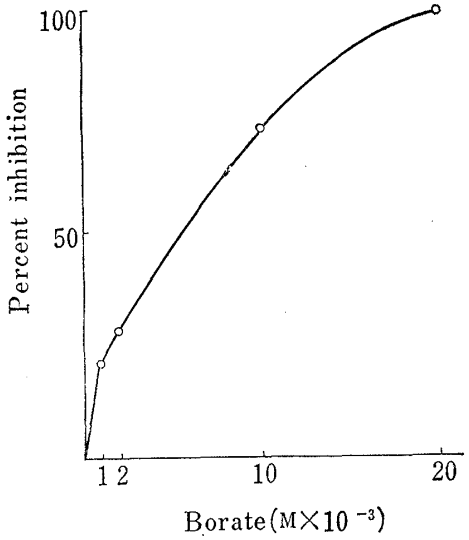

Fig. 3. Inhibition Rates of Glyceraldehydephosphate Dehydrogenase by Various Concentrations of Borate

Substrate; 0.002M GAP
pH; 8.5
Temp.; at room temperature

The activity of GAP dehydrogenase was reduced extensively by borate proportionately with its concentrations. The results were shown in Fig. 2. Fig. 3 illustrated the relationship between concentrations of borate and inhibition rates of GAP dehydrogenase, that is, the inhibition rates were 20% and 100% at 1×10^{-3}M and 2×10^{-2}M of borate, respectively.

Concerned with lactate dehydrogenase, on the other hand, no appreciable effect of borate on the activity was observed as described in Fig. 4.

For the activity of G6P dehydrogenase as shown in Fig. 5, data exhibited that the slight effects and 20% inhibition at 0.01M and 0.02M of borate concentration, respectively observed.

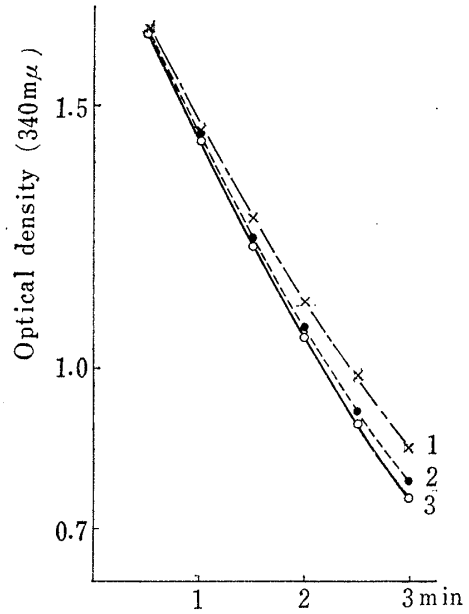

Fig. 4. Effects of Borate on Lactate Dehydrogenase

curve 1, 0.02M borate; curve 2, 0.01M borate; curve 3, control.
0.033mmole pyruvate was used as substrate, and the enzyme activities were measured at pH 7.4, at room temperature by the method of Kornberg.[9]

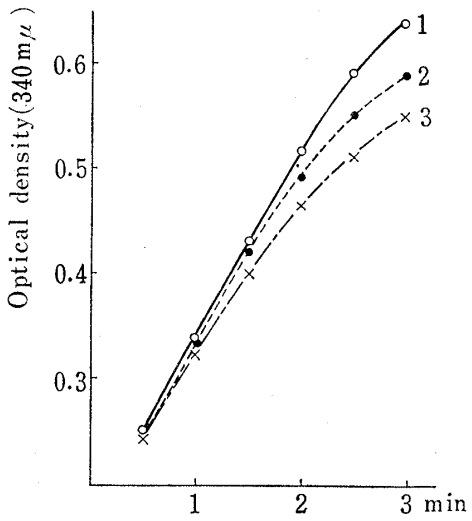

Fig. 5. Effects of Borate on Glucose-6-Phosphate Dehydrogenase

curve 1, control; curve 2, 0.01M borate; curve 3, 0.02M borate. 0.67 mM G6P was used as substrate, and the enzyme activities were measured at pH 7.5 at room temperature by the method of Kornberg, *et al.*[10]

Discussion

In the first paper[6] of this series, it was reported that orally administered borate distributed in some organs, including blood, of rabbit and guinea pig. Furthermore, by *in vitro* experiments, it was also elucidated[1] that borate was permeable into red blood cell and resulted in accumulation of MeHb and inhibition of glycolysis. According to Jandel, *et al*,[12] it was suggested that for red blood cell the first step to death was to be the oxidation of hemoglobin to MeHb, so that this work was designed to elucidate the toxic action of borate to blood.

As illustrated in Fig. 1, the spontaneous reduction of MeHb developed artificially by nitrite was obviously retarded in the presence of borate. It was also clearly indicated that the inhibition by borate depended upon the concentration of borate. Namely, 2.7, 3.4, and 5.0 times of MeHb were sustained being compared with control at the concentrations of 0.01M, 0.02M, and 0.04M borate after five hours respectively.

However, as compiled in Table I, the inhibition by borate was greatly recovered by the addition of NADH, on the other hand, it was slight restoration by the addition of NADPH.

It is suggested at the present time that the enzymatic reduction of MeHb depends upon the reductive potentials of either NADH generated in the Embden-Meyerhof anaerobic pathway or NADPH generated in the hexose monophosphate shunt.[13-15]

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13) H.R. Gutmann, B.J. Jandorf, and O. Bodansky, *J. Biol. Chem.*, **169**, 145 (1947).
14) O. Bodansky, *Pharmacol. Rev.*, **3**, 144 (1951).
15) M. Kiese and B. Weis, *Arch. Exper. Path. Pharmacol.*, **202**, 493 (1943).

In red blood cell under physiological conditions, it was suggested that NADH-linked pathway played major role for the reduction of MeHb being compared with NADPH-linked pathway.[16–19]

The experiments with enzyme fractions from bovine blood indicated that GAP dehydrogenase activity was greatly inhibited in the presence of borate as shown in Fig. 2 and 3.

For the activity of lactate dehydrogenase, no appreciable inhibition was observed by the treatment with borate. The inhibition of G6P dehydrogenase activity by borate was not so severe as like in the case of GAP dehydrogenase as described in Fig. 5, that is, slight effect at 0.01M borate and 20% inhibition at 0.02M borate were observed.

Data, as shown in Fig. 2, suggested that the accumulation of MeHb caused by borate might be mainly due to the restricted generation of NADH caused by borate-inhibited GAP dehydrogenase.

These results were supported by the previous reports from our laboratories that borate inhibited anaerobic glycolysis[20] and GAP dehydrogenase activity[21] in liver homogenate.

From these results, the cause of accumulation of MeHb by borate might be said that the major of accumulated MeHb were due to the deficiency of NADH necessary for the enzymatic reduction of physiologically occurred MeHb. Considerations of the accumulation of MeHb and inhibition of enzymatic activities, especially important for energy metabolism in red blood cell, by borate, warned us as to chronic poisoning of borate.

16) Q.H. Gibson, *Biochem. J.*, **42**, 13 (1948).
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21) T. Misawa, H. Kaneshima, and M. Akagi, *Chem. Pharm. Bull.* (Tokyo), **14**, 467 (1966).