Effects of Boranes upon Tissues of the Rat
I. Aspartate Aminotransferase and Lactic Dehydrogenase[1] (34791)

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The boron hydrides, or boranes, are a class of highly reactive compounds whose high heats of combustion have made them useful as high-energy propellants. This application, in addition to the use of boron in steel alloys, has led to a number of industrial accidents. Although extremely toxic, the basic mechanism of action of the boranes has not been clarified.

In the reported cases of accidental exposure of humans to boranes the effects included signs and symptoms of nervous system toxicity such as dizziness, weakness, slurred speech, headaches, and muscular incoordination (1, 2). Rats and dogs exposed to decaborane ($B_{10}H_{14}$) developed signs interpreted as further evidence that borane toxicity was primarily due to its effects upon the central nervous system (3).

Because the effects of boranes resemble those of reserpine, a number of workers studied biogenic amine metabolism and found a striking depletion of brain norepinephrine and serotonin following decaborane exposure (4, 5). However, monoamine oxidase inhibitors effective in reversing reserpine toxicity did not consistently ameliorate the effects of borane upon the levels of biogenic amines. Furthermore, there was no correlation between the effectiveness of a given monamine oxidase inhibitor in maintaining brain amine levels and its ability to relieve the toxic effects of borane (6).

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Because these earlier approaches to the problem of decaborane toxicity had not led to the elucidation of the basic mechanism of action of the boranes, and because the only enzyme known to be inhibited in the tissues of the treated animals was a pyridoxal-dependent enzyme (7), we began a study of several enzyme systems in the tissues of animals to examine the possibility that the primary effect of the boranes was due to the inhibition of pyridoxal enzymes, rather than a specific effect upon catecholamine metabolism in the brain. We found earlier that decaborane inhibited aspartate aminotransferase (EC 2.6.1.1) in several tissues of the rat (8). The current study was designed to investigate the effect of depletion of endogenous pyridoxal upon this inhibition, and to determine whether certain enzymes other than those dependent upon pyridoxal as cofactor are inhibited by decaborane.

Materials and Methods. Pyridoxal deficiency. Male Sprague-Dawley rats were randomly divided into two groups: one group was given free access to Purina laboratory chow and water, the other only to a pyridoxine-free chow supplied by the California Biochemical Corporation. After 2 to 3 weeks the rats fed the pyridoxine-deficient diet developed the characteristic stigmata of pyridoxine deficiency; the borane study was begun 6 weeks after the animals were selected for study and started on the appropriate diet. Five rats in each group were injected intraperitoneally with decaborane, 20 mg/kg of body weight, as a solution in corn oil. Five control animals in each group were injected with a similar amount of corn oil. Sixteen hr after injection the animals were sacrificed and the liver,

kidneys, brain, and heart quickly removed, frozen in liquid nitrogen, and stored at —25° for 1–3 days until the time of assay. All further procedures were carried out at 4°.

The tissues were homogenized in a solution of cold buffered sucrose (9) with a Ten-Broeck glass homogenizer. The homogenates were centrifuged at 700*g* in a Sorvall RC2-B for 20 min and the resultant supernatant was re-centrifuged at 20,000*g* for 30 min. The supernatant fluid obtained by the second centrifugation was used for the enzyme assays.

Enzyme assays. The lactic dehydrogenase activity of the supernatant fluid was assayed in duplicate by the method of Bergmeyer *et al.* (10) following dilution in phosphate buffer (0.05 *M*, pH 7.5). The assay mixture contained 0.88 μmoles of sodium pyruvate, 150 μmoles of sodium phosphate, and 0.6 μmoles of NADH in a volume of 3 ml (pH 7.5).

Aspartate aminotransferase (AAT) activity of a second aliquot of the supernatant fluid was measured in duplicate by the method of Bergmeyer and Bernt (11). The assay mixture contained 700 μmoles of *l*-aspartate, 0.6 μmoles of NADH, 25 mg of malic dehydrogenase (Mann Research Labs), and 0.1 ml of diluted tissue supernatant fluid. The reaction was started by adding 20 μmoles of α-ketoglutarate to bring the final volume to 3 ml and transamination was measured by the initial rate of decrease in absorbance at 340 mμ. Each tissue was reassayed in duplicate with the addition of 18 μ of pyridoxal phosphate to the incubation mixture.

The absorbance measurements were performed in a Beckman DU monochromator fitted with a Gilford Model 220 absorbance indicator and recorder. The samples and the assay mixtures were thermally equilibrated in the reservoir of a Gilson water bath prior to assay. The cuvette chamber was waterjacketed and the temperature was maintained at 25° ± 0.5° with the same circulating water bath. The assays were performed in duplicate and no pair of assays differed by more than 5%. The molar extinction coefficient of NADH was taken as 6.22×10^6 mole^{-1} liter^{-1} (12).

Protein content was determined by a modification of the biuret method and the enzyme activities expressed in terms of micromoles of NAD reduced/min/g of protein.

Time course. Twenty-four male Sprague-Dawley rats having free access to Purina chow were injected intraperitoneally with decaborane 20 mg/kg as a solution in corn oil. The animals were sacrificed at intervals of 4, 16, and 24 hr following the injection, and the tissues were removed as before for the aspartate aminotransferase and LDH assays.

Results. Aspartate aminotransferase. Rats maintained on the pyridoxal deficient diet showed the expected reduction in AAT activity (Table I). However, addition of pyridoxal phosphate to the assay mixture not only restored activity, but resulted in a striking increase in activity compared to that in tissues from the control rats on the normal diet. The increment in enzyme activity over the normal tissues ranged from 40% (heart and kidney) to 138% (liver).

Normal rats injected with decaborane showed a much greater reduction in AAT activity than did the rats maintained on the pyridoxal-deficient diet. The loss in activity following decaborane treatment was greatest in the liver (70%), but was observed in all the tissues (Table II). Moreover, there was no restoration of enzyme activity when pyridoxal phosphate was added to these tissue extracts (Table I). Further, tissue extracts from the decaborane-treated rats were dialyzed 16 hr against a phosphate buffer (50 m*M*, pH 7.6); assay of the dialysate with added pyridoxal gave no restoration of the AAT activity.

When rats on the pyridoxal-deficient diet were injected with decaborane, AAT activity in the liver assayed without added pyridoxal was not significantly lower than the assay (without added pyridoxal) in the normal tissue ($0.2 > p > 0.1$) (Table I). AAT activity in the livers of decaborane-treated pyridoxal-deficient rats was, in fact, greater than in those pyridoxal-deficient rats that were not treated with decaborane ($p < 0.1$). Addition of pyridoxal to the tissue extracts from pyridoxal-deficient decaborane-treated rats restored AAT activity in every organ studied

TABLE I. Liver Aspartate Aminotransferase Activity.[a]

	No added pyridoxal				Pyridoxal added			
	No.	Control	No.	Decaborane	No.	Control	No.	Decaborane
Regular diet	1)	763	1)	217	1)	747	1)	216
	2)	755	2)	192	2)	600	2)	194
	3)	716	3)	157	3)	613	3)	158
	4)	596	4)	167	4)	659	4)	169
	5)	831	5)	243	5)	661	5)	250
Mean		732		195[b]		656		197[b]
SEM		38		16		25		17
Pyridoxal-deficient diet	1)	487	1)	548	1)	1475	1)	1406
	2)	576	2)	734	2)	1270	2)	1110
	3)	404	3)	636	3)	1440	3)	1623
	4)	388	4)	660	4)	1666	4)	1209
	5)	305			5)	1940		
Mean		432		644[c]		1558		1337[d]
SEM		46		38		114		113

[a] Expressed as International Units (μM NADH oxidized/min)/g of soluble protein. p value comparing control animals with decaborane-treated animals in each set: [b] $p < .001$; [c] $p < .01$; [d] $0.3 < p < 0.2$.

to levels at least as great as in the untreated normal tissues (Table II). With the exception of the kidney, the activity in the tissues from decaborane-treated pyridoxal-deficient rats assayed with exogenous pyridoxal gave values equivalent to those of the pyridoxal-deficient rats not treated with the borane. The activity in all these tissues was greater in every case than in the rats maintained on a normal diet prior to the injection of the decaborane.

The time course study, illustrated in Fig. 1, shows that the effect of decaborane upon the AAT activity is maximal within 4 hr following the injection of the animal, and that there is no further change during the

TABLE II. Mean Values of Tissue Enzyme Levels.[a,j]

	Control (mean ± SE)	Control diet + decaborane (mean ± SE)	Pyridoxal deficient (mean ± SE)	Pyridoxal deficient + decaborane (mean ± SE)
Aspartate aminotransferase[b]				
Liver	656 ± 26	198 ± 17[c]	1559 ± 114[f]	1337 ± 113[g,i]
Heart	1932 ± 60	1263 ± 55[c]	2680 ± 115[f]	2411 ± 197[i]
Kidney	585 ± 36	457 ± 47[d]	821 ± 22[f]	524 ± 29[h]
Brain	1154 ± 30	958 ± 52[e]	1876 ± 56[f]	1927 ± 118[i]
Lactic dehydrogenase				
Liver	1464 ± 77	1297 ± 60	1558 ± 112	1321 ± 87
Heart	3472 ±118	1806 ±112[c]	3593 ± 367	1714 ± 26[h]
Kidney	826 ± 38	581 ± 37[d]	765 ± 56	555 ± 27[g]
Brain	1098 ± 38	654 ± 39[c]	1124 ± 83	1150 ± 36[i]

[a] Expressed as μmoles/min/mg of soluble protein.

[b] Assayed with added pyridoxal phosphate.

[j] p values comparing (i) control group to normal diet with decaborane: [c] $< .001$; [d] $< .01$; [e] $< .05$; (ii) pyridoxal-deficient diet to controls: [f] $< .001$; all others, NS; (iii) pyridoxal-deficient diet with decaborane to pyridoxal-deficient diet: [g] $< .02$; [h] $< .001$; (iv) pyridoxal-deficient diet with decaborane to control diet with decaborane: [i] $< .001$; all others, NS.

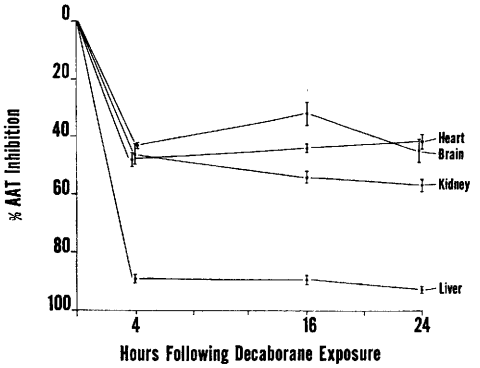

FIG. 1. Time course of AAT inhibition in normal rats injected with decaborane. The enzyme activities were determined (μmoles/min/g of tissue), and expressed as percentage activity relative to normal controls.

subsequent 20-hr period.

Lactic dehydrogenase. Injection of normal animals with decaborane resulted in reductions in LDH activity in all the tissues studied (Table II). The loss in activity, although not significant in the liver, was striking in the kidney (30%), brain (40%), and heart (48%). Measurement of LDH activity in the tissues of the untreated pyridoxal-deficient animals showed no loss in activity. However, injection of the pyridoxal-deficient animals with decaborane caused reductions in LDH activity in the liver (15%), heart (52%), and kidneys (28%) that were almost exactly the same amount as the inhibition in the rats maintained on the regular diet. The LDH activity of the brain, which was reduced 40% by decaborane in the rats on a regular diet, was not reduced by decaborane in the pyridoxal-deficient rats.

Discussion. The effectiveness of the borane in blocking AAT is evident when the values for the decaborane-treated animals are compared to AAT in the livers of the rats on the pyridoxal-deficient diet (Table I); decaborane caused a much greater loss in activity than did the dietary deficiency. Neither addition of pyridoxal nor dialysis of the tissue preparation and reassay with added pyridoxal gave any recovery in AAT activity, indicating that the inhibition is not due to lack of cofactor nor to the presence of some competitive inhibitor of AAT. We assume that the loss of AAT activity is due to some irreversible change in the apoenzyme and considered that this might be due to the selective inhibition of AAT synthesis by the tissues. If synthesis of AAT apoenzyme were blocked by borane, then one would expect an exponential decay in AAT activity with time as degraded apoenzyme is not replaced. On the contrary, our data show that the decrement of AAT activity lost by each organ following borane treatment is complete within the first period of observation (4 hr following injection) and that AAT activity then remains constant for the next 20 hr (Fig. 1). We conclude therefore that the borane primarily acts upon that AAT enzyme already synthesized at the time of injection.

One well-known property of the boranes is their reductive power. We have considered this property as a possible mechanism for the inactivation of AAT in these tissues. Pyridoxal enzymes are especially vulnerable to chemical reduction because the apoenzyme is linked to the pyridoxamine residue by an unstable double bond (a Schiff base). It has been demonstrated that reduction of this double bond irreversibly inactivates the enzyme and results in a stable covalent bond linking a lysine residue in the active site of the apoprotein with the pyridoxamine group (13, 14). The deficiency in pyridoxal cofactor, with the result that a significant amount of AAT enzyme would lack the pyridoxamine residue, should lower the susceptibility of the AAT (and other pyridoxal enzymes) to reduction by borane. This interpretation may be given to our data where the total apoenzyme assayed with the addition of pyridoxal is almost as great, with the exception of the kidney, in the borane treated pyridoxal-deficient animals as in the untreated pyridoxal-deficient animals.

The rats on the pyridoxal-deficient diet had a lower tissue AAT activity than the control animals. However, the finding that AAT activity may be restored by exogenous pyridoxal to levels greater than in control tissues indicates that there is no deficiency in the apoenzyme, but only in the cofactor. This suggests that the lack of pyridoxal stim-

ulates the synthesis by the tissue of protein apoenzyme in an attempt to overcome the deficiency in enzyme activity. This effect, an increase in transaminase activity of the tissue of the pyridoxine deficient animal when assayed with added cofactor, has been observed with tyrosine transaminase in the rat liver, though the increase was much smaller than we observed in this study (15, 16).

Our data showing that pyridoxal enzymes are inhibited by borane, and that the degree of inhibition is related to the pyridoxal residue, are consistent with the earlier studies of brain metabolism following borane exposure. The depletion by borane of brain amines could be accounted for by the inhibition of the aromatic amino acid decarboxylases required for the synthesis of serotonin and of norephonephrine, Indeed, Merritt and Sulkowski (7) found that 5-hydroxytryptophan decarboxylase activity was inhibited 50–60% in the brain of rats injected with decaborane.

The reduction in the activity of LDH, an enzyme that does not require pyridoxal, may be due to an indirect effect of the borane upon the tissue. However, it seems worth noting that the general pattern of inhibition in the various tissues generally follows the distribution of the relative amounts of H and M subunits of LDH within the organs studied here (17). The possibility exists that the LDH isozyme characteristic of the skeletal muscle and liver is less susceptible to borane reduction than the isozyme found in the heart.

Summary. Boron hydrides inhibit AAT in the liver, heart, brain, and kidneys of normal rats. Decaborane does not cause a significant loss in activity in the tissues of pyridoxal-deficient rats. The AAT activity, when assayed with sufficient exogenous pyridoxal, is greater in the tissues from the pyridoxal-deficient animals than in the tissues from the normal animals. LDH, an enzyme that does not require pyridoxal, is moderately inhibited in the heart and kidney of both normal and pyridoxal-deficient animals, and in the brain of normal animals.

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