Effects of Riboflavin on Boric Acid Toxicity

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Abstract □ Chronic feeding of rats with 1% boric acid impairs growth more in those on zero riboflavin than in those receiving 133 mcg. riboflavin/100 g. diet. Guinea pigs on a diet containing 28 mcg. riboflavin/100 g. diet show skin changes and a significant mortality when fed 1% boric acid. Toxicity is minimal in riboflavin-repleted animals (1.6 mg./100 g. diet) fed the same level of boric acid. Chicks receiving riboflavin in excess of the accepted growth requirement show a decreased incidence of boric acid toxicity. Intraperitoneal injection of 2-^{14}C-riboflavin results in a significantly greater urinary excretion of labeled riboflavin in boric acid-fed animals than in controls for both rats and guinea pigs. Studies *in vitro* show that the binding of riboflavin to serum proteins is reduced by borate, even in very low concentrations. It is concluded that riboflavin depletion can be induced by borate.

Keyphrases □ Boric acid toxicity—*in vivo* effect of riboflavin, rats, guinea pigs, chicks □ Riboflavinuria—borate-treated rats, guinea pigs, chicks □ Riboflavin—*in vivo* effect on boric acid toxicity, *in vitro* serum protein binding □ Urinary excretion, riboflavin—effect of boric acid administration □ Serum protein binding to riboflavin—effect of serum dialysis against borate buffer

Interspecies and intraspecies variations in sensitivity to the toxicity of boric acid have been well documented. Individual and familial susceptibility to boric acid poisoning occurs in man, with wide variations in the lethal dose for babies and other subjects (1, 2). Pfeiffer *et al.* (3) investigated acute borate toxicity in rats, mice, guinea pigs, and dogs and found that guinea pigs were the most susceptible of these animals.

Numerous published accounts showed that boric acid reacts with polyhydroxyl compounds to form complexes, as cited in the 1951 review by Zittle (4). Frost (5) indicated the riboflavin–borate complex by noting that riboflavin was more soluble in aqueous borate solution than in water alone. Wadke and Guttman (6) also showed that the ribityl side chain was involved in the complex formation. Boric acid injected into the yolk sac of embryonated chicken eggs acts as a teratogenic substance, and skeletal malformations are produced which are characteristic of riboflavin deficiency (7, 8). Landauer (9), who originally made these observations in the chick embryos, later showed that striking breed differences existed in the dose required to induce the deformities and that chick embryos from strains with high riboflavin requirements showed the highest incidence of skeletal abnormalities.

Adrian (10) found that boric acid inhibited the growth of *Lactobacillus casei* and *L. arabinosus* and that this inhibition was reversed by addition of riboflavin to the growth media. Subsequently, he showed that urinary output of riboflavin was potentiated by boric acid in the mature rat.

The main purpose of the present investigation was to show the effects of boric acid intake on growing rats, guinea pigs, and chicks receiving low, maintenance, and high levels of dietary riboflavin. The investigation also was designed to determine whether or not Adrian's (10) observation of boric acid-induced riboflavinuria in the rat could be confirmed with the sensitive and more specific isotope techniques. Finally, an attempt was made to see if such riboflavinuria could be due to interference of borate with the binding of the vitamin by serum protein so that renal excretion would be potentiated.

Table I—Diet Patterns of Rats and Guinea Pigs in Flavin–Borate Studies

Group	Diet Pattern	
		Rat
I	R_0B_0	Riboflavin, zero; boric acid, zero
II	R_0B_1	Riboflavin, zero; boric acid, 1.0%
III	R_1B_0	Riboflavin, 133 mcg./100 g. diet; boric acid, zero
IV	R_1B_1	Riboflavin, 133 mcg./100 g. diet; boric acid, 1.0%
		Guinea Pig
I	$R_{28\ mcg.}B_0$	Riboflavin, 28 mcg./100 g. diet; boric acid, zero
II	$R_{28\ mcg.}B_1$	Riboflavin, 28 mcg./100 g. diet; boric acid, 1.0%
III	$R_{1.6\ mg.}B_0$	Riboflavin added, 1.6 mg./100 g. diet; boric acid, zero
IV	$R_{1.6\ mg.}B_1$	Riboflavin added, 1.6 mg./100 g. diet; boric acid, 1.0%

EXPERIMENTAL[1]

Animal Studies—In both rat and guinea pig experiments, animals were studied during the period of active growth.

Rat—Sixteen male Holtzman rats, 26 days old and 70 g. average starting weight, were divided into four groups of four rats each according to the diet pattern. Their basic diet was the riboflavin-free mixture. This diet was fed alone to the first group, while the second received this diet with the addition of 1% boric acid. The third group was given the basic diet, supplemented with riboflavin to supply 133 mcg./100 g. of food; the fourth received the riboflavin-containing diet to which 1% boric acid had been added (Table I).

Food and water were supplied *ad libitum*. Feed intake and weight gains were measured on alternate days. Animals were inspected daily for gross evidence of toxicity. After 6 days of adaptation to the experimental diets, rats were transferred to metabolic cages. At this time, two animals from each group were injected intraperitoneally with 1 ml. of 2-^{14}C-riboflavin dissolved in 0.9% sodium chloride solution in a concentration of 4.8 μc./ml., with a total riboflavin concentration of 50 mcg./ml. Another two animals were injected with only 0.9% saline. Urine was then collected for 8 days so that the urinary excretion of total radioactivity could be assayed for sequential 48-hr. periods. The volumes of all urine samples collected. were measured, and 0.1-ml. aliquots were added to Bray's solution (11) to measure radioactivity in a liquid scintillation spectrometer[2] Corrections for quenching were made.

[1] 2-^{14}C-Riboflavin (61 mc./m*M*) was obtained from Amersham/Searle. Boric acid A.R. was purchased from Mallinckrodt Chemical Works. The basic rat diet was obtained from Nutritional Biochemicals Corp. The pelleted guinea pig diets were supplied by General Biochemicals.

[2] Packard Tri-Carb.

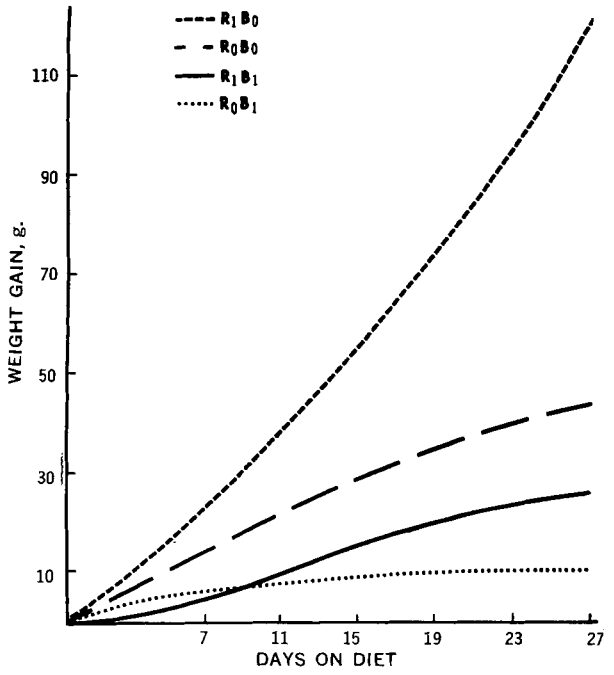

Figure 1—*Growth curves of rats. Key: B_0, no borate; B_1, borate added to the diet; R_0, riboflavin-free diet; and R_1, riboflavin supplied in the diet.*

The identity of the radioactive material in the urine was determined by spotting samples on Whatman No. 1 paper, carrying out chromatographic separation in a butanol–acetic acid–water (2:1:1) solvent system, scanning the chromatogram for radioactive spots using a strip scanner[3], and identifying the labeled compounds by R_f values.

Two weeks after the initial isotopic tracer studies, the animals were again injected with 2-^{14}C-riboflavin. Urine collection and assays of urinary radioactivity were repeated, and then the animals were sacrificed. The livers were kept. The duration of study was 27 days. Residual radioactivity in the livers was measured by counting aliquots of homogenates as previously described (12).

Guinea Pig—The experimental design of investigations utilizing guinea pigs of the English Shorthair strain was similar but not identical.

Sixteen 22-day-old animals, with starting weights in the range of 150–190 g., were divided into four groups of four each and fed pelleted diets. Diets of the first and second groups contained 28 mcg. riboflavin/100 g. food, with the second group receiving 1% boric acid in addition. The third and fourth groups were given the basic diet supplemented with riboflavin to supply 1.6 mg./100 g., and the latter group of animals was fed this high riboflavin diet with 1% boric acid (Table I). Food and water were supplied *ad libitum*. After 1 week of adaptation to experimental diets, two animals from each group were injected with 1 ml. of 2-^{14}C-riboflavin dissolved in 0.9% sodium chloride solution in a concentration of 4.8 μc./ml., with a total riboflavin concentration of 50 mcg./ml. The animals were then put in the plastic metabolic cages. Urine was collected for 4 days for sequential 24-hr. periods. The remaining two animals in each group were housed in the maintenance cages and injected with 1 ml. of 0.9% saline solution. Feed intake and weight gains were measured on alternate days. The procedure of measuring the urinary radioactivity was the same as in the rat studies. The identity of the radioactive material in the urine was determined by paper chromatographic assay using the butanol–acetic acid–water (2:1:1) solvent system as for the rat.

Two weeks after the initial administration of 2-^{14}C-riboflavin, the same procedure was repeated and then the animals were sacrificed. The residual radioactivity of livers was measured as described for the rat (12). In addition, total flavins of the livers were determined using an adaptation of the fluorometric method of Burch *et al.* (13).

[3] Nuclear-Chicago Actigraph III.

Chick—White Leghorn chicks were employed in a further investigation to elucidate the role of riboflavin in the prevention of boric acid toxicity. Thirty birds, 6 days old and of 55 g. average weight, were divided into five diet groups. The first group received a powdered chick diet containing 7 mg. of riboflavin/kg. The second received this diet with the addition of 0.8% boric acid. The third was given a diet containing 14 mg. of riboflavin/kg. with 0.8% boric acid. The fourth received a diet containing 28 mg. of riboflavin/kg. with 0.8% boric acid, and the fifth group was given a diet containing 28 mg. of riboflavin/kg. but without boric acid. Chicks were housed six birds to a cage. Food and water were supplied *ad libitum*. The experiment was continued for 25 days, during which time the food intake and weight gains were measured three times a week and the adverse effects of the boric acid on the chicks were recorded.

Experiments *In Vitro*—Blood was obtained from decapitated adult Holtzman rats that had been maintained on a 25% casein diet containing maintenance levels of riboflavin (80 mcg./100 g.). Serum was separated by centrifugation and used in two-phase equilibrium dialyses carried out in a darkened cold room at 1.1° (34° F.) (14). Aliquots of serum were dialyzed against either 0.2 *M* phosphate buffer alone or a phosphate buffer of the same molarity containing 0.001 or 0.01 *M* borate. All buffers were adjusted to pH 7.3. The dialysis sacs contained serum diluted with the complementary buffer (1:1), and the fluid volume in the sacs was one-tenth of that in the external solution.

Dialysis was continued for 21 hr. with continuous gentle agitation on a magnetic mixer. At the end of this time, the serum samples were dialyzed against the 0.2 *M* phosphate buffer to which 2-^{14}C-riboflavin had been added. Dialysis was carried out under the same conditions as those used for the first phase of the study and continued for 24 hr. During this time, 0.1-ml. aliquots of the serum were removed from the sacs sequentially and placed in counting vials to which 10 ml. of Bray's solution was added; radioassay was carried out in the liquid scintillation spectrometer. Radioassay of the external solution was carried out at zero time and at the end of the 24 hr. The protein content of the serum was determined by the biuret method. Specific activities of the boric acid-treated and control serums were calculated (disintegrations per minute per milligrams protein), and the uptake of the 2-^{14}C-riboflavin by the serum proteins was computed from zero time until equilibrium had been reached. After correction for volume changes, the difference between the radioactivity of the external and internal solutions at equilibrium was accepted as the bound 2-^{14}C-riboflavin. By applying the known specific activity of the original 2-^{14}C-riboflavin solution,

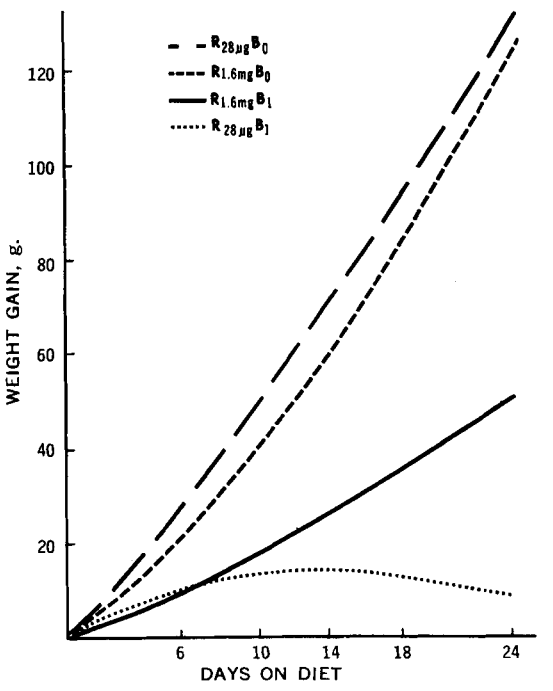

Figure 2—*Growth curves of guinea pigs. Key: B, borate; R, riboflavin; and the subscripts indicate presence (1), absence (0), or the amount in mg. or mcg./100 g. of diet.*

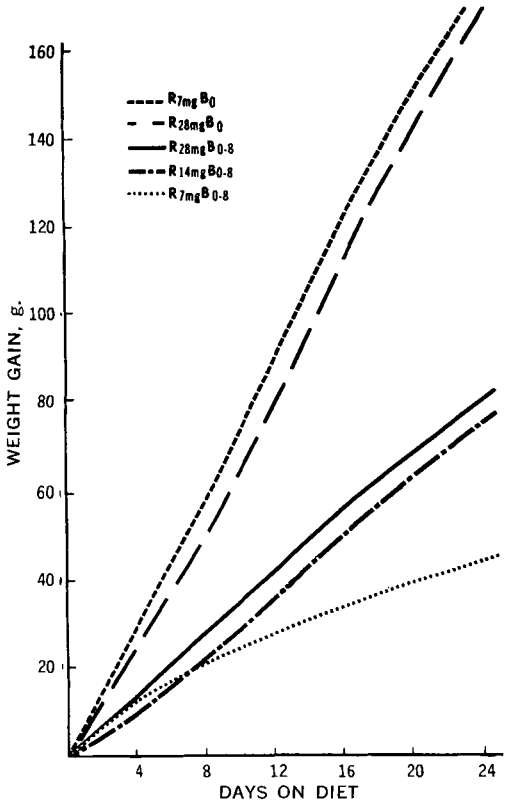

Figure 3—*Growth curves of chicks. The additions of borate (B) and riboflavin (R) are as designated in Figs. 1 and 2.*

values were expressed as riboflavin bound per milliliter serum or per milligram protein.

RESULTS

Boric acid induced growth retardation in rats. The weight gains of animals on riboflavin-deficient diets were less than those receiving maintenance levels of this vitamin. Animals that were riboflavin depleted and also those that were fed boric acid showed the smallest weight gain (Fig. 1). Apart from the growth inhibition, the rats showed no gross evidence of boric acid toxicity nor any specific signs which could be attributed to riboflavin deficiency.

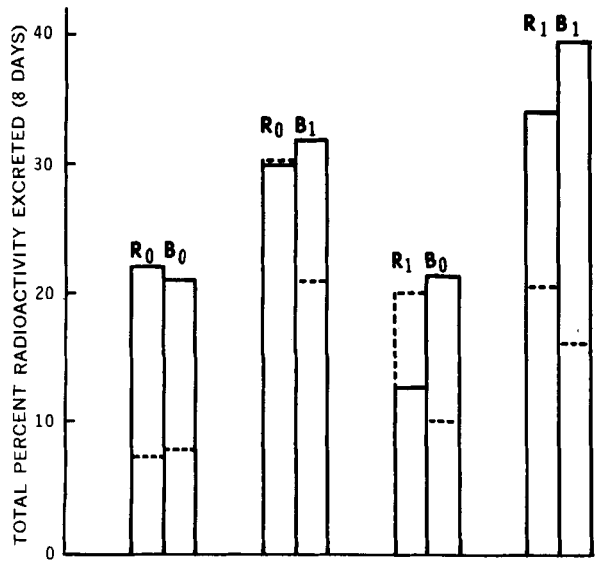

Figure 4—*2-^{14}C-Riboflavin excreted in rat urine as percent of injected radioactivity. Key: □, first injection; and ▨, second injection.*

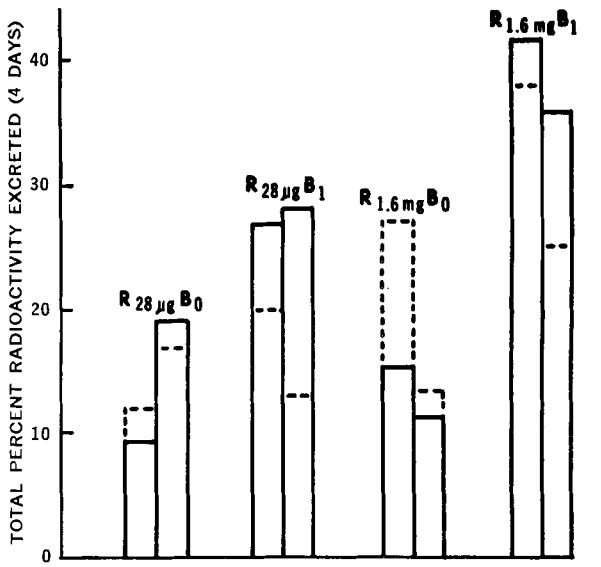

Figure 5—*2-^{14}C-Riboflavin excreted in guinea pig urine as percent of injected radioactivity. Key: □, first injection; and ▨, second injection.*

Guinea pigs on the riboflavin-supplemented diet showed similar weight gains and feed efficiency to those on the basic diet. Boric acid impaired the growth of animals on diets supplemented with riboflavin and completely inhibited growth of those on the low riboflavin diet (Fig. 2). Among animals receiving boric acid, all those on the low riboflavin diet showed roughness of their hair as well as dermatitis and edema of their feet during the terminal week of study. Two animals in this group died on the 17th and 18th days of the 24-day experiment. Animals on the riboflavin-supplemented diet which were fed boric acid showed no coat changes, and only one animal had detectable edema of the feet which was observable on the day of sacrifice.

Chicks receiving riboflavin in excess of the accepted requirement of this vitamin for growth showed a decreased incidence of boric acid toxicity. Doubling the level of riboflavin in the chick diets improved weight gains, but further improvement in growth was not attained by increasing riboflavin intake above this level (Fig. 3). All chicks fed boric acid showed feather changes characterized by easy pluckability and spontaneous loss; these changes appeared on or about the 10th day in birds on the diet containing 7 mg./kg. riboflavin. Chronic boric acid feeding was associated with death of these birds between the 12th and 22nd days of the 25-day study; premortal signs included weakness and falling in the cage.

Excretion of Flavin Radioactivity—After the first injection of 2-^{14}C-riboflavin, the accumulative urinary excretion of the labeled compound showed group differences which were similar in the rats

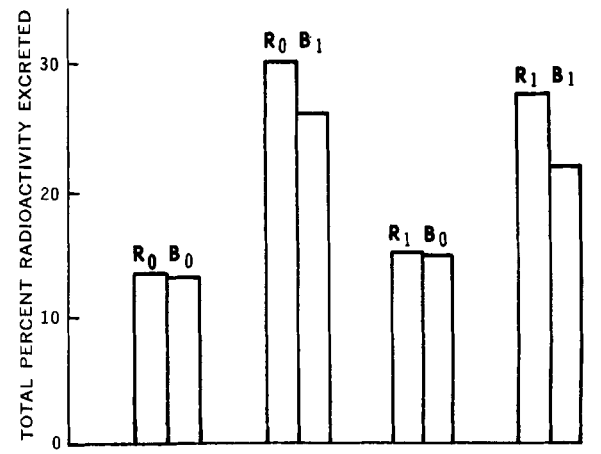

Figure 6—*2-^{14}C-Riboflavin excreted in rat urine as percent of total of two injections.*

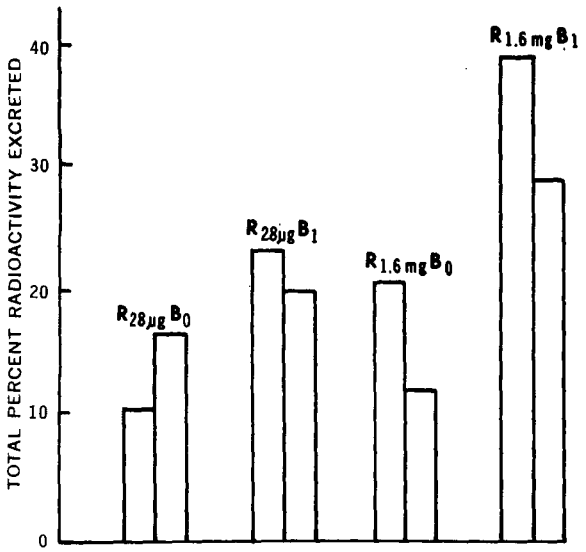

Figure 7—*2-^{14}C-Riboflavin excreted in guinea pig urine as percent of total of two injections.*

and in the guinea pigs. Animals receiving boric acid excreted a significantly greater percentage of the administered radioactivity than those on the riboflavin-supplemented or riboflavin-"deficient" diets alone, the excretion being highest in boric acid-fed animals on riboflavin supplements (Figs. 4 and 5). After the second injection of 2-^{14}C-riboflavin, intergroup differences in the urinary excretion of the labeled compound were less; and in the guinea pigs on the low riboflavin diet, there was no difference in the excretion of radioactivity between boric acid-fed animals and the control group. However, the total excretion of two injections was higher in the boric acid-fed groups (Figs. 6 and 7).

Radiochromatographic studies of the urine showed one spot which had the R_f value of 2-^{14}C-riboflavin. No riboflavin products could be detected, although the limitations of the method cannot rule out their presence in trace amounts.

Riboflavin in Liver—In rats, while the percentage of the administered radioactivity remaining in the liver was marginally affected by the diet, the residual radioactivity of the liver (expressed as disintegrations per minute per gram fresh tissue) was greater in animals that had received boric acid, being highest in the riboflavin-depleted group that had received the drug (Table II). Since the liver weights of the animals on boric acid were significantly less than those of the control groups, these findings showed that the capacity of the liver to retain riboflavin was unaffected by boric acid treatment.

In the guinea pigs, the percentage retention of radioactivity by the liver was highest in the control group on the low riboflavin intake and lowest in the control group on the riboflavin-supplemented diet (Table III). When the residual radioactivity of the liver was expressed as disintegrations per minute per gram fresh tissue, only those animals that had had riboflavin supplementation without boric acid exhibited diminished retention, indicating that in these the liver was already saturated with the vitamin. The total flavin in the guinea pig

Table II—Residual ^{14}C in the Livers of Rats

Group	Diet Pattern	Liver Weight, g.	Percent of Injection	d.p.m. × 10^3/g. Liver
I	R_0B_0	7.4	10.89	327
		5.2	10.01	429
II	R_0B_1	2.0	11.07	1248
		2.4	10.74	998
III	R_1B_0	7.7	7.89	230
		6.7	10.45	348
IV	R_1B_1	2.3	7.76	753
		2.4	9.01	838

Table III—Residual ^{14}C in the Livers of Guinea Pigs

Group	Diet Pattern	Liver Weight, g.	Percent of Total Injection	d.p.m. × 10^3/g. Liver
I	$R_{28\ mcg.}\ B_0$	10.8	10.83	213
		14.6	16.41	242
II	$R_{28\ mcg.}\ B_1$	4.9	4.78	209
		4.5	6.47	305
III	$R_{1.6\ mg.}\ B_0$	11.5	2.61	49
		11.1	1.76	34
IV	$R_{1.6\ mg.}\ B_1$	11.6	7.30	134
		5.2	6.81	282

livers was markedly decreased in animals that had received boric acid, but differences were accounted for by the smaller size of the livers in these groups (Table IV).

Studies *In Vitro*—In the experiments *in vitro*, it was shown that uptake of 2-^{14}C-riboflavin by serums that had been dialyzed against borate-containing buffer was greater than uptake of the labeled vitamin by serums that had been dialyzed against phosphate-control buffers. Borate buffers of very low molarity (0.001 *M*) had a significant effect on the subsequent uptake of 2-^{14}C-riboflavin, so that the amount taken up by the serum proteins was increased after equilibrium had been reached. The effect of a higher concentration of borate (0.01 *M*) seemed not to be significantly different from the effect with a low concentration (0.001 *M*) (Fig. 8). These results indicate that borate removes riboflavin from binding sites on serum proteins and that binding is reversible upon removal of borate.

DISCUSSION

It has been shown that growth retardation induced in rats, guinea pigs, and chicks by boric acid is dependent at least in part on riboflavin intake. In studies utilizing 2-^{14}C-riboflavin, it was demonstrated that the urinary excretion of riboflavin is increased by boric acid feeding. This result was not due to the smaller size of the animals which results after some time on the borate-containing diets, since at the time of the first injection the body weights of the animals of all groups were similar. There was no significant difference after the second injection, because the excretion of riboflavin is proportional to dietary and tissue level of this vitamin (15, 16).

The mechanism producing this riboflavinuria was elucidated in the studies *in vitro*, in which it was shown that the binding of riboflavin to serum proteins is reduced by borate, even in very low concentrations. In previous studies by Frost (5) and Weygand (17), who investigated riboflavin–borate complexes, it was found that such complexes have full biological activity. Nevertheless, if as has been assumed in the present investigations, riboflavin is detached by boric acid from binding sites for the vitamin on serum proteins through formation of flavin–borate complexes, then the vitamin would be available for renal excretion. This situation is analogous to the avian recessive disorder of renal riboflavinuria, in which it was shown by Winter (18) that there is a deficiency or absence of the normal flavin-binding protein in the serum and egg yolk as well as a deficiency of egg albumin flavoprotein. In this genetic disease,

Table IV—Flavin in the Livers of Guinea Pigs

Group	Diet Pattern	Liver Weight, g.	Total Flavin, mcg./g. Liver	Total Flavin, mcg./Liver
I	$R_{28\ mcg.}\ B_0$	12.9	10.7	138.0
		11.2	9.6	107.5
II	$R_{28\ mcg.}\ B_1$	5.3	14.5	76.8
		4.6	16.9	87.7
III	$R_{1.6\ mg.}\ B_0$	11.2	14.9	166.9
		14.4	9.1	131.0
IV	$R_{1.6\ mg.}\ B_1$	5.2	8.7	45.2
		8.5	11.0	93.5

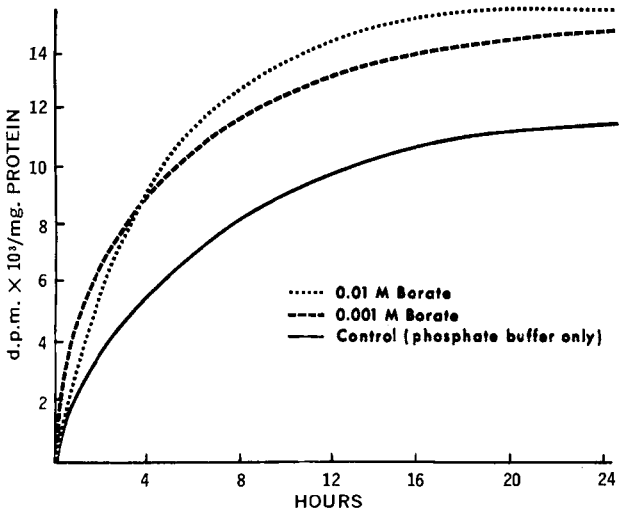

Figure 8—*Uptake of 2-^{14}C-riboflavin by rat serums in equilibrium dialysis.*

massive urinary loss of the vitamin occurs, and its deposition in developing eggs is diminished (19). Progeny of the affected females become severely riboflavin deficient and usually die at about the 13th day of incubation.

Absorption of boric acid increases the riboflavin requirements for growing animals to compensate for the vitamin drain incurred by the presence of the drug in the body. The capacity of the liver to retain riboflavin seems not to be affected by boric acid treatment.

Chronic toxicity of boric acid is in part determined by riboflavin status; that is, animals that have a low intake or are progressively riboflavin depleted show the more severe signs of intoxication. Conversely, riboflavin intake in excess of requirements can confer some protection against the adverse effects of the drug.

Since it was previously shown that boric acid forms complexes with sugars and mucopolysaccharides, inactivates certain enzymes, and produces pathological changes in the kidney and CNS (3, 4, 20–22), it is apparent that increasing riboflavin intake alone does not afford protection against poisoning.

Predisposition to boric acid intoxication can be due either to genetic factors, such as in the mutant chick (18, 19), or to dietary factors, as has been demonstrated in the present study. In either case, one variable which may determine the outcome of boric acid absorption is the ability of the animal to retain riboflavin. This has been conclusively shown in three different experimental animals; it is projected that it may also be true in man.

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