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Liver Nonprotein Sulfhydryl in Vitamin B_6-Deficient Rats: Effects of Age, Feeding, and Fasting.* (28515)

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(Introduced by G. M. Briggs)
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Hsu *et al*(1,2) reported that liver and erythrocyte glutathione increased in Vit. B_6-deficient rats. In contrast, Williams *et al*(3) found that Vit. B_6-deficient rats fed an amino acid diet showed a small but significant decrease in liver nonprotein sulfhydryl (NPSH), which is principally glutathione(4). Possible reasons for the apparent differences in the reported effect of Vit. B_6 deficiency on liver glutathione are the age of the rats, composition of the diet, and type of control used for comparison. The Vit. B_6-deficient rats of Williams *et al* were 6-7 weeks old, were fed an amino acid diet containing 1.5% nitrogen, 0.56% methionine, and 0.22% cystine, compared with pair-fed controls, and not fasted before the analyses. The deficient rats of Hsu *et al*(1) were fed a casein-sucrose diet, presumably were compared with *ad libitum*-fed controls, and may have been fasted(5).

Since the metabolism of pair-fed controls may vary considerably from that of *ad libitum*-fed animals(6,7), it was possible that the NPSH and glutathione content of the pair-fed controls might be greater than that of the *ad libitum*-fed controls. Consequently, the concentration of these compounds in the liver in Vit. B_6-deficient rats might appear reduced if compared with the pair-fed controls but elevated if compared with the *ad libitum*-fed controls.

The following experiments were done (a) to compare the liver NPSH content of Vit. B_6-deficient rats with those of both pair-fed and *ad libitum*-fed controls and (b) to determine whether the age of the deficient rats, duration of the deficiency, and fasting significantly changed liver NPSH concentration.

Methods and materials. Animals and diets. Male rats of the Long-Evans strain raised in our laboratory were used in Experiments 1-3. Both male and female rats were used in Experiment 4. Liver NPSH values were similar for both sexes. The rats were weaned at 3 weeks of age (45-55 g), caged individually in galvanized screen-wire cages, and fed a casein-sucrose diet†, except for the group in

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† Composition, g/100 g diet: "vitamin-free" casein (Nutritional Biochemicals Corp., Cleveland), 20.0; cottonseed oil, 5.0; salts (U.S.P. 14, Nutritional Biochemicals Corp., Cleveland), 4.0 or 3.5 (*Fed. Proc.*, 1963, v22, 261); choline bitartrate, 0.18; powdered sucrose to 100. Two ml of a 20% ethanol solution of B vitamins and menadione were fed 3 times

Exp. 3. These rats were fed a commercial ration‡ until they weighed 150 g when they were transferred to the casein diet. The dams of the rats in Exp. 1-3 were fed a pyridoxine-deficient diet§ 1 week before weaning of the young.

Vit. B_6 deficiency was created by omitting pyridoxine from the vitamin supplement†. The rats were considered deficient and sacrificed for analyses when they failed to gain more than 3 g in 1 week. At this stage of depletion they also showed a large decrease in serum glutamic-oxaloacetic transaminase‖. Food intake of the pair-fed controls was restricted to the food intake of littermates in the deficient group.

Beck *et al*(8) observed that liver NPSH showed a diurnal variation which was most probably related to the eating habits of *ad libitum*-fed rats since the highest NPSH levels occurred in the morning, with a gradual decrease throughout the day. To avoid this variation in the present experiments, the rats were sacrificed at the same time each morning. The pair-fed controls were also fed between 9-10 p.m. the evening before so that they would have received their food at the time when the *ad libitum*-fed rats were eating.

Analytical. Liver NPSH was determined by the method of Grunert and Phillips(9) as modified by Gerwing and Long(10). In this modification the nitroprusside solution is added before the sodium cyanide solution, a procedure which gives a more stable color than the original method. The livers were frozen in dry ice, and then 100-200 mg were homogenized in 3.0 ml of ice-cold 3% metaphosphoric acid and 1.0 ml of water in a Potter-Elvehjem homogenizer and filtered in the cold. All analyses were completed within one day after the livers were homogenized.

Results and discussion. The results are presented in Table I. In Exp. 1, the deficient rats were approximately 7-8 weeks old at time of sacrifice and had been fed the deficient diet for 3-4 weeks. The concentration of liver NPSH in the deficient rats did not differ significantly from the values for either the pair-fed or the *ad libitum*-fed controls.

In Exp. 2 the rats were 6 weeks old when Vit. B_6 depletion was started, and the deficient diet was fed for more than 9 weeks. Again, there was no significant difference between the NPSH values for the deficient rats and the *ad libitum*-fed controls. Liver NPSH values in both the deficient and control groups in this experiment were similar to those found in the younger rats in Exp. 1.

The decrease in liver NPSH in Vit. B_6-deficient rats previously reported by Williams *et al*(3) occurred in rats given an amino acid diet containing 9.4% protein (1.5% nitrogen) and 0.56% L-methionine. The decrease in liver NPSH may have been related to the low protein content of the diet since no significant decrease was observed in the present experiments with Vit. B_6-deficient rats fed a 20% casein diet. Barford and Eden(11) found that liver NPSH levels in rats fed a 10% casein diet were significantly lower than in rats fed a 20% casein diet. Ashwood-Smith and Smith(12) also reported that the levels of glutathione in several areas of the brain were reduced in Vit. B_6-deficient rats fed a low protein diet.

In Exp. 3, the effect of fasting (17 hours

weekly to provide the following intakes in micrograms per rat per day, except where noted: thiamine hydrochloride, 40; riboflavin, 40; niacinamide, 170; calcium-D-pantothenate, 510; D-biotin, 8.6, folic acid, 8.5; vitamin B_{12}, 0.20; menadione, 50; pyridoxine hydrochloride, 61 (50 μg pyridoxine). Two drops of a solution of vit. A, D, and E in cottonseed oil were fed 3 times weekly to provide 32 IU of vit. A, 3 IU of vit. D_2, and 162 μg of DL-alpha-tocopheryl acetate per rat per day.

‡ Diablo Labration for Rats and Mice, Diablo Animal Laboratories, Berkeley, Calif.

§ Composition, g/100 g diet: "vitamin-free" casein (Nutritional Biochemicals Corp., Cleveland), 36.0; fat (Primex, Proctor & Gamble, Cincinnati), 8.0; salts (U.S.P. 14, 1950, Nutritional Biochemicals Corp., Cleveland), 6.0; a concentrate of vit. A, D, and E in cottonseed oil, 3.2; a concentrate of B-vitamins in sucrose, 2.0; powdered sucrose, 44.8. The concentrates supply the following amounts of vitamins per 100 g diet; vit. A, 3200 IU; vit. D_2, 282 IU; DL-alpha-tocopheryl acetate, 16 mg; thiamine hydrochloride, 0.9 mg; riboflavin, 0.9 mg; calcium-D-pantothenate, 7.0 mg; niacinamide, 3.4 mg; D-biotin, 0.2 mg; folic acid, 0.2 mg; menadione, 1.0 mg; vit B_{12}, 4 μg.

‖ M. A. Williams, G. Tsung, unpublished observations.

TABLE I. Effect of Vitamin B_6 Deficiency and Fasting on Liver NPSH in Rats Fed a 20% Casein Diet.

Exp No.	B_6	No. rats	Age at start (wk)	Days on diet	Final wt avg	Feeding routine	Liver NPSH μmoles/100 g wet liver
1	—	12	3	21-28	81	pair-fed	669 ± 106
	+	12	"	" "	112	" "	629 ± 164
	—	6	"	" "	86	ad libitum	672 ± 89
	+	6	"	" "	180	" "	699 ± 109
2	—	6	6	66	259	" "	712 ± 46
	+	6	"	"	345	" "	757 ± 13
3	—	6	3	26	69	" "	614 ± 55
	+	6	"	"	137	" "	730 ± 57
	—	7	"	19	56	" " then fasted	429 ± 22*
	+	7	"	"	111	" " " "	270 ± 30
4	—	5	3	67-69	115	" "	667 ± 55
	+	4	"	" "	262	" "	726 ± 71
	—	6	"	68-70	112	" " then fasted	563 ± 38
	+	5	"	" "	237	" " " "	589 ± 35

* Significant difference, $P<.01$.

from 5 p.m. to 10 a.m.) was tested in young rats. Although Hsu *et al*(1,2) did not state whether their rats were fed or fasted before the NPSH determinations, Hsu and Chow (5), in a study of the effect of thiamine deficiency on the level of tissue glutathione, fasted the rats before NPSH determination. Thus, it was possible that Hsu *et al*(1,2) had determined liver NPSH in fasted animals, and that the increase reported in liver NPSH occurred only in fasted Vit. B_6-deficient rats.

The results of Exp. 3 support this possibility. In the *ad libitum*-fed controls, fasting 17 hours decreased liver NPSH by 63% in comparison with the nonfasted values (from 730 ± 57 μmoles/100 g to 270 ± 30 μmoles/100 g). In the Vit. B_6-deficient group, however, fasting produced only a 28% decrease in comparison with the nonfasted values (from 614 ± 55 μmoles/100 g to 439 ± 22 μmoles/100 g).

Thus, on the basis of the fasted values in Exp. 3, Vit. B_6 deficiency appeared to increase liver NPSH in rats fed the deficient diet for 3 weeks. When compared to the nonfasted values, however, the "increase" appears to be the lack of a decrease, rather than a true increase. This view is consistent with the observation that the synthesis of liver glutathione, as measured by incorporation of glycine-2-C^{14} or glutamic acid-1-C^{14}, was not increased in Vit. B_6-deficient rats(2). A different response has been reported in Vit. B_{12} deficiency. O'Dell *et al*(13) found no difference in liver NPSH between fasted deficient and control rats although there was a significant decrease in liver NPSH in nonfasted deficient rats.

The effect of fasting was again tested in Exp. 4 with rats which had been fed the Vit. B_6-deficient diet for approximately 10 weeks. Under these conditions, however, fasting 17 hours caused some reduction in liver NPSH in both control and deficient rats in comparison with the nonfasted groups. The fasting levels of the deficient and control groups, however, were similar (563 ± 38 μmoles/100 g *vs* 589 ± 35 μmoles/100 g). The NPSH values of the nonfasted deficient and control groups did not differ significantly. Thus, the effect of Vit. B_6 deficiency on liver NPSH in fasted rats appears to vary with the age of the rats and/or duration of the deficiency.

Summary. Liver nonprotein sulfhydryl (NPSH) values in nonfasted Vit. B_6-deficient rats fed a 20% casein diet were similar to the values in nonfasted pair-fed or *ad libitum*-fed controls. Liver NPSH was altered by fasting, but the effect of fasting varied with the age of the animal. Fasting reduced liver NPSH by only 28% in rats depleted of Vit. B_6 for 19-26 days after weaning, but caused a 63% reduction in the liver NPSH in the controls of the same age. This effect produced an apparent "increase" in the liver NPSH in the fasted deficient rats. In rats fed the experimental diet for approximately 70 days, fasting caused similar decreases in

NPSH in both the deficient and the control groups.

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Antinuclear Factor in Sensitized Lymph and Serum from Skin Homografted Dogs.* (28516)

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The transfer of transplantation immunity with sensitized lymphoid cells has been considered as strong evidence that homograft rejection is a form of delayed-type or cellular hypersensitivity. However, recognition that transferred, sensitized cells are capable of producing humoral antibody(1,2) as well as the failure to observe apparently significant numbers of such cells in grafts undergoing rejection(3,4) disallows for absolute exclusion that the host *vs* graft reaction may be mediated by a humoral factor or factors. Najarian and Feldman noted accelerated rejection of skin homografts in mice(4) and guinea pigs (5) and Kretschmer and Perez-Tamayo(2) in rabbits when transferred, sensitized lymphoid cells were enclosed in cell-impenetrable millipore chambers. The former have also recently observed rejection of skin homografts with the transfer of the clear supernate obtained from sonicated, sensitized lymphoid cells to the graft site(6). The active material had many of the characteristics of a gamma globulin. This information as well as the recognized inconsistencies concerning the demonstration of transplantation antibodies in serum indicates the pertinence of investigating lymph of homografted animals for possible humoral factors which may play a role in homograft rejection.

The purpose of this report is to record the occurrence of an antinuclear factor as demonstrated by the indirect fluorescent antibody technic in the supernate of lymph in approximately 50% of dogs sensitized with a full-thickness skin graft.

Materials and methods. Mongrel dogs of both sexes weighing 13-23 kg were utilized in all experiments. Animals were maintained on Ken-L-Ration and water *ad lib.* Full-thickness homografts or autografts of skin were applied to the inner aspect of the right thigh with aseptic technic. Lymph fistula was produced 3-14 days following grafting in recipient and donor dogs anesthetized with pentobarbital sodium (24 mg/kg). Utilizing sterile surgical technic the muscles in the anterior cervical triangle were severed above the manubrium and the jugulo-subclavian junction exposed. A long piece of polyethylene tubing (PE 90-190) or silastic tubing (X-7002-062) was inserted into the thoracic

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