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Influence of Vit. B_{12} and Sulfur Amino Acids on Liver Glutathione Level in Mice. (23839)

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Several recent reports suggest the existence of a relation between Vit. B_{12} and sulfhydryl metabolism. Dubnoff showed this vitamin to play a role in the reduction of S-S groups(1). Ling and Chow(2) found low non-protein sulfhydryl values in rat erythrocytes deficient in cobalamin, while Register(3) reported low levels in both erythrocytes and livers in B_{12}-deficient rats as compared to the corresponding control animals. In previous studies, we observed low values of reduced glutathione (GSH) in erythrocytes of rats, raised on a diet deficient in Vit. B_{12}(4); nonetheless, liver levels in both rats and mice kept under these conditions were normal(5,6). In Sanguinetti and Marchetti's experiments, the liver GSH in B_{12}-deficient rats was even higher than in the controls(12).

In the present paper the effect of Vit. B_{12} and sulfur amino acids on the GSH level of livers of mice is further studied.

Materials and methods. 300 adult albino mice of our stock were used. Those used for B_{12}-supplemented groups had been kept on complete commercial rat stock ration containing about 3 μg/100 g of this vitamin. Those deficient in Vit. B_{12} had been raised for several generations on a soybean-corn ration (diet 1, Table I). The performance of this deficient stock has been studied in detail(7). All animals used were between 10 and 14 weeks old when the respective experiments were started. At this age, the large weight differences existing at weaning between the B_{12}-deficient and normal mice have practically disappeared, all weighing between 22-25 g. The mean weight at weaning at 28 days of the deficient animals was 12 g, while that of the controls was 17 g. Care was taken that all B_{12}-deficient mice had a weaning weight between 11 and 13 g and continued on the deficient diet until the start of the experi-

TABLE I. Composition of Experimental Diets.†

No.	Soy meal	Bean meal	Corn meal	Starch	Sesame oil	Salts USP XII No. 2	Vit. mixture*
1	46		46		5	2	1
2		50		42	5	2	1
3				92	5	2	1

* Containing/100 g of diet: Thiamine-HCl, 0.3 mg; riboflavin, 0.3 mg; pyridoxine-HCl, 0.2 mg; Ca-pantothenate, 0.2 mg; niacin, 2 mg; folic acid, 0.025 mg; biotin, 0.01 mg; p-aminobenzoic acid, 25 mg; inositol, 10 mg; choline-HCl, 100 mg.

† 0.2% of percomorphum oil and 0.2% of wheat germ oil added.

TABLE II. Liver Glutation in Adult Mice Kept on Different Experimental Diets with and without Vit. B_{12}.

			Not deficient*		Deficient†	
No.	Exp. diet	Days on diet	No. of animals	Liver GSH, mg/100 g	No. of animals	Liver GSH, mg/100 g
1	Stock	Since birth	15	267 ± 12 ‡		
2	No. 1 Soy bean-corn	*Idem*			13	266 ± 9 ‡
3	*Idem* + 3 μg/100 g of B_{12}	"	15	256 ± 7		
4	No. 2 Methionine-low	30 days	18	72 ± 2.3	25	89 ± 2.4§
5	*Idem* + .3% d,l-methionine	30	16	256 ± 10	20	244 ± 9
6	No. 3 Protein-free	1	5	129 ± 12	5	111 ± 4
7	*Idem*	2	8	99 ± 6	4	97 ± 7
8	No. 2 Methionine-low	1	12	88 ± 6	12	72 ± 2.3‖
9	Fasting	1	6	175 ± 7	11	151 ± 6 ¶
10	"	2	7	175 ± 22	8	165 ± 10
11	No. 3 Protein-free	4	18	89 ± 7	20	68 ± 5 **
12	*Idem* + .25% l-cysteine HCl	4	7	206 ± 22	14	218 ± 11
13	" + .2% l-cystine	4	7	241 ± 17	21	234 ± 14
14	" + .25% d,l-methionine	4	8	190 ± 17	5	212 ± 14

* Animals raised and kept on complete stock ration until start of exp.
† " " " " " " B_{12} deficient soy bean-corn ration until start of exp.
‡ Stand. error of the mean.
§ Difference from corresponding B_{12} supplemented control group significant; $t = 5.0$, $p < .01$.
‖ *Idem* " $t = 2.5$, $p = .02$.
¶ " " $t = 2.8$, $p < .02$.
** " " $t = 2.4$, "

ments. Other deficiency symptoms include delayed maturity and high mortality of litters. In diet 2, cooked, dried, and ground black beans (*Phaseolus vulgaris*) were used as the protein source, as these pulses are low in methionine and adequate in all other essential amino acids(8). Liver GSH was determined in duplicate by a modification(6) of the method of Grunert and Phillips(9) and the results were calculated as mg of reduced glutathione (GSH) 100 g of fresh tissue, although it is understood that they rather represent the non-protein sulfhydril (NPSH).

Results. In the first 3 series of Table II are presented the results of experiments on the influence of Vit. B_{12} on GSH levels in mice kept since birth on soy bean-corn rations with or without added Vit. B_{12}, or on a stock diet. No differences between experimental groups could be found in the liver values (Table II, Nos. 1-3).

The liver GSH values of animals eating the methionine-low bean diet, supplemented with methionine, were normal, whether or not Vit. B_{12} was added (Table II, No. 5). When rats were fed the methionine-deficient diet without B_{12} for one month, they had significantly higher liver GSH values than the corresponding group, which had been supplemented with B_{12} (Table II, No. 4).

In other experiments normal and B_{12}-deficient mice were given a protein-free, or methionine-low diet for 1 or 2 days, or were fasted. The methionine-low diet caused the liver GSH levels to drop more rapidly and to lower values than either the protein-free diet or fasting (Groups 6-10). The last treatment was the least effective in lowering these levels. Comparing the Vit. B_{12}-deficient groups with their respective controls, a tendency can be observed for the former to have lower GSH values than the latter, although these differences were statistically significant only in 2 cases, with the methionine-low, and one of the fasting groups.

In another experiment, the influence of sulfur amino acids on GSH was studied in B_{12}-deficient and normal mice. Only the protein-free diet fed for 4 days caused a significantly greater drop in liver GSH levels in animals deficient in Vit. B_{12} than in the controls. Mice kept on similar rations, but supplemented with cysteine, or cystine, or methionine in equivalent amounts, had nearly normal values, whether or not they were deficient in Vit. B_{12} (Groups 11-14). There was no

significant difference between the diets supplemented with any one of the 3 amino acids in their action on liver GSH.

Discussion. In the present experiments, a Vit. B_{12} deficiency reduced hepatic GSH levels only in mice fed for short periods on the protein-free, or methionine-low diets, or fasted. This suggests that the influence of some stress factor (low intake of sulfur amino acids) may be important in revealing the effect of Vit. B_{12} on GSH in the liver.

Vit. B_{12} had no effect on GSH levels of mice fed an otherwise complete diet. This is evident from the data of Table II. Vit. B_{12} also had no effect on the liver GSH levels of mice fed protein free diets supplemented with sulfur amino acids (Series 10-14, Table II). It is difficult to compare these results with those published by Register(3) who found different GSH levels between B_{12}-deficient and supplemented, but otherwise normal rats, as he used stock animals which were put on a B_{12}-low diet for the experiments, while the controls were injected with large amounts of that vitamin. This fact could produce some influence on food consumption or some other unnoticed effects. In our experiments, all the B_{12}-deficient animals were born from deficient dams, and kept on the deficient diet, while the controls came from normal mothers and had never consumed a B_{12}-deficient diet.

The mice kept for 4 weeks on the methionine-low diet had higher liver GSH levels when they were deficient in Vit. B_{12}, than those which were not (Table II, Group 4). This unexpected observation may be comparable to some results of Register and Bartlett (11) who found that exposure to cold or injection of epinephrine raises the liver GSH of methionine-deficient rats, although these treatments lower the liver sulfhydryl in normally fed animals. Recently, Sanguinetti and Marchetti found liver GSH in B_{12}-deficient rats 35% higher than in the controls (12).

The low values found in mice after the consumption of the bean diet are in accordance with the role of the sulfur containing amino acids on the glutathione level(13) and the low methionine content of these pulses(8). We did not observe the slow rise of liver NPSH in mice kept on sulfur amino acid-low diets from the low values after 24 hr to higher values after 2-3 weeks, described by Beck and Bianconi(14).

The methionine-low diet had the most pronounced effect on liver GSH. After only 24 hours on this ration, the values had dropped to nearly as low a level as after 1 month of the same dietary treatment (Table II Groups 4 and 8); fasting caused a much less significant change, while the effect of the protein-free ration was intermediate. These results are in accordance with the relation of glutathione to carbohydrate metabolism, and would suggest also a role of this tripeptide in protein metabolism.

A relation between Vit. B_{12} and methionine synthesis(15) as well as cystine reduction(16) has been postulated; it was therefore of interest to study the effect of the different sulfur amino acids in glutathione formation in mice consuming the protein-free diet. The results presented in No. 11-14, Table II would not indicate an impaired utilization of cystine, as compared to cysteine or methionine in the B_{12}-deficient animals. Anderson and Stekøl likewise found normal incorporation of cystine into liver GSH in B_{12}-deficient rats(17).

In order to control the deficiency of the B_{12}-low experimental groups, Vit. B_{12} was determined in the pooled samples of livers and kidneys of 6 control and 6 deficient mice of the present experiments (series 1 and 2, Table II), using a *L. leichmannii* method with the results presented in Table III. The differences observed between the deficient and control groups are of the same order as those found in rats(5). The control groups had Vit. B_{12} liver values similar to those found by other authors(18). The liver GSH values of the mice of Groups 1 and 2 in Table II were

TABLE III. Vitamin B_{12} in Livers and Kidneys of Adult Mice.

Diet	Vit. B_{12}, $\mu g/g$, in Livers	Kidneys
B_{12}-deficient*	.152	.92
Control†	.949	2.27

* Weaning wt 12.1 g.
† " " 17.2 g.

similar to those found in rats kept on the corresponding diets although the Vit. B_{12} organ levels in the latter were much lower(5).

The present results indicate that the relation between Vit. B_{12} and reduced liver glutathione is not a simple one and that the effect of the latter on the former is only evident under special experimental conditions.

Summary. No differences were found in liver soluble GSH levels between Vit. B_{12}-deficient and control mice, kept on otherwise normal diets, or on protein-free, or methionine-low rations supplemented with sulfur amino acids. When kept for a short period on diets free of protein, or low in methionine, or fasted, mice deficient in Vit. B_{12} had lower hepatic GSH levels than the corresponding controls. After 1 month on a methionine low diet, these levels were higher in B_{12}-deficient animals than in the corresponding controls. Cysteine, cystine, and methionine were equally effective in maintaining the soluble liver GSH at high levels in B_{12}-deficient or supplemented mice consuming protein-free diets.

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Development of a Possible *in vitro* Assay for Intrinsic Factor.*† (23840)

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Miller, Raney, and Hunter(1,2) reported an enhancement of Co^{60}-labeled Vit. B_{12} uptake by rat liver slices in the presence of hog intrinsic factor concentrate (HIFC), and this finding has been confirmed by Latner and Raine(3), and Herbert and London(4). The latter workers(4,5) reported that enhancement of Co^{60}-B_{12} uptake by HIFC was calcium-dependent, reversible to an appreciable degree by ethylene-diamine-tetraacetate (disodium Versenate®), and occurred in the cold as well as at 37.5°C. Most enhancement by HIFC occurred in first hour of incubation(5). Further studies on the mechanism of the enhanced uptake of Co^{60}-B_{12} by rat liver slices in the presence of HIFC are here reported, along with the development of a possible *in vitro* assay for intrinsic factor

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