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EFFECT OF CARBON TETRACHLORIDE ADMINISTRATION ON THE METABOLISM OF VITAMIN B_{12} IN THE RAT*

I. PLASMA B_{12} LEVEL

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The liver has been known as the main storage organ of vitamin B_{12} and also the site where the main functions of this vitamin are carried out. It has also been demonstrated by *Harte et al*[1] in rat and by *Glass et al*[2] in humans that the liver takes up a large portion of radioactive vitamin B_{12} introduced into the body by oral and parenteral routes. The liver, therefore, seems to be the organ which essentially regulates the metabolism of this vitamin.

It has recently been reported[3,4] that some of the liver enzymes are increased in the plasma when the liver is damage. This increase was thought to be accounted for by liberation of that particular enzyme from the damaged liver cells. It has been observed by the author[5] that serum B_{12} levels are increased in some of the patients with liver diseases. *Davis* and *Chow*[6] also demonstrated an abrupt decrease of vitamin B_{12} binding capacity in serum in the very early stage of viral hepatitis. Therefore, it seemed of interest to investigate the the role of the liver in utilization and metabolism of vitamin B_{12} by determining plasma levels and urinary excretion of this vitamin in experimental liver damage. Such a study may supply further informations on the relationship between the liver and vitamin B_{12}. In this study, carbon tetrachloride which has been widely used as hepatotoxic agent was employed to produce acute parenchymatous injury of the liver. The influence of the administration of this agent on the plasma B_{12} levels in rat was first studied and the results are presented in this communication.

EXPERIMENTAL

Rats–Approximately year old male rats of *McCollum* strain were used in experiments 1 and 2. They were raised on the stock diet containing cow liver. In experiment 3, half of rats were placed on soybean diet and the other half on casein diet at weaning, and were raised for 6 months before used. All the animals were kept in individual cages through experimentation.

* This work was done in the laboratory of the Department of Biochemistry, Johns Hopkins School of Hygiene and Public Health.

Diets–The three diets used in this study were designed to produce rats with different degrees of vitamin B_{12} supplementation. The stock diet contained sufficient amount of B_{12}. Although vitamin B_{12} was omitted from both soybean and casein diet, it has been known that even purified casein is not quite free of this vitamin and the rats raised on casein diet grow better than those on soybean meal.

Composition of diets–Stock diet consisted of: 30 % wheat, 20 % maize, 18 % rolled oats, 12.5 % whole milk powder, 12.5 % skimmed milk powder, 4 % casal, 2 % cow liver powder, 0.5 % NaCl, supplemented with 120 g $CaCO_3$, 50 g Fe citrate and 20 g $CuSO_4$ per 100 lbs of diet. Soybean diet: 62 % soybean meal, 29.5 % sucrose, 4 % salts mixture IV,[7] and 4.5 % corn oil. The vitamin supplement for each kg diet consists of 2.0 mg thiamine, 3.0 mg riboflavin, 2.5 mg pyridoxine, 20 mg calcium pantothenate, 50 mg niacin, 100 mg inositol, 0.1 mg biotin, 210 mg p-aminobenzoic acid, 0.2 mg folic acid and 1 g choline HCl, 210 mg percomorph oil, 23 mg vit. E, 2.1 mg vit. K, 12,600 units vit. A, and 1,785 units vit. D. Casein diet: 20 % vitamin free casein, 71.5 % sucrose, 4 % salts mixture IV, and 4.5 % corn oil. The vitamin supplement was the same as in soybean diet.

Administration of CCl_4:CCl_4 was dissolved in olive oil and injected intraperitoneally. The dosage of CCl_4 varied from 0.035 to 0.1 ml per 100 mg of body weight, and CCl_4 olive oil mixture was prepared in such a way that the amount of injection was 1 ml per 400 gm body weigh of rat. The controls also recieved equivalent amounts of olive oil intraperitoneally.

Microbiological determination of vitamin B_{12} in plasma, serum and liver: Heparinized plasma was obtained by cardiac puncture under light ether anesthesia. The plasma was added directly to *Skeggs'* media[8] at the levels of 0.025, 0.05 and 0.075 ml per tube which contained 5 ml of media. The growth of Lactobacillus leichmannii 4797 was measured titrimetrically after 70 hour incubation at 37°C. The details of this technique have been described elsewhere.[9]

For the measurement of alkali stability of vitamin B_{12} activity, serum was processed according to the procedure of *Okuda et al*,[10] and the protein-free supernatant was analyzed for both total and alkali stable activities. The alkali treatment was done by heating the serum extract at 100°C for 30 minutes at NaOH concentration of 0.2 N.[11] The neutralized aliquots were measured for B_{12} activity with a series of standard tubes containing comparable concentrations of salt.

The liver was frozen immediately at necropsy, and subsequently homogenized in the *Potter-Elvehjem* homogenizer. The 0.5 % homogenate was further diluted with distilled water and assayed directly without extraction by titrimetric measurement.

Experiment 1: Eighteen rats were divided into three groups, one group serving

as control and the other two receiving 0.035 and 0.07 ml CCl_4 per 100 gm body weight respectively. Twenty-four hours and seven days after the injection of CCl_4, all survived animals were bled and the plasma was analyzed for vitamin B_{12}. At the end of the experiment, the liver was removed by necropsy and analyzed for B_{12} content.

Experiment 2: In order to clarify whether the increase in B_{12} activity in plasma was due to vitamin B_{12} itself or due to some other substances with B_{12} like activity, the second experiment was designed to measure the alkali stable factors in serum. Three groups, 5 rats in each, were given olive oil alone, 0.05 and 0.1 ml CCl_4 per 100 gm body weight respectively. Twenty-four hours after the administration of CCl_4, animals were totally bled and serum was analyzed for total and alkali stable B_{12} activities.

Experiment 3: In this study, the relation of the original levels of plasma B_{12} to the increase of B_{12} following the administration of CCl_4 was studied. Two groups of rats were raised on B_{12} free soybean diet, while the other two groups were fed 20 % casein diet for six months. One group out of the two groups on each diet was given twice 0.035 ml CCl_4 per 100 gm body weight in two days. Twenty-four hours after the last injection, the animals were bled and their plasma was separated for B_{12} determination.

Results and Discussion

The results shown in Table 1 clearly demonstrate that CCl_4 poisoned rats had elevated B_{12} levels in plasma 24 hours after the administration of CCl_4 Since multiple bleedings by heart puncture had been avoided, the plasma B_{12} level before CCl_4 administration was considered to be represented by that of the control

Table I

Effect of CCl_4 administration on plasma and liver B_{12} levels in rat

Dose of CCl_4 (ml/100g B.W)	Av. Body Wt. Before CCl_4 Administ. (gm)	24 Hours after CCl_4 Administ.		7 Days after CCl_4 Administ.	
		Body Wt. (gm)	Plasma B_{12} (mγ/ml	Plasma B_{12}(mγ/ml)	Liver B_{12} (mγ/gm)
0	400	410(+10)	0.656±0.024	0.775±0.025	202±57
0.035	408	404(− 4)	0.871±0.082	0.809±0.031	
0.070	397	383(−14)	1.174±0.105	0.857±0.025	190±26

Each group had 5 rats ; ±Standard error of the mean

plasma taken 24 hours after CCl_4 treatment. Somewhat stoichiometric relation was indicated between the CCl_4 dose and the increase of B_{12} in plasma. At the dose of 0.07 ml of CCl_4, the B_{12} level was almost doubled as compared to the con-

trol 24 hours after CCl_4 administration. The CCl_4 intoxication was also manifested by hyperbilirubinemia and the decrease in body weight of the rats. The once elevated B_{12} levels were lowered to the near-normal levels in 7 days. The difference in B_{12} level between the 24 hour and the 7 day specimens of the control is not significant, since they were assayed for vitamin B_{12} in two separate batches. B_{12} assay yields to some extent a batch-to-batch fluctuation in the results.

There was no demonstrable difference in liver B_{12} content between the control and 0.07 ml CCl_4 group. It is not very likely that the liver B_{12} levels in CCl_4 treated animals were decreased at the height of injury and were normalized during the course of recovery. Because, the increase of B_{12} in plasma was very little as compared to the liver B_{12} concenration, and this would not account for any detectable decrease in liver B_{12} concentration, even though urinary excretion of the plasma B_{12} was taken into consideration.

In experiment 2, a similar finding as in the previous experiment was obtained (Table II). Instead of plasma, serum was used in this experiment, and the serum was deproteinated to minimize the buffering effect of serum. The difference in processing of serum and technique of B_{12} assay would probably explain the

TABLE II

Effect of CCl_4 administration on total and alkali stable B_{12} activity of the serum.

Dose of CCl_4 (ml/100g Body wt.)	No. of Pats	Body Wt. Before CCl_4 Administr. (gm)	24 Hours after CCl_4 Administration		
			Body Wt. (gm)	Total B_{12} Activity (mγ/ml Serum)	Alkali-stable B_{12} Activity(mγ/ml Serum)
0	5	399	390(− 9)	0.401±0.008	0.210±0.016
0.05	5	388	357(−31)	0.659±0.059	0.235±0.008
0.10	5	401	378(−23)	0.637±0.051	0.236±0.012

somewhat lower values for serum B_{12} as compared to those in experiment 1. Although alkali treatment has been employed by many investigators to inactivate vitamin B_{12} and make correction for false B_{12} activity, a question has recently been raised[12, 13] as to the quantitative relation of the alkali labile factor to the total B_{12} activity. It is, therefore, quetionable that the alkali stable activity which constituted a considerable portion of the total B_{12} activity was not B_{12}. It is more likely that some of the alkali stable activity was due to vitamin B_{12}. Regardless of the nature of the measured alkali stable B_{12} activity, there was not any difference in the total alkali stable activity between the control and the experimental group. This finding obviously eliminates an argument that the increased serum B_{12} levels of CCl_4 treated animals are derived from substances other than

vitamin B_{12} which are also likely to appear in the blood stream following liver cell damage.

The results in Table III revealed low basal plasma B_{12} levels in the soybean fed rats, 0.05 mγ/ml on an average, in contrast with 0.75 mγ/ml in the casein fed animals. This difference in plasma B_{12} levels is compatible with the different growth rate of rats between those two groups as indicated by the body weight in this table. The CCl_4 administration did not bring about an increase of plasma B_{12} level in the soybean group, whereas there was an increase in the casein group following CCl_4 treatment as was the case in the preceding two experiments.

TABLE III

Effect of CCl_4 administration on plasma B_{12} level of rats on different diets

Dose of CCl_4 (ml/100 g Body Wt.)	On Casein Diet				On Soybean Diet			
	No. of Rats	Before-	24 Hours After-CCl_4		No. of Rats	Before-	24 Hours After-CCl_4	
		Body Wt. (gm)	Body Wt. (gm)	Plasma B_{12} (mγ/ml)		Body Wt. (gm)	Body. Wt. (gm)	Plasma B_{12} (mγ/ml)
0	5	368	365	0.75±0.05	5	279	272	0.50±0.03
0.07*	5	354	320	0.99±0.07	5	284	253	0.50±0.03

* In two doses

Since soybean meal has been widely used for the production of vitamin B_{12} deficient rat, it was expected that soybean fed rats had not received exogenous supply of B_{12} and had been depleted of this vitamin by the time they were used for this experiment. Therefore, it might be of interest to postulate that there is a mobilizable type of B_{12} in the normal liver, and that the B_{12} deficient animals lack in this type of liver B_{12} which is readily liberated into the blood upon injury of the liver cells. If this hypothesis should be extended, it might also be interesting to presume that this mobilizadle type of B_{12} is loosely bound in the cell, and CCl_4 injured cells can no longer hold it. Since CCl_4 induces extensive parenchymatous damage including necrosis at the doses employed, plasma B_{12} level should jump up if most of the liver cell B_{12} was liberated into the blood. Liver cell B_{12}, probably bound to polypeptide,[14] may not be readily tranported into the blood stream by necrosis. Only this postulated mobilizable B_{12}, which constitutes a minute portion of total liver B_{12}, would be released into the blood to result elevated plasma B_{12} level.

As based on the data presented, it should be expected that some of the acute liver diseases in humans might bear a similar pattern of vitamin B_{12} metabolism, and that determination of plasma B_{12} level might aid in diagnostic procedure and better evaluation of the liver conditions.

CONCLUSION

Plasma vitamin B_{12} levels were increased following administration of carbon tetrachloride in the normal rat, and this increase was due to vitamin B_{12} but not due to other substances. In vitamin B_{12} deficient rat, however, carbon tetrachloride did not result a demonstrable increase of plasma B_{12} level. The liberation of liver B_{12} by liver cell injury was thought to account for the elevated level of plasma B_{12}.

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