for 24 hours. (5) One 15 mg troche decreases bacterial counts significantly for at least one hour. (6) A second troche administered one hour after the first adds considerably to the antibacterial effect.

We wish to express our appreciation to Miss Constance M. Marginot for her technical assistance; and to Mrs. Joan Keyser for the statistical handling of our data.

Received July 30, 1951. P.S.E.B.M., 1951, v78.

Liver Storage of Vitamin B_{12} by the Rat.* (19139)

HAROLD E. SCHEID, MONA M. ANDREWS, AND B. S. SCHWEIGERT.

From the Division of Biochemistry and Nutrition, American Meat Institute Foundation, and Department of Biochemistry, University of Chicago, Ill.

Assays for vit. B_{12} by the use of rat and chick growth, or microbiological methods, are well known. Although available information (1,2) indicates that the amount of vit. B_{12} in the liver or other organs was lower for animals fed a vit. B_{12} deficient diet as compared to a supplemented diet, it was of interest to extend these studies and to determine the vit. B_{12} potency of the livers of rats fed graded amounts of vit. B_{12}. Further, the liver storage of the vitamin, when fed as the crystalline vitamin or as a food, could be evaluated as a method for determining the vit. B_{12} potency of foods and could be compared with data obtained by the microbiological and animal growth methods. These studies have been conducted, and data have also been obtained on the effect of the level of vit. B_{12} fed on the liver weight to body weight ratio.

Experimental and results. Weanling male rats of the Holtzman strain were fed a basal ration (corn-soybean oil meal-iodinated casein) without added vit. B_{12} as a depletion ration for a period of 2 weeks. The rats were then randomized into groups and fed either 0, 3, 6, 9, 15 or 24 μg of crystalline vit. B_{12} per kilo of ration or crude supplements of unknown vit. B_{12} potency for a period of 4 weeks. The ration and procedure used have been described previously(3). Seven rats were used in each group. After completion of the rat growth assay, all animals from selected groups were retained for studies on the storage of vit. B_{12} in the liver. The animals were sacrificed by decapitation, the livers removed and weighed. Two gram samples were homogenized in a Waring blendor with 50 ml of 0.1 M phosphate buffer, pH 6.8, for one minute, then autoclaved for 5 minutes at 15 lb pressure. After autoclaving, the samples were cooled, made to 100 ml volume, filtered and stored in the refrigerator. Aliquots of these samples were taken for microbiological assay according to the method previously described(4,5). The enzymatic digestion step was eliminated since investigations with short-term autoclaving of the samples as compared to subsequent enzyme

TABLE I. Vitamin B_{12} Content of Rat Livers As Assayed Using *Lactobacillus leichmannii.*

μg B_{12} added/kg ration	μg B_{12}/ g liver*	μg B_{12}/ liver	Liver wt (% of body wt)
0	.026 ± .005	.200	6.20
3	.030 ± .003	.306	6.49
6	.037 ± .011	.361	6.09
9	.047 ± .009	.474	5.77
15	.048 ± .006	.442	5.20
24	.066 ± .017	.621	5.00

* Mean and stand. dev.

* We are indebted to Merck and Co. for supplying the vit. B_{12} used in these studies. Journal Paper No. 45, American Meat Institute Foundation.

1. Lewis, U. J., Register, U. D., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 590.

2. Richardson, L. R., Witten, P. W., and Couch, J. R., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 265.

3. Scheid, H. E., McBride, B. H., and Schweigert, B. S., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 236.

4. Scheid, H. E., and Schweigert, B. S., *J. Biol. Chem.*, 1950, v185, 1.

5. Scheid, H. E., and Schweigert, B. S., *J. Biol. Chem.*, 1951, v193, 299.

TABLE II. Vitamin B_{12} Activity of Liver With and Without Alkali Treatment.

Material	Test organism	Sample treatment: Autoclaved pH 7, μg/g	Sample treatment: Autoclaved Alkali, μg/g
Beef liver	*L. leichmannii*	.77	.146
	Euglena gracilis	.74	0
Rat liver (B_{12} deficient diet)	*L. leichmannii*	.04	.031
	Euglena gracilis	.02	0
Rat liver (B_{12} supplemented diet)*	*L. leichmannii*	.068	.057
	Euglena gracilis	.054	0

* 24 μg of vit. B_{12}/kg of diet.

treatment gave identical results on three assays. Duplicate analyses were made for each liver and *Lactobacillus leichmannii 327* (ATCC 7831) was used as the test organism.

The vit. B_{12} potency of the liver (0.066 ± .017 μg/g) for the group fed an optimum level of vit. B_{12} was considerably higher than that for the basal group (0.026 ± .005 μg/g) as shown in Table I. In a subsequent experiment, livers from rats fed the basal diet and basal diet plus 24 μg B_{12} per kg diet were analyzed for vit. B_{12} in a similar manner. In this experiment, the basal group averaged 0.046 μg/g of vit. B_{12} per g of liver, and the supplemented group averaged 0.108 μg. These values for the B_{12} potency of rat livers are approximately 1/10 to 1/20 those observed for beef liver(5).

Since values for the vit. B_{12} potency of rat liver appeared low compared to that of beef liver, it was of interest to determine how much of the B_{12} potency in these samples may be attributed to desoxyribosides. Hoffman, *et al.*(6) showed that vit. B_{12} was destroyed by heating with 0.2 N NaOH at 100°C for 30 minutes and that under these conditions the vit. B_{12} activity of the desoxyribosides of thymine, guanine, and hypoxanthine was not affected. They also found that about 97% of the total microbiological activity of liver extract disappeared after alkali treatment. Rat liver was subjected to a similar treatment: Two g samples were homogenized in 40 ml 0.1 N NaOH and autoclaved at 15 lb pressure for 30 minutes. The samples were neutralized with 2 N HCl, made to 100 ml volume and filtered. Aliquots were then taken for microbiological analyses. Rat liver homogenates treated with alkali were found to retain an appreciable amount of vit. B_{12} activity for *L. leichmannii* (Table II). When rat liver plus crystalline vit. B_{12} was so treated, the added B_{12} was destroyed, about 80% of the vit. B_{12} potency of beef liver was destroyed, but the vit. B_{12} activity of rat liver was retained. It is known that *L. leichmannii* responds to thymidine and desoxyribosides (7-9), and since rat liver contains alkali stable substances with vit. B_{12} activity, it is likely that the organism measured desoxyriboside activity after alkali treatment. Vit. B_{12} complexes that may be present in liver that are stable to alkali could also explain these findings.

This problem was further investigated by the use of the algal flagellate *Euglena gracilis*. Hutner, *et al.*(10) have shown that thymidine does not replace the vit. B_{12} requirement of this organism. The medium used in these studies was that of Robbins, *et al.*(11) with the exception of NH_4VO_3 which was not included. The method of assay was essentially that of Hutner,† *et al.*(10). The organism was cultured on an agar slant containing

6. Hoffmann, C. E., Stokstad, E. L. R., Hutchings, B. L., Dornbush, A. C., and Jukes, T. H., *J. Biol. Chem.*, 1949, v181, 635.

7. Shive, W., Eakin, R. E., Harding, W. M., Ravel, J. M., and Sutherland, J. E., *J. Am. Chem. Soc.*, 1948, v70, 2299.

8. Snell, E. E., Kitay, E., and McNutt, W. S., *J. Biol. Chem.*, 1948, v175, 473.

9. Shive, W., Ravel, J. M., and Harding, W. M., *J. Biol. Chem.*, 1948, v176, 991.

10. Hutner, S. H., Provasoli, L., Stokstad, E. L. R., Hoffmann, C. E., Belt, M., Franklin, A. L., and Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 118.

11. Robbins, W. J., Hervey, A., and Stebbins, M. E., *Bull. of the Torrey Bot. Club*, 1950, v77, 423.

0.5% peptone, 0.1% liver extract, 0.2% sodium acetate and 1.5% agar. The inoculum was prepared in the same medium (pH 7) without the agar. An assay period of 6 days was used, and the amount of growth determined by measuring the turbidity of the cultures with the use of a Coleman Junior Spectrophotometer.

Fresh rat and beef liver homogenates with and without alkali treatment were assayed with *Euglena*. The results indicated that the values for livers from vit. B_{12}-deficient rats were consistently lower in vit. B_{12} than those for livers from rats fed an optimum level of vit. B_{12}. The values obtained by the vit. B_{12} *Euglena* method of assay for rat or beef liver treated with alkali were negligible. The results for fresh beef and rat liver with this method of assay compared very well with the values found by the *L. leichmannii* assay (Table II).

These studies indicate that application of the procedure of alkali treatment to low potency samples, that retain a large portion of the vit. B_{12} activity after alkali treatment for *L. leichmannii*, may not provide information that can be reliably interpreted. Other studies(12) have shown that the desoxyribonucleic acid level of livers from vit. B_{12}-supplemented rats is 10-20% higher than for deficient rats. Thus, the increases observed in the vit. B_{12} activity of rat liver when the amount fed was increased can be only partly explained by increased desoxyriboside activity. Although the data obtained after alkali treatment are not definitive, the results for *L. leichmannii* and *Euglena*, as well as the rat assay(1), show that the vit. B_{12} stored in the liver is related to the vit. B_{12} intake.

It is of interest that the liver weight was greater per unit of body weight for animals fed the low levels of crystalline vit. B_{12}. Among the deficient animals, the liver weight was found to be 6.2% of the body weight, while the percentage for the B_{12}-supplemented group was 5.0 (Table I). These data are in agreement with those observed in another experiment in which the percentage of body weight that was liver weight was 6.6 for animals fed the basal diet and 5.2 for animals fed the basal diet plus 24 μg vit. B_{12} per kg.‡ The percentage for animals fed the supplemented diet with the food intake restricted to that of the basal group was 5.0.

By the use of the liver storage method of assay, values for fresh beef round, pork ham, lamb leg, kidney, liver, and injectable liver extract have been determined. In the cases of pork and lamb, the data for vit. B_{12} are in close agreement with those for similar samples studied earlier using the microbiological technic. The values for the remainder of the crude materials tend to be somewhat higher. Details of these studies will be reported at a future date.

Summary. The storage of vit. B_{12} in rat liver was investigated after feeding rats graded levels of crystalline vit. B_{12} or of crude supplements. The vit. B_{12} potency was determined with *L. leichmannii* after short-term autoclaving of the liver homogenates at pH 7.0. The vit. B_{12} potency was shown to increase as the level fed was increased. The values were only 1/10 to 1/20 those for beef liver, however, and the vit. B_{12} potency of rat liver was not reduced appreciably by alkali treatment. Studies on the desoxyribonucleic acid levels in rat livers and studies with *Euglena gracilis* assays for vit. B_{12} indicated that the vit. B_{12} activity remaining, as determined with *L. leichmannii* after alkali treatment, may be due to the presence of alkali stable forms of vit. B_{12} as well as to desoxyribosides. The liver weight was shown to be greater per unit of body weight for the rats fed the vit. B_{12}-deficient diet as compared to the optimum diet.

† We are indebted to Dr. S. H. Hutner for providing a culture of *Euglena gracilis*.

12. Rose, I. A., and Schweigert, B. S., *Abstracts, Am. Chem. Soc. meeting*, Chicago, September, 1950, p55C.

‡ Rose, I. A., and Schweigert, B. S., unpublished data.

Received October 10, 1951. P.S.E.B.M., 1951, v78.