does not show changes in association with early or minimal lesions of cancer.

2) The test may aid in excluding the diagnosis of advanced but non-terminal malignancy, or in the discovery of these lesions when they present themselves in obscure form.

3) Since antitrypsin values appear to be closely related to the growth of seminoma of the testis, it suggested that the test may have unique prognostic value in the study of this condition.

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Rat Growth Assay for Vitamin B_{12}. (17346)

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The method of assay for vitamin B_{12} herein described was developed using a diet based on purified casein. The diet was made to contain 0.05% iodinated casein (Protamone) as a metabolic stimulant and 0.5% sulfaguanidine to inhibit bacterial synthesis of vitamins. Weanling rats, depleted on this diet for about 7 days, show a depression of growth rate which is counteracted by administration of vitamin B_{12}. The rate of response within a critical range is proportional to the amount of vitamin B_{12} administered, either as the purified vitamin or as concentrates of the vitamin, such as liver extract.

It is well known that liver extract and vit. B_{12} show much higher activity by injection than by oral administration in the pernicious anemia patient. We were interested in the present study to determine whether a difference could be shown between the effects of oral and intramuscular administration of the vitamin. We were interested also to determine the comparative effects of single doses at the beginning of the assay period versus divided doses throughout the assay period. Following completion of these studies, Emerson[1] reported that vitamin B_{12} is equally active by either the oral or subcutaneous route in daily doses ranging from 0.0625 to 0.5 μg per rat day, somewhat higher than the range we have studied.

Register, Ruegamer and Elvehjem[2] reported a quantitative response in rat growth in their assay in the range of 0.025 to 0.1 U.S.P. units per rat day in the form of commercial liver extract. Direct comparison with vit. B_{12} activity was not made in the Wisconsin report. We were interested to determine with a sample liver extract whether 1 μg of vit. B_{12} would correspond fairly closely by rat assay to I U.S.P. unit, a figure previously suggested by Rickes *et al.*,[3] to be in approximately the correct range.

Experimental. Weanling rats, 35-45 g, of mixed sex were placed on a vitamin B_{12} low diet for depletion. The B_{12} depletion diet has the composition shown in Table I. In preliminary assays S.M.A vitamin test casein was further purified by the method of solution and reprecipitation of Novak and Hauge.[4] Later we found that vitamin test casein gave satisfactory results without further purification.

The rate of weight gain is about 12-15 g during the second week on the depletion diet and diminishes to about one-half this rate during the third week of depletion. The depletion period used ranged from 7 to 14 days. The 7-day period is preferred because of the increased mortality which occurs on more ex-

1 Emerson, G. A., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 392.

2 Register, U. D., Ruegamer, W. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1949, **177**, 129.

3 Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K., *Science*, 1948, **107**, 396.

4 Novak, A. F., and Hauge, S. M., *J. Biol. Chem.*, 1947, **174**, 647.

TABLE I.
Vitamin B_{12} Depletion Diet.

	g/100 g
Purified casein	18
Cystine	0.2
Dextrin	69
Salt mixture No. 1 (U.S.P.)	4
Cellu-flour	2
Agar	1.5
Primex	5
CLO	1
Choline	0.1
Sulfaguanidine	0.5
Protamone (iodinated casein)	0.05
	mg/100 g
Riboflavin	3
Thiamine • HCl	3
Inositol	20
Niacin	3
Pyridoxine • HCl	5
Biotin	.01
Folic acid	.01
Calcium pantothenate	5
p-Amino benzoic acid	5
Menadione	0.5

tended depletion. Groups of about 50 rats are depleted at one time with about 16 rats to a cage. Following depletion, the rats are placed in individual cages with raised floors. Six rats of mixed sex are used for each assay level. As a check on the rat assay, experiments were run, using the dietary regimen and procedure for assay of the animal protein factor (zoopherin), as described by Zucker and Zucker.[5] Following the preferred method described by these authors, we fed diet P60, based on 60% cottonseed meal to the mother rats during gestation and lactation, and to the offspring to 28 days of age when the curative assay was begun. Five male rats were used in each group.

Oral vs. Injection Administration. Three vit. B_{12} preparations studied were as follows: a concentrate of vit. B_{12}, 25 μg per cc, supplied for investigational use by Merck and Company (designated Concentrate No. 1); a concentrate of vit. B_{12} prepared in this laboratory (H.H.F.) to contain any desired concentration of vit. B_{12} as determined by microbiological assay (designated Concentrate No. 2) and Cobione (Merck), 10 μg crystalline vit. B_{12} per cc. Ampoules of Concentrate No. 1 were used as the standard throughout. A lot of these ampoules was assayed microbiologically by Eleanor Willerton in our laboratories against a lot of material distributed by Merck and Co. as a microbiological assay standard. By repeated comparisons, ampoules of Concentrate No. 1 were found to contain very close to 25 μg of vit. B_{12} per cc.

Experiments were devised to compare the activity of vitamin B_{12} added directly to the diet and given orally, or by injection, in single or multiple doses. For direct addition to the diet, solutions of vit. B_{12} at a level of about 1 μg per cc were mixed with about 20 g of starch. The starch was then dried in air at 50-60°C and added to one kg of the basal diet. Results of assays wherein vit. B_{12} was added directly to the diet are shown in Table II.

For comparison of oral versus injection dosage under conditions of multiple dosage, vit. B_{12} solutions were made to contain from .33 to 1.0 μg per cc. Intramuscular injections and oral feedings of 0.23 cc per dose were then made 3 times weekly over the 2-week assay period at various assay levels. When comparing the effect of single oral or injection doses, appropriate critical concentrations of B_{12} were administered the first day of assay corresponding to a range of .025 to .075 μg per rat day for the 14-day period. The results of the different modes of administration are shown in Table III.

Experiments were designed also to relate the activity of vitamin B_{12} in a very preliminary way with the labeled A.P.A. potency of liver extract preparations of known clinical activity. The microbiological assay method of Skeggs, Huff, Wright and Bosshardt[6] provided a further basis for correlation. Aseptic addition of the liver extract supplements to the microbiological medium was used to avoid destruction of the vit. B_{12} which, according to Stokstad *et al.*,[7] occurs on autoclaving.

The results of the comparison assays be-

[5] Zucker, L. M., and Zucker, T. F., *Arch. Biochem.*, 1948, **16**, 115.

[6] Skeggs, H. R., Huff, J. W., Wright, L. D., and Bosshardt, D. K., *J. Biol. Chem.*, 1948, **176**, 1459.

[7] Stokstad, E. L. R., Dornbush, A. C., Franklin, A. L., Hoffman, C. E., Hutchings, B. L., and Jukes, T. H., *Fed. Proc.*, 1949, **8**, 257.

TABLE II.
Direct Addition of Vitamin B_{12} to the Diet.

Exp.	Supplement	Vit. B_{12}* per kg diet, μg	Avg 2 wk wt gain and std. error,† g
1	None	0	21‡
	Cobione	5	39 ± 3.5
	,,	10	53 ± 3.7
	,,	30	49.4 ± 4.2
2	None	0	17.6 ± 6.3
	Vit. B_{12} concentrate No. 1	5	25.3 ± 0.2
	Vit. B_{12} concentrate No. 1	15	39.3 ± 5.5
3	None	0	18.1 ± 3.1
	Vit. B_{12} concentrate No. 1	10	38.5 ± 3.9
	Vit. B_{12} concentrate No. 2	10	37.2 ± 4.3
	Vit. B_{12} concentrate No. 2a	10	40.6 ± 2.2

* The preparations used were checked for vitamin B_{12} content by microbiological assay, one against the other, and against a Merck microbiological assay standard. See text.

† Standard error = $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

‡ Only 3 of 6 rats survived.

TABLE III.
Vitamin B_{12} Assays—Injection and Oral Administration.

Exp.	Supplement	Avg 4 wk wt gain and range, g
Assay method of Zucker and Zucker	None	35.4 (31- 41)
	Vit. B_{12} conc. No. 1, 10 μg per kg diet	123.2 (113-140)
	Vit. B_{12} conc. No. 1, = .05 μg daily by mouth*	110.1 (90-138)
	Vit. B_{12} conc. No. 1, = .05 μg daily by inj.*	123.6 (108-140)
	Vit. B_{12} conc. No. 2, = .05 μg daily by inj.*	115.0 (110-120)
		Avg 2 wk wt gain and stand. error
Method in study	None	18.2 ± 4.5
	Vit. B_{12} conc. No. 1, 0.35 μg inj. (single dose)	26.0 ± 2.9
	Vit. B_{12} conc. No. 1, 0.35 μg oral (single dose)	28.5 ± 5.5
Method in study	None	17.7 ± 3.7
	Vit. B_{12} conc. No. 1, = .033 μg daily by inj.*	27.3 ± 9
	Vit. B_{12} conc. No. 1, = .067 μg daily by inj.*	35 ± 3.7
	Vit. B_{12} conc. No. 2, = .1 μg daily by inj.*	41.1 ± 3.3

* Injections and oral dosings—3 times weekly to supply daily equivalent shown.

tween vit. B_{12} and 2 injectable liver extract preparations are shown in Table IV. The microbiological assay of Liver Extract No. 1 indicated a vit. B_{12} content of one μg per U.S.P. unit This value appears to be confirmed by the rat assay. Liver Extract No. 2, which showed a minimal clinical response, was found to contain only .22 μg of vit. B_{12} per estimated U.S.P. unit by microbiological assay. Good agreement with this low value was indicated by the rat assay response. A resumé of experience in this laboratory in the preparation of experimental liver extracts for clinical trial has indicated in general that preparations low in vit. B_{12} are correspondingly low in clinical activity.

Discussion. An advantage of the method described herein is the ability to use animals weaned directly from mothers on stock diet, without special dietary control of the parent animals. This is true at least for animals reared in our own stock colony. Weanling rats

TABLE IV.
Vitamin B_{12} Rat Assay—Liver Extracts.

Exp.	Supplement	Avg wt gain and range 2 wk, g	3 wk, g	4 wk, g
Assay method of Zucker and Zucker	None	44	54	74 (59- 84)
	Vit. B_{12} conc. No. 1, 5 μg/kg diet	63	80	100 (85-128)
	Liver extr. No. 1, 5 U.S.P. units/kg diet	65	80	98 (78-116)
				Avg 2 wk wt gain and stand. error, g
Method in study	None			17.7 ± 2.7
	Vit. B_{12} conc. No. 1, = .05 μg daily (i.m.)*			36.8 ± 4.4
	Liver extr. No. 1, + .05 U.S.P. unit daily (i.m.)*			34.6 ± 3.6
Method in study	None			17.7 ± 3.7
	Vit. B_{12} conc. No. 1, = .1 μg/day			41.1 ± 3.3
	Liver extr. No. 1 = 0.1 U.S.P. unit/day (i.m.)* (Equal to .1 μg B_{12} by *L. leichmannii* assay)			44.8 ± 5
	Liver extr. No. 2 = 0.2 U.S.P. unit/day (i.m.)* (Equal to .044 μg B_{12} by *L. leichmannii* assay)			34.5 ± 4.2

* Injections made intramuscularly 3 times per week in doses to supply the daily equivalent shown.

from outside sources have not been tried.

The depletion period following weaning must remain, to some extent, a matter of judgment, depending on the condition of various lots of animals. It appears necessary to deplete the animals until the controls gain an average of only about 20 g during the 2-week period of assay. This has held true with a 7-14 day depletion in all but one of 12 assays in this laboratory involving about 600 rats.

No significant difference has been noted between the response of male and female rats in these studies, and precautions other than equal pairing as to sex do not appear necessary or desirable on the basis of convenience. Although there is considerable variation in response among individual animals, as shown by the fairly high standard errors (Tables II-IV), the significance of the results also appears high because of the distinct spread in increments between the control and test groups. In a few instances assays have been extended for 3 weeks. In these cases the animals supplemented with vit. B_{12} continued to gain at a steady rate, whereas the control animals showed a small and constantly declining rate of weight gain, plus an increasing mortality. Occasional control rats gain weight rapidly without any supplement. We surmise that these unusual animals have a high rate of intestinal synthesis of vit. B_{12}, similar to the fast growing rats described by Hartman, Dryden and Cary,[8] and that neither the sulfaguanidine nor iodinated casein affect these particular rats nearly as much as they do the general run of test animals. In the above-mentioned single assay run in which the animals were not sufficiently depleted before assay, the control group gained weight nearly as rapidly the first 2 weeks as the supplemented groups. The groups were continued another week during which the supplemented groups gained considerably more weight than the controls, indicating that depletion of the control group had finally taken effect.

Our findings are in accord with those of Lillie, Denton and Bird[9] with chicks indicating that a single administration of vitamin B_{12} at the beginning of assay meets the needs of deficient animals for growth for at least an ensuing 2-week period. Thus the assay may be simplified to involve only one administration of concentrated materials, such as liver extract, which lend themselves to this technic. An advantage of the single dosage technic is found in supplying the deficient

[8] Hartman, A. M., Dryden, L. P., and Cary, C. A., *Fed. Proc.*, 1949, **8**, 205.

[9] Lillie, R. J., Denton, C. A., and Bird, H. R., *J. Biol. Chem.*, 1948, **176**, 1477.

animals the full supplement at a time most conducive to overcoming the acute phase of the deficiency.

The present data indicate that vit. B_{12} is about equally effective by oral or injection administration in promoting growth of young rats on highly specialized depletion diets. The deficiency of vit. B_{12} induced in rats by these rather extreme dietary means does not, therefore, appear to evoke a failure in utilization of the vitamin, but rather a true deficiency and enhanced need for the vitamin itself.

The following additions to the diet failed to yield a comparable growth response, and even appeared to have an inhibitory effect: 5-10% purified casein, 5-10% of various primary-grown and brewer's yeasts, and additional folic acid. The addition of thymidine, 25-50 mg per kg diet, betaine hydrochloride 0.5%, or additional choline 0.1% likewise did not stimulate growth in absence of vit. B_{12}.

The growth promoting action of vit. B_{12} in rations containing iodinated protein has recently been reviewed.[10] Betheil and Lardy[11] have further compared the effectiveness of vit. B_{12}, whole liver substance and extracts high in APA activity, as growth promoting materials for hyperthyroid rats. The ration used by these authors was also based on purified casein, but did not contain a sulfa drug. It is worthy of note that mortality is high among our control animals and that death is generally preceded by appearance of hemorrhage about the nose and paws. Vit. B_{12} clearly prevents this syndrome and has a marked effect toward longevity under the dietary conditions imposed.

The question of the equivalence of vit. B_{12} with U.S.P. anti-pernicious anemia units can only be answered by assays in human pernicious anemia patients. Microbiological and animal assays may serve as a useful guide, however, toward this goal. The data presented provide some evidence that concentrated liver extracts which show a correlation by microbiological assay of 1 U.S.P. unit approximately equal to 1 μg of vit. B_{12}, show a similar approximate equivalence by rat assay.

Summary. The growth response of vit. B_{12} depleted rats under the conditions studied is proportional to the vit. B_{12} administered in the critical range of .025-.1 μg per rat day. Oral and injection administration of the vitamin at critical levels yield approximately equal growth responses. Addition of the vitamin to the diet, multiple dosing or single dosing the first day of assay also appear roughly equivalent. A preliminary correlation between the results of microbiological and rat assays as applied to injectable liver extracts was obtained.

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[10] *Nutrition Reviews*, 1949, **7**, 183.

[11] Betheil, J. J., and Lardy, H. A., *J. Nutrition*, 1949, **37**, 495.

Mucoproteins of Human Plasma. III. Electrophoretic Studies of Mucoproteins from Perchloric Acid Filtrates of Plasma.* (17347)

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It has been shown[1] that the "proteose" of human plasma is a mixture of mucoproteins with isoelectric points which are low in comparison with those of the principal plasma proteins. The present communication deals in more detail with the electrophoretic behav-

* The electrophoresis studies were supported by a grant from the United States Public Health Service and the isolation studies were supported by the American Cancer Society. Contribution No. 224 from the Department of Biochemistry and Nutrition, University of Southern California, School of Medicine.

[1] Winzler, R. J., Devor, A. W., Mehl, J. W., and Smyth, I. M., *J. Clin. Invest.*, 1948, **27**, 609.