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Stimulation of Vit. B_{12} Uptake in Tissue Slices by Intrinsic Factor Concentrate.* (23388)

O. NEAL MILLER AND FRED M. HUNTER

Department of Biochemistry and the Nutrition and Metabolism Research Laboratory, Department of Medicine, Tulane University School of Medicine, New Orleans, La.

For several years we have held the view that intrinsic factor may influence absorption of vit. B_{12} by tissues other than the intestinal mucosa. Currently it is held that intrinsic factor is a mucopolypeptide of about 5,000 molecular weight(1). This size would not preclude the possibility of its being absorbed into the blood stream and hence becoming available for possible action on all tissues. However, no reports have yet appeared that demonstrate the effect of intrinsic factor on peripheral tissues.

Data are presented that demonstrate the stimulatory action of intrinsic factor on uptake of vit. B_{12} by slices of rat liver† or kidney *in vitro*. It appears that these observations may form the basis for an *in vitro* assay of intrinsic factor.

Materials and methods. Wistar strain, weanling rats were fed a diet of the following percentage composition: vitamin-free casein, 22; sucrose, 68.5; salts mixture (U.S.P. No. 2), 4.15; crisco, 4; iodinated casein, 0.04 or 0.05; L-cystine, 0.2; choline, 0.1. Each 100 g of diet contained α-tocopherol, 2.5 mg; menadione, 50 μg; vit. A. 75 I.U.; vit. D, 25 I.U.; riboflavin, 600 μg; thiamin, 400 μg; pyridoxine hydrochloride, 400 μg; calcium pantothenate, 1600 μg; niacin amide, 2000 μg; paraaminobenzoic acid, 10 mg; inositol, 20 mg; biotin, 10 μg; and folic acid 20 μg. In diets *B* and *D* vit. B_{12}, 2 μg/100 g diet was added. Iodinated casein was not added to diets *C* and *D*, (Table II). After 4 weeks on the diets the animals in groups A and C showed depressed growth rates and animals from all groups were ready for experiments. Rats were sacrificed by decapitation and the liver quickly removed and cooled on cracked ice. Slices were prepared 0.5 mm thick, blotted on filter paper, weighed on torsion balance before being placed in 7 ml Warburg flasks, and incubated at 37°C. Each flask contained a total volume of 3 ml of buffer pH 7.4 described by Hastings *et al.*(2). The radioactive vit. B_{12}, intrinsic factor concentrate‡ and other additives were made up in buffer solution. The system was equilibrated with gas (5% CO_2, 95% O_2) and vit. B_{12} tipped-in from the side arm after temperature equilibrium had been reached. After incubation, the slices were removed from the flask,

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† Preliminary report, *Fed. Proc.*, 1957, v16, 393.

‡ Intrinsic factor concentrate was prepared from hog mucosa and was supplied by Abbott Laboratories, North Chicago, Ill. This preparation had a biological oral dose potency of about 5 mg/day.

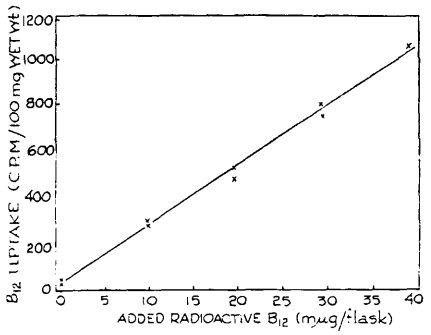

FIG. 1. Uptake of vit. B_{12} by rat liver slices plotted against varying amounts of radioactive vit. B_{12} added to each flask. No intrinsic factor concentrate added.

washed 10 sec. in 5 ml of buffer, and placed in cuvettes for assay in center-well scintillation counter. Cobalt^{60} labeled activity vit. B_{12}§, μc/μg, was diluted to 0.05 μc/ml. Potassium pyruvate was prepared(3) and added to the incubation mixture at a final concentration of 60 mM l. The data are reported as counts/minute (C.P.M.)/100 mg tissue wet weight, corrected for background. Livers were analyzed for vit. B_{12} content using the *L. leishmannii* ATCC 7380 as the test organism. Homogenates were prepared and digested with pancreatin for 24 hours at 37°C. The values are reported in mμg/g wet weight of liver and have been corrected for non-vit. B_{12}-like materials by reporting only the alkali-labile values.

Results. Fig. 1 shows the results of an experiment in which the effect of vit. B_{12} concentration on vit. B_{12} uptake by liver slices is demonstrated. Remarkably good agreement is obtained between duplicate determinations, and uptake is proportional to concentration of vitamin over the range tested during a 4 hour incubation.

The effect of washing tissue slices after incubation, upon uptake of vit. B_{12} was determined. (Fig. 2). Slices of liver were incubated 4 hours, removed from the flask and washed 10, 60 or 180 seconds in buffer. Washing control slices for these intervals reduced uptake of B_{12} only slightly, whereas slices to which 100 μg of intrinsic factor concentrate had been added lost a significant amount during the first 60 seconds. However, a significant stimulation in uptake remained even after the longest wash period. Wash periods of 1 or 3 minutes seemed to reduce the net stimulation due to added intrinsic factor concentrate and for this reason a 10 sec. wash period was selected for routine use. Incubation of slices in medium with radioactive B_{12} for 10 sec. followed by washing for 10 sec. exhibited minimal uptake, 48 $\pm$ 3 C.P.M/100 mg. Furthermore, it was found that incubating slices in medium without radioactive B_{12} for 4 hours prior to tipping in radioactive vitamin, did not increase amount absorbed by the slice in 10 seconds.

The influence of time on uptake of vit. B_{12} is demonstrated in Fig. 3. Uptake by control slices essentially reached maximum after 1 hr. of incubation. However, when 100 μg of intrinsic factor concentrate was added, the uptake was more than twice that of control slices after 4 hours.

Stimulation of vit. B_{12} uptake appears to be a function of the amount of intrinsic factor concentrate added in Fig. 4. As little as 5 μg and as much as 100 μg appear to stimulate

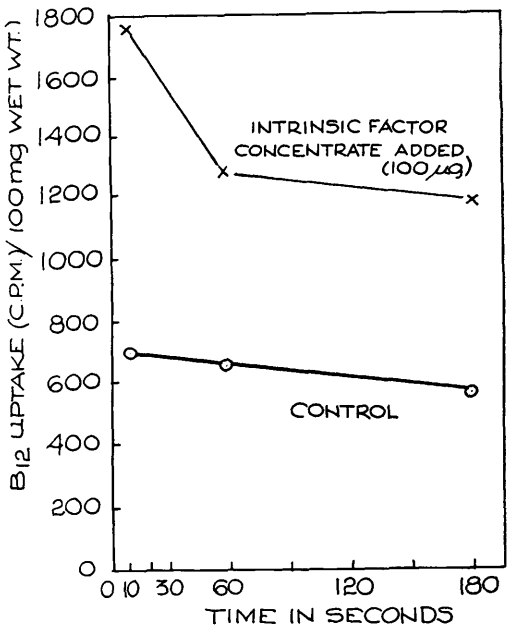

FIG. 2. Uptake of vit. B_{12} by rat liver slices plotted against length of time taken to wash the slice free of adsorbed radioactive vit. B_{12}.

§ Cobalt^{60} labeled vit. B_{12} was furnished by Merck and Co., Rahway, N. J.

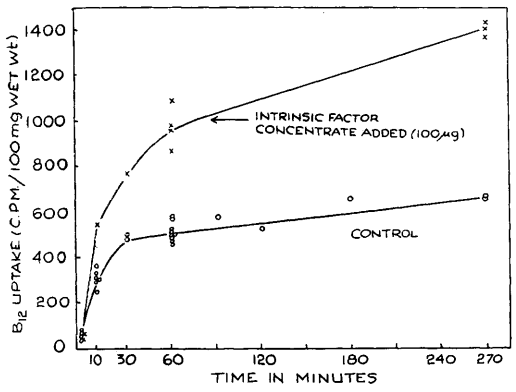

FIG. 3. Uptake of vit. B_{12} by rat liver slices plotted against length of incubation. Stimulatory effect of intrinsic factor concentrate is demonstrated.

the uptake of vit. B_{12}. Increased amounts (500 to 1000 μg) of this preparation caused inhibition in uptake, presumably because the concentrate bound the radioactive vit. B_{12}, thus preventing its uptake by the slice.

To determine whether or not stimulation of vit. B_{12} uptake of liver slices is related to the intrinsic factor content of the concentrate, the experiments summarized in Table I were done. The data are not conclusive but they do suggest that stimulation of B_{12} uptake is a function of intrinsic factor. For example, preheating intrinsic factor concentrate in boiling water for 30 minutes negates the stimulation. Pink-protein,‖ a cobalamin protein complex which has no intrinsic factor activity (4), has no stimulatory effect. Crystalline pepsin and lysozyme had no effect although crude gelatin may have had a slight effect.

TABLE I. Specificity of Intrinsic Factor Concentrate for Stimulating Uptake of Radioactive Vit. B_{12} by Rat Liver Slices.

Additions to flask*	No. of exp.†	C.P.M. per 100 mg	% of control
None	20	870	100
Intrinsic factor concentrate	16	1395	160
Intrinsic factor concentrate, heated	10	1065	113
"Pink protein"	6	960	105
Crystalline pepsin	6	1096	103
Lysozyme	6	1154	108
Crude gelatin	4	1277	119

* Each addition was made 100 μg/flask.
† 4 hr incubations.

‖ "Pink-protein" was supplied by Dr. K. W. Thompson of Organon Inc., Orange, N. J.

For most experiments, slices were prepared from livers of rats reared on a diet devoid of added vit. B_{12} and including 0.04 or 0.05% iodinated casein, diet *A*. However, a study was made to determine whether or not it was necessary to use hyperthyroid-vit. B_{12}-deficient rats. In Table II data are shown which compare liver slices from rats given 4 different diets. Intrinsic factor concentrate will stimulate uptake of vit. B_{12} by liver slices from rats receiving any one of the 4 diets, but uptake in the absence of intrinsic factor concentrate is less in normal animals than hyperthyroid rats.

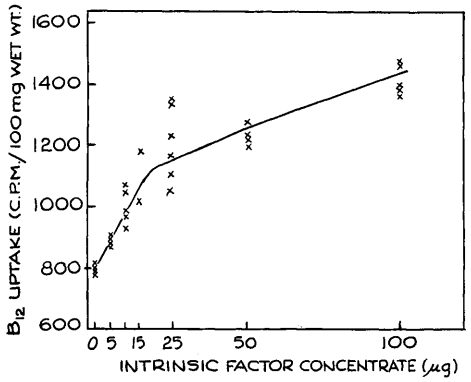

FIG. 4. Uptake of vit. B_{12} by rat liver slices plotted against amount of intrinsic factor concentrate added to each flask.

Uptake of vit. B_{12} by kidney slices is also stimulated by intrinsic factor concentrates. Control slices absorbed 562 $\pm$ 43 C.P.M./100 mg but in the presence of 100 μg intrinsic factor concentrate 789 $\pm$ 86 C.P.M./100 mg was absorbed. The stimulation was statistically significant at the 5% level.

Discussion. Data presented demonstrate that intrinsic factor concentrates will stimulate the uptake of vit. B_{12} by slices of liver and kidney. The data indicate that this stimulation is a function of intrinsic factor in the concentrates used inasmuch as pink-cobalamin-protein, that stimulated the combining of vit. B_{12} on serum proteins(5), had no effect in this system. Furthermore, concentrates that were preheated in boiling water for 30 minutes lost their stimulatory action. Lysozyme, which has been shown previously(6)

TABLE II. Effect of Diet on Vit. B_{12} Uptake by Slices of Rat Liver.

Diet	Iodinated casein	Vit. B_{12}	No. of exp.*	C.P.M./100 mg of liver: Control	C.P.M./100 mg of liver: Intrinsic factor concentrate added, 100 μg
A	+	—	6	957 ± 71†	1397 ± 71
B	+	+	6	953 ± 83	1535 ± 29
C	—	—	10	626 ± 34	1249 ± 93
D	—	+	10	620 ± 21	1337 ± 82

* 4 hr incubations. † Stand. error of mean.

to bind B_{12} and make it unavailable to microorganisms, is without intrinsic factor activity by bioassay and in the system described above. This suggests that the binding of vit. B_{12} *per se* may not be the only mechanism involved whereby intrinsic factor stimulates the uptake of vit. B_{12} by liver slices. In some instances binding of vit. B_{12} may prevent stimulation owing to competition between the material and the slice of tissue.

It was anticipated that vit. B_{12}-deficient rat liver slices would be necessary to show an effect of intrinsic factor on uptake of vit. B_{12}. Actually, stimulation of B_{12} uptake was observed in slices from rats given each of the 4 diets tested; the only difference being in the uptake of vitamin by control slices. Hyperthyroidism seems to cause increased uptake of radio-B_{12} quite unrelated to the presence or absence of vit. B_{12} in the diet, or the level of vit. B_{12} in the liver. Inasmuch as the vit. B_{12} level in the liver was depressed from 87 ± 9 (diet B; B_{12} supplemented) to 30 ± 15 mμg/g in animals given a diet to which no B_{12} was added (diet A), it seems that the effect noted was probably unrelated to the level of vit. B_{12} in the liver.

The technic described is capable of detecting small amounts of intrinsic factor concentrate and further refinements and experience may make it the most sensitive assay for intrinsic factor thus far reported in the literature. The development of a specific and sensitive *in vitro* method for determination of intrinsic factor may aid in its isolation, identification, and also in elucidating its physiological action.

Recently we have demonstrated in our laboratory that homogenates of liver will combine vit. B_{12} and this can be stimulated by addition of intrinsic factor concentrate. When these homogenates are centrifuged at 25,000 x g, stimulation of vit. B_{12}-combining was observed only in the supernatant fraction and none in the particulate fraction which sedimented under these conditions. Full details of these experiments will be published subsequently. These data are interpreted to indicate that intrinsic factor is absorbed into the cells of the liver slice and thus exerts its stimulatory action intracellularly rather than exerting a non-specific membrane adsorption phenomenon.

It has been demonstrated previously that intrinsic factor concentrates and gastric juice will stimulate vit. B_{12}-combining on certain of the plasma proteins(7). For this action of intrinsic factor to be of physiological importance it was postulated(5) that intrinsic factor is absorbed into the blood stream where it acts not only to conserve the dietary vit. B_{12} by combining it with serum proteins but also facilitates the absorption of vit. B_{12} by peripheral tissues in the same manner as it facilitates absorption of vit. B_{12} from the gut. Evidence is presented here in support of this hypothesis.

Summary. It was demonstrated that intrinsic factor concentrate stimulates the uptake of radioactive vit. B_{12} in slices of rat liver and kidney, *in vitro*. Evidence presented indicates the specificity of this stimulatory action. The stimulation is a function of concentration of intrinsic factor concentrate added, and may therefore be the basis of an *in vitro* assay for intrinsic factor. It was not necessary to use vit. B_{12} deficient tissues to show the stimulation effect and it was observed that hyperthyroidism caused increased uptake of vit. B_{12} in the absence of added intrinsic factor concentrate. A brief discussion of the possible function of intrinsic factor was

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Plasma Protein Changes in Parakeets with Pituitary Tumors.* (23389)

HANS G. SCHLUMBERGER† AND ROBERT L. WALL

Departments of Pathology and Medicine, College of Medicine, Ohio State University, Columbus

Among the commonest tumors of the parakeet *Melopsittacus undulatus,* are adenomas and carcinomas of the pituitary(1). Over 160 cases have been examined in this laboratory, and in the past 4 years one of the tumors has been carried through 14 passages by subcutaneous transplantation(2). Both primary tumors and transplants often produce marked obesity and hyperglycemia. Bioassays of the transplanted tumors suggest the presence of somatotropin, but they have been negative for other pituitary hormones(2). The plasma proteins show a striking quantitative change in the electrophoretic pattern (Fig. 1), and a qualitative difference from the normal after ultracentrifugation.

Materials and methods. Tumors to be transplanted were minced in 0.9% NaCl and injected beneath the skin of the breast. Cortisone, total body x-irradiation, or other form of pretreatment of recipient birds was never used. Total plasma protein and blood sugar were determined by a microphotometric application of the Folin-Ciocalteu procedure(3) and by the Folin-Wu technic(4) respectively. Parakeets with primary pituitary tumors were exsanguinated by cardiac puncture. Clotting was prevented with heparin in most instances; in several cases no heparin was used and the electrophoretic pattern of the serum so obtained compared with that of plasma. The method of paper electrophoresis did not demonstrate a significant difference between serum and plasma. No separate peak corresponding to fibrinogen could be detected in the plasma, a finding similar to that observed in mice(5) and fowls(6). Birds with transplanted pituitary tumors were also exsanguinated, particularly if blood sugar determinations and ultracentrifugation were to be carried out on the same sample. When serial protein determinations were made on a single bird, blood was collected in heparinized capillary tubes from a wing vein. Plasma was applied in amounts of 10 cu mm to Whatman 3-mm filter paper that measured 10 x 14 cm, providing space for simultaneous testing of several samples. Using a conventional vertical principle electrophoretic cell designed by one of us (R.W.), Veronal buffer at pH 8.6, u 0.05 with 5% glycerine, a current of 150-170 volts delivering 15-20 milliamperes was applied to the paper for 4 hours. After heat fixation the papers were stained with Bromphenol blue for protein and Sudan black B for lipoprotein. The papers were then cut into strips each bearing the pattern of a single sample, and scanned with a Spinco Analytrol that permitted simultaneous area integration. Pooled human serum served as a control and mobility reference. The protein fractions in parakeet sera were not isolated, therefore their dye-binding capacities could not be de-

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† Present address, Medical Center, University of Arkansas, Little Rock.