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Effect of Highly Purified Vitamin B_{12} Binders from Human Gastric Juice on B_{12} Absorption in Rats. (27928)

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There is considerable evidence to suggest that species specificity exists among the intrinsic factors (IF's) of various animals(1,2, 3). Inasmuch as chemically pure IF has not been available, and contamination of some of the preparations by an inhibitory impurity has been indicated(4,5), species specificity has yet to be established with exclusion of impurities. Such studies would contribute to the understanding of the chemical nature of IF. Although some investigators(3,6,7) failed to demonstrate IF activity in human gastric juice when applied on rat intestine, the possibility still remains that purified human IF might be active in the rat, as suggested by Taylor *et al*(8).

Gräsbeck and his coworkers have recently been successful in purification of IF from human gastric juice to the extent that doses of the order of 50 μg of their preparations are active in pernicious anemia patients(9, 10). They further demonstrated the presence of at least 2 B_{12} binding components in gastric juice, one migrating slowly, the other rapidly in immunoelectrophoresis(11). The slow binder possesses IF activity(10) whereas some preparations of the rapid binder have no IF activity. The purpose of the present investigation was to test in rats these purified fractions of human gastric juice which do not contain impurity to any physiologically significant extent.

Materials and methods. Young adult rats of the Wistar strain were used. Cobalt-60* labeled cyanocobalamin (Co^{60}-B_{12}) with the original specific activity of 1 μc/μg was employed either diluted isotopically 5-fold for fractionation of IF from human gastric juice, or undiluted for absorption in the rat loop.

Purification of human IF. Purification was carried out by a series of column chromatographic procedures on diethylaminoethyl (DEAE) and carboxymethyl cellulose and dextrans (Sephadex®)(9,10). The purified binders were homogeneous in immunoelectrophoresis in which rabbit antigastric juice antiserum was allowed to diffuse against the purified fraction that had been subjected to agar gel electrophoresis. They are named S and R (from "slow" and "rapid" electrophoretic mobility). Both are in the form of their B_{12} complexes, being saturated but not oversaturated with Co^{60}-B_{12}. In the present preparations, the ratio of protein to B_{12} was about 64 μg/μg B_{12} in the former and 210-240 μg/μg B_{12} in the latter. They were prepared and stored frozen in 5 mM phosphate buffer of pH 5.5, diluted with a physiological saline and used in the dose of 30 mμg with respect to the bound Co^{60}-B_{12}, because of its low specific activity. This was the smallest dose possible.

Hog and rat IF. Two hog stomach preparations of WES #727 and #942† which are active at a dose of a few mg in pernicious anemia patients were used. Rat IF concentrate was prepared from rat stomach mucosa

* Kindly supplied by Merck Co., Rahway, N. J.

† Generously furnished by Dr. L. Ellenbogen, Lederle Laboratories, Pearl River, N. Y.

in a form of saline extract(7), and a purified fraction was obtained with a DEAE cellulose column(12). The latter was used only for intravenous injection. Because the human preparations were in the form of B_{12}-complexes, B_{12} binding capacities of the animal preparations were determined first by exhaustive dialysis(13), then Co^{60}-B_{12} was mixed with the amounts of IF just sufficient to render the former all bound.

Loop assay of IF activity. The loop was prepared *in situ* in the rat out of the small intestine distal to the duodenojejunal junction, according to the technic already described(7). Such a loop has no access to endogenous IF and is suited for assay of IF. IF activity was taken as the capacity of the test material to enhance absorption of physiological doses of Co^{60}-B_{12} above that obtained without an additive. Absorption, or transport of B_{12} past the intestinal serosa, was measured from the decrease of radioactivity in the loop 24 hours after administration of the dose containing Co^{60}-B_{12}.

Hepatic uptake following intravenous injection. The IF preparations containing Co^{60}-B_{12} were injected into the tail vein, the rats were killed 5 minutes later, and uptake of radioactivity by the liver and kidneys was determined by the procedure already reported(13).

Radiometric measurement. The loop, liver, kidneys, and entire carcass were dissolved with the aid of KOH and heating, made up to 30 ml volumes, or 100 ml in the case of the carcass, and subjected to gamma-scintillation counting. Washing of the loop was concentrated to the same volume and processed similarly. Under the conditions used, Co^{60}-B_{12} combined with human binders gave a net count of 280 cpm/30 mμg of B_{12} above the background of 50 cpm. The isotopically undiluted Co^{60}-B_{12} yielded about 5-fold counts.

Results. Absorption from loop. Loops were prepared and divided into 5 groups. S and R were administered in a dose of 30 mμg with respect to bound Co^{60}-B_{12}. The same Co^{60}-B_{12} employed for their preparation was used also in the dose of 30 mμg in the control as well as in the groups for rat and hog IF. The 24-hour absorption (Table I) showed that while Co^{60}-B_{12} alone gave absorption of about 15% and rat IF about 40%, absorption afforded by R and hog IF was lower than the control of Co^{60}-B_{12} alone. S yielded an absorption which was significantly higher than the control and close to that obtained with rat IF. The absorption measurement was double-checked with determination of radioactivity in the whole carcass without the loop.

TABLE I. Absorption of Co^{60}-B_{12} Bound to Human Binders and Animal IF's from the Rat Intestinal Loop.

Materials	No. of loops	Radioactivity in loop after 24 hr (% of dose)	Absorption (% of dose)
None (control)	4	85.9 ± 2.0*	14.1
S	4	69.4 ± .6	30.6
R	3	95.1 ± .5	4.9
Rat IF	4	60.3 ± 3.1	39.7
Hog IF (WES #942)†	3	90.2 ± .7	9.8

Each dose contained 30 mμg Co^{60}-B_{12}.
* Mean ± standard error.
† .1 mg.

In view of the finding that R-bound Co^{60}-B_{12} was absorbed to a lesser extent than free Co^{60}-B_{12} in the control, an attempt was made to see whether this preparation had an inhibitory effect on absorption of B_{12} that was not bound to this fraction. To this end, 10 mμg of Co^{60}-B_{12} (isotopically undiluted) was applied to the loop with or without an optimal amount of rat IF (capable of binding 15 mμg B_{12}), immediately followed by administration of R containing 30 mμg Co^{60}-B_{12}. These groups were compared with those receiving 10 mμg Co^{60}-B_{12} alone, R alone and 10 mμg Co^{60}-B_{12} plus rat IF (15 mμg binding). If R exerted inhibition, the measured absorption in counts should differ from that calculated from the individual components given separately. The results indicated otherwise and R did not reduce absorption of Co^{60}-B_{12} whether or not homologous IF was administered.

Hepatic uptake. Human fractions containing 30 mμg Co^{60}-B_{12}, and hog IF (0.1 mg of WES #727) and purified rat IF mixed with the same amount of Co^{60}-B_{12} were injected into the tail veins of rats. Hepatic and renal

TABLE II. Uptake by Liver and Kidneys Following Intravenous Injection of Human Binders and IF Preparations Combined with Co^{60}-B_{12}.

Materials	No. of rats	Uptake (% of dose) Liver	Kidneys
30 mμg Co^{60}-B_{12}	3	9.8	11.4
Rat IF*	3	50.2	4.6
S	3	33.9	11.3
R	2	36.3	9.9
Hog IF (WES #727)	3	89.2	1.2

Each preparation contained 30 mμg Co^{60}-B_{12}.
* Purified fraction.

uptakes of radioactivity measured 5 minutes after injection (Table II) showed that Co^{60}-B_{12} bound to hog IF was almost completely taken up by the liver, and to rat IF less completely. Hepatic uptakes of S and R were smaller than those with hog and rat IF, yet larger than the control which received 30 mμg Co^{60}-B_{12} alone.

Discussion. The human IF fraction which corresponds to the "slow" binding component(11) exhibited an IF activity while the other fraction ("rapid" binder) had no activity when tested with the rat intestinal loop. The latter fraction (R), however, exerted no inhibitory effect upon absorption of B_{12} that was not combined with it. It appears that the B_{12} molecules bound to R were not readily liberated in the intestine and the R-B_{12} complex lacked IF activity in the rat. This fraction was similar to purified hog IF in its behavior in the rat intestine. The reported failures(3,6,7) to demonstrate IF action with human gastric juice in the rat were probably due to R that was not saturated with B_{12}, and possibly due to other non-IF contaminants. Even if R was saturated with a test dose of B_{12}, the portion of the dose shared by the slow binder would be too small to distinguish itself in absorption. By analogy, the previously speculated species specificity might be related to impurities which predominate IF action under certain conditions. A reasonable assumption may be that hog IF, upon further removal of impurity, might also prove active in rats. Whether binding impurities (R in human gastric juice) are degradation products of IF (S) is not clear, but remains as a possibility (14).

The difference in hepatic uptake among human binders and hog and rat IF following intravenous administration was probably due to differences in size of the B_{12} binding molecules and amount of macromolecular substances in the preparations. This phenomenon of hepatic uptake is not directly related to IF activity of the substance in the intestine and reflects no species specificity(13, 15).

Summary. Two highly purified vit. B_{12}-binder fractions prepared as their Co^{60}-B_{12} complexes from human gastric juice were tested in rats for intrinsic factor activity and other biological properties. The fraction corresponding to the electrophoretically rapid binder showed no IF or inhibitory effect, while the other fraction which has a higher B_{12} binding capacity exhibited IF activity upon Co^{60}-B_{12} which was bound to it. The previous failures to demonstrate IF activity with human gastric juice in the rat may be attributed to the former which behaves as a B_{12} binding impurity.

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