Intrinsic Factor-Mediated Radio-B12 Uptake in Sequential Incubation Studies Using Everted Sacs of Guinea Pig Small Intestine: Evidence that IF is not Absorbed into the Intestinal Cell* (33390)

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Insufficient data are presently available concerning the mechanism whereby intrinsic factor (IF) mediates the absorption of vitamin B12 to allow firm conclusions as to whether or not intrinsic factor remains on the mucosal cell surface, enters the cell along with B12, or passes through the cell into the portal circulation (1–3).

The present studies were designed to determine whether IF bound to everted sacs of guinea pig small intestine as the IF-B12 complex would release its B12 to the mucosa, remain available to bind in specific fashion (not exchange) a second isotope of B12, and also remain available to interact with IF antibody.

Materials and Method. The B12-^{60}Co (sp act. 1.4 μCi/μg) and B12-^{57}Co (sp act. 3.66 μCi/μg) were generously supplied by Dr. Elmer Alpert of Merck Sharp and Dohme.[3] The IF used in all the experiments was National Formulary Hog Intrinsic Factor, lot no. 6043. IF-antibody sera obtained from 2 patients with P.A. were employed in these studies; one serum contained high titer "blocking" antibody activity without detectable "precipitating" antibody activity, the other contained high titer "precipitating" (binding) antibody activity and moderate "blocking" activity (4). In order to detect significant IF-blocking antibody activity (5) of the P.A. serum with the purified hog IF used in these studies, it was necessary to employ concentrations of IF between 50 and 100 μg/0.1 ml of serum. Vitamin B12 concentrations were determined by microbiologic assay with the *Euglena gracilis* (6); the total B12-binding capacity of the IF preparation was determined by equilibrium dialysis (7) and the specific B12 binding due to IF was measured by charcoal "dialysis" in the presence of anti-IF-blocking antibody (5). The amount of B12-^{60}Co and B12-^{57}Co in the intestinal sacs were determined *after the final incubation step* by a Nuclear Chicago twin scaler gamma spectrometer (model 4222). When B12-isotope content (B12-^{60}Co) was determined in the incubating and rinsing solutions, the volume of the latter was reduced fiftyfold; this was accomplished by first binding all the B12-isotope in the rinsing solution(s) with IF and then reducing the volume by pressure dialysis in Visking tubing. In all experiments adult guinea pigs weighing between 450 and 500 g were employed.

Experimental procedure. Each guinea pig was killed by a sharp sudden blow on the head and the entire small intestine was removed immediately, flushed with and immersed in cold saline. Eight 10-cm everted sacs, 4 proximal and 4 distal, were prepared from each animal using the technique of Strauss and Wilson (8). Each sac was placed in a 50-ml Erlenmeyer flask containing 5 ml of Krebs–Henseleit solution with 200 mg/100 ml of glucose (and 1.3 mmole of calcium at pH 7.4); unless otherwise stated, one, two, or three successive incubation periods, each of 0.5 hr duration were performed at 37° in a Dubnoff metabolic shaker. In all experiments (Table I) the first period of incubation (in-

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TABLE I.

Expt. no.	Inc. no.	Type expt.[a]	B12 (ng/sac)	Sera: Normal (0.1 ml)	Sera: IF-Ab: Blocking (0.1 ml)	Sera: IF-Ab: Precipitating (0.1 ml)	EDTA (0.2 m*M*, 0.2 ml)	B12 uptake (pg/sac): Proximal	B12 uptake (pg/sac): Distal
1	I	B12-^{60}Co-IF	4.6	—	—	—		B12-^{60}Co 115	230
	II	B12-^{57}Co	4.6	—	—	—	—	B12-^{57}Co 100	225
2	I	B12-^{60}Co-IF	4.6	—	—	—	—	B12-^{60}Co 85	220
	II	B12-^{57}-Co (0°)	4.6	—	—	—	—	B12-^{57}Co 85	80
3	I	B12-^{60}Co-IF	4.6	—	—	—	—	B12-^{60}Co 100	225
	II	B12-^{57}Co	4.6	+	—	—	—	B12-^{57}Co 90	220
4	I	B12-^{60}Co-IF	4.6	—	—	—	—	B12-^{60}Co 105	225
	II	B12-^{57}Co	4.6	—	+	—	—	B12-^{57}Co 100	20
5	I	B12-^{60}Co-IF	4.6	—	—	+	—	B12-^{60}Co 100	26
	II	B12-^{57}Co	4.6	—	—	—	—	B12-^{57}Co 95	20
6	I	B12-^{60}Co-IF	4.6	—	—	—	+	B12-^{60}Co 120	80
	II	B12-^{57}Co	4.6	—	—	—	—	B12-^{57}Co 100	30
7	I	B12-^{60}Co-IF	4.6	—	—	—	—	B12-^{60}Co 105	220
	II	B12-^{57}Co	4.6	—	—	—	+	B12-^{57}Co 140	85
8	I	B12-^{60}Co-IF	4.6	—	—	—	—	B12-^{60}Co 105	230
	II	B12-^{57}Co	4.6	—	—	—	+	B12-^{57}Co 140	70
	III	New sacs	—	—	—	—	—	70	185
9	I	B12-^{60}Co-IF	4.6	—	—	—	—	B12-^{60}Co 100	220
	II	B12-^{57}Co	4.6	—	—	—	+	B12-^{57}Co 145	85
	III	New sacs	—	—	—	+	—	70	40

[a] Unless otherwise indicated the experiments were performed at 37°.

cubation I) contained 4.6 ng of B12-^{60}Co which was completely bound by IF. One hundred percent binding was accomplished by quantitating through equilibrium dialysis, the precise amount of IF necessary to bind the 4.6 ng of B12-^{60}Co. Tests of the final preparation for the presence of both free B12-^{60}Co and IF prior to the experiments detected no excess of either. After the first incubation period (incubation I) the sacs were removed from the flasks, each was thoroughly rinsed in 200 ml of cold saline and placed in new incubation flasks containing 5 ml of incubating solution plus 4.6 ng of vitamin B12 labeled with a different isotope, B12-^{57}Co. The second incubation was then carried out (incubation II). During the second incubation periods various manipulations were performed (Table I). The sacs were then removed from the flasks, each was rinsed in 200 ml of cold saline, and the ends were cut off and discarded. Each sac was blotted dry on absorbent paper and placed directly in a counting tube for differential determination for the two B12-isotope activities. The results reported for each experiment are derived from the mean cpm for the 4 proximal and the 4 distal sacs from 4 guinea pigs (SD = ± 9.8% and ± 8.2% of the mean for proximal and distal sacs, respectively). An occasional group of guinea pigs showed somewhat greater than "normal" radio-B12 uptake in first, second, or both incubation periods and for uniformity these values are not included in the results reported here.

Results and interpretation. Three main studies were carried out to evaluate possible "nonspecific" changes in the procedure that

might influence or confuse the results obtained concerning IF-mediated B12 uptake. First, to determine the amount of vitamin B12 taken up by processes not mediated by added IF, studies were made with only B12-^{60}Co, 4.6 ng/sac, for incubation I and only B12-^{57}Co, 4.6 ng/sac, for incubation II (not illustrated). Proximal sacs took up and retained 35 pg of B12-^{60}Co and 30 pg of B12-^{57}Co; distal sacs, 55 pg of B12-^{60}Co and 50 pg of B12-^{57}Co, respectively. All susequent experiments are corrected for this B12 uptake (not mediated by added IF) by subtracting the appropriate values from the total B12-^{60}Co and B12-^{57}Co activities. No microbiologically detectable amounts of vitamin B12 (endogenous) were found in incubation media exposed to either proximal or distal sacs for a 1-hour period without added radio-B12.

Second, to determine the amount of IF binding to sacs when the IF was not complexed with radio-B12, proximal and distal sacs were incubated with IF alone for 0.5 hr at 37° and, after rinsing, subsequently incubated with 4.6 ng of B12-^{57}Co and 0.5 hr at 37° (not tabulated). The B12-^{57}Co uptake values were 45 pg/proximal and 65 pg/distal sac. These results indicate the IF does not firmly attach itself to intestinal sacs in the absence of B12 and are similar to the findings of Strauss and Wilson (9).

A third type of control experiment was performed to determine the extent of "non-specific" molecular exchange between the B12-isotopes in the two incubation sequences. Two separate sets of proximal and distal loops were incubated in flasks containing 4.6 ng of B12-^{60}Co-IF for 0.5 hr (incubation I) followed by rinsing of the sacs (rinse 1) and subsequent incubation of the two sets of proximal and distal sacs in: (a) flasks containing the buffer and glucose *with* 4.6 ng of B12-^{57}Co and (b) flasks containing the buffer and glucose *without* B12-^{57}Co. Subsequently all sacs were thoroughly rinsed with saline (rinse 2) and the amount of B12-^{60}Co was determined in the pooled *second* incubating solutions and second (rinse 2) saline solutions. The amounts of B12-^{60}Co detected under conditions (a) above were 55 pg for proximal sacs and 85 pg for distal sacs and the amounts of B12-^{60}Co detected under conditions (b) were 65 pg for proximal and 90 pg for distal sacs.

These comparative values for B12-^{60}Co excluded the possibility that the B12-^{57}Co uptake values in distal sacs after incubation II (Table I, Expt. 1) could be accounted for by "isotope exchange."

Sequential incubations of 4.6 ng of B12-^{60}Co-IF for period I followed by 4.6 ng of B12-^{57}Co in period II (Table I, Expt. 1) produced a B12-^{60}Co uptake of 115 pg/proximal and 230 pg/distal sac and a B12-^{57}Co uptake of 100 pg/proximal and 225 pg/distal sac, respectively. When this experiment was repeated with the second incubation period (incubation II) performed at 0° (Table I, Expt. 2) the B12-^{57}Co uptake in the distal sacs was only 80 pg/sac; no significant change in uptake by proximal sac was observed. Addition of 0.1 ml of normal serum (B12 level, 200 pg/ml) to incubation II (Table I, Expt. 3) did not significantly alter either proximal or distal B12-^{57}Co uptake (compared to Expt. 1). However, addition of 0.1 ml of P.A. serum containing potent IF-"blocking" antibody (B12 level 195 pg/ml) to incubation II (Table I, Expt. 4) produced a significant (205 pg) reduction in distal sac uptake (compared to Expt. 1); proximal B12-^{57}Co uptake was not affected by "blocking" antibody in incubation II and B12-^{60}Co uptake in proximal and distal sacs in incubation I was not affected by "blocking" antibody in incubation II.

These experiments (Table I, Expts. 1–4) suggested that the uptake of the second isotope (B12-^{57}Co) in the second incubation sequence was dependent on: (i) the native presence of an intact receptor for the IF–B12 complex in distal but not proximal intestinal sac, (ii) a temperature of 37° for metabolic activity, and (iii) steric availability of an attachment site for B12 on the IF molecule since IF-"blocking" antibody nearly abolished distal sac uptake of B12-^{57}Co in incubation II.

Experiment 5 (Table I) demonstrates the effect of addition of 0.1 ml of P.A. serum containing high titer IF-"precipitating" anti-

body (B12 level 185 pg/ml) to incubation I. Distal sac B12-^{60}Co uptake was decreased from 230 pg/sac (Expt. 1) to 26 pg/sac with no appreciable change in proximal sac uptake; distal sac B12-^{57}Co uptake (incubation II) was decreased to 20 pg/sac with no change in proximal sac uptake.

These results taken in comparison to results of Expt. 1 indicate that the distal sac (B12-^{57}Co) uptake of the second B12-isotope in incubation II was dependent upon the prior attachment of the IF–B12 (B12-^{60}Co) complex in incubation I, and the subsequent availability of its original B12 binding site in incubation II.

Experiments 6 through 9 (Table I) depict the effects of the addition of NaEDTA to period I or II of the incubation sequence. Addition of 0.2 m*M* NaEDTA to incubation I (Expt. 6) resulted in a decrease of distal sac B12-^{60}Co uptake from 230 pg/sac to 30 pg/sac and a decrease in B12-^{57}Co (incubation II) uptake in distal sacs of from 225 pg/sac to 30 pg/sac (compared to Expt. 1). Proximal sac uptakes of B12-^{60}Co and B12-^{57}Co were not significantly affected. Addition of the EDTA to the second incubation period (incubation II) (Expt. 7) was associated with a decrease in distal sac B12-^{57}Co uptake of from 225 pg/sac to 85 pg/sac and inexplicably resulted in a reproducible slight increase in proximal sac uptake. The addition of EDTA to incubation II did not demonstrably affect B12-^{60}Co (incubation I) uptake in proximal or distal sacs. Experiments 8 and 9 (Table I) utilized the same experimental procedure as in Expt. 7 with one additional step; this consisted of incubating a series of new (fresh) proximal and distal sacs in the incubating fluids of the second incubation sequence (incubation II) after the original sacs had been removed. Experiment 8 (Table I) depicts the results of this additional sequence; the B12-^{57}Co uptake in the new sacs was 70 pg/proximal and 185 pg/distal sac. In Expt. 9, 0.1 ml of P.A. serum containing potent IF-"precipitating" antibody was added to the media in period III along with the new sacs; this resulted in a reduction of the B12-^{57}Co uptake in the fresh distal sacs from 185 pg/sac (Expt. 8) to 40 pg/sac; proximal sac uptake was not affected by "precipitating" antibody in the third incubation sequence (compare with Expt. 8).

The results of the last four (Expts. 6–9) experiments suggest that: (i) IF in incubation II must be bound to distal sac mucosa since its dissociation (? release) by EDTA resulted in a decreased uptake of the second B12 isotope (B12-^{57}Co) by distal sacs. (ii) following dissociation (? release) of IF by EDTA in the second incubation period, the IF retains biologic activity for this system since it mediates the uptake of the second isotope by the new distal sacs in the third incubation period. (iii) the uptake of the second isotope (B12-^{57}Co) in the new sacs (third incubation sequence, Expt. 8) was IF-dependent since it was markedly inhibited in the presence of IF-"precipitating" antibody (Expt. 9), and (iv) since the uptake of the first B12 isotope (B12-^{60}Co) that occurred in the first incubation sequence (incubation I) was not affected by EDTA in the second incubation sequence (incubation II), this isotope of B12 must have been completely transferred to the distal mucosal cells before incubation II leaving IF available for interaction with EDTA or the second B12 isotope (B12-^{57}Co) as depicted in Expts. 7 and 8.

Discussion. Conditions requisite for IF-mediated transfer of radio-B12 in everted sacs have been previously defined (8–12). Cooper (12) demonstrated that radio-B12 uptake in everted sacs follows saturation kinetics and that the uptake appeared to involve 2 steps (12, 13), the first calcium dependent and non-energy requiring and the second, noncalcium dependent but requiring metabolic activity. Donaldson and co-workers (14) using intact brush borders from hamster small intestine have demonstrated that homologous IF by itself is capable of attachment to the brush border and is then able to bind radio-B12 to the brush border. In our experiments with everted sacs of guinea pig intestine and hog IF, as well as those of others (9), preincubation of the sacs with IF alone failed to facilitate radio-B12 uptake in a subsequent incubation. Rothenberg (using guinea pig ileum and human IF) has recent-

ly identified and partially characterized a macromolecular factor in dialyzed extracts of ileum which is capable of binding in specific fashion IF and radio-B12-IF (15). He has suggested that this factor may be the postulated intestinal receptor for intrinsic factor (10).

Our studies are interpreted to indicate that IF–B12 complex is attached to a specific receptor site in the distal small intestine since B12 uptake of distal but not proximal sacs can be blocked by EDTA and by IF-"precipitating" antibody. After the IF–B12 complex (saturated with B12) attaches to the specific receptor sites, additional molecules of B12 (second isotope) can be bound by the IF molecule. The binding of the second B12-isotope can be prevented by the so-called "blocking" antibody of IF and yet is dependent upon metabolic activity (uptake decreased at 0°) of the distal sac. These observations constitute evidence that the binding of additional molecules of B12 (second isotope) is IF-dependent. EDTA when present in the second incubation period, produced a decreased uptake of the second B12-isotope. That this was due to dissociation from receptor sites of IF molecules that retained biologic activity for this system was documented by the IF-dependent attachment of the second B12 isotope to a *new* distal intestinal sac. Once again, the attachment of the second isotope was dependent upon specific receptor sites in the new distal sacs and reattachment could be prevented by the serum containing high titer IF-"precipitating" antibody (and moderate titer "blocking" antibody). It should be noted here that when, under conditions of Expts. 8 and 9, the IF was dissociated from the first sac (distal) and became available not only for (i) binding the second isotope, (ii) attachment to the new distal sac, and (iii) interacting with precipitating antibody, the amount of B12 (first isotope) present in the first sac was unchanged. This would indicate that the IF molecule had promoted the transfer of the B12 originally bound to it to the mucosal cell (distal).

The simplest mechanism of action for IF activity based upon the above findings is as follows: IF, saturated with radio-B12, effects a specific attachment to the distal small intestine. Dependent upon cellular metabolic activity, the IF–B12 complex is somehow altered so that additional B12 can be bound by a specifically IF-dependent uptake process (decreased by EDTA, and IF-"blocking" antibody). Expts. 7, 8, and 9 demonstrated that after EDTA brings about partial release of intrinsic factor from the distal sac, B12 is left behind. Secondly, Expts. 8 and 9 demonstrated that intrinsic factor retains biologic activity for this system and specificity of activity. In the light of these two observations, it is most likely that alteration of the IF–B12 complex is a result of transfer of B12 (first isotope) to the mucosal cell. A B12-binding site then becomes available for the attachment of the second B12 isotope. Although present evidence makes it unlikely that a valence change of IF for B12 is brought about by binding of the complex to the intestinal receptor, amplification of the recent studies of Highley and Ellenbogen (16) with respect to *in vivo* behavior of IF (monomer) and IF–B12 complex (dimer) might more precisely determine this point. However the alteration occurs, it is clear that within the time duration of these experimental conditions, IF remains available to bind additional B12 and to interact with the IF-"blocking" antibody. Because of the known specifics of B12 binding and absorption and because of the large size of the IF-"blocking" antibody [in this instance, known to be a 7 S gamma globulin (4)], the most plausible conclusion is that the IF molecule remains on the surface of the intestinal cell and is not absorbed into the cell while mediating B12 uptake. Moreover, steric considerations would then make it unlikely that pinocytosis as previously suggested plays a role in B12 absorption.

Cooper (17) recently reported studies using extracts of human distal ileum during B12-^{57}Co absorption and concluded that the B12-^{57}Co had the *in vivo* and *in vitro* characteristics of IF-bound vitamin B12; however, the *in vitro* immunologic behavior of this B12-^{57}Co differed from IF-bound vitamin B12 by virtue of its failure to coprecipitate

with antibody ("precipitating") to IF. These studies did not resolve the precise location (outside or inside the cell) of the B12-^{57}Co in the ileal extracts.

Summary. Sequential incubation studies using everted sacs of guinea pig small intestine and vitamin B12 labeled with two different isotopes of cobalt, ^{60}Co and ^{57}Co, showed that IF-saturated with B12, could effect specific attachment of the complex to distal sacs. In a subsequent incubation IF could: (i) mediate in specific fashion (not exchange) the uptake of additional B12, (ii) interact with IF-"blocking" antibody, and (iii) be partially dissociated by EDTA from the sac (in a form biologically active for this system and retaining specificity of activity) leaving its B12 attached to the mucosa. These results were interpreted to indicate that, in this experimental system, IF is not absorbed into the intestinal cell during the B12 uptake that is mediated by intrinsic factor.

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The Metabolism of Acetate-1-^{14}C in the Day-Old Chicken (33391)

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It has been repeatedly demonstrated that a large variety of animals develop ketosis when their diet contains a high proportion of lipid relative to carbohydrate (1). However, the chick embryo (2) as well as growing chicks (3, 4) can utilize diets essentially devoid of carbohydrates without developing ketosis. Since the enzymes necessary for acetoacetate biosynthesis are demonstrable in chick liver (5) and ketosis can be produced if fatty acids furnish all nonprotein calories (4), the lack of ketosis in the chicken can not be attributed to an inability to synthesize ketone bodies.

Ketosis could be avoided, however, if the chick could perform a net synthesis of carbohydrate from acetate via some pathway which is not physiologically important in other animals (6) but may become important in the developing chick where lipid is the only available energy source. Three possible pathways which could account for a net synthesis of carbohydrate from acetate are: (a) initial

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