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Research Article

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Kinetics of the Attachment of Intrinsic Factor-Bound Cobamides to Ileal Receptors

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ABSTRACT To determine whether the molecular configuration of vitamin B_{12} influences the attachment of intrinsic factor-vitamin B_{12} complex to ileal microvillous membrane receptor sites, we have examined the kinetics of uptake of intrinsic factor-bound cyanocobalamin by brush borders and microvillous membranes isolated from guinea pig ileum, and have compared this uptake with that of intrinsic factor alone and with that of intrinsic factor complexed with various analogs of cyanocobalamin.

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We then investigated the attachment of intrinsic factor-bound cyanocobalamin to ileal receptor. Attachment to microvillous membranes showed saturation kinetics with a dissociation constant of 0.25 nM. Attachment was rapid and was 70% complete within 5 min; the second-order rate constant for attachment was 1.3×10^6 M^{-1} s^{-1}. The half-time for dissociation of intrinsic factor-bound cyanocobalamin from the ileal receptor was approximately 35 min. Free intrinsic factor inhibited the attachment of intrinsic factor-bound cyanocobalamin, but the rate of attachment of free intrinsic factor was slower than that of intrinsic factor bound to cyanocobalamin. When intrinsic factor was complexed with various analogs of cyanocobalamin, the affinities of these complexes for ileal microvillous membranes were similar to that of intrinsic factor-bound cyanocobalamin. These findings suggest that the molecular configuration of vitamin B_{12} is not a major determinant in the interaction between intrinsic factor-bound vitamin B_{12} and its ileal receptor site.

INTRODUCTION

Intrinsic factor (IF)[1] binds vitamin B_{12} in a macromolecular complex (IFB_{12}) and promotes uptake of the vitamin by small bowel mucosa (1). We previously showed that IFB_{12} attaches to specific receptor sites on microvillous membranes isolated from the distal half, but not the proximal half, of hamster small intestine

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[1] *Abbreviations used in this paper:* (Ade)CN-Cba, α-(adenyl) cobamide cyanide; AdoCbl, adenosylcobalamin (coenzyme B_{12}); CN-Cbl, cyanocobalamin; CN-Cbl(OH)°, α-(5,6-dimethylbenzimidazole)-cobamic acid a,b,c,d,g-pentaamide cyanide; (CN,OH)Cbi, cyanoaquocobinamide; IF, intrinsic factor; KRB, Krebs-Ringer bicarbonate; MeCbl, methyl cobalamin; OH-Cbl, hydroxocobalamin.

(2). Although hamster IF promotes vitamin B_{12} uptake by brush borders and microvillous membranes prepared from hamster ileum, human IF is ineffective (2). On the other hand, human IF enhances intestinal uptake of the vitamin in the guinea pig (3, 4). To study human IF, we prepared microvillous membranes from guinea pig ileum, and demonstrate here that these membranes contain receptor sites for vitamin B_{12} bound to human IF.

Previous studies indicate that attachment of IF to its receptor site occurs rapidly, is not impaired by metabolic inhibitors, and requires calcium ions and a pH higher than 6.0 (5, 6). Nevertheless, quantitative data concerning the kinetics of binding of IF to its receptor are lacking. Furthermore, there is little information on the relationship between the binding of B_{12} by IF and the interaction of IF with receptor. In particular, controversy exists as to whether IF, in the absence of vitamin B_{12}, competes with IFB_{12} for ileal surface receptors (7, 8). In the present investigation, we have systematically examined the kinetics of IFB_{12} uptake by guinea pig brush borders and have compared attachment of IFB_{12} with that of IF alone and with that of IF complexed with various analogs of vitamin B_{12}.

METHODS

Intrinsic factor IF. Gastric juice was obtained from patients with peptic disease who required Histalog-stimulated tests of gastric secretion (Eli Lilly & Co., Indianapolis, Ind.). Pepsin was inactivated as described previously (9). A single large pool of gastric juice from several patients was divided into small portions and stored at −20°C. The total CN-Cbl (cobalamin) binding capacity of this pool was 81.5 ng/ml, as determined by exhaustive dialysis (10). Serum containing blocking antibody (11) to IF abolished 97% of this total binding capacity. The remaining 3% of binding was shown by chromatography on DEAE cellulose to be due to "R binder" (12).

Cobamides. ^{60}Co-labeled CN-Cbl (2.8 mCi/mg) was purchased from E. R. Squibb & Sons, New York. Unlabeled CN-Cbl and adenosylcobalamin (AdoCbl) were purchased from Sigma Chemical Co., Inc., St. Louis, Mo. Methylcobalamin (MeCbl) was synthesized by the alkylation of cob(I)alamin with methyl iodide, as described by Johnson, Mervyn, Shaw, and Smith (13). Hydroxocobalamin (OH-Cbl) was prepared by aerobic photolysis of an aqueous solution of methylcobalamin (14). The solution of aquocobalamin so prepared was taken to dryness on a rotary evaporator to remove volatile products derived from the methyl group.

α-(Adenyl)cobamide cyanide [(Ade)CN-Cba; "pseudo B_{12}"] was obtained from the microorganism that synthesizes ethanolamine ammonia-lyase, a B_{12}-dependent enzyme (15). When isolated from the microorganism, this enzyme carries a substantial quantity of bound Ado:(Ade)AdoCba. The cobamide was released from the enzyme by treatment with acid-ammonium sulfate (15) and was photolyzed to aquo-α-(adenyl)cobamide. Liquified phenol was used to extract the cobamide from the aqueous acid-ammonium sulfate phase. The phenol layer was washed, and the cobamide was extracted back into the water by the technique of Barker (16). To convert the aquo-α-(adenyl)cobamide into α-(adenyl)cobamide cyanide, a small crystal of NaCN was added to the solution, after which the solution was acidified with 0.1 N HCl. The product was extracted into the phenol, the phenolic solution washed twice with water to remove excess NaCN, and the cobamide was extracted back from phenol into water as described above.

α-(5,6-dimethylbenzimidazole)-cobamic acid a,b,c,d,g,-pentaamide cyanide [cyanocobalamin minus the e-amide group; CN-Cbl(OH)^e] was prepared by the method of Bernhauer, Wagner, Beisbarth, Rietz, and Vogelmann (17). Cyanocobalamin (3 mg) was dissolved in 5 ml of 0.02 N HCl. This solution was permitted to stand at room temperature for 4 days, after which the products were extracted into phenol and then back-extracted into water, as described by Barker (16). The B_{12} derivatives were then applied to Whatman 3MM paper and subjected to descending chromatography for 24 h. Chromatograms were developed with water-saturated *sec*-butanol: glacial acetic acid (99:1), containing 20 mg KCN/100 ml. The component with the highest mobility ($R_{CN\text{-}Cbl} = 1.7$), identified as the desired compound from the data of Bernhauer, et al. (17), was eluted with water. Removal of the last traces of KCN was accomplished by extraction into phenol, followed by back-extraction into water as described above.

Cyanoaquocobinamide [(CN,OH)Cbi] was prepared from aquocobinamide formed by the aerobic photolysis of cobinamide coenzyme. The latter was the generous gift of Prof. J. M. Wood. No attempt was made to separate the corrin from the adenosyl derivatives formed during photolysis (18). Aquocobinamide was converted to cyanoaquocobinamide by the technique used for the conversion of aquo-α-(adenyl)cobalamin to α-(adenyl)cobalamin cyanide.

The concentrations of unlabeled cobamides were determined spectrophotometrically at 367 nm after conversion to the cobamide dicyanides by photolysis, followed by the addition of a crystal of solid KCN to the cuvette. The molar extinction coefficient was taken as 30,400 $cm^2\ mol^{-1}$ (19).

Isolation of brush borders and microvillous membranes. Brush borders were isolated from guinea pig intestine as previously described for the hamster (2). All procedures were carried out in a cold room at 4°C. Guinea pigs weighing 300–400 g were killed by a blow to the head. The small bowel was immediately removed and divided into proximal and distal halves. Each half was washed separately by flushing with cold 5 mM EDTA in 0.15 M sodium phosphate buffer, pH 7.0. The mucosa was expressed from the washed intestinal segments by pressing on the serosal surface of the bowel with a glass rod. Mucosa from either the proximal or the distal half of four to six intestines was pooled in 100 ml of EDTA solution and homogenized in a Waring Blendor (Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.) for 25 s. The speed and time of homogenization required for optimal brush border preparations was determined experimentally and found to be the same as previously described for the hamster (2). The homogenate was filtered through No. 2 bolting silk (Tobler, Ernst & Trabler, Inc., New York), and the filtrate was centrifuged in an International refrigerated centrifuge (Damon/IEC Div., Damon Corp., Needham Heights, Mass.) at 1,500 rpm for 10 min. The pellet was washed twice by carefully resuspending it in cold EDTA solution and centrifuging at 1,500 rpm to remove lighter contaminating particles. The final pellet

was resuspended in cold EDTA solution and centrifuged at 500 rpm for 1 min to remove any remaining nuclei and intact cells. The supernate was then centrifuged for 10 min at 2,500 rpm and the precipitate was resuspended in Krebs-Ringer bicarbonate (KRB) buffer at pH 7.4. Phase contrast microscopy was used to assess the purity of each brush border preparation.

Microvillous membranes were prepared from brush borders by disruption with Tris, followed by density gradient centrifugation as described by Eichholz and Crane (20). After density gradient centrifugation, only a broad C band containing microvillous membranes was observed. The C^1 band seen in hamster preparations (2, 20) was not evident. The purity of microvillous membranes was established by electron microscopy, as previously described (2).

Binding of [^{60}Co]CN-Cbl to IF. All binding experiments were done in 0.9% saline buffered at pH 7.4 with 0.01 M potassium phosphate buffer. Portions of the gastric juice pool were thawed and diluted with the buffer to give a final concentration of 0.19 mg of protein/ml. Binding was measured in test tubes by placing 2 ml of this dilute gastric juice into a cellulose dialysis bag (Arthur H. Thomas Co., Philadelphia) and dialyzing this against 2 ml of buffered saline that contained free [^{60}Co]CN-Cbl in concentrations ranging from 0.3 to 8.0 nM. Preliminary experiments with saline inside the dialysis bag showed that equilibrium was attained when tubes were rotated on a wheel at 15 rpm for 18 h at room temperature. For each binding assay, equilibration was monitored by including tubes with saline instead of gastric juice inside the bag. After dialysis, 1-ml portions from the bag and from the outside fluid were counted in a Packard Auto-Gamma detector (Packard Instrument Co., Inc., Downers Grove, Ill.). The concentration of unbound CN-Cbl was equal to that measured in the outside fluid. The amount of CN-Cbl inside the bag was equal to the sum of the unbound CN-Cbl plus that bound to IF. In calculating the binding of CN-Cbl to IF, the assumption was made that the concentration of protein inside the bag did not change during the course of the dialysis.

Inhibition of [^{60}Co]CN-Cbl binding to IF by cobamides. Equilibrium dialysis was carried out as described above, but in these experiments dilute gastric juice was dialyzed against a series of solutions containing a fixed amount of the cobamide under investigation, together with increasing concentrations of [^{60}Co]CN-Cbl. The K_i for the analog was determined either with the aid of the Scatchard plot (21), or by means of the following equation, which is derived in the Appendix:

$$\frac{[I_t]}{\frac{K_L[L]}{Y} - K_L[L] - 1} = \frac{Y}{K_L[L]}[E_t] + K_i$$

$[I_t]$ = total concentration of inhibitor; $[E_t]$ = total concentration of IF; K_L = association constant for the formation of the CN-Cbl·IF complex; $[L]$ = concentration of [^{60}Co]-CN-Cbl; and Y = fractional saturation of total IF binding sites.

Preparation of the cobamide·IF complexes used in binding studies with ileal receptor. An excess of [^{57}Co]CN-Cbl was added to thawed portions of the gastric juice pool and incubated at room temperature for 30 min. The mixture was then dialyzed at 4°C for 48 h against two changes of 3 liters each of 0.9% saline to remove excess unbound B_{12}. Suitable dilutions of the dialyzed material were prepared and stored at −20°C. Complexes of IF with vitamin B_{12} analogs were similarly prepared and diluted. Saturation of all available binding sites by the analog was established by the observation that when [^{60}Co]CN-Cbl was added to the preparation, no ^{60}Co was bound to IF. Free IF was prepared by incubation and dialysis of gastric juice as described above, except that cobamide was omitted.

Complexes of vitamin B_{12} with non-IF binding proteins on human saliva, gastric juice, and serum were prepared by DEAE column chromatography (12) after saturation of binders with [^{57}Co]CN-Cbl.

Removal of cobamide-binding proteins from the IF preparation by affinity chromatography. In certain experiments, a preparation of IF was used from which cobamide-binding proteins were specifically removed by passage through a vitamin B_{12}-Sepharose column prepared by the method of Allen and Majerus (22). For these experiments, the IF preparation was subjected to chromatography as follows: Two columns were poured, one containing 0.2 ml of vitamin B_{12}-Sepharose (the affinity column), the other containing 0.2 ml 3,3′-diaminopropylamine-Sepharose that had not been coupled to vitamin B_{12} (the control column). Each column was washed with 5 ml of 0.1 M potassium phosphate buffer, pH 7.4. 2 ml of IF (original CN-Cbl binding capacity 28.3 ng/ml)[2] was then passed through each column. After chromatography, the CN-Cbl binding capacities of the IF from the control and affinity columns were 27.5 and < 0.001 ng/ml, respectively. The preparation from the control column was diluted with 0.155 M NaCl to a CN-Cbl binding capacity of 2.0 ng/ml. The IF-depleted preparation was diluted to the same extent.

Vitamin B_{12} uptake by tissue preparations. Freshly prepared mucosal homogenates, brush borders, or microvillous membranes were resuspended in cold KRB. Volumes were adjusted so that tissue protein concentrations were 0.6–0.9 mg/ml for brush borders and 0.1–0.2 mg/ml for microvillous membranes. Tissue protein concentration was determined by the method of Lowry, Rosebrough, Farr, and Randall (23). 1-ml portions of suspension were added to 16 × 250-mm polyethylene counting tubes containing, unless otherwise indicated, 2 ng of free or IF-bound [^{57}Co]CN-Cbl. The reaction mixtures were adjusted to 5 ml with KRB and incubated in a shaking water bath at 37°C. After incubation, an excess of cold KRB was added, and the tubes were centrifuged at 2500 rpm for 10 min at 4°C. The precipitate was washed twice in cold KRB, and the radioactivity taken up by the tissue was determined in a Packard Autogamma detector.

For measurements of attachment of IFB_{12} to membrane receptors at early time intervals, the free IFB_{12} was separated from the attached complex by filtration. Reaction mixtures contained brush borders, IF-bound [^{57}Co]CN-Cbl, and KRB as described above. Incubations were performed in a Millipore filtration apparatus containing a Millipore glass fiber filter (2.5 cm diameter, Millipore Corp., Bedford, Mass.). For the incubation, the apparatus was placed in the mouth of a sidearm flask connected by a rubber hose to a continuously running water aspirator. During the incubation the filtration apparatus was isolated from the vacuum by a pinch clamp on the hose. The incubation was terminated by removing the clamp, causing the soluble portion of the incubation mixture to be drawn rapidly into the

[2] The experiments using this material were performed many months after the rest of the experiments reported in this paper were completed. In that time, the CN-Cbl binding capacity of the IF preparation fell to 35% of its initial value.

flask. Immediately, the brush borders retained on the filter were washed with 25 ml of cold KRB. The filter was then dried and placed in a polyethylene tube for determination of radioactivity.

Binding of analog·IF complexes to guinea pig receptor was measured by incubating fresh preparations of brush borders for 1 h with increasing concentrations of [^{57}Co]-CN-Cbl complex in the presence of a quantity of analog·IF complex containing 1.5 pmol of analog. Incubations were performed in KRB solution as described above. Brush borders were separated from the soluble portion of the reaction mixture by centrifugation. In each set of experiments, paired control tubes without analog·IF complex were incubated to determine the K_d for the complex between receptor and [^{57}Co]CN-Cbl·IF complex in the absence of analog·IF complex.

Protection from photolysis. All manipulations of Co-alkyl cobamides were conducted in dim light, and all equilibrium dialyses were conducted in the dark, to prevent destruction of these compounds by light.

RESULTS

The interaction between cobamides and human intrinsic factor

Binding of CN-Cbl to IF. The binding of CN-Cbl to IF showed saturation kinetics. A Scatchard plot of the binding data (Fig. 1) shows that the IF present in 1 mg (23) of gastric juice protein bound 17 pmol of CN-Cbl. Binding in excess of this amount may have been due to the small concentration of R binder known to be present in the IF preparation used. Considerable deviation from linearity was observed at low concentrations of CN-Cbl, when the IF was less than half saturated. Whether this deviation from linearity was a true property of the interaction between IF and CN-Cbl or an artifact of the assay was not investigated further. The dissociation constant (K_d) for the CN-Cbl·IF complex was calculated from the linear portion of the Scatchard plot and was found to be 0.066 nM. This figure agrees with that previously reported by McGuigan (24).

Binding of other cobamides to IF. The binding of vitamin B_{12} analogs to IF was determined by competition experiments. Preliminary experiments showed that the

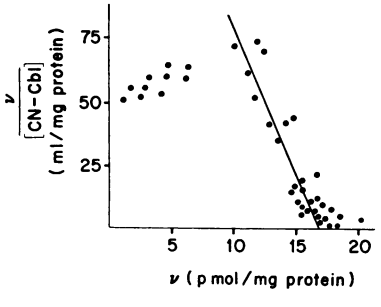

FIGURE 1 Scatchard plot of the binding of [^{60}Co]CN-Cbl to IF.

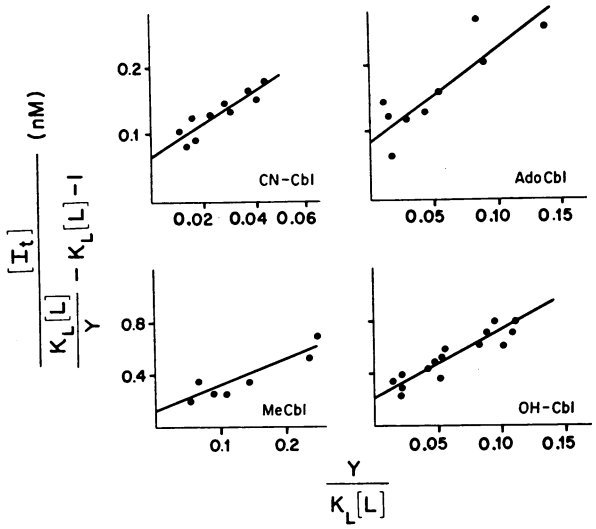

FIGURE 2 Binding of high-affinity B_{12} analogs to IF. Regression lines were fitted by the method of least squares, using 14 $(nM)^{-1}$ as the value of K_L. For further details, see text.

analogs fell into two categories: those whose affinities for IF were similar to that of CN-Cbl and those with an affinity much lower than that of CN-Cbl. Binding constants for those B_{12} derivatives in the former category were calculated by the equation derived in the Appendix. Fig. 2 shows data plotted according to this equation for these "high-affinity" analogs. The K_i obtained from the y-intercept of each plot is given in Table I. When unlabeled CN-Cbl was used as a high-affinity inhibitor of the binding of [^{60}Co]CN-Cbl, the K_i (0.067 nM) was virtually identical to the K_d of CN-Cbl as measured directly by equilibrium dialysis (0.066 nM), an observation supporting the validity of the equation under the experimental conditions. The values of K_i for the rest of the high-affinity analogs indicate that the affinity of each of these analogs for IF is similar to that of CN-Cbl.

The slopes of the regression lines (Table I) obtained with CN-Cbl, MeCbl, and AdoCbl agree closely with the concentration of IF in the system (1.6 nM, calculated on the basis of the total volume of the system, in-

TABLE I

Inhibition Constants of High-Affinity Vitamin B_{12} Analogs

Analog	Slope	K_I
	nM	*nM*
[^{60}Co]CN-Cbl	—	0.066*
CN-Cbl	2.3	0.067
OH-Cbl	4.9	0.23
MeCbl	1.8	0.13
AdoCbl	1.4	0.093

* K_d measured directly by equilibrium dialysis.

TABLE II

Inhibition Constants for Low-Affinity Cobamide·IF Complexes

Cobamide	R_{CN-Cbl}*	K_i
		nM
(Ade)CN-Cba	0.39	9.9
CN-Cbl(OH)[e]	1.56	33
(CN,OH)Cbi	1.85	>10,000

* R_{CN-Cbl} = mobility of cobamide/mobility of CN-Cbl.

cluding fluid both inside and outside the dialysis bag). This finding indicates that each of the analogs inhibits binding of [^{60}Co]CN-Cbl to IF in a simple competitive manner (see Appendix). With OH-Cbl, however, the slope of the regression line was 4.9 nM, suggesting that with this analog, inhibition was not strictly competitive.

For those analogs with a low affinity for IF, it was possible to determine the dissociation constants by Scatchard plots, because at levels required to show inhibition, the concentration of unbound analog closely approximated total analog concentration. Because the apparent affinity of these compounds for IF was so low, the question was raised as to whether the inhibition demonstrated by these preparations might not actually be due to contamination with trace amounts of unlabeled CN-Cbl. To investigate this possibility, these analogs were tested both before and after repurification by paper chromatography. For this purpose, Whatman 3MM paper was used. Chromatograms were developed for 24 h by the descending technique with a solvent system consisting of *sec*-butanol/glacial acetic acid/water/10% (wt/vol) KCN = 75/1/25/0.1 (vol/vol). The analogs were eluted with water, desalted by extraction into phenol and back-extraction in water, and again tested for their ability to inhibit the binding of CN-Cbl to IF. Table II shows the R_{CN-Cbl} values and the inhibition constants for the repurified analogs. From the R_{CN-Cbl} values it is apparent that the repurification procedure was suitable for removing contaminating CN-Cbl from the analog preparations. The dissociation constants of (Ade)CN-Cba and CN-Cbl(OH)[e] were found to be unchanged by purification, whereas the apparent inhibition observed with the original preparation of (CN, OH)Cbi was abolished by purification. Thus, (Ade)-CN-Cba and CN-Cbl(OH)[e] appear to bind to IF, albeit with a comparatively low affinity, while (CN, OH) Cbi does not bind to IF at concentrations as high as 10^{-5} M.

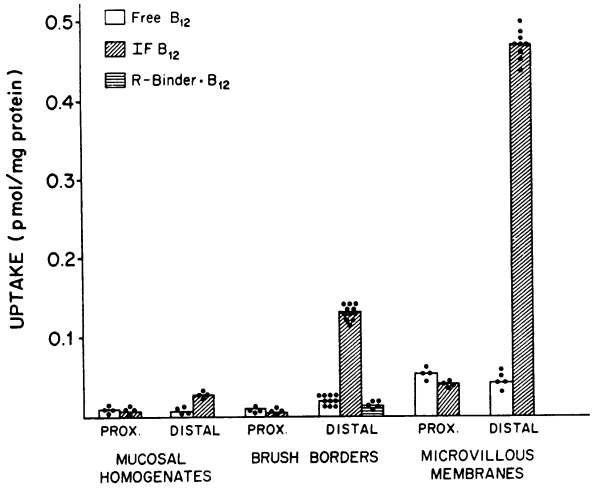

FIGURE 3 Binding of [^{57}Co]CN-Cbl by preparations from guinea pig intestinal mucosa. Points represent results of individual experiments and bars the mean values. The same pool of neutralized human gastric juice was used in all experiments.

The binding of the IF·cobamide complex to the ileal receptor

Attachment of CN-Cbl·IF to guinea pig ileal receptor. Human IF enhanced the uptake of [^{57}Co]CN-Cbl by all preparations obtained from the distal half of guinea pig small bowel (Fig. 3). In terms of uptake per milligram of tissue protein, brush borders took up four times as much IFB_{12} as crude mucosal homogenates, while uptake of microvillous membranes was 16 times that of homogenates. By contrast, uptake of IFB_{12} by tissue preparations from proximal small intestine was consistently less than that of the free vitamin. On the other hand, salivary, gastric, and serum vitamin B_{12}-binding proteins lacking IF activity (R binders) were found to be completely ineffective in promoting attachment of [^{57}Co]-CN-Cbl to distal brush borders.

Attachment of human IFB_{12} complex to ileal microvillous membranes displayed saturation kinetics. From a reciprocal plot of uptake versus concentration of IFB_{12} (Fig. 4), the dissociation constant for the reaction IFB_{12}

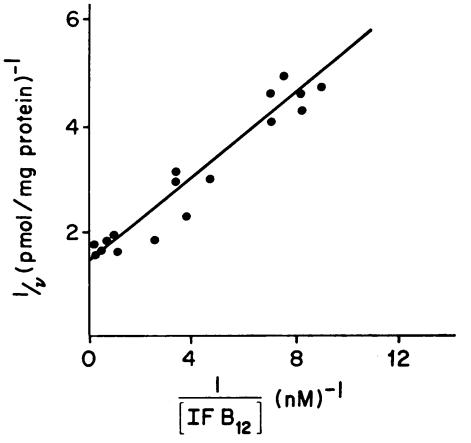

FIGURE 4 Reciprocal plot of binding of IFB_{12} to microvillous membranes as a function of IFB_{12} concentration.

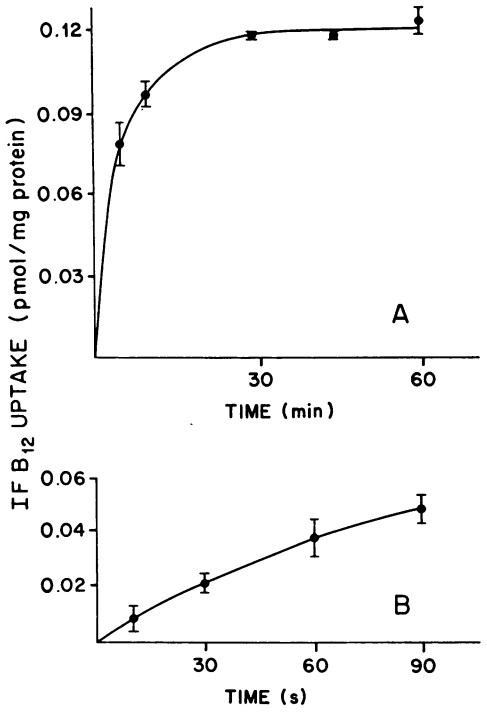

FIGURE 5 Rate of uptake of IFB_{12} by brush borders. Uptake by brush borders of [^{57}Co]CN-Cbl bound to IF was measured as a function of time. Reaction mixtures contained 1.15 nM IFB_{12} complex and a quantity of brush border suspension containing 1 mg tissue protein. Error bars indicate ±1 SD. A, Attached IFB_{12} separated by centrifugation (see Methods); B, Early uptakes measured by separating attached IFB_{12} by filtration (see Methods).

+ receptor $\rightleftharpoons$ IFB_{12}·receptor was determined to be 0.25 nM. Similar values were obtained for attachment of human IFB_{12} complex to mucosal homogenates and brush borders (0.21 nM and 0.30 nM, respectively).

The rate of attachment of human IFB_{12} complex to brush border receptor was determined by measuring uptake at a given concentration of IFB_{12} as a function of time. The first experiments, in which centrifugation was used to separate unattached IFB_{12} from IFB_{12} bound to receptor, showed that the binding reaction was about 70% complete at 5 min (Fig. 5A). The initial rate of attachment was determined more precisely by measuring uptake at earlier time intervals. For this purpose a rapid filtration assay was developed (see Methods). From measurements made over the first 90 s (Fig. 5B), an initial rate of uptake of approximately 0.05 pmol/mg protein per min was seen at a concentration of IFB_{12} complex of 1.15 nM. From the data of Fig. 4, the total binding capacity of microvillous membranes for IFB_{12} at a concentration of 1.15 nM is 0.55 pmol/mg protein. The second-order rate constant for the attachment of IFB_{12} to receptor is therefore 1.3×10^6 M^{-1} s^{-1}. From this value and the previously determined thermodynamic dissociation constant, the rate of dissociation of IFB_{12} from the receptor can be calculated to be 1.2 h^{-1}.

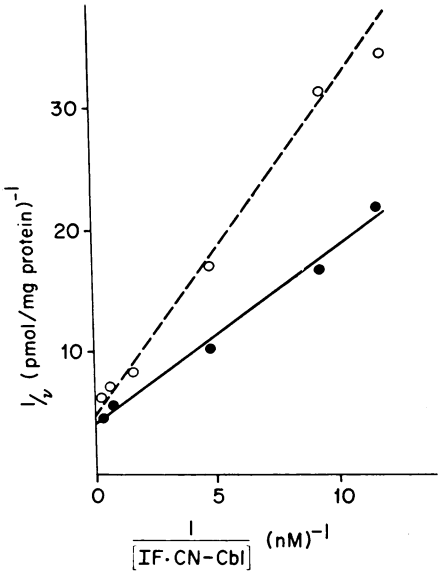

FIGURE 6 Inhibition by OH-Cbl·IF of the attachment of [^{57}Co]CN-Cbl·IF to the ileal receptor.

Attachment to ileal receptor of complexes between IF and other B_{12} derivatives. Nonradioactive CN-Cbl·IF complex competitively inhibited uptake of [^{57}Co]CN-Cbl·IF complex by brush borders isolated from guinea pig ileum. The K_i with unlabeled CN-Cbl calculated from this experiment (0.30 nM) was the same as the K_d of [^{57}Co]CN-Cbl·IF for the ileal receptor. Reciprocal plots of binding data showed that all of the other cobamide-IF complexes tested also inhibited attachment of [^{57}Co]CN-Cbl·IF complex to the receptor competitively. As an example, Fig. 6 shows a reciprocal plot of the effect of OH-Cbl·IF complex on the attachment of [^{57}Co]CN-Cbl·IF complex. The calculated inhibition constants for the various cobamide·IF complexes are presented in Table III. Complexes of IF with OH-Cbl, MeCbl, AdoCbl, and CN-Cbl(OH)[e] had the same affinity as CN-Cbl·IF complex for the receptor, while the

TABLE III

Inhibition of the Attachment of [^{57}Co]CN-Cbl·IF to Receptor by Analog·IF Complexes

Analog	K_i
	nM
CN-Cbl	0.30
OH-Cbl	0.22
MeCbl	0.29
AdoCbl	0.15
CN-Cbl(OH)[e]	0.46
(Ade)CN-Cba	0.99

TABLE IV

Inhibition by Free IF of the Attachment of IFB_{12} Complex to Brush Border Receptor

Preliminary incubation	Final incubation	Uptake
		pmol/mg tissue protein
—	IFB_{12}	0.118
IF	IFB_{12}	0.081
—	IF and IFB_{12}	0.124
—	B_{12}	0.015
IF	B_{12}	0.023

During the preliminary incubation, brush borders were incubated for 1 h at room temperature in 5 ml of KRB, which, where indicated, contained 1.5 pmol free IF. Brush borders were then centrifuged, washed in KRB, and resuspended in 5 ml KRB containing 1.5 pmol of either free [^{57}Co]CN-Cbl or [^{57}Co]CN-Cbl complexed with IF (IFB_{12}) and, where indicated, with 1.5 pmol of free IF. After this final incubation for 1 h at 37°C, uptake of radioactivity was determined by the method of centrifugation (see Methods).

K_i for (Ade)CN-Cbl·IF complex was somewhat less than that of the other cobamide complexes.

Attachment of free IF to the ileal receptor. To determine whether IF not bound to vitamin B_{12} attaches to the ileal surface, we measured the uptake of radioactive IFB_{12} complex by distal brush borders in the presence and absence of free IF. In these experiments, uptake of IFB_{12} complex by brush borders preincubated for 1 h with free IF was compared with uptake when IFB_{12} and free IF were added simultaneously. The results (Table IV) show that inhibition of IFB_{12} uptake by an equivalent amount of free IF is observed if the brush borders are preincubated with the free IF.

TABLE V

Failure of IF Preparation Passed through a Vitamin B_{12}-Sepharose Column to Inhibit the Attachment of IFB_{12} Complex to Brush Border Receptor

IF preparation		
Preliminary incubation	Final incubation	Uptake
		pmol/mg tissue protein
—	—	0.016±0.004
—	Control	0.120±0.017
—	IF-depleted	0.012±0.003
Control	Control	0.052±0.002
IF-depleted	Control	0.125±0.010

IF-depleted and control IF preparations were made as described in Methods. Experiments were conducted as described in Table IV. Uptakes represent the mean±SD of three experiments.

Although competition by free IF could explain the reduction in IFB_{12} uptake observed in these experiments, it was also possible that inhibition by other constituents of the IF preparation could account for these findings. To investigate this alternative, experiments were performed testing the ability of IF preparation specifically depleted of cobamide-binding proteins by passage through a vitamin B_{12}-Sepharose column to inhibit IFB_{12} uptake. The results (Table V) show that preincubation of brush borders with IF preparation passed through a vitamin B_{12}-Sepharose column had no effect on brush border IFB_{12} uptake. IF preparation passed through a B_{12}-free Sepharose column, on the other hand, inhibited brush border IFB_{12} uptake approximately as much as IF preparation not subjected to chromatography. These results are consistent with the notion that free IF, rather than another constituent of the IF preparation, is responsible for the reduction in IFB_{12} uptake that follows preincubation of ileal brush borders with the preparation.

The extent of inhibition of IFB_{12} uptake was found to depend on the duration of preincubation of brush borders with free IF. Fig. 7 shows that inhibition of IFB_{12} uptake by free IF develops moderately rapidly during the preincubation, reaching its fullest extent by about 20 min. The rate at which inhibition appears is roughly comparable to the rate at which the IFB_{12} complex attaches to receptor in the absence of free IF (cf. Fig. 5*a*).

Although inhibition of IFB_{12} uptake was not observed when equal amounts of IFB_{12} complex and free IF were simultaneously added to brush borders, inhibition was observed under these conditions with larger

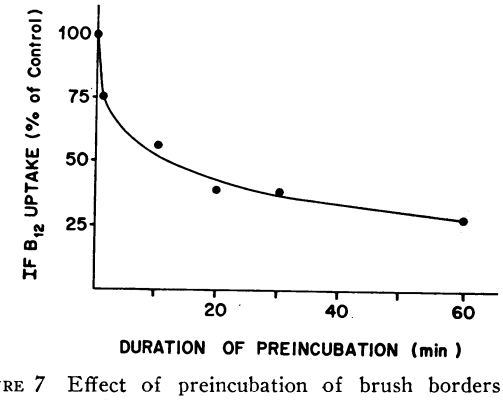

FIGURE 7 Effect of preincubation of brush borders with free IF on subsequent brush border uptake of radioactive IFB_{12}. Reaction mixtures containing brush borders (1 mg tissue protein) in 5 ml KRB were incubated with 1.5 pmol free IF at room temperature for the times indicated. In control tubes brush borders were incubated in KRB without free IF. [^{60}Co]CN-Cbl·IF (1.5 pmol) was then added and the incubation was continued for an additional hour. Uptake of IFB_{12} by brush borders was then determined by centrifugation (see Methods). Uptakes are expressed as percent of control values. Error bars indicate ±1 SD.

amounts of free IF. Fig. 8 shows inhibition of IFB_{12} uptake as a function of the concentration of free IF. When brush borders were not preincubated with free IF, slight but significant interference with IFB_{12} uptake was observed at free IF/IFB_{12} ratios as low as 2. The extent of interference increased with increasing concentrations of free IF. Preincubation of brush borders with free IF greatly augmented the interference with IFB_{12} uptake at each concentration of free IF.

The results of experiments to determine the nature of the inhibition of IFB_{12} uptake by free IF are shown in Fig. 9. While uptake data obtained in the absence of free IF could be fitted well to a Michaelis-Menten plot by a nonlinear least squares method, the data obtained in the presence of free IF could not be so fitted within the limits of experimental error. This finding indicates that inhibition by free IF under these experimental conditions is neither competitive, noncompetitive, nor mixed, but instead displays an unconventional pattern. Perhaps the unusual nature of the inhibition may be explained by proposing that true equilibrium was not attained during the course of the incubation. In any event, that significant inhibition was effected by 2.3 nM IF indicates that the binding of free IF to the ileal receptor is tight.

DISCUSSION

Previous studies have shown that several cobalamins prevent binding of CN-Cbl to IF (25–27), and are themselves absorbed by an IF-dependent mechanism (28–31). Derivatives with an altered corrin ring or benzimidazole moiety did not appear to inhibit binding of CN-Cbl to IF (32), and appeared to be poorly absorbed by humans and experimental animals (33). These studies indicated in a qualitative way a relationship between B_{12} structure, its binding to IF, and its subsequent absorption.

Quantitative data from the present investigation and from previous investigations reported by Hippe, Haber, and Olesen (34), Hippe and Oleson (35), and Allen and Mehlman (36) accord with the results of the more qualitative studies referred to above. In the present studies, the affinity of CN-Cbl for IF was not significantly altered by substitution at the -CN position, suggesting that the ligand at this position is not involved in the binding process. The e-amido group on the corrin ring, however, appears to play a significant role in binding to IF, since removal of this single amide group to form CN-Cbl(OH)^e markedly reduces affinity. The base coordinated to the cobalt is apparently critical in the binding process. Replacement of the dimethylbenzimadole moiety of CN-Cbl by adenine [CN-Cbl → (Ade)-CN-Cba] greatly reduced affinity, while removal of base [CN-Cbl → (CN,OH)Cbi] rendered the derivative incapable of competing with CN-Cbl for binding

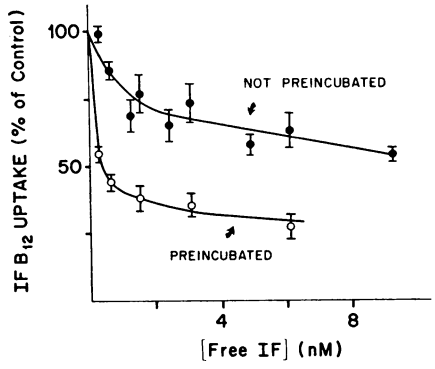

FIGURE 8 Inhibition of IFB_{12} uptake as a function of the concentration of free IF. Reaction mixtures contained brush borders (1 mg tissue protein), [^{57}Co]CN-Cbl·IF (1.5 pmol), and free IF at the concentrations indicated. In one set of experiments (●—●), all constituents were added at the same time. In the other set of experiments (○—○), brush borders in KRB were incubated with free IF for 1 h at 37°C. [^{57}Co]CN-Cbl·IF was then added and the incubation was continued for an additional hour. For each set of experiments, control tubes were incubated under precisely the same conditions except that no free IF was included in the reaction mixtures. Uptake of complex by brush borders was determined by the method of centrifugation (see Methods). Uptakes are expressed as percent of control values. Error bars indicate ±1 SD.

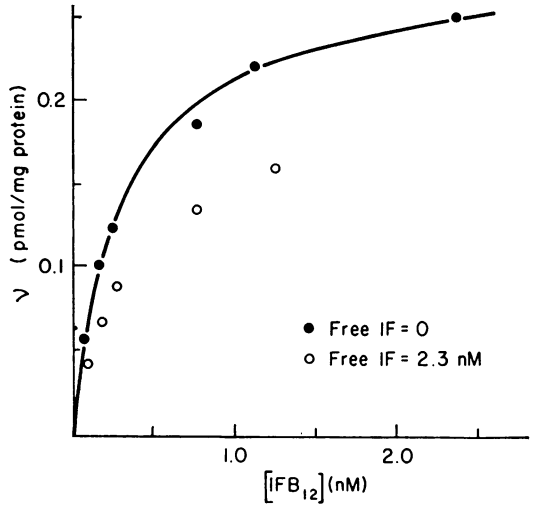

FIGURE 9 Kinetics of inhibition of the attachment of IFB_{12} complex to brush border receptor by free IF. Reaction mixtures contained brush borders (1 mg protein), 5 ml KRB, IFB_{12} complex at the concentrations shown, and, where indicated, 2.3 nM free IF. There was no preliminary incubation. Reaction mixtures were incubated at 37°C for 1 h. Uptake of IFB_{12} complex by brush border receptor was then determined by the method of centrifugation (see Methods).

The curve drawn through the closed circles was calculated by a nonlinear least-squares fit of the data obtained in the absence of free IF to the Michaelis-Menten equation. The parameters obtained from this curve-fitting procedure were as follows: $K_M = 0.31$ nM, $v_M = 0.28$ pmol/mg protein.

to IF. These results support the model of IF-B_{12} binding proposed by Gräsbeck (37), who suggested that the B_{12} molecule fits into a pit on the globular IF molecule with the nucleotide facing inward and the -CN side of the planar corrin ring facing outward.

The present studies also demonstrated the uptake of the complex between cobamide and human IF by the guinea pig ileal receptor. Previous workers have shown that human IF promotes uptake of vitamin B_{12} by everted sacs (3) and homogenates (4) of guinea pig ileum. In our study, vitamin B_{12} bound to human IF was preferentially taken up by whole mucosal homogenates, brush borders, and microvillous membranes prepared from the distal but not the proximal half of guinea pig small bowel. Binding of IFB_{12} complex per milligram of tissue protein increased as the relative amount of microvillous membrane in the preparation increased. Similar dissociation constants were determined for the attachment of IFB_{12} complex to each of the mucosal preparations (0.21 nM, 0.30 nM, and 0.25 nM for whole mucosal homogenate, brush borders, and microvillous membranes, respectively). Although human IF enhanced attachment of vitamin B_{12}, other proteins that bind vitamin B_{12} but that lack intrinsic factor, i.e., R binders (9) prepared from human gastric juice, saliva, and serum, were totally ineffective. Thus, as in the hamster (2), intestinal receptors specific for IFB_{12} complex are located on microvillous membranes of absorptive cells in the guinea pig ileum. The advantage of using microvillous membranes from guinea pigs rather than from hamsters is that human IF can be studied.

The rate constant for the dissociation of IFB_{12} from the receptor indicates a half-time for dissociation of about 35 min. This value suggests that the lifetime of the complex is sufficiently long to permit the vitamin to be slowly transported into the mucosal cell. There is considerable evidence that transport of the vitamin across the intestine is a slow process (38).

Although the rate of dissociation is slow, the rate of attachment of IFB_{12} to receptor is rapid. Since this rate of attachment is proportional to the concentration of IFB_{12} in the incubation medium, a half-time for the attachment process would be difficult to interpret in physiological terms. A physiological interpretation is provided by a comparison of the second-order rate constant determined in our experiments with the maximum possible second-order rate constant; namely, the rate constant of a reaction in which velocity is limited by the rate of diffusion of the reactants. Such a comparison shows that about 1% of all encounters between IFB_{12} and receptor are productive of attachment.

Controversy exists as to whether or not IF not complexed with vitamin B_{12} (free IF) is able to attach to the ileal receptor. Herbert (7) concluded from experiments in which everted intestinal sacs were sequentially incubated first with free IF and then with IFB_{12} complex that free IF was capable of attaching to the ileal receptor. On the other hand, attachment of free IF could not be demonstrated by Strauss and Wilson (8), who simultaneously incubated everted sacs with free IF and IFB_{12}. From the results of the present study, it is apparent that under the usual experimental conditions, attachment of IFB_{12} takes considerable time to reach completion (Fig. 5*a*). It therefore seemed possible that the discrepant findings concerning uptake of free IF by intestinal tissue might be related to the time required for free IF to attach to the ileal receptor. The results described above indicate that when brush borders are preincubated with free IF, subsequent uptake of IFB_{12} is inhibited (Table IV), and that the extent of inhibition is related to the duration of preincubation (Fig. 7). The possibility that the reduction of IFB_{12} uptake is an effect of a nonspecific inhibitor in the IF preparation, rather than an effect of free IF, is unlikely (Table V).

It thus appears that free IF attaches to the ileal receptor. This attachment process, however, occurs more slowly than for IFB_{12}. In addition to free IF, each of the analog·IF complexes tested was able to compete with CN-Cbl·IF for the receptor. Since the affinities of the analog·IF complexes for the receptor varied by less than a factor of three, it appears that the nature of the cobamide bound to IF has little effect on the interaction between IF and its receptor.

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APPENDIX

Measurement of the K_i for the inhibition of binding of [^{60}Co]CN-Cbl to IF by B_{12} analogs. In a system consisting of a small molecular ligand and a macromolecule to which it binds, a second compound that binds to the macromolecule and thereby prevents the binding of the first ligand is called a competitive inhibitor. This situation can be represented by the equilibria:

$$E + L \overset{K_L}{\rightleftharpoons} EL$$

$$E + I \overset{K_X}{\rightleftharpoons} EI$$

The equation which relates the extent of saturation of the macromolecule with ligand to the concentrations of ligand and inhibitor is:

$$Y = \frac{1}{1 + \frac{1}{K_L[L]} + \frac{K_X[I]}{K_L[L]}}$$

where Y is the fractional saturation of the macromolecule, K_L and K_X are the *association* constants for ligand and inhibitor, respectively, and $[L]$ and $[I]$ the the concentrations of the *unbound* species.

Ordinarily, the concentrations of both ligand and inhibitor are large relative to the concentration of macromolecule, so the concentration of unbound species is approximately equal to its total concentration. Under such circumstances, the total concentration of ligand and inhibitor can be used with the above equation to determine binding constants. However, in the present experiments, the total concentration of ligand was always of the same order of magnitude as that of the macromolecule, and the total concentration of inhibitor was frequently so. Moreover, while the concentration of unbound ligand was known from the experimental data, the concentration of unbound inhibitor could not be measured directly. For experiments in which the inhibitor concentration was large compared to the concentration of macromolecule and ligand, so that $[I_t] \sim [I]$, the inhibition constant could be determined by plotting the data according to Scatchard (14); the slope of the line, which in the absence of inhibitor is equal to $-1/K_L$, becomes equal to $-1/K_L(1+K_X[I_t])$ in the presence of inhibitor at a total concentration of $[I_t]$. However, when both ligand and inhibitor are present at concentrations of the same order of magnitude as the macromolecule, the Scatchard plot fails. An equation which permits the K_t to be determined under these conditions was derived as follows. The equilibria are

$$[EL] = K_L[E][L] \tag{1}$$

$$[EI] = K_X[E][I] \tag{2}$$

$$= K_X[E]([I_t] - [EI]), \tag{3}$$

where K_L, K_X, $[L]$, $[I]$, and $[I_t]$ are defined as before, (K_L and K_X being expressed in reciprocal concentration) and $[E]$, $[EL]$, and $[EI]$ are the concentrations of free macromolecule, macromolecule-ligand complex, and macromolecule-inhibitor complex, respectively. Solving Eq. 3,

$$[EI] = \frac{K_X[E][I_t]}{1 + K_X[E]} \tag{4}$$

Adding $[E]$ to both sides of Eq. 4 and rearranging,

$$[EI] + [E] = \frac{[E](1 + K_X[E] + K_X[I_t])}{1 + K_X[E]} \tag{5}$$

Expressing the concentrations of the various species of macromolecule in terms of fractional saturation, since

$$[E] + [EL] + [EI] = [E_t] \tag{6}$$

where $[E_t]$ is the total concentration of macromolecule, then by definition

$$Y = \frac{[EL]}{[E_t]} \tag{7}$$

and

$$1 - Y = \frac{[E] + [EI]}{[E_t]} \tag{8}$$

Dividing Eq. 7 by Eq. 8,

$$\frac{[EL]}{[E] + [EI]} = \frac{Y}{1 - Y} \tag{9}$$

$$= \frac{K_L[L](1 + K_X[E])}{1 + K_X[E] + K_X[I_t]} \tag{10}$$

where Eq. 10 is obtained from the left-hand side of Eq. 9 by appropriate substitution from Eq. 1 and 5.

Solving for $[E]$ between Eq. 1 and 7 yields the expression

$$[E] = \frac{Y[E_t]}{K_L[L]} \tag{11}$$

Substituting into Eq. 10,

$$\frac{Y}{1 - Y} = \frac{K_L[L] + K_X Y[E_t]}{1 + \frac{K_X Y[E_t]}{K_L[L]} + K_X[I_t]} \tag{12}$$

In this expression, all values except K_X can be directly obtained from the experimental data. When the reciprocal of this equation is taken and both sides are multiplied by $K_L[L]$, then

$$\frac{1 - Y}{Y} K_L[L] = \frac{K_L[L] + K_X Y[E_t] + K_L K_X[L][I_t]}{K_L[L] + K_X Y[E_t]}$$

$$= 1 + \frac{K_L K_X[L][I_t]}{K_L[L] + K_X Y[E_t]} \tag{13}$$

$$= 1 + \frac{K_X[I_t]}{1 + Y\frac{K_X[E_t]}{K_L[L]}}$$

Rearranging,

$$\frac{1 - Y}{Y} K_L[L] - 1 = \frac{(1 - Y)K_L[L] - Y}{Y}$$

$$= \frac{K_X I_t}{1 + Y\frac{K_X[E_t]}{K_L[L]}} \tag{14}$$

On taking the reciprocal and rearranging again,

$$\frac{[I_t]}{\frac{K_L[L]}{Y} - K_L[L] - 1} = \frac{Y}{K_L[L]}[E_t] + \frac{1}{K_X} \tag{15}$$

When the left-hand side of this equation is plotted against $Y/K_L[L]$, a line is obtained whose y-intercept is equal to $1/K_X$, that is, to the dissociation constant (K_t) for the inhibitor.

This equation contains two internal checks on the appropriateness of the model to the physical situation. First, plotting the data according to Eq. 15 should produce a straight line. Second, the slope of the line should be $[E_t]$, a quantity whose value is known from direct measurement.

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