Effects of Ethylenediaminetetraacetate and Metal Ions in Intestinal Absorption of Vitamin B_{12} in Man and Rats. (30431)

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It has been proposed, based mainly on *in vitro* studies using rat(1,2) and guinea pig(3) intestines, that intrinsic factor (IF)-mediated absorption of vit B_{12} (B_{12}) requires Ca ions. However, evidence in intact animals to support this hypothesis is insufficient. In man, Gräsbeck and Nyberg(4) administered ethylenediaminetetraacetate (EDTA) by mouth together with radioactive B_{12} and demonstrated reduction of absorption which was reversed by addition of Ca. Using gastrectomized rats, Abels *et al*(5) failed to find any effect of EDTA on absorption of B_{12} administered with rat gastric juice. They maintained that in the rat system, heterologous IF behaves differently from rat IF and that some of the results obtained with *in vitro* studies might not be applicable to normal physiology. Our study likewise showed that hog IF had no activity in rat intestine *in vivo*(6). Rice *et al* presented data indicating lack of influence of EDTA on B_{12} absorption in rats(7). This paper presents our study carried out in human and rat intestines *in vivo* to elucidate the effects of EDTA in relation to certain metal ions in B_{12} absorption.

Material and methods. Schilling test following intraduodenal administration. Patients with no advanced disease or gastrointestinal disorder were used, and a duodenal tube was passed about 90 cm under fluoroscope after overnight fasting. The duodenal route was used to avoid the stomach which would introduce variables such as the amount of gastric juice and cause a delay in the passage of the test dose. Two ml of aqueous solution of cyanocobalamin-Co^{60}* (B_{12}-Co^{60}) were instilled followed by washing with 5 ml saline, and after graded time intervals, 25 or 50 ml of 4% Na_2-EDTA of pH 7.2 were instilled. B_{12}-Co^{60} was so prepared that each dose contained 0.18 to 0.5 μc. Two hours after the dose of B_{12}-Co^{60}, 1 mg of unlabeled B_{12} was injected intramuscularly for flushing, and 24-hour urine specimens were subjected to gamma scintillation counting. The effects of metal ions were studied by infusing an aqueous solution of their salts in the volume of 20 ml, 5 minutes after EDTA. The amount of salt was such that it would combine with 1.5 g EDTA in test tubes.

Rat intestinal loop. Adult rats of the Wistar strain were used. A washed loop of small intestine was prepared *in situ* by operation as described(6). Such a loop has a normal blood supply and is suited for IF assay, because it is devoid of endogenous IF, and the enhancement of absorption of a test B_{12} dose by coadministered IF is a direct indication of the activity. A mixture of 10 mμg of B_{12}-Co^{60} and homologous IF concentrate equivalent of 30 mμg B_{12} binding, an amount which would yield maximal absorption, was injected into the loop and the decrease of radioactivity in the loop was determined after 24 hours as the measure of absorption.

Source of IF and measurement of B_{12} binding power. Gastric juice was aspirated from healthy volunteers and was immediately neutralized, frozen and used within one week. For rat studies, chromatographically concentrated homologous IF was used. A large batch of crude saline extract of rat stomach mucosae was first prepared, and partial purification of IF was carried out with a diethylaminoethyl cellulose column as detailed elsewhere(8). When the column was eluted at pH 7.2 by increasing the ionic strength, only the effluent containing B_{12} binding power had the IF activity as studied by the loop technique, and the activity was proportional to the binding power in the effluent. For this reason, the binding power was used as a measure for IF in this study. The B_{12} binding power of human gastric

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TABLE I. Effect of EDTA on Absorption of B_{12}-Co^{60} Administered Intraduodenally, as Determined by Schilling Test.

Dosage			
B_{12}-Co^{60} (μg)	EDTA (g)	No. of subjects	Urinary excretion (%)
.2	0*	6	15.7 ± 2.6 †
	2	5	1.5 ± .2
	1	7	8.0 ± 1.1
250	0*	3	.85 ± .27
	2	3	.73 ± .39

EDTA was administered immediately after B_{12}-Co^{60}.

* 50 ml of water, instead of EDTA, was instilled.

† Standard error of mean.

juice and purified rat IF was measured by dialysis(8) as well as the resting cell method (9). With fresh human gastric juice, although the binding does not represent IF itself, it provides a measure of the amount to be used with B_{12}.

Results. The results of the Schilling test following intraduodenal administration of 2 doses of B_{12}-Co^{60} with or without EDTA are shown in Table I. Administration of 2 g of EDTA in a 4% solution immediately after B_{12}-Co^{60} abolished absorption of a physiological dose of 0.2 μg, whereas 1 g of EDTA inhibited absorption only partially. No inhibition was obtained with 2 g EDTA when used with a supraphysiological dose of 250 μg of B_{12}-Co^{60}.

Table II represents the effects of various cations on EDTA inhibition studied under the same conditions, using 2 g EDTA. When administered 5 minutes after EDTA, Ca, Mg and Sr ions counteracted EDTA completely; the effect of Zn was equivocal, and ferrous and ferric ions did not reduce EDTA inhibition.

TABLE II. Effect of Various Metal Ions on EDTA Inhibition of B_{12} Absorption.

Ion*	Chemical form and amount	No. of subjects	Urinary excretion (%)
—	— (control)	5	1.5 ± .2
Ca^{++}	$CaCl_2 \cdot 6H_2O$, 1.2 g	3	17.9 ± .6
Mg^{++}	$MgSO_4 \cdot 6H_2O$, 1.3 g	3	15.5 ± .7
Sr^{++}	$Sr(C_3H_5O_3) \cdot 3H_2O$, 1.5 g	3	16.1 ± .9
Zn^{++}	$ZnSO_4 \cdot 7H_2O$, 1.3 g	4	6.2 ±1.9
Fe^{++}	$FeSO_4 \cdot 7H_2O$, 1.0 g	5	4.0 ± .5
Fe^{+++}	Ferric ammonium citrate, 1.2 g	3	3.2 ± .7

* Administered 5 min after 2 g of EDTA in the duodenum.

In a third experiment, the time interval was determined that was necessary for B_{12} to go through an absorptive process where EDTA no longer inhibited absorption. Two doses, 0.2 μg and 2.0 μg, of B_{12}-Co^{60} were first instilled intraduodenally and, after 15, 45, 90 and 180 minutes, 2 g of EDTA were administered. The results showed that even 90 minutes interval was not long enough and it was after about 3 hours that the EDTA inhibition was no longer demonstrable (Table III).

TABLE III. Time Intervals and EDTA Inhibition of Absorption of B_{12} Administered Intraduodenally.

Dose of B_{12}-Co^{60} (μg)	Interval between B_{12}-Co^{60} and EDTA* (min)	No. of subjects	Urinary excretion (%)
.2	0	5	1.5 ± .2
	45	4	2.7 ± .7
	90	2	5.7
	180	2	13.0
2.0	0	2	1.9
	15	1	1.0
	45	3	1.6
	90	5	4.7 ±1.4
	180	4	12.1 ±5.3

* 2 g.

The fourth experiment was designed to see whether or not a shortage of IF along the path of B_{12} was responsible for EDTA inhibition, and whether or not abundant supply of IF would cause earlier absorption after which chelation would be without effect. Fresh human gastric juice in amounts exceeding the B_{12} dose in terms of mμg binding, was instilled with B_{12}-Co^{60}, or alone followed by B_{12}-Co^{60} 45 minutes later, and EDTA was given 45 minutes after B_{12}-Co^{60}. The choice of this interval was based on the rat study below. The results were still the same and gastric juice showed no effect (Table IV).

In the rat loop (Table V), absorption of 10 mμg B_{12}-Co^{60} was 14% (5 rats) and addition of IF increased it to 55% (5 rats). Administration of 1 ml of 0.1 M EDTA immediately following B_{12} plus IF reduced absorption to 6%. Interposition of 3 minutes between B_{12} plus IF and EDTA increased

TABLE IV. Effect of Gastric Juice Administered Prior to EDTA on Inhibition of B_{12} Absorption by EDTA.

Order of administration	No. of subjects	Urinary excretion (%)
G.J.* B_{12}-Co^{60}†—(45 min)—EDTA‡	4	3.8 ± 2.7
G.J.—(45 min)—B_{12}-Co^{60}—(45 min)—EDTA	3	1.7 ± 1.0

* 30 ml fresh human gastric juice, capable of binding 250-400 mμg B_{12}. † 0.2 μg. ‡ 2 g.

TABLE V. Effect of EDTA on B_{12} Absorption from Rat Intestinal Loop with the Aid of Homologous IF.

Order of administration	Absorption (%)	Organ uptake (%) Liver	Kidneys
B_{12}	14 ± .5	1.3 ± .2	1.2 ± .2
B_{12} + IF	55 ± 2.8	8.5 ± .9	14.8 ± 1.2
B_{12} + IF + EDTA	6	.3	.9
B_{12} + IF — 3 min — EDTA 1 ml	19	1.1	5.2
B_{12} + IF — 3 min — EDTA 1 ml + $CaCl_2$ .5 ml	54	7.0	12.3
B_{12} + IF — 45 min — EDTA 1 ml	43	4.0	10.5
EDTA .5 ml — 3 min — B_{12} + IF	16	.6	4.0
EDTA 1 ml — 6 min — B_{12} + IF	10	.4	1.8
" — " — B_{12} + IF + $MgSO_4$ .5 ml	43	7.8	14.2
" — " — B_{12} + IF + $CaCl_2$ .5 ml	52	7.5	13.0

Figures are averages of 2 loops except for first 2 controls.
B_{12}—10 mμg; IF—equivalent of 30 mμg binding; EDTA—0.1 M; $MgSO_4$ and $CaCl_2$—0.2 M.

absorption somewhat, and a 45-minute interval almost completely annulled the EDTA inhibition. Alteration of the order of administration with a 6-minute interval between 1 ml EDTA and B_{12} plus IF did not change the EDTA effect. With the latter sequence of administration, addition of 0.5 ml of 0.2 M $CaCl_2$ and $MgSO_4$ to B_{12} plus IF counteracted EDTA.

Effect of EDTA on B_{12} binding power of IF was studied as follows: crude rat stomach extract and a chromatographically purified fraction of rat IF, the activity of which had been assayed, were mixed with EDTA, incubated for 30 minutes and the binding power of the mixture was determined by dialysis. The quantitative ratio was such that IF equivalent of 50 mμg B_{12} binding was mixed with EDTA at its final concentration of 0.02 M. The results showed that EDTA slightly but not significantly reduced the binding capacity of IF preparations.

Discussion. These data clearly demonstrated that EDTA interferes with IF-mediated absorption of B_{12}. No such inhibition was obtained with a supraphysiological dose of B_{12}. Absorption of large doses of B_{12} does not involve IF(10), and the negative results of Rice *et al*(7) were probably due to the dosage used. It was further indicated that chelating capacity of 2 g of EDTA, but not 1 g, was sufficiently large to remove practically all Ca ions, if temporarily, from the intestinal canal of man. Calculation of the dosage of salts in the study of cation effect was based on this observation, and the study showed Ca, Mg and Sr to be equally effective in counteracting EDTA. Neither ferrous nor ferric ions had such effect, and the effect of Zn was questionable. Since Zn has a much stronger affinity for EDTA than Ca, it is possible that part of EDTA-bound Ca was exchanged with Zn, making Ca available to absorption.

The studies on time intervals disclosed that if EDTA was administered after B_{12}-Co^{60}, the inhibition was reduced much faster in the rat. About 45 minutes in the rat loop and 3 hours in man seemed to be necessary to avoid the EDTA effect.

When B_{12} plus IF and EDTA were placed in the rat loop without a long interval, ab-

sorption was markedly inhibited. It would not take much time for EDTA to be conjugated by polyvalent ions in the loop, and some of the B_{12} should have a chance of absorption after excess Ca ions start entering the gut, provided IF kept its activity. Further studies are required to see if EDTA would not directly affect IF or damage the mucosal receptor site. As far as the binding capacity of rat IF preparations was concerned, EDTA did not appreciably reduce it in the test tube.

The time study demonstrated a long interval required for absorption to become unaffected by chelation, suggesting that the reaction involving Ca and possibly Mg is a slow and reversible process. As Herbert(1) first suggested, such divalent cations might act as a bridge to aid mucosal adsorption of IF-B_{12} complex(11), but the evidence has been meager. The failure to demonstrate any effect of gastric juice administration in the duodenum is in line with the assumption that IF activity is present in adequate quantities in the human intestinal canal(12).

Summary. The effect of metal chelation on intestinal adsorption of B_{12} was studied in man and in rats. When a physiological dose of B_{12}-Co^{60} was instilled through an indwelling tube into the duodenum in man, immediately followed by 2 g of EDTA in a dilute solution, absorption was reduced markedly as studied by the Schilling test. Administered shortly after EDTA, Ca^{++}, Mg^{++}, Sr^{++}, but not Fe^{++}, Fe^{+++}, or Zn^{++} counteracted the inhibition by EDTA. Similar results were obtained with rat intestinal loops. When there was interval between the dosage of B_{12}-Co^{60} and EDTA, about 3 hours in man and 45 minutes in the case of rat loop were necessary for the absorption to become unaffected by EDTA.

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Effect of Hyperthyroidism on Intestinal Absorption of Calcium in the Rat.* (30432)

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Several manifestations of abnormal calcium metabolism have been observed in hyperthyroidism. Hypercalcuria(1-3), and, less frequently, hypercalcemia(4-8) have been found. Rarefied bone has been observed, most often in hyperthyroidism of long duration(1,4,9-12). In the past this laboratory studied 2 patients with long-standing recurrent Graves' disease associated with demineralized bone. In both patients, serum calcium was normal, serum alkaline phosphatase was elevated, and urinary calcium excretion was low, even on a high calcium diet. These observations led to consideration of the possibility that the

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