Cycloheximide Effect on Vitamin B_{12} Absorption and Intrinsic Factor Production in the Rat* (33768)

S. D. J. YEH AND M. E. SHILS

Division of Medical Research, Sloan-Kettering Institute, New York, N. Y. 10021

Investigations of the effects of various inhibitors of protein synthesis on gastrointestinal function has revealed impairment of vitamin B_{12} absorption in rats treated with actinomycin D or colchicine (1) but not with tetracycline (2). Cycloheximide which is a potent and rapidly acting inhibitor of protein synthesis (3) was found to impair absorption of iron and of long-chain triglycerides and fatty acids (4–6). Its effect on vitamin B_{12} absorption and on intrinsic factor production is reported below.

Materials and Methods. Leucine-1-^{14}C incorporation into tissue protein (7) and vitamin B_{12} absorption *in vivo* (2) were determined by previously described methods. In these experiments the term vitamin B_{12} connotes B_{12}-^{57}Co. Absorption was also studied in rat isolated intestinal loops. These were prepared under light ether anesthesia by ligating the pylorus and ileocecal region with silk sutures. Intrinsic factor preparations were made either from neutralized gastric juice (as described in appropriate tables) obtained from pylorus-ligated rats or by homogenizing gastric mucosa from normal rats in 4 parts of 0.9% NaCl. Vitamin B_{12}–intrinsic factor complex was prepared by introducing 100 mμg of vitamin B_{12} into pylorus-ligated cycloheximide-treated and control rats. Four hr later the contents were aspirated and treated as indicated in Table IV prior to testing in isolated loops. The various intrinsic factor preparations with vitamin B_{12} were introduced into the duodenal lumen through a 27-gauge needle and the abdomen was closed with silk sutures. Four hr later, the animals were sacrificed and the entire gastrointestinal tract or the isolated loops were quickly removed and washed with 50 ml of 0.9% NaCl. Luminal contents and washings, intestinal wall and various organs were measured for ^{57}Co radioactivity (2). Uptake of vitamin B_{12} was also measured in the everted sacs of hamster intestine by the method of Strauss *et al.* (8). Specimens of pooled gastric juice collected from cycloheximide-treated or control rats were dialyzed and lyophilized as indicated in Table V and their intrinsic factor activities were tested in isolated loops of normal rats.

Results. Cycloheximide resulted in a marked inhibition of leucine incorporation into the proteins of various tissues including stomach and small intestine (Table I). When vitamin B_{12} was given intragastrically, rats given cycloheximide were noted to have a significant delay in the passage of the vitamin out of the stomach. This was apparent 1 hr after B_{12} was given and 3 hr after cycloheximide (0.5 mg/kg); however, between the third and sixth hours almost all of the vitamin had entered the small intestine (Table II). When the dose of cycloheximide was increased fivefold, 34.6 $\pm$ 4.7% of the vitamin radioactivity was present in the stomach 6 hr later (8 hr after cycloheximide) as compared to 0.03 $\pm$ 0.01% in the controls. The delayed gastric emptying was associated with decreased radioactivity in the liver and kidneys (Table II). In this experiment with 0.5 mg of cycloheximide, the organ radioactivity in the treated rats at 6 hr was only about one-third that of the controls despite the fact that almost all of the vitamin had entered the small intestine (Table II). This observation suggested that a reduction of vitamin B_{12} absorption had occurred in addition to delayed gastric passage. Balance studies combined with measurements of residual activity in the luminal contents of various portions of the GI tract indicated that in the 8 hr following B_{12} administration (10.5 hr after cycloheximide) the absorption of the vitamin was only two-thirds that of control rats (43.5 $\pm$

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TABLE I. Effect of Cycloheximide on Leucine Incorporation.

Cycloheximide (mg/kg)	Specific activities of proteins (dpm/mg)					
	Plasma	Liver	Kidneys	Stomach	Small bowel	Large bowel
0 (6)[a]	944 ± 53	666 ± 44	437 ± 22	1500 ± 95	1205 ± 65	846 ± 52
0.5 (6)	392 ± 14[b]	269 ± 9[b]	116 ± 10[b]	594 ± 27	719 ± 27[b]	451 ± 24[b]

[a] ( ) = number of rats; females of 200 g were given cycloheximide i.p. 3 hr prior to the i.p. injection of 5 μCi of *dl*-leucine-1-^{14}C and were sacrificed 4 hr later (7-hr postcycloheximide).

[b] $p < 0.01$.

3.24% versus 29.3 ± 5.31% in 6 control and 6 treated rats, respectively, $p < 0.05$). Radioactivity in the liver and kidney was proportionately less in the treated rats. Uptake of labeled vitamin B_{12} by liver and kidneys was not influenced by cycloheximide when vitamin B_{12} was injected intraperitoneally and animals sacrificed 4 hr later; 5.8 ± 0.49% and 4.5 ± 0.24% of the injected dose was present in the livers of 6 control and 6 treated rats, respectively, and 16.2 ± 0.55 and 17.4 ± 0.39%, respectively, in the kidneys.

The effect of cycloheximide upon the absorption of vitamin B_{12} by the small bowel was investigated utilizing the isolated small intestine *in situ*. Vitamin B_{12} alone or with sources of intrinsic factor (either gastric mucosa homogenate or gastric juice) from *untreated* rats was introduced into the duodenum of the isolated loops of control and cycloheximide-treated rats. The latter had significantly less vitamin B_{12} absorption on the basis of radioactivity present in the lumen, liver, and kidneys (Table III). The absorption of some vitamin B_{12} by the first two groups in this experiment which were not given intrinsic factor is attributed to the presence of some endogenous intrinsic factor in the loop. The data in this experiment indicate that cycloheximide had an inhibitory effect on small bowel absorption unrelated to intrinsic factor production.

Quantitative aspects of intrinsic factor production were investigated by comparing the potency of gastric juice obtained from cycloheximide-treated and control rats in enhancing vitamin B_{12} absorption from the isolated loops of small bowel of normal rats. In the first type of study, intrinsic factor–vitamin B_{12} complex was obtained from donor rats previously given cycloheximide or saline as described in Table IV. Two ml of this preparation equal to 20% of the gastric contents were introduced into duodenum of normal rats with isolated loops to test the potency of the intrinsic factor produced by both groups of animals. The gastric juice from the treated

TABLE II. Effect of Cycloheximide on Gastric Emptying and Absorption of Vitamin B_{12}.

Cycloheximide (mg/kg)	After B_{12} (hr)	^{57}Co radioactivity (% dose)		
		Stomach content	Liver	Kidneys
0 (4)[a]	1	5.91 ± 2.10	0.19 ± 0.03	0.03 ± 0.01
0.5 (4)	1	30.61 ± 9.08*[b]	0.10 ± 0.01*	0.03 ± 0.01
0 (4)	3	0.08 ± 0.02	1.59 ± 0.07	1.70 ± 0.13
0.5 (4)	3	21.80 ± 4.40**	0.39 ± 0.06**	0.30 ± 0.06**
0 (4)	6	0.07 ± 0.03	2.03 ± 0.52	3.87 ± 0.55
0.5 (4)	6	0.62 ± 0.10**	0.64 ± 0.12*	1.27 ± 0.32**

[a] ( ) = number of rats; females of 200 g were used and sacrificed at various hours after intragastric administration of 20 mμg of vitamin B_{12}-^{57}Co which was given 2 hr after cycloheximide injection. The percentage dose in the liver and kidneys was calculated after subtracting the labeled vitamin remaining in the stomach.

[b] * $p < 0.05$; ** $p < 0.01$ in comparing control and treated rats at same time interval.

TABLE III. Effect of Cycloheximide on Vitamin B_{12} Absorption from Intestinal Loops with Intrinsic Factor (IF) from Normal Rats.

Cycloheximide (mg/kg)	IF	^{57}Co radioactivity (% dose)			
		Loop content	Loop wall	Liver	Kidneys
0 (6)[a]	0[b]	77.0 ± 4.77	15.0 ± 2.78	0.49 ± 0.057	0.60 ± 0.073
0.5 (7)	0	85.6 ± 2.29	10.6 ± 2.09	0.27 ± 0.029**[c]	0.40 ± 0.052*
0 (6)	Mucosa[c]	58.3 ± 3.93	28.1 ± 3.83	0.98 ± 0.204	0.86 ± 0.114
0.5 (6)	Mucosa	74.1 ± 2.33**	24.2 ± 1.77	0.26 ± 0.042**	0.44 ± 0.092*
0 (8)	Gastric juice[d]	59.0 ± 2.86	20.9 ± 1.31	1.94 ± 0.206	1.67 ± 0.268
0.5 (8)	Gastric juice	72.1 ± 1.22**	17.1 ± 0.77*	0.77 ± 0.070**	0.70 ± 0.047**

[a] ( ) = number of rats; fasted females of 200 g.

[b] Two hr after cycloheximide injection 20 mμg of vitamin B_{12}-^{57}Co were introduced into duodenal lumen of isolated small intestine *in situ*.

[c] A 20-mμg dose of vitamin B_{12}-^{57}Co and mucosal homogenate from normal rat stomach were given.

[d] A 17.4-mμg dose of vitamin B_{12}-^{57}Co and 0.6 ml of gastric juice from normal rats were used. Animals were sacrificed 5 hr after introduction of vitamin B_{12} into the intestinal loops.

[e] *, **, same as footnote *b* in Table II.

rats was consistently less effective in promoting vitamin B_{12} absorption as compared to controls as indicated by the various parameters tested (Table IV).

When similar absorption experiments were conducted with the intrinsic factor–B_{12} complex being administered on the basis of equal amounts of solids rather than percentage of gastric secretion, there was less difference between control and treated animals in the potency of their respective intrinsic factors (Table V). While there was usually no significant difference in any parameter tested in experiments of this type, there was a consistent trend for liver and kidney radioactivity to be less when the gastric juice from the cycloheximide-treated rats was utilized. The pooled data of liver uptake of 18 controls and 19 treated rats from 3 such experiments was significantly different (1.24 ± 0.127 vs 0.89 ± 0.064%, $p < .05$). The potency of these lyophilized gastric juice preparations was also tested in everted sacs of hamster distal small intestine incubated in 10 ml Krebs–Ringer bicarbonate buffer at pH 7 at 37° for 1 hr. When this system was tested with gastric juice from control rats at 0, 0.05, 0.1, and 0.25 mg of solids and 0.4 mμg of vitamin B_{12}-^{57}Co per ml, the uptakes were 0.69, 0.70, 1.15, and 1.90%, respectively. In a typical experiment with 0.1 mg/ml the control material resulted in 1.28 ± 0.123% uptake where-

TABLE IV. Intrinsic Factor (IF) Activity in Gastric Juice Obtained from Control and Cycloheximide-Treated Rats (as tested in normal rats with isolated loops).

Source of IF	^{57}Co radioactivity (% dose)			
	Loop content	Loop wall	Liver	Kidneys
Control (6)[a]	53.6 ± 2.81	29.9 ± 1.26	2.61 ± 0.190	2.91 ± 0.340
Treated (6)	70.7 ± 3.84[b]	25.8 ± 2.32	1.37 ± 0.109[b]	1.40 ± 0.117[b]

[a] ( ) = number of rats; females of 150 g. Intrinsic factor–vitamin B_{12} complex was obtained from donor rats by introducing 100 mμg of vitamin B_{12}-^{57}Co into their stomachs following pylorus ligation and aspirating the gastric juice 4 hr later; the pH was adjusted to 12 with 2 *N* NaOH for 20 min to inactivate pepsin and then to pH 7 with 1 *N* HCl, a uniform volume of 10 ml was made with water and the material was stored at —20° until used. Two ml were injected into the duodenum of normal rats with isolated loops.

[b] $p < 0.01$.

TABLE V. Potency of Lyophilized Gastric Juice (IF) on Equi-weight Basis from Normal and Cycloheximide-Treated Rats.

Source of IF	^{57}Co radioactivity recovered (% dose)			
	Loop content	Loop wall	Liver	Kidneys
Control (7)[a]	70.6 ± 2.84	16.9 ± 1.70	1.23 ± 0.123	1.17 ± 0.159
Treated (7)	72.0 ± 2.48	19.1 ± 2.01	0.89 ± 0.137	0.85 ± 0.186

[a] ( ) = number of rats; males of 200 g. Gastric juice was collected from control and cycloheximide-treated rats for 4 hr after pylorus ligation; the pH was adjusted to 12 with 2 *N* NaOH for 20 min and brought to 7 with 1 *N* HCl. The material was dialyzed against distilled water for 18 hr at 4° with 4 changes of the bath, then centrifuged at 3000 rpm at 4° for 10 min; the supernatant was lyophilized. Twenty mμg of vitamin B_{12} were added to 5 mg of lyophilized gastric juice in a total volume of 1 ml of water and introduced into the duodenum of normal rats with isolated loops which were sacrificed 4 hr later.

as that from cycloheximide-treated rats had 0.84 ± 0.055% uptake ($p < 0.01$).

Discussion. Cycloheximide reduced the absorption of ^{57}Co, presumably as intact vitamin B_{12}, by three different actions; namely, slowing the normal rate of gastric emptying, decreasing the amount of intrinsic factor secreted in a given time and interfering with absorption in the small intestine.

The cause of delayed gastric emptying with cycloheximide is unknown. As might be expected, it occurred to an even greater extent when fat was present (6) and is associated with decreased gastric volume, pepsin, and acidity (9). Gastric juice from cycloheximide-treated rats is less active in its intrinsic factor activity than that from controls. This is most clearly revealed in the studies where proportional volumes were used. However, the trend was the same but to a lesser degree when equal amounts of a lyophilizate of gastric solids were compared. These data are interpreted as indicating that intrinsic factor was a smaller proportion of the material; however, synthesis of inactive intrinsic factor cannot be ruled out. Cycloheximide appears to have an inhibitory effect on both parietal and chief cell functions which may involve decreased protein synthesis on a general scale by virtue of its interference with transfer of activated amino acid from amino acyl-sRNA into ribosomes (10). Synthesis of intrinsic factor—a glycoprotein of molecular weight of about 60,000 (11)— may be inhibited by this same mechanism.

The basis of the inhibitory action of cycloheximide on vitamin absorption in the small bowel is also speculative. The mechanism of transfer of vitamin B_{12} into and across the mucosal cells is unknown. Interference with the production of a carrier or transport protein is possible and recently a protein differing in its properties from intrinsic factor was reported to occur at the receptor site during active vitamin B_{12} absorption (12). It will be of interest to determine whether this protein occurs in normal amounts following cycloheximide administration.

Cycloheximide is added to actinomycin D and colchicine as powerful inhibitors of protein synthesis capable of interfering with vitamin B_{12} absorption. Whether these actions are directly related requires further study.

Summary. Cycloheximide given parenterally to rats delays gastric emptying of vitamin B_{12}-^{57}Co, decreases the secretion rate of intrinsic factor and inhibits absorption of the vitamin in the small intestine even in the presence of intrinsic factor.

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Effect of External Potassium and Ouabain on Sodium Efflux from Frog Sartorius Muscle* (33769)

W. McD. Armstrong (Introduced by E. E. Selkurt)

Department of Physiology, Indiana University School of Medicine, Indianapolis, Indiana 46202

It is well known that the efflux of Na ions from frog skeletal muscle depends upon the external K concentration (1). It is also well established that this K stimulated efflux of Na, as well as the net exchange of Na and K ions in this tissue, is inhibited by the cardiac glycoside ouabain (G-strophanthin) and its aglycone strophanthidin (2–7). The inhibitory effects of cardiac glycosides and aglycones on Na and K transport appear to be quite general and have been demonstrated in a large variety of cells and tissues (8).

In several instances, it was found that the inhibitory effects of cardiac glycosides and related substances can be reversed by increasing the external K concentration (9–12). It has been suggested that, in some cells at least, cardiac glycosides in low concentrations compete with K for a site in the cell membrane which is implicated in the active transport of Na, though quantitative demonstration of a competitive relationship has proved difficult (9–11).

Despite the widespread use of cardiac glycosides in studies of Na and K transport in frog skeletal muscle, comparatively few studies were made of the interrelationship between the K. concentration of the external medium and the inhibitory effect of these substances. The present paper is concerned with some aspects of this interrelationship.

Materials and Methods. Pairs of freshly dissected sartorius muscles weighing about 70–80 mg from a single frog (*Rana pipiens*) were immersed overnight at 5° in K-free Ringer's containing 120 meq of Na/liter. The Na was labeled with a tracer amount of ^{24}Na (obtained as $^{24}NaCl$ in HCl solution from the Oak Ridge National Laboratory). Following this immersion, the muscles were carefully blotted, weighed, and washed out for 3 hr at room temperature (K25°) in 5-ml aliquots of nonradioactive Ringer's solutions containing 104 meq of Na/liter. These aliquots were subsequently assayed for ^{24}Na using a well scintillation detector.

The washout procedure for each pair of companion muscles was as follows. One member of the pair was kept in K-free Ringer's throughout. Its companion was first washed out in an identical K-free solution for 1 hr. During the second hour it was transferred to successive aliquots of a solution containing 104 meq of Na/liter together with a constant amount of KCl. Finally it was washed out for a third hour in a solution containing 1×10^{-6} *M* ouabain[1] but identical in other re-

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[1] This concentration of ouabain was chosen because, although it is high enough to cause virtually