Evidence Against Preferential Intestinal Absorption of Physiologic Quantities of Liver-Bound Vitamin B_{12} by Patients with Pernicious Anemia

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IN 1959 it was reported[1] that the fecal excretion of radiovitamin B_{12} bound in pig liver (liver-bound vitamin B_{12}) by patients with pernicious anemia was much less than that of free radiovitamin B_{12}. These findings suggested either preferential absorption of the liver-bound vitamin B_{12} or a nonspecific delayed intestinal passage (i.e., fecal excretion) of this material. Subsequent experiments,[2] which suggested the presence of intrinsic factor in bile, raised a third possibility—that the preferential absorption of liver-bound vitamin B_{12} may have been caused by the presence of variable quantities of intrinsic factor in the preparation. On the contrary, enhanced absorption of liver-bound radiovitamin B_{12} could not be demonstrated in either gastrectomized rats[3] or in patients with intestinal malabsorption.[4] Other studies[5] indicate that coenzyme vitamin B_{12} represents the majority of the cobamide in human liver, and that cyanocobalamin and hydroxycobalamin, previously isolated from liver, are mainly artefacts produced by chemical decomposition of coenzyme vitamin B_{12}. Liver-bound vitamin B_{12} present in noncyano form,[6] because of its high affinity to various proteins, might well be excreted more slowly than cyanocobalamin. Earlier, it had been speculated that liver-bound vitamin B_{12} and coenzyme vitamin B_{12} may be the same substance.[7] The present studies, in which measurements of the absorption of liver-bound vitamin B_{12} were made by four methods, would tend to support the possibility that delayed fecal excretion may have explained the initial results.

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MATERIALS AND METHODS

Six subjects with pernicious anemia in remission with minimal therapy (30 μg. of cyanocobalamin given intramuscularly monthly) and one subject with untreated pernicious anemia were selected for study on the metabolic ward of the Thorndike Memorial Laboratory. Hog intrinsic factor concentrate (HIFC) was used in a dose of 50 mg. in order to augment vitamin B_{12} absorption when so indicated.

Preparation of Liver

The liver was prepared by giving a two week old pig injections of a total of 19 μg. Co^{60}-B_{12} (approximate specific activity 1 μc. per μg.) in divided doses during forty-one days, and, after a ten day rest period, 0.33 μg. Co^{58}-B_{12} (specific activity approximately 150 μc. per μg.) in three divided doses. The pig was killed five days after the final injection. Gamma spectrometry six days after the sacrifice showed 0.024 μc. Co^{58} and 0.0034 μc. Co^{60}

per gm. liver, wet weight. The microbiologic activity as measured by E. gracilis[8] was 0.147 μg. per gm. wet weight. This figure represents the *total* activity, i.e., the extractable activity of 0.119 μg. per gm. multiplied by 100/81, since only 81 per cent of the total radioactivity was removed from the liver by our extraction procedure.[9] The extractable microbiologic activity as measured by L. leichmanii 313, ATCC 7,830[10,11] was 0.11 μg. per gm. The specific activity of the liver-bound vitamin B_{12} was 0.19 μc. per μg., based on the more specific E. gracilis assay. Three hundred grams of the liver was passed through the coarse and then the fine grate of a kitchen vegetable grater in a cold room at 3°c. This grated pig liver was then thoroughly mixed to ensure uniformity. (Similar uniformity of all samples was ensured in the original study by passing the liver through a meat grinder and thoroughly mixing the ground liver prior to preparing test aliquots.)

For *in vivo* studies, 5 parts of grated pig liver were thoroughly mixed with 7.5 parts of finely minced onion, 9 parts of Heinz tomato ketchup, and a liberal amount of salt and pepper. The mixture was divided into individual portions of 43 gm. each, wrapped in aluminum foil, and then frozen. Each portion contained 10 gm. of liver and 1.47 μg. of vitamin B_{12}.

For *in vitro* studies, 10 gm. of the pig liver were minced, homogenized for one minute with 10 ml. of 0.9 per cent sodium chloride in a Waring Blendor, and centrifuged at 3,000 r.p.m. for ten minutes. One milliliter aliquots of the supernate, which counted 1,353 c.p.m. above the background of 125 c.p.m., were tested without and with added normal human gastric juice as a source of intrinsic factor in both a rat liver homogenate[12] and a guinea pig gut homogenate[13] system.

Administration of Liver

Each morning the patients participating in the study were served with freshly thawed individual portions of the liver and condiment mixture on a slice of white bread, in lieu of breakfast. One milligram of non-radioactive-vitamin B_{12} then was administered intramuscularly to each subject undergoing the Schilling test; this was followed by a second injection of 1 mg. of nonradioactive vitamin B_{12} the next morning.[14] Control tests (Schilling and Glass tests) were performed with 2 μg. vitamin B_{12} containing 0.25 μc. Co^{60}.

Measurement of Absorption

Complete urine collections were obtained for the two twenty-four–hour periods immediately following the oral dose in the subjects to be tested (Schilling test). Each twenty-four–hour urine collection was boiled down to 200 ml. over a gas flame, using 1 ml. of a 1:10 dilution of Tween 80* to prevent foaming. The amount of radioactivity in the 200 ml. urine concentrate was then determined using a large well-type scintillation detector. A large probe detector, usually used for determining the I^{131} radioactivity uptake by the thyroid gland, was used to determine radiovitamin B_{12} uptake by the liver in three subjects.[15]

The patient with untreated pernicious anemia was given an individual portion of 1.47 μg. liver-bound vitamin B_{12} daily for ten days.

TABLE I

Urinary Excretion of Radioactive Vitamin B_{12} Administered Liver-Bound or Free, without or with Added Intrinsic Factor

Patient	% of Ingested Radioactive Vitamin B_{12} Excreted in 48 Hours			
	After Liver-Bound Vitamin B_{12}		After Free Cyanocobalamin	
	Alone	+ HIFC*	Alone	+ HIFC
1	0.7	...	1.6	12.3
2	0.9	...	3.6	24.0
3	0.9	...	0	9.8
4	0.8	5.0	0.86	8.0
5†	...	...	1.5	6.2

* Hog intrinsic factor concentrate.
† Untreated pernicious anemia.

RESULTS

As indicated in Tables I and II, none of the subjects studied absorbed significant amounts of radioactive vitamin B_{12} bound in pig liver, unless it was administered with HIFC.

The patient with untreated pernicious anemia (hematocrit 15.5 per cent) had no response to a ten-day course of therapy with daily doses of liver bound vitamin B_{12} orally. In fact, serum iron rose from 193 to 303 μg. per 100 ml. during this therapy, instead of falling sharply as it does when adequate therapy is given.[16,17] This patient subsequently had a hematologic response (11.3 per cent reticulo-

* Polyoxyethylene sorbitan mono-oleate, Ajax Powder Co., Wilmington, Delaware.

TABLE II
Hepatic Uptake of Radioactive Vitamin B_{12} Administered Liver-Bound or Free, without or with Added Intrinsic Factor

Patient	% Increase over Baseline Radioactivity of Liver Projection			
	After Liver-Bound Vitamin B_{12}		After Free Cyanocobalamin	
	Alone	+ HIFC*	Alone	+ HIFC
5	0†	...	...	...
6	0	38.2	2.1	13.1
7	0	13.2	10.2	85.7

* Hog intrinsic factor concentrate.

† This count obtained eight days after the tenth daily dose of liver-bound vitamin B_{12}. At this time, although there was no radioactivity in the liver, the left lower quadrant of the abdomen counted twice background.

cytes on the fifth day) after starting therapy with 0.1 μg. of vitamin B_{12} given intramuscularly daily.[18] This suggests that the patient absorbed less than 0.1 μg. of the 1.47 μg. of liver-bound vitamin B_{12} administered daily. Reticulocytes did not rise above 0.5 per cent at any time during the ten days of liver ingestion.

Radioactive vitamin B_{12} in a saline homogenate of pig liver was not taken up *in vitro* by either rat liver homogenate or guinea pig gut homogenate. Added human gastric juice did not bring about such adsorption, suggesting that the vitamin B_{12} was still bound to non-intrinsic factor materials and unavailable for binding to intrinsic factor. When liver-bound vitamin B_{12} is given orally, however, its absorption is enhanced by intrinsic factor (Tables I and II), indicating that it is freed from its non-intrinsic factor bond in the gastrointestinal tract and then bound to intrinsic factor, as occurs with vitamin B_{12} in other foods.[19]

Note in Tables I and II that the absorption of liver-bound vitamin B_{12} is not enhanced as much by intrinsic factor as is that of free vitamin B_{12}. This was previously found to be true for the absorption of coenzyme vitamin B_{12}[7] as compared to that of free vitamin B_{12}. This would tend to support the possibility that liver-bound vitamin B_{12} and coenzyme vitamin B_{12} may be the same substance, as previously speculated.[7]

COMMENTS

There are several points of difference between the present study and the original study: (1) In the original study, because of the low specific activity of vitamin B_{12} in the liver, only two of the ten patients with pernicious anemia received amounts of liver-bound vitamin B_{12} which were close to the physiologic level. In the present study, the specific activity of the radiovitamin B_{12} bound in the liver was increased by a factor of 20, allowing the use of physiologic quantities (1.47 μg.) in all seven subjects. "Physiologic" quantities are defined as orally administered doses of vitamin B_{12} up to 2 μg. The absorption of such doses is intrinsic factor-dependent. When "supraphysiologic" doses are used, a small aliquot appears to be absorbed by a mass action phenomenon.[20] Liver-bound vitamin B_{12} given in supraphysiologic quantities seems to be partly retained by patients with pernicious anemia.[1]

(2) Radiovitamin B_{12} bound in a different pig liver was used in the present study. If the biliary radicles of liver contained intrinsic factor, for which some evidence has been presented,[2] there may be variable quantities of intrinsic factor in different livers. Morphologically, both the original pig liver and the present one were normal. However, the original pig liver contained 0.56 μg. of vitamin B_{12} per gm. which is about the normal concentration,[21] whereas the present one contained only 0.147 μg. per gm. by E. gracilis assay. The relatively lower concentration of vitamin B_{12} per gram of the present liver would suggest that such intrinsic factor as was available for binding the vitamin would need to be present in free form in only one-fourth the concentration necessary in the original pig liver. However in the saline homogenate of liver tested *in vitro* there was no evidence of intrinsic factor.

(3) In the original study, the fecal excretion test was the measure of vitamin B_{12} absorption. The major possible source of error was the possibility of incomplete collections of feces. Fecal excretion studies were performed in two

TABLE III

Retention of Liver-Bound and Free Vitamin B_{12} in Patients with Pernicious Anemia in Relapse and Remission

Form in Which Vitamin B_{12} Was Given	Case No.*	Relapse (% retained)	Case No.*	Remission (% retained)	Degree of Significance Between Means†
Liver-bound vitamin B_{12}	3	69	7	40	$0.10 > p > 0.05$
	8	45	16	16	
	9	91	20	2	
	17	18	21	0.7	
	18	36			
	19	19			
	M ± SE	46.3 ± 11.8		14.43 ± 9.12	
Free vitamin B_{12}	8	4	16	2	$0.10 > p > 0.05$
	9	9	20	1	
	17	1.2	21	0.4	
		13			
	18				
	19	13			
	M ± SE	8.04 ± 2.38		1.13 ± 0.47	

* Taken from Table III in the original publication.[1]

† Because of the smallness of the samples the formula:

$$t^2 = \frac{(\bar{x}_1 - \bar{x}_2)^2 (n_1 + n_2 - 2)}{(1/n_1 + 1/n_2)(S_1 - S_2)}$$ was used, where, $\bar{x}$ = mean, n = number of cases, $S = \Sigma(x - \bar{x})^2$.

of the seven subjects of the present study. The results were contradictory to the other studies in indicating good absorption of not only liver-bound vitamin B_{12} but also of free cyanocobalamin made up to have the same specific activity. The apparent good absorption was most probably due to incomplete collections of feces. As indicated in Table II (patient 5) liver-bound vitamin B_{12} may be retained in the colon for a longer time than cyanocobalamin.[22] This may explain the original results.

The Schilling test data of the present study, which are subject to the possible errors of incomplete urine collection and of preferable retention of noncyano forms of cobalamin[23–28] did not indicate preferential absorption of liver-bound vitamin B_{12} over free cyanocobalamin in subjects with pernicious anemia. Furthermore, neither the patients tested by the hepatic radioactivity uptake method nor the subject tested by therapeutic trial (neither of which modality is subject to the possibly invalidating errors which may occur with the Schilling test) indicated any evidence for preferential absorption of liver-bound vitamin B_{12}.

(4) In the original study, six patients were in relapse whereas all but one of the present patients were in remission. It is possible that absorption by diffusion of vitamin B_{12} is higher in patients with acute vitamin B_{12} deficiency.[29] A breakdown of the group studied originally into those in relapse and those in remission seems to support this hypothesis (Table III). The retention of both unbound cyanocobalamin and liver-bound vitamin B_{12} is higher in the relapse group, although the differences are not quite significant statistically. Such a mechanism may also explain the response of some patients with pernicious anemia to small (5 μg. thrice daily) oral doses of cyanocobalamin.[30]

SUMMARY

The absorption of physiologic quantities (1.47 μg.) of liver-bound vitamin B_{12} was compared with the absorption of free cyanocobalamin (2 μg.) in seven subjects with pernicious anemia. Urinary excretion of radiovitamin

B_{12} was determined in five subjects, hepatic uptake in three subjects, and therapeutic trial in one subject. There was no evidence for preferential absorption of liver-bound vitamin B_{12}. Liver-bound vitamin B_{12} absorption was enhanced by intrinsic factor, suggesting it becomes free of its liver bond in the intestine.

In vitro studies, using both a liver homogenate and a guinea pig gut homogenate system, also indicated no preferential uptake of liver-bound vitamin B_{12}.

The apparent preferential absorption of liver-bound vitamin B_{12} in the original study, may be explainable, as considered in the original report, by delayed excretion rather than enhanced absorption of liver-bound vitamin B_{12}, which is in a noncyano form, or to possible increased absorption by diffusion in acute vitamin B_{12}-deficiency of the supraphysiologic quantities of vitamin B_{12} administered. No such increased absorption by diffusion was demonstrated in the patient undergoing therapeutic trial in the present study, however, using a "physiologic" quantity of liver-bound vitamin B_{12}.

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Original Communications

Perspectives in Nutrition

Erratum

In the article entitled "Evidence Against Preferential Intestinal Absorption of Physiologic Quantities of Liver-Bound Vitamin B_{12} by Patients with Pernicious Anemia" by L. W. Sullivan, V. Herbert and P. Reizenstein (*Am. J. Clin. Nutrition*, 11:568, 1962), the figure 13.1 in Table II is incorrect; it should be 131.