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Intrinsic Factor Studies VI. Competition for Vit. B_{12} Binding Sites Offered by Analogues of the Vitamin.* (23548)

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The mechanism by which intrinsic factor, a component of normal human gastric juice, enhances absorption of vit B_{12} from the gut has not yet been elucidated. Recently reported evidence(1,2) indicates that vit B_{12} which is bound to gastric juice is absorbed preferentially over unbound vitamin. This observation suggests that binding of vit B_{12} is an essential property of intrinsic factor. However, the ability to bind vit B_{12} is an attribute of several biologic substances which do not possess intrinsic factor activity(3-7). As far as we are aware, there are no published reports of preparations possessing intrinsic factor activity but lacking the property of binding B_{12}. It is possible that binding of vit B_{12} by intrinsic factor is a unique process as compared to binding of the vitamin by substances lacking intrinsic factor activity. To determine if the vit B_{12} binding mechanism of intrinsic factor is more fastidious than that of other vit B_{12} binding proteins, we have measured competition offered by certain vit B_{12} analogues for vit B_{12} binding sites of several biologic fluids. This is a report of such observations.

The effect of an excess of one vit B_{12} analogue, pseudovit B_{12}, on radioactive vit B_{12} binding by human gastric juice has been reported(7). Binding of vit B_{12} was only slightly reduced in the presence of a 50-fold excess of the pseudovitamin. It was concluded, then, that this analogue presents little competition for vit B_{12} binding sites of gastric juice. Gregory and Holdsworth(8) have made somewhat analogous observations on preferential binding of vit B_{12} by an intrinsic factor preparation derived from hog mucosa.

Materials and methods. Vit B_{12} analogues. Ten crystalline analogues of vit B_{12}† were studied. Three of the analogues,‡ sulfatocobalamin (SU-B_{12}), nitrocobalamin (NI-B_{12}), and chlorocobalamin (CL-B_{12}), hold sulfate, nitrate, or chloride ions, respectively, in lieu of the cyanide ion(9). In 4 other B_{12} analogues, desdimethylbenziminazole B_{12} (DDMBI-B_{12}),§ 5(6)-trifluoromethylbenziminazole B_{12} (TFMBI-B_{12}),§ 5(6)-hydroxybenziminazole B_{12} (HBI-B_{12})‖ or Factor III, and 5(6)-aminobenziminazole B_{12} (ABI-B_{12}),§ the side chains on the benziminazole moiety differ from those 2 methyl groups filling comparable loci in B_{12}(10,11). If adenine occupies the position filled by 5,6-dimethylbenziminazole in the B_{12} molecule, the analogue is known as pseudovit B_{12}(pseudo-B_{12})¶ (12,13), the sample of which used for this project was entirely free of B_{12}. Lactam (lactam-B_{12})§ and lactone (lactone-B_{12})§ forms result from cyclization of the acetamide

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† Vit. B_{12} (cyanocobalamin) = B_{12}.

‡ Kindly donated by C. Rosenblum, Merck and Co.

§ Kindly donated by E. L. Smith and K. E. Fantes, Glaxo Labs., England.

‖ Kindly donated by K. Bernhauer, Aschaffenburger Zellstoffwerke AG., Germany.

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TABLE I. Failure of $B_{12}Co^{60}$ Molecules to Dissociate from Gastric Juice (GJ) Binding Sites in Presence of Excess Non-radioactive Vitamin.

Aliquot	Order of addition (x = 10 mμg/ml)	% retention $B_{12}Co^{60}$ after dialysis
1	1x $B_{12}Co^{60}$ GJ*	96
2	1x $B_{12}Co^{60}$ 50x B_{12} GJ*	7.9
3	50x B_{12} GJ* 1x $B_{12}Co^{60}$*	4.5
4	1x $B_{12}Co^{60}$ GJ* 50x B_{12}*	87
5	1x $B_{12}Co^{60}$ 50x B_{12} H_2O	.2

* One hr of equilibration at room temperature followed addition of this substance. Refer to Fig. 1 for B_{12} binding capacity.

chain on ring B of B_{12}. The preparation of the 2 latter compounds, free from traces of the other or of B_{12}, has been described(14). Analogues SU-B_{12}, NI-B_{12}, CL-B_{12}, and pseudo-B_{12} were weighed on a micro-balance, dissolved in fresh 0.85% saline, and autoclaved. DDMBI-B_{12}, TFMBI-B_{12}, HBI-B_{12}, lactam-B_{12}, and lactone-B_{12} were dissolved in aqueous 0.5% phenol and the optical density of each solution determined in a Beckman DU Spectrophotometer at 361 mμ. The concentration of each analogue was calculated on the assumption that the $E^{1\%}_{1\,cm}$ at 361 mμ is the same as that for B_{12}. Only one of the analogues, ABI-B_{12}, was received in solution. When adding 50-fold amounts of analogues. their molecular weights were considered to equal that of B_{12}. The same B_{12} analogue stock solutions were used throughout the observations reported herein. *Biologic substances.* Gastric juice was collected after histamine stimulation from at least 15 donors, pooled, and processed as previously described (15). Boiled gastric juice was prepared by placing a sample in a test tube in boiling water for 10 minutes. Saliva was collected from one person prior to meals and filtered through coarse sintered glass. Colostrum was collected from 7 post-partum mothers and pooled. Liver and kidney extracts were prepared by homogenizing the tissue in water in a high speed grinder-mixer. The supernate from such a homogenate was dialyzed against water. Liver extract required centrifugation and filtration after dialysis to yield a relatively clear preparation. Intrinsic factor concentrate (IFC)** was from hog gastric mucosa. All of these materials, when in solution, were stored at −12°C. *Assay.* Binding of radioactive B_{12}†† by biologic substance was determined quantitatively by comparing amounts of radioactivity before and after dialysis in Visking tubing against running tap water for 60 hours(7). The effect of excess analogue on B_{12} binding was tested by adding a sample of biologic material to a tube containing a measured amount of radioactive B_{12} (1 ×) and 50 times that amount of B_{12} analogue (50 ×). This order of addition of materials was varied for the experiments documented in Table I. By observing per cent retention of radioactive B_{12} and by comparing this percentage with that in a "control" tube; *i.e.*, a tube containing biologic material and 1 × radioactive B_{12} only, it was known to what extent excess analogue had affected B_{12} binding. When water rather than biologic substance was added to the same amount of 1 × $B_{12}Co^{60}$ and 50 × B_{12}, retention of $B_{12}Co^{60}$ after dialysis was less than one per cent.

Results. I. *B_{12} binding capacity.* When increasing amounts of B_{12} are added to gastric juice, the amount of B_{12} bound increases until a maximum or plateau is reached (Fig. 1). When large excesses of B_{12} are added, the binding capacity is slightly increased. Boiling gastric juice reduces markedly, but does not eliminate, binding of B_{12} (Fig. 1). The B_{12} binding curves for saliva, colostrum, serum, and aqueous liver and kidney extracts are shown in Fig. 1 and 2. Saliva binds as much B_{12} per mg protein as does gastric juice but it is known(16) that saliva does not act as intrinsic factor. The B_{12} binding curve of liver or kidney is a straight line rather than an

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†† Radioactive B_{12} = $B_{12}Co^{60}$, a portion of which was donated by C. Rosenblum, Merck & Co.

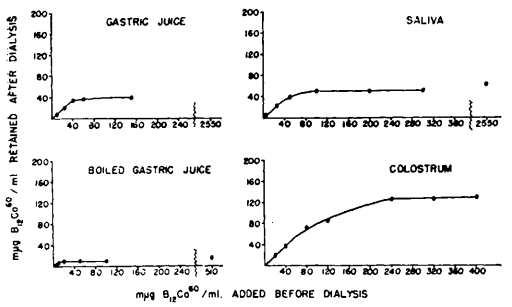

FIG. 1. Vit. B_{12} binding capacity of pooled neutralized normal human gastric juice (Biuret protein = 1.2 mg/ml), saliva (Biuret protein = 0.9 mg/ml), and colostrum (Biuret protein = 77 mg/ml).

ascent to a plateau. In accord with a report by Bird and Hoevet(3) lysozyme does not prevent B_{12} from passing out of the dialysis bag.

II. *Stability of B_{12} binding.* To determine the degree of dissociability of gastric juice-bound B_{12} in the presence of excess vitamin, the experimental plan outlined in Table I was devised. It is apparent that, given a chance for prior binding, most of the $B_{12}Co^{60}$ molecules remain bound even in the presence of a 50-fold excess of non-radioactive vitamin (aliquot 4). If there had not been any exchange of vitamin molecules in this aliquot, however, per cent retention would have equalled that in aliquot 1. Postdialysis radioactivity in aliquot 3 was less than in the second test aliquot. If there had not been *any* exchange of vitamin molecules in the third aliquot, essentially no radioactivity would have survived dialysis. The amount of B_{12} added, 500 mμg/ml, greatly exceeded the binding capacity of gastric juice, and thus should have prevented binding of $B_{12}Co^{60}$ added subsequently. The differences between the data in aliquots 2 and 3 are reproducible. A *second experiment,* which strengthened this observation, was done as follows: An amount of $B_{12}Co^{60}$ known to saturate all B_{12} binding sites at the 1 × level (40mμg/ml) was added to gastric juice samples and allowed to equilibrate at room temperature for one hour. Next, 50 times that amount of non-radioactive B_{12} was added and allowed to equilibrate at 4°C for varying lengths of time, 2 minutes to 71 hours, before dialysis. (The low temperature was maintained to avoid possible deterioration of intrinsic factor.) Retention of $B_{12}Co^{60}$ was not lessened as the time of equilibration before dialysis was increased. Stated in another way, these results demonstrate that complete equilibrium between previously bound $B_{12}Co^{60}$ and a subsequently added excess of non-radioactive B_{12} occurred within a few minutes after addition of the latter.

III. *Competition for B_{12} binding sites by analogues.* If the amount 1 × $B_{12}Co^{60}$ is not sufficient to saturate all B_{12} binding sites of gastric juice, competition for those sites offered by an analogue may not be manifest. This was clearly seen in one experiment, the results of which are listed in Table II. It has been established that pseudo-B_{12} offers slight competition for B_{12} binding sites of gastric juice(7). However, when the amount of $B_{12}Co^{60}$ added was not sufficient to saturate the B_{12} binding sites, competition by pseudo-B_{12} was not observed. (Compare aliquots 1 and

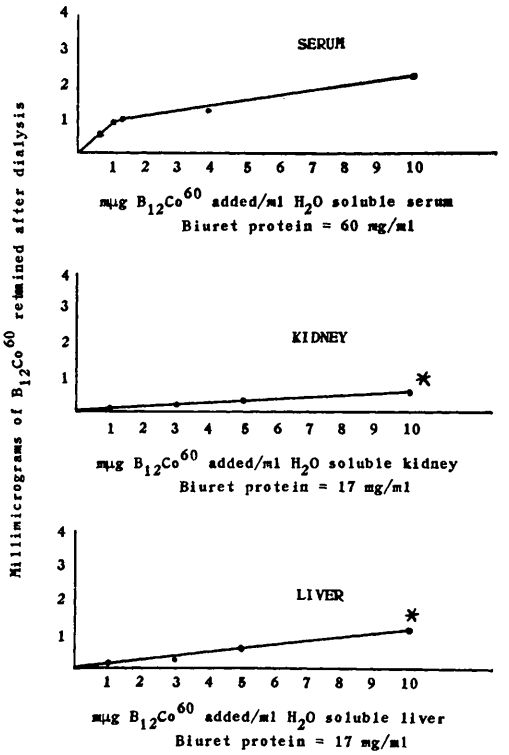

FIG. 2. B_{12} binding by normal human serum and aqueous kidney and liver extracts.

* Slope is similar when abscissa is extended to 100 mμg/ml.

TABLE II. Variation in Competition Offered by Pseudo-B_{12} for B_{12} Binding Sites of Gastric Juice (GJ) Depending upon Amount of $B_{12}Co^{60}$ Added.

Aliquot	Substances to which GJ* added: 1x $B_{12}Co^{60}$ (mμg/ml)	Substances to which GJ* added: 50x pseudo-B_{12} (mμg/ml)	% retention $B_{12}Co^{60}$ after dialysis	Calculated competition, %†
1	10		85	
2	10	500	85	.9
3	25		85	
4	25	1250	83	2.4
5	40		87	
6	40	2000	67	22
7	60		61	
8	60	3000	49	20
9	150		26	
10	150	7500	21	21

* Refer to Fig. 1 for B_{12} binding capacity.
† See text for calculation.

2; 3 and 4.) When the B_{12} binding sites were saturated, reduced retention of $B_{12}Co^{60}$ in the presence of excess pseudo-B_{12} resulted. (Compare aliquots 5 and 6.)

Included in Tables II, IV, and V, are figures for "calculated competition." This is a numerical expression of per cent reduction of the quantity of $B_{12}Co^{60}$ bound in the presence of excess analogue. The calculation is as follows:

Let A = mμg $B_{12}Co^{60}$ bound in *absence* of analogue

Let B = mμg $B_{12}Co^{60}$ bound in *presence* of analogue

Then $\frac{A-B}{A} \times 100$ = calculated competition %

From such calculations a constant value for competition is obtained in spite of increasing quantities of $B_{12}Co^{60}$. (Compare aliquots 6, 8 and 10 of Table II.) Similar observations were noted in other experiments. Thus, it was necessary to determine B_{12} binding capacity of each biologic sample prior to testing competition offered by B_{12} analogues for the B_{12} binding sites. The binding capacity is considered as that point at which there is a sharp break in the binding curve determined as in Fig. 1.

A. *Gastric juice.* Competition for B_{12} binding sites of gastric juice offered by SU-B_{12}, NI-B_{12} and CL-B_{12} was investigated. Representative data, comprising Table III, demonstrate that competition by these analogues for the B_{12} binding sites is similar to that offered by excess B_{12}. Further evidence for the similarity of binding of SU-B_{12} and B_{12} by gastric juice was obtained from the observation that binding of radioactive sulfatocobalamin‡‡ resembled binding of $B_{12}Co^{60}$; both the slope of the curve and the maximum amount bound were similar. Results of studying competition for B_{12} binding sites of gastric juice offered by DDMBI-B_{12}, TFMBI-B_{12}, HBI-B_{12}, ABI-B_{12}, lactam-B_{12}, lactone-B_{12} and pseudo-B_{12} are listed in Table IV. These analogues present differing amounts of competition for the B_{12} binding sites, pseudo-B_{12} offering least competition. Lactam-B_{12} and lactone-B_{12} are poor competitors. Excess non-radioactive B_{12} does not offer 100% calculated competition for the B_{12} binding sites because observed binding capacity increases slightly with higher vitamin concentration (Fig. 1).

B. *Intrinsic factor concentrate.* The analogues are more effective competitors for the binding sites of the hog mucosal preparation than for those of human gastric juice (Table V).

C. *Saliva.* Most of the analogues present competition similar to that offered by B_{12} for the B_{12} binding sites (Table IV).

D. *Colostrum.* Pseudo-B_{12} is not as successful a competitor for the B_{12} binding sites as is DDMBI-B_{12} or B_{12} but, yet, is a more effective one than in gastric juice (Table IV).

TABLE III. Competition for B_{12} Binding Sites of Gastric Juice (GJ) Offered by SU-B_{12}, NI-B_{12}, or CL-B_{12}.

Aliquot	Substances to which GJ added: 1x* $B_{12}Co^{60}$	50x B_{12}	50x SU-B_{12}	50x NI-B_{12}	50x CL-B_{12}	% retention $B_{12}Co^{60}$ after dialysis
1	+	—	—	—	—	82
2†	+	+	—	—	—	2.6
3	+	—	+	—	—	3.2
4	+	—	—	+	—	2.5
5	+	—	—	—	+	2.7

* x = 50 mμg/ml GJ. Refer to Fig. 1 for B_{12} binding capacity.
† When water rather than GJ was added to the same amounts of 1x $B_{12}Co^{60}$ and 50x B_{12}, % retention of $B_{12}Co^{60}$ after dialysis equalled 0.3.

‡‡ Kindly donated by C. Rosenblum.

TABLE IV. Analogue Competition for B_{12} Binding Sites of Certain Biologic Substances.

Aliquot	Substances to which biologic sample added 1x	50x	Gastric juice* (x = 40 mμg/ml) % retention $B_{12}Co^{60}$ after dialysis	Calc. competition, %	Saliva* (x = 50 mμg/ml) % retention $B_{12}Co^{60}$ after dialysis	Calc. competition, %	Colostrum* (x = 120 mμg/ml) % retention $B_{12}Co^{60}$ after dialysis	Calc. competition, %	Serum† (x = 1 mμg/ml) % retention $B_{12}Co^{60}$ after dialysis	Calc. competition, %
1	$B_{12}Co^{60}$		88		91		86		94	
2	"	B_{12}	2.6	97	2.4	97	3.6	96	9.5	90
3	"	DDMBI-B_{12}	7.9	91	2.7	97	3.9	95	11	88
4	"	TFMBI-B_{12}	11	87	2.6	97			9.2	90
5	"	HBI-B_{12}	13	85	3.7	96			11	88
6	"	ABI-B_{12}	16	82	3.6	96			15	84
7	"	lactam-B_{12}	30	66	12	87	15	83	33	65
8	"	lactone-B_{12}	61	30	10	90	26	69	53	44
9	"	pseudo-B_{12}	72	18	5.6	94	17	81	9.2	90

* Refer to Fig. 1 for B_{12} binding capacity.

† Refer to Fig. 2 for B_{12} binding.

E. *Serum.* Pseudo-B_{12} offers as much competition as does B_{12} for the binding sites of serum (Table IV).

Discussion. If binding of B_{12} in the presence of excess analogue is not changed, then that analogue is not offering any competition for the B_{12} binding sites. The analogue, pseudo-B_{12}, added in 50-fold excess of $B_{12}Co^{60}$, offers roughly 20% competition for B_{12} binding sites of gastric juice in most cases. It is known that the only difference between pseudo-B_{12} and B_{12} is that the 5,6-dimethylbenziminazole moiety of the latter is replaced by adenine(12,13). This modification strikingly reduces the ability of the molecule to compete for B_{12} binding sites of gastric juice. When the benziminazole moiety is present in the molecule but lacks the 2 methyl groups at positions 5 and 6 (DDMBI-B_{12}), there is almost complete competition for the B_{12} binding sites (Table IV). Rosenblum, Davis, and Chow(18) have recently reported that DDMBI-B_{12} is very poorly absorbed in man. Therefore, it is apparent that the great selectivity of absorption of cyanocobalamin by the human is not solely dependent upon selective binding by intrinsic factor.

TABLE V. Analogue Competition for B_{12} Binding Sites of Intrinsic Factor Concentrate (IFC).

Aliquot	Substances to which IFC added 1x*	50x	% retention $B_{12}Co^{60}$ after dialysis	Calc. competition, %
1	$B_{12}Co^{60}$		60	
2	"	B_{12}	1.4	98
3	"	DDMBI-B_{12}	2.0	97
4	"	TFMBI-B_{12}	2.3	96
5	"	HBI-B_{12}	2.0	97
6	"	ABI-B_{12}	2.0	97
7	"	lactam-B_{12}	5.0	92
8	"	lactone-B_{12}	6.2	89
9	"	pseudo-B_{12}	6.2	89

* x = 30 mμg/0.01 mg IFC.

The fact that boiled gastric juice, known to lack intrinsic factor activity, maintains considerable preference for B_{12}(7) is evidence that selective binding of B_{12} is not equivalent to intrinsic factor activity. One possible explanation is that the intrinsic factor molecule has several functional groups which are necessary for physiologic activity. The binding may be due to a portion of the molecule which is only partially affected by boiling. The decreased binding capacity of boiled gastric juice may result from covering of some of the sites during boiling.

The B_{12} binding sites of saliva, colostrum, and serum exhibited markedly less preference for B_{12} in the presence of a 50-fold excess of pseudo-B_{12} than did those of gastric juice (Table IV). In fact, pseudo-B_{12} and B_{12} are equally successful competitors for B_{12} binding sites of saliva and serum. These data are interpreted as demonstrating qualitative differences in the B_{12} binding substances of these sources. It is known that these 3 substances lack intrinsic factor activity(2,16,17).

Another possibility is that each biologic material known to bind B_{12} contains a different ratio of "specific" and "non-specific" B_{12} binding substances, in some cases the latter exceeding the former to such a degree that

any specificity for B_{12} is masked. This conjecture might explain why differing preference patterns for B_{12} in the presence of certain B_{12} analogues were expressed by gastric juice and IFC (Tables IV and V). Discrepancies between B_{12} binding and intrinsic factor activity may be due to varying amounts of B_{12} binding substances other than intrinsic factor.

Summary and conclusions. 1) This article reports some observations on the general nature of vit B_{12} binding by gastric juice and certain other biologic substances. The majority of vit B_{12} molecules bound by gastric juice did not dissociate in the presence of excess vitamin molecules added subsequently. 2) Our data show competition offered by certain vit B_{12} analogues for the cyanocobalamin binding sites of gastric juice, intrinsic factor concentrate, saliva, colostrum, and serum. Of these substances, gastric juice exhibited the greatest preference for cyanocobalamin in the presence of excess analogue. The intrinsic factor concentrate tested was much less fastidious than gastric juice. Saliva, colostrum, and serum exhibited little selectivity in their B_{12} binding mechanisms. 3) Occurrence of a sulfate, nitrate, or chloride ion in lieu of the cyanide ion in the cobalamin did not diminish competition for cyanocobalamin binding sites of gastric juice.

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Effects of Sodium and Potassium upon Cardiac Glycogen Fractions of the Rat Heart.*† (23549)

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A medium low in sodium and potassium stimulates glycogenesis in the rat heart(1) and similarly, a high potassium concentration initiates glycogenolysis(2). It has also been reported that a solution low in potassium will

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