

# Effect of *pH* changes on the binding of vitamin B<sub>12</sub> by intrinsic factor

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**SYNOPSIS** The binding of vitamin B<sub>12</sub> by human gastric juice has been found to be *pH* dependent. Maximum binding occurs between *pH* 6.5 and 10. Outside this *pH* range the vitamin B<sub>12</sub>-binding ability of human gastric juice decreases and at *pH* below 2 or above 12.2 this drops sharply to about 10 to 15% of the maximum. Three commercial hog intrinsic factors have been found to give a similar response to *pH* changes. The *pH*-dependent binder in human gastric juice has been shown to be intrinsic factor by the addition of intrinsic factor-blocking antibody. About 10% of vitamin B<sub>12</sub> bound by human gastric juice is not bound by intrinsic factor and is not *pH* dependent. The reduction in the vitamin B<sub>12</sub>-binding capacity of human gastric juice induced by an adverse *pH* is reversed by neutralization. The physiological and clinical significance of these observations is discussed and their relevance to various procedures *in vitro* noted.

Intrinsic factor is now generally recognized as a substance which is necessary for the normal absorption of vitamin B<sub>12</sub> in man. It has been shown to be secreted by the parietal cells of the stomach but its chemical identity is still not certain although it has been reported to be a glycoprotein with a molecular weight equal to some multiple of 50,000 (Grasbeck, 1969). The exact mode of action of intrinsic factor in the normal absorption of vitamin B<sub>12</sub> is not known, but it is known to be a very potent binder of vitamin B<sub>12</sub>. The mechanism by which intrinsic factor binds vitamin B<sub>12</sub> and what part of the intrinsic factor molecule is involved are not known.

In this paper we report the results of our studies on the effect of *pH* on the ability of human gastric juice intrinsic factor to bind vitamin B<sub>12</sub>. This problem was studied because intrinsic factor has been used in a number of unbuffered assay procedures *in vitro* for serum vitamin B<sub>12</sub> (Lau, Gottlieb, Wasserman, and Herbert, 1965; Raven, Robson, Walker, and Barkhan, 1969), serum intrinsic factor blocking antibody (Gottlieb, Lau, Wasserman, and Herbert, 1965; Ungar, 1967), and in the estimations of intrinsic factor content of human gastric juice (Ardeman and Chanarin, 1963; Gottlieb *et al*, 1965). The *pH* of the various parts of the alimentary tract may also be important in the absorption of B<sub>12</sub> *in vivo* in normal and abnormal states.

## Materials

### <sup>57</sup>CO-VITAMIN B<sub>12</sub> (<sup>57</sup>CO-B<sub>12</sub>)

This was obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England. Preparations of specific activities from 10  $\mu\text{Ci}/\mu\text{g}$  to 20  $\mu\text{Ci}/\mu\text{g}$  were used. Each batch was diluted to a concentration of 1,000 pg/ml and stored at  $-20^{\circ}\text{C}$  in 10 ml aliquots.

### HUMAN GASTRIC JUICE

Human gastric juice was obtained from a large number of non-achlorhydric patients undergoing histamine test meals. Mucus and cellular debris was removed by centrifugation and the supernatant was then depepsinized by adjusting it to *pH* 10 with NaOH, incubated at room temperature for 20 minutes, neutralized with HCl, and stored at  $-20^{\circ}\text{C}$  until required. The pooled human gastric juice was found to have a vitamin B<sub>12</sub>-binding capacity of about 20 ng/ml at *pH* 7.4. Its intrinsic factor activity was demonstrated *in vivo* by its ability to correct vitamin B<sub>12</sub> malabsorption in two patients with proven pernicious anaemia. As a working solution, human gastric juice was diluted so that the amount used would maximally bind about 80% of the total <sup>57</sup>Co-B<sub>12</sub> added in the various test procedures.

### INTRINSIC FACTOR BLOCKING ANTIBODY

Intrinsic factor blocking antibody serum was obtained

from a pernicious anaemia patient. The intrinsic factor blocking antibody activity in this patient's serum was detected and assayed by a previously described method (Ungar, 1967). It was found to have a titre of 57 ng units/ml.

#### HUMAN GASTRIC JUICE TREATED WITH INTRINSIC FACTOR BLOCKING ANTIBODY

One ml of human gastric juice was mixed with 1 ml of intrinsic factor blocking antibody serum, then incubated at room temperature for 10 minutes so that all its intrinsic factor activity was blocked. It was then diluted with distilled water to the required concentration for each experiment.

#### COMMERCIAL HOG INTRINSIC FACTOR

The following three different commercial hog intrinsic factor preparations were tested.

##### *Nutritional biochemical intrinsic factor 10 x*

This was obtained from the Nutritional Biochemicals Corp, Cleveland, Ohio. A stock aqueous solution of 0.075 g% was made up and stored at -20°C. The vitamin B<sub>12</sub>-binding capacity of this solution was 200 ng/ml at pH 7.4.

##### *National Formulary intrinsic factor*

This was obtained from the National Formulary, American Pharmaceutical Association, Washington, D.C. A stock aqueous solution of 0.15g% was made up and stored at -20°C. The vitamin B<sub>12</sub>-binding capacity of this solution was 100 ng/ml at pH 7.4.

##### *Lederle intrinsic factor*

This was obtained from the Lederle Laboratories Division, American Cyanamid Comp., Pearl River, N.Y. A saturated aqueous stock solution was made up and stored at -20°C. The vitamin B<sub>12</sub>-binding capacity of this solution was found to be 57 ng/ml at pH 7.4.

#### ALBUMIN-COATED CHARCOAL

This was prepared by mixing equal volumes of a 5% aqueous suspension of Norit A neutral pharmaceutical grade decolorizing carbon (Amend Drug and Chemical Co., Inc., N.Y.), and a 1% aqueous solution of bovine albumin (Armour fraction V, Armour Pharmaceutical Company Ltd., England). Albumin-coated charcoal was prepared freshly for each assay since we have previously noted an appreciable deterioration on storage (O'Neill, Streeter, and Shum, 1968).

#### BUFFER SOLUTION

Sorensen's phosphate buffer was used. pH values

outside the range of this buffering system were obtained using different concentrations of NaOH and HCl.

#### Methods

#### EFFECT OF pH ON VITAMIN B<sub>12</sub> BINDING BY HUMAN GASTRIC JUICE AND HOG INTRINSIC FACTOR

Experiments were set up according to the protocol shown in Table I to cover a wide pH range. The radioactive <sup>57</sup>Co-B<sub>12</sub> solution was brought to the required pH and then the intrinsic factor source was added. This was allowed to incubate at room temperature for 30 minutes and then the albumin-coated charcoal was added. This reagent acts as a 'molecular sieve' and adsorbs free vitamin B<sub>12</sub> but not the bound form (Lau *et al*, 1965). The coated charcoal was removed by centrifugation and the radioactivity in the supernatant determined. The counts from the control series were counts due to unbound <sup>57</sup>Co-B<sub>12</sub> not taken down by the coated charcoal at the various pH levels (usually about 1-2% of the total counts added, irrespective of the pH). The difference between the test count and the control count was a direct measure of the amount of <sup>57</sup>Co-B<sub>12</sub> bound to intrinsic factor at each pH. In each experiment the highest 'net count', ie, test count minus control count, was taken as 100% binding capacity; the other net counts from the same experiment were then expressed as a percentage of this highest net count.

#### EFFECT OF pH ON VITAMIN B<sub>12</sub> BINDING BY HUMAN GASTRIC JUICE TREATED WITH INTRINSIC FACTOR BLOCKING ANTIBODY

This was carried out as in Table I with two additional series. One series had human gastric juice treated

	Test Series	Control Series
<sup>57</sup> Co-B <sub>12</sub> (1,000 pg/ml)	1.0 ml	1.0 ml
Buffer solution/N NaOH/N HCl	8.5 ml	8.5 ml
Intrinsic factor source (human gastric juice or hog intrinsic factors)	0.5 ml	—
Distilled water	—	0.5 ml
	Mix, stand at room temperature for 30 minutes; take 5.0 ml aliquot for pH determination	
Coated charcoal	3.0 ml	3.0 ml
	Mix, centrifuge at 4,000 rpm (3,500g) for 20 minutes; take 5.0 ml of clear supernatant for radioactivity determination <sup>1</sup>	
Counting result	Test count	Control count

Table I Protocol for experiments

<sup>1</sup>This was carried out in an automatic Nuclear-Chicago, well-type scintillation counter.

with intrinsic factor blocking antibody in place of the intrinsic factor source; the other series had intrinsic factor blocking antibody serum instead of the intrinsic factor source. Subtracting the supernatant radioactivity of the intrinsic factor series from the supernatant radioactivity of the human gastric juice treated with intrinsic factor blocking antibody series gave a measure of the <sup>57</sup>Co-B<sub>12</sub> bound to the non-intrinsic factor component of human gastric juice at each pH.

#### REVERSIBILITY OF pH EFFECT ON VITAMIN B<sub>12</sub> BINDING BY HUMAN GASTRIC JUICE

This was performed as in Table I with the following modifications. After the human gastric juice had been incubated with the <sup>57</sup>Co-B<sub>12</sub> mixture for 30 minutes at the various pHs, each solution of the test series was divided into two equal portions. Portion 1 was neutralized and an equal volume of distilled water added to portion 2. Further distilled water was then added so that all tubes of both portions had the same final volume. The pH of all tubes was then determined. After 30 minutes' incubation at room temperature coated charcoal was added. The net counts of each tube from portion 1 were taken as 100% binding capacity and the net counts of portion 2 from the corresponding tubes were expressed as a percentage of this.

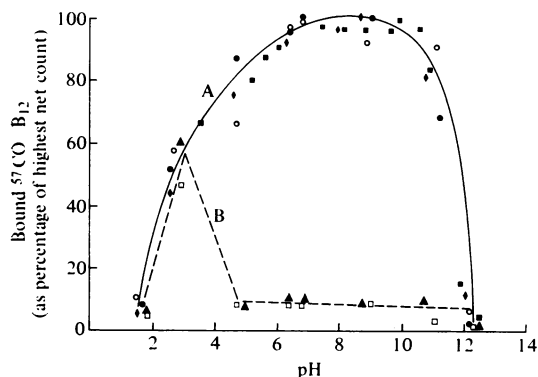


Fig. 1. Effect of pH on the binding of vitamin B<sub>12</sub> by human gastric juice (curve A) and by human gastric juice treated by intrinsic factor blocking antibody (curve B). ○, ●, ■, ◆ = results of four separate experiments for curve A. □, ▲ = results of two separate experiments for curve B.

## Results

### EFFECT OF pH ON VITAMIN B<sub>12</sub> BINDING BY HUMAN GASTRIC JUICE AND HOG INTRINSIC FACTOR PREPARATIONS

The results of four separate experiments of the effect of pH on vitamin B<sub>12</sub> binding by human gastric juice appear in Fig. 1 (curve A). Agreement between these experiments was satisfactory. It can be seen that maximal binding of vitamin B<sub>12</sub> by human gastric juice occurs in the pH range 6.5-10. Outside this optimal pH range the binding capacity of human gastric juice drops and at pH values below 1.6 and above 12, the binding of vitamin B<sub>12</sub> by human gastric juice drops below 10% of its maximum binding capacity. We have used acetate buffer as well as NaOH and HCl solutions to control the pH changes and have observed similar results. This therefore rules out the possibility that our observations may be due to the effect of Na<sup>+</sup>, K<sup>+</sup>, or phosphate ions.

The results of similar experiments using hog intrinsic factor are shown in Figure 2. There were no apparent differences in the pH sensitivity of three brands of hog intrinsic factor tested. The pattern of pH dependence shown by hog intrinsic factor resembles the effect of pH on human gastric juice except that at pH values between 1.4 and 3.5 the

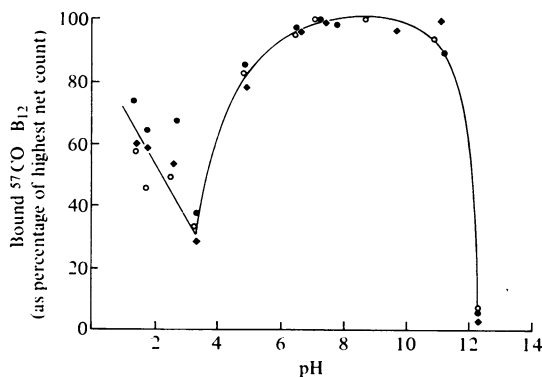


Fig. 2. Effect of pH on the binding of vitamin B<sub>12</sub> by hog intrinsic factor preparations. ○ = Nutritional biochemical intrinsic factor; ◆ = National Formulary intrinsic factor, and ● = Lederle intrinsic factor.

vitamin B<sub>12</sub>-binding pattern of the hog intrinsic factors was rather irregular.

#### EFFECT OF pH ON VITAMIN B<sub>12</sub> BINDING BY HUMAN GASTRIC JUICE TREATED WITH INTRINSIC FACTOR BLOCKING ANTIBODY

The results of two experiments are shown in Figure 1 (curve B). When added to human gastric juice in excess the intrinsic factor blocking antibody would block all the intrinsic factor activity and any vitamin B<sub>12</sub> bound may be attributed to the non-intrinsic factor vitamin B<sub>12</sub> binders. It can be seen that, at least for pH levels above 5, the amount of <sup>57</sup>Co-B<sub>12</sub> bound by the non-intrinsic factor binders is not significantly altered by changes in pH. The fact that the serum had no effect on human gastric juice vitamin B<sub>12</sub> binding at pH levels below 4 probably indicates that the antibody is not active at these low levels (McGuigan, 1967). This implies that the pattern of vitamin B<sub>12</sub> binding by human gastric juice in response to pH changes is due to the effect of pH on human intrinsic factor.

#### REVERSIBILITY OF pH EFFECT ON VITAMIN B<sub>12</sub> BINDING BY HUMAN GASTRIC JUICE

This experiment was designed to determine whether, *in vitro*, the binding ability of human gastric juice is irreversibly destroyed by subjecting it to extremes of pH. The results (Table II) suggest that exposure of human gastric juice to non-optimal pH values for 30 minutes results in a loss of vitamin B<sub>12</sub> binding capacity which is almost completely restored by neutralization.

pH	B <sub>12</sub> -binding Capacity of Human Gastric Juice (%Δ) <sup>1</sup>	
	Before Neutralization	After Neutralization
2.1	0.6	100
2.7	24.2	100
3.2	45.8	100
10.1	98.3	100
10.5	85.7	100
11.8	10.2	100

Table II Reversibility of pH effect on binding of B<sub>12</sub> by human gastric juice

<sup>1</sup>These are arbitrary figures calculated by taking the amount of <sup>57</sup>Co-B<sub>12</sub> bound after neutralization as 100%.

#### Discussion

The present studies show that human gastric juice binding of vitamin B<sub>12</sub> is pH dependent. Between pH 6.5 and 10 the binding capacity is maximal and is fairly constant; outside this pH range, however, the binding capacity decreases. When the pH is below

1.6 or above 12.2, less than 10% of the maximal binding occurs. In the pH range of 5 to 12, the presence of intrinsic factor blocking antibody removed 85-90% of the total binding ability, indicating that the predominant vitamin B<sub>12</sub> binder in human gastric juice is intrinsic factor. Below pH 4 the presence of intrinsic factor blocking antibody did not significantly reduce the vitamin B<sub>12</sub> binding ability of human gastric juice. This is not unexpected since a number of antibody-antigen complexes dissociate at such low pHs and the intrinsic factor blocking antibody was therefore probably not active. The vitamin B<sub>12</sub> binding ability of the non-intrinsic factor fraction of human gastric juice did not appear to be affected by pH. Apparently it is the intrinsic factor itself, rather than the non-intrinsic factor fraction of human gastric juice, which is affected by pH changes.

It was shown that, in the pH range 3.5-12.2, three commercial hog intrinsic factor preparations responded in a similar manner to human gastric juice. Below pH 3.5, however, they respond differently to human gastric juice. This unexpected phenomenon has not been investigated further. It might be due to the presence of other non-intrinsic factor proteins which may bind vitamin B<sub>12</sub> at such low pHs or the freeing of intrinsic factor binding sites which are not usually available at other pH levels in these manufactured reagents.

Goldberg and Fudenberg (1969) supported our suggestion that the vitamin B<sub>12</sub> binding ability of intrinsic factor is pH dependent (O'Neill *et al.*, 1968). They found that this increased when the pH was increased from 2 to 9. They, however, did not extend their studies to higher pH values and consequently did not report any inhibitory effects at pHs above 12.2. However, using equilibrium dialysis, McGuigan (1967) reported that there was no significant difference in the vitamin B<sub>12</sub> binding by human gastric intrinsic factor at pH 4.5, 7.4, and 11. This appears to be inconsistent with our results, but due to the differences in techniques and in the assessment of results, it is not apparent whether a genuine inconsistency exists.

These effects of pH on intrinsic factor might explain some of the abnormalities of vitamin B<sub>12</sub> absorption previously reported in man. Veeger, Abels, Hellemans, and Neiweg (1962) and Le Bauer, Smith, and Greenberger (1968) reported that in some patients with pancreatic insufficiency and a low intestinal pH, vitamin B<sub>12</sub> malabsorption was not due to a lack of intrinsic factor but could be corrected by the administration of sodium bicarbonate. Shimoda, Saunders, and Rubin (1967) also reported that in a case of Zollinger-Ellison syndrome malabsorption of vitamin B<sub>12</sub> was corrected when

acidity of the upper small intestine was neutralized. The gastric juice of this patient was shown to exhibit normal intrinsic factor activity. Furthermore, an adverse effect *in vitro* of acid pH on vitamin B<sub>12</sub> uptake by everted sacs of rat small intestine has been reported (Herbert, 1959).

It appears that a neutral or slightly alkaline pH condition in the intestine is probably necessary for normal vitamin B<sub>12</sub> absorption. Whether this pH effect is concerned solely with the optimal binding of vitamin B<sub>12</sub> by intrinsic factor is still not clear. It is possible that intraluminal neutrality may also be necessary for the normal attachment of the intrinsic factor-vitamin B<sub>12</sub> complex to the intestinal mucosal cells.

The reversibility experiments (Table I) indicate that intrinsic factor is not destroyed when subjected to extreme pH values for 30 minutes. This implies that the intrinsic factor molecule, or at least its binding site or sites, remains intact under those conditions. This is not unexpected as, under normal physiological conditions, intrinsic factor is subjected to a wide spectrum of pH changes through the gastrointestinal tract.

Our results suggest that a certain ionic conformation of the binding site or sites of the intrinsic factor molecule facilitates maximum binding of vitamin B<sub>12</sub>. It is further suggested that this ionic conformation is acquired at neutral or slightly alkaline pH. The exact mode of the binding mechanism, however, still remains to be elucidated.

The effect of pH on the binding of vitamin B<sub>12</sub> by intrinsic factor appears to have been neglected in a number of assay procedures *in vitro* for serum vitamin B<sub>12</sub> (Lau *et al*, 1965; Raven *et al*, 1969), serum intrinsic factor blocking antibody (Gottlieb *et al*, 1965; Ungar, 1967), and intrinsic factor in human gastric juice (Ardeman and Chanarin, 1963; Gottlieb *et al*, 1965). These assay methods do not use a buffering system to control the pH and it is therefore not surprising that inconsistent results have sometimes been reported (Lau *et al*, 1965; Raven, Walker, and Barkhan, 1966). We have recently reported on the reproducibility of a modified

isotope dilution vitamin B<sub>12</sub> assay using a buffering system to maintain the pH within a narrow range (Shum, Streeter, and O'Neill, 1970).

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