

A simple assay of intrinsic factor-vitamin B₁₂ complex employing the binding intrinsic factor antibody

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SYNOPSIS The reaction between binding intrinsic factor antibody and intrinsic factor-vitamin B₁₂ complex has been studied. Initially in the zone of antibody excess, the relationship between the amount of antigen present and the amount of antigen-antibody complex adsorbed onto zirconium phosphate gel was linear. With increasing amounts of antigen, the curve departed from linearity and reached a plateau. The linear portion of the reaction forms the basis of a simple and reproducible assay for quantitating intrinsic factor to which vitamin B₁₂ has already been bound. The assay provides a method for studying the fate of intrinsic factor-vitamin B₁₂ complex during digestion and absorption. In two normal subjects given radioactive vitamin B₁₂ orally, aspiration of ileal contents showed that only 50 to 70% of the radioactivity was bound to intrinsic factor at that level.

The study of the physiology of vitamin B₁₂ absorption has been hampered by the lack of a simple assay for human intrinsic factor-vitamin B₁₂ complex. The activity of human intrinsic factor-radioactive vitamin B₁₂ complex can be measured *in vivo* in patients with pernicious anaemia (Heinle, Welch, Scharf, Meacham, and Prusoff, 1952; Schilling, 1953; Glass, Boyd, Gellin, and Stephanson, 1954; Baker and Mollin, 1955). However, this requires the cooperation of patients with pernicious anaemia, is time consuming, and comparatively insensitive. Human intrinsic factor-vitamin B₁₂ complex can also be estimated *in vitro* by techniques employing ileal segments, everted ileal sacs, or small intestinal homogenates (Wilson and Wiseman, 1954; Wolff and Nabet, 1961; Sullivan, Herbert, and Castle, 1962; Boass and Wilson, 1963; Castro-Curel and Glass, 1963). These techniques have the disadvantage that they are specialized and difficult to apply to large numbers of samples.

The recognition of two types of intrinsic factor antibodies, the blocking antibody which prevents the combination of vitamin B₁₂ with intrinsic factor, and the binding antibody which unites either with intrinsic factor or intrinsic factor-vitamin B₁₂ complex (Garrido-Pinson, Turner, Crookston, Samloff, Miller, and Segal, 1966), has made possible the development of specific *in vitro* intrinsic factor assays.

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A variety of techniques employing the blocking antibody have been described, but these are only applicable to free intrinsic factor and cannot measure intrinsic factor already bound to vitamin B₁₂ (Ardeman and Chanarin, 1963; Gottlieb, Lau, Wasserman, and Herbert, 1965; Hansen, Miller, and Tan, 1966). The binding antibody, because it combines with the intrinsic factor-vitamin B₁₂ complex, is potentially capable of quantitating the latter. To date, the only ways of measuring intrinsic factor-vitamin B₁₂ complex, employing the binding antibody, have been the electrophoretic retention method (Jeffries and Sleisenger, 1963; Bardhan, Wangel, and Callender, 1967) and the competitive inhibition of the antibody using sodium sulphate precipitation (Rothenberg, 1966). These are rather complicated techniques and do not appear to have gained wide acceptance. Moreover, in practice, these techniques have only been advocated as methods for measuring intrinsic factor activity, and their potential in quantitating the intrinsic factor-vitamin B₁₂ complex has not been exploited.

This communication reports studies on the combination of the binding antibody with intrinsic factor-vitamin B₁₂ complex and the use of this reaction in a simple and sensitive *in-vitro* assay for intrinsic factor-vitamin B₁₂ complex. The assay has been employed in a preliminary study of the fate of intrinsic factor-vitamin B₁₂ complex in the intestine.

Materials and Methods

The technique is based on the fact that zirconium gel adsorbs protein complexes at or below their isoelectric point. The vitamin B₁₂ binders in gastric juice, including intrinsic factor, are quantitatively adsorbed onto the gel at pH 5.0, but at pH above 5.5 the negative charge of the intrinsic factor and other gastric juice binders increases and they are not adsorbed onto the gel (Hansen *et al*, 1966). When the binding type antibody is attached to the intrinsic factor-vitamin B₁₂ complex, the isoelectric point changes and it is adsorbed onto the gel at pH 6.25 (Jacob, Hansen, and Miller, 1966). Substances in solution not adsorbed onto the gel, including free radioactive vitamin B₁₂, can be removed by washing the gel with buffer.

Zirconium phosphate gels at pH 5.0 and pH 6.25 were prepared by the method of Hansen *et al* (1966). ⁵⁷Co-labelled radioactive vitamin B₁₂ (specific activity 70 μc/μg) was obtained from the Radiochemical Centre, Amersham. This was diluted before use with non-radioactive vitamin B₁₂ to give a specific activity of approximately 8 μc/μg. Radioactive measurements were carried out in a Packard automatic gamma scintillation counter, at least 10 000 counts being accumulated in each case.

Sera containing the binding and blocking antibody were obtained from four patients with pernicious anaemia. The titre of the antibodies in these sera was measured by zirconium gel assay (Hansen *et al*, 1966; Jacob *et al*, 1966). Control sera were obtained from healthy adults with no demonstrable intrinsic factor antibodies. To prevent radioactive vitamin B₁₂ from binding to transcobalamin II in the serum and being adsorbed to the gel (Hansen, Tan, and Jacob, 1967), the vitamin B₁₂ binding sites of the antibody sera and the control sera were saturated by adding excess non-radioactive vitamin B₁₂ (5 ng/ml). When the sera were used as a source of blocking antibody, the unbound vitamin B₁₂ was removed by albumin-coated charcoal prepared as described by Gottlieb *et al* (1965). The removal of excess unlabelled vitamin B₁₂ was unnecessary when the serum was used as a source of binding antibody because the serum was added to the system after radioactive vitamin B₁₂ was combined with intrinsic factor.

Gastric juices were obtained from healthy adult volunteers and patients with pernicious anaemia. The fasting juice was discarded. An intramuscular injection of pentagastrin (6 μg/kg body weight) was given and the juice aspirated at 15, 30, and 45 minutes thereafter. The juice was immediately filtered through glass wool, the pH adjusted to 10.0 with 1N NaOH to inactivate the pepsin and 30 minutes later brought back to 7.4 by the addition of 1N HCl. The

juice was divided into aliquots and stored at -20°C until further use.

The intrinsic factor content of each sample of gastric juice was estimated by the use of the binding antibody in the assay described below and by the blocking antibody employing either albumin-coated charcoal (Gottlieb *et al*, 1965) or zirconium gel at pH 5.0 (Hansen *et al*, 1966).

Results

OBSERVATIONS ON THE COMBINATION OF BINDING ANTIBODY WITH INTRINSIC FACTOR-VITAMIN B₁₂ COMPLEX

To determine whether the combination of the binding antibody with intrinsic factor-vitamin B₁₂ complex could be used for assay of the complex, a detailed study of the reaction was undertaken using a fixed amount of antibody and increasing amounts of the complex. Radioactive vitamin B₁₂ was added to gastric juice in excess of its total binding capacity to saturate all available intrinsic factor molecules. The juice was diluted 1 in 10 with 0.15M NaCl and duplicate amounts ranging from 0.05 to 1.0 ml were added to two sets of test tubes. To one set, 0.1 ml of serum containing 6.5 ng units of the binding antibody was added, and to the other set 0.1 ml of control serum was added. Ten ml of zirconium gel (pH 6.25) was then added to each tube. The tubes were mixed in a vortex mixer, centrifuged at 1000 g for five min and the supernatants discarded. The gel was washed three times with 15 ml of 0.1M am-

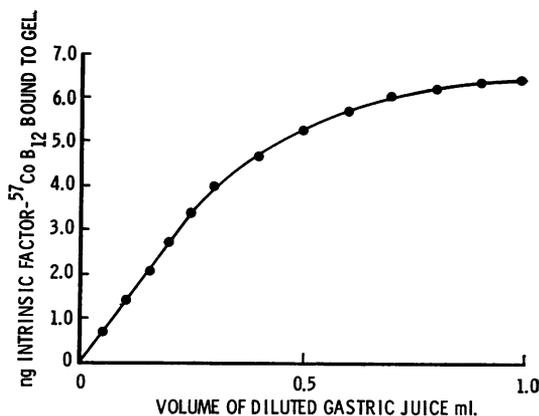


Fig. 1 Relationship between volume of gastric juice containing excess ⁵⁷Co vitamin B₁₂ and the amount, in ng units, of intrinsic factor-⁵⁷Co vitamin B₁₂ bound to zirconium gel at pH 6.25. The gastric juice was diluted 1 in 10. The amount of antibody was kept constant throughout at 6.5 ng units.

monium acetate buffer at pH 6.25. The radioactivity adsorbed onto the gel in the tube with antibody serum minus that in the corresponding tube with the control serum represented the amount of intrinsic factor- ^{57}Co vitamin B_{12} -antibody complex adsorbed onto the gel. The results are shown in Figure 1. Initially, the relationship between the amount of gastric juice and the amount of intrinsic factor- ^{57}Co vitamin B_{12} -antibody complex adsorbed onto the gel was linear up to about 3.4 ng units. As the amount of intrinsic factor- ^{57}Co vitamin B_{12} complex was further increased, the amount of radioactivity bound to the gel for a given increment in intrinsic factor-vitamin B_{12} complex decreased. Finally a plateau was reached when the amount bound to the gel was equal to the antibody content of the serum. Increasing the intrinsic factor-vitamin B_{12} complex still further produced no change in the amount of radioactivity bound to the gel.

THE ASSAY

It has been found that the relationship between the amount of gastric juice saturated with ^{57}Co vitamin B_{12} used and the amount of intrinsic factor- ^{57}Co vitamin B_{12} adsorbed onto the gel is always linear up to a value equivalent to approximately half the antibody content of the volume of serum employed. Using a volume of serum containing 5 ng units, the relationship is therefore linear to at least 2 ng units of intrinsic factor- ^{57}Co vitamin B_{12} complex, although, at times, the linear range may extend further (Fig. 2). The protocol of a typical assay, for 1 volume of gastric juice to which excess ^{57}Co vitamin B_{12} has been added, is shown in Table I, and the value obtained from this, together with values obtained from other volumes of the gastric juice, is shown in Figure 2.

When this assay is used simply to quantitate intrinsic factor in gastric juice, a single dilution of gastric juice giving a value within the linear range is adequate. For gastric juices with a probable normal content of intrinsic factor 0.1 ml of a 1 in 10 dilution gives adequate results. Juices which are likely to

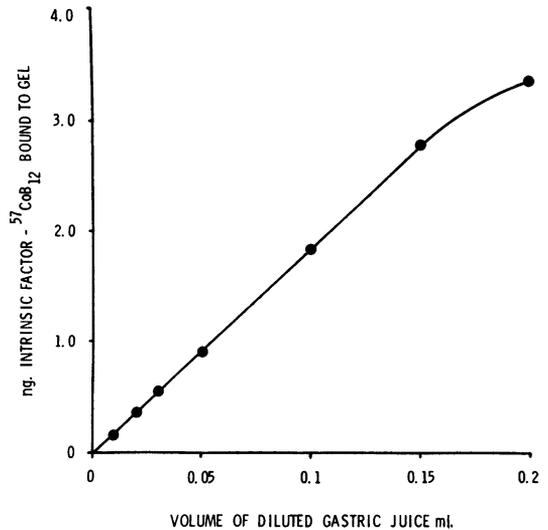


Fig. 2 Assay of intrinsic factor- ^{57}Co vitamin B_{12} complex of a normal gastric juice, diluted 1 in 10, to which an excess of radioactive vitamin B_{12} has been added. The amount of antibody serum employed contained 5 ng units of binding antibody. The graph is linear up to 2.8 ng units of intrinsic factor- ^{57}Co vitamin B_{12} . The point at 0.01 ml of gastric juice is that referred to in the protocol in Table I.

contain less than 20 ng units/ml are assayed undiluted.

REPRODUCIBILITY

The intrinsic factor of one gastric juice (measured as intrinsic factor- ^{57}Co vitamin B_{12} complex) was determined by this assay 12 times in one day. The values ranged from 158 to 163 ng units/ml with a coefficient of variation of 0.8% (Table II). Another gastric juice was stored in multiple aliquots and one of these was assayed in duplicate on eight different

| 0.15M NaCl (ml) | Gastric Juice $^{57}\text{CoB}_{12}$ Diluted 1 in 10 (ml) | Control Serum (ml) | Antibody Serum ¹ (ml) | Zirconium Gel pH 6.25 (ml) | ng $^{57}\text{CoB}_{12}$ Bound to Gel | ng $^{57}\text{CoB}_{12}$ Bound by Binding Antibody ² |
|--------------------|--|--------------------------|--|----------------------------------|--|---|
| 1.0 | 0.01 | — | 0.1 | 10 | 0.19 | 0.18 |
| 1.0 | 0.01 | — | 0.1 | 10 | 0.18 | 0.17 |
| 1.0 | 0.01 | 0.1 | — | 10 | 0.01 | — |
| 1.0 | 0.01 | 0.1 | — | 10 | 0.01 | — |

Table I Assay protocol¹

¹Only 1 volume of gastric juice- ^{57}Co vitamin B_{12} is shown; the protocol for other volumes is identical.

²The antibody serum used contained 50 ng units of binding antibody per ml.

³ng units adsorbed onto gel using antibody serum minus the amount adsorbed using control serum. In practice, because of the high degree of reproducibility, duplicates may not be necessary.

days. The values ranged from 145 to 162 ng units/ml with a coefficient of variation of 3.8% (Table II).

| Gastric Juice A | | Gastric Juice B | |
|-----------------|-------------|-----------------|-------------|
| Date | ng units/ml | Date | ng units/ml |
| 13-8-71 | 158 | 17-8-71 | 157 |
| 13-8-71 | 159 | | 159 |
| 13-8-71 | 161 | 18-8-71 | 148 |
| 13-8-71 | 163 | | 150 |
| 13-8-71 | 160 | 19-8-71 | 145 |
| 13-8-71 | 161 | | 146 |
| 13-8-71 | 161 | 20-8-71 | 157 |
| 13-8-71 | 163 | | 154 |
| 13-8-71 | 161 | 23-8-71 | 154 |
| 13-8-71 | 161 | | 154 |
| 13-8-71 | 160 | 24-8-71 | 146 |
| 13-8-71 | 162 | | 147 |
| | | 27-8-71 | 162 |
| | | | 162 |
| | | 31-8-71 | 158 |
| | | | 156 |

Table II Reproducibility of the assay carried out on the same day (gastric juice A) and on eight different days (gastric juice B)

COMPARISON WITH OTHER ASSAYS

The intrinsic factor content of 21 specimens of gastric juice, as measured by the present assay for intrinsic factor-vitamin B₁₂ complex, was compared with the results of intrinsic factor measurement using zirconium gel at pH 5.0 and the blocking anti-

body (Hansen *et al*, 1966). The results are shown in Figure 3. There is good correlation between the two assays (r = 0.99) although the binding antibody method gives a slightly lower value (approximately 5%) than the blocking antibody method.

The present assay was also compared with the albumin-coated charcoal assay (Gottlieb *et al*, 1965) (Fig. 4). Again there is good correlation between the results of the two assays (r = 0.99), although the binding antibody method gives values about 12% lower.

USE OF ASSAY IN MEASURING INTRINSIC FACTOR-⁵⁷CO VITAMIN B₁₂ IN INTESTINAL JUICE

Two volunteers, with normal vitamin B₁₂ absorption and normal secretion of intrinsic factor, were intubated orally with a fine radioopaque polyvinyl tube. When the distal end of the tube was in the upper ileum, a 1 µg dose of ⁵⁷Co vitamin B₁₂ (1 µc) was given by mouth. Commencing 45 minutes later, ileal juice was aspirated at intervals with a syringe and the radioactivity of the aspirates monitored. The two specimens containing the most radioactivity were centrifuged at 4°C at 10 000 g for one hour and the supernatants removed. None of the sediments contained any significant amounts of radioactivity. The radioactivity in the supernatants was present in

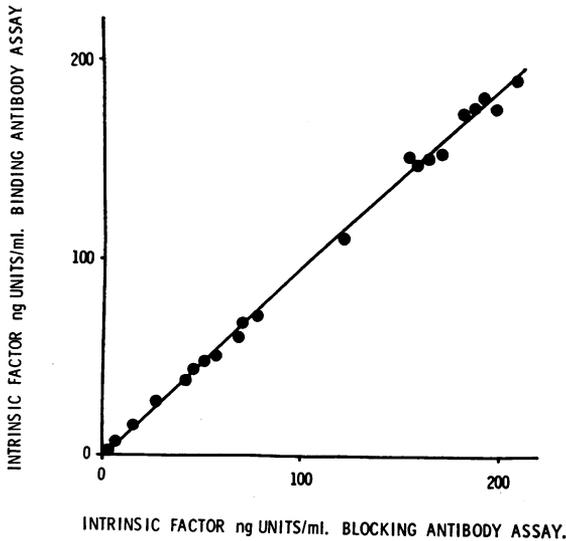


Fig. 3 Intrinsic factor content of 21 specimens of gastric juice measured by the present assay (binding antibody assay) compared with the measurements by the blocking antibody zirconium gel assay (r = 0.99) (y = 0.96x - 0.71).

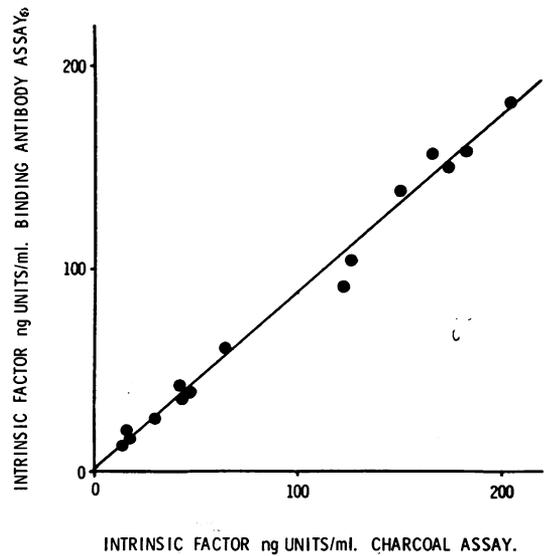


Fig. 4 Intrinsic factor content of 15 specimens of gastric juice measured by the present assay (binding antibody assay) compared with the measurements by the blocking antibody charcoal assay (r = 0.99) (y = 0.87x + 0.76).

| | Time Specimen Obtained (hr after dose) | Total ng ⁵⁷ CoB ₁₂ /ml Aspirate | Unbound ⁵⁷ CoB ₁₂ Removed by Albumin- coated Charcoal | Bound ⁵⁷ CoB ₁₂ | |
|-----------|--|---|---|---|--|
| | | | % of Total | Intrinsic Factor- ⁵⁷ CoB ₁₂ Complex | ⁵⁷ CoB ₁₂ Attached to Non-intrinsic Factor Binders |
| Patient A | 1.5 | 9.8 | 18 | 52 | 30 |
| | 2.5 | 1.8 | 11 | 55 | 33 |
| Patient B | 1.0 | 1.6 | 19 | 69 | 12 |
| | 1.5 | 0.2 | 25 | 65 | 10 |

Table III Results of study of vitamin B₁₂ binding on ileal juice obtained after feeding radioactive vitamin B₁₂

at least three forms: 'unbound' radioactivity which was measured and removed by treatment with albumin-coated charcoal; intrinsic factor-⁵⁷Co vitamin B₁₂ complex which was measured by the present assay; and ⁵⁷Co vitamin B₁₂ bound to unknown non-intrinsic factor binders, which was estimated by subtracting the other two quantities from the total amount of radioactivity in the supernatants. Table III shows the results of this study on two specimens of ileal juice from each of two patients and Fig. 5 the results of the intrinsic factor-vitamin B₁₂ complex assay on one of the specimens.

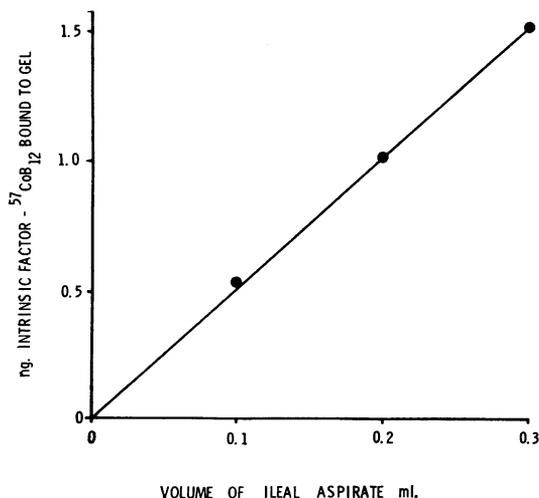


Fig. 5 Assay of intrinsic factor-⁵⁷Co vitamin B₁₂ complex aspirated from ileum of a normal subject 1.5 hours after an oral dose of 1 µg of ⁵⁷Co vitamin B₁₂. Serum containing 5 ng units of binding antibody was employed. 0.1 ml of aspirate contained 0.51 ng units of intrinsic factor-⁵⁷Co vitamin B₁₂ complex.

Discussion

A detailed study of the relationship between the binding antibody and intrinsic factor-vitamin B₁₂

complex does not appear to have been previously undertaken. The results shown in Fig. 1 are typical of the combination of an antigen to an antibody which occurs in varying proportions. Using the initial part of the zone of antibody excess, the results given in this paper indicate that the reaction can be used to assay intrinsic factor-vitamin B₁₂ complex. On a given day, the assay has a high degree of reproducibility. When carried out on different occasions, on aliquots of the same specimen, the variation is slightly greater. The reasons for this are not yet clear but the variations are not such as to interfere with the value of the assay.

The intrinsic factor content of gastric juices measured by the present assay tends to be a little lower than the results obtained using the two assays employing the blocking type antibody although there is an excellent correlation between the assays. Bardhan *et al* (1967) also noticed that the results of the charcoal assay using the blocking antibody were higher than their results using the binding antibody with the electrophoretic retention technique. Schade, Feick, Imrie, and Schilling (1967) have shown that, at room temperature, blocking antibody can displace some of the vitamin B₁₂ already bound to intrinsic factor. It is possible that in the present assay the blocking antibody present in the binding antibody serum displaced some of the radioactive vitamin B₁₂ from its combination with intrinsic factor, and so reduced the amount of radioactivity bound to the gel. This would result in a lower intrinsic factor measurement with the binding antibody. It is also perhaps not surprising that the binding and blocking assays give slightly different results, since they depend on two different antibodies, the one active at the site of vitamin B₁₂ attachment to intrinsic factor and the other at some other site(s) on the intrinsic factor molecule. Of the two assays with the blocking antibody, the charcoal method gave the higher values. The explanation for this is not known. It was observed, however, that minor variations in the assay conditions, such as changing the brand of charcoal, or the amount of non-intrinsic factor

protein in the system, produced alterations in the estimation of total vitamin B₁₂ binding and in the apparent intrinsic factor content when blocking antibody was added to the system. There is no evidence as to which of the assays gives a better measure of the physiological activity of intrinsic factor in promoting vitamin B₁₂ absorption. Any of the three assays appears to be satisfactory for the estimation of intrinsic factor in routine clinical practice.

The present assay has been shown to be simple and reproducible and to give a good measure of intrinsic factor. In addition, it has the great advantage that it can be used to measure intrinsic factor to which radioactive vitamin B₁₂ has already been bound. It is therefore capable of being used to quantitate accurately intrinsic factor-vitamin B₁₂ complex such as is sometimes used in the diagnosis of pernicious anaemia and other vitamin B₁₂ malabsorptive states.

The most important aspect of the present assay is its potential for the study of the physiology and pathology of vitamin B₁₂ absorption. The preliminary results of the study of the fate of intrinsic factor-vitamin B₁₂ complex in two subjects demonstrate this application of the assay. The findings are of interest in that only 50 to 70% of administered radioactivity recovered from the ileum was bound to intrinsic factor. This percentage was the same in each of the two specimens obtained from each patient, even though in patient A the second specimen was obtained one hour after the first. This may well explain why in normal subjects a test dose of radioactive vitamin B₁₂ is never completely absorbed. Further studies are in progress.

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