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IMMUNOASSAY OF GASTRIC INTRINSIC FACTOR AND THE TITRATION OF ANTIBODY TO INTRINSIC FACTOR

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SUMMARY

A modification of the charcoal method for the detection and titration of antibody to intrinsic factor in serum and for the assay of intrinsic factor in human gastric juice is described. The antibody was detected in a titre greater than 5 ng units/ml in 52% of the sera of 162 patients with Addisonian pernicious anaemia. False positive results may occur if the serum is withdrawn within 24 hr of the parenteral administration of normal therapeutic doses of vitamin B_{12}.

In the analysis of human gastric juice, the charcoal method of immunoassay clearly distinguishes between intrinsic factor and other vitamin B_{12} binding substances. A good correlation was observed between the gastric acid secretion, the ability to absorb oral radioactive vitamin B_{12} in the Schilling test, and the assay of intrinsic factor in the gastric juice. Intrinsic factor as an antigen is stable in acid and alkali media but is destroyed by incubation at 56°C for 30 min. A secretion of less than 100 ng units intrinsic factor in the gastric juice during the post-histamine hour is not compatible with adequate vitamin B_{12} absorption and is indicative of Addisonian pernicious anaemia.

A good correlation was observed between the immunoassay and the biological activity of preparations of hog intrinsic factor. To correct the absorption of oral vitamin B_{12} in the Schilling test in patients with pernicious anaemia 300–600 ng units hog intrinsic factor are required. It is suggested that hog intrinsic factor preparations should be defined in terms of nanogram units intrinsic factor per milligram.

The development of a satisfactory *in vitro* method for the assay of intrinsic factor would provide an important advance towards the isolation and characterization of this elusive substance. It would also prove helpful in following the natural history of the disease process that culminates in the condition of Addisonian pernicious anaemia. One of the most

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promising developments has been the assay of intrinsic factor using immunological techniques.

Antibody to intrinsic factor was first identified *in vitro* by Jeffries, Hoskins & Sleisenger (1962) using an electrophoretic retention technique. The methods described by Abels *et al.* (1963) and by Ardeman & Chanarin (1963) depend upon the observation that intrinsic factor binds vitamin B_{12}; in the presence of antibody to intrinsic factor the binding of vitamin B_{12} to intrinsic factor is inhibited. These latter two methods are essentially similar except that Abels and his colleagues use dialysis to remove free vitamin B_{12} from the system while charcoal is used for this purpose by Ardeman & Chanarin (1963). Roitt, Doniach & Shapland (1964) have demonstrated that the methods using dialysis or charcoal detect antibody to the vitamin B_{12}-binding site on the intrinsic factor molecule while the electrophoretic method relates to a different antigenic determinant on the intrinsic factor molecule. Both Jeffries *et al.* (1962) and Abels *et al.* (1963) have shown a correlation between the presence of antibody to intrinsic factor in the serum as detected by their respective *in vitro* techniques and the ability of that serum to inhibit the action of exogenous intrinsic factor in the absorption of vitamin B_{12} in patients with Addisonian pernicious anaemia.

The purpose of the present communication is to describe a modification of the method of Ardeman & Chanarin for the detection and titration of antibody to intrinsic factor in human serum and for the titration of intrinsic factor in human gastric juice. The amount of intrinsic factor secreted following histamine stimulation has been measured in a variety of patients and the results correlated with the patient's ability to secrete gastric acid and to absorb oral vitamin B_{12} and with the presence or absence of antibody to intrinsic factor in the serum. Using this method of assay, the intrinsic factor content of a number of preparations of hog antral mucosa has been determined and the results compared with the biological activity of these preparations.

DETECTION AND TITRATION OF ANTIBODY TO INTRINSIC FACTOR

Screening test for antibody to intrinsic factor

The procedure used for the detection of antibody to intrinsic factor in human serum is given in Table 1.

The tests were done in disposable plastic 10 ml test tubes with plastic stoppers (Stayne Laboratories Ltd, High Wycombe, Bucks., England). The phosphate buffer consists of 0·5 M-NaH_2PO_4 brought to pH 7·0 with 10 N-NaOH and diluted 1:50 with 0·85% saline before use.

Gastric juice from a patient with duodenal ulcer provides a convenient source of intrinsic factor; the most suitable specimen is usually obtained in the first 40 min following stimulation by histamine or gastrin (Irvine, 1965a). The concentration of intrinsic factor per millilitre of the gastric juice should be $\geqslant$ 75 ng units/ml (*vide infra*). The juice can be stored at —20°C for at least several months without loss of the antigenic properties of intrinsic factor.

In order to standardize the degree of mixing the specimens were placed in a Rolamix apparatus (Luckham Ltd, London, England) for the time intervals indicated.

Lyopholized ^{57}Co vitamin B_{12} (Cyanocobalamin) was obtained from the Radiochemical Centre, Amersham, England, in 10 μc ampoules with a specific activity of 1·0 $\mu c/\mu g$. To prepare a stock solution the contents of one ampoule were dissolved in 25 ml buffer to give a concentration of 400 ng ^{57}Co vitamin B_{12} per ml. This was further diluted 1:10 before use. ^{57}Co vitamin B_{12} was used in preference to ^{60}Co vitamin B_{12} because of the higher efficiency of detecting the γ-radiation. ^{57}Co has no β-radiation and the intensity and energy of its γ-radiation is less than that of ^{60}Co; these properties combined with the shorter half-life (270 days as opposed to 5·27 years) makes ^{57}Co vitamin B_{12} less hazardous than ^{60}Co vitamin B_{12} in the event of contamination of personnel or equipment.

Instead of using activated charcoal in powdered form (Ardeman & Chanarin, 1963; Irvine, 1965b, c), it was found that more reproducible results were obtained if the activated charcoal (British Drug Houses, Poole, England) is made into a 20% suspension in buffer. In this way clumping of the charcoal is reduced.

TABLE 1. Detection of antibody to intrinsic factor in serum

	Test		Standard	Controls	
	1*	2	3*	4*	5*
Phosphate buffer (ml)	1·4	1·4	2·5	1·5	2·0
Standard gastric juice (ml)	0·1	0·1	—	—	—
Normal serum (ml)	0·5	—	—	0·5	—
Test serum (ml)	—	0·5	—	—	—
	Mix for 10 min				
20 ng ^{57}Co vitamin B_{12} (1·0 $\mu c/\mu g$) in buffer (ml)	0·5	0·5	0·5	0·5	0·5
	Mix for 20 min				
20% charcoal in buffer (ml)	0·5	0·5	—	0·5	0·5
	Mix for 2 min, centrifuge, count activity in supernatant				

*These tubes need only be included once in each battery of tests in which many test sera (tube 2) are studied.

A well-type scintillation counter with a $1\frac{3}{4} \times 2$ in. sodium iodide crystal was used and each sample was counted for a period of 100 sec. By reference to the standard the number of counts equivalent to 1 ng ^{57}Co vitamin B_{12} was determined. The amount of antibody to intrinsic factor in the test serum is represented by the difference in counts between tubes 1 and 2 (see Table 1). The titre of intrinsic factor antibody in the serum is calculated as the inhibition of vitamin B_{12} binding in nanograms that would be produced by 1 ml of the test serum. The titre is expressed in ng units/ml. Using the reagents as shown in Table 1 the titre of antibody to intrinsic factor is given by the formula:

$$\frac{\text{Count in tube 1} - \text{Count in tube 2}}{\text{Standard} - \text{Background counts}} \times 40.$$

The variation in counts in Table 1, if repeated throughout a series of experiments, is ±2%. This degree of error is equivalent to less than 1 ng of vitamin B_{12} binding. In the analysis of a large number of test sera it was found that there was no significant variation in the vitamin B_{12} binding properties of normal serum in the absence of gastric juice as compared to serum from patients with pernicious anaemia or with thyroid disease or from a large number of hospital control patients. It is therefore not necessary to compare the vitamin B_{12} binding properties of normal serum and test serum in the absence of intrinsic factor. The standard and background counts are estimated at the beginning and at the end of each series of experiments as a control of the counting apparatus. Tubes 4 and 5 are included as controls for the absorptive properties of the charcoal. They ensure that the charcoal will remove more than 99% of free vitamin B_{12} (tube 5) but will not absorb the small amount of vitamin B_{12} that is bound to serum in the absence of intrinsic factor (tube 4).

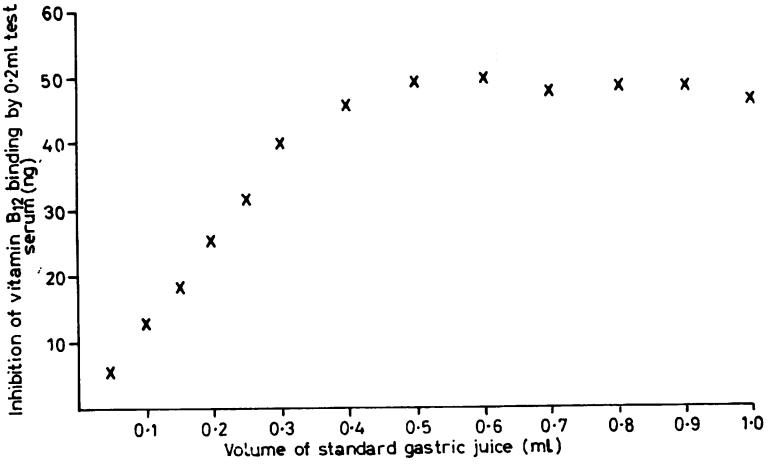


FIG. 1. Titration of serum B.1249 for antibody to intrinsic factor at the vitamin B_{12} binding site using increasing amounts of gastric juice in the presence of a constant amount of the test serum.

Tubes 1, 3, 4 and 5 need be included once only in each battery of tests. This greatly simplifies the method and allows the analysis of large numbers of test sera. The number of tubes required is $x+4$, where x is the number of sera to be tested.

The procedure shown in Table 1 serves as a screening test for the presence of antibody to intrinsic factor. Sera that give an inhibition of vitamin B_{12} binding of >5 ng units/ml are regarded as positive for intrinsic factor antibody. According to the amount of intrinsic factor in the gastric juice used in the test, this screening procedure will titrate the antisera to between 10 and 20 ng units/ml. Sera giving maximum inhibition of vitamin B_{12} binding that is possible within the limits of this screening test require to be titrated.

Titration of antibody to intrinsic factor

Sera were titrated for antibody to intrinsic factor by determining the inhibition of vitamin B_{12} binding by a constant amount of the test serum in the presence of increasing

amounts (0·05–1·00 ml) of a standard human gastric juice containing ⩾100 ng units intrinsic factor/ml. The volume of the gastric juice at each stage was made up to 1·0 ml with buffer. An example of such a titration is shown in Fig. 1. The procedure adopted was as described in Table 1 except that 0·5 ml buffer and 1·0 ml serum were used and tube 1 was included at each stage of the assay. In tube 2, 0·2 ml test serum was diluted in 0·8 ml pooled normal human serum. 400 ng ^{57}Co vitamin B_{12} (specific activity 0·1 $\mu c/\mu g$) in 1·0 ml buffer was added to each tube. The procedure for absorption with charcoal was as described above. The serum studied in Fig. 1 gave a titre of 250 ng units intrinsic factor antibody per ml. The titration curve indicates that the results are highly reproducible and that the reaction is between a single antigen and antibody.

For the routine titration of sera for antibody to intrinsic factor it is only necessary to

TABLE 2. Routine titration of antibody to intrinsic factor in serum

	Test		Standard	Controls	
	1*	2	3*	4*	5*
Phosphate buffer (ml)	1·0	1·0	3·0	2·0	2·5
Standard gastric juice (ml)	1·0	1·0	—	—	—
Normal serum (ml)	0·5	—	—	0·5	—
Test serum (ml)	—	0·5	—	—	—
	Mix for 10 min				
400 ng ^{57}Co vitamin B_{12} (0·1 $\mu c/\mu g$) in buffer (ml)	1·0	1·0	1·0	1·0	1·0
	Mix for 20 min				
20% charcoal in buffer (ml)	0·5	0·5	—	0·5	0·5
	Mix for 2 min, centrifuge, count activity in supernatant				

*These tubes need only be included once in each battery of tests in which many sera may be titrated.

determine the level of the plateau in the titration curve shown in Fig. 1. The procedure for routine titration is shown in Table 2. A control test using a standard antiserum of high titre is included in each set of titrations to verify the upper limit of the titration which the system will detect. Test sera with titres approaching this limit require to be titrated again using 0·1 ml test serum diluted in 0·4 ml normal human serum.

The titres of intrinsic factor antibody (vitamin B_{12} binding site) in the sera of 162 patients with Addisonian pernicious anaemia are shown in Fig. 2. The antibody was present in a titre of >5 ng units/ml in 52% and the titres ranged from 5·4 to 790 ng units/ml.

False positive results for antibody to intrinsic factor

In the *in vitro* system a high level of stable vitamin B_{12} in the test serum may compete with the ^{57}Co vitamin B_{12} for the available binding sites and so produce a false positive result. In order to test this possibility, serum was obtained from a normal subject (W.J.I.)

immediately before and at 2, 4, 8, 24, 48 and 72 hr after an intramuscular injection of 1000 μg vitamin B_{12} (Cytamen, Glaxo). The titre of antibody to intrinsic factor was <5 ng units/ml (negative) before the injection, but rose to 27 ng units/ml at 2 hr and fell to 14 ng units/ml at 4 hr and was again less than 5 ng units/ml at 8 hr and thereafter. The corresponding figures for the serum vitamin B_{12} levels determined by microbiological assay (Girdwood, 1960) were 0·3, 20, 6, 2·5, 1·3 and 0·8 ng/ml, respectively. A patient with treated pernicious anaemia, whose serum was negative for antibody to intrinsic factor 6 days after 1000 μg intramuscular vitamin B_{12}, showed a titre of 12 ng units/ml 6 hr after an intramuscular injection of 1000 μg Cytamen. In two other patients with pernicious anaemia, but with negative tests for antibody to intrinsic factor, the duration of a false positive result following 1000 μg Cytamen intramuscularly was limited to 24 hr.

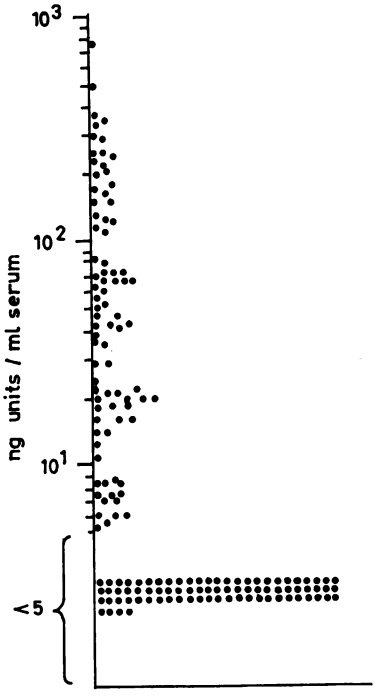


FIG. 2. The incidence and titres of antibody to intrinsic factor (vitamin B_{12} binding site) in the sera of 162 patients with Addisonian pernicious anaemia. The antibody was present in a titre of greater than 5 ng units/ml in 52% of the patients.

TITRATION OF INTRINSIC FACTOR IN HUMAN GASTRIC JUICE

In the assay of the intrinsic factor content of a sample of gastric juice it is advantageous to use a serum with a high titre of antibody to intrinsic factor. This ensures that in the majority of assays the limiting factor is the amount of intrinsic factor in the sample of gastric juice and not the amount of antibody to intrinsic factor that had been used. Where the recorded amount of intrinsic factor in the sample of gastric juice approaches the known amount of antibody in the standard antiserum, the test must be repeated using either a higher titre antiserum or the gastric juice should be diluted.

In order to obtain a supply of suitable standard antisera a small panel was made of fit, co-operative patients with high titres of intrinsic factor antibody in their sera who were agreeable to donating 200–400 ml of blood every 6 months for the purpose of this research. Care was taken to ensure that the donor had not received an injection of vitamin B_{12} within the previous 24 hr for the reason discussed above. The donor whose serum was used for the majority of assays was one of the few patients with antibody to intrinsic factor in the serum but who had no evidence of malabsorption of vitamin B_{12} and who was not receiving vitamin B_{12} therapy.

Each of the standard antisera were titrated for antibody to intrinsic factor by the method illustrated in Fig. 1 and also by the single tube method described in Table 2. Sera with antibody titres $\geqslant 100$ ng units/ml were considered suitable for use as standard antisera.

Figs. 3–5 show the titration of intrinsic factor in three selected samples of gastric juice. The

TABLE 3. Immunoassay of intrinsic factor in human gastric juice

	Test		Controls			Standard
	1	2	3*	4*	5*	6*
Phosphate buffer (ml)	1·0	1·0	1·5	1·5	2·5	3·0
Test gastric juice (ml)	0·5	0·5	—	—	—	—
Normal serum (ml)	1·0	—	—	1·0	—	—
Standard anti-serum (ml)	—	1·0	1·0	—	—	—
	Mix for 10 min					
400 ng ^{57}Co vitamin B_{12} (0·1 μc/μg) in buffer (ml)	1·0	1·0	1·0	1·0	1·0	1·0
	Mix for 20 min					
20% charcoal in buffer (ml)	0·5	0·5	0·5	0·5	0·5	—
	Mix for 2 min, centrifuge, count activity in supernatant					

*These tubes need only be included once in each battery of tests in which many samples of gastric juice may be assayed.

amount of antigen has been kept constant at 1·0 ml test gastric juice and the amount of standard antiserum progressively increased in tube 2 (see Tables 1 and 2) from 0·0 to 0·5 ml.

In Fig. 3 the gastric juice is from a patient with duodenal ulcer who had a high secretion of gastric acid (34 mEq HCl) during the hour following a subcutaneous injection of histamine (0·04 mg/kg body weight). The inhibition of vitamin B_{12} binding (the difference in counts between tube 1 and 2 divided by the number of counts equivalent to 1 ng ^{57}Co vitamin B_{12}) increases with the addition of increasing amounts of standard antiserum until all the intrinsic factor available has reacted with the antibody. Thereafter, the addition of further quantities of antiserum has no effect on the vitamin B_{12} binding. In this sample of gastric juice most of the vitamin B_{12} binding could be inhibited by the antiserum.

In contrast, the sample of gastric juice used in Fig. 4 is from a patient with Addisonian pernicious anaemia. The difference between the vitamin B_{12} binding in the presence of normal serum and in the presence of standard antiserum is insignificant. There is almost a

Fig. 3

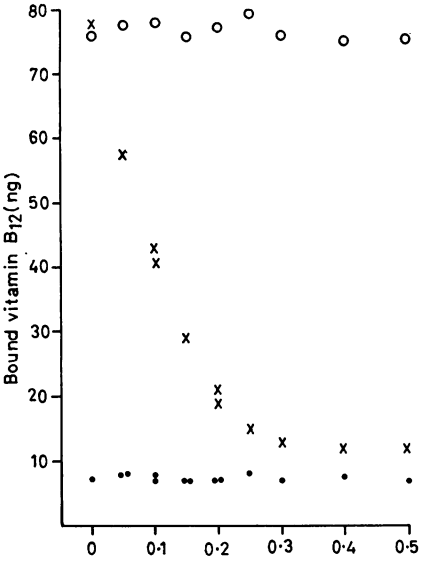
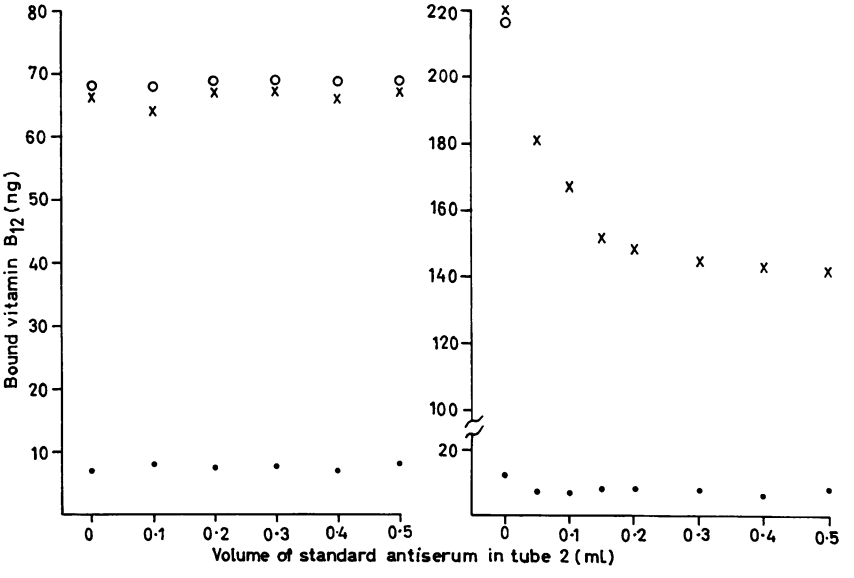


Fig. 4

Fig. 5



FIGS. 3–5. The experimental procedure in these figures was the same as shown in Tables 1 and 2 but using the following quantities of reagents: Tube 1 (○): 1·5 ml buffer, 1 ml test gastric juice, 1 ml pooled normal human serum. Tube 2 (×): 1·5 ml buffer, 1 ml test gastric juice, increasing amounts of standard anti-serum made up to 1·0 ml with pooled normal human serum. Tube 4 (●): 2·5 ml buffer, no gastric juice, 1 ml pooled normal human serum. 200 ng ^{57}Co vitamin B_{12} (0·1 μc/μg) was added to each tube, to the standard and to the controls. 0·5 ml 20% charcoal suspension in buffer. Individual legends are given at the foot of the facing page.

comparable amount of vitamin B_{12} binding per ml gastric juice but this is all due to non-specific vitamin B_{12} binding substances that do not have the physiological function nor the antigenic properties of intrinsic factor.

In Fig. 5 the same procedure had been used to analyse the gastric juice from a patient with histamine-fast achlorhydria but in whom there was no evidence of malabsorption of oral vitamin B_{12}. This sample of gastric juice contains a very high content of vitamin B_{12} binding substances, but only about one-third of the vitamin B_{12} binding capacity is due to the presence of intrinsic factor.

In order to have a rapid assay for the intrinsic factor content of gastric juice it is necessary to detect the flat portion of the curve (×) illustrated in Figs. 3–5. This can be done using a two tube assay, together with a single standard and single charcoal controls. The count in tube 1 (normal serum) should not approach that of the standard and the units of intrinsic factor antigen recorded in the volume of gastric juice under test should not approach the number of ng units of antibody to intrinsic factor that are present in the standard antiserum used. If these limitations are reached it is necessary to dilute the sample of gastric juice and repeat the test. The procedure for the rapid assay of intrinsic factor content of samples of gastric juice is then as shown in Table 3. Before this procedure was adopted as the routine laboratory test for assay of intrinsic factor in gastric juice further studies were done concerning the absorptive properties of charcoal.

Absorptive properties of charcoal

The effect of prolonging the duration of mixing with charcoal in the penultimate stage of each procedure was studied over a period of 2–40 min. The results in the assay of a specimen of gastric juice for intrinsic factor are shown in Fig. 6.

Within this period of study there is little change in the amount of ^{57}Co vitamin B_{12} absorbed by the charcoal suspension. In particular, the amount of binding of vitamin B_{12} to serum that was inhibited by the presence of antibody to intrinsic factor was essentially

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FIG. 3. Titration of vitamin B_{12} binding substances in the pooled gastric juice secreted during the post-histamine hour in a male aged 42 years with duodenal ulcer. Secretion of HCl during the hour following i.m. 0·04 mg histamine/kg body weight = 34 mEq. Most of the binding of vitamin B_{12} can be inhibited by the anti-serum. Intrinsic factor content of this gastric juice is estimated at 64 ng units/ml.

FIG. 4. Titration of vitamin B_{12} binding substances in pooled gastric juice secreted during the post-histamine hour from a female aged 69 years with Addisonian pernicious anaemia. Only a small proportion of the binding of vitamin B_{12} can be inhibited by the anti-serum; the gastric juice contains a high concentration of vitamin B_{12} binding substances that do not have intrinsic factor activity. Intrinsic factor content is estimated at 3 ng units/ml gastric juice.

FIG. 5. Titration of vitamin B_{12} binding substances in the pooled gastric juice secreted during the post-histamine hour in a female aged 27 years with histamine-fast achlorhydria, but a normal level of serum vitamin B_{12} and a normal Schilling test of vitamin B_{12} absorption. Note the change in scale in the ordinate in comparison to Figs. 3 and 4. Due to the high concentration of vitamin B_{12} binding substances in this juice 400 ng ^{57}Co vitamin B_{12} had to be used for the first set of points when no antiserum was present in the system. The binding of up to 80 ng vitamin B_{12} per ml gastric juice can be inhibited by the antiserum. The gastric juice contains a high concentration (140 ng units/ml) of non-specific vitamin B_{12} binding substances. The intrinsic factor content of this gastric juice is therefore 80 ng units/ml.

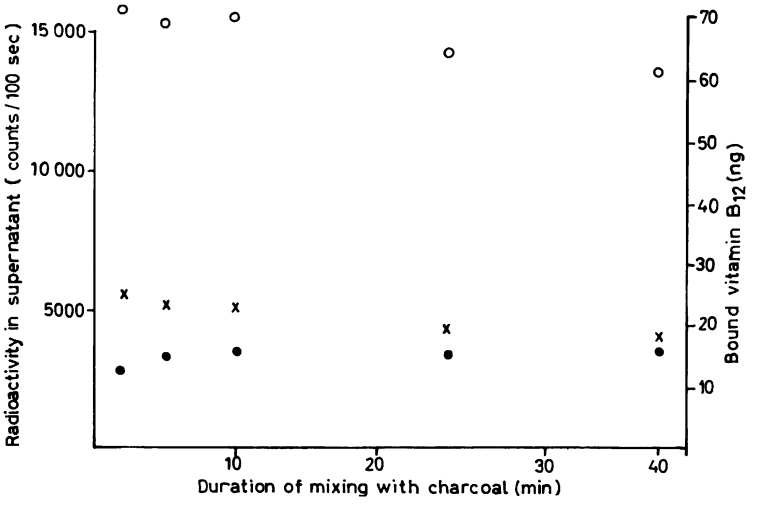

FIG. 6. The effect of prolonging the period of absorption with charcoal in the immunoassay of intrinsic factor in gastric juice. The experimental procedure was as shown in Table 2 but with varying times allowed for absorption with charcoal. The same standard gastric juice and the same standard antiserum was used throughout this experiment. Tube 1 (○); Tube 2 (×); Tube 3 (●). The amount of intrinsic factor per ml of the gastric juice was estimated as 46·8, 46·8, 47·9, 45·6 and 43·8 ng units/ml using 2, 5, 10, 25 and 40 min incubation with charcoal, respectively.

constant irrespective of the duration of absorption with charcoal. The figures obtained for the intrinsic factor content of this particular sample of gastric juice after mixing with charcoal for 2, 5, 10, 25 and 40 min were 46·8, 46·8, 47·9, 45·6 and 43·8 ng units/ml respectively. A mixing time of 2 min was therefore adopted as standard procedure.

TABLE 4. Comparison of the absorptive properties of charcoal in buffer and in 3% bovine albumin. The experimental procedure was as described in Table 3. The standard is shown separately. The complete assay was repeated six times at each stage and the results are presented as the mean ± 1 standard deviation

Tube No.	20% charcoal in buffer counts/100 sec ± 1 SD	20% charcoal in 3% albumin counts/100 sec ± 1 SD
1	14783 ± 139	16707 ± 184
2	2395 ± 104	2959 ± 40
3	2070 ± 60	3479 ± 130
4	1960 ± 75	3147 ± 177
5	204 ± 25	1880 ± 80
Intrinsic factor content	132·5 ± 0·7 ng units/ml	145·5 ± 2·1 ng units/ml

Radioactive standard = 75·181 ± 402 counts/100 sec.
Background = 89 ± 8 counts/100 sec.

According to Herbert *et al.* (1964) the charcoal is effective in selective absorption by virtue of the fact that it becomes coated with protein and thereafter acts as a molecular sieve that will only absorb small molecules such as free vitamin B_{12} but not vitamin B_{12} that is bound to protein. A study was therefore made in order to determine whether it was necessary to coat the charcoal with protein (e.g. bovine albumin) before adding it to the system or whether there was sufficient protein in the system already without incorporating this step. The titration of intrinsic factor content of a sample of gastric juice and the titration of antibody to intrinsic factor in a sample of serum was therefore done using a 20% suspension of uncoated charcoal in phosphate buffer and using a 20% suspension of charcoal in 3% bovine albumen solution and phosphate buffer. The results in the titration of intrinsic factor in gastric juice are shown in Table 4.

In the presence of albumin coated charcoal the counts were higher in all tubes including tube 5 which contained only ^{57}Co vitamin B_{12} in buffer prior to the addition of the charcoal. The difference in counts for the various tubes with and without albumin were fairly consistent in tubes 1, 3, 4 and 5 but not in tube 2; hence the discrepancy in the assay figures (132·5 ng units/ml as opposed to 145·5 ng units/ml). The reproducibility was not improved by the use of albuminized charcoal. The procedure shown in Table 3 for the rapid assay of intrinsic factor in gastric juice was therefore adopted as routine.

Reproducibility of the immunoassay of intrinsic factor

The variations in counts recorded by the scintillation counter in tubes 1 and 2 of Table 3 was ± 1–2%. Test samples of gastric juice, particularly from achlorhydric patients may contain very low concentrations of intrinsic factor which may or may not be associated with high concentrations of other vitamin B_{12} binding substances. However, using ^{57}Co vitamin B_{12} with specific activity of 0·1 $\mu c/\mu g$ the counts recorded in tubes 1 and 2 were always greater than 1000 in 100 sec. The variation in the assay of intrinsic factor was found to be ± 3 ng units/ml gastric juice. The percentage variation was therefore maximal in specimens of gastric juice that contain minimal amounts of intrinsic factor and is minimal in specimens that contain high concentrations of intrinsic factor.

Studies on the stability and on the filtration of intrinsic factor antigen

Using the method of immunoassay shown in Table 3 it was demonstrated that intrinsic factor lost its antigenic properties as a result of incubation at 56°C for 30 min. After this treatment no intrinsic factor could be detected by immunoassay in a sample of gastric juice that previously gave a titre of 130 ng units/ml.

The titre of intrinsic factor as determined by immunoassay was not significantly influenced by incubation at pH less than 2·0 for 2 hr at room temperature; the titre was the same ($\pm 1·5$ ng units/ml) as in an aliquot of the same sample of gastric juice that was immediately neutralized to pH 7·0 and in another aliquot that was taken up to pH 10·0 for 20 min and then brought to pH 7·0 and kept at room temperature for 2 hr. There was no evidence of loss of intrinsic factor antigen on standing at room temperature (20°C) for 2 hr.

Filtration through a sterilizing Carlson asbestos pad (type EK, John C. Carlson, Ltd, Barnoldswick, Lancs., England) in a Seitz filter removed intrinsic factor and other vitamin B_{12} binding substances. There was no loss of intrinsic factor activity after Seitz filtration

through a clarifying Carlson asbestos pad (Type K) or through a Berkefeld filter (British Berkefeld Filters Ltd, Tonbridge, Kent, England).

Results of immunoassay of intrinsic factor in human gastric juice

The secretion of intrinsic factor during the post-histamine hour was studied in eighty-seven patients suffering mainly from duodenal ulcer, thyroid disease or pernicious anaemia. Gastric aspiration was performed as described previously (Irvine, 1965a) and the histamine

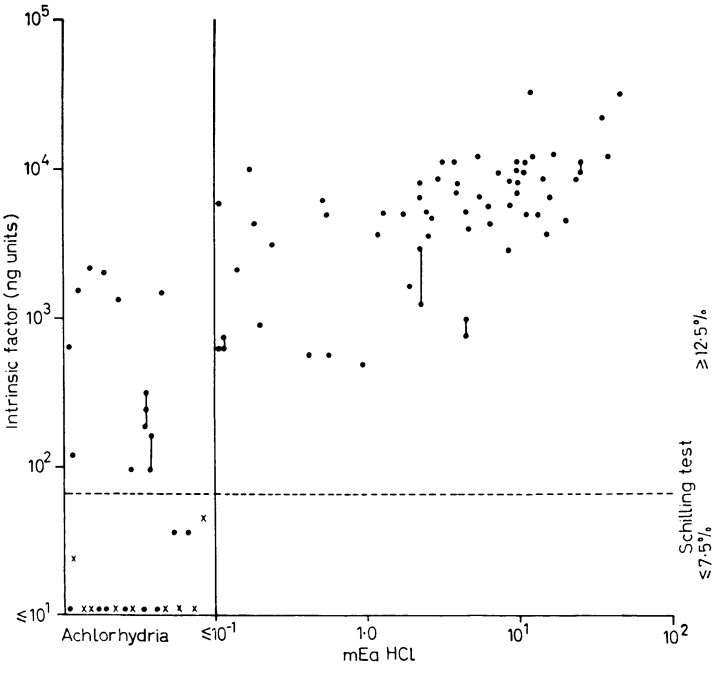

FIG. 7. The secretion of intrinsic factor and of HCl in the gastric juice during the post-histamine hour. The histamine was given subcutaneously or intramuscularly in a dose of 0·04 mg/kg body weight. One hundred and three assays of intrinsic factor were made on eighty-seven samples of gastric juice from eighty-seven patients. In nine instances the variation between repeat assays was not sufficiently large to be discernible on the graph. Patients with achlorhydria, those with malabsorption of oral vitamin B_{12} in the Schilling test and those with antibody to intrinsic factor in the serum are indicated. ×, Patients serum positive for antibody to intrinsic factor; ●, patients serum negative for antibody to intrinsic factor.

was given by subcutaneous or intramuscular injection in a dose of 0·04 mg/kg body weight (Kay, 1953). The secretion of gastric acid during the post-histamine hour in this patient population varied from histamine-fast achlorhydria (pH ⩾ 6·0) to 34 mEq HCl (titrated with 0·1 N-NaOH using methyl red as indicator).

In Fig. 7 the secretion of intrinsic factor is correlated with the secretion of gastric HCl in the same samples of juice and with the patient's ability to absorb oral vitamin B_{12} in the Schilling test (Schilling, 1953). In patients with evidence of malabsorption of vitamin B_{12} it was shown that the Schilling test could be corrected when repeated with the simultaneous

administration of hog intrinsic factor. The serum of each patient was tested for antibody to intrinsic factor by the methods shown in Tables 1 and 2. There is a broad correlation between the secretion of intrinsic factor and the secretion of gastric acid during the post-histamine hour. In general terms, the higher the secretion of gastric acid the higher the secretion of intrinsic factor. Patients with evidence of malabsorption of vitamin B_{12} in the Schilling test all had a secretion of intrinsic factor in the post-histamine hour of less than 80 ng units. Patients with achlorhydria but normal Schilling tests of vitamin B_{12} absorption had $\geqslant 100$ ng units intrinsic factor in the gastric juice secreted during the post-histamine

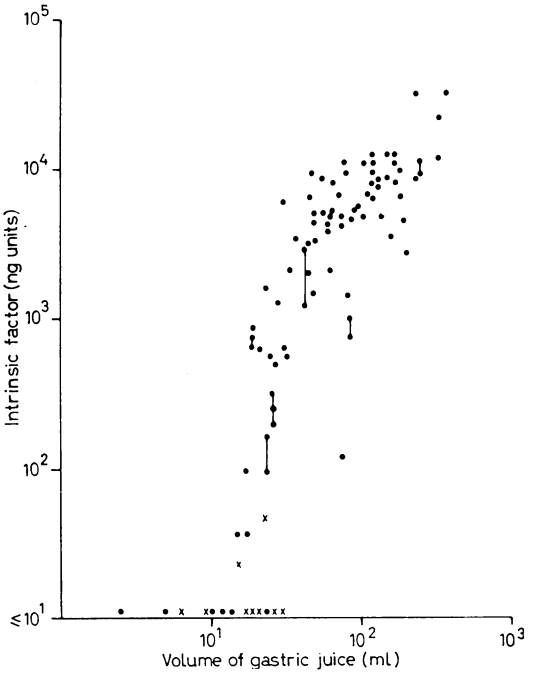

FIG. 8. The intrinsic factor content correlated with the volume of gastric juice secreted during the post-histamine hour in the same group of patients as in Fig. 7. ×, Patients serum positive for antibody to intrinsic factor; ●, patients serum negative for antibody to intrinsic factor.

hour. Patients that were capable of secreting acid in response to histamine had an intrinsic factor secretion of $\geqslant 500$ ng units in the post-histamine hour.

Fig. 8 refers to the same group of patients as shown in Fig. 7 and correlates the volume of the gastric secretion with the secretion of intrinsic factor during the post-histamine hour. The reduction in intrinsic factor secretion is not entirely dependent upon a reduced volume of secretion.

The immunoassay of intrinsic factor was repeated on sixteen of the samples of gastric juice included in Figs. 7 and 8. In nine instances the variation in results was insufficient to be demonstrable on the graph; in the other seven instances the repeat analyses on the same gastric juice are shown by lines joining the corresponding points.

In this population of patients the presence of antibody to intrinsic factor in the serum

was invariably associated with histamine-fast achlorhydria and malabsorption of vitamin B_{12} in the Schilling test that was correctable by exogenous intrinsic factor.

The pattern of secretion of intrinsic factor following the subcutaneous or intramuscular injection of 0·04 mg/kg histamine has been previously described (Irvine, 1965a; Wangel & Callender, 1965). The secretion of intrinsic factor is maximal during the first 30 min and then falls off more rapidly than the volume or the acidity of the gastric juice. Fig. 9 shows

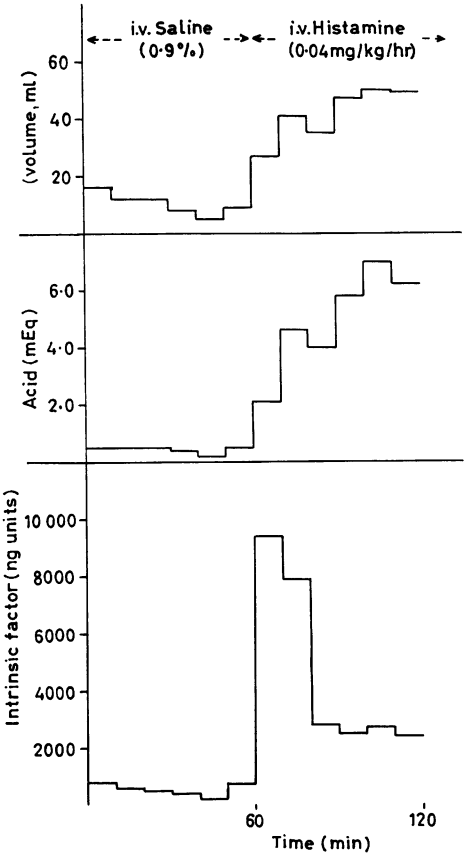

FIG. 9. The volume, acidity and intrinsic factor content of the gastric secretion in response to a constant intravenous infusion of histamine, 0·04 mg/kg body weight/hr.

that when histamine was given by constant intravenous infusion a very prompt release of intrinsic factor into the gastric juice occurred within the first 10 min and was maintained to some extent during the second 10 min. Thereafter the secretion of intrinsic factor was at a lower level but greater than during the basal 1 hr. In contrast, the volume and the acidity of the gastric juice rose progressively during the 1 hr of the infusion. In two other subjects similarly studied the secretion of intrinsic factor returned to near basal levels during a second hour of histamine infusion although the acid secretion was maintained.

ANALYSIS OF PREPARATIONS OF HOG INTRINSIC FACTOR

Commercial preparations of hog intrinsic factor were assayed biologically by determining how much of the preparation was required in order to correct the absorption of oral vitamin B_{12} in patients with Addisonian pernicious anaemia. The method of assay is based on the recommendations of the Anti-anaemic Preparations Advisory Board of the U.S. National Formulary (11th edition, 1960) and is described in Table 5. The method consists of a series of Schilling tests each separated by a minimum of 2 days control period with intramuscular flushing doses of 1000 μg vitamin B_{12} given on each day. Serial 24 hr collections of urine were counted in a large ring Geiger counter for their content of the isotope and the result expressed as a percentage of the dose administered. Urine collections from 48 to 72 hr after the oral administration of ^{57}Co vitamin B_{12} either contained no radioactivity or only negligible amounts.

TABLE 5. Procedure for the *in vivo* assay of preparations of hog intrinsic factor

Day	^{58}Co vitamin B_{12} (μg)	Parenteral vitamin B_{12} (μg)	Intrinsic factor preparation	
1	0·5	1000	—	Baseline response
2	—	1000	—	
3	—	1000	—	
4	0·5	1000	Test 1	Sample response
5	—	1000	—	
6	—	1000	—	
7	0·5	1000	Test 2	Sample response
8	—	1000	—	
9	—	1000	—	
10	0·5	1000	Test 3	Sample response
11	—	1000	—	
12	—	1000	—	

The same commercial preparations of hog intrinsic factor were assayed using the *in vitro* method. The appropriate weight of the intrinsic factor preparation was dissolved in phosphate buffer and the procedure was thereafter the same as in Table 3, substituting the solution of intrinsic factor for gastric juice. Seven preparations of intrinsic factor were assayed both by the *in vivo* method and by the *in vitro* technique. In the *in vitro* assay each preparation of hog intrinsic factor was assayed at least four times. The comparison between the *in vitro* and the *in vivo* assay of preparation WES-818 is shown in Fig. 10. In the *in vivo* assay of this preparation three patients with pernicious anaemia were studied. By the *in vitro* technique 1 mg of preparation WES-818 was found to contain 120 ng units of intrinsic factor, and in the *in vivo* assay between 2·5 and 5·0 mg of the preparation were sufficient to correct the Schilling test in all three patients with Addisonian pernicious anaemia.

In Table 6 the results of the *in vivo* assay of the seven preparations of hog intrinsic factor are compared with the *in vitro* assay determinations of intrinsic factor content. There is an inverse relationship between the minimum weight of the preparation that is effective in

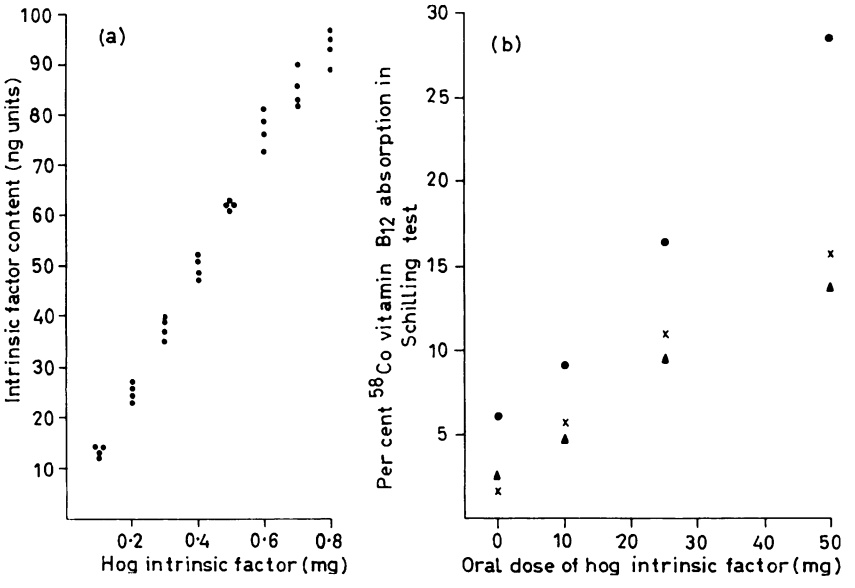

FIG. 10. A comparison between the *in vitro* (a) and the *in vivo* (b) assay of hog intrinsic factor preparation WES—818. (b) ●, pernicious anaemia patient 1; ×, pernicious anaemia patient 2; ▲, pernicious anaemia patient 3.

the *in vivo* assay and the content of intrinsic factor per milligram of the preparation as determined by the *in vitro* assay. It is estimated that 300–600 ng units hog intrinsic factor are required to correct the Schilling test in patients with pernicious anaemia.

TABLE 6. Analysis of preparations of hog intrinsic factor

Hog intrinsic factor preparation	Minimum weight effective by *in vivo* assay (mg)	Content of intrinsic factor by *in vitro* assay	
		ng units/mg	% of total vitamin B_{12} binding substances
3908C—46—1	0·5	720 ± 10*	21
WES—818	2·5–5·0	120 ± 4	31
WES—942	5	98 ± 4	25
Revai—189	10	52 ± 3	26
L129	25	26 ± 5	17
934C—186—1	10–15	28 ± 3	36
48743—129	50	8 ± 4	7

*The results of the *in vitro* assay are shown as the mean ± 1 SD of at least four assays.

DISCUSSION

The charcoal method for the detection and titration of antibody to intrinsic factor in the serum and for the titration of the intrinsic factor content of gastric juice or of preparations of hog intrinsic factor is both simple and rapid. It is well suited to a hospital routine laboratory that is familiar with the use of isotopes. The results are highly reproducible and the shape of the titration curves indicate that only one antigen–antibody reaction is involved. Roitt *et al.* (1964) showed that the charcoal method (Ardeman & Chanarin, 1963) and the dialysis method (Abels *et al.*, 1963) both determine the presence of antibody to intrinsic factor at the vitamin B_{12} binding site, while the electrophoretic retention method of Jeffries *et al.* (1962) detects the presence of an antibody to intrinsic factor that is specific for a site remote from that of vitamin B_{12} binding. In a batch of thirty-one sera from patients with pernicious anaemia, Roitt *et al.* (1964) noted that the charcoal and the dialysis methods gave the same incidence of positive results. Charcoal is, however, a more convenient method of removing free vitamin B_{12} from the *in vitro* system than is dialysis.

The observation by Herbert *et al.* (1964) that protein is required in the test system in order to allow the charcoal to selectively absorb unbound vitamin B_{12} is confirmed but it was found to be unnecessary to use albumin-coated charcoal if serum in the quantities recommended in Tables 1–3 was already present in the *in vitro* system. The period of absorption with charcoal is not critical. Shorter incubation times before and after adding the ^{57}Co vitamin B_{12} may well prove satisfactory but were not studied during the present investigation. In order to avoid false positive results for antibody to intrinsic factor it is important to verify when obtaining the sample of serum that the patient has not received parenteral vitamin B_{12} during the preceding 24 hr. The incidence of antibody to intrinsic factor (vitamin B_{12} binding site) in a titre of greater than 5 ng units/ml serum was found to be 52% in patients with proven Addisonian pernicious anaemia. In a group of eighty-seven patients (Figs. 7 and 8) suffering predominantly from duodenal ulcer, thyroid disease or pernicious anaemia, nine had antibody to intrinsic factor in the serum and all these nine had a histamine-fast achlorhydria and evidence of malabsorption of vitamin B_{12} in a Schilling test that was correctable by the simultaneous administration of exogenous intrinsic factor. This is in keeping with the previous observation that only a small minority of patients with antibody to intrinsic factor (vitamin B_{12} binding site) in the serum have adequate absorption of oral vitamin B_{12} (Irvine, 1965b, c). It remains to be seen whether the few patients with intrinsic factor antibody in the serum and adequate vitamin B_{12} absorption will progress to latent or frank pernicious anaemia during the course of months or years. The clinical value of antibody to intrinsic factor (vitamin B_{12} binding site) as a rapid serological screening test for pernicious anaemia can be summarized in the statement that it need give few false positive results but that the incidence of false negative results is high at 40–50%. The second antibody to intrinsic factor, as detected by the electrophoretic retention method, has a low incidence of positivity in the sera of patients with pernicious anaemia at 20–30% (Jeffries *et al.*, 1962). Moreover this antibody tends to occur when antibody to intrinsic factor at the vitamin B_{12} binding site is also present (Rice & Irvine, unpublished observations). These factors limit the diagnostic usefulness of the Jeffries type of intrinsic factor antibody.

The titre of antibody to intrinsic factor as determined by the charcoal method ranged up

to 790 ng units/ml serum. Sera with a titre of $\geqslant 100$ ng units/ml are suitable for use as standard antisera in the assay of the intrinsic factor content of samples of human gastric juice. In order to obtain sufficient standard antisera it is advisable to form a small panel of blood donors among patients with treated pernicious anaemia who are in good health, have high titres and who are co-operative.

The charcoal method for immunoassay of intrinsic factor in human gastric juice clearly distinguishes between intrinsic factor and other vitamin B_{12} binding substances that are frequently present in gastric juice. It gives results that are in keeping with the patient's gastric secretion of acid and the patient's vitamin B_{12} metabolism as studied by the Schilling test. Other *in vitro* methods for the assay of intrinsic factor in gastric juice have been based on the finding that vitamin B_{12} uptake by rat liver slices is enhanced by intrinsic factor (Miller & Hunter, 1957) and on the observation that vitamin B_{12} uptake by everted sacs of guinea-pig ileum is facilitated by human intrinsic factor (Strauss & Wilson, 1960; Wolff & Nabet, 1962). Because of uncertainty as to its physiological meaning, relative insensitivity to human intrinsic factor, and the occasional failure of the system to detect intrinsic factor in gastric juice containing free acid (Herbert, 1959), the liver assay has not been widely used in the study of human intrinsic factor. Sullivan, Herbert & Castle (1963) have developed the guinea-pig ileum method by using pooled lots of homogenized guinea-pig intestinal mucosa to avoid the variabilities of the everted sacs of guinea-pig ileum. The ratios of vitamin B_{12}: intrinsic factor: receptors are, however, somewhat critical if reproducible results are to be obtained (Herbert & Castle, 1961). Jeffries, Sleisenger & Benjamin (1963) have modified their electrophoretic technique so as to identify and measure human intrinsic factor in gastric juice. However, the charcoal method is probably the most sensitive, reproducible, and easily performed of the assays for intrinsic factor that are presently available.

The clinical value of the charcoal method of intrinsic factor assay is indicated by the clear difference in the estimated secretion of intrinsic factor in achlorhydric patients with and without evidence of malabsorption of oral vitamin B_{12} in the Schilling test (Fig. 7). A gastric secretion of less than 80 ng units intrinsic factor in the hour following subcutaneous or intramuscular histamine, 0·04 mg/kg body weight in a standard Kay test (Kay, 1953), is indicative of Addisonian pernicious anaemia provided the gastric aspiration has been adequately performed. The secretion of intrinsic factor in response to histamine (Ardeman, Chanarin & Doyle, 1964) and to gastrin (Irvine, 1965a; Wangel & Callender, 1965) in the diagnosis of Addisonian pernicious anaemia is discussed more fully in a separate paper (Irvine *et al.*, 1965).

A further application of a satisfactory method for the *in vitro* assay of intrinsic factor is the study of the physiology of intrinsic factor secretion. During a constant infusion of histamine it was observed that the peak in the secretion of intrinsic factor occurred at the beginning of the infusion but soon fell to a steady level. This steady level was appreciably higher than the secretion of intrinsic factor during the basal hour when saline was being infused at the same rate. In contrast, the volume and the acidity of the gastric juice progressively increased during the 1 hr period of histamine infusion, as described by Lawrie, Smith & Forest (1964). It would therefore seem likely that, as a result of stimulation with histamine, intrinsic factor may be washed from the crypts into the gastric juice or that a reserve of intrinsic factor within the cells of the gastric mucosa may be rapidly released into the gastric

juice. This phase appears to be followed by a steady rate of intrinsic factor secretion dependent upon continued stimulation, but this latter phase in the secretion pattern was not so clearly seen in two other patients similarly studied.

The lack of species specificity of antibody to intrinsic factor is such that the serum of a patient containing the antibody in a suitable titre can be used for the titration of preparations of hog antral mucosa for intrinsic factor activity. A good correlation was found between the intrinsic factor activity of hog preparations determined by the charcoal method of immunoassay and the activity of these preparations when tested *in vivo*. It was found that some 300–600 units of hog intrinsic factor were required to correct the absorption of oral vitamin B_{12} in patients with Addisonian pernicious anaemia. It is therefore now possible to characterize the activity of any given preparation of hog intrinsic factor in terms of units of intrinsic factor activity per milligram of the preparation. It is suggested that in using hog intrinsic factor for diagnostic purposes a minimum dose of 1000 ng units should be regarded as acceptable.

In view of the ease and precision with which the charcoal method of immunoassay can distinguish between intrinsic factor and non-specific vitamin B_{12} binding substances, this technique should be of considerable value in the separation of intrinsic factor using extraction procedures. Finally, the possibility is raised that the application of immunological techniques may themselves lead to the eventual isolation of intrinsic factor.

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