A comparison of two methods for the immunoassay of intrinsic factor

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SYNOPSIS The intrinsic factor content of 263 samples of gastric juice was determined by immunoassay using charcoal absorption and by immunoelectrophoresis on acrylamide gel. There was good correlation between the results of the two techniques and, with a few exceptions, both made it possible to predict which patients would have malabsorption of radioactive vitamin B₁₂.

Of the two methods, immunoassay using charcoal absorption generally gave the higher readings. Further study showed that this could not be attributed to the recognized difference in the principles of the two methods but that it was more likely to be due to the dissimilarity of the experimental conditions.

The two commonly used methods for immunoassay of intrinsic factor are the charcoal absorption assay (Ardeman and Chanarin, 1963) and electrophoresis on starch gel (Jeffries and Sleisenger, 1963). Recently, Bardhan, Callender, and Spray (1966) have modified the latter technique by substituting acrylamide gel for starch gel to produce a quicker and more accurate assay system.

In this paper the results of immunoassay of intrinsic factor using charcoal absorption are compared with those obtained by immunoelectrophoresis on acrylamide gel.

METHODS

Gastric juice was collected as previously described (Wangel and Callender, 1965a). Specimens were collected after an overnight fast, during basal conditions, and after stimulation of gastric secretion.

The stimulants used were histamine acid phosphate 0.04 mg./kg. subcutaneously in 68 subjects, and a combination of gastrin I and II in a dose of 3 Leo units per kilogram intramuscularly (Leo Laboratories, Copenhagen) in 31 subjects. One subject received 15 units of soluble insulin intravenously, resulting in a fall of the blood sugar to less than 20 mg. per 100 ml. after 30 minutes, and one received 25 mg. of methacholine (acetyl- β -methylcholine chloride) subcutaneously in three divided doses at intervals of 15 minutes (Jeffries and Sleisenger, 1965) as the gastric stimulant.

IMMUNOELECTROPHORESIS This was carried out on

acrylamide gel as described previously (Bardhan *et al.*, 1966). In this assay normal serum containing antibody to intrinsic factor is added to a mixture of gastric juice and ⁵⁷Co-B₁₂ which has been previously incubated. Intrinsic factor-vitamin B₁₂ complex migrates anodally during electrophoresis; combination with antibody prevents this migration.

IMMUNOASSAY USING CHARCOAL ABSORPTION This was done according to the principle of Ardeman and Chanarin (1963) as previously described (Wangel and Callender, 1967). ⁵⁸Co-B₁₂ was added to mixtures of gastric juice plus normal serum and gastric juice plus serum containing antibody to intrinsic factor. The unbound vitamin was removed by absorption with activated charcoal.

In both assay methods, the ratio of radioactive vitamin B₁₂ to gastric juice was the same, *i.e.*, 400 μ g. vitamin B₁₂ to 1 ml. gastric juice neutralized to pH 7.0. The final results were corrected for the dilution resulting from neutralization of the gastric juice with *N* NaOH.

RESULTS

With a few exceptions there was good correlation between the results of the two methods for specimens of gastric juice collected after an overnight fast, during basal conditions, and after stimulation with histamine or gastrin (Fig. 1). The agreement between the two methods was closest for measurements made on specimens collected after stimulation (Table I).

The subjects were divided on the basis of acid secretion in the hour following stimulation into the following groups: those with achlorhydria, those who

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FIG. 1a.

FIG. 1b.

FIG. 1. The concentration of intrinsic factor ($\mu\text{g. units/ml. of gastric juice}$) measured by immunoelectrophoresis (EP) and by immunoassay using charcoal absorption (CA). (a) Histamine-stimulated gastric secretion; (b) gastrin-stimulated gastric secretion.

secreted between 0.1 and 9.9 mEq. HCl, and those who secreted 10 mEq. HCl or more. This division was made regardless of the stimulant used. Figure 2 shows that the mean amounts of intrinsic factor secreted rose with increasing acid secretion. In all groups im-

munoassay using charcoal absorption gave consistently higher values than the electrophoretic technique. By the latter method, nine patients with proven pernicious anaemia (cases 1-9, Table II) secreted from 0 to 50 units in the hour following stimulation while

TABLE I

CORRELATION OF THE VALUES FOR THE CONCENTRATION OF INTRINSIC FACTOR MEASURED BY IMMUNOELECTROPHORESIS AND IMMUNOASSAY USING CHARCOAL ABSORPTION

Gastric Juice Collected	Number of Samples	Correlation Coefficient r	P
A After overnight fast	69	0.689	<0.001
B During basal conditions	95	0.705	<0.001
C Following stimulation			
With histamine	68	0.832	<0.001
With gastrin	31	0.813	<0.001

immunoassay using charcoal absorption gave a range of 0 to 540 units per hour for the same patients.

As measured by immunoelectrophoresis, 18 out of 26 patients with achlorhydria secreted less than 170 units of intrinsic factor per hour in response to stimulation. Twelve of these 18 subjects had overt or latent pernicious anaemia and absorbed 30% or less of an oral dose of 0.5 μ g. of $^{58}\text{Co-B}_{12}$ measured by whole-body counting (Warner and Oliver, 1966; Callender, Witts, Warner, and Oliver, 1966) (cases 1-12, Table II). In a further two patients with latent pernicious anaemia (FD and BR, Table II), who secreted 160 and 170 units of intrinsic factor respectively, the absorption of $^{58}\text{Co-B}_{12}$ was not reduced to this extent but was clearly enhanced by exogenous intrinsic factor. Of the

remaining four, two were relatives of patients with pernicious anaemia and in one of them the absorption was in the low normal range (50%). Another patient (MM, Table II) absorbed 60% of a dose of 0.5 μ g. $^{58}\text{Co-B}_{12}$, but the prednisolone she was receiving for rheumatoid arthritis may have influenced the absorption (Gordin, 1959; Kristensen and Friis, 1960). This patient's serum contained antibody to intrinsic factor, and during the past four years the serum B_{12} level has varied between 78 μ g. and 100 μ g. per ml. The fourth patient (WY) had a gastrojejunostomy; the gastric juice was contaminated by considerable reflux through the stoma, but the normal serum folate and the response to exogenous hog intrinsic factor showed that the low absorption of radioactive vitamin B_{12} was in fact due to a lack of intrinsic factor.

By the charcoal assay 19 of 26 patients with achlorhydria secreted less than 550 units of intrinsic factor in the hour following stimulation. Twelve of these 19 subjects, with latent or overt pernicious anaemia (cases 1-12, Table II), absorbed 30% or less of a dose of 0.5 μ g. $^{58}\text{Co-B}_{12}$ measured by whole-body counting. In another five (FD, BR, EE, MM, and WY, Table II), the intrinsic factor secretion was also within the range observed for overt or latent pernicious anaemia (0 to 170 units) when measured by immunoelectrophoresis. One patient (FT) who absorbed 52.8% of 0.5 μ g. $^{58}\text{Co-B}_{12}$ but showed no increased absorption with added exogenous intrinsic factor, secreted 540 units of intrinsic factor measured by charcoal assay. He had complete gastric atrophy and a serum B_{12} level of only 80 μ g. per ml.

With one exception all subjects whose total acid output was 10 mEq.HCl or more per hour secreted more than 2,400 units of intrinsic factor as measured by immunoelectrophoresis and more than 4,400 units as measured by immunoassay using charcoal absorption (Fig. 3). The exception was the 19-year-old daughter of a patient with pernicious anaemia. She appeared to have a selective impairment of intrinsic factor secretion in that she secreted 330 ml. of gastric juice containing a total of 17.3 mEq.HCl in response to gastrin. However, intrinsic factor could not be demonstrated by immunoelectrophoresis and the charcoal assay gave the low value of 3.8 units per ml. of gastric juice and a total of 1,250 units. The serum from this girl did not contain antibodies to gastric parietal cells or to intrinsic factor.

Charcoal assays were found to give higher readings when compared with the electrophoretic method regardless of whether histamine, gastrin, or insulin had been used to enhance gastric secretion (Table III). In the single case in which Methacholine had been used as the stimulant this difference was not apparent. The difference in the results of the two assay techniques was small for specimens with a low content of intrinsic

FIG. 2. The mean secretion of intrinsic factor in the hour following stimulation in three groups of patients.

TABLE II

SECRETION OF INTRINSIC FACTOR IN THE HOUR FOLLOWING GASTRIC STIMULATION AND ABSORPTION
OF $^{58}\text{Co-B}_{12}$ IN ACHLORHYDRIC SUBJECTS

No.	Patient	Intrinsic Factor Secretion Measured by		$^{58}\text{Co-B}_{12}$ Absorption ¹ (%)	Diagnosis	Comments
		Immunoelectro- phoresis	Immunoassay using Charcoal Absorption			
1	I.C.	0	540	3	P.A.	
2	H.A.	0	20	7.9	P.A.	
3	M.C.	0	70	0	P.A.	
4	J.F.	0	70	0	P.A.	
5	J.T.	20	480	0	P.A.	
6	M.S.	20	60	19.5	P.A.	
7	J.C.	30	30	16	P.A.	
8	G.S.	40	30	30	P.A.	
9	W.H.	50	0	9.1	P.A.	
10	D.J.	0	0	23.9	L.P.A.	
11	T.W.	0	480	29	L.P.A.	
12	C.B.	20	140	19.6	L.P.A.	
13	F.D.	160	520	42.5	L.P.A.	Serum B_{12} 29 $\mu\text{g.}/\text{ml.}$; complete gastric atrophy; with 50 mg. H.I.F.C. $^{58}\text{Co-B}_{12}$ absorption increased to 60.7% With 50 mg. H.I.F.C. absorption of $^{58}\text{Co-B}_{12}$ increased to 83% Circulating G.P.C.A.B. present On treatment with prednisolone
14	B.R.	170	290	39.8	L.P.A.	
15	E.E.	0	370	50.1	P.A.R.	
16	M.M.	30	60	60.1	Rheumatoid arthritis	
17	W.Y.	50	0	18.2	Gastrojejunostomy plus vagotomy	Serum B_{12} 240 $\mu\text{g.}/\text{ml.}$; serum folate 7 $\mu\text{g.}/\text{ml.}$; serum iron 30 $\mu\text{g.}/\text{ml.}$ With 1,500 units human I.F. absorption 74% Circulating G.P.C.A.B. present
18	D.M.	120	600	63.6	P.A.R.	
19	F.T.	350	540	52.8	Gastric atrophy	Serum B_{12} 80 $\mu\text{g.}/\text{ml.}$ No increase in $^{58}\text{Co-B}_{12}$ absorption with 50 mg. H.I.F.C. Circulating G.P.C.A.B. present
20	E.B.	410	780	Not done	P.A.R.	
21	E.S.	480	750	51.8	Rheumatoid arthritis	
22	R.H.	510	690	65	P.A.R.	Circulating G.P.C.A.B. present
23	E.P.	590	1,030	63.9	Gastric atrophy	
24	V.E.	880	560	48	Rheumatoid arthritis	Atrophic gastritis
25	W.S.	920	0	65	Iron-deficiency anaemia	
26	E.S.	1,210	2,020	53.6	Myxoedema	

¹Percentage $^{58}\text{Co-B}_{12}$ absorption refers to the percentage of a dose of 0.5 $\mu\text{g.}$ (0.5 $\mu\text{C.}$) $^{58}\text{Co-B}_{12}$ retained in the body on the 14th day after administration, measured by whole-body counting

P.A. = pernicious anaemia

L.P.A. = latent pernicious anaemia

H.I.F.C. = hog intrinsic factor concentrate

P.A.R. = relative of patient with pernicious anaemia

G.P.C.A.B. = gastric parietal cell antibody (detected by immunofluorescence)

TABLE III

CONCENTRATION OF INTRINSIC FACTOR IN GASTRIC JUICE COLLECTED FOR ONE HOUR AFTER STIMULATION

Stimulant Used

	Gastrin			Histamine			Gastrin			Histamine			Insulin			Mecholyl		
	E.P.	E.P. Variant	C.A.	E.P.	E.P. Variant	C.A.	E.P.	E.P. Variant	C.A.	E.P.	E.P. Variant	C.A.	E.P.	E.P. Variant	C.A.	E.P.	E.P. Variant	C.A.
No. of assays	6	6	6	11	11	6	13	4	9	6	6	3	6	6	3	6	6	5
Standard deviation	2.280	0.272	1.149	5.210	5.946	1.699	7.631	5.859	14.387	7.151	5.451	4.158	2.963	4.630	7.808	5.702	6.259	2.320
Mean	20.1	18.9	22.5	22.8	23.8	27.2	78.0	89.8	139.3	56.0	54.9	85.8	125.3	114.8	147.7	74.9	74.5	79.0
Statistics	t = 1.934 P > 0.05			t = 0.419 P > 0.05			t = 0.028 P > 0.05			t = 27.055 P < 0.001			t = 4.681 P < 0.001			t = 1.528 P > 0.05		
				t = 2.653 P > 0.05			t = 6.560 P < 0.001						t = 25.697 P < 0.001					

E.P. = immunoelectrophoresis

C.A. = immunoassay using charcoal adsorption

E.P. Variant = immunoelectrophoresis after $^{58}\text{Co-B}_{12}$ is added to pre-incubated mixtures of gastric juice and normal serum, and gastric juice and serum containing antibody to intrinsic factor, i.e., the same sequence as in the charcoal assay

factor but considerable for those with a high content (Table III).

Further experiments were carried out to ascertain whether the difference in the results of the charcoal and electrophoretic techniques could be attributed to the fact that a different sequence of incubation of gastric juice, antibody, and vitamin B₁₂ was employed in the two methods. For this purpose the intrinsic factor content of six specimens of gastric juice was assayed by three methods, viz., the charcoal and electrophoretic techniques as already described and, in addition, gel electrophoresis of gastric juice incubated with serum containing antibody *before* the addition of ⁵⁷Co-B₁₂. The sequence of incubation in this variant of the electrophoretic technique was thus identical with that of the charcoal assay. Table III shows that the results of the electrophoretic method were not influenced by altering the sequence of incubation and that the difference between this method and the charcoal assay remained.

The correlation between the total intrinsic factor and acid secreted in the hour following stimulation with either histamine or gastrin was significant (Table IV).

TABLE IV
CORRELATION BETWEEN THE AMOUNTS OF
INTRINSIC FACTOR AND ACID SECRETED IN THE
HOUR FOLLOWING GASTRIC STIMULATION

Secretory Stimulant Used	Assay Method			
	Immunoelectrophoresis		Immunoassay with Charcoal Absorption	
Histamine n = 68	r = 0.790	P < 0.001	r = 0.870	P < 0.001
Gastrin n = 31	r = 0.832	P < 0.001	r = 0.879	P < 0.001

DISCUSSION

For practical purposes, immunoassay using charcoal absorption is simpler than immunoelectrophoresis and therefore preferable for routine clinical use. Both methods make it possible to predict in most cases whether or not an individual will have impaired absorption of vitamin B₁₂.

Nevertheless, a few subjects who on the basis of either of the assays would have been expected to have impaired absorption of the vitamin had normal ab-

sorption tests. Such exceptions have also been noted by others (Ardeman, Chanarin, Jacobs, and Griffiths, 1966).

The degree of correlation between the results of the two methods was highest for samples of gastric juice obtained after stimulation and lowest for the overnight specimens, while samples collected during basal conditions occupied the intermediate position (Table I). This may simply reflect the relative degree of contamination of the gastric juice with swallowed saliva and mucus.

The charcoal assay gave higher values for the concentration of intrinsic factor both in single assays of 263 different samples of gastric juice and in repeated assays on single specimens. It has been suggested that the formation of an intrinsic factor-vitamin B₁₂ complex may make some of the antigenic sites on the intrinsic factor molecule unavailable for combination with specific antibody. This linkage might be the reason why values obtained by immunoelectrophoresis, where such a sequence is involved, are lower than those obtained by immunoassay using charcoal absorption where linkage of antigen and antibody is complete before radiovitamin B₁₂ is added (Ardeman, Chanarin, and Berry 1965). However, our results indicate that the availability of antigenic sites is not affected by the binding of vitamin B₁₂ to intrinsic factor, since the results obtained by immunoelectrophoresis were not markedly altered by allowing the antibody to interact with intrinsic factor before the addition of vitamin B₁₂ (Table III). We feel that the difference in the results of the two assay methods is more likely to be due to the dissimilarity of the experimental conditions of the two techniques.

Ardeman (1965), showed that there was a significant correlation between the secretion of acid and of intrinsic factor during basal conditions. Our results show that there is an even closer correlation between the secretion of acid and of intrinsic factor after stimulation of gastric secretion. Similar results were obtained by Rødbro, Christiansen, and Schwartz (1965).

Both immunoassay using charcoal absorption and immunoelectrophoresis are sensitive and reproducible methods. The former is, because of its simplicity, in our opinion the method of choice for routine clinical use. The latter is of particular value as a research technique because it can be used to measure intrinsic

factor by two different principles under identical experimental conditions.

ADDENDUM

Subsequent work (Roger Hall and K.D.B.) has shown that the similarity of results obtained by the electrophoretic method and its variant was attributable to the fact that the pool of serum containing antibody to intrinsic factor fortuitously had high titres of both 'blocking' and 'binding' antibody (Schade, Abels, and Schilling, 1967).

When a serum containing a low titre of 'binding' antibody but with a high titre of 'blocking' antibody was used, the results obtained by electrophoresis were lower than those obtained by the electrophoretic variant method and by charcoal immunoassay.

The basic findings in this paper remain unchanged; however, we wish to emphasize that, to duplicate these results, it would be necessary to use a pool of serum containing high titres of both 'blocking' and 'binding' antibody to intrinsic factor.

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