STUDIES ON ANTIBODY TO GASTRIC INTRINSIC FACTOR

A thesis submitted for the degree of Doctor of Philosophy in the Faculty of Medicine, University of London.

by

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Abstract.

This thesis itemises findings which support the belief that antibodies against intrinsic factor within the gastro-intestinal tract, play a critical role in the pathogenesis of pernicious anaemia.

A method is described for the release and identification of antibody in the gastric juice against intrinsic factor. The release of antibody was studied over a range of pH, under conditions of antigen excess and using progressively smaller quantities of antibody.

This method was used in an investigation of patients with pernicious anaemia. Out of fifty-three subjects studied, antibody was identified in the gastric juice of twenty-nine. There was no correlation in these subjects of antibody in the gastric juice with antibody in the serum.

The antibody findings in the serum and gastric juice were related to vitamin B_{12} absorption studies, employing hog intrinsic factor. Patients without antibodies in the gastric juice or serum were found to absorb vitamin B_{12} normally when provided with extraneous hog intrinsic factor. Vitamin B_{12} absorption was impaired in those patients who exhibited intrinsic factor antibody in the gastric juice.

In addition, eleven patients were studied who did not have pernicious anaemia but in whom antibodies against intrinsic factor were identified in the serum. All but one of these subjects lacked antibody from the gastric juice. All continued to absorb vitamin B_{12} normally.

The relative importance of antibody in the serum and in the gastric juice is discussed in relation to their respective roles in the pathogenesis of pernicious anaemia.

The relevant literature is reviewed and the work herein discussed in relation to the findings of other workers in this field.

INDEX

Section 1.

INTRODUCTION

1.1 The Intention.

There is indirect evidence to suggest that the impaired absorption of vitamin B_{12} which characterises pernicious anaemia is partly due to the action of organ-specific auto-antibodies.

Intrinsic factor is produced by gastric parietal cells in the normal human subject. It is required to mediate vitamin B_{12} absorption by the distal small bowel. Deficiency of intrinsic factor may be the result of reduced production; alternatively intrinsic factor may be made functionally inert by antibody. When the available intrinsic factor falls beneath a critical level, vitamin B_{12} is no longer adequately absorbed and the clinical consequences of vitamin B_{12} deficiency supervene.

The purpose of this thesis is to investigate the incidence, role and importance of antibody to intrinsic factor within the gastro-intestinal tract in the pathogenesis of pernicious anaemia.

1.2 Historical survey.

The term "pernicious anaemia" has been preserved out of deference to tradition. The transposition of the diagnostic criteria from the bed-side to the laboratory has resulted in the unsatisfactory situation where a diagnosis of pernicious anaemia may now be made in a patient who is not anaemic and in whom a pernicious outcome can almost always be forstalled.

Biermer in 1872 coined the term "Progressive pernicious anaemia" to describe the clinical features in a group of patients resembling the cases originally reported by Thomas Addison in 1855. Quincke (1877) described macrocytes in the peripheral blood. Increased cellularity of the bone marrow, expansion of the haemopoietic marrow and megaloblastic red cell precursors were described respectively by Pepper (1975), Cohnheim (1876), Ehrlich and Lazarus (1898).

The diagnosis of pernicious anaemia was almost certainly authentic in the cases documented by Osler and Gardner (1877), Leichtenstern (1884), Russell et al (1900), who described characteristic disturbances of neurological function coexistent with the anaemia.

The stomach was first intuitively implicated in the pathogenesis of pernicious anaemia by Austin Flint (1860). This speculation was subsequently given authority by Fenwick's observations of atrophy of the stomach in four subjects manifesting the clinical features of pernicious anaemia (1870). Histopathological changes of the mucosa in fixed gastric material were described by Faber and Bloch (1900). Cahn and von Mehring (1886) noted the absence of acid from the gastric juice and Martius (1897), Levine and Ladd (1921) and Hurst (1923) established this finding as the rule in pernicious anaemia. Gomperts and Vorhaus (1925) described the persistent absence of acid despite gastric stimulation with histamine.

Castle's classical experiments (1929-30) implicated lack of an intrinsic factor from the gastric juice in pernicious anaemia which was normally required for the absorption of an essential haematinic in liver. Precise in vitro methods for the measurement of this component of gastric juice have been developed and the original term "intrinsic factor" has persisted. (Miller and Hunter 1957; Wilson et al 1959; Sullivan et al 1963; Jefferies and Sleisenger; Abels et al 1963; Ardeman and Chanarin 1963).

Intrinsic factor is a glycoprotein (Grasbeck et al 1962) produced in man by the gastric parietal cells (Hoedmaker et al 1964). These cells are mainly localised in the fundus of the stomach.

Intrinsic factor is normally secreted in more than one-hundred fold excess of that required for normal vitamin B_{12} absorption (Ardeman et al 1964).

1.3 A review of the literature on the pathogenesis of pernicious anaemia.

A. THE GASTRIC LESION:

The introduction of the flexible gastric biopsy tube by Wood et al (1949) has made it possible to correlate gastric appearances in life with tests of gastric function.

Severe gastric mucosal atrophy or atrophic gastritis with histamine- or gastrin-fast achlorhydria in the gastric juice are the sine qua non for a diagnosis of Addisonian pernicious anaemia. However the presence of such findings does not provide cast iron evidence for the diagnosis or even imply that it is imminent. The degree of atrophy or inflammation seen in the gastric mucosa and submucosa provides no criterion for predicting whether the patient concerned maintains normal vitamin B_{12} absorption or has gross impairment of this function. Furthermore it cannot be determined from the gastric mucosal histopathology alone, which subjects are or will become vitamin B_{12} deficient with the attendant clinical consequences (Shiner and Doniach 1957; Williams et al 1958; Fisher et al 1967).

The distinction between pernicious andaemia and haematologically uncomplicated atrophic gastritis is made principally on symptoms. Patients with pernicious anaemia attend mainly with symptoms of anaemia or neuropathy whereas patients with simple atrophic gastritis are usually asymptomatic. Nonetheless clinical and

laboratory study has identified differences in these two groups of patients and the follow-up studies of Wood et al (1964) over two to fourteen years and Siurala et al (1966) over ten to fifteen years do not suggest a merging or a continuous deterioration in more than twenty five per cent of those subjects who did not initially have pernicious anaemia. It thus appears that in uncomplicated gastric atrophy and chronic atrophic gastritis, whilst the gastric secretion is impaired, in the majority there is a persistence of intrinsic factor production which is sufficient to mediate normal vitamin B_{12} absorption. This is in turn adequate to prevent megaloblastic haemopoiesis and the neurological sequelae of vitamin B_{12} deficiency.

B. THE ROLE OF AUTO-ALLERGY.

1. Clinical evidence.

Mollin and Ross (1954) and Glass et al (1954) described patients with pernicious anaemia who had initially responded well to oral administration of heterologous preparations of intrinsic factor but both subsequently relapsed. This form of treatment was introduced by Sharp (1929) and Wilkinson (1930). Wilkinson (1949) later reported relapses in fifteen out of 441 patients thus treated over a six year period and he attributed this to their failure to continue with their medication. However a high incidence of relapse in patients who continued to take their oral preparations was observed by Blackburn et al (1955), Bastrup-Madsen (1957), Lowenstein et al (1957), Killander (1957 and 1958) and Berlin et al (1958).

Kristensen et al (1957) were unable to reverse relapse by increasing the dose of hog pyloric mucosa, whereas Berlin et al (1958) obtained remission by using a large increase in the amount of hog intrinsic factor administered.

Schwartz et al (1957) established that relapse during treatment was due to impaired vitamin B_{12} absorption when this was given with hog intrinsic factor, while human intrinsic factor continued to mediate normal vitamin B_{12} absorption in these patients.

2. In vivo studies in man:

Schwartz (1958) identified a serum component in thirteen subjects with pernicious anaemia who had become refractory to hog intrinsic factor. When these sera were incubated with hog intrinsic factor and vitamin B_{12} prior to administration to pernicious anaemia subjects who were normally responsive to hog intrinsic factor, there was a marked impairment of vitamin B_{12} absorption.

Schwartz (1958) also described three other subjects who had become resistant to hog intrinsic factor and whose serum did not interfere with vitamin B_{12} absorption through this agency. Conversely some of the sera manifesting inhibitory activity against hog intrinsic factor were from patients who continued to absorb vitamin B_{12} when administered with hog intrinsic factor. Thus a serum inhibitor of intrinsic factor was not the sole cause of the intestinal blockade on vitamin B_{12} absorption, where resistance to the effects of hog intrinsic factor had developed. Schwartz (1958) suggested that this inhibitor might be an antibody and sought to elicit an antibody to this antigen by immunising rabbits (see section 1.3 B4).

Subsequently Taylor (1959) demonstrated that the serum of nine out of nineteen previously untreated patients with pernicious anaemia or patients who had received only vitamin B_{12} injections, also had a component in their serum which intefered with the ability

of hog intrinsic factor to mediate vitamin B_{12} absorption in pernicious anaemia subjects. Taylor (1959) also noted that in three of his cases the level of serum inhibitor declined or disappeared after four weeks treatment with hydrocortisone and in the one subject tested had returned to its original level one week after withdrawal of hydrocortisone. From these findings he inferred that this inhibitor was an immune globulin. Schwartz (1960) reported similar findings in untreated cases of pernicious anaemia and identified the inhibitor as a serum globulin.

Kaplan et al (1963) immunised two volunteers who had pernicious anaemia, with hog intrinsic factor and Freund's adjuvant. Whilst antibodies against hog intrinsic factor appeared in the sera of both these patients, hog intrinsic factor was still effective in augmenting vitamin B_{12} absorption. These observations supported the contention of Schwartz (1954) that antibody in the serum was not the cause of resistance to hog intrinsic factor.

3. Eliciting the gastric lesion in animals:

Spontaneously occurring gastric atrophy is infrequently encountered in mammals. Gastric atrophy has been induced in dogs with homologous and human gastric juice (Smith et al 1958; Fixa et al 1964). However Sircus et al (1963) employing similar methods failed to induce such gastric lesions. Hausamen et al (1969) gave intravenous injections of gamma globulin fractions from rabbit antisera against guinea pig gastro-intestinal antigens, to guinea pigs. They observed acute inflammation in the stomachs of these animals at necropsy, within twenty-four hours of the injection.

Krohn (1968) showed that dogs immunised with homologous or autologous gastric preparations developed chronic gastric mucosal inflammation and atrophy. Andrada et al (1969) reported the induction

of chronic atrophic gastritis in three monkeys injected with homologous gastric mucosal suspensions and complete Freund adjuvant.

4. Eliciting gastric antibodies in animals:

Taylor and Morton (1958) prepared antisera in rabbits against hog intrinsic and human intrinsic factor. Incubation of these antisera with the appropriate intrinsic factor resulted in impairment of their capacity to mediate vitamin B_{12} absorption in pernicious anaemia subjects. Gullberg and Kistner (1962) using a gel diffusion technique, demonstrated precipitation of antisera prepared in rabbits with human intrinsic factor.

Krohn (1968) using the immunofluorescent techniques of Coons (1958), demonstrated parietal cell antibodies in the serum of his immunised dogs which appeared concurrently with the development of chronic gastric inflammation and atrophy. Subsequently these animals developed antibodies against a canine gastric vitamin B_{12} binder. Krohn (1968) provided indirect evidence that this macro-molecule corresponded to canine intrinsic factor. Indeed, dog serum containing antibody to this gastric component cross-reacted with human intrinsic factor, interfering with its augmentation of vitamin B_{12} absorption in a subject with pernicious anaemia. Nevertheless, studies of vitamin B_{12} absorption in these experimental animals showed only moderate impairment of vitamin B_{12} absorption. Thus despite the serum inhibitor the intrinsic factor function persisted unimpaired.

Hausamen et al (1969) in their experiments (see 1.3 B3) employing a direct immunofluorescent technique, demonstrated antibodies localised on the gastric parietal cells of guinea pigs.

5. Antibodies against intrinsic factor in the serum in man:

Lowenstein et al (1961) applied the method of Stavitsky and Arquilla (1958) to coating bis-diazotised benzidine treated rabbit erythrocytes with intrinsic factor in order to identify antibodies to intrinsic factor by a haemagglutination technique. In the same paper Lowenstein et al introduced the electrophoretic retention technique to identify antibodies against hog intrinsic factor in complex with vitamin B_{12}. In this test the electrophoretic mobility of the intrinsic factor: vitamin B_{12} complex was retarded by sera containing antibody, evidently combining with a component of the gamma globulin fraction instead of migrating unimpeded. This technique was applied by Jefferies et al (1962) to a study of the sera from 44 patients with pernicious anaemia who had either received no treatment or therapy only with parenteral vitamin B_{12}. This technique gave positive results for antibody in nine (20%). The antibody was localised within the globulin fraction of the serum. The correlation with in vivo testing for antibody was good.

This test became the prototype of many modified tests in which the antigen was a complex of intrinsic factor with vitamin B_{12} (Taylor et al 1962; Jacob and Schilling 1966).

A further group of in vitro tests were developed in which intrinsic factor alone was the antigen and the presence of antibody in the test serum was determined by its ability to inhibit vitamin B_{12} binding to the intrinsic factor (Abels et al 1963; Ardeman and Chanarin 1963; Gottlieb et al 1965; Hansen et al 1966). These methods vary principally in the technique whereby unbound vitamin B_{12} is extracted. They can be applied to the identification and quantitation of antibody to intrinsic factor or to the assay of intrinsic factor in the gastric juice.

A third variety of test employs animal tissue slices or homogenates. In these tests positive sera interfere with the ability of intrinsic factor to bind vitamin B_{12} onto the tissue concerned(Wilson et al 1959; Sullivan et al 1963; Ashworth et al 1967).

Roitt et al (1964) tested sera for antibodies against intrinsic factor by representative tests from the first two groups cited above. They showed that antibody to intrinsic factor alone was found with greater frequency than was antibody to the complex of intrinsic factor with vitamin B_{12}. This discrepancy was first believed to represent a methodological rather than an immunological difference. However Roitt et al (1964) investigated a large number of sera from subjects with pernicious anaemia and they showed that whilst most sera were either positive or negative by both types of test used, some of their sera were consistently positive by one test and negative by the other. Furthermore, when antibody was identified by both tests there was frequently a disparity of titre. This led Roitt et al (1964) to suggest the existence of two separate forms of antibody against intrinsic factor. This speculation has been supported by the findings of Schade et al (1967) who showed that the antibody functions could be differently adsorbed. Garrido Pinson et al (1966) and Samloff et al (1968) have reported similar findings.

The two antibodies against intrinsic factor have been variously named:

a) Type 1. Blocking antibody (blocking: not to be confused with the generic use of the term, as when describing the prevention of synthesis of reaginic antibodies). Antibody to the vitamin B_{12} binding site.

b) Type II. Precipitating antibody. Antibody to the intrinsic factor vitamin B_{12} complex.

Thus in confirmed cases of pernicious anaemia the incidence of antibodies to intrinsic factor in the serum is, in decreasing order of frequency:

Neither antibody	40%
Both antibodies	40%
Type I antibody only	20%
Type II antibody only	0%

(Ardeman and Chanarin 1963; Irvine 1965; Fisher and Taylor 1965; Ardeman 1966; Wangel et al 1968; Samiloff et al 1968; Chanarin 1969).

Examples of type 2 antibody alone have been only sporadically documented (Abels and Schilling 1964; Roitt et al 1965; Garrido Pinson et al 1966; Schwartz 1967; Samiloff et al 1968), and the claims quoted in the first two of the above references have since been rescinded. The relative frequency of type 1 antibody is suggested to reflect greater antigenicity of the vitamin B_{12} binding site, than elsewhere on the intrinsic factor molecule (Samiloff et al 1968)

6. Antibodies against parietal cells in the serum in man:

A more uniform impression of the immunological disturbance in pernicious anaemia is conveyed by the incidence of antibodies to the gastric parietal cells. These may be demonstrated by either complement fixation or by immunofluorescent methods (Taylor et al 1962). The incidence of parietal cell antibodies in pernicious anaemia, identified by immunofluorescent methods, is about 85% (Chanarin 1969). Antibody against intrinsic factor is seldom found in the absence of parietal cell antibody. However, Doniach and Roitt (1964) reported cases of pernicious anaemia in juvenile subjects in whom intrinsic factor antibodies were present in the absence of parietal cell antibodies. In many of these children there were

multiple coexistent endocrine disorders in addition to the gastric lesion. Possibly these patients represented a particular group. Fisher and Taylor (1969), using techniques of differential adsorption suggested that the different distribution of fluorescence might be due to the different nature of the antibodies being identified. They suggested that a particular distribution of fluorescence on the parietal cell represented antibody to intrinsic factor which could be adsorbed out with the appropriate antigen, whereas another distribution represented antibody against certain microsomal components of the parietal cell.

7. Clinical associations:

Studies on the coexistence of pernicious anaemia with other conditions of possible or probable auto-allergic pathogenesis, have provided evidence of clinical association in a higher number than predicted by chance.

The incidence of pernicious anaemia in Grave's disease is 30%, whereas in primary myxoedema and Hashimoto's disease it is 11% (Chanarin 1969; collected figures). Hyperthyroidism is more common or more commonly diagnosed than hypothyroid states, nonetheless among patients with pernicious anaemia hypothyroidism is more commonly encountered (Wilkinson 1963). In patients with pernicious anaemia the incidence of thyroid disease is between 4-15% (Wilkinson 1963; Doniach et al 1963; Wangel et al 1968).

Vitiligo is found with increased frequency in patients with generalised thyroid disease (Morgans 1964), in pernicious anaemia (Allison and Curtis 1955), and in Addison's disease (Dunlop 1963; Meecham and Wyn Jones 1967).

The association between pernicious anaemia and Addison's disease of the adrenal cortex is derived from relatively small series (Goudie et al 1966; Blizzard et al 1966; Blizzard et al 1967; Irvine et al 1967). It is interesting to speculate that it may not have been entirely coincidence which led Addison (1855) to include a clinical description of "pernicious anaemia" as a preamble in a discourse upon the supra-renal capsules.

The evidence for prevalence of pernicious anaemia in idiopathic hypoparathyroidism comes also mainly from single cases reports, with the exception of the paper of Blizzard et al (1966).

Neither clinical association of diabetes mellitus nor rheumatoid arthritis with pernicious anaemia is striking, although some documented series are suggestive (Sundberg and Grunberg 1960; Wilkinson 1963; Rousso and Cruchaud 1966; Wangel et al 1968; Partridge and Duthie 1963; Irvine et al 1970). Numerous studies have established that subjects with perrdcious anaemia frequently have relatives with the same condition (Murphy 1939; Stamos 1940; Romei 1952; Mosbech 1953; Callender and Denborough 1957; Cox 1962; Wangel et al 1968). There are case reports of identical twins who developed pernicious anaemia more or less concurrently and other reports where only one of a non-identical twin pair developed pernicious anaemia. Among fifteen families in which both parents had pernicious anaemia there were thirty-six offspring of whom only one had pernicious anaemia. Callender and Denborough (1957) studied 308 relatives of subjects with pernicious anaemia. In 8% they found achlorhydria, a low serum vitamin B_{12} and subnormal vitamin B_{12} absorption.

Likewise there is an increased incidence of thyroid disease in the relatives of subjects with pernicious anaemia (Callender and Denborough 1957; Wangel et al 1968).

8. Serological studies in man.

Studies on sera from subjects with pernicious anaemia have revealed antibodies to the microsomal cell constituents and the products of organs incriminated in the associated clinical disorders (1.3 B7), Marksen and Moore 1962; Taylor et al 1962; Solomon and Blizzard 1963; Doniach et al 1965; Irvine et al 1965). Thyroid antibodies are found in 55% of pernicious anaemia sera and in the sera from 50% of their relatives. Parietal cell antibodies are present 84% of pernicious anaemia sera and about fifteen percent of matched controls, whereas they are positive in 36% of pernicious anaemia relatives, about 30% of patients with thyroid disase and 20% of the relatives of patients with thyroid disease (Te Velde et al 1964; Doniach et al 1965; Evans et al 1967).

Parietal cell antibody in the serum is almost invariably associated with some degree of chronic inflammation in the gastric mucosa and submucosa and some impairment of secretory function (Irvine et al 1965; Taylor 1966). However this impairment of secretion is seldom sufficient to compromise vitamin B_{12} absorption and the presence of a parietal cell antibody in the serum of a subject is not a declaration that pernicious anaemia will develop.

9. Antibody against intrinsic factor in the serum: the final insult or an innocent bystander?

Throughout the immunological characterisation of pernicious anaemia the question has persisted whether antibody against intrinsic factor circulating in the serum plays a participating role.

Four reports have been published concerning the transplacental passage of antibodies against intrinsic factor of class IgG from young women with pernicious anaemia to their foetuses in utero. At birth the neonates had detectable antibody against intrinsic factor circulating in the serum. Two groups (Bar Shany and Herbert 1967; Goldberg et al 1967) reported transitory impairment of intrinsic factor production whilst the maternal antibody persisted within the infants' circulation. However in two less comprehensive studies neither Charache and McIntyre (1966) nor Fisher and Taylor (1967) showed any impairment of intrinsic factor production. In none of the above-mentioned studies was an antibody against intrinsic factor identified within the neonatal gastric juice.

Possibly the clearest evidence that antibody against intrinsic factor in the serum is not implicated in the pathogenesis of pernicious anaemia comes from the studies of Schiller et al (1965) and Ardeman et al (1966) in which type 1 antibody against intrinsic factor was identified in the serum of selected patients with generalised chronic thyroid disease who did not have pernicious anaemia. Te Velde et al (1964) described a similar finding in one relative of a subject with pernicious anaemia. All these people maintained normal vitamin B_{12} absorption and correspondingly did not have pernicious anaemia. Follow up over a period of five years, by Rose et al (1970), on the five cases of Ardeman et al (1966) and a further five cases of Dr. D. Doniach (unpublished) has not shown any progression towards the gastrointestinal or haematological counterparts of pernicious anaemia.

Herbert and Kaplan (1961) suggested that intrinsic factor was absorbed with vitamin B_{12} and was taken up from the portal venous blood into the liver. However Cooper and White (1968) showed that intrinsic factor was limited to the gastro-intestinal tract. They

were able to identify labelled vitamin B_{12} in the portal venous blood after intrinsic factor mediated absorption through the small bowel mucosa but were unable to identify intrinsic factor by an immune technique. Yamaguchi et al (1970) labelled intrinsic factor with radioactive chromium and were also unable to show any evidence that it had been absorbed. If the conclusions drawn from these findings are correct, then antibody responsible for interfering with the function of intrinsic factor would have to confront this antigen in the region of its activity, that is, within the upper gastro-intestinal tract.

The characterisation of serum antibodies against intrinsic factor as class IgG by Ardeman (1966), Bernier and Hines (1967), suggests that this antibody originates from immunocytes subserving the tissues and the blood rather than being a remote expression of localised gastro-intestinal antibody synthesis, where mainly antibody of class IgA is produced (Crabbe et al 1965).

There is circumstantial evidence that an inhibitor to intrinsic factor may act within the gastro-intestinal tract of subjects with pernicious anaemia. It has been observed by a number of workers that the mean vitamin B_{12} absorption in pernicious anaemia after augmentation by intrinsic factor remains beneath the range encountered in normal subjects (Callender and Evans 1955; Ellenbogen et al 1955; Schilling et al 1955; Ellenbogen and Williams 1956; Oxenhorn et al 1957; Ardeman and Chanarin 1965). The consensus findings were that in the Schilling test a mean 20% of an oral dose of 1 mcg was excreted in the urine of normal subjects within twenty-four hours, whereas in subjects with pernicious anaemia tested with human intrinsic factor

the mean figure was 12% and with hog intrinsic factor, 14% (Chanarin, 1969). These differences were noted in patients irrespective of the presence or absence of antibody against intrinsic factor in the serum (Ardeman and Chanarin 1965). Ardeman and Chanarin (1965) noted that intrinsic factor mediated vitamin B_{12} absorption was lower in pernicious anaemia subjects than in subjects after partial gastrectomy. There are a number of allowances which must be made before deriving conclusions from differences in vitamin B_{12} absorption in different groups. It must be clear whether urine collections were made on the ward or from out-patients. The nature of the operative procedure employed for the gastrectomy is of importance, as the Polya gastrectomy leaves a blind loop which predisposes to the proliferation of organisms within the small bowel. Tabaqchali et al (1966) found that patients who had high bacterial counts in the jejunum had impaired vitamin B_{12} absorption which was not corrected with extraneous intrinsic factor.

The iron status of the groups compared was also of importance as Williams et al (1962) have shown that iron deficiency may compromise vitamin B_{12} absorption. Furthermore the vitamin B_{12} status of patients is of importance. Transient failure of intrinsic factor to mediate vitamin B_{12} absorption, which was later corrected by vitamin B_{12} therapy was reported by Brody et al (1966). Further similar cases have been reported by Haurani et al (1964) and Lawrence (1966). Conversely there is evidence that vitamin B_{12} absorption through the agency of extraneous intrinsic factor may be supressed by saturation of the subject with therapeutic vitamin B_{12}. Harwood and Forshaw (1967) showed that of vitamin B_{12} absorption fell in subjects who had previously received large parenteral doses of vitamin B_{12}.

Ardeman and Chanarin (1965a) have compared the potentiating effects of heterologous and homologous intrinsic factor on vitamin B_{12} absorption in pernicious anaemia subjects. They found that the heterologous (hog) intrinsic factor was more effective than human sources of intrinsic factor. However Ungar (1969) failed to show any differences between the abilities of hog and human intrinsic factor to mediate vitamin B_{12} absorption.

Ardeman and Chanarin (1965c) explained their findings by suggesting that an antibody against intrinsic factor was active in the gastrointestinal tract, also that the avidity of this antibody was greater for the homologous antigen-human intrinsic factor than it was for the hog intrinsic factor. There was nonetheless some degree of cross-reactivity, explaining the failure of hog intrinsic factor to elevate the mean vitamin B_{12} absorption in pernicious anaemia to the levels observed in normal patients and in patients after gastrectomy.

10. <u>The immunological character of subjects with uncomplicated atrophic gastritis</u>:

In a study of patients with biopsy proven atrophic gastritis unattended by megaloblastic anaemia Doniach and Reitt (1964) found that the incidence of parietal cell antibodies was 62% (females only) whereas Glass et al (1968) in a similar study observed positive results for parietal cell antibodies in only 24%. Whittingham et al (1969) studied 80 relatives of ten probands with simple atrophic gastritis who had no parietal cell antibodies. These relatives showed an entirely different incidence of pernicious anaemia, thyroid disease, Addison's disease, vitiligo, premature greying of the hair and

auto-antibodies to gastric and thyroid antigens when compared with the relatives of subjects with pernicious anaemia.

11. The effect of cortico-steroids:

Case reports have been published of clinical and haematological remission of patients with pernicious anaemia, whilst receiving either steroids or ACTH (Lowenstein et al 1951; Doig et al 1957; Gordin 1959). An improvement of vitamin B_{12} absorption was described in the majority of patients treated in this way by Frost and Goldwein (1958), Kristensen and Friis (1960 a and b), Ardeman and Chanarin (1965b), Jefferies et al (1966) and Wall et al (1968). This improvement was evident within a week of starting this treatment and a return to the status quo had taken place within three months of withdrawal.

Steroid treatment did not change the volume of the gastric secretion (Rodbro et al 1967). There was return of intrinsic factor in the majority and acid secretion in 15% (Kristensen and Friis 1960b). Jefferies (1965) Ardeman and Chanarin (1965b), Rodbro et al (1967) noted a reappearance of gastric parietal cells in biopsies taken from steroid treated patients. Such treatment also resulted in a decline of the titre of antibody to intrinsic factor in the serum (Jefferies 1965; Ardeman and Chanarin 1965b; Charache et al 1968) but an improvement in vitamin B_{12} absorption preceded any detectable change in titre of the serum intrinsic factor antibody (Ardeman and Chanarin 1965b). Furthermore the improvement in vitamin B_{12} absorption which followed steroid treatment was equally marked in patients with or without antibodies to intrinsic factor in their serum.

12. Antibodies against intrinsic factor in the alimentary tract:

Fisher et al (1965) were the first to demonstrate the presence of auto-antibodies in the gastric juice. They identified antibodies against parietal cells in the gastric juice from the subjects with pernicious anaemia. These workers subsequently (1966) identified type 1 antibody against intrinsic factor in the gastric juice of five out of fourteen subjects with

pernicious anaemia. In one of these subjects with the antibody in the gastric juice, no antibody against intrinsic factor was detected in the serum. Schade et al (1966) identified an antibody of type 2 in the gastric juice of one patient by in vivo and in vitro methods. The antibody was also detected in the serum of this patient.

Carmel and Herbert (1966) investigated serum and gastric juice from seventeen patients with pernicious anaemia and they found that in only two were they unable to detect an antibody against intrinsic factor at all. They found predominantly type 1 antibody in the serum and type 2 in the gastric juice. However, their findings were presented in an abstract and no details of their methods and clinical material have subsequently appeared.

Carmel and Herbert (1967) also investigate salivary secretions from three subjects with pernicious anaemia. They found a type 2 antibody to intrinsic factor in one specimen, which they identified as an immunoglobulin of class IgA.

Bauer et al (1968), using an autoradiographic technique, demonstrated antibody against a complex of intrinsic factor with vitamin B_{12}, coating submucosal mononuclear cells in gastric biopsies from two out of ten subjects investigated, with pernicious anaemia. Both of the subjects also had type 2 antibodies in their serum.

Antibody against intrinsic factor is of particular interest because it may participate in the pathogenesis of pernicious anaemia. Its presence in the gastro-intestinal tract may be the cardinal feature discriminating haematologically uncomplicated chronic atrophic gastritis from pernicious anaemia. If antibody is responsible for interfering with the function of residual but otherwise adequate intrinsic factor, then this must take place within the gastrointestinal tract. There it might function as the critical limiting factor in vitamin B_{12}

absorption. Such a role would implicate the antibody in the pathogenesis of vitamin B_{12} deficiency, even if it is not the cause of gastric lesion.

1.4 The Hypothesis.

In pernicious anaemia an antibody against intrinsic factor is synthesised by immunocytes in the submucosa of the upper alimentary tract. In the stomach it forms a complex with the residual intrinsic factor still produced by the few remaining parietal cells. In simple gastric atrophy, unattended by the presence of antibodies against intrinsic factor, the output of intrinsic factor is still adequate to subserve normal vitamin B_{12} absorption. When antibody to intrinsic factor is formed locally in the gastrointestinal tract it forms a complex with intrinsic factor, thereby interfering with its ability to mediate vitamin B_{12} absorption. When the criteria for a diagnosis of pernicious anaemia are met this is in association with the presence of antibodies against intrinsic factor in the gastrointestinal tract.

Since the antibody and intrinsic factor may coexist in the form of a complex within the gastric secretion, the total antibody content of an aspirated sample may only be detected after steps have been taken to separate the components of such a complex.

This thesis is concerned with describing a technique for separating intrinsic factor from its antibody and the application of this technique to the investigation of gastric juice samples from patients known to have pernicious anaemia.

1.5 Release of type 1 antibody against intrinsic factor and its identification. Principle and method.

A. THE PRINCIPLE:

Type 1 antibody will combine with intrinsic factor and thereby prevent access of vitamin B_{12} to its binding site on this molecule.

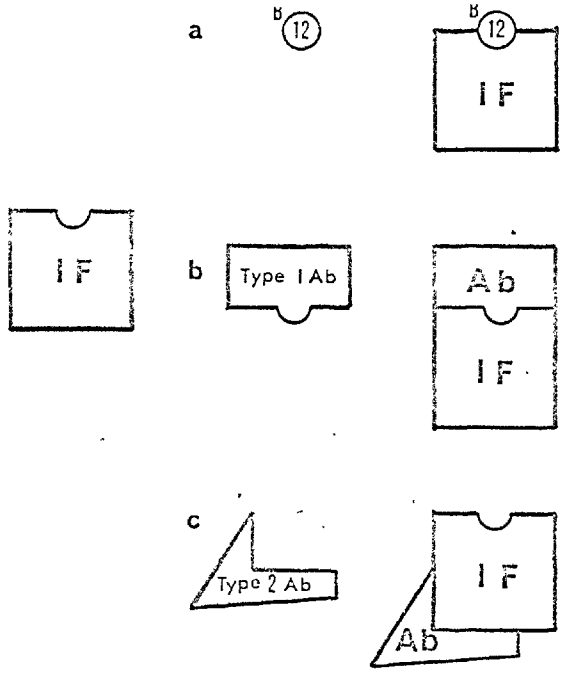


Figure 1.

Representation of intrinsic factor combining with:
a) vitamin B_{12}
b) Type I antibody
c) Type II antibody

A serum or gastric juice sample which interferes with the ability of normal gastric juice to combine with vitamin B_{12} may contain a type 1 antibody.

About thirty per cent of subjects with pernicious anaemia have some intrinsic factor in their gastric juice. All human gastric juice samples contain some macromolecules which bind vitamin B_{12}. Intrinsic factor is only one such component.

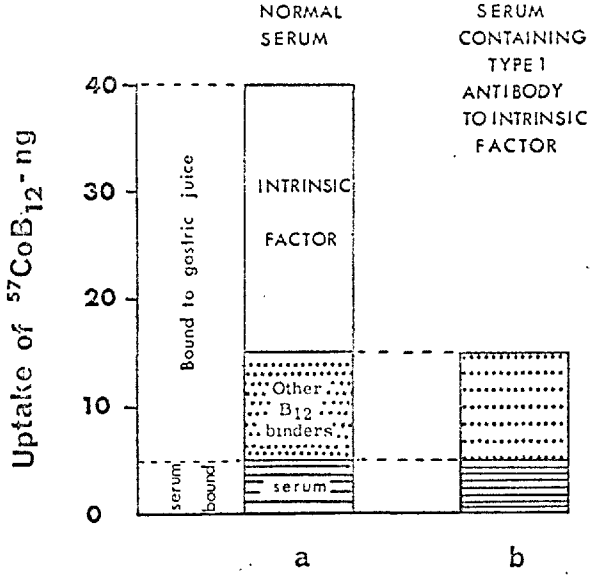


Figure 2.

The binding of vitamin B_{12} by normal gastric juice in the presence of: a) normal serum
b) serum containing Type I antibody to human intrinsic factor

The possible combinations of intrinsic factor and antibody which speculatively may be found in the gastric juice of a subject with pernicious anaemia are illustrated:

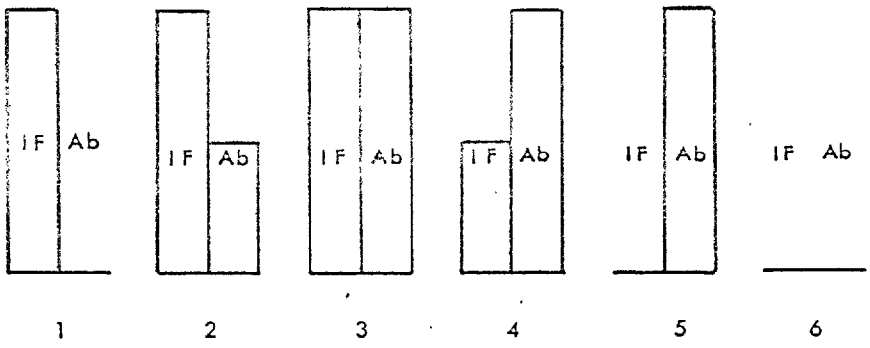


Figure 3.

Possible combinations of intrinsic factor and antibody against intrinsic factor, which may be found in the gastric juice of a subject with pernicious anaemia.

1. Residual intrinsic factor alone.
2. Intrinsic factor in excess of antibody.
3. Equal amounts of intrinsic factor and antibody.
4. Antibody in excess of intrinsic factor.
5. Antibody alone.
6. Neither intrinsic factor nor antibody.

In 4 and 5, where antibody is either present in excess of intrinsic factor or is present alone, it may be identified directly. However if antibody is present only in combination with intrinsic factor it cannot be identified on direct assay. Some preliminary measures are thus necessary in order to separate the antibody from intrinsic factor and thereafter to prevent their re-combination.

If gastric juice specimens are to be tested for the presence of an antibody to intrinsic factor, it is first necessary to:

a) Saturate all of the vitamin B_{12} binders since this will increase the sensitivity of a subsequent assay for free antibody.

b) Separate the antibody from any residual intrinsic factor.

c) Make the intrinsic factor antigenically inert so that it will neither recombine with antibody nor interfere with the subsequent assay of the released antibody.

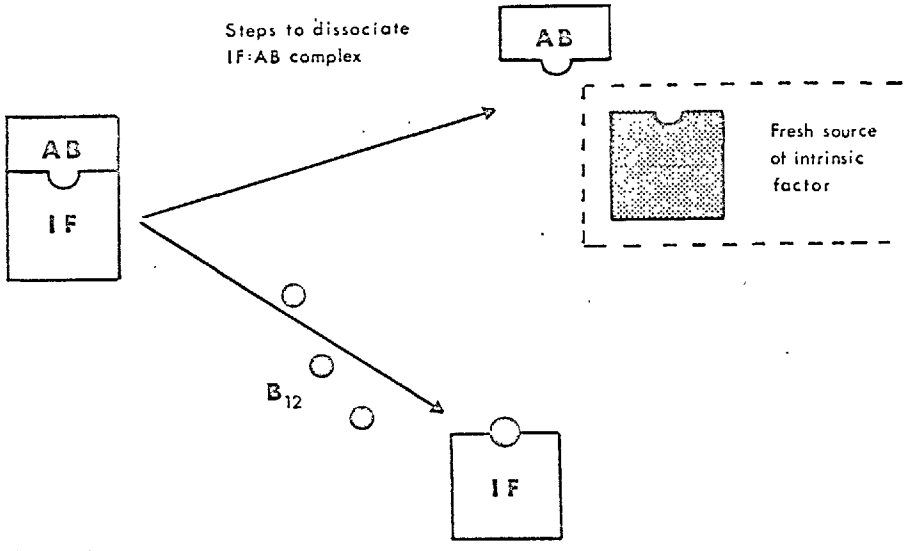


Figure 4.

Principle of method for release of antibody from intrinsic factor followed by its identification.

B. DESCRIPTION OF THE METHOD:

1. Release of the antibody.

Two samples of gastric juice being examined are incubated with an "excess" of unlabelled vitamin B_{12}. The pH in one of these samples is reduced and in the second sample the volume is adjusted accordingly whilst preserving neutral pH. Samples of normal gastric juice and of normal gastric juice with type 1 antibody added serve as negative and positive controls, respectively.

Under the conditions described, antibody present will separate from intrinsic factor and vitamin B_{12} will attach to any binding sites thereby exposed.

After fifteen minutes the pH is returned to neutral. Excess "free" vitamin B_{12} is removed by adsorption onto activated charcoal, which is then removed by centrifugation.

2. Identification of the antibody.

The clear supernatant is now tested for the presence of "free" antibody. It is decanted into a sample of normal gastric juice with a known intrinsic factor content. After incubation an excess of radioactive labelled vitamin B_{12} is added. This will only combine with the available binding sites of the normal gastric juice, since the sites of the test gastric juice samples are already occupied by non-radioactive vitamin B_{12}. The "free" radioactive vitamin B_{12} is then adsorbed with charcoal. After centrifugation the supernatant radioactivity is measured. If antibody is present in the treated gastric juice sample it will combine with the intrinsic factor in the normal gastric juice and thereby reduce its capacity to bind the radioactive vitamin B_{12}.

C. THE UNITS:

A unit of intrinsic factor is that amount which will bind 1 ng of vitamin B_{12}. A unit of type 1 antibody against intrinsic factor will prevent the binding of 1 ng of vitamin B_{12} by 1 unit of intrinsic factor.

D. ABBREVIATIONS:

Type 1 antibody against intrinsic factor in this thesis will therefore be referred to as antibody.

Where other antibodies are considered the term "antibody" will be qualified.

pH 3.2 buffer refers to 0.2 molar citrate-phosphate buffer (see appendix I).

Na_2HPO_4 refers to 0.2 molar disodium hydrogen phosphate.

IF = intrinsic factor.

PA = pernicious anaemia.

Section 2.

MATERIALS, METHODS AND SUBJECTS STUDIED.

2:1 Materials.

A. NORMAL GASTRIC JUICE.

This was aspirated by intermittent gastric suction from hospital subjects with normal gastric function, from patients with duodenal ulcer undergoing pre-operative investigation, and from normal young volunteers. Aspiration was conducted under resting conditions and also after stimulation with a standard dose of pentagastrin (Peptavalon. I.C.I. 6ugm per Kg).

The aspirated gastric juice was centrifuged at 7000 r.p.m. for twenty minutes at 10°C in an M.S.E. fixed axis centrifuge. The supernatant was filtered through dental gauze. The pH was measured, using a direct reading, glass electrode pH meter (Electrical Instruments Ltd) and a small aliquot was removed and titrated with N/10 NaOH to measure the total acid content.

The gastric juice was brought to pH 10 with 40% NaOH, left for twenty to thirty minutes to destroy pepsinogen (Grsbeck et al 1962) and then returned to pH 7.0 - 8.0 by the addition of 6 Normal HCl. The gastric juice was stored in 20 ml aliquots at -20°C until used.

B. NORMAL PLASMA:

This was obtained in the frozen state, as "Fresh Frozen Plasma", from the National Blood Transfusion Service. It was thawed and stored in 20 ml aliquots at -20°C until used.

C. SERUM CONTAINING ANTIBODY AGAINST INTRINSIC FACTOR:

Serum with a high titre of antibody to intrinsic factor was taken from patients with pernicious anaemia, who attended Haematology Clinics at St. Mary's Hospital, Paddington. Subjects with an antibody titre of more than 200 units per ml were bled about 300 ml at six monthly intervals. The serum was stored in 20 ml aliquots at -20°C until used.

D. VITAMIN B_{12}:

1. Non radioactive: Ampoules of 100 ug and 1000 ug of Cobalamin (Payne and Byrne -- Cyanocobalamin) were stored away from the light at room temperature, and diluted appropriately in isotonic saline before use.

2. Radioactive: Vitamin B_{12} labelled with a radioactive cobalt (^{57}Co) was obtained from the Radiochemical Centre at Amersham. The stock solutions for in vitro work were prepared by introducing 10uCI of the ^{57}Cobalt vitamin B_{12} into 200 ml of distilled water, to which non-radioactive vitamin B_{12} had been added. The final concentration of vitamin B_{12} was 400 ng per ml, with a radioactive concentration of 50 nCI per ml. This stock solution was dispensed into 25 ml capacity plastic tubes and stored at -20°C in lead containers until used.

The working solution was made up by diluting the stock solution with an equal volume of isotonic saline. Thus 0.5 ml of the working solution contained 100 ng of vitamin B_{12} with a radioactivity of 12.5 nCI. The radioactive concentration was 125 nCI per ugm of vitamin B_{12}.

E. ANTIGLOBULIN SERA:

a) Equine anti-human globulin (Netherland Red Cross: Code PHO - 13 - P_2).

b) Sheep anti-human serum IgA (xBOH) supplied by the Department of Immunological Pathology, University of Birmingham Medical School.

c) Wellcome sheep anti-human serum IgA.

d) Sheep anti-chain serum.

e) Horse anti-human IgG.

F. CHARCOAL.

British Drug Houses Activated Charcoal was employed to separate unbound vitamin B_{12} from that complexed with biological macromolecules.

The charcoal was heated for two hours at 160°C in a dry heat sterilising oven. The charcoal was then dispensed as a dry powder or after initial suspension in normal serum. For each 100 ng of charcoal, 1 ml of normal serum was added. After thorough mixing the suspension was centrifuged at 3000r.p.m. for twenty minutes and the clear supernatant serum was discarded. The deposited charcoal was resuspended and washed in isotonic saline and after further centrifugation was resuspended in isotonic saline to provide a concentration of 100 mg charcoal per ml of suspension. 1 ml volumes were dispensed.

G. APPARATUS:

a) pH meter: Direct reading glass electrode pH meter (Electrical Instruments Ltd).

b) High speed refrigerated centrifuge (MSE).

c) Packard Tri-carb well-type liquid scintillation spectrometer.

2.2 Methods.

A. THE DETECTION OF FREE TYPE 1 ANTIBODY AGAINST INTRINSIC FACTOR IN THE GASTRIC JUICE:

Two ml (or less made up to 2 ml with isotonic saline, if no more was available) of test gastric juice was dispensed into 3 ml of isotonic saline in a 20 ml capacity clip-glassed bottle (figure 5).

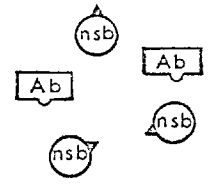


Figure 5.

The components of gastric juice sample lacking intrinsic factor

400 ng of non-radioactive vitamin B_{12} was then added in 0.5 ml. This combined with the binding sites in the gastric juice, thereby making them inert for the subsequent steps (figure 6).

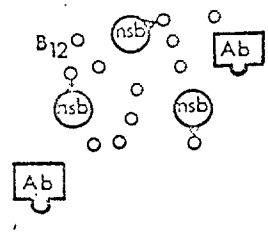


Figure 6.

After addition of vitamin B_{12}

The mixtures was shaken and left at 37°C for one hour. Then one ml of a saline suspension of serum coated charcoal was added and the mixture was shaken for two minutes. The charcoal adsorbed vitamin B_{12} which was not bound to macromolecules in the gastric juice (section 3.20). Thus when the charcoal was removed only vitamin B_{12} bound to gastric-juice macromolecules remained in the supernatant (figure 7).

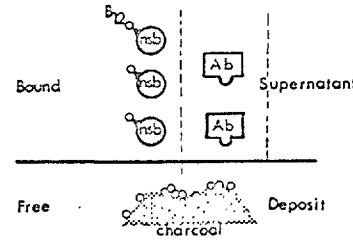


Figure 7.

Adsorption of unbound vitamin B_{12}

The suspension was centrifuged at 3000 r.p.m. for ten to fifteen minutes and the clear supernatant was decanted into 0.5 ml of normal gastric juice. Any free antibody would combine with the intrinsic factor in the normal gastric juice and thereby occlude the vitamin B_{12} binding site. This was incubated at room temperature for two hours and then 0.5 ml of radioactive vitamin B_{12} was added (100 ng ^{57}Cobalt vitamin B_{12}).

When antibody was absent the vitamin B_{12} was bound to the normal gastric juice in the negative control. Where antibody was present it occluded the binding site on intrinsic factor and caused an impairment of vitamin B_{12} binding.

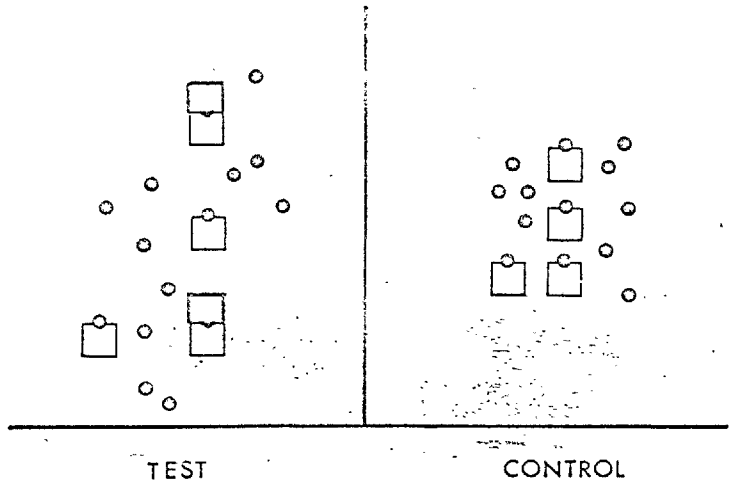


Figure 8.

Inhibition of B_{12} binding as measure of antibody

This was incubated for a further fifteen minutes before the addition of 1 ml of a saline suspension of activated charcoal, which adsorbed unbound radioactive vitamin B_{12}.

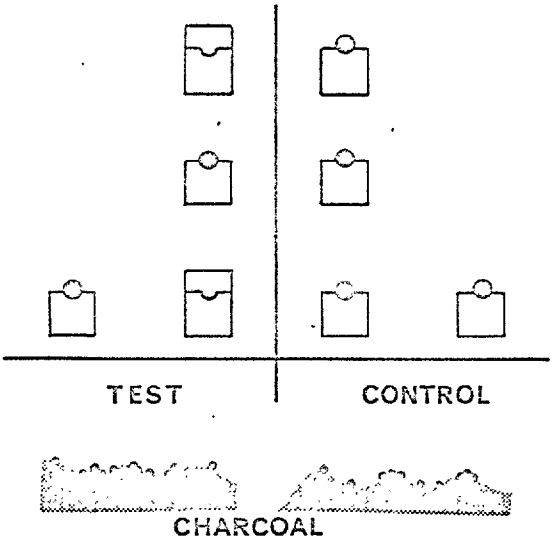


Figure 9. Unbound B_{12} adsorbed on charcoal

The suspension was centrifuged for fifteen minutes at 3000 r.p.m. and the supernatant was decanted into 10 ml capacity, calibrated pyrex tubes for radioactive counting.

A straight-forward assay of the intrinsic factor content of the normal gastric juice was concurrently performed. The unbound radioactive vitamin B_{12} was separated by addition of saline suspended charcoal and not by addition of charcoal powder to the mixture of gastric juice and normal serum, as originally described by Ardeman and Chanarin (1963).

The following controls were treated likewise:

1. Negative control: normal gastric juice alone.

2. Positive control: normal gastric juice seeded with serum known to contain antibody against intrinsic factor.

3. Recovery control: serum containing antibody against intrinsic factor in isotonic saline.

The radioactivity in the clear supernatants were related to that in the radioactive standard containing 100 ng of labelled vitamin B_{12} in the appropriate volume of saline.

B. THE RELEASE AND IDENTIFICATION OF TYPE 1 ANTIBODY ALREADY BOUND TO GASTRIC INTRINSIC FACTOR.

Two ml of a mixture of normal gastric juice and serum containing antibody was dispensed into 20 ml capacity glass bottles as described. 400 ng of non-radioactive vitamin B_{12} was then added in 0.5 ml, in order to saturate the vitamin B_{12} binding sites and make them inert in the subsequent steps.

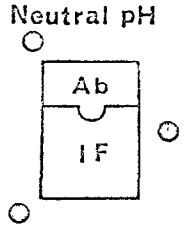


Figure 10.

Gastric juice containing antibody and intrinsic factor

One ml of 0.2 M phosphate-citrate buffer was added, creating a pH between 3.0 and 3.2. The mixture shaken and incubated at 37°C for fifteen minutes, permitting the separation of antibody from intrinsic factor and the combination of vitamin B_{12} with intrinsic factor at the site thereby exposed.

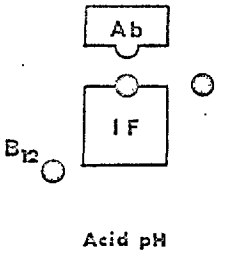


Figure 11.

Release of antibody from intrinsic factor. As pH falls vitamin B_{12} combines with intrinsic factor

Two ml of 0.2 molar Na_2HPO_4 was then added, returning the pH to neutral. This was incubated for 45 minutes at 37°C.

One ml of a saline suspension of serum treated charcoal was then added to adsorb the unbound vitamin B_{12} and the subsequent steps to identify the antibody were as described in section 2.2 A.

The steps employed for the release of antibody from intrinsic factor and its identification are illustrated in figure 12.

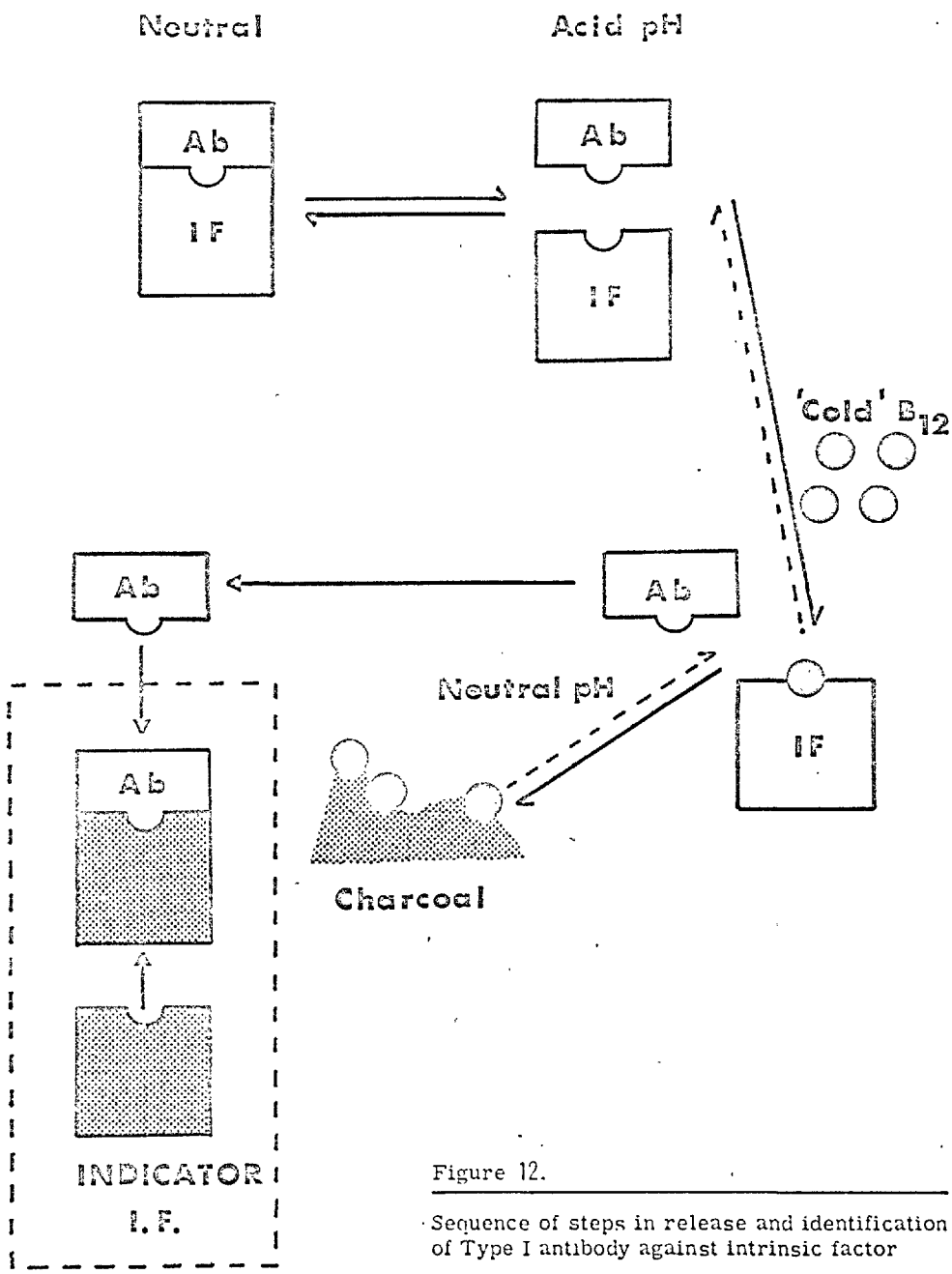


Figure 12.

Sequence of steps in release and identification of Type I antibody against intrinsic factor

C. MODIFIED GASTRIC ASPIRATION IN ACHYLIA GASTRICA:

Gastric juice was aspirated by standard methods through a Ryle's tube, before and after stimulation with betazole hydrochloride (Histalog: Lilly) or or pentagastrin (Peptavalon I.C.I.). More recently, when the yield of gastric aspirate was small, 250-300 ml of buffered isotonic saline was instilled into the stomach through the Ryle's tube in 20-30 ml quantities and aspirated. The washings were dialysed against buffered saline for twenty-four hours at 4°C and concentrated to about one tenth of the original volume by vacuum dialysis through "visking" tubing. The specimens were treated and tested as described in sections 2.2 A and B.

D. RADIOACTIVE COUNTING.

The supernatant samples containing radioactive vitamin B_{12} were placed in 10 ml capacity, calibrated pyrex tubes and these were placed in screw-topped cellulose acetate containers. The radioactivity was measured in a Packard auto-gamma spectrometer. Samples were counted for 5-10 minutes at about 3000-4000 counts per minute above background.

E. LEUCOCYTE MIGRATION INHIBITION FACTOR:

This test was undertaken as a possible measure of cell mediated immunity against gastric antigens. The technical details of this test are described in Appendix IV.

2.3 Clinical Material.

A. PATIENTS STUDIED:

Investigations were performed upon pool samples of normal gastric juice aspirated from medical students at St. Mary's Hospital Medical School. Gastric juice samples were obtained from fifty-three patients

with pernicious anaemia. Most of these samples were taken from patients undergoing current investigation at St. Mary's Hospital during the period of this study. Further samples were from patients with pernicious anaemia whose gastric juice had been stored at -20°C since aspiration between 1964 and 1967. A few samples were obtained during the course of this study also from:

Paddington General Hospital (now St. Mary's Hospital, Harrow Road, London, W.9).

Edgeware General Hospital, Middlesex.

St. Bartholomew's Hospital, London, E.1.

Middlesex Hospital, London, W.1.

Further samples of gastric juice were obtained from patients:

a) with no evidence of gastric mucosal disorder.

b) after partial gastrectomy.

c) with atrophic gastritis or gastric atrophy, who did not show impaired vitamin B_{12} absorption.

d) with thyroid gland dysfunction who were found to have a type 1 antibody against intrinsic factor circulating in their serum. In all of these patients the vitamin B_{12} absorption was normal.

B. CRITERIA FOR THE DIAGNOSIS OF ADDISONIAN PERNICIOUS ANAEMIA:

The following criteria were a requisite for the diagnosis of pernicious anaemia:

a) Histamine-fast or pentagastrin-fast achlorhydria.

b) Absent or rudimentary intrinsic factor in the gastric juice after stimulation with histamine or pentagastrin.

c) Evidence of impaired vitamin B_{12} absorption.

d) Improvement of vitamin B_{12} absorption when the oral dose of vitamin B_{12} is given with an extraneous source of intrinsic factor.

There are many other features frequently observed in pernicious anaemia but these are widely encountered in megaloblastic anaemias from a variety of causes and fail to specify the nature of the primary lesion.

It should be emphasised that the criteria listed are principally concerned with specifying the presence and nature of the gastric lesion and that these may permit a diagnosis of pernicious anaemia at a time when the haematological or neurological consequences are not overt.

Section 3.

THE IDENTIFICATION OF TYPE 1 ANTIBODY AGAINST INTRINSIC FACTOR IN THE GASTRIC JUICE.

3.1 Introduction.

Many macromolecular components of gastric juice bind vitamin B_{12} (Grasbeck 1956; Gullberg and Olhagen 1959; Glass et al 1962; Grasbeck et al 1962; Glass 1963). It is therefore necessary to expose a sample of gastric juice to an excess of vitamin B_{12} in order to occupy all of the vitamin B_{12} binding sites. After removal of the unbound vitamin B_{12} the test mixture may be decanted into a source of normal gastric juice in order to detect antibody. Unbound vitamin B_{12} must be entirely removed because even traces will interfere with the subsequent steps.

The gastric aspirates from about one third of subjects with pernicious anaemia contain residual detectable intrinsic factor (Ardeman and Chanarin 1965a). Therefore antibody in the gastric juice may occasionally elude detection if it co-exists in the form of a complex with its antigen. Correspondingly measures have been employed to separate this antibody from the antigen in order to permit its identification. One suitable measure for separating antibody from antigen, where the physico-chemical forces effecting union are principally electrostatic, is to increase the hydrogen ion concentration (Boyd 1956).

Since B_{12} and the antibody compete for the same site on intrinsic factor at neutral pH, intrinsic factor can combine either with the antibody or with B_{12}. A fall in pH will result in separation of intrinsic factor from antibody. If this separation occurs in the presence of vitamin B_{12}, this vitamin will combine with the newly exposed binding sites on intrinsic factor as the union between intrinsic factor and vitamin B_{12} is principally due to physico-chemical forces other than electrostatic (i.e: hydrophobic, van der Waal's, dipolar etc).

Accordingly when the pH is returned to neutral and the physical conditions would permit the recombination of type 1 antibody with intrinsic factor, this cannot take place as the binding site is now occupied by vitamin B_{12}, and thus the antibody remains free.

After removal of the remaining "free" vitamin B_{12}, the specimen is tested for the presence of antibody by the technique of Ardeman and Chanarin (1963).

The purpose of this section is to consider the effect on the assay system of variations in the conditions under which the reagents are allowed to react. The adsorbing properties of charcoal are considered first in relation to vitamin B_{12} and type 1 antibody and thereafter variables in the whole experimental system are assessed.

3.2 Charcoal as an adsorbing agent.

A. ACTIVATED CHARCOAL AND VITAMIN B_{12}.

Charcoal was used at two stages in the procedure:

a) in order to adsorb unbound non-radioactive cyanocobalamin which had been added to saturate the vitamin B_{12} binding components of the test gastric juice.

b) to adsorb unbound radioactive ^{57}Cobalt vitamin B_{12} which was added as part of the test system for measuring the presence of type 1 antibody.

The presence of intrinsic factor antibody is shown by its ability to interfere with radioactive vitamin B_{12} binding by normal gastric juice. It was essential that the removal of free non-radioactive vitamin B_{12} be complete. If residual unbound vitamin B_{12} remained in the sample then on introduction into sample of normal gastric juice (used to assay the free antibody) it would bind preferentially to intrinsic factor and thereby reduce the number of

sites available for binding by radioactive vitamin B_{12}. In this system residual unbound non-radioactive vitamin B_{12} would be responsible for a fall in radioactive vitamin B_{12} binding, indistinguishable from that caused by antibody against intrinsic factor.

The following experiments were undertaken in order to evaluate the efficiency of charcoal as an adsorbent for vitamin B_{12}.

1. <u>The adsorption of ^{57}Cobalt vitamin B_{12} by activated charcoal</u>.

<u>Procedure</u>:

Measured quantities of radioactive vitamin B_{12} were added to 8 ml volumes of saline. 100 mg of powdered charcoal was added to each preparation, shaken and left at room temperature for 2-3 minutes. The suspensions were centrifuged at 3000 r.p.m. for ten minutes and then the radioactivity in the decanted supernatant was measured and compared with a radioactive standard containing 100 ug radioactive vitamin B_{12}. The residual vitamin B_{12} in the supernatant after adsorption by charcoal was measured.

Charcoal was tested in various forms for the efficiency of its ability to adsorb vitamin B_{12}.

The charcoal was tested :

i. As powder. 100 mg.

ii. Suspended in normal serum 100 mg/ml.

iii. Suspended in isotonic saline, after first being mixed and incubated with normal serum. 100 mg/ml.

Results:

Table I

Initial B_{12} content (ng).	B_{12} (ng), remaining after adsorption by charcoal as:		
	Powder	serum suspension	saline washed
100	0.8	1.0	0.8
200	2.0	1.25	1.25
300	2.5	2.0	1.5
400	2.5	2.7	2.5
500	3.0	2.0	2.5
1000	4.0	4.0	3.5
2000	6.0	6.0	4.0

Conclusions:

Charcoal was an avid adsorbent for vitamin B_{12} and that 100 mg in any of the three forms tested was able to remove more than 99% of vitamin B_{12} in quantities up to 2000 ng from solution.

2. The rate of vitamin B_{12} adsorption by activated charcoal in serum suspension.

Procedure:

Activated charcoal was suspended in normal serum (100 mg/ml). 1 ml quantities of this suspension were dispensed into tubes containing 200 ng of ^{57}cobalt vitamin B_{12} in 8 ml of saline. These mixtures were left for varying time before centrifugation. The results express[illegible] the total residual vitamin B_{12} in the supernatant.

Results:

Table 2

Time of exposure and the adsorption of vitamin B_{12} by serum suspended charcoal.

Time (minutes)	Supernatant B_{12} content (ng).
1	1.0
10	0.9
20	1.0
30	0.7
60	0.8

Conclusion:

The adsorption of 200 ng of vitamin B_{12} by 100 mg of activated charcoal is virtually complete within one minute.

3. The stability of adsorbing properties of activated charcoal in serum suspension.

Procedure:

Activated charcoal was suspended in normal serum (100 mg/ml) and left at room temperature on the bench for specified periods before being added to ^{57} cobalt vitamin B_{12} in saline for two minutes. The suspension was centrifuged at 3000 r.p.m. for fifteen minutes and the supernatant radioactivity was measured.

Result:

Table 3

The effect of time in serum suspension on the ability of activated charcoal to adsorb vitamin B_{12}.

Time (minutes)	Supernatant vitamin B_{12} (ng).
1	0.8
15	0.8
30	0.8
60	1.0

Conclusion:

The adsorbing capacity of the activated charcoal was sustained for 60 minutes in serum suspension.

4. The effect of sequential additions of serum suspended charcoal.

Procedure:

Serum suspended charcoal (100 mg/ml) was added to radioactive vitamin B_{12} in saline. The vitamin B_{12} remaining was measured after one addition of charcoal and after two such additions which were separated by two minutes.

Results:

Table 4

Total vitamin B_{12}. (ng)	Residual vitamin B_{12} in supernatant after:	
	One charcoal addition	Two charcoal additions.
1000	4.0	3.5
2000	5.0	4.5

Conclusions:

The radioactivity which remained in the supernatant after one exposure to activated charcoal was not further reduced after a second similar exposure to charcoal. Possibly a balance is struck between the vitamin B_{12} adsorbed onto the charcoal and that which remains in the supernatant. With increasing quantities of vitamin B_{12} further additions of charcoal would have limited power to complete the adsorption.

5. Comments.

1. Activated charcoal was an effective adsorbent of unbound vitamin B_{12} whether administered as powder, in serum suspension or in isotonic saline after incubation with normal serum.

2. Two additions of serum suspended charcoal were no more effective in clearing unbound vitamin B_{12} than was a single addition.

3. Up to 500 ng of vitamin B_{12} could be satisfactorily extracted with only a small supernatant residue.

4. Increasing the duration of exposure to the charcoal preparation for up to one hour made no appreciable difference to the order of vitamin B_{12} extraction.

5. When the charcoal had remained suspended in serum for up to one hour at room temperature, it made no difference to its function as an adsorbent.

B. ACTIVATED CHARCOAL AND TYPE 1 ANTIBODY AGAINST INTRINSIC FACTOR.

Serum proteins attach themselves to the surface of charcoal particles. This can discriminate between large and small molecules. It is possible that in addition to adsorbing "free" vitamin B_{12}, the charcoal might also remove some of the antibody from suspension.

In order to determine whether type 1 antibody was lost during incubation with a charcoal preparation the following test was performed:

Procedure:

Serum containing type 1 antibody of known titre was added to saline. Serum from five subjects was thus dispensed in triplicate. Charcoal was added to two of the samples, either as a serum suspension in saline after incubation with serum. In the third sample the volume was made up with saline.

After two minutes incubation with charcoal the samples were centrifuged at 3000 r.p.m. for fifteen minutes and the supernatants were decanted into samples of normal gastric juice of known intrinsic factor content, in order to measure the antibody present by the usual method.

Result:

Table 5

	Antibody detected (units)		
		charcoal treated	
Serum sample	neat sample	serum suspension	saline washed
A	15.	11.0	11.0
B	9.5	6.5	7.0
C	10.0	8.0	8.0
D	12.0	9.0	8.0
E	7.0	4.5	5.0

Comment:

A fall in identifiable antibody took place after exposure to charcoal. This fall was fairly consistent among the samples tested:

Table 6

Sample	per cent antibody lost in charcoaling.
A	27
B	26
C	20
D	29
E	28

Antibody loss is probably due to adsorption of antibody onto charcoal.

C. COATED CHARCOAL AND THE SEPARATION OF FREE FROM BOUND VITAMIN B_{12}.

1. Introduction.

Charcoal is widely used in immunoassay techniques for discriminating between molecules of different sizes. It is used in peptide and protein hormone assays as well as assays for intrinsic facotr and antibody against intrinsic factor.

Since its function is critical in the assay herein described it is considered appropriate to itemise some of the properties and functions of charcoal in the techniques in which it is employed. Gottlieb et al (1965) suggested that the charcoal particles might be conceived as a "micro-sponge". Charcoal is an avid adsorbent of a large variety of molecules. When coated with plasma, blood fractions,

plasma expanders or other materials, charcoal will continue to adsorb molecules of lesser molecular size than the coating particles and will reject molecules equal to or of greater size than that of the coating molecules (Gottlieb et al 1965). In the immunoassay systems employed for hormones, specific antibodies bind to radioactivity labelled hormone present and the complex is too large to gain access to coated charcoal and remains in the supernatant (Herbert 1969). In studies of gastric juice the vitamin B_{12} which combines with macromolecules in the gastric juice is inaccessible to the coated charcoal.

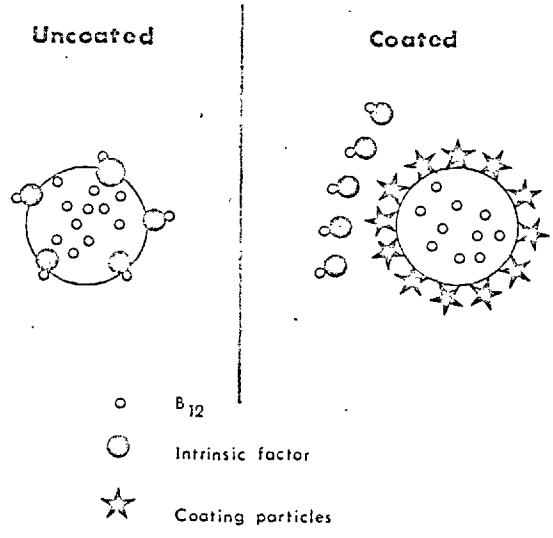


Figure 13.

Molecular discrimination by coated charcoal

In order to discriminate between molecules of different sizes in a mixture, it is important that the charcoal is introduced into the mixture previously coated with the appropriate molecules. In the assay system for intrinsic factor introduced by Ardeman and Chanarin (1963), powdered charcoal is introduced into the mixture containing gastric juice, serum and vitamin B_{12}. The vitamin B_{12} is present partly bound to macromolecules and partly free. In this system it is assumed that in order to discriminate between bound and free vitamin B_{12} the charcoal must become instantaneously coated with the serum proteins. However rapidly this coating takes place it would be remarkable if it was complete before any of the bound vitamin B_{12} had become taken up by the charcoal particles.

2. A comparison of pre-coated and uncoated charcoal in the intrinsic factor assay.

Procedure:

Normal gastric juice samples samples were incubated with normal serum and with serum known to contain antibody against intrinsic factor. After fifteen minutes ^{57} cobalt vitamin B_{12} was added. Duplicate preparations were made of all samples thus tested. In the one preparation unbound vitamin B_{12} was extracted by the addition of charcoal in powder form, and unbound vitamin B_{12} was removed from the duplicate preparations by charcoal in saline suspension which had previously been incubated with normal serum (100 mg charcoal in 1 ml of serum). The suspensions were centrifuged at 3000 r.p.m. for fifteen minutes and the radioactivity was measured in the clear supernatant.

Results:

Table 7

Sample	ng of $^{57}CoB_{12}$ in supernatant	
	"naked charcoal"	Precoated charcoal
A + normal serum	48.0	67.0
A + Type 1 Ab.	14.5	18.0
B + normal serum	54.5	65.5
B + Type 1 Ab.	13.5	24.5
C + normal serum	43.0	60.0
C + Type 1 Ab.	10.0	13.5

Comment:

The supernatant radioactivity is higher after adsorption with precoated charcoal. This suggests that powdered charcoal adsorbs some of the bound B_{12} before becoming adequately coated to discriminate between bound and free vitamin B_{12}. These figures result in a higher estimate of intrinsic factor in the duplicates tested with pre-coated charcoal.

Pre-coated charcoal was used in the studies herein itemised, because:

a) it was easier to separate by centrifugation than charcoal added in powder form, some of which settled on the surface of the suspension or adhered to the sides of the tubes and contaminated the decanted supernatant.

b) the above experiments showed that it was less able to absorb large molecules and therefore more likely to leave antibody molecules free in the supernatant.

3. The preparation of pre-coated charcoal:

The required amount of powdered charcoal was introduced into normal serum, at a concentration of 100 mg charcoal per ml of serum. This was stirred until the charcoal was in suspension. The suspension was left for ten minutes at room temperature and then centrifuged at 3000 r.p.m. for fifteen minutes. The supernatant serum was discarded and the charcoal resuspended in isotonic saline, stirred and centrifuged again at 3000 r.p.m. The supernatant was again discarded and the charcoal deposit resuspended in a volume of isotonic saline so that there was 100 mg of charcoal per ml of suspension. This was kept on the bench at room temperature and used in the assay within the subsequent hour. Where the tests extended beyond the period of an hour the charcoal was freshly prepared.

3.3 Antibody against intrinsic factor.

A. THE EFFECT OF pH:

Since intrinsic factor and type 1 antibody will separate as the pH is brought down through a critical range, it was necessary to identify the effect of exposure to a range of pH for increasing periods on the function of the antibody alone.

1. Antibody stability over pH range 7.5 - 2.0.

Procedure:

Samples of serum with a known titre of antibody against intrinsic factor were introduced into 0.2 M phosphate citrate buffer over the pH range 7.5 - 2.0 at room temperature. After precisely fifteen minutes the pH was returned to neutral by adding 0.2 M disodium-hydrogen phosphate. Each sample was tested for the remaining functioning antibody by the method of Ardeman and Chanarin (1963).

Result:

In four different serum samples tested, fifteen minutes exposure to pH 3.0 made no appreciable difference to the antibody detected. Below pH 2.5 however there was a fall in antibody titre.

Table 8

Antibody detected expressed as percentage of the content in control samples preserved at neutral pH.

pH	Serum samples:			
	A	B	C	D
7.5	100	100	100	100
6.0	90	100	95	95
5.0	97	98	95	96
4.0	93	97	95	100
3.8	100	98	95	96
3.6	100	98	92	96
3.4	100	98		98
3.2	100	97		100
3.0	100	97	95	94
2.5		88	84	85
2.0		72	77	75

2. The effect of the duration of exposure to pH 2.3

Procedure:

Serum which was known to contain antibody against intrinsic factor was placed in a glycine-sulphuric acid buffer at pH 2.3. Aliquots were extracted and tested for antibody at stated intervals:

Results:

Table 9

Time (minutes)	Antibody activity, expressed as percentage of that in control sample maintained at neutral pH.
5	87
10	81
15	78
30	71
45	57
60	45

Comment:

There was a progressive and steady decline of antibody activity after increasing exposure to pH 2.3

3. The relative effects of low pH and the duration of exposure on type 1 antibody.

Procedure:

Similar volumes of serum containing antibody against intrinsic factor were decanted into:

a) isotonic saline

b) 0.2 M phosphate citrate buffer at pH 3.2

c) 0.2 M phosphate citrate buffer at pH 3.0

Aliquots were removed at stated intervals and decanted into 0.2 M disodium-hydrogen phosphate, at neutral pH. The samples were then tested for antibody content by the usual method.

Results:

Table 10

Units of antibody detected after incubation.

Sample	pH	Duration (minutes) at pH stated 15	30	60
1	3.0	14.5	16.0	16.0
2		4.5	4.0	6.0
3		5.0	6.5	6.0
4		10.0	11.0	12.0
1	3.2	15.0	16.0	17.0
2		4.0	4.0	5.0
3		5.0	5.0	7.0
4		10.0	10.0	12.5
1	7.5	14.0	16.0	16.0
2		4.0	3.5	5.0
3		5.0	5.0	7.0
4		10.0	10.0	12.0

Comment:

In the four serum samples tested there was no evidence of decline in antibody activity after exposure for up to one hour at pH 3.0.

B. THE RELATIVE RATES OF COMBINATION OF INTRINSIC FACTOR WITH VITAMIN B_{12} AND WITH ANTIBODY.

1. Combination of normal gastric juice with vitamin B_{12} at neutral pH:

Ten ml of neutralised normal gastric juice was introduced into 5 ml of saline at 4°C. The vitamin B_{12} binding capacity of this gastric juice had been previously tested and was about 70 ng/ml. 5 ml of ^{57}cobalt vitamin B_{12} (200 ng/ml) were rapidly dispensed into the gastric juice. The total mixture thus contained 700 vitamin B_{12} binding units and 1000 ng of vitamin B_{12}. This was mixed and 1 ml aliquots were removed with a "Summit" spring loaded syringe and delivered at stated intervals into glass bottles containing serum coated charcoal in saline. The coated charcoal adsorbed the unbound vitamin B_{12}, thus curtailing the combination of gastric juice binders with vitamin B_{12}. The suspension was shaken for two minutes, centrifuged at 3000 r.p.m. for ten minutes and the clear supernatant was decanted into calibrated tubes for radioactive counting.

2. The combination of normal gastric juice with vitamin B_{12} at pH 3.2.

A similar experiment was carried out to test the combining rate of normal gastric juice with vitamin B_{12} at pH 3.2. 10 ml of normal gastric juice were added to 5 ml of 0.2 molar phosphate-citrate buffer at pH 3.2 ^{57}cobalt vitamin B_{12} was added as described above. One ml aliquots were transferred at stated intervals into a suspension of serum coated charcoal in 0.2 M disodium hydrogen phosphate. The suspension was shaken, centrifuged and decanted as itemised above.

Results:

The results of a typical experiment are shown:

Table 11

Vitamin B_{12} bound ml of incubation mixture.

Time	pH 7.5	pH 3.2
	ng. B_{12} bound	
5 seconds	40	38
10 "	42	40
15 "	46	44
20 "	45	44
30 "	43	44
40 "	44	44
50 "	44	44
60 "	44	41
10 minutes	47	47

Comment:

There was no difference in the rate or order of combination between normal gastric juice and vitamin B_{12} at pH 7.5 and pH 3.2. These findings conflict with Goldberg and Fudenberg's claim (1969) that the binding of vitamin B_{12} by intrinsic factor becomes impaired as the pH fell.

3. The effects of pH on the intrinsic factor assay systems as a whole:

In view of the claim by Goldberg and Fudenberg (1969) that normal gastric juice showed a decline of vitamin B_{12} binding with falling pH, an intrinsic factor assay by the method of Ardeman and Chanarin (1963) was performed on samples of normal gastric juice over the pH range 7.5 - 2.3 in 0.2 M phosphate citrate buffer.

Results:

Table 12

Gastric juice with:

pH	Normal Plasma	P.A. Plasma	ng B_{12} bound	
7.5	+	-	40	27
	-	+	13	
4.0	+	-	38	26
	-	+	12	
3.8	+	-	30	16
	-	+	14	
3.6	+	-	26	12
	-	+	14	
3.4	+	-	28	13
	-	+	15	
3.2	+	-	25	12
	-	+	13	
3.0	+	-	24.5	11.5
	-	+	13	
2.3	+	-	16.5	3.0
	-	+	13.5	

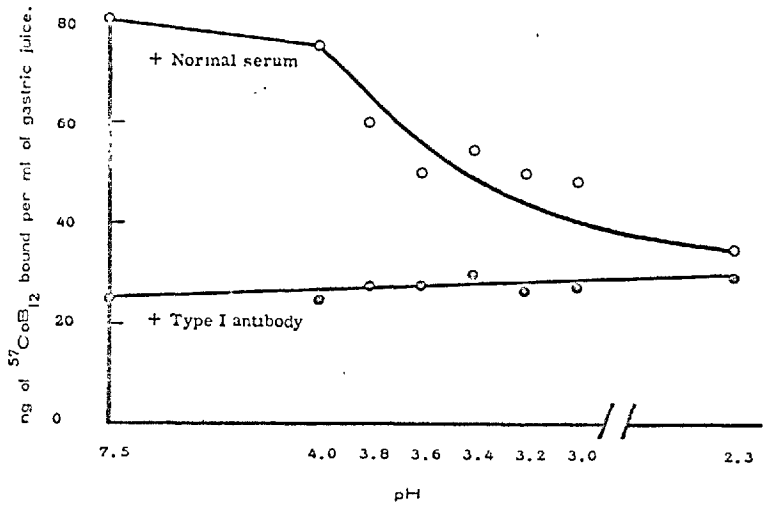


Figure 14.

Results of intrinsic factor assay performed over pH range 7.5 - 2.3

Comment:

The fall in the amount of vitamin B_{12} remaining after charcoal adsorption at low pH is due to the charcoal adsorbing less selectively with lowering of pH. The alternative explanation offered by Goldberg and Fudenberg (1969) that intrinsic factor fails to bind vitamin B_{12} at low pH is not borne out by the findings represented in the previous section. It was shown that intrinsic factor bound with vitamin B_{12} over a wide range of pH.

In view of the disparity between the combining rates of gastric juice with vitamin B_{12}, and with antibody which became more evident as the pH fell, it was decided to employ this change in physical conditions in order to separate pre-complexed antibody from intrinsic factor. However the identification of liberated antibody required a return to neutral pH, since the assay system had been shown to be affected by low pH. Clearly a return to neutral pH would permit recombination of intrinsic factor with antibody. This could be prevented by the addition of vitamin B_{12} when the pH was still low, since vitamin B_{12} could combine with the intrinsic factor, obstructing the site for the antibody, which thus remained free in the solution.

4. Combination of the intrinsic factor in normal gastric juice with Type 1 antibody at neutral pH.

Procedure:

10 ml of normal gastric juice known to bind about 80 ng vitamin B_{12} per ml were added to 6.7 ml of saline. Then 3.3 ml of serum was added which contained about 200 units of Type 1 antibody per ml. One ml samples were removed with a "Summit" spring loaded syringe and decanted at stated intervals into bottles containing saline and 100 ng ^{57}Cobalt vitamin B_{12}. Binding with vitamin B_{12} curtailed further combination with antibody whilst leaving the intrinsic factor antibody complex undisturbed. After incubation at room temperature for ten minutes, activated charcoal was added to adsorb the unbound vitamin B_{12}. This was shaken and then centrifuged at 3000 r.p.m. for ten minutes. The supernatant was decanted into calibrated glass tubes for radioactive counting.

5. Combination of intrinsic factor with Type 1 antibody at pH 3.2.

Procedure:

A similar experiment was set up to test the rate of combination between the intrinsic factor of the normal gastric juice with type 1 antibody at pH 3.2. 10 ml of normal gastric juice was mixed with 6.7 ml of 0.2 M phosphate-citrate buffer at pH 3.2. 3.3 ml of plasma containing an antibody to intrinsic factor was added as before. 1 ml samples were removed from the mixture with a "Summit" spring loaded syringe and decanted into 0.2 M Na_2HPO_4 containing 100 ng of ^{57}Cobalt vitamin B_{12}. After incubation for ten minutes at room temperature, these samples were treated as above.

Results: The results of a typical experiment are shown.

Table 13

Type 1 antibody bound by gastric juice in an ml of incubation mixture:

Time	at neutral pH	at pH 3.2
	ng Units of antibody bound	
5 seconds	22	7
10 "	27	10
15 "	29	11
20 "	30	15
30 "	32	19
40 "	33	21
50 "	33	21
60 "	36	22
10 minutes	38	35

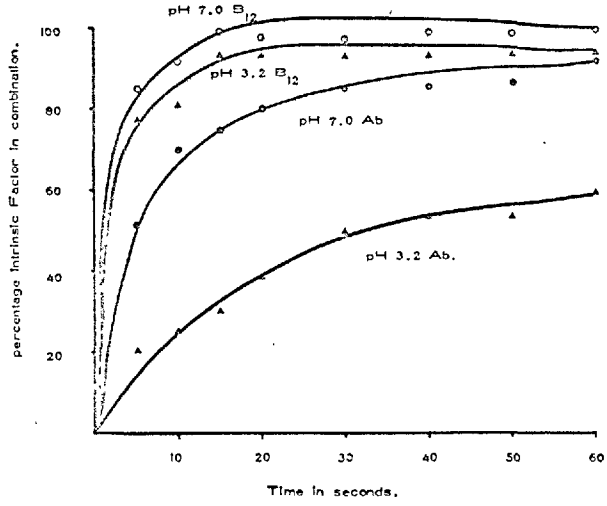


Figure 15.

Comparison of rates of combination of intrinsic factor with:
a) vitamin B_{12} } neutral pH
b) antibody against intrinsic factor } pH 3.2

Comment:

The rate of combination of intrinsic factor and type 1 antibody was dependent upon hydrogen ion concentration, whereas binding between gastric juice and vitamin B_{12} was independent of pH within the range selected.

6. Competitive Combination of Intrinsic Factor with Vitamin B_{12} and with type 1 Antibody.

The measurements of combining rates presented in items 1 and 2 of this section presuppose that when aliquots are decanted into charcoal or vitamin B_{12} solution, the reaction between the components of the initial mixture is instantaneously curtailed. That is: where gastric juice and vitamin B_{12} are studied, expulsion into charcoal is responsible for immediate adsorption of unbound vitamin B_{12}. Where gastric juice is mixed with antibody, that expulsion into vitamin B_{12} curtails further combination of intrinsic factor with antibody. Clearly the steps taken to stop the reaction must proceed at their own rate. Thus, in concluding that antibody combines with intrinsic factor more slowly than does B_{12}, allowance must be made for the fact that B_{12} also takes time to combine with gastric juice binders and the difference may be more apparent than real. This is likely to more important at pH 7.0 than at pH 3.2.

In order to test the relative rates of combination between gastric intrinsic factor with either vitamin B_{12} or antibody to intrinsic factor, the gastric juice was introduced into a mixture containing both ^{57}Cobalt vitamin B_{12} and antibody. The quantities were such that nanograms of vitamin B_{12} corresponded in number to ng units of antibody.

Procedure:

0.5 ml $^{57}CoB_{12}$ (100 ng) and 0.5 ml serum containing antibody was prepared. Controls contained each of these in saline. Normal gastric juice was added. The combination of these components with intrinsic factor was measured after 15 minutes at pH 7.0 and at pH 3.2. A fall in B_{12} binding was held to represent combination of intrinsic factor with antibody.

Results: The results in a typical experiment were:

1. Gastric juice alone bound 50 ng vitamin B_{12}.
2. B_{12} bound by non-specific binders in gastric juice 14 ng.
3. Intrinsic factor presents 36 units.
4. Gastric juice added to antibody and vitamin B_{12}.

   Binding to vitamin B_{12} :-

   (a) pH 7.0 : 38

   (b) pH 3.0 : 46.5

Since non-specific binders account for 14 ng of B_{12} in both (a) and (b), the vitamin B_{12} bound to intrinsic factor was:-

(a) 24 ng.

(b) 32.5 ng.

The intrinsic factor available for combination in both tests was 36 units. Thus at pH 7.0 24/36 = 2/3 of the intrinsic factor bound to vitamin B_{12}; 12/36 = 1/3 of the intrinsic factor was bound to antibody. Where antibody and vitamin B_{12} were present in equivalent proportions, intrinsic factor takes up 2 parts of B_{12} and only 1 part of antibody.

At pH 3.0; 32.5/36 = 90% units of intrinsic factor bound to vitamin B_{12}; 3.5/36 units of intrinsic factor combined with antibody (10%). Thus at pH 3.0 where antibody and vitamin B_{12} were present in equivalent proportions, intrinsic factor combined with nine times as much B_{12} as antibody.

Although combination of intrinsic factor with vitamin B_{12} or with type 1 antibody are mutually exclusive, since they compete for the same binding site, physical conditions may be adapted to virtually exclude antibody from forming a complex with intrinsic factor.

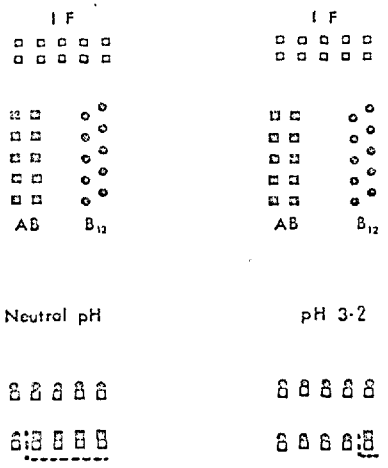


Figure 16.

Relative combination of intrinsic factor with vitamin B_{12} and antibody at neutral pH and pH 3,2

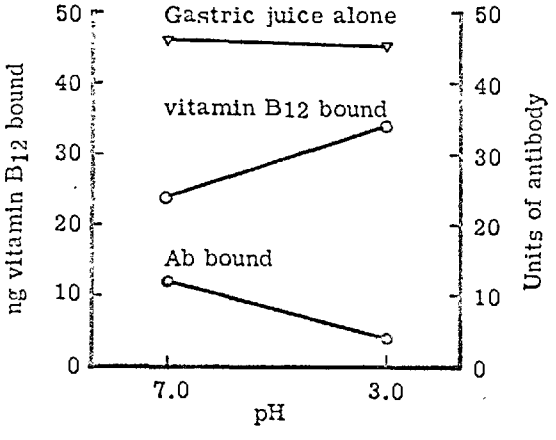


Figure 17.

Effect of pH on relative binding of intrinsic factor to vitamin B_{12} and Type I antibody

C. COMPARISON OF INTRINSIC FACTOR ASSAY IN SALINE AND PHOSPHATE-CITRATE BUFFER AT NEUTRAL pH.

During the steps employed to dissociate intrinsic factor from type 1 antibody 0.2 M phosphate-citrate buffer pH 3.2 was added and subsequently the pH was returned to neutral by addition of 0.2 M Na_2HPO_4. Since these steps altered the ionic content and molar concentrations in the final mixtures in the measurement of vitamin B_{12} binding by normal gastric juice, intrinsic factor assays in saline and "phosphate-citrate buffer" were performed and the results compared.

Procedure.

1 ml of 0.2 M phosphate-citrate buffer pH 3.2 was added to 3.0 ml 0.2 M Na_2HPO_4. A further 2 ml of saline were added. Controls were added as shown below. An intrinsic factor assay was performed in this medium and also in isotonic saline.

Table 14

(Volumes in ml)

	TESTS		CONTROL	
Saline	2.0	2.0	6.0	6.0
pH 3.2 buffer (0.2M)	1.0	1.0	-	-
Na_2HPO_4 (0.2M)	3.0	3.0	-	-
Gastric juice	0.5	0.5	0.5	0.5
Normal plasma	1.0	-	1.0	-
P.A. plasma	-	1.0	-	1.0
^{57}Co vitamin B_{12}	0.5	0.5	0.5	0.5
Charcoal	100mg	100mg	100mg	100mg

Results:

There was no evident difference in results of the intrinsic factor assay as performed in a neutral mixture of neutralised citrate-phosphate buffer or performed in saline.

Section 4.

STUDIES ON THE RELEASE OF ANTIBODY FROM INTRINSIC FACTOR.

4.1 Differential thermal lability.

A. NORMAL GASTRIC JUICE:

Intrinsic factor assays were performed on aliquots of normal gastric juice, after incubation in a water bath at temperatures from 45°C - 65°C for periods up to 60 minutes.

Results:

Table 15.

The thermal stability of intrinsic factor and non-specific binders in gastric juice, expressed as percentage residual activity of that in the unheated sample.

Temperature	Component	0	15	30	60
65°C	Intrinsic factor	100	nil	nil	nil
	Non-specific binders	100	nil	nil	nil
60°C	Intrinsic factor	100	nil	nil	nil
	Non-specific binders	100	nil	nil	nil
50°C	Intrinsic factor	100	62	50	29
	Non-specific binders	100	42	40	35
45°C	Intrinsic factor	100	84	76.5	70
	Non-specific binders	100	70	65	52

Comment:

After fifteen minutes at temperatures above 60°C, no intrinsic factor or non-specific vitamin B_{12} binding remained detectable. At 50°C the initial decline of non-specific binding activity was more rapid than that of intrinsic factor but intrinsic factor deterioration was progressive whereas that of the non-specific binders was limited.

At 45°C the deterioration of non-specific binders was also more rapid than that of intrinsic factor.

B. SERUM INTRINSIC FACTOR ANTIBODY:

Serum containing an antibody against intrinsic factor was incubated in a water bath for periods up to 60 minutes at 50°C and 60°C. 0.1 ml samples were removed at stated intervals and introduced into saline and normal gastric juice, in order to test for the remaining antibody. 0.9 ml of normal plasma was added to all the sample tubes in order to permit selective charcoal adsorption of unbound vitamin B_{12}. The subsequent procedure was that described for the detection of antibody.

Results:

Table 16.

Antibody activity remaining after heating, expressed as percentage of the antibody present in the unheated control.

	Time (Minutes)			
Temperature	0	15	30	60
50°C	100	94	94	91
60°C	100	85	91	85

Comment:

There was only slight deterioration of antibody over sixty minutes at 60°C.

This contrasted with the thermal lability of the vitamin B_{12} binding molecules in normal gastric juice, which were destroyed during fifteen minutes exposure to 60°C.

C. RECOVERY OF ANTIBODY FROM COMPLEX WITH INTRINSIC FACTOR BY HEATING:

Normal gastric juice was mixed with serum from a subject with pernicious anaemia which was known to contain an antibody against intrinsic factor. The volumes chosen were such as to provide an equivalent number of units of intrinsic factor and antibody. This mixture was incubated at room temperature for twenty minutes. One ml aliquots were taken to measure the free intrinsic factor and the free detectable antibody. Thereafter the mixture was incubated in a water bath at 60°C. One ml aliquots were taken at fifteen minutes and thirty minutes to measure:

a) the vitamin B_{12} binding.

b) detectable antibody which might have been released by the elevation of temperature.

Comment:

It was shown that vitamin B_{12} binding of normal gastric juice was reduced to nil by heating to 60°C for fifteen minutes. Correspondingly the presence of antibody would be shown when a test preparation inhibited the vitamin B_{12} binding of a normal gastric juice sample to which it had been added. The total vitamin B_{12} binding of 1 ml of test mixture together with 0.5 ml of normal gastric juice, declined after heating at the same rate as the fall in vitamin B_{12} binding of the heated sample alone. Therefore no antibody could be detected by these means, after it had been in complex with intrinsic factor.

4.2 Alterations in hydrogen ion concentration.

A. INTRODUCTION

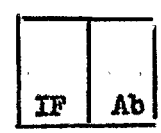
It was shown in section 3.3 that intrinsic factor binding to vitamin B_{12} was not affected by a fall in the pH to 3.2, whereas the combination of intrinsic factor with type 1 antibody was impaired. The purpose of the subsequentinvestigations was to determine whether pre-complexed antibody could be separated from intrinsic factor by increasing the hydrogen ion concentration, also the rate and order of such separation and the degree of recovery of the antibody introduced.

B. THE RELEASE OF TYPE 1 ANTIBODY FROM INTRINSIC FACTOR.

Procedure:

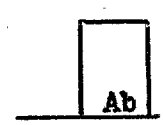
Serum was titrated for antibody against intrinsic factor by the method of Ardeman and Chanarin (1963). In the particular experiment set out below the antibody titre of the serum was 120 units per ml. The intrinsic factor content in a sample of pooled normal gastric juice was measured by the same method. The content was 62 units per ml. The following mixtures were made:

A. 4 ml gastric juice. 248 units IF
2 ml serum 240 units Ab.



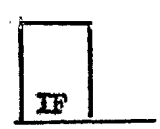
A

B. 4 ml isotonic saline
2 ml serum 240 units Ab



B

C. 4 ml gastric juice 248 units IF



C

These mixtures were incubated at room temperature for twenty minutes to allow the antibody to react with intrinsic factor.

Thereafter 1000 ng of non-radioactive cyanocobalamin in 1 ml of saline was added to each mixture in order to saturate the vitamin B_{12} binding sites present and also to provide an excess of vitamin B_{12} so that it could combine with sites on intrinsic factor previously occupied by antibody which had been released.

One ml was withdrawn from each mixture to test for antibody content at neutral pH. Then four ml of 0.2 M phosphate—citrate buffer at pH 3.2 was added to the remaining six ml in the three mixtures in order to permit separation of the antibody from its pre-complexed state with intrinsic factor. One ml aliquots were transferred at stated intervals directly into 2 ml of 0.2 M Na_2HPO_4 in order to return the pH to neutral. This permitted measurement of free antibody as described in section 2.2.B.

The 1 ml aliquots comprised 0.6 ml aliquots of the original incubation mixture and 0.4 ml of the added phosphate citrate buffer. In order to have an equivalent amount from the control mixtures 0.6 ml aliquots were taken from these and decanted into a neutral mixture of 0.4 ml M phosphate-citrate buffer and 2.0 ml of Na_2HPO_4. The samples from mixtures A, B and C were then all treated identically.

Protein coated charcoal suspended in 1 ml saline was added to all the specimen tubes and shaken in order to adsorb all the unbound vitamin B_{12}. After 1-2 minutes these suspensions were centrifuged at 3000 r.p.m. for ten minutes and the clear supernatants were decanted. The free antibody present was measured.

In a typical experiment 240 units of antibody were present in mixture A and B. One ml was removed from the incubation mixture prior to the addition of buffer in order to measure the free antibody present. Since the mixtures were originally of 6 ml volume, the antibody remaining was: 240-240/6=200 units. Buffer was added to

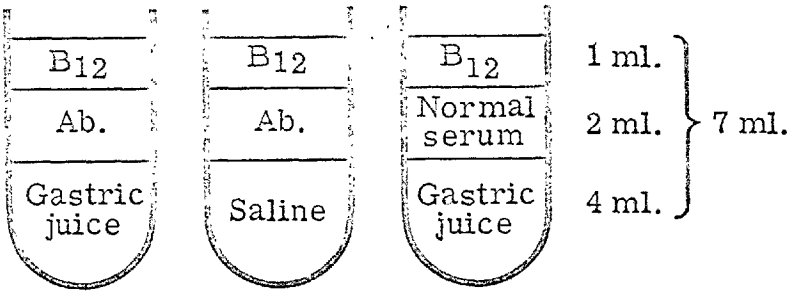
a total volume of 10 ml and one ml aliquots were removed and tested for free antibody at stated intervals. Each of these aliquots was thus calculated to contain 20.0 units of antibody in combination with intrinsic factor before processing.

The procedure is outline in table 17.

Table 17.

The components of test and control mixtures in measurement of antibody.

MIXTURES



CONTROLS

1 ml. aliquots decanted into neutral buffer

TESTS (6 ml.)
4.0 ml. 0.2 m. buffer at pH 3.2
Incubated at 37°C
1 ml. aliquots removed and decanted into 0.2 m. Na_2HPO_4 at stated intervals

Free vitamin B_{12} adsorbed by charcoal
Supernatant decanted into normal gastric juice
100 ng. $^{57}CoB_{12}$ added
Unbound $^{57}CoB_{12}$ adsorbed by charcoal

Impairment of radioactive vitamin B_{12} binding is a measure of free antibody in treated sample

Results: Recovery of antibody from complex with intrinsic factor.

Table 18 shows the percentage of bound antibody which was released from combination with intrinsic factor. The release of antibody in sera 2, 5 and 9 was tested only once whereas with the remaining sera this was tested more than once to determine the reproducibility of the method.

Table 18.

Antibody containing sera (1 to 9) added to standard gastric juice.

	1	2	3	4	5	6	7	8	9
Total Antibody (ng units)	15	32	15.5	21	25	30	16.5	22.5	13.5
Free Antibody in mixture	5.5	6	0	0	2	3.5	0	0	0
Antibody bound	9.5	26.0	15.5	21	23	26.5	16.5	22.5	13.5
Antibody released	5.5	20.5	11.5	16.5	18	23	10.5	14.5	8.0
Recovery (%)									
Experiment a.	58	79	74	78	78	87	64	64	60
b.	54			76.5	75	90	83	60	
c.	62					88	82		
d.							85		

Comment:

This between 54 - 90% of antibody added to gastric juice could be made to dissociate from intrinsic factor in a form detectable by the standard assay described for intrinsic factor antibody. With the same serum the method appeared to be reasonably reproducible.

C. THE RATE OF RELEASE OF ANTIBODY FROM INTRINSIC FACTOR AT pH 3.2.

Procedure:

Normal gastric juice and serum containing antibody against intrinsic factor were mixed (A), in such volumes as to introduce corresponding units of intrinsic factor and antibody. This mixture was incubated at room temperature for 20 minutes. Preparations were also made containing serum with antibody alone (B) and normal gastric juice with normal serum (C). These were included in order to test the effects of the subsequent steps on the independent components of mixture A. The mixtures were then placed in a water bath at 37°C and non-radioactive vitamin B_{12} was added in excess of the binding capacity of the components of the mixtures. The subsequent steps were as outlined in table 17.

Results:

The rate of release of precomplexed antibody in a typical experiment is shown in figure 18.

In three similar experiments employing different sera containing antibody against intrinsic factor, the rate of release was greatest during the first five minutes at pH 3.2 and no further release was obtained after fifteen minutes.

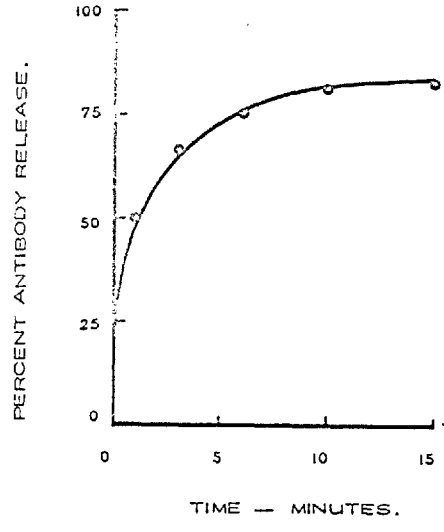


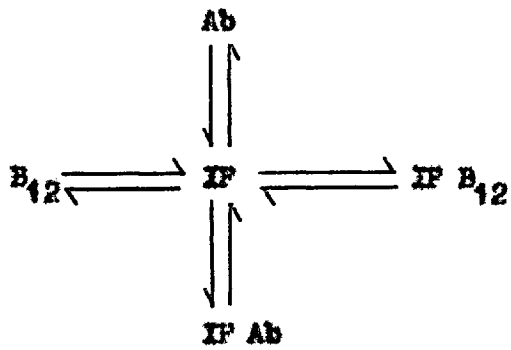
Figure 18

Rate of antibody release at pH 3.2 room temperature

4.3 The intrinsic factor-antibody complex.

A. INTRODUCTION.

In mixture, intrinsic factor, type 1 antibody and vitamin B_{12} are in a state of equilibrium:



The purpose of the following studies was to define the physical conditions so that, in an incubation mixture containing intrinsic factor in complex with type 1 antibody, the vitamin B_{12} will exchange with the antibody at their common binding site on the intrinsic factor molecule:

$$\text{IF Ab} + B_{12} \rightleftharpoons \text{IF } B_{12} + \text{Ab}$$

B. THE EFFECT OF TIME AND TEMPERATURE ON THE INTRINSIC FACTOR-ANTIBODY COMPLEX IN THE PRESENCE OF VITAMIN B_{12}.

Procedure:

Normal gastric juice and serum containing antibody were incubated together for twenty minutes at room temperature. The respective volumes were such that a slight excess of free intrinsic factor remained detectable.

100 ng of ^{57}Cobalt vitamin B_{12} was added to each of three such incubation mixtures. The specimens at neutral pH were left at 4°C, room temperature and 37°C for 48 hours. Samples of gastric juice alone were incubated at these temperatures for 48 hours to serve as controls.

At the end of 48 hours the bound ^{57}Cobalt vitamin B_{12} was measured in the incubation mixtures and in the gastric juice controls.

Table 19.

MIXTURES INCUBATED FOR 48 HOURS AT TEMPERATURES SPECIFIED.

	Test	Control
Gastric juice	+	+
Isotonic saline		
Serum with antibody	+	
Normal serum		+
$^{57}CoB_{12}$	+	+

Results:

Titration of serum showed that 26 units of antibody were present in the initial mixtures.

There was a considerable decline in the vitamin B_{12} binding capacity of the normal gastric juice alone, over 48 hours at 37°C by contrast with 4°C.

At 4°C 13.5% of the antibody was free in the presence of unbound vitamin B_{12} whereas at room temperature 65% of the antibody had been released.

The release of antibody was related to the rise in vitamin B_{12} binding since they displace each other from the same site on intrinsic factor.

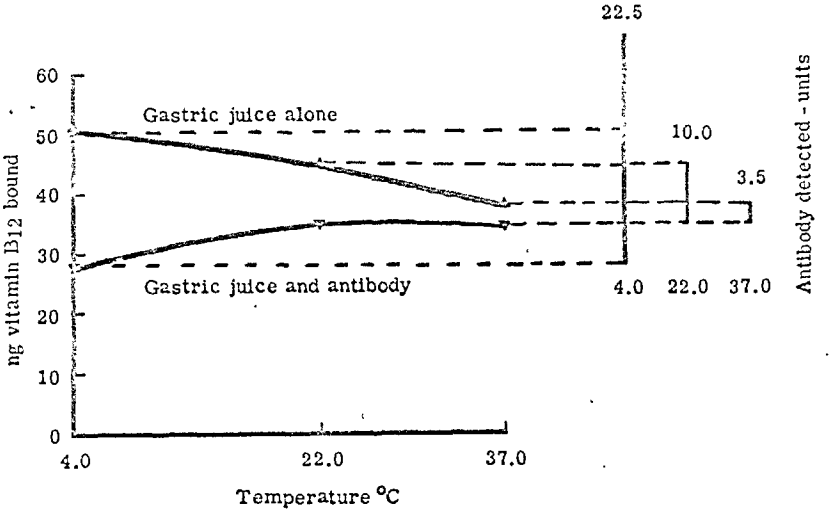


Figure 19.

Exchange of Type I antibody and vitamin B_{12} over 48 hours at 4.0, 22.0, 37 °C

C. COMBINED MEASUREMENT OF ANTIBODY RELEASED AT LOW pH BY OBSERVING THE RISE IN VITAMIN B_{12} BINDING AND BY DIRECT TITRATION OF ANTIBODY.

In the previous experiment it was assumed that the difference in vitamin B_{12} binding after 48 hours at 37°C and 4°C was a measure of antibody release. It has been shown (in section 4.2) that antibody release may be hastened by increasing the hydrogen ion concentration.

In order to show whether the rise in vitamin B_{12} binding corresponded to antibody released, when the pH was lowered to 3.2, the following tests were performed:

Procedure:

Six samples of normal gastric juice to which antibody against intrinsic factor had been added were each separated into equal volumes of 1 ml. Buffer (pH 3.2) was added to each of the six sample pairs. In one of the sample pair, the change in vitamin B_{12} binding was measured after fifteen minutes exposure to pH 3.2. In the second specimen from the sample pair the antibody release after fifteen minutes at pH 3.2 was measured by the method described in section 3.2B.

Results:

Table 20.

Correlation of Antibody Release with Elevation of Vitamin B_{12} Binding at pH 3.2.

Sample	Units of Antibody Added	Units of Antibody Released	Rise in Vitamin B_{12} Bound (ng)
1	17.0	15.0	19.0
2	16.0	13.5	21.5
3	14.5	9.0	11.0
4	13.5	8.0	7.0
5	13.5	8.0	9.0
6	4.0	0	0.0

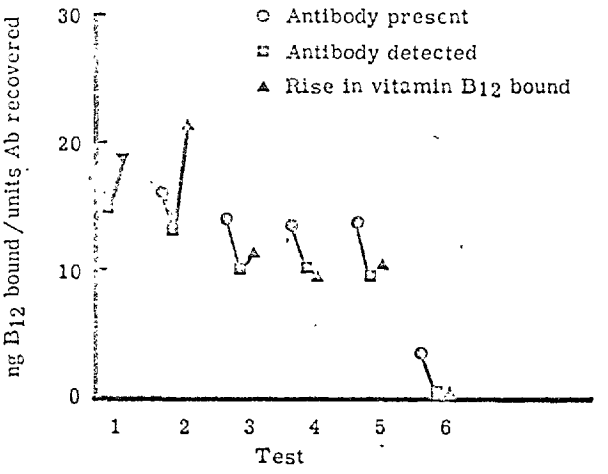


Figure 20.

Comparison between vitamin B_{12} bound and antibody release

Comment:

Antibody recovery was consistently less than 100% of that added, varying from zero to 88%. Mean recovery was 68%. By and large the concomitant rise in vitamin B_{12} binding was higher than the antibody recovered, varying from zero to 134%, mean 86%. In two tests for alteration in vitamin B_{12} binding the results were higher than anticipated; they were lower than predicted in four of the tests. There are a number of explanations for the variation in degree of recovery of antibody from different subjects. Antibodies in a serum to a specific antigen may exhibit heterogeneity in respect of their susceptibility to an alteration in physical conditions. The antibody molecules to intrinsic factor may have heterodeneous avidity (binding constant) for intrinsic factor. These antibodies may correspondingly vary in the relative proportion which may be released from combination with the antigen as the hydrogen ion concentration is increased. Also increasing proportions of antigen relative to antibody, will result in an increased strength of binding.

In order to test the effect of antigen excess upon antibody recovery, the recovery was measured in specimen mixtures with graded antigen excess.

D. ANTIBODY RECOVERY FROM INTRINSIC FACTOR:ANTIBODY COMPLEX IN THE PRESENCE OF INTRINSIC FACTOR EXCESS.

Procedure:

Sera from three subjects with antibody to intrinsic factor in high titre were incubated for 20 minutes with normal gastric juice. The volumes of gastric juice and serum were calculated to provide:-

(a) Equivalent proportions of intrinsic factor units and units of antibody.

(b) A two-fold excess of intrinsic factor over antibody.

(c) A four-fold excess of intrinsic factor over antibody.

After incubation at pH 3.2 in the presence of a known excess of non-radioactive vitamin B_{12}, the antibody release was measured as previously described (section 3.2B).

Results:

Table 21.

Serum Sample	I.F./Ab	Units Ab Added	Units Ab Recovered	% Recovery
1	1 : 1	21	17	78
	2 : 1	18.5	14	76
	4 : 1	23.5	10	42.5
2	1 : 1	18	14.5	81
	2 : 1	18	14.0	78
	4 : 1	18.5	11.5	62
3	1 : 1	22.5	12.5	56
	2 : 1	22	12.0	55
	4 : 1	22.5	8.5	38

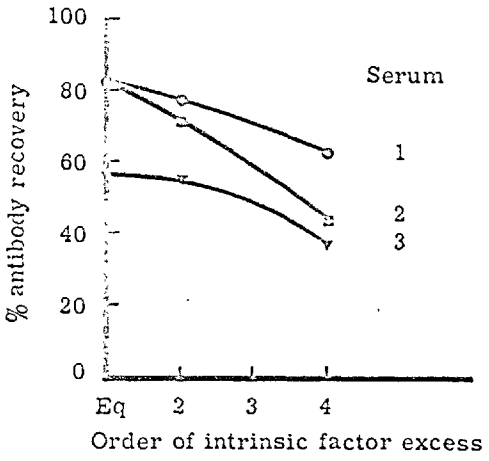


Figure 21.

Effect of intrinsic factor excess on antibody recovery

Comment:

All three curves of declining recovery of antibody with increasing antigen excess show a greater fall with the four-fold excess than would be expected if the initial decline in recovery from equivalent to two-fold excess of intrinsic factor were sustained.

E. ANTIBODY RECOVERY FROM INTRINSIC FACTOR EXCESS OVER A WIDER RANGE.

Samples of serum K.R. Number 2 (in previous section) were incubated with progressively increasing quantities of normal gastric juice.

Antibody controls were also prepared of serum in a corresponding volume of isotonic saline. The relative volumes employed were derived from the intrinsic factor content of the normal gastric juice and the antibody titre of the serum. The relative units of intrinsic factor to units of antibody ranged from 1 : 4 to 1 : 20.

Results:

Table 22.

Serum : K.R.

IF/Ab	Units of Antibody Predicted	Units of Antibody Recovered	Units of Neat Antibody	% Recovery
4 : 1	15.5	11.5	15.5	75
6 : 1	11.0	7.0	11.5	61
8 : 1	9	4.5	8.5	53
10 : 1	7.5	3.5	6.5	54
15 : 1	5.5	2.5	7.0	36
20 : 1	4.0	1.5	5.0	30

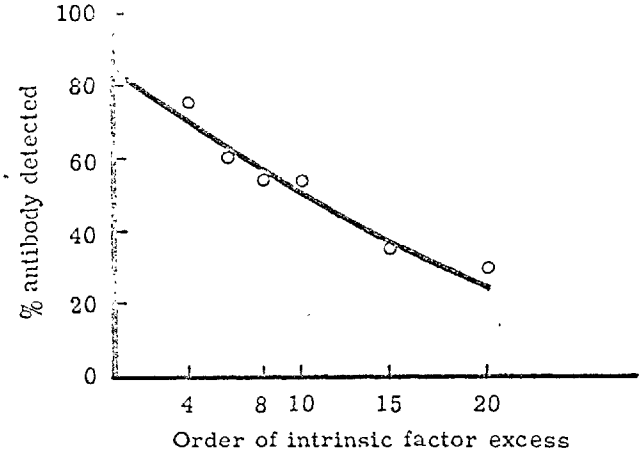


Figure 22.

Antibody detected from scaled increase of intrinsic factor excess

F. THE RECOVERY OF DECREASING QUANTITIES OF ANTIBODY FROM A CONSTANT QUANTITY OF INTRINSIC FACTOR.

In order to establish whether the declining antibody recovered was related to increasing excess of intrinsic factor over antibody present or was simply due to the reduced amount of antibody present an experiment was performed to compare recovery when:-

(a) decreasing antibody was present in constant quantities of normal gastric juice.

(b) increasing dilutions of antibody were made in complex with an equivalent number of units of intrinsic factor in saline.

Procedure:

0.5 ml samples of gastric juice (intrinsic factor concentration: 28 units per half ml) were incubated with volumes of serum, containing antibody, in increasing quantities. Antibody controls were also made with similar volumes of serum containing antibody, in 0.5 ml of saline.

A further mixture of antibody and normal gastric juice was prepared containing equivalent units of antibody and intrinsic factor. Aliquots of increasing volume were taken from the test and control mixtures and decanted into normal saline to make a total volume of 0.5 ml. The steps described in section 3.2B were employed to separate intrinsic factor from antibody and to identify the released antibody.

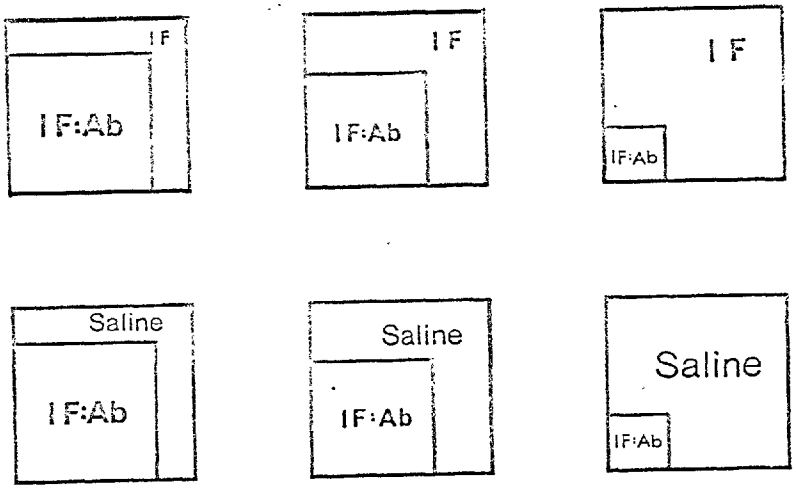


Figure 23.

Recovery from decreasing quantities of antibody

Results:

Table 23.

A. Intrinsic Factor Present : 28 Units.

Units of Antibody Present	Units of Antibody Recovered	%
5	2.0	40
7	2.5	35.5
9	4.0	44.5
11.5	7.0	61.0
15.5	11.5	74.0

Table 24.

B. Intrinsic Factor Present : as antibody.

Units of Antibody Present	Units of Antibody Recovered	%
4	2.5	62.5
7	5.0	71.5
12	10.0	83.5

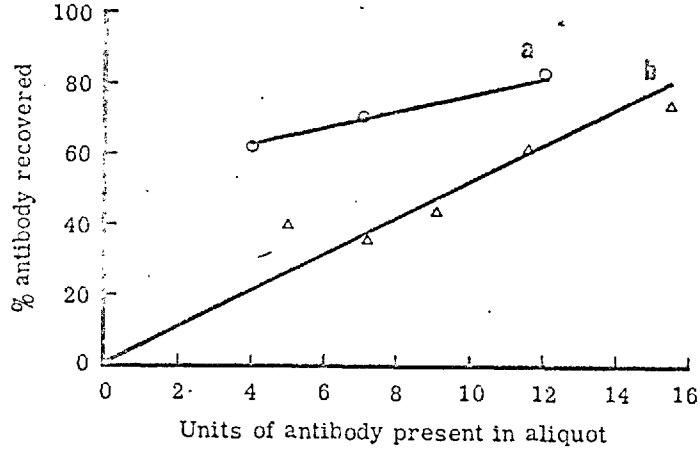


Figure 24.

Recovery from decreasing quantities of antibody

Comment:

Where the units of antibody and intrinsic factor were similar, the antibody recovery remained high with decreasing units of these components. Where the quantity of antibody was progressively smaller with identical content of intrinsic factor, antibody recovery was lower than obtained with the conditions described above.

There are evidently two factors which determine the efficiency of recovery of the antibody from an excess of intrinsic factor. One is the absolute quantity of antibody present - proportionately less antibody is recovered where less antibody is present. The second is the order of intrinsic factor excess: the recovery from declining amounts of antibody is lower in the presence of intrinsic factor excess.

One factor common to both systems is the loss of some identifiable antibody during the process of charcoaling to remove the unbound vitamin B_{12}.

In the presence of excess intrinsic factor, antibody complexed with intrinsic factor molecules may be a lot more strongly bound and be less susceptible to the methods employed for dissociation, whereas in the system of simple dilution the complexes of antibody and intrinsic factor are bound in a variety of strengths.

When the pH is lowered, antibody is released from intrinsic factor and vitamin B_{12} combines with the sites thereby vacated.

$$IF + B_{12} + \text{Antibody} \text{ ------ } IF{:}B_{12} + \text{Antibody}.$$

As this remains an equilibrium reaction, the amount of free intrinsic factor depends on the total intrinsic factor present in the mixture. Correspondingly this proportion of "free" intrinsic factor remains available for combination with antibody when the pH is returned to neutrality.

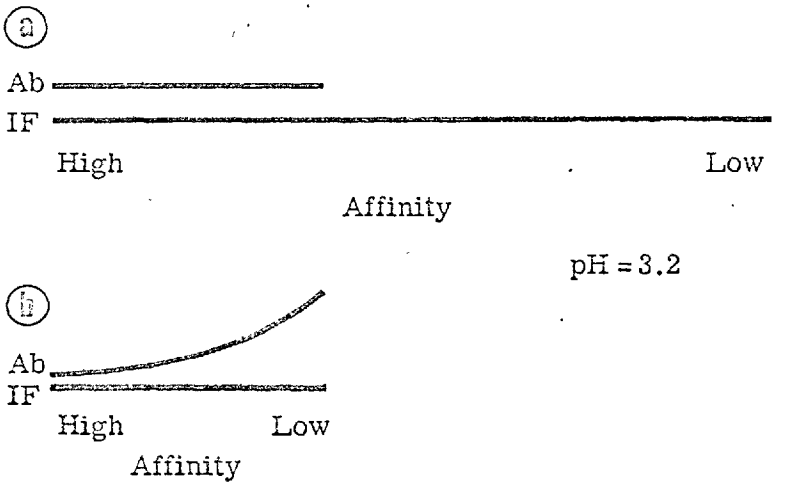


Figure 25.

Binding of intrinsic factor with antibody at pH 3.2

G. RATE OF ANTIBODY RELEASE FROM INTRINSIC FACTOR OVER RANGE pH 3.0 - 7.0.

To measure the relative importance of the hydrogen ion concentration upon the rate and order of antibody release in the presence of an excess of non-radioactive vitamin B_{12}, recovery experiments were performed on aliquots from a single pool of mixture of normal gastric juice, serum with an intrinsic factor antibody and non-radioactive vitamin B_{12}. These aliquots were incubated with 0.2M citrate-phosphate buffer at pH 3.0, 4.0, 5.0 and 7.0 respectively.

Procedure:

Normal gastric juice was mixed with serum containing an antibody to intrinsic factor in high titre. After incubation at room temperature for 20 minutes, an excess of cold vitamin B_{12} was added. This mixture was divided into four sub-samples. To each of these samples 0.2 M citrate-phosphate buffer was added to reach pH 3.0, 4.0, 5.0 and 7.0 respectively.

After fifteen minutes aliquots of these sub-samples were expelled into 0.2 M Na_2HPO_4 returning the pH to neutrality. Unbound vitamin B_{12} was adsorbed with a saline suspension of serum coated, active charcoal. After centrifugation at 3000 r.p.m. for 10 minutes the supernatant tested for antibody released as outlined in section 3.2B. Appropriate controls were treated likewise:-

(a) Antibody control : test plus saline.

(b) Gastric juice control : gastric juice plus normal serum.

Results:

Total antibody in test samples : 19 units.

Free antibody present : nil.

Therefore antibody bound to intrinsic factor : 19 units.

Free Antibody Identified in Control Mixtures:

(a) Saline plus test serum : 19 units

(b) Gastric juice plus normal serum : nil

(c) Gastric juice plus test serum : nil

Table 25.

Antibody Recovery (%)

Time (Minutes)	pH 3.0	4.0	5.0	7.0
1	63	37	10	nil
5	74	53	26	nil
10	82	68	31.5	nil
15	84	74	34	nil

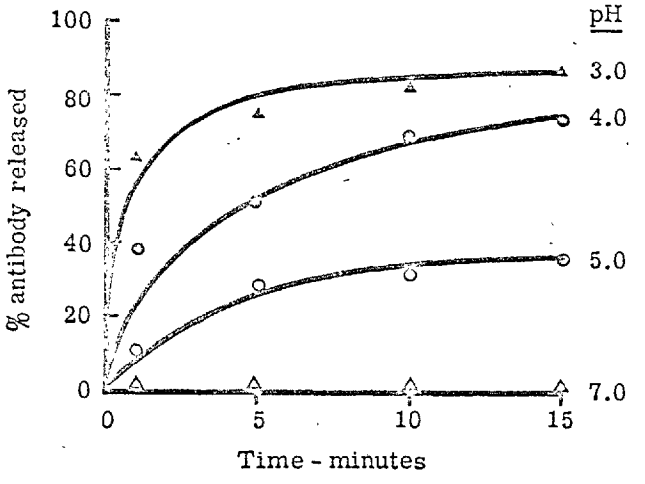


Figure 26.

Rates of release of antibody over pH range 3.0 - 7.0

H. THE CRITICAL EFFECT OF HYDROGEN ION CONCENTRATION.

When exposing a complex of intrinsic factor and antibody to a fall in pH there are two components determining the recovery of antibody. One is the release of antibody from intrinsic factor and the other is deterioration of antibody at the selected pH. Whilst native antibody was able to withstand pH 3.0 for up to 60 minutes with no appreciable deterioration, it may have had a different susceptibility to the increased hydrogen ion concentration during and after separation from intrinsic factor. Since only the resultant recovery could be measured, tests were performed on complexes of intrinsic factor and antibody over a wide pH range with particular attention between pH 3.0 - 4.0.

Procedure:

Normal gastric juice and serum containing antibody were mixed. After incubation at room temperature for 20 minutes, 0.5 ml aliquots, calculated to contain 23 units of intrinsic factor and 13 units of antibody were decanted into 200 ng of non-radioactive vitamin B_{12}. After 20 minutes 1 ml of 0.2 M citrate-phosphate buffer was added to reach the pH specified. This was incubated at 37°C for 15 minutes. Then 3 ml of 0.2 M Na_2HPO_4 was added, returning the pH to neutrality. The procedure for removal of unbound vitamin B_{12} and for the detection of antibody was as described in section 3.2B.

Results:

Table 26.

pH	Antibody Recovered	% Recovered
7.5	nil	0
7.0	nil	0
6.5	nil	0
6.0	nil	0
5.5	nil	0
5.0	nil	0
4.5	0.5	4.0
4.0	2.5	19.0
3.8	6.0	46.0
3.6	5.5	42.5
3.4	6.5	50.0
3.2	10.0	77.0
3.0	8.5	65.0
Negative Control	Nil	0
Antibody Control	13.0	100

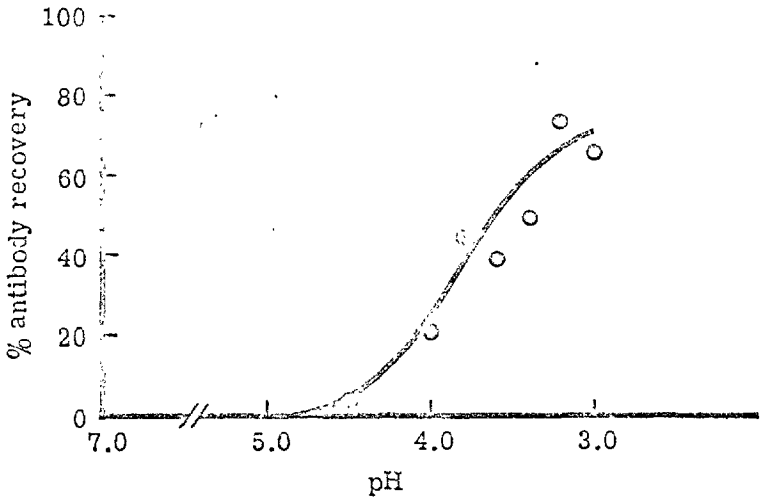


Figure 27. Antibody release and pH

4.4. The effect of Bile-Stained Gastric Juice on Intrinsic Factor Content of a Normal Gastric Juice Sample.

Temperley et al (1969) described non-specific, heat stable components of normal duodenal juice which inhibited intrinsic factor. In order to test this, the effect of a bile-stained sample of gastric juice on the intrinsic factor content of a normal gastric juice specimen was tested.

Procedure:

Normal gastric juice was incubated with the bile-stained sample for one hour: two volumes of normal gastric juice plus one volume of the bile-stained sample. These were kept at room temperature. After one hour the sample was tested for its intrinsic factor concentration. Control samples were similarly tested:-

Two volumes of normal gastric juice plus one volume of saline.

Two volumes of saline plus one volume of the bile-stained sample.

The test sample and control samples were then stored for 48 hours at -20°C and the intrinsic factor content measured again to see if any deterioration had taken place on storage.

Results:

Table 27.

Gastric juice	Serum Added	mg Vitamin B_{12} Bound.	Intrinsic Factor (Units)
Normal	Normal	40.0	30.5
"	Ab	9.5	
Bile-Stained	Normal	5.0	0
	Ab	5.0	
Mixture of normal and bile-stained at 1 hour	Normal	43.5 - 5.0 = 38.5	26.5
	Ab	17.0 - 5.0 = 12.0	
Mixture after storage	Normal	38.0	26.0
	Ab	12.0	

Comment:

Mixing with bile-stained gastric juice resulted in a fall of the intrinsic factor content of a normal gastric juice sample. This fall was registered within an hour and no further decline was observed after frozen storage and thawing.

4.5. In vitro interference with vitamin B_{12} binding by intrinsic factor.

A. INTRODUCTION.

There are components in the gastro-intestinal secretions which may interfere with vitamin B_{12} binding by intrinsic factor or may destroy the intrinsic factor. Bile-stained gastric juice for example inhibits the union of vitamin B_{12} with intrinsic factor, as demonstrated in the previous section (4.4.). Residual unadsorbed unlabelled vitamin B_{12} would have a priority of access to intrinsic factor and would obstruct sites for radioactive vitamin B_{12}, thereby creating confusion in recognising the presence of intrinsic factor antibody. In order to confirm that impaired vitamin B_{12} binding by intrinsic factor was due to antibody, a specific antibody inhibition test was required.

B. ANTIBODY INHIBITION WITH ANTIHUMAN GLOBULIN.

Procedure:

Samples of pernicious anaemia serum with a known antibody content were introduced into saline. Specified volumes of horse anti-human IgG were added. Normal serum was added to control tubes. The mixtures were incubated at 37°C for two hours and then for 48 hours at -50°C. They were thawed and decanted into 0.5 ml samples of normal pooled gastric juice, so that antibody could combine with intrinsic factor. After 20 minutes, 100 ng of radioactive vitamin B_{12} was added. Normal serum was also added to make the total serum volume added up to 1.0 ml. After a further 20 minutes unbound vitamin B_{12} was adsorbed with activated charcoal.

Procedure:

Table 28.

Sample	Control	Volumes in ml Tests			
		1	2	3	4
PA Serum	0.1	0.1	0.1	0.1	0.1
Anti IgG		0.1	0.2	0.4	1.0
Normal Serum Added in Final Step	1.0	0.9	0,8	0.6	nil

Results:

Table 29.

Specimen	Units of Antibody Detected	Units of Antibody Inhibited
Control	22	
Anti IgG Tests		
0.1	21	1.0
0.2	20	2.0
0.4	18.5	3.5
1.0	10.0	12.0

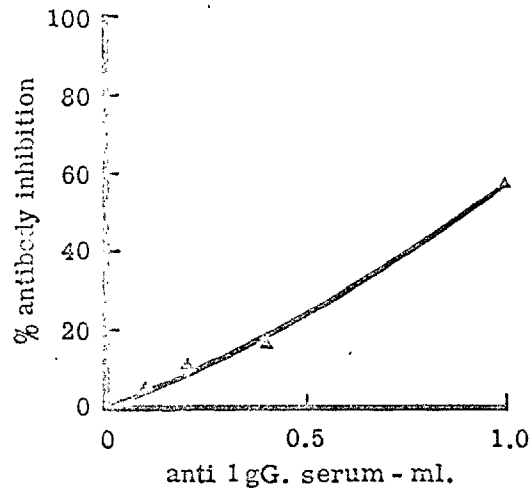


Figure 28.

Inhibition of Type I antibody by antihuman 1gG. serum

Comment:

The ability of anti-human globulin to interfere with the intrinsic factor inhibition by the test serum, confirmed that the inhibition was due to an antibody. This test could furthermore be used to identify the antibody class of inhibitors found in gastric juice from pernicious anaemia subjects (see section 5).

4.6. THE EFFECT OF DEPEPSINISING THE GASTRIC JUICE UPON RECOVERY OF ANTIBODY TO INTRINSIC FACTOR.

A. INTRODUCTION.

The antibody activity of an immunoglobulin is evidently unaffected by digestion with pepsin (Misonoff et al 1960). Pepsin in the presence of cysteine causes cleavage of the immunoglobulin molecule as shown in figure 29.

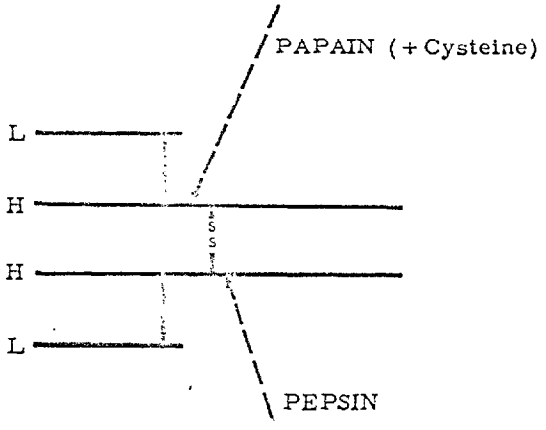


Figure 29.

Sites of clearage of immunoglobulin molecule by Pepsin and Papain

The Fab pieces are unaffected by pepsin whereas the Fc piece is digested. The antibody activity is however a function of the Fab pieces and this should therefore remain unaffected.

Pepsin shows two pH peaks of optimum activity. One between pH 1.6 - 2.4; the second between pH 3.3 - 4.0 (Bell, Davidson and Scarborough 1968). Hydrolytic activity due to pepsin does not continue beyond the range: pH 0.7 - 4.5 (Bockus 1963). Exposure to pH 10 for thirty minutes reduced pepsin activity to a negligible order once the gastric juice is returned to the optimal pH range Grasbeck et al (1962).

In order to test the effect of pepsin upon intrinsic factor antibody activity, the antibody was incubated in normal active gastric juice and in pepsin inactivated gastric juice.

B. THE EFFECT OF PEPSIN ON ANTIBODY AGAINST INTRINSIC FACTOR.

Procedure:

Normal gastric juice which had been brought to neutral pH was divided into two samples of equal volume. In one sample the pH was raised to 10 with 40% NaOH. This was left at room temperature for 30 minutes in order to inactivate the pepsinogen. Thereafter 6 normal HCl was added, returning the pH to neutrality. The volumes of these reagents which were added were extremely small. The second sample was left at neutral pH.

Serum containing antibody to intrinsic factor was added to the depepsinised gastric juice and to normal gastric juice. The relative volumes were such that intrinsic factor was present in two-fold excess over the antibody. Antibody controls were prepared with serum and saline. An excess of non-radioactive vitamin B_{12} was added to all mixtures.

The subsequent steps for release and identification of antibody against intrinsic factor were as outlined in section 3.2B.

Procedure:

Table 30.

Reagents	Volume in ml. (except where otherwise stated) Tests		Controls	
	Straight	Depepsinised	Serum	Serum
Saline			0.5	0.5
Gastric Juice	0.5	0.5		
Antibody	0.1	0.1	0.1	0.1
Cold B_{12}	400ng	400ng	400ng	400ng
pH 3.2 buffer	1.0	1.0	1.0	1.0
Na_2HPO4	3.0	3.0	3.0	3.0
Charcoal suspension	1.0	1.0	1.0	1.0
$^{57}CoB_{12}$	100ng	100ng	100ng	100ng
Charcoal suspension	1.0	1.0	1.0	1.0
Sample	1	2	3	4

Results:

Table 31.

Specimen	ng $^{57}CoB_{12}$ bound	Units of Antibody liberated
Normal gastric juice	62.5	
NGJ + Antibody	20.0	
NGJ + Sample (1)	40.0	22.5
NGJ + Sample (2)	40.5	22.0
NGJ + Sample (3)	36.0	26.5
NGJ + Sample (4)	33.0	29.5

(NGJ = Normal pool gastric juice).

Recovery

$$\frac{\text{Normal gastric juice + Antibody}}{\text{Serum control at pH 7.0}} = {}^{22.5}/_{29.5} = 76.5\%$$

$$\frac{\text{Depepsinised gastric juice + Antibody}}{\text{Serum control at pH 7.0}} = {}^{22}/_{29.5} = 75\%$$

Since some deterioration of antibody took place in the serum control at pH 3.2, the recovery of antibody from complex with intrinsic factor, making allowance for deterioration of antibody at pH is expressed as :-

$$76.5 \times 29.5/26.5 = 85\%$$

$$75 \times 29.5/26.5 = 83.5\%$$

$$\text{Antibody deterioration} : \frac{29.5 - 26.5}{29.5} = 10\%$$

Comment:

Depepsinisation made no difference to the recovery of antibody from complex with intrinsic factor.

4.7 Release of type II antibody.

The technique for release and identification of type I antibody has the advantage that once vitamin B_{12} occupies the binding site on intrinsic factor the antibody can no longer recombine. No specific function has been localised to the zone of intrinsic factor with which type II antibody combines. Since the only other recognised function of intrinsic factor is to bind with mucosal receptors in the ileum, it may be that the type II antibody is directed against a portion of the intrinsic factor molecule responsible for this function.

The simplest method for detecting type II antibody against intrinsic factor was the ammonium sulphate precipitation technique introduced by Jacob and Schilling (1966). Tests performed by this method confirmed that the precipitated radioactivity bore a linear relationship to the amount of type II antibody present. Figure 30 shows the results of a representative experiment.

However, when attempts were made to release and identify antibody from union with intrinsic factor, a fall in pH was not found to increase recovery and that precipitated radioactivity was of a relatively low order compared with control serum samples (figure 31).

This method was unsatisfactory when applied to gastric juice samples because of the negligible precipitation with ammonium sulphate. Furthermore this system did not lend itself to tests for antibody specificity with mono-specific antiglobulin antisera.

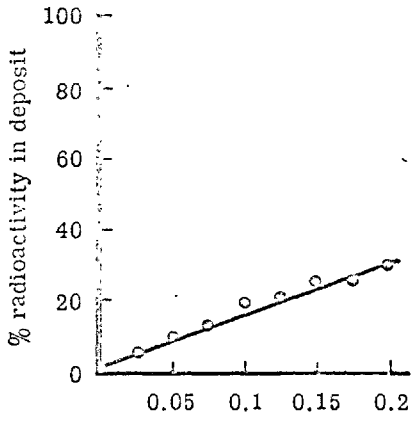


Figure 30.

Radioactive deposit by ammonium sulphate precipitation

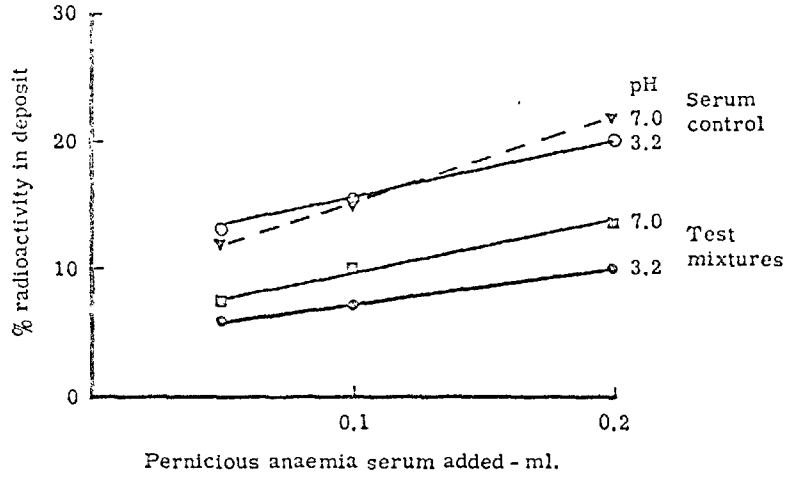


Figure 31.

Recovery of Type II antibody from combination with intrinsic factor

SECTION 5

ANTIBODY IN THE GASTRIC JUICE AGAINST CASTLES INTRINSIC FACTOR.

5.1. Addisonian Pernicious Anaemia.

A. INTRODUCTION.

Speculation on the role of auto-immunity in the pathogenesis of pernicious anaemia is complicated by the finding that a large minority of patients with this condition have no detectable antibody to intrinsic factor in their serum. A further problem is that occasional subjects, particularly those suffering from associated organ specific, auto-immune diseases, may have an antibody to intrinsic factor circulating in their system, whilst maintaining normal vitamin B_{12} absorption.

Intrinsic factor fulfils its functions of binding vitamin B_{12} and mediating absorption of vitamin B_{12} across the mucosal surface of the distal ileum. If an antibody is to interfere with these functions then it must be detectable within the gastro-intestinal secretions.

The purpose of this section was to determine the presence and the incidence of antibodies to intrinsic factor within the gastric juice of pernicious anaemia subjects. This involved the identification of free antibody and the release of antibody from union with intrinsic factor. The pilot studies itemised in section 4 have shown that separation of intrinsic factor from the type I antibody can be enhanced by lowering the pH into a critical range between pH 3.0 - 4.0.

B. CLINICAL MATERIAL.

Patients with Addisonian Pernicious Anaemia: (see appendix II)

Samples of gastric juice were taken from patients as a routine diagnostic procedure in order to determine the cause of a megaloblastic anaemia or a low serum vitamin B_{12} level. These samples were collected as detailed in section 2.2C. Samples were obtained from patients under

current investigation at St. Mary's Hospital, Paddington, W.2. during the period of the present study and further specimens collected at the same hospital during the preceding period, 1963 - 1967, were obtained from cold storage (-20°C) for similar testing.

Occasional samples of gastric juice were also received from other hospitals (see appendix II).

C. PERNICIOUS ANAEMIA.

1. Diagnostic criteria: A diagnosis of pernicious anaemia was made with confidence in patients with evidence of impaired vitamin B_{12} absorption, in whom extraneous intrinsic factor served to improve this function. This clearly defined the diagnosis in terms of gastric dysfunction rather than in terms of the consequential haematological changes, which are indistinguishable from megaloblastic haemopoiesis, from whatever cause.

a) Blood findings:

Patients initially underwent further investigation for macrocytic anaemia with megaloblastic haemopoiesis in the bone marrow.

b) Serum findings:

Levels of vitamin B_{12} below 180 pg/ml were suggestive of vitamin B_{12} deficiency, of which pernicious anaemia was the most likely cause. The finding of an antibody to intrinsic factor in the serum, strongly supported this diagnosis.

c) Gastric findings:

Histamine fast or pentagastrin fast achlorhydria were the sine qua non for a diagnosis of pernicious anaemia. Radio-immune assay for intrinsic factor performed upon the aspirated samples (Ardeman and Chanarin 1963) showed either an absence or the presence of intrinsic factor in only small amounts.

d) Clinical findings:

Associated organ-specific auto-immune disease, a family history of pernicious anaemia or organ specific auto-immune disease, were supportive evidence for the diagnosis.

e) Therapeutic findings:

Response to a therapeutic course of vitamin B_{12} was taken as evidence of a pre-existing state of vitamin B_{12} deficiency.

2. Antibody to intrinsic factor in the gastric juice of patients with pernicious anaemia.

The gastric juice specimens from fifty-three patients with pernicious anaemia were studied. A radio-immune assay of the intrinsic factor content was performed on all gastric juice specimens by the method of Ardeman and Chanarin (1963).

Tests for antibody to intrinsic factor were made by the method described in section 3.2B.

Overt antibody was detected in eighteen out of fifty-three specimens tested (34%). Antibody was found in a further eleven specimens (21%) only after steps had been taken to release antibody from complex with intrinsic factor. Thus the overall incidence of antibody to intrinsic factor in these samples of gastric juice was 29 out of 53 (55%)

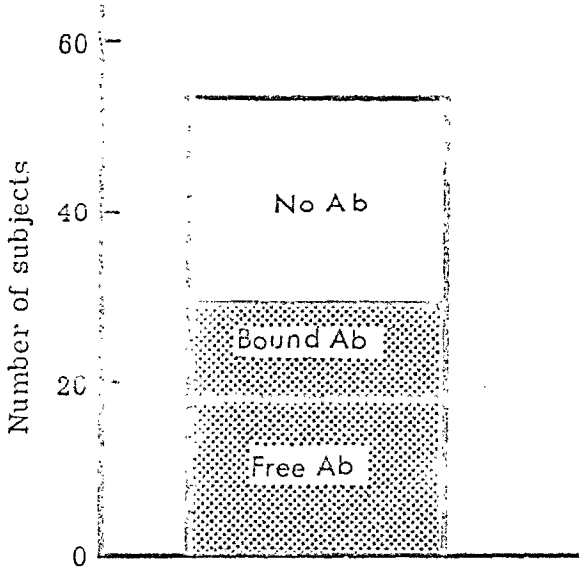


Figure 32.

The incidence of antibody against intrinsic factor in the gastric juice of 53 patients with pernicious anaemia

Table 32.

Antibody against intrinsic factor detected in gastric juice of fifty-three subjects with pernicious anaemia.

Case	Units of antibody detected Free	Total	Case	Units of antibody detected Free	Total
1	4	5.5	28		0
2		0	29		0
3	4	4	30	0	5.0
4	0	6	31		0
5	3	7	32	0	8
6		0	33		0
7		0	34		0
8	7	10.5	35		0
9	0	8	36	0	10.0
10		0	37	4.5	7.0
11	6	11	38		0
12		0	39	0	5.5
13	5.5	4.5	40	0	4.0
14	0	4	41	5	9.0
15		0	42	0	8.0
16	19.5	21.5	43		0
17	0	6.5	44	4	9
18		0	45	4	4.0
19		0	46		0
20	7.5	8.0	47	7.0	10.5
21		0	48		0
22		0	49	0	5.5
23	0	3.5	50	0	4.0
24		0	51	6	8.0
25		0	52		0
26		0	52	4	8.0
27	0	3.5			

3. Correlation of antibody to intrinsic factor in serum and in gastric juice.

Of the fifty-three patients with pernicious anaemia from whom gastric juice was obtained, the serum contained an antibody to intrinsic

factor in 24 (45.5%), contained no such antibody in 27 (51%) and in the two remaining cases no serum sample was obtained (3.5%).

Of the twenty-four subjects with an antibody in the serum, fourteen had an antibody in the gastric juice and ten did not. Of the twenty-seven subjects with no antibody in the serum, fifteen had an antibody in the gastric juice and twelve did not. Of the two remaining cases with no information concerning the serum, one had detectable antibody in the gastric juice and one did not.

(The correlation is shown in table 33. For case breakdown see appendix V).

Twelve subjects out of a total of fifty-three had no detectable antibody in either the serum or in their gastric juice (23%). In thirty-none patients (74%) antibody to intrinsic factor was detected in either serum or in the gastric juice or in both.

Table 33

Correlation between antibody against intrinsic factor in the serum and gastric juice

SERUM \ GASTRIC JUICE	POS	NEG	
POS	14	10	24
NEG	15	12	27
	29	22	Total 51

P = 0.98

Comment:

In order to test whether there was any correlation between the presence of antibody in the serum and in the gastric juice, the probability of such a distribution being due to chance was evaluated by the "chi squared" test: P = 0.98. There was thus no significance in this correlation.

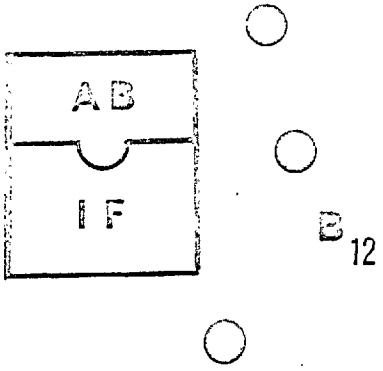
4. Effect of antisera on intrinsic factor inhibition.

a) Principle.

It can be seen in the preceding section that a component in many of the gastric juice specimens from subjects with pernicious anaemia tested, interfered with vitamin B_{12} binding by normal gastric juice. This inhibition was considered due to an antibody. In order to test this, the effects of multiple and specific anti-human sera upon a limited number of samples containing an inhibitor were observed.

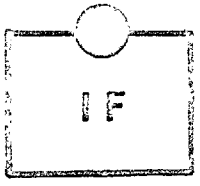
These observations were limited to eight samples, where the volume of aspirated gastric juice was sufficient. Of these eight samples, three were obtained from patients without and five from patients with an antibody to intrinsic factor in the serum. All of the samples had shown the presence of an inhibitor to intrinsic factor on prior testing by the method described in section 3.2B.

1.
'Cold' vitamin B_{12} added to gastric juice

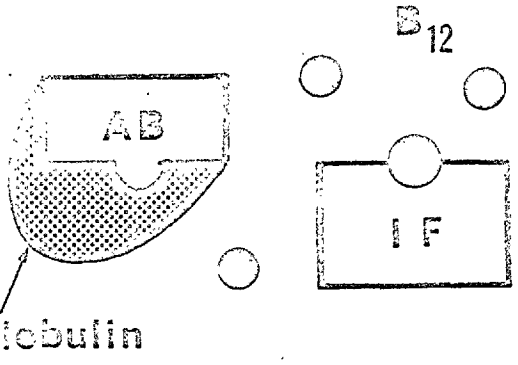


2.
pH lowered to 3.2 with phosphate citrate buffer

3.
pH returned to neutral with Na_2HPO_4



4.
Antiglobulin serum added



5.
Thereafter the steps were as described in section 3.2 B, for removing unbound vitamin B_{12} and identifying free antibody

Figure 33 .

Antiglobulin inhibition

b) Procedure, results and comments.

The tests were performed to identify free and bound antibody and to observe the effects of:

a) Horse anti-whole human serum.

b) Specific horse anti-IgG.

c) Specific sheep anti IgA.

(for sources and specifications of these antisera see Section 2.1E).

Where sufficient volume was available, six separate one ml samples of test gastric juice were dispensed into tubes containing non-radioactive vitamin B_{12}. 0.2 buffer at pH 3.2 was added to five of the preparations and incubated in a water bath at 37°C for fifteen minutes. This step would permit separation of antibody, where present, from intrinsic factor.

Thereafter 0.2 Na_2HPO_4 was added returning the pH to neutrality. A mixture of pH 3.2 buffer and 0.2 M Na_2HPO_4 was added to the sixth tube, preserving neutral pH and thus permitting identification of any free antibody.

The respective antisera were added to three of the five mixtures exposed to a fall in pH. Normal serum was added to the fifth samples as an antiserum control.

All mixtures were incubated in a water bath at 37°C for two hours. The unbound vitamin B_{12} was extracted from all specimens by the addition of serum coated charcoal in saline. The mixtures were shaken for two minutes and then centrifuged for fifteen minutes at 3000 r.p.m. The supernatants were decanted into samples of normal gastric juice to detect the presence of antibody and hence to observe whether antibody inhibition occurred in those samples incubated with antiserum.

Results:

Table 34.

Sample	antiserum	ng B_{12} bound	Antibody	Inhibition
0.5 normal gastric juice	None	31.5	-	-
TEST 1		13.0	18.5	
"	IgA	26.0	5.5	13.0
TEST 2		16.0	15.5	
"	IgA	30.5	1.0	14.5
TEST 3		26.0	5.5	
"	AHG	31.0	0.5	5.0
"	IgA	31.5	0.0	5.5
TEST 4		22.0	9.5	
"	AHG	21.5	10.0	
"	IgA	25.5	6.0	3.5
TEST 5		19.0	13.5	
"	AHG	23.5	8.5	4.0
TEST 6		28	3.5	
"	AHG	34	0.0	3.5
TEST 7		26	5.5	
"	IgG	25	6.5	0.0
	IgA	28	3.5	2.0
TEST 8		29	2.5	
"	IgG	29	2.5	0.0

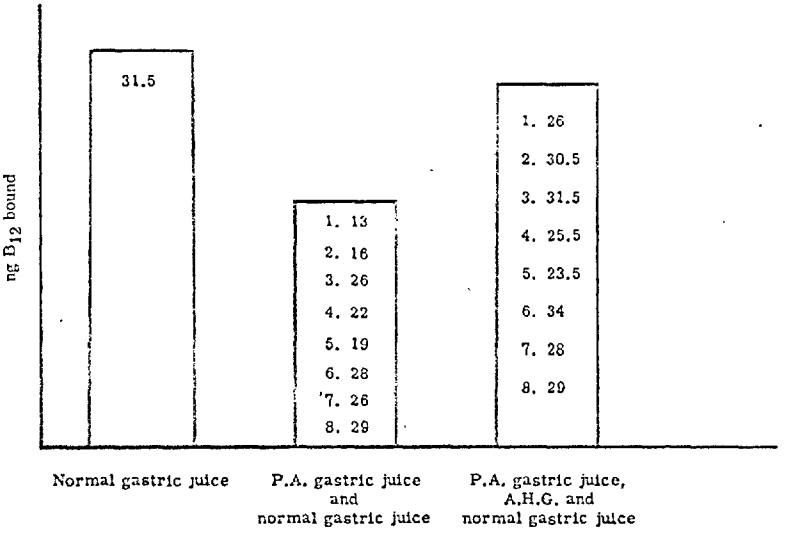


Figure 34.

Antiglobulin inhibition of gastric juice antibody

Comment:

In two cases the intrinsic factor inhibition was entirely counteracted after incubation with IgA antiserum (tests 2 and 3). In a further three cases a partial effect was seen after incubation with IgA antiserum (tests 1, 4 and 7). In one case (test 6) total inhibition occurred after incubation with AHG and in another (test 5) AHG caused partial inhibition only of the antibody. In one case, (test 8), an IgG antiserum failed to inhibit the "antibody".

Thus in five of the eight specimens tested, the inhibitor was either partially or entirely identified as an IgA immunoglobulin. In a further two it was an unspecified immunoglobulin, inhibited by an anti-whole human serum. In one case the inhibitor was not blocked by IgG antiserum and insufficient gastric juice was left to test the effect of anti-IgA.

5. Effects of lowering pH on vitamin B_{12} binding.

a) Principle: Preliminary experiments performed with normal gastric juice, seeded with antibody against intrinsic factor, had shown a fairly close correlation between antibody released upon lowering the pH and the rise in vitamin B_{12} binding (section 4.3/ figure 23). A small number of gastric juice specimens from pernicious anaemia subjects were tested at neutral pH and after exposure to pH 3.2 in order to observe any differences in vitamin B_{12} binding.

b) Procedure, results and comment: Two samples were taken from each gastric juice specimen and incubated with radioactive vitamin B_{12}. 0.2 M pH 3.2 buffer was added to one tube for fifteen minutes, after which the pH was returned to neutrality with 0.2 M Na_2HPO_4.

A corresponding mixture of pH 3.2 buffer and Na_2HPO_4 at neutral pH were added so that both tubes had identical contents and similar volumes. Unbound radioactive vitamin B_{12} was adsorbed by serum coated charcoal in saline and the supernatant radioactivity was measured.

Results:

Table 35.

Sample	ng of ^{57} Cobalt vitamin B_{12} bound	
	pH 7.0	pH 3.2
Normal gastric juice	27	24.5
Specimen 1 *	45	47.5
2	45	44
3 *	45	46.5
4 *	21	23.0
5	41.0	38
6	19	18.0

(* Antibody detected in gastric juice).

Comments:

These results are not striking but there is a tendency for those gastric juice samples in which an antibody was detected to show a rise in vitamin B_{12} binding after exposure to a low pH, whereas the normal gastric juice showed a slight impairment of vitamin B_{12} binding and those specimens without demonstrable antibody also showed a decline in vitamin B_{12} binding.

6. Augmented vitamin B_{12} binding: Occasional samples of gastric juice from subjects with pernicious anaemia not only failed to show an inhibitor to vitamin B_{12} binding but consistently augmented binding to a higher level than encountered in the negative antibody control preparation. In order to determine whether augmentation could be curtailed and an antibody thereafter detected, test gastric juices were mixed with normal gastric, prior to testing for the presence of an inhibitor by the method described in section 3.2B.

In three out of the five samples thus treated, the augmented vitamin B_{12} binding no longer occurred. In the remaining two it was still observed but somewhat reduced. Antibody was not detected in any of these samples after these steps ahd been employed.

Enhancement of vitamin B_{12} binding by intrinsic factor has been encountered in radio-isotope dilution methods for vitamin B_{12} assay. Rothenberg (1961) noted that serum was responsible for enhanced vitamin B_{12} binding by intrinsic factor and Raven et al (1969) made similar observations.

5.2. Intrinsic factor antibody and vitamin B_{12} absorption.

A. INTRODUCTION.

Vitamin B_{12} absorption studies using extraneous intrinsic factor were performed on most of the subjects with pernicious anaemia, whose gastric juice was examined during the course of this investigation. Where samples were received from hospitals other than St. Mary's Hospital, Paddington, this information was occasionally unavailable.

Vitamin B_{12} absorption mediated by extraneous intrinsic factor was measured in 29 of the 53 subjects (Rose and Chanarin 1970). The small size of this sample is because this correlation was undertaken retrospectively. The correlations which have been assessed are between:

a) Presence or absence of antibody against intrinsic factor in the serum and the results of intrinsic factor mediate absorption of 1 ug of vitamin B_{12}.

b) Presense or absence of antibody against intrinsic factor in the gastric juice and the results of intrinsic factor mediated absorption of 1 ug of vitamin B_{12}.

c) Absence of antibody against intrinsic factor from both serum and gastric juice and the results of intrinsic factor mediated absorption of 1 ug of vitamin B_{12}.

d) Correlation of the age of onset and sex of patient with the presence of antibody against intrinsic factor in the serum.

B. MEASUREMENT OF VITAMIN B_{12} ABSORPTION.

This was performed by the urinary excretion method of Schilling (1953): after the patient had voided his urine, 1000 ugm of cyanocobalamin was injected intramuscularly. 1 ug of ^{57}cobalt vitamin B_{12} was mixed with 20 mg of hog intrinsic factor (Lederle WES 818) dissolved in saline. This was swallowed. A meal was provided thirty minutes later. Urine was collected for the subsequent twenty-four hours and a blood sample was taken 8-10 hours after the oral dose had been given.

The urinary excretion of vitamin B_{12} was derived from the ratio of the radioactivity of an aliquot of urine and the radioactivity of a standard prepared from an aliquot of the oral dose. The result was expressed as a percentage of the oral dose of radioactive vitamin B_{12} which was excreted during the succeeding twenty-four hours.

C. CORRELATION BETWEEN ANTIBODY AND VITAMIN B_{12} ABSORPTION.

1. Serum antibody and vitamin B_{12} absorption.

Table 36

Intrinsic factor antibody not detected in serum		Intrinsic factor antibody present in serum.	
Case	Vitamin B_{12} absorption with IF.	Case	Vitamin B_{12} absorption with IF.
4	12.8	1	3.8
8	10.7	2	10.3
11	10.4	3	13.4
16	11.1	5	10.4
18	22.0	6	10.7
24	18.6	13	5.4
28	16.2	15	17.7
33	15.8	19	15.8
35	20.3	21	19.0
36	5.4	23	10.2
40	15.5	25	12.6
43	23.0	27	10.2
47	9.0	39	5.5
48	12.5		
49	8.5		
50	15.0		

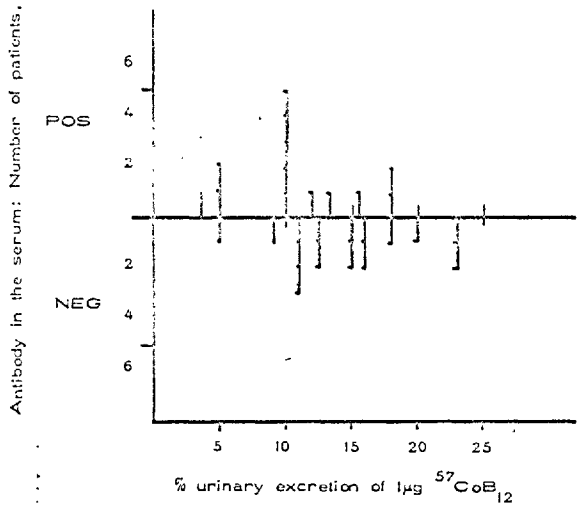


Figure 35.

Correlation of intrinsic factor mediated vitamin B_{12} absorption with antibody to intrinsic factor in the serum

Table 31

Correlation between results of intrinsic factor mediated vitamin B_{12} absorption tests and serum antibody

Vitamin B_{12} excretion.*	SERUM POS	SERUM NEG	
> 15	3	8	11
< 15	10	7	17
	13	15	P = 0.1

Comment:

In order to test whether correlation between serum antibody and vitamin B_{12} absorption was significant the chi squared test was applied: P = 0.1., which is not significant.

2. Gastric juice antibody and vitamin B_{12} absorption.

Table 38

Correlation of gastric juice antibody status and results of vitamin B_{12} absorption with intrinsic factor.

Intrinsic factor antibody NOT detected in gastric juice.		Intrinsic factor antibody PRESENT in gastric juice.	
Case	Vitamin B_{12} absorption with IF	Case	Vitamin B_{12} absorption with IF
2	10.3	1	3.8
6	10.7	3	13.4
15	17.7	4.	12.8
18	22.0	5	10.4
19	15.8	8	10.7
21	19.0	11	10.2
24	18.6	13	5.4
25	12.6	16	11.1
28	16.2	23	10.2
33	15.8	27	10.2
35	20.3	36	5.4
43	23.0	39	5.5
		40	15.5
		47	9.0
		48	12.5
		49	8.5
		50	15.0

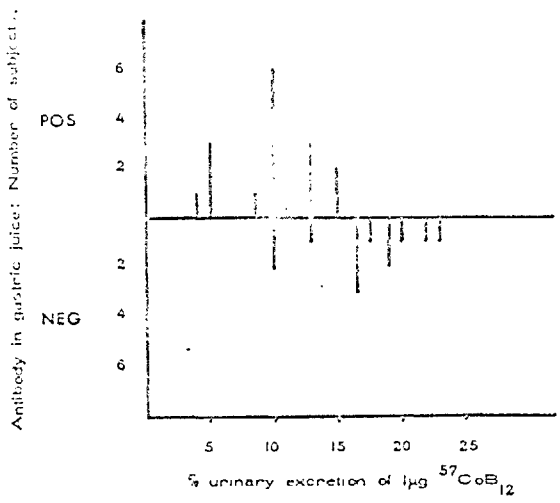


Figure 36.

Correlation of vitamin B_{12} absorption with antibody against intrinsic factor in gastric juice

Table 39

Correlation between results of intrinsic factor mediated vitamin B_{12} absorption tests and gastric juice antibody (* percentage urinary excretion of 1 µg. $^{57}CoB_{12}$ taken by mouth)

Vitamin B_{12} excretion.*	Gastric juice antibody POS	NEG	
>15	0	9	9
<15	17	3	20
	17	12	P = 0.001

Comment:

When this correlation was tested by the chi squared test it was shown to be significant $p = 0.01$.

3. Correlation between antibody in either gastric juice and/or serum and vitamin B_{12} absorption.

Table 40

Intrinsic factor antibody NOT detected		Intrinsic factor antibody PRESENT in serum, gastric juice or both	
Case	Vitamin B_{12} absorption with IF.	Case	Vitamin B_{12} absorption with IF
18	22.0	1	3.8
24	18.6	2	10.3
28	16.2	3	13.4
33	15.8	4	12.8
35	20.3	5	10.4
43	23.0	6	10.7
		8	10.7
		11	10.2
		13	5.4
		15	17.7
		16	11.1
		19	15.8
		21	19.0
		23	10.2
		25	12.6
		39	5.5
		40	15.5
		47	9.0
		48	12.5
		49	8.5
		50	15.0

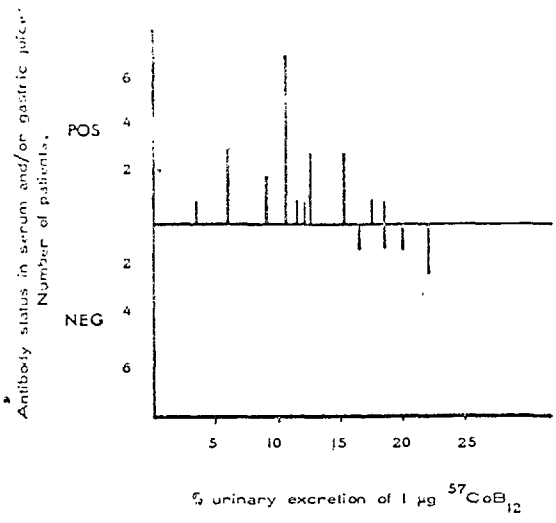


Figure 37.

Correlation of vitamin B_{12} absorption and antibody against intrinsic factor in serum and/or gastric juice

Table 41

Correlation between results of intrinsic factor mediated vitamin B_{12} absorption and antibody status

(* percentage urinary excretion of 1 µg $^{57}CoB_{12}$ taken by mouth)

Vitamin B_{12} excretion.*	ANTIBODY POS	ANTIBODY NEG	
≥ 15	5	5	10
< 15	18	0	18
	23	5	$P < 0.01$

Comment:

The distribution of this pairing was significant. By the chi squared test: P = 0.01

D. CORRELATION BETWEEN AGE OF ONSET OF PERNICIOUS ANAEMIA AND ANTIBODY STATUS.

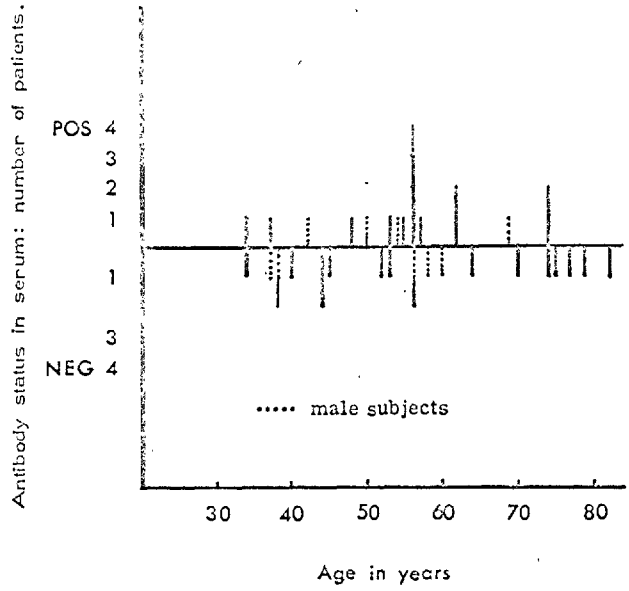


Figure 38.

Antibody status age and sex

Table 42

Correlation between serum antibody status and age

Age in years.	SERUM POS	SERUM NEG	
< 55	8	10	18
> 55	10	11	21
	18	21	P > 0.1

Comment:

Dividing the patients by age into a group younger than 55 years at diagnosis and a second group of 55 years or more, there was no significant difference in the distribution of patients with or without antibodies to intrinsic factor. P = 0.1 by the chi squared test.

E. SUMMARY OF FINDINGS.

A strong correlation was shown between the presence of antibodies against intrinsic factor in the gastric juice and a relative impairment of vitamin B_{12} absorption through the agency of hog intrinsic factor.

Those subjects in whom no antibody was identified in either serum or gastric juice showed the highest levels of vitamin B_{12} absorption when given with hog intrinsic factor.

No significant correlation was shown between serum anitbody and vitamin B_{12} absorption with hog intrinsic factor. Age of onset did not determine the antibody status.

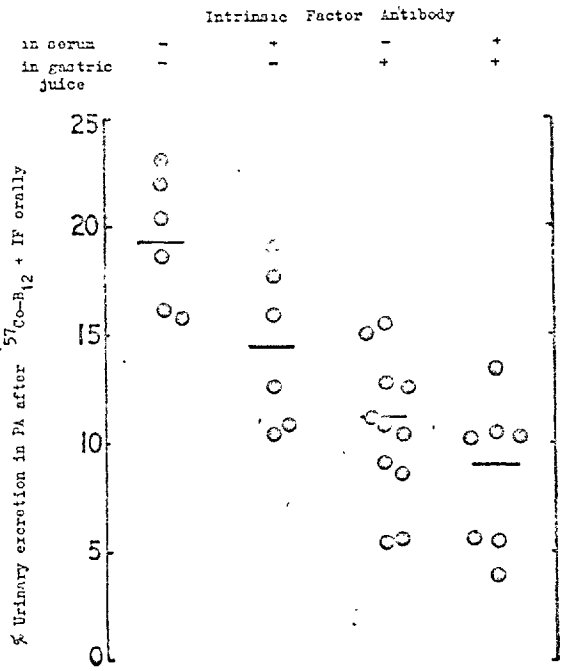


Figure 39

The relationship between antibody status and vitamin B_{12} absorption mediated by hog intrinsic factor.

SECTION 6.

THE SIGNIFICANCE OF INTRINSIC FACTOR ANTIBODIES IN THE ABSENCE OF PERNICIOUS ANAEMIA.

6.1 Patients with auto allergic Thyroid disease.

A. INTRODUCTION.

Antibodies to intrinsic factor in the serum are virtually exclusive to pernicious anaemia and are found in 55% of subjects with this condition. This antibody may however occasionally be found in the serum of patients with unimpaired vitamin B_{12} absorption.

Most of the subjects described in the literature, who have an intrinsic factor antibody circulating in the serum in the absence of pernicious anaemia have had an associated organ-specific, auto-allergic disease.

There is good reason to believe that intrinsic factor antibodies are important in the development of vitamin B_{12} deficiency since the great majority of cases in whom such antibodies can be demonstrated have pernicious anaemia. The antibody has been shown by in vivo studies in man to prevent intrinsic factor mediated vitamin B_{12} absorption (Schwartz 1960) and, furthermore, in pernicious anaemia it is present in the gastro-intestinal tract where it can react with intrinsic factor. On the other hand patients with simple atrophic gastritis do not have intrinsic factor antibodies in the serum (Coghill et al 1965) and the small amount of intrinsic factor produced by the atrophic mucosa is usually sufficient to potentiate vitamin B_{12} absorption (Whiteside et al 1964).

A small number of examples have been recorded of the presence of intrinsic factor antibodies in serum in the absence of pernicious anaemia. Antibodies to intrinsic factor have been detected in the serum of a patient with atrophic gastritis (Jeffries et al 1962) in two relatives of patients with pernicious anaemia (te Velde et al 1964; Sharpstone and James 1965) in patients with thyroid disease (Ardeman et al 1966; Schiller et al 1968) and in diabetes mellitus (Ungar et al 1967; Irvine et al 1969, 1970). Single examples of such an antibody have been documented by Irvine (1965)

te Velde et al (1966) and Wangel and Schiller (1966).

If the development of pernicious anaemia in the majority of patients requires an intrinsic factor antibody then the long term follow up of patients who initially had such an antibody but were still able to absorb vitamin B_{12} normally, is of particular interest. It is important to know whether in time such patients develop pernicious anaemia. If they do not, then how do they differ from patients with this disease? It may be that the relevant antibodies are those secreted into the gastric juice, or that the difference may lie in the degree of cell-mediated immunity which the patient develops to intrinsic factor. Recent findings suggest that the leucocyte migration test reflects this component of the auto-immune response in several diseases (Bendixen et al 1969).

A group of patients with thyroid disease and intrinsic factor antibody in serum were followed for 3 to 7 years. Their vitamin B_{12} status, gastric secretion and auto-antibodies including test for migration inhibition factor were investigated.

B. CLINICAL MATERIAL.

Patients were derived from two sources. In 1964, 249 subjects who were attending a thyroid clinic were investigated for evidence of vitamin B_{12} deficiency and for the presence of antibodies against intrinsic factor. Nine patients had intrinsic factor antibodies and initial observations were reported by Ardeman et al (1966). Another 14 such patients with intrinsic factor antibody in serum were collected at the Middlesex Hospital over a period of years and these patients were studied through the courtesy of Dr. D. Doniach. Follow-up was planned

to determine whether malabsorption of vitamin B_{12} developed by carrying out either urinary excretion (Schilling) tests with an oral dose of 1.0 ug of ^{57}Co-labelled vitamin B_{12}, or measuring the amount of ^{57}Co-labelled vitamin B_{12} retained by whole body counting, one week after a similar oral dose. Initially most patients were seen at 6 monthly intervals and later annually.

C. INVESTIGATIONS.

1. Serum vitamin B_{12} levels were assayed by microbiological assay using L.leichmannii or by saturation analysis and a full blood count was usually carried out at each visit.

2. Gastric secretion was studied initially in 6 patients with intrinsic factor antibody in serum but without pernicious anaemia and finally in all but one patient using an augmented dose of histamine and more recently pentagastrin (6 ug per kg) as the stimulant to gastric secretion. Gastric juice was examined for the presence of acid, intrinsic factor (Ardeman and Chanarin 1963) and intrinsic factor antibodies by the method described in section 3.2B.

3. Antibodies against intrinsic factor in serum were titrated by the method of Ardeman and Chanarin (1963), and against the gastric parietal cells by an immunofluorescent method. Thyroid antibodies were estimated using methods described by Roitt and Doniach (1969).

D. CLINICAL FINDINGS.

Of the 23 patients with thyroid disease studied, 12 were found to have early pernicious anaemia (Group 1) and 11 absorbed vitamin B_{12} normally (Group 2). All but three of the patients were women and all had normal haemoglobin levels. The patients with early pernicious anaemia had a macrocytic peripheral blood film, the marrow when examined showed megaloblastic haemopoiesis, the serum vitamin B_{12} level was

borderline or low, there was a histamine fast achlorhydria in all 6 cases examined and all had malabsorption of vitamin B_{12}. Seven patients in whom the absorption test was repeated with added intrinsic factor showed an enhanced vitamin B_{12} retention. All had intrinsic factor antibodies in serum. The patients with pernicious anaemia are not considered further in this section but were considered in section 5. The patients in Group 2 were seen at more or less regular intervals, the duration of follow-up being 6 to 7 years in 6, 5 years and in 2 and 3 years in 3.

Table 43

FOLLOW-UP	
YEARS	CASES
7	1
6	5
5	2
4	
3	3
2	

There were interesting differences between these groups of patients in the nature of the thyroid disease and in the family history (table 44). Six of the 7 patients with Hashimoto's thyroiditis or primary myxoedema had pernicious anaemia. By contrast only 6 out of 16 patients with thyrotoxicosis had pernicious anaemia the other 10 having serum intrinsic factor antibodies with a non-progressive atrophic gastritis. A family history of other auto-immune disorders was noted in only 4 out of the 12 pernicious anaemia patients but was present in 9 out of the 11 patients in Group 2.

Table 44

COMPARISON OF TWO GROUPS OF PATIENTS WITH THYROID DISEASE AND SERUM ANTIBODIES TO INTRINSIC FACTOR

	DIAGNOSIS	
	1. EARLY PA	2. ATROPHIC GASTRITIS
NUMBER OF PATIENTS	12	11
SEX RATIO	1M/11F	2M/9F
MEAN AGE (YEARS)	56	54
ASSOCIATED DISEASES		
Thyrotoxicosis	6	10
Hashimoto & primary myxoedema	6	1
Addison's disease	0	1
Myasthenia gravis	1	0
Rheumatoid arthritis	1	0
Diabetes mellitus	1	0
Vitiligo or early greying	0	4
FAMILLY HISTORY OF AUTOIMMUNE DISEASE		
Thyroid disease	4	7
Pernicious anaemia	1	3
Addison's disease	0	1
Rheumatoid arthritis	0	1
Vitiligo or early greying	0	2

E. INITIAL LABORATORY FINDINGS.

All subjects in group 2 had a normal haemoglobin level and the appearance of the stained blood film was normal. The serum vitamin B_{12} level was normal in all 7 patients in whom this was carried out initially. Six had gastric juice aspirated. Five showed the presence of acid and one (case 1) had a histamine-fast achlorhydria with a low intrinsic factor output. Nevertheless she absorbed vitamin B_{12} normally, as did all the other patients tested and her serum vitamin B_{12} level was normal. In addition all the patients had antibody in serum against human intrinsic factor and against gastric parietal cells, and 9 out of 10 patients tested had thyroid antibodies. The titres of gastric parietal cell fluorescence were unusually high in 6 of the 8 patients where serial dilutions had been made but thyroid antibodies were present in low concentrations as seen in Graves' disease except for two patients with complement fixation titres of 32 and 128 respectively.

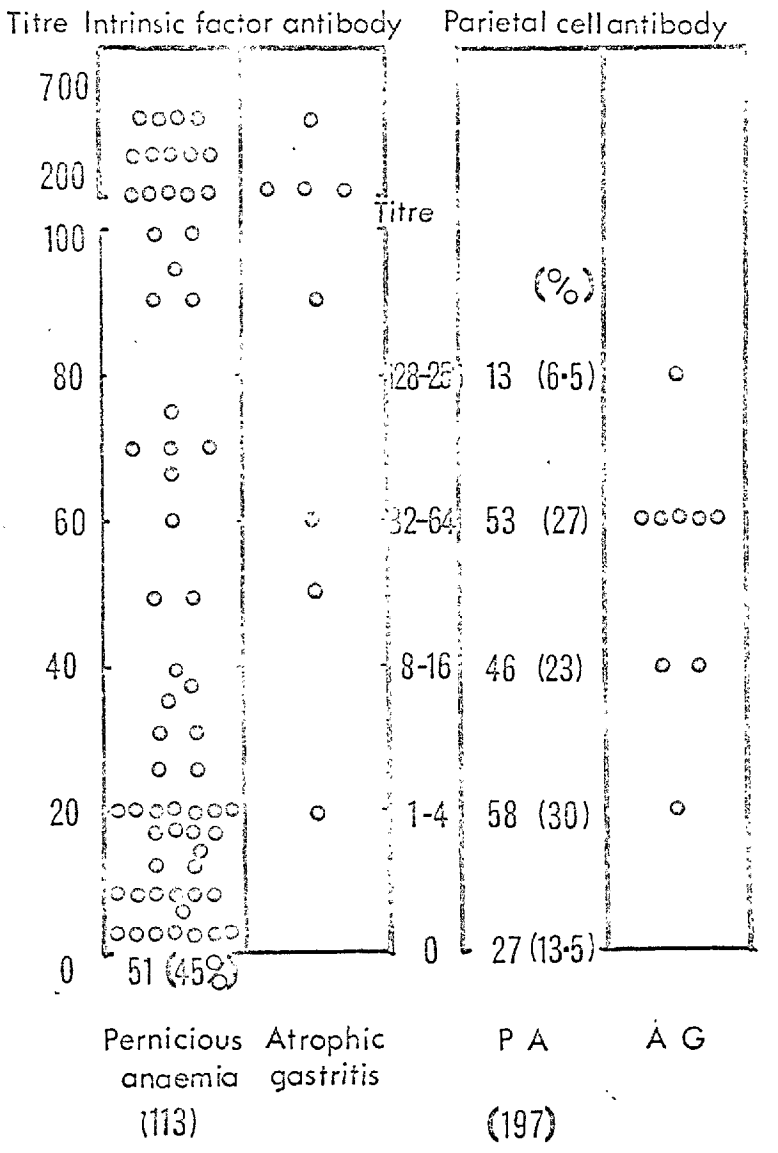


Figure 40

Relative titres of antibodies in subjects with pernicious anaemia and "non-progressive gastritis". (By courtesy of Dr. D. Doniach).

F. LABORATORY FINDINGS AFTER FOLLOW-UP.

Re-examination after 3 to 7 years showed the blood picture to be unchanged. The serum vitamin B_{12} concentration remained normal although this could have been influenced by the injection of 1000 ug of vitamin B_{12} given to some of the patients once or twice yearly in the performance of Schilling tests. The absorption of vitamin B_{12} was unchanged in 7 patients who had regular tests and was also normal in the remaining 4 who were only tested at the end of this period although they were known to have had intrinsic factor antibodies in serum for 2 to 6 years. In particular, case 1 still absorbed vitamin B_{12} normally although 6 years previously she already had a histamine-fast achlorhydria, severe atrophic gastritis on biopsy and residual amounts of intrinsic factor that were not significantly greater than those encountered in some patients with pernicious anaemia. Nevertheless the Schilling test excretion increased from 12.4 to 19.4% on addition of intrinsic factor.

In three of the 11 patients intrinsic factor antibodies disappeared from the plasma (Appendix III). In case 1 the intrinsic factor antibody titre was between 60 to 80 units during 1965 and 1966 but thereafter declined and was no longer detectable after 1968. Parietal cell antibodies persisted in all 11 patients without significant changes in titre. Thyroid antibodies were still present in 8 patients and case 3 who also had Addison's disease and a premature menopause had both adrenal and ovarian antibodies detected by immuno-fluorescence as well.

Intrinsic factor antibodies in gastric juice were looked for in ten patients (one refused) by the method described in Section 3.2B. This was absent from 9 samples but present in significant titre in case 7,

The gastric juice in this patient in 2 studies had a pH of 2.0 after pentagastrin and an intrinsic factor concentration of 22 units per ml. Only 10 ml of juice was aspirated and hence the estimate for total secretion over an hour may be unreliable. Following dissociation of antibody from intrinsic factor, the gastric juice was found to have an antibody titre of 7 units per ml. This antibody was largely neutralised by the addition of anti-IgA immunoglobulin serum and only to a small extent by the additon of an anti-IgG serum.

G. LEUCOCYTE MIGRATION TEST. (By courtesy of Dr. J. Brostoff).

This was carried out in 10 of the patients in Group 2 and in 2 additional cases of simple atrophic gastritis in thyroid patients without serum intrinsic factor antibody (see Appendix IV). For comparison 10 cases of known pernicious anaemia were tested as well as 8 patients with early pernicious anaemia associated with thyroid disease (group 1). Eleven laboratory staff were included as normal controls. The results are shown :

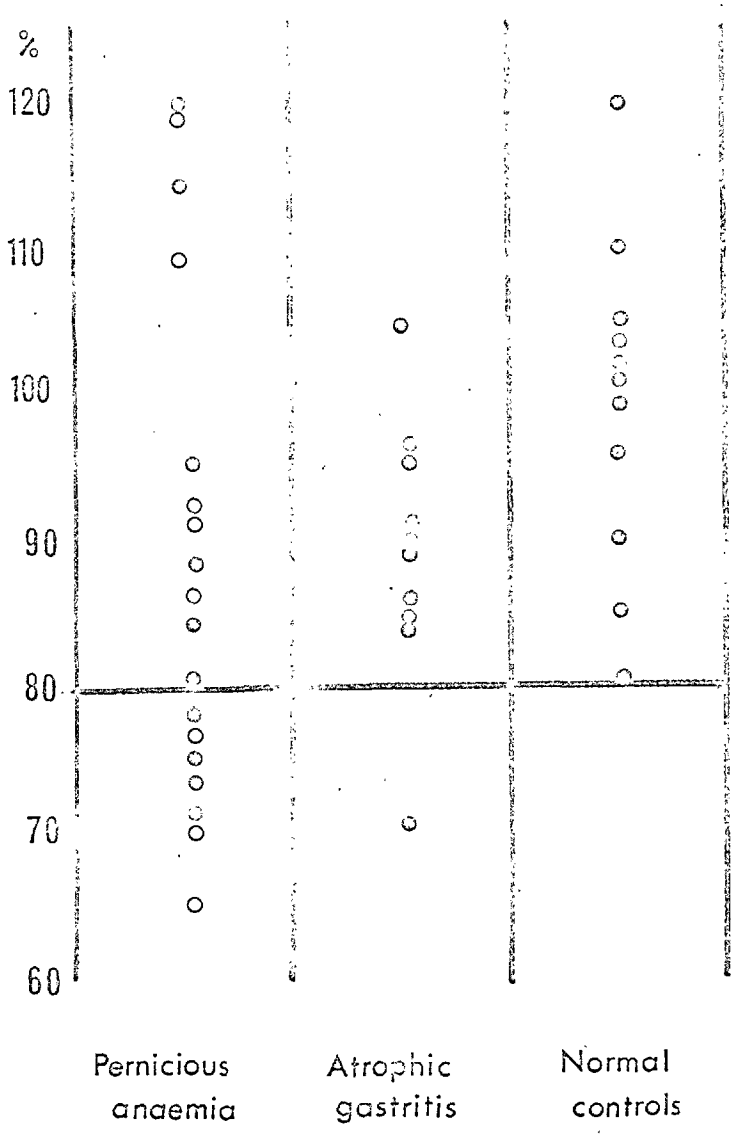


Figure 41

Results of leucocyte migration test in the presence of gastric intrinsic factor.
(By courtesy of Dr. Jonathan Brostoff).

The test was considered positive when cultures containing intrinsic factor showed a migration area of less than 80% that of control chambers. Seven of the 18 patients with early or overt pernicious anaemia showed an inhibition of leucocyte migration in the presence of intrinsic factor while only 1 of 11 cases in Group 2 gave a positive test. In addition, one patient with Hashimoto's disease, normal gastric function parietal cell antibodies but without intrinsic factor antibodies in the serum gave a positive result (migration 79%). The other positive case (migration 70%) was the only patient in Group 2 who had intrinsic factor antibodies in both blood and gastric juice and who showed the most striking familial incidence of thyrotoxicosis, pernicious anaemia and early greying of the hair.

The individual differences are illustrated in the following two brief summaries:

a) One patient, a sixty-one year old lady with Grave's disease, had histamine and pentagastrin-fast achlorhydria. She had a low intrinsic factor concentration and the output of intrinsic factor during the thirty minutes after stimulation was within the range of pernicious anaemia.

The findings in her gastric juice did not change appreciably over the six years of follow-up. Her vitamin B_{12} absorption, measured by urinary excretion of a 1 ugm dose of ^{57}Cobalt vitamin B_{12} was normal and unchanged (1964 : 12.4%; 1970 : 13.0%). When first seen, the patient had an antibody against intrinsic factor in her serum at a titre of 60 units/ml; However, on three separate occasions since 1968 this antibody has been undetectable. The gastric juice of this patient did not contain any antibody to intrinsic factor and correspondingly the impaired output of intrinsic factor has remained adequate to subserve normal vitamin B_{12} absorption.

b) The second patient of interest was a fifty-six year old lady with Graves' disease. She had a strong family history of pernicious anaemia and thyroid disorder. Antibody to intrinsic factor was found in both her serum and in her gastric secretion after measures had been taken to release the antibody from combination with intrinsic factor. This inhibitor was identified as an immunoglobulin of class IgA, by antiserum inhibition (see section 5.1C4).

In this patient sufficient excess of free intrinsic factor was still produced, despite the presence of antibody, to mediate normal vitamin B_{12} absorption.

Also, the test for leucocyte "migration inhibition factor" was positive in this one subject and negative in the remaining ten.

H. COMMENT.

In nine of the ten subjects in this group, auto-allergic insult to the thyroid gland was associated with hyperthyroidism.

Although eight of these ten patients showed a strong predisposition to pernicious anaemia, with striking family histories and serological overlap, none of them developed pernicious anaemia during the period of follow-up, nor have they shown a deteriorating vitamin B_{12} absorption.

Whilst the absence of antibody from the gastric juice may be of significance, as may be also the negative results obtained for inhibition of leucocyte migration in the presence of intrinsic factor, the findings in case b) show that pernicious anaemia will not occur when the gastric output of intrinsic factor is in excess of locally synthesised antibody. Furthermore, where acid secretion persists and the pH remained low antibody and intrinsic factor will be free and do not react. Hence the intrinsic factor is still available to take up vitamin B_{12} and is still able to mediate vitamin B_{12} absorption.

The leucocytes in the gastric submucosa may behave differently to leucocytes in the peripheral blood, since the circulating leucocytes in about 50% of pernicious anaemia cases show no inhibition of migration whereas case b) showed inhibition but did not have pernicious anaemia.

It is clear that no single factor so far identified can determine whether pernicious anaemia will develop in an inherently predisposed subject.

6.2. Miscellaneous Hospital Subjects.

Gastric juice was tested from nine subjects with a variety of gastro-intestinal disorders and from two patients with megaloblastic haemopoiesis in whom no disorder of gastro-intestinal function was identified. Antibody to intrinsic factor was not demonstrated in the serum of any of these subjects. Samples from patients following gastrectomy were not studied as it was found that bile stained samples caused impairment of vitamin B_{12} binding which could not be inhibited by antisera.

No inhibition of vitamin B_{12} binding by normal gastric juice was caused by these gastric juice samples.

SECTION 7.

DISCUSSION AND CONCLUSIONS.

7.1 Release and detection of antibody against intrinsic factor in the gastric juice.

A. INTRODUCTION.

The method employed for the release and detection of type I antibody against intrinsic factor was described in detail in section 3.2B.

The procedure for release was undertaken in order to detect antibody in samples where intrinsic factor and antibody were present in relative quantities such as to evade detection by more straightforward means.

The following factors were of importance in the release of antibody from intrinsic factor.

1) Type 1 antibody and vitamin B_{12} are mutually exclusive vis a vis their binding to intrinsic factor.

2) At neutral pH, room temperature and isotonic solution, intrinsic factor will form a durable complex with either vitamin B_{12} or with type 1 antibody, depending on which is first introduced. This phenomenon is of fundamental importance in the immunological techniques for assay of intrinsic factor.

3) Vitamin B_{12} binds strongly and rapidly with intrinsic factor over a wide pH range.

4) Type 1 antibody combines less rapidly and less strongly with intrinsic factor as the pH falls.

5) With a fall in pH there is a shift in the equilibrium of the reaction:

$$IF{:}Ab \rightleftharpoons IF + Ab$$

to the right. Correspondingly the combination between intrinsic factor and vitamin B_{12} continues unimpaired until most of the antibody is released.

6) When the pH is returned the newly released antibody is excluded from its original combining site and can be identified by testing against a fresh source of intrinsic factor.

B. ALTERNATIVE METHODS.

Fisher et al (1966) first identified type 1 antibody in the gastric juice against intrinsic factor by an indirect method in five out of fourteen subjects with pernicious anaemia (36%). They considered antibody to be present when the vitamin B_{12} bound by a mixture of normal gastric juice and test gastric juice was less than the total of that bound to each component sample. The figure of 36% positive results corresponds to the incidence of free antibody in the gastric juice identified in the present study (30%). However, Fisher et al (1966) did not consider concealed antibody.

The only other method so far employed to release and identify precomplexed antibody is acidic gel filtration. Goldberg and Bluestone (1970), testing the concept introduced by Rose and Chanarin (1969), were able to identify free antibody in six samples, and antibody after release in a further four out of a total of twelve samples from pernicious anaemia patients. Of the two subjects with no demonstrable antibody in the gastric juice, one had agammaglobulinaemia. They also found no correlation between their findings in the serum and in the gastric juice.

7.2 Intrinsic factor antibodies in the gastrointestinal tract.

A. TYPE OF ANTIBODY.

The investigations of Fisher et al (1966) identified antibody of type 1 in five out of fourteen subjects with pernicious anaemia. No tests were performed to show type 2 antibody. However, Schade et al (1966) demonstrated type 2 antibody in the gastric juice of one patient,

whereas no tests were performed for type 1.

In a further study Baur et al (1968) identified type 2 antibody coating submucosal immunocytes in the gastric biopsies of two out of twelve subjects with pernicious anaemia. Again, no search was made for type 1 antibody.

The only correlation of the incidences of type 1 and type 2 antibody in the gastric juice has been made by Goldberg and Bluestone (1970). Of their twelve cases, ten had type 1 antibodies and one of those ten also had type 2 antibody in the gastric juice.

It is worth mentioning that whilst type 1 antibody may be more easily identified in the gastric juice, it is less likely to be of importance in obstructing intrinsic factor function than type 2 antibody, since it cannot interfere with absorption of the complex of intrinsic factor. Type 2 antibody may interfere with intrinsic factor in the intestine, down to the distal ileum, whereas type 1 antibody can only unite with intrinsic factor in the absence of vitamin B_{12}.

B. SITE.

Carmel and Herbert (1967) have described intrinsic factor antibody in the saliva of a single case with pernicious anaemia. The identification of antibody in the gastric juice has been described previously. Baur et al (1968) using autoradiographic methods, showed localisation of cell bound antibody in the submucosa of two gastric biopsies from pernicious anaemia subjects.

C. CLASS OF ANTIBODY.

The results of Fisher et al (1966) give no information concerning the immunoglobulin class of the intrinsic factor antibodies which they

identified in the gastric juice. Goldberg and Bluestone (1970) found that the gastric juice antibodies against intrinsic factor, were mainly IgG, whereas out of eight samples in the present study, which were investigated for immunoglobulin class, in five at least, a component of the antibody activity could be inhibited by an IgA antiserum.

D. INCIDENCE.

Fisher et al (1966) identified free antibody in the gastric juice of five out of fourteen subjects with pernicious anaemia (36%). This corresponds to the figure obtained for free antibody in this study. Goldberg and Bluestone (1970) found free antibody in six of their twelve subjects (50%) and in a further four (33%) after release from intrinsic factor. Thus in their study 40% of the subjects with antibody against intrinsic factor were identified only after release of the pre-complexed molecule, whereas in the present study 45% of the positive results were obtained only after release.

In the present study antibody was not identified in twenty-four out of fifty-three samples of gastric juice from subjects with pernicious anaemia. The following suggestions may explain this failure:

a) Type 1 antibody was absent from the gastric secretion. Possibly such antibody is principally located in the gastric mucosa and submucosa. Associated and as yet undetermined factors may be responsible for its presence in the gastric secretion. Baur et al (1968) have shown localisation of antibody on the surface of mononuclear cells in two subjects with pernicious anaemia. They did not comment on the gastric secretion of these subjects, but both had antibody in the serum.

b) Possibly the appearance and disappearance of antibodies against intrinsic factor in the serum and gastric juice represent phases in the pathogenesis of pernicious anaemia. The persistence of intrinsic factor

may be required to provoke production and assist transport of the antibody from its site of synthesis into the gastric secretion.

c) Antibody is principally synthesised further down the small bowel, where submucosal lymphoid tissue is more abundant.

d) Type 2 antibody is synthesised preferentially in the gastro-intestinal tract (Carmel and Herbert 1966) and unlike the serum counterpart, it is produced independently of type 1 antibody. Carmel and Herbert's (1966) findings were presented in an abstract which has not since received elaboration in a detailed paper. Their observations cannot correspondingly be commented upon further.

e) The method employed was insufficiently sensitive. Goldberg and Bluestone (1970) using a similar method reported a higher incidence of positive results. Their findings lend support to the evidence submitted in this thesis, that intrinsic factor and antibody frequently coexist in the gastric secretion of patients with pernicious anaemia. Their method is possibly more sensitive. However their criteria for claiming presence of antibody were less stringent than those employed in the present study. A further shortcoming is that their method is time consuming and laborious, requiring about one week for the processing of a single gastric juice sample.

7.3 Intrinsic factor antibodies in the serum and gastric juice. Relative importance and significance in pathogenesis of pernicious anaemia.

A. INTRODUCTION.

The amount of vitamin B_{12} that is absorbed in patients with gastritis depends upon the amount of intrinsic factor that is available. This in turn is related to the number of remaining parietal cells and to the presence of factors that interfere with action of intrinsic factor. Under particular circumstances these factors can be an

abnormal bacterial flora in the small gut, fish tape-worm, impaired capacity of ileal cells for vitamin B_{12} absorption and antibody against intrinsic factor. In the early phases of pernicious anaemia the latter factor is of particular importance.

B. ANTIBODY IN THE SERUM.

There is strong evidence to suggest that antibody against intrinsic factor in the serum is not actively responsible for the pathogenesis of pernicious anaemia.

Half of the patients with Addisonian pernicious anaemia do not have intrinsic factor antibody in their serum. Ungar et al (1967) found a significant correlation between the duration of the disease and the incidence of antibody, suggesting that given time, all patients with pernicious anaemia would develop detectable circulating antibody against intrinsic factor. Dige-Petersen et al (1967) described a correlation between the gastric submucosal lymphocytic infiltration seen on histology of biopsy specimens and the incidence of antibody.

Fisher et al (1967) observed a more profound impairment of vitamin B_{12} absorption by the Schilling test in subjects with serum antibodies than those without antibodies against intrinsic factor. However most of their patients without antibodies against intrinsic factor did not have bona fide pernicious anaemia but chronic atrophic gastritis. Thus their claims are not substantiated.

Most workers have however been unable to identify differences between patients with and without intrinsic factor antibodies in the serum. There are no records to date of patients with established pernicious anaemia in whom intrinsic factor antibody has appeared in

the serum after initially being declared negative, nor is there a record of a patient with such an antibody later losing it. There are however cases in which considerable spontaneous fluctuations of antibody titre occur (Ungar et al 1967) and circumstances where such fluctuations may be induced (Taylor, 1959; Ardeman and Chanarin, 1965/6). The only documented cases in which there has been a spontaneous alteration of intrinsic factor antibody status are included in this thesis (section 6.1F). In these three subjects an antibody was initially identified which disappeared during the period of follow-up. None of these patients however had pernicious anaemia.

One finding of note was that the patients who did not have pernicious anaemia but who had intrinsic factor antibodies in their serum tended to have higher titres both of this antibody, parietal cell antibodies and thyroid antibodies than did bona fide pernicious anaemia subjects.

The evidence for the effects of antibody in neonates is equivocal. Four cases have been described in which mothers with pernicious anaemia, who had circulating IgG antibodies against intrinsic factor have given birth to infants in whom this antibody was initially detected in the cord blood and in whom the antibody titre subsequently declined. In two of the neonates, described by Bar Shany and Herbert (1966) and by Goldberg et al (1967) there was total failure of intrinsic factor secretion which returned to normal as the titre of serum antibody fell. In the two other cases the intrinsic factor secretion of the infants was normal (Charache and McIntyre 1966; Fisher and Taylor 1967).

Finally it is important to emphasise that of those patients without pernicious anaemia, who had serum antibodies to intrinsic factor, none have developed pernicious anaemia. They have been studied over a period from 3-7 years. Thus where intrinsic factor is being produced, it remains inaccessible to inhibition by antibody in the serum.

C. INTRINSIC FACTOR IN THE GASTRIC JUICE.

About thirty per cent of subjects with pernicious anaemia have detectable intrinsic factor in their gastric secretion. This is however inadequate to mediate vitamin B_{12} absorption. But there are subjects with uncomplicated atrophic gastritis in whom the intrinsic factor output is of a similar low level, who continue to absorb vitamin B_{12} normally (Adams et al 1964).

Croft et al (1966) provide evidence that the gastric mucosa in atrophic gastritis is actively proliferating. A balance is struck between proliferation and destruction. The coexistence of intrinsic factor and antibody may be an expression of these conflicting processes.

If antibody in the serum itself participates in the impairment of intrinsic factor function then intrinsic factor would need to pass across the mucosal surface into the portal circulation. There is no evidence that it does so. Cooper and White (1968) using an immunological technique, and Yammaguchi et al (1970) who labelled intrinsic factor with ^{51}Chromium were unable to show that intrinsic factor entered the portal circulation.

Thus intrinsic factor performs its function within the gastro-intestinal tract and if an antibody interferes with this function it must also be localised there.

D. ANTIBODY IN THE GASTRIC JUICE.

The relative paucity of published studies upon antibody to intrinsic factor in the gastric juice of patients with pernicious anaemia gives the impression that this is a rare finding. However the findings presented in this thesis are evidence that this is the rule rather than the exception in pernicious anaemia.

Localisation of antibody in the gastrointestinal tract may be a crucial factor in the pathogenesis of Addisonian pernicious anaemia in some subjects. Nevertheless it is evidently not the sine qua non for this condition to develop. Furthermore the antibody may be detected in gastric juice in a patient without pernicious anaemia, as seen in section 6.1F. Gastrointestinal antibody plays a crucial note only when the intrinsic factor output has fallen below a critical level. In the absence of this antibody, these levels of intrinsic factor output may remain adequate. Where severe gastric atrophy or atrophic gastritis coexist with intrinsic factor antibody within the gastro-intestinal tract, the stage is set for pernicious anaemia. These may be independently determined factors in the pathogenesis of the disease. Yates and Cooper (1968) using an indirect method were unable to show evidence in seven patients with pernicious anaemia that antibody in the gastrointestinal tract was interfering with the function of intrinsic factor.

However, the correlation between intrinsic factor mediated vitamin B_{12} absorption in patients with and without antibodies in the gastric juice, described in section 5.2, was highly significant. This lends support to the belief that antibody in the gastrointestinal tract does indeed interfere with intrinsic factor.

E. LEUCOCYTE "MIGRATION INHIBITION FACTOR".

If the leucocyte migration test which detects the production of "migration inhibition factor" by sensitised lymphoid cells reflects cellular immunity to intrinsic factor, this was present in about 40% of tested patients with overt or latent pernicious anaemia but was only positive in a single patient belonging to group 2 (see section 6.1). This same patient also showed intrinsic factor antibodies in her gastric juice. This test is still being evaluated and possibly results could be improved by using different quantities of antigen or by setting up some chambers with gastric microsomal antigen. These preliminary results however suggest a difference in the degree of cellular hypersensitivity between patients with pernicious anaemia and those with a gastritis in which greater numbers of functioning parietal cells have persisted.

7.4 Conclusions.

Clinical and serological findings in the families of subjects with pernicious anaemia provide evidence that the predisposition to form organ-specific auto-antibodies is inherited. However, this is only one component in the pathogenesis of pernicious anaemia. Further, as yet unknownfactors determine the order of gastric atrophy and rate of gastric mucosal cell turnover.

Organ specific auto-antibodies in the serum in high titre may accompany only mild atrophy of the organ concerned. They are thus not responsible for the severity of the process.

Possibly the predisposition to forming circulating antibodies against gastric antigens may be intimately associated with susceptibility to environmental agents responsible for gastric atrophy.

It is proposed that the following factors are responsible for pernicious anaemia:

1) an inherited predisposition to form organ specific auto-antibodies.
2) the gastro-intestinal localisation of cells producing antibodies against intrinsic factor.
3) possibly the persistence of a residual intrinsic factor output as an antigenic stimulus to antibody synthesis.
4) susceptibility to causes of gastric atrophy or atrophic gastritis.
5) impaired parietal cell regeneration possibly related to parietal cell antibody to cell bound antibody.
6) an excess of antibody produced in the upper gastro-intestinal tract, over and above the residual intrinsic factor output.

Appendix I

MATERIALS AND METHODS (GENERAL).

A. MATERIALS.

1. Glassware. All glassware was washed in tapwater, left in 5% chromic acid for 3-6 hours, thoroughly rinsed in distilled water and dried in an oven at 110°C.

2. Storage of materials. All fluid materials including solutions of B_{12}, gastric juice, plasma and serum were stored in aliquots at -20°C.

3. Hog intrinsic factor concentrate. This was obtained in powder form. (Lederle Laboratories. Code WES 818). A dose of 20mg was used in the augmented Schilling test.

4. Citrate-phosphate buffer. This mixture was made up according to McIlvaine (1921). Buffer solutions ranging from pH 2.2 - 8.0 were obtained by adding varying amounts of 0.2M disodium phosphate to 0.1M citric acid.

5. pH meter. A direct reading glass electrode pH meter (Electronic Instruments Ltd., model 23A) was used for measuring the hydrogen ion concentration of gastric juice samples.

B. METHODS.

1. Intrinsic factor assay (Ardeman and Chanarin 1963). Six ml. saline and 0.5 ml. of the gastric juice being tested were dispensed into two glass bottles. 1 ml. of pooled normal serum was added to one tube and 1 ml. of a serum containing antibody to intrinsic factor was added to the second tube. Serum controls were also prepared. The mixtures were shaken and left at room temperature for ten minutes. Thereafter ^{57}Cobalt-vitamin B_{12} was added to each tube (100 ug in 0.5 ml). These mixtures were shaken and left at room temperature for twenty minutes. The free vitamin B_{12} was removed by the addition of charcoal and the residual radioactivity in the supernatant was measured. The results

were expressed as ng units of vitamin B_{12} binders.

The values for the corresponding serum controls were subtracted.

2. Detection of antibody to gastric intrinsic factor in the serum (Ardeman and Chanarin 1963). The method was similar to that described above. However the gastric juice was taken from pooled normal samples with a known intrinsic factor concentration. In addition to tubes containing normal serum and plasma containing antibody, a further tube included gastric juice (0.5ml) saline (6.0ml) and serum from the test subject (1.0ml). Antibody was considered present when the vitamin B_{12} binding of the normal gastric juice was significantly blocked by the addition of the test serum.

3. Vitamin B_{12} absorption.

i. Urinary excretion tests (Schilling 1953). Absorption of vitamin B_{12} was measured by its excretion in the urine during the twenty-four hours following the oral dose.

Each dose of 1.1 ug of ^{57}Cobalt vitamin B_{12} was made up in 25 ml. of distilled water. The "urine" standard was prepared by adding 0.2ml. of this dilution to 20ml. of distilled water in a volumetric flask. A further 0.2ml. was taken from the original dilution and added to 4.8ml. of distilled water in a universal bottle and used as a "plasma" standard (see below). These dilutions were always prepared within twenty-four hours of performing the test.

After an overnight fast the patient voided urine. 1mg. of non-radioactive hydroxycobalamin was given by intramuscular injection. Immediately thereafter the radioactive dose of vitamin B_{12} was taken by mouth.

Urine was collected into darkened glass winchester bottles without preservative, for twenty-four hours from the time of taking the oral dose.

The results were expressed as a percentage of the total radioactivity in the oral dose which appeared in the urine during the twenty-four hours of collection.

ii. Plasma level of radioactive vitamin B_{12}. As an ancillary measure 10 ml. of blood was taken into lithium heparin between 8-10 hours after administration of the oral dose. The radioactivity in the plasma was also expressed as a percentage of the radioactivity in the oral dose and calculated by the formula :

$$\frac{\text{plasma counts} \times 8}{\text{plasma standard counts} \times 5} \times 100 = \%$$

This was done primarily to detect errors in the urinary excretion method due to urine loss or omission of intramuscular vitamin B_{12}.

iii. Method using whole body counting. Those patients studied at Middlesex Hospital, whose details are included in section 6, were investigated by a whole body counting methods as indicated in the text. The radioactivity remaining in the patient. One week after taking an oral dose of ^{58}Cobalt vitamin B_{12} was taken as a measure of absorption.

4. Vitamin B_{12} levels in the serum were measured by microbiological assay using Lactobacillus leishmanii.

Saturation analysis was employed to measure serum B_{12} levels in patients at Middlesex Hospital included in section 6.

5. Thyroid antibodies were demonstrated by tanned red cell agglutination (Middlesex Hospital).

6. Gastric parietal cell antibodies were identified by an immunofluorescent method and by complement fixation.

7. Haemoglobin was estimated in the blood by the cyanmethaemoglobin method.

APPENDIX II.

PATIENTS STUDIED WITH PERNICIOUS ANAEMIA.

Case	Sex	Age	Serum Ab	Gastric juice Ab	B_{12} absorption		Units IF in gastric juice
					B_{12} alone	B_{12} with Intrinsic factor	
1	M	42	Pos	Pos	0	3.8	0
2	F	48	Pos	Neg	1.9	10.3	0
3	F	62	Pos	Pos	2.4	13.4	4
4	F		Neg	Pos	0.2	12.8	0
5	M	68	Pos	Pos	0.8	10.4	0
6	F	57	Pos	Neg	1.7	10.7	0
7	F	69	Neg	Neg	3.6	---(M.H.)	1
8	F	43	Neg	Pos	2.6	10.7	0
9	F	34	Pos	Pos	---	---(E.G.H.)	6
10	M	50	Pos	Neg	---	---(Dementia)	0
11	M	56	Neg	Pos	1.4	10.4	4
12	F	34	Neg	Neg	---	---	1
13	M	54	Pos	Pos	0	5.4	0
14	F	56	Pos	Pos	---	---	0
15	F	74	Pos	Neg	1.2	17.7	0
16	F	79	Neg	Pos	1.2	11.1	2
17	M		Pos	Pos	5.4	---	0
18	F	74	Neg	Neg	2.0	22.0	0
19	F	56	Pos	Neg	4.3	15.8	7
20	M		Pos	Pos	2.2	---	0
21	F	[illegible]	Pos	Neg	7.2	19.0	0
22	M		Neg	Neg	---	---	0
23	F	56	Pos	Pos	2.7	10.2	0
24	F	75	Neg	Neg	1.2	18.6	0
25	F	74	Pos	Neg	---	12.6	7
26	M	37	Neg	Neg	---	---	0
27	F	56	Pos	Pos	0.5	10.2	0

Case	Sex	Age	Serum Ab	Gastric juice Ab	B_{12} absorption B_{12} alone	B_{12} with Intrinsic factor	Units IF in gastric juice
28	M	58	Neg	Neg	0	16.2	0
29	F	70	---	Neg	---	---(P.G.H.)	0
30	F	54	Neg	Pos	1.8	---(E.G.H.)	0
31	F	86	Pos	Neg	---	---(E.G.H.)	1
32	M	60	---	Pos	---	---(P.G.H.)	0
33	F	82	Neg	Neg	1.8	15.8	0
34	M		Neg	Neg	---	---(P.G.M.S.)	0
35	F	38	Neg	Neg	8.1	20.3	0
36	F	78	Neg	Pos	0.3	5.4	0
37	F	62	Neg	Pos	6.0	---(M.H.)	0
38	F	40	Neg	Neg	---	---(W.M.H.)	7
39	M	56	Pos	Pos	2.0	5.5	0
40	F	55	Neg	Pos	0.5	15.5	6
41	F	45	Neg	Pos	---	---(st.Barts)	0
42	---	---	Neg	Pos	---	---(St.Stevens)	0
43	M	38	Neg	Neg	0.3	23.0	0
44	F	62	Pos	Pos	8.8 (wbc)	---(M.H.)	0
45	F	55	Pos	Pos	6.9	---(M.H.)	0
46	F	53	Pos	Neg	7.6 (wbc)	---(M.H.)	0
47	F	60	Neg	Pos	3.5	9.0	2
48	F	43	Neg	Pos	0.5	12.5	2
49	F	64	Neg	Pos	0	8.5	3
50	M	72	Neg	Pos	2.8	15.0	0
51	F	39	Pos	Pos	14 (wbc)	---(M.H.)	0
52	M	14	Pos	Neg	---	---(M.H.)	0
53	F	52	Neg	Pos	---	---(Cardiff)	0

APPENDIX III

PATIENTS WITH AUTOALLERGIC THYROID DISEASE

AND INTRINSIC FACTOR ANTIBODIES IN THE SERUM.

TABLE 45

Autoimmune disease and serum antibody titres in patients with thyroid disease and intrinsic factor antibodies followed 3 to 7 years (Group 2).

CASE	OTHER AUTOIMMUNE DISEASE	% leucocyte migration with gastric juice (normal 80-120)	SERUM ANTIBODIES TO *									
			INTRINSIC FACTOR		PARIETAL CELL Fluorescence Titre		THYROID					
							TRC	CYTO	CF	TRC	CYTO	CF
			1	2	1	2		1			2	
1	Sister, aunt thyrotoxic	91	60	-	+	8	..	..	..	-	-	-
2	Nil	83	75	115	64	128	-	-	-	-	-	-
3	Post-op myxoedema, Addison's disease, infertility with adrenal/ ovarian antibodies; Aunt thyrotoxic	89	10	130	512	256	-	+	-	-	++	16
4	post-op myxoedema	85	250	100	64	64	20	++	-	-	++	16
5	post-I^{131} myxoedema; mother goitre	104	50	90	32	64	-	++	4	-	-	-
6	post-I^{131} myxoedema; mother pernicious anaemia; aunt thyrotoxic	83	700	120	64	64	20	++	128	40	++	32
7	post-I^{131} myxoedema; rheumatic heart disease, smooth tongue - 5 years, mother goitre; father, cousin pernicious anaemia; sister thyrotoxic with rheumatoid arthritis; aunt thyrotoxic; early greying (3 relatives)	70	+	50	8	1	80	++	-	320	++	-
8	Mother goitre	..	+	+	+	64	40	++	-	10	++	-
9	Grey hair at 22 years; sister thyrotoxic	95	+	20	+	128	10	++	-	20	++	-
10	Grey hair at 17 years	89	+	-	16	64	160	++	32	40	++	128
11	Vitiligo; sister pernicious anaemia	96	+	135	32	128	640	++	8	320	++	8

* 1 = Initial finding; 2 = most recent finding; TRC = tanned red cell agglutination for thyroglobulin antibodies; cyto = immunofluorescence for microsomal antibodies; CF = complement fixation for microsomal antibodies; .. indicates no titration done; - negative; figures indicate highest serum dilution giving a + result.

TABLE 46

Gastric secretion, vitamin B_{12} absorption and serum vitamin B_{12} level in patients with thyroid disease and serum intrinsic factor antibodies followed for 3 to 7 years (Group 2)

CASE	SEX/AGE	DIAGNOSIS	FOLLOW-UP YEARS	Hb. G/100 ml.	Gastric Juice - post-stimulant: ph 1*	ph 2	IF conc. units/ml 1	IF conc. units/ml 2	IF/hr. units 1	IF/hr. units 2	IF antibody	B_{12} absorption 1	B_{12} absorption 2	Serum vitamin B_{12} pg/ml 1	Serum vitamin B_{12} pg/ml 2
1	F/61	Graves'	6	13.4	7.9	7.4	3	6	200	138	absent	12.4	13.0	380	340
2	F/45	Myxoedema	6	15.0	1.1	1.2	66	94	10,100	11,400	absent	20.5	20.5	350	360
3	F/35	Graves'	6	14.2	3.0	2.4	52	52	1,200	1,040	absent	25.2	34.5	260	290
4	M/48	Graves'	6	14.8	5.2	4.9	52	49	3,000	1,600	absent	16.3	15.0	180	180
5	M/55	Graves'	6	15.0	1.3	..	159	..	9,900	..	absent	25.0	31.0	190	270
6	F/54	Graves'	7	13.6	..	1.6	..	42	..	1,260	absent	..	56(w)	..	850
7	F/56	Graves'	5	14.8	2.0	2.0	..	22	..	220	present	21.0	66(w)	610	610
8	F/55	Graves'	3	13.6	..	1.6	..	36	..	1,440	absent	..	35(w)	..	230
9	F/57	Graves'	5	13.0	..	2.0	..	30	..	180	absent	..	64(w)	..	375
10	F/59	Graves'	3	13.2	..	4.0	..	33	..	1,980	absent	..	50(w)	..	260
11	F/66	Graves'	3	14.2	..	..	..	..	..	..		13.0	65(w)	308	
Normal range				13.0	2		14-147 mean 55		2,000-25,000 mean 9,000			10 35(w)		170-1,000 (mean 400)	

w = Vitamin B_{12} absorption by a whole body counting technique. Result as % oral dose retained at 7 days.

All other tests by urinary excretion (Schilling) method.

* 1 = Initial finding; 2 : most recent findings.

APPENDIX IV

METHOD OF LEUCOCYTE MIGRATION INHIBITION TEST.

(By courtesy of Dr. Jonathan Brostoff).

25 ml of heparinised venous blood was allowed to sediment at 37°C in sterile plastic tubes for 1 hour. The supernatant, containing mostly leucocytes, was removed with a siliconised pipette, centrifuged at 125g for 5 minutes and the cell pellet was washed three times in Eagles tissue culture medium. The washed leucocytes were then aspirated into 120 mm x 1 mm capillary tubes, the ends sealed with warm paraffin wax and centrifuged at 150g for 10 minutes. The capillary tube was broken at the fluid-cell interface and the cells placed in a circular migration chamber 18mm diameter, 5mm depth, containing human gastric intrinsic factor in tissue culture fluid. Experiments were performed in quintuplicate and control chambers set up without antigen. The area of migration was assessed after 17 hours incubation by placing the chambers in a photographic enlarger, projecting the image onto heavy paper and outlining the migration area which was then cut out and weighed. The migration was calculated from the mean area in the presence of intrinsic factor as a percentage of that in the control cultures lacking antigen. 1 Inhibition of leucocyte migration was considered significant when the leucocyte migration in the presence of intrinsic factor was less than 80% of the mean control.

APPENDIX V

TABLE SHOWING ANTIBODY STATUS OF PATIENTS WITH PERNICIOUS ANAEMIA.

Table 47

Correlation between antibody against intrinsic factor in the serum and gastric juice. Breakdown of cases.

SERUM	GASTRIC JUICE: POS		GASTRIC JUICE: NEG	
POS	1	23	2	46
	3	27	6	52
	5	39	10	
	9	44	15	
	13	45	19	
	14	51	21	
	17		25	
	20		31	
NEG	4	42	7	35
	8	47	12	38
	11	48	18	43
	16	49	22	
	30	50	24	
	36	53	26	
	37		28	
	40		33	
	41		34	

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Dissociation of Intrinsic Factor from its Antibody: Application to Study of Pernicious Anaemia Gastric Juice Specimens

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Summary: Intrinsic factor antibody may sometimes be concealed in the gastric secretions of pernicious anaemia subjects, being complexed with residual amounts of intrinsic factor.

A method is described for dissociating intrinsic factor from its antibody. Antibody to the vitamin-B_{12}-binding site of intrinsic factor was identified in 16 (57%) out of 28 samples of pernicious anaemia gastric juice after dissociation but in only 10 before dissociation. There was no clear relationship between the incidence of antibody in the serum and in the gastric juice of these patients.

Introduction

Antibody to Castle's intrinsic factor is detectable in the serum of 55% of patients suffering from Addisonian pernicious anaemia (Taylor, 1959 ; Ardeman and Chanarin, 1963). There is good reason to suppose that this antibody may interfere with intrinsic factor production as well as prevent its function in vitamin-B_{12} absorption, and these aspects have been reviewed by Chanarin (1968). Intrinsic factor antibodies are of at least two types. The commoner type reacts with that portion of the intrinsic factor molecule which links with vitamin B_{12}, thus preventing union with this vitamin (synonyms: vitamin-B_{12}-binding-site antibody ; blocking antibody ; type I antibody). The other type of antibody reacts with intrinsic factor evidently elsewhere than on the vitamin-

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B_{12}-binding site (synonyms: complex antibody; precipitating antibody; type II antibody). Two general types of in-vitro test have been devised to identify these two kinds of antibody. Antibody localized in the gastrointestinal tract is likely to be of greater importance in pernicious anaemia patients than the antibody in the serum, since the antibody can interfere with intrinsic factor function only at sites of confrontation. As intrinsic factor is probably not absorbed, this encounter must take place in the gastrointestinal lumen or at the mucosal surface.

Fisher *et al.* (1966) found intrinsic factor antibody in the gastric juice of 5 out of 14 patients with pernicious anaemia, including one who did not have such an antibody in the serum. Others have investigated the gastric juice from patients in whom additional intrinsic factor failed significantly to improve the absorption of vitamin B_{12}. Gastric juice antibody was present in the case reported by Schade *et al.* (1966) and in all nine cases reported by Herbert *et al.* (1967).

These observations have been concerned with free antibody in the gastric juice. Intrinsic factor secretion is often not abolished in pernicious anaemia, and small amounts are present in about one-third of the patients (Ardeman and Chanarin, 1965). If intrinsic factor antibody is present in the gastric juice samples from such patients, it is likely to be concealed in a complex with intrinsic factor and hence it will not be demonstrable by direct examination. The purpose of this study was to devise a means whereby such antibody could be released from intrinsic factor and then assayed by a standard procedure. This method would then be used for the detection of antibody in gastric juice samples from pernicious anaemia patients.

Materials

Normal human gastric juice obtained from several donors was pooled, depepsinized, restored to neutral *p*H, and frozen in aliquots at $-20°$ C. until used. The vitamin-B_{12}-binding capacity and intrinsic factor titre were measured by the method of Ardeman and Chanarin (1963).

Gastric juice from subjects with pernicious anaemia was aspirated by nasogastric tube for 30 minutes before and for one hour after stimulation with either histamine or pentagastrin (Peptavalon). These samples were centrifuged, filtered, and stored at $-20°$ C. until tested.

Serum from five subjects with pernicious anaemia, containing an antibody to the vitamin-B_{12}-binding site of intrinsic factor, in titres ranging from 100 to 250 units/ml. were used in the pilot studies. The antibody titre was measured by the

charcoal adsorption method of Ardeman and Chanarin (1963). These samples were stored at −20° C. in aliquots until used.

$^{57}CoB_{12}$ (Radiochemical Centre, Amersham) with a specific activity of 25 mμCi/ml. and diluted with non-radioactive cyanocobalamin to give a final concentration of 200 mμg./ml. Activated charcoal (British Drug Houses) was prepared by heating at 160° C. for two hours and then mixed with normal plasma at a concentration of 100 mg./ml., immediately before use. Measurements of γ-ray emission were made in a Packard, well-type scintillation counter. *p*H was measured on a direct reading glass electrode *p*H meter (Electronic Instruments Limited.)

Methods

(*a*) *Velocity of Combination of Intrinsic Factor with Vitamin B_{12} and with Antibody to Vitamin-B_{12}-binding Site.*—(1) Estimates were made of the speed of combination of normal gastric juice samples with vitamin B_{12}. A 10-ml. volume from a pool of five normal gastric juice samples (binding about 80 mμg. of vitamin B_{12}/ml.) was mixed with 5 ml. of $^{57}CoB_{12}$ (200 mμg./ml.). One-millilitre aliquots were expelled into saline containing serum-coated charcoal, which absorbed the unbound vitamin B_{12}, thus curtailing the combination. After centrifugation the supernatant radioactivity was expressed as a percentage of the radioactivity observed 10 minutes after mixing, by which time no further vitamin B_{12} was bound. (2) Ten millilitres of the gastric juice pool was mixed with 4 ml. of serum containing intrinsic factor antibodies (250 units of antibody per ml.). One-millilitre aliquots were removed at stated intervals and expelled into saline containing 100 mμg. of $^{57}CoB_{12}$. After 10 minutes serum-coated charcoal was added to extract the unbound vitamin B_{12}. After centrifugation at 3,000 r.p.m. for 10 minutes the activity of the supernatant was measured. Antibody combination was expressed as a percentage of the value observed after 10 minutes. These studies were carried out at *p*H 7·5, 3·8, and 3·2.

(*b*) *Effect of pH on Combination of Intrinsic Factor and Antibody Against Vitamin-B_{12}-binding State of Intrinsic*

TABLE I.—*Test and Control Preparations to Measure Dissociation of Antibody from Complex With Intrinsic Factor*

	Test	Controls		
Gastric juice	2·5 ml. (125 u. intrinsic factor)	2·5		
Saline			2·5	2·5
Pernicious anaemia serum	0·5 ml. (100 u. Ab)		0·5	
Normal serum ..		0·5		0·5

Factor.—This was studied in mixtures of serum containing antibody to intrinsic factor and with normal gastric juice. Suitable controls were also set up as indicated in Table I. The quantities chosen were such that no free antibody could be detected. After incubation at room temperature for 30 minutes the test mixture and controls were exposed to the sequence of steps outlined below. The *p*H was reduced to 3·2 with 0·2 M disodium-phosphate/citric-acid buffer. Cyanocobalamin was added in about tenfold excess of the binding capacity of the components. The mixture was agitated for 15 minutes. The unbound vitamin B_{12} was then removed by two additions of 100 mg. of serum-coated charcoal, added at an interval of three minutes. The mixture was again shaken, centrifuged at 3,000 r.p.m. for 10 minutes, and the supernatant was then tested for antibody by the method of Ardeman and Chanarin (1963). The charcoal control was set up to confirm complete removal of the unbound vitamin B_{12} excess.

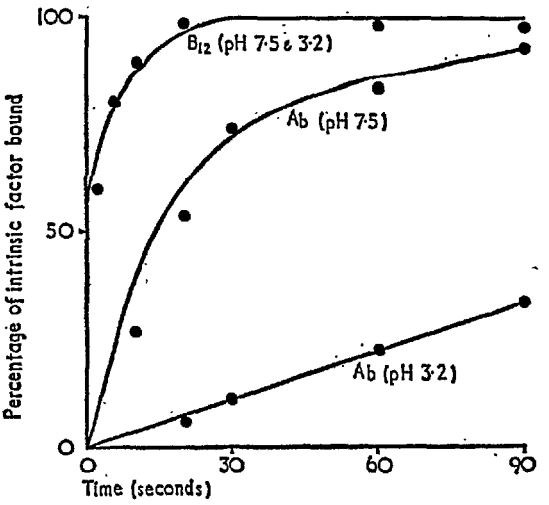
(*c*) *Dissociation of Antibody from Sites of Intrinsic Factor Other than those Reacting with Vitamin* B_{12}.—Mixtures containing normal gastric juice (100 units of intrinsic factor) and serum with intrinsic factor antibody in increasing quantities were lowered to *p*H 3·2 for 15 minutes with 0·2 M sodium-phosphate/citrate buffer. These were decanted into 1-ml. volumes of normal gastric juice complexed with $^{57}CoB_{12}$. The antibody was precipitated with 40% saturated NH_4SO_4 (Jacob and Schilling, 1966) and radioactivity in both supernatant and deposit were measured. The radioactivity in the deposit was expressed as a percentage of total radioactivity.

(*d*) *Pernicious Anaemia Gastric Juice.*—Manœuvres applied to these specimens were the same as the sequence outlined in section (*b*).

(*e*) *Suppression of Antibody with Anti-human Serum.*—An anti-human serum preparation was used (Netherlands Red Cross Transfusion Service PHO—13—P2) ; also a specific anti-human serum IgA (X80H kindly supplied by Dr. R. Drew, Department of Immunological Pathology, University of Birmingham). These were mixed with gastric juice specimens found to have an antibody to intrinsic factor. After incubation at 37° C. for two hours they were frozen at −20° C. for 24 hours and then thawed and tested for antibody as described.

Results

(*a*) *Reaction Velocities* (see Chart).—(1) *Intrinsic Factor and Vitamin* B_{12}: The combination was extremely rapid both at room temperatures and at 4° C. Vitamin B_{12} binders were 60% saturated in two seconds and 100% saturated within



Speed of combination of intrinsic factor with vitamin B_{12} and of intrinsic factor with antibody to vitamin-B_{12}-binding site.

20 seconds. There was no significant difference in the rate of binding at *p*H 7·5, *p*H 3·2, and *p*H 3. (2) *Intrinsic Factor and Antibody*: At *p*H 7·5 50% of the antibody had combined by 20 seconds and complete combination occurred only after 90 seconds. At *p*H 3·2 30% of the antibody united with intrinsic factor after 90 seconds.

(*b*) *Dissociation of "Vitamin-B_{12}-binding-site Antibody" from Intrinsic Factor.*—Five separate sera were mixed with normal gastric juice and tested as described. Under optimum conditions between 75 and 100% of antibody added to normal gastric juice was recovered. The degree of dissociation was measured over the *p*H range from 7·5 to 3·2. A *p*H of 3·2 was chosen since this *p*H gave maximum dissociation without a significant degree of denaturation of released antibody. A fall in antibody activity occurred below *p*H 3·0 when the duration of exposure was greater than 15 minutes.

(*c*) Endeavours to dissociate "non-binding-site" antibody by lowering the *p*H to 3·2 and then permitting random reassociation at neutral *p*H with intrinsic factor-$^{57}CoB_{12}$ provided no convincing evidence that this antibody behaved similarly to the vitamin-B_{12}-binding-site antibody under these conditions. Correspondingly, application of this manœuvre has provided no evidence concerning the presence of this antibody in gastric juice from pernicious anaemia cases.

juice specimens from subjects with pernicious anaemia and 17

(*d*) *Pernicious Anaemia Gastric Juice.*—Twenty-eight gastric

control specimens were treated and tested, as described, for the release and identification of antibody to the vitamin-B_{12}-binding site. Of the 28 pernicious anaemia gastric juices, nine were from patients with an antibody to intrinsic factor in the serum and 11 from subjects without such an antibody. Of the 28 gastric juice specimens, 16 gave positive results for the presence of antibody. Of these 16, 8 were from patients with a serum antibody and 8 from patients without (Table II). Among the 17 controls, including four specimens from patients with autoimmune thyroid disorders, who had serum antibody

TABLE II.—*Correlation of Incidence of Gastric Juice Antibody with Serum Antibodies to Intrinsic Factor*

			Gastric Juice Intrinsic Factor Antibodies	
			+	−
Serum intrinsic factor antibodies ..	*M*	+	8	3
	M	−	8	9
			16	12

to intrinsic factor without evidence of impaired vitamin-B_{12} absorption, no positive results for gastric juice antibody were obtained. A general observation among the pernicious anaemia samples was that dissociation showed more antibody than was found on simple testing and that where some intrinsic factor was found in the sample, antibody was demonstrated only after dissociation.

(*e*) In three gastric juice specimens found to have an antibody to intrinsic factor mixture with an equal volume of anti-human serum caused a clear drop in antibody activity. This observation was likewise made on using specific anti IgA serum, thus identifying the antibody in these specimens as of class IgA.

Discussion

Free antibody in pernicious anaemia gastric juice was present in 10 out of 28 samples ; in six more cases antibody could be demonstrated on dissociation from intrinsic factor. In those with free antibody the dissociation procedure produced some rise in titre of antibody. Antibody was present in the gastric juice of four patients in the absence of serum antibody ; Fisher *et al.* (1966) described one similar case. This emphasizes that it is local gastrointestinal antibody which is of importance in impairing vitamin-B_{12} absorption, particularly since serum antibody may be present and in no way interferes with vitamin-B_{12} absorption.

In the present group of 28 patients antibody was found in the serum or gastric juice of 19 (68%). It cannot, however, be concluded that no antibody was present in the remaining 32% in whom we failed to find it.

Observations on the transplacental transmission of antibody to intrinsic factor suggest that this antibody may suppress intrinsic factor production in the infant without itself appearing in the gastric juice. Fisher and Taylor (1967), however, failed to observe any impairment of infantile intrinsic factor production as a consequence of transplacental passage of intrinsic factor antibody. This antibody may therefore be synthesized in the gastric submucosa and function locally in the mucosa (Bar Shany and Herbert, 1966 ; Goldberg *et al.*, 1967) and either not appear in the gastric secretion or be found only in small quantities. Endeavours to identify antibody in concentrates of pernicious anaemia gastric juice, where the undiluted sample evidently contained no antibody, have failed. Antibody may be directed against other sites of the intrinsic factor molecule than the vitamin-B_{12}-binding site. Such antibodies were not identified in gastric juice by the procedures described.

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INTRINSIC-FACTOR ANTIBODIES IN ABSENCE OF PERNICIOUS ANÆMIA

3–7-Year Follow-up

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Summary Eleven out of twenty-three patients with thyroid disease and intrinsic-factor (I.F.) antibodies in serum had an atrophic gastritis which did not progress to pernicious anæmia over a period of 3–7 years. In these patients the absorption of vitamin B_{12} remained normal, and the amount of acid and I.F. in the gastric secretion remained unchanged. Ten out of these eleven patients had thyrotoxicosis and nine had a family history of other autoimmune disorders. The other twelve patients with I.F. antibodies had early pernicious anæmia. Six had thyrotoxicosis and six had Hashimoto's thyroiditis or primary myxœdema. Four of these patients had a family history of thyroid disease or pernicious anæmia. It is concluded that the patients with I.F. antibody and a non-progressive atrophic gastritis differ from patients with pernicious anæmia in that the I.F. antibody appears in serum at a relatively early phase in the evolution of gastritis when adequate amounts of I.F. are still being produced. They also differ from patients with pernicious anæmia in the absence (with one exception) of I.F. antibody in gastric juice. The leucocyte-migration test, done to assess cellular immunity to I.F., was positive in 40% of pernicious-anæmia cases but in only one patient followed for 5 years without progression to pernicious anæmia in spite of I.F. antibodies in serum and gastric juice.

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Introduction

ANTIBODIES against human gastric intrinsic factor (I.F.) have been found in the serum of 55% of patients with pernicious anæmia.[1] Many patients have such an antibody in the gastric secretion [2,3] or in the stomach wall.[4] There is good reason to believe that I.F. antibodies are important in the development of vitamin-B_{12} deficiency since the great majority of patients with such antibodies have pernicious anæmia. In man the antibody prevents I.F.-mediated vitamin-B_{12} absorption [5] and in cases of pernicious anæmia it is present in the gastrointestinal tract where it can react with intrinsic factor. On the other hand, patients with simple atrophic gastritis do not have I.F. antibodies in the serum [6] and the small amount of I.F. produced by the atrophic mucosa is usually sufficient to potentiate vitamin-B_{12} absorption.[7]

In a few cases I.F. antibodies in serum have been recorded in the absence of pernicious anæmia. Antibodies to intrinsic factor have been detected in the serum of a patient with atrophic gastritis,[8] in two relatives of patients with pernicious anæmia,[9,10] and in patients with thyroid disease,[11–13] and in diabetes mellitus.[14,15] Single cases of antibody in patients without pernicious anæmia have been recorded.[16–18]

If the development of pernicious anæmia in most cases requires an I.F. antibody then the long-term follow-up of patients who initially had such an antibody but were still able to absorb vitamin B_{12} normally is of special interest. Will such patients, in time, develop pernicious anæmia? If not, how do they differ from patients with this disease? It may be that the relevant antibodies are those secreted into the gastric juice, or that the difference may lie in the degree of cell-mediated immunity which the patient develops to I.F. The leucocyte-migration test may reflect this component of the autoimmune response in several diseases.[19–21]

We have now followed a group of patients with thyroid disease and I.F. antibody in serum for 3–7 years. Their vitamin-B_{12} status, gastric secretion, and autoantibodies including test for migration-inhibition factor are reported.

Patients and Methods

Patients

Patients were selected from two sources. In 1964, two hundred and forty-nine patients attending a thyroid clinic were investigated for evidence of vitamin-B_{12} deficiency and

for I.F. antibodies. Nine had I.F. antibodies.[12] Another fourteen patients with I.F. antibody in serum was collected at the Middlesex Hospital over a period of years. Follow-up was designed to determine whether malabsorption of vitamin B_{12} developed by doing either urinary excretion (Schilling) tests with an oral dose of 1·0 μg. of ^{57}Co-labelled vitamin B_{12} or by measuring the amount of ^{58}Co-labelled vitamin B_{12} retained by whole-body counting 1 week after a similar oral dose. At first, most patients were seen at 6-monthly intervals and later annually.

Methods

Serum-vitamin-B_{12} levels were assayed by microbiological assay using *Lactobacillus leichmannii* or by saturation analysis,[22] and a full blood-count was usually done at each visit.

Gastric secretion was studied initially in six patients in group II and finally in all but one patient using an augmented dose of histamine or, more recently, pentagastrin (6 μg. per kg.) as the stimulant to gastric secretion. Gastric juice was examined for the presence of acid, I.F.,[23] and I.F. antibodies.[24]

I.F. antibodies in serum were titrated by the method of Ardeman and Chanarin,[23] and against the gastric parietal cells by the immunofluorescent method. Thyroid antibodies were estimated by methods previously described.[25]

Leucocyte-migration test.—25 ml. of heparinised venous blood was allowed to sediment at 37°C in sterile plastic tubes for 1 hour. The supernatant, containing mostly leucocytes, was removed with a siliconised pipette, centrifuged at 125*g* for 5 minutes, and the cell pellet washed three times in Eagle's tissue-culture medium. The washed leucocytes were then aspirated into 120 mm. × 1 mm. capillary tubes, the ends sealed with warm paraffin wax, and centrifuged at 150*g* for 10 minutes. The capillary tube was broken at the fluid-cell interface and the cells placed in a circular migration chamber 18 mm. diameter, 5 mm. depth [26] containing human gastric I.F. in tissue-culture fluid. Experiments were performed in quintuplicate and control chambers set up without antigen. The area of migration was assessed after 17 hours' incubation by placing the chambers in a photographic enlarger, projecting the image on to heavy paper, and outlining the migration area which was then cut out and weighed. The percentage migration was calculated from the mean area in the presence of I.F. as a percentage of that of the control cultures lacking antigen. Inhibition of migration was considered significant when the leucocyte migration in the presence of I.F. was less than 80% that of the mean control.

Results

Clinical Features

Of the twenty-three patients studied, twelve were found to have early pernicious anæmia (group I) and

TABLE I—GASTRIC SECRETION, VITAMIN-B_{12} ABSORPTION, AND SERUM-VITAMIN-B_{12} IN PATIENTS WITH THYROID DISEASE AND SERUM I.F. ANTIBODIES FOLLOWED UP FOR 3–7 yr. (GROUP II)

Case	Sex	Age	Diagnosis	Follow-up (yr.)	Hb (g. per 100 ml.)	Gastric juice (post-stimulant)							Vitamin-B_{12} absorption		Serum-vitamin-B_{12} (pg. per ml.)	
						pH		I.F. (units per ml.)		I.F. per hr. (units)		I.F. anti-body				
						1	2	1	2	1	2		1	2	1	2
1	F	61	Graves'	6	13·4	7·9	7·4	3	6	200	138	Absent	12·4	13·0	380	340
2	F	45	Myxœdema	6	15·0	1·1	1·2	66	94	10,100	11,400	Absent	20·5	20·5	350	360
3	F	35	Graves'	6	14·2	3·0	2·4	52	52	1200	1040	Absent	25·2	34·5	260	290
4	M	48	Graves'	6	14·8	5·2	4·9	52	49	3000	1600	Absent	16·3	15·0	180	180
5	M	55	Graves'	6	15·0	1·3	..	159	..	9900	..	Absent	25·0	31·0	190	270
6	F	54	Graves'	7	13·6	..	1·6	..	42	..	1260	Absent	..	56 (w)	..	850
7	F	56	Graves'	5	14·8	2·0	2·0	..	22	..	220	Present	21·0	66 (w)	610	610
8	F	55	Graves'	3	13·6	..	1·6	..	36	..	1440	Absent	..	35 (w)	..	230
9	F	57	Graves'	5	13·0	..	2·0	..	30	..	180	Absent	..	64 (w)	..	375
10	F	59	Graves'	3	13·2	..	4·0	..	33	..	1980	Absent	..	50 (w)	..	260
11	F	66	Graves'	3	14·2	..	..	..	..	..	..	..	13·0	65 (w)	308	610
Normal range	..	..	..	..	>13·0	<2	..	14–147 (mean 55)		2000–25,000 (mean 9000)		.. ..	>10 >35 (w)		170–1000 (mean 400)	

w = Vitamin-B_{12} absorption by a whole-body counting technique. Result as % oral dose retained at 7 days. All other tests by urinary excretion (Schilling) method. 1 = Initial finding. 2 = Most recent findings.

TABLE II—COMPARISON OF TWO GROUPS OF PATIENTS WITH THYROID DISEASE AND SERUM ANTIBODIES TO I.F.

—	Group I (early pernicious anæmia)	Group II (atrophic gastritis)
No. of patients	12	11
Sex ratio (M/F)	1/11	2/9
Mean age (yr.)	56	54
Associated diseases:		
Thyrotoxicosis	6	10
Hashimoto or primary myxœdema..	6	1
Addison's disease	0	1
Myasthenia gravis	1	0
Rheumatoid arthritis	1	0
Diabetes mellitus	1	0
Vitiligo or early greying	0	4
Family history of autoimmune disease:		
Thyroid disease	4	7
Pernicious anæmia	1	3
Addison's disease	0	1
Rheumatoid arthritis	0	1
Vitiligo or early greying	0	2

eleven absorbed vitamin B_{12} normally (group II). All but three of the patients were women, and all had normal hæmoglobin levels. The patients with early pernicious anæmia had a macrocytic peripheral blood-film, the marrow showed megaloblastic hæmopoiesis, the serum-vitamin-B_{12} level was borderline or low, there was a histamine-fast achlorhydria in all six cases examined, and all had malabsorption of vitamin B_{12}. Seven patients in whom the absorption test was repeated with added I.F. showed an enhanced vitamin-B_{12} retention. All had I.F. antibodies in serum. The patients with pernicious anæmia are not considered further in this paper. The patients in group II were seen at more or less regular intervals, the duration of follow-up being 6 to 7 years in six, 5 years in two, and 3 years in three (table I).

There were interesting differences between these groups of patients in the nature of the thyroid disease and in the family history (table II). Six of the seven patients with Hashimoto's thyroiditis or primary myxœdema had pernicious anæmia. By contrast only six out of sixteen patients with thyrotoxicosis had pernicious anæmia the other ten having serum-I.F.-antibodies with a non-progressive atrophic gastritis. A family history of other autoimmune disorders was noted in only four out of the twelve pernicious-anæmia patients but was present in nine out of the eleven patients in group II (tables I and II).

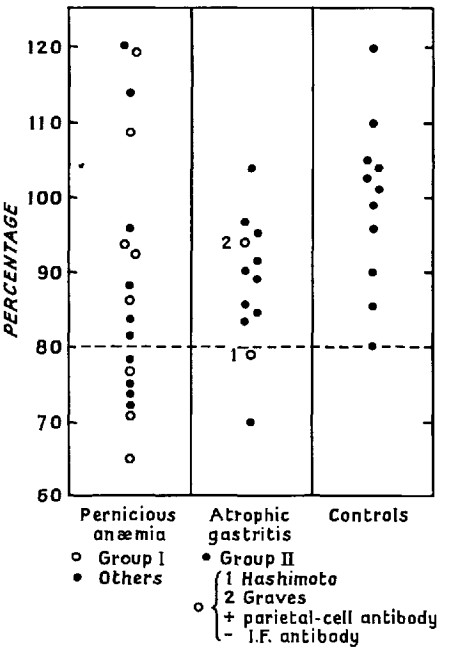

Fig. 1—Results of a test for " migration inhibition factor " in eighteen patients with pernicious anæmia, including eight in group I, eleven controls, and twelve patients with atrophic gastritis (including ten patients in group II).

Initial Findings

All patients in group II had a normal hæmoglobin level and a normal stained blood-film (table II). The serum-vitamin-B_{12} level was normal in all seven patients in whom this was carried out initially. Six had gastric juice aspirated. Five showed the presence of acid and one (case 1) had a histamine-fast achlorhydria with a low I.F. output. Nevertheless she absorbed vitamin B_{12} normally, as did all the other patients tested, and her serum-vitamin-B_{12} was normal. All the patients had antibody in serum against human I.F. and against gastric parietal cells, and nine out of ten patients tested had thyroid antibodies. The titres of gastro-parietal-cell fluorescence were unusually high in six of the eight patients where serial dilutions had been made, but thyroid antibodies were present in low concentrations as seen in Graves' disease except for two patients with complement fixation titres of 32 and 128 respectively (table III, fig. 2).

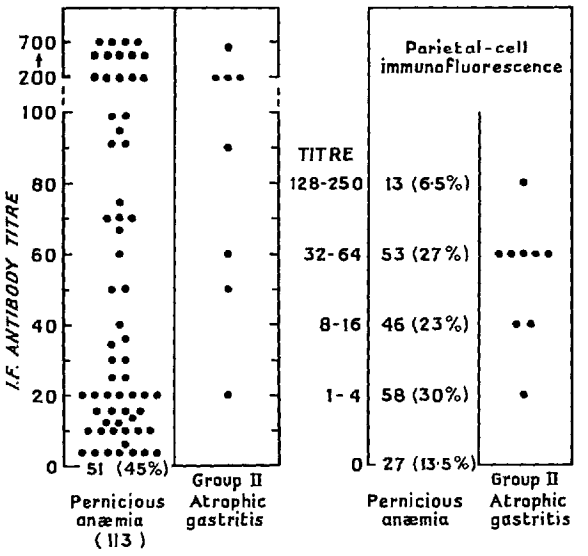

Fig. 2—Serum antibody titres against the gastric parietal cells in 197 patients with pernicious anæmia and nine patients in group II (atrophic gastritis with I.F. antibodies) and against gastric intrinsic factor in 113 patients with pernicious anæmia and eight patients in group II.

Subsequent Findings

Re-examination after 3–7 years showed the blood picture to be unchanged. The serum-vitamin-B_{12} remained normal although this could have been influenced by the injection of 1 mg. vitamin B_{12} given to some of the patients once or twice yearly during Schilling tests (table I). The absorption of vitamin B_{12} was unchanged in seven patients who had regular tests and was also normal in the remaining four who were only tested at the end of this period although they were known to have had I.F. antibodies in serum for 2–6 years. In particular, patient 1 still absorbed vitamin B_{12} normally although 6 years previously she already had a histamine-fast achlorhydria, severe atrophic gastritis on biopsy, and residual amounts of I.F. that were not significantly greater than those encountered in some patients with pernicious anæmia. Nevertheless the Schilling test excretion increased from 12·4 to 19·4% on addition of I.F.

In three of the eleven patients I.F. antibodies disappeared from the plasma (table III). In case 1 the I.F. antibody titre was between 60 and 80 units during 1965 and 1966 but thereafter declined, and was no

TABLE III—AUTOIMMUNE DISEASE AND SERUM ANTIBODY TITRES IN PATIENTS WITH THYROID DISEASE AND I.F. ANTIBODIES FOLLOWED UP FOR 3–7 yr. (GROUP II)

Case	Other autoimmune disease	% leucocyte migration with gastric juice (normal 80–120)	Serum antibodies to *									
			I.F.		Parietal cell (fluorescence titre)		Thyroid					
			1	2	1	2	T.R.C. (1)	Cyto (1)	C.F. (1)	T.R.C. (2)	Cyto (2)	C.F. (2)
1	Sister, aunt thyrotoxic	91	60	—	+	8	..	..	..	—	—	—
2	Nil	83	75	115	64	128	—	—	—	—	—	—
3	Postoperative myxœdema, Addison's disease, infertility with adrenal/ovarian antibodies; aunt thyrotoxic	89	10	130	512	256	—	+	—	—	++	16
4	Postoperative myxœdema	85	250	100	64	64	20	++	—	—	++	16
5	Post-^{131}I myxœdema; mother goitre	104	50	90	32	64	—	++	4	—	—	—
6	Post-^{131}I myxœdema; mother pernicious anæmia; aunt thyrotoxic	83	700	120	64	64	20	++	128	40	++	32
7	Post-^{131}I myxœdema; rheumatic heart-disease, smooth tongue—5 yr. Mother goitre; father, cousin pernicious anæmia; sister thyrotoxic with rheumatoid arthritis; aunt thyrotoxic; early greying (3 relatives)	70	+	50	8	1	80	++	—	320	++	—
8	Mother goitre	..	+	+	+	64	40	++	—	10	++	—
9	Grey hair at 22 yr.; sister thyrotoxic	95	+	20	+	128	10	++	—	20	++	—
10	Grey hair at 17 yr.	89	+	—	16	64	160	++	32	40	++	128
11	Vitiligo; sister pernicious anæmia	96	+	135	32	128	640	++	8	320	++	8

* 1 = Initial finding; 2 = most recent finding; T.R.C. = tanned red-cell agglutination for thyroglobulin antibodies; Cyto = immunofluorescence for microsomal antibodies; C.F. = complement fixation for microsomal antibodies; + indicates no titration done; − negative; figures indicate highest serum dilution giving a + result.

longer detectable after 1968. Parietal-cell antibodies persisted in all eleven patients without significant change in titre. Thyroid antibodies were still present in eight patients and case 3 who also had Addison's disease and a premature menopause had both adrenal and ovarian immunofluorescence as well.

I.F. antibodies in gastric juice were looked for in ten patients (one refused). This was absent from nine samples but present in significant titre in case 7. The gastric juice in this patient in 2 studies had a pH of 2·0 after pentagastrin and an intrinsic factor concentration of 22 units per ml. Only 10 ml. of juice was aspirated and hence the estimate for total secretion over an hour may be unreliable. After dissociation of antibody from I.F., the gastric juice was found to have an antibody titre of 7 units per ml. This antibody was largely neutralised by the addition of anti-IgA serum and only to a small extent by the addition of an anti-IgG serum.

Leucocyte-migration Test

This was done in ten of the patients in group II and in two additional cases of simple atrophic gastritis in thyroid patients without I.F. antibody in serum. For comparison ten cases of known pernicious anæmia were tested as well as eight patients with early pernicious anæmia from group I. Eleven laboratory staff were included as controls. The results are shown in fig. 1. The test was considered positive when cultures containing I.F. showed a migration area of less than 80% that of control chambers. Seven of the eighteen patients with early or overt pernicious anæmia showed an inhibition of leucocyte migration in the presence of intrinsic factor while only one of eleven cases in group II gave a positive test. In addition, one patient with Hashimoto's disease, normal gastric function, parietal-cell antibodies but without I.F. antibodies in the serum gave a positive result (migration 79%). The other positive result (case 7, migration 70%) was in the only patient in group II who had I.F. antibodies in both blood and gastric juice and who showed the most striking familial incidence of thyrotoxicosis, pernicious anæmia, and early greying of the hair.

Discussion

The amount of vitamin B_{12} absorbed by patients with gastritis depends upon the amount of I.F. available, and this in turn is related to the number of remaining parietal cells and to the presence of factors

that interfere with action of I.F. Under certain circumstances these factors can be an abnormal bacterial flora in the small gut, fish tapeworm, impaired capacity of ileal cells for vitamin-B_{12} absorption, or antibodies against I.F. Probably, only I.F. antibody is important, at least in the early phases in pernicious anæmia. In this disorder a very low or even absent I.F. secretion coupled with gastrointestinal antibody to I.F. together prevent vitamin-B_{12} absorption.

All but one of the patients in this study (group II) maintained an adequate though often reduced I.F. output. We showed that the serum antibody reacted with the patients own I.F. by giving the patients the oral dose of ^{57}Co-vitamin B_{12} with 10 ml. of the patients own serum. Two such tests in case 2 reduced the urinary excretion in the Schilling test from 20% to 4%.

Even patient 1, who had only very small amounts of residual I.F., was able to utilise this to the full and so maintain normal vitamin-B_{12} absorption presumably because of the absence of local gastrointestinal antibody. Rose [27] found I.F. antibodies in twenty-four out of fifty-two gastric-juice samples from pernicious anæmia patients by an acid elution method and Goldberg and Bluestone [28] detected these antibodies in ten out of twelve cases by effecting the dissociation of antigen-antibody complexes in acid acrylic-gel medium. By contrast such an antibody was present in only one of the ten gastric-juice samples from the patients in group II of our series. This patient (case 7) had acid in her gastric secretion and an excess of free I.F. which allowed her to absorb vitamin B_{12} normally when tested on three occasions in the past 5 years, despite the I.F. antibodies.

If the leucocyte-migration test indeed reflects cellular immunity to I.F. by virtue of the production of " migration inhibition factor " by sensitised lymphoid cells in vitro, then this was present in about 40% of patients with overt or latent pernicious anæmia but was only positive in a single patient belonging to group II. This same patient also showed I.F. antibodies in her gastric juice. These preliminary results suggest a difference in the degree of cellular hypersensitivity between patients with pernicious anæmia and those with a gastritis in which greater numbers of functioning parietal cells have persisted.

There were significant differences in antibody titres in these groups of patients. The titres of I.F. anti-

body in the serum in the patients in group II were relatively high when compared with the titres generally encountered in pernicious anæmia (fig. 2). Similarly the titres of parietal-cell antibodies observed in the patients in group II were somewhat higher than those generally found in pernicious anæmia (fig. 2). In myxœdema microsomal-antibody titres also tend to be lower than those found in many cases of focal thyroiditis associated with Graves' disease or found in symptom-free relatives of patients with Hashimoto's disease.

The patients in group II also differed from those with early pernicious anæmia in the nature of their thyroid disease since most of the Hashimoto or primary myxœdema patients with I.F. antibodies had pernicious anæmia whereas most of the thyrotoxic patients had a milder gastric lesion. Thyrotoxic patients who have gastric antibodies also generally have evidence of active focal thyroiditis reflected in the almost constant presence of cytoplasmic thyroid antibodies, sometimes in high titre, and in the high incidence of post-operative and post-^{131}I myxœdema in this group. These patients also had an unusually high frequency of familial thyroid and gastric autoimmunity with other signs such as vitiligo,[29] early greying of the hair, and, in one case, an associated autoimmune adrenalitis accompanied by infertility and early menopause, the serum containing an antibody to steroid-producing cells in the adrenal cortex, cross-reacting with ovary and testis.[30,31] The striking familial tendency to auto-immunity may be responsible in the patients in group II for the appearance of I.F. antibodies at a relatively early period in the evolution of the gastritis when a significant number of functioning gastric parietal cells and an adequate though reduced intrinsic factor output are still present. It is possible that these patients are not a homogenous group and that some, especially patients 1 and 7, might progress to pernicious anæmia at a later date. However, it is equally possible that the final precipitating factor lacking in these patients may be IgA antibody produced by lymphoid cells in the wall of the gastric mucosa where it immediately neutralises any I.F. produced, or that cell-mediated hypersensitivity is also a necessary factor in the complete destruction of the gastric mucosa.

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STUDIES ON ANTIBODY TO GASTRIC INTRINSIC FACTOR.

I regret that a number of errors escaped detection before the above mentioned thesis was bound and submitted:

Page.	Error	Correction.
12	Biermer coined the term Progressiver Perniciöser Anämie.	
13	Jeffries and Sleisenger (1963)	
15	their	continue with medication.
	Berlin et al didn't show remission but observed a rise in vitamin B_{12} absorption.	
16	and Tayler and Morton (1958) sought to elicit an antibody.	
20	differently	differentially.
22	30%	3%
25	..on the four cases of Ardeman et al (1966) and a further seven cases of Dr. D. Deniach...	
27	Ardeman and Chanarin	(1965 e)
	of	(emit.)
28		(human intrinsic factor)
	62%	61%
37	therefore	henceforth.
40	d) sheep anti-chain	sheep anti gamma chain.
	(this should in any case have been omitted since it was not employed).	
41	clip glassed bottle	glass clip-topped bottle.
43	2nd para: ...as in the negative control.	

Page	Error	Correction.
48		Edgware.
59		factor.
61	samples	omit.
64	0.2 M glycine-sulphuric acid was employed to create pH 2.5 and 2.0. (ie: not phosphate-citrate buffer).	
71	The first paragraph should not be included at this point but should come after p.75, at the end of the section. This really represents a conclusion.	
75	pH 3.0	3.2
76	fig 17 pH 3.0	3.2
78	neutral	omit.
83	C. 2 ml isotonic saline.	
84	aliquots	omit.
	Protein	serum.
	6 ml	7 ml 240-240/7=206 units.
85		outlined.
90	Table 19 omit isotonic saline.	
93		heteregeneous.
95	fig 21	serum 2. 1. 3.
99.	fig 24	A & B wrongly marked.
102	After fifteen minutes	At stated intervals...
111	fig 29 legend	cleavage.
113	Table 30: straight	normal.
122	Table 32	53. No 14, total Ab: 6.
123		nine

Page	Error	Correction.
131	First para:	normal gastric juice,
132		mediated.
139	fig 38	2.
140		P 0.1
143	Ungar et al	(1968)
145		leichmanii.
146	considered	included.
150	three	two
151		addition.
153		ug
158		to neutral.
159	First para: insertion at margin.	
	3rd para: badly expressed, see supplementary sheet.	
160	45%	38%
163	1965/6	1965 e.
	three	two.
	none	neither
167	5)	or to cell bound antibody.
171		leichmanii.

p. 159: 3rd paragraph.

It is worth mentioning that although type 1 antibody is more easily identified in the gastric juice, it is unable to interfere with intrinsic factor: B_{12} complex. Where intrinsic factor fails to assist with vitamin B_{12} absorption by the small bowel type 2 antibody is more likely to be implicated.