VITAMIN B$_{12}$-BINDERS IN HUMAN BODY FLUIDS

I. ANTIGENIC AND PHYSICO-CHEMICAL CHARACTERISTICS

J. HURLIMANN AND CECILE ZUBER

Institut Universitaire de Biochimie, Lausanne

SUMMARY

Three factors binding vitamin B$_{12}$ are detected in human gastric juice by autoradiographs of immunoelectrophoresis. Binder 1 is present only in gastric juice and gastric mucosa extract and corresponds to intrinsic factor. Binder 2 is present also in other excretions such as bile, lactoserum, saliva, in serum and leucocyte extracts. Binder 3 present in saliva and gastric juice in lower quantity than binder 2, seems derived from this last one by attachment of sialic acid molecules.

There is no immunological identity between intrinsic factor and the other binders without intrinsic factor activity. Binder 3 is resistant to proteolysis *in vivo* and *in vitro*. Binder 2 is modified by peptic digestion *in vivo* but not *in vitro*. Binder 1 is insensitive to proteolysis if saturated by vitamin B$_{12}$, but loses its binding capacity and/or immunological properties if the digestion takes place before fixation of vitamin B$_{12}$.

The three binders seem to have the same affinity for vitamin B$_{12}$ as judged by dialysis experiments, and the same molecular weight of 60,000 as judged by volume exclusion on Sephadex G-200.

INTRODUCTION

In the body fluids of man several components are able to bind vitamin B$_{12}$ (Glass, 1963). One of them, the intrinsic factor (IF) in addition facilitates the absorption of vitamin B$_{12}$ in the small intestine. The physico-chemical characteristics of the IF and of the B$_{12}$-binders without intrinsic factor activity (non-IF binders) have been studied on components highly purified but not completely pure (Gräsbeck, Simons & Sinkkonen, 1965). These characteristics differ according to the authors or to the techniques used such as in the case of molecular weight (Kubo, Kakei & Glass, 1965; Rothenberg, 1966a; Chosy & Schilling, 1963) and the sensitivity to proteolysis (Gräsbeck, Simons & Sinkkonen, 1966; Uchino, Schwartz & Glass, 1964; Okuda & Fujii, 1966).

Correspondence: Dr J. Hurlimann, Institut Universitaire de Biochimie, Bugnon 21, Lausanne 1005, Switzerland.

Few immunological studies of these components have been made (Gullberg & Kistner, 1962; Hurlimann & Scheidegger, 1962; Simons & Gräsbeck, 1963; Hurlimann, 1963; Simons *et al.*, 1964; Gullberg, 1966). However, precise data concerning their antigenic determinants could be interesting since in some patients with pernicious anaemia, antibodies were found which combine with and inactivate intrinsic factor (Schwartz, 1958; Taylor, 1959; Gullberg *et al.*, 1966).

The present experiments were made to study the antigenic characteristics of human B_{12}-binders. These characteristics were determined by autoradiographs of immuno-electrophoresis and double diffusion tests of various materials to which vitamin B_{12} labelled with ^{57}Co was added. Several body fluids were examined and compared using various antisera. The influence of proteolysis, denaturation and of dialysis on the antigenic properties of the components was studied and molecular weight estimated.

MATERIALS AND METHODS

Gastric juice

Gastric juice was collected by gastric intubation following histamine stimulation. It was macroscopically free of saliva, bile or blood. After centrifugation at 3000 g for 15 min and dialysis against distilled water for 48 hr, it was lyophilized. Gastric juice from twenty normal adults was pooled.

Gastric juice from ten normal adults was collected during its intragastric neutralization according to the technique of Gullberg & Olhagen (1959). Proteolysis of the gastric juice proteins was prevented by instillation into the stomach of phosphate buffer, ionic strength 0·2, pH 8·0. These gastric juices were handled as described above and called buffered gastric juice.

Normal human serum

This was collected from normal adults, pooled and lyophilized after dialysis against distilled water.

Saliva

This was collected from ten normal adults after stimulation with chewing-gum, dialysed against distilled water, lyophilized and pooled.

Lactoserum

Lactoserum was obtained from human milk by ultracentrifugation at 40,000 rev/min for 40 min. It consisted of the liquid between the precipitate on the bottom of the tube and the fatty layer on the top. After dialysis against distilled water the lactoserum was lyophilized.

Leucocyte extract

The leucocytes were separated from 80 ml of citrated blood by sedimentation in PVP solution according to the technique described by Payne (1964). The liquid rich in leucocytes was frozen and thawed three times, then centrifuged at 3000 g for 15 min. The supernatant, after dialysis against distilled water, was lyophilized.

Gastric mucosa extract

Fragments from stomach obtained after gastrectomy for duodenal ulcers were washed in phosphate buffer 0·15 M, pH 8·0. The gastric mucosa was dissected from the deep planes and ground in phosphate buffer 0·15 M, pH 7·4, at 4°C in a Silverson mixer (Silverson, London, England). After centrifugation, the water-soluble extract was dialysed against distilled water and lyophilized.

Antisera

These were obtained by immunization of rabbits of various strains, weighing 2·5–3·5 kg. The antigen solution in saline was mixed with an equal part of complete Freund's adjuvant (Difco, Detroit, Michigan). The emulsion was injected into the hind footpads the 1st week and then into four sites in the musculature once a week. The rabbits were bled out usually 4–7 weeks after the beginning of the immunization. The amount of injected protein was the same every week: 10 mg for saliva, gastric juice, buffered gastric juice and gastric mucosa, 30 mg for human serum.

In some experiments antisera were absorbed with various antigens. The antiserum was mixed with the solution of antigen, put in a waterbath at 37°C for $\frac{1}{2}$ hr, then overnight at 4°C and centrifuged at 3000 *g* at 4°C for 20 min.

Immunoelectrophoresis

The micromethod of Scheidegger (1955) was followed with slight modifications, using the LKB apparatus 6800 (LKB, Stockholm, Sweden).

Double diffusion in agar

The microtechnique on microscopic slides was used with 2% agar in barbital buffer 0·025 M, pH 8·2.

Autoradiographs

The lyophilized materials to be analysed were dissolved in a solution of vitamin B_{12} labelled with ^{57}Co (Cyanocobalamin, ^{57}Co, 1 μc/μg, The Radiochemical Centre, Amersham, England). One milligram of lyophilized powder was put in 0·012 ml of H_2O with 50 ng of cyanocobalamin (^{57}Co).

These mixtures were submitted to immunoelectrophoresis or to double diffusion test in agar. Usually the solution was put once in an antigen well of a volume of 1·5 μl, but in some experiments the antigen well had a volume of 4, 7 or 14 μl. After immunoelectrophoresis and double diffusion, the slides were washed in saline, dried and stained with Amido Black. They were then placed in contact with photographic film (Kodak professional Royal Pan 400 ASA) for 14 days at room temperature. The film was developed with DK 60a (Kodak) for 4 min and fixed with Metafix (Kodak).

Identification of esterase

The method of Nachlas *et al.* as reported by Lison (1960) was used. The immunoelectrophoretic slides after washing and drying were incubated 5 min in a solution of 1-naphthyl acetate $2·5 \times 10^{-4}$ M in a veronal buffer 0·05 M, pH 7·8, with 4% of Fast Blue B. The slides were washed in 2% acetic acid and dried. The precipitation lines with esterase activity were stained dark red.

I

Chromatography on DEAE-cellulose

Columns of DEAE-cellulose (Whatman, Balston Ltd, England) $50 \times 2 \cdot 4$ cm were used and the chromatography was performed using a stepwise gradient of three phosphate buffers: $0 \cdot 01$ M, pH $7 \cdot 4$; $0 \cdot 1$ M, pH $6 \cdot 2$ and $0 \cdot 3$ M, pH $4 \cdot 8$ (Method 1). In some experiments the chromatography was performed with phosphate buffer at a constant pH of $7 \cdot 4$ and a stepwise gradient of molarity of $0 \cdot 07$ M, $0 \cdot 3$ M, $0 \cdot 35$ M and $0 \cdot 4$ M (Method 2).

The lyophilized material to be chromatographed was dissolved in distilled water in the proportion of 60 mg powder/ml water. The solution was dialysed overnight against the starting buffer and applied to the column, using a ratio of 60 mg of lyophilized material per gram of DEAE-cellulose. Fractions were pooled according to peaks of optical density at 280 mμ, dialysed against distilled water and lyophilized.

Filtration on Sephadex

The lyophilized material was dissolved in phosphate buffered saline pH $7 \cdot 4$ and passed through a column of Sephadex G-200 (Pharmacia, Uppsala, Sweden) equilibrated with phosphate buffered saline (volume of sample 3 ml, column $110 \times 2 \cdot 4$ cm, flow rate 13 ml/hr). The column was calibrated according to Andrews (1965) by filtration of bovine serum albumin (Pentex, Kankakee, Illinois), human fibrinogen (Hoffmann-La Roche, Basle, Switzerland), Blue Dextran 2000 (Pharmacia, Uppsala, Sweden), apoferritin and rabbit IgG. Apoferritin was prepared from horse ferritin twice crystallized (Pentex) by treatment with Desferal (Ciba, Basle, Switzerland) and dialysis against saline with $0 \cdot 8\%$ sodium hydrosulphite. Rabbit IgG was obtained from rabbit serum by precipitation with ammonium sulphate at 33% saturation with subsequent chromatography on DEAE-cellulose with phosphate buffer $0 \cdot 01$ M, pH $7 \cdot 4$.

The elution volumes of these various materials were determined according to optical density peaks at 280 mμ. The elution volumes were plotted against log molecular weight of the proteins; the calibration curve thus established was used for the determination of molecular weight of the B_{12}-binders.

Dialysis assay

Samples of materials in solution with radioactive vitamin B_{12} were put in Visking tubings and dialysed against saline for 5, 24, 48 and 72 hr. After dialysis the samples were adjusted to the same volume and compared by immunoelectrophoretic autoradiography to an undialysed sample.

Digestion with enzymes and denaturation

Various lyophilized materials in solution with labelled vitamin B_{12} were digested with pepsin (three times crystallized, Nutritional Bioch. Corp., Cleveland, Ohio) or neuraminidase free of proteases (Behringwerke, Marburg, Germany).

Neuraminidase was added in the proportion of 4 units/mg lyophilized powder. The mixture was incubated at 37°C for 24 hr in acetate buffer $0 \cdot 05$ M, pH $5 \cdot 5$, containing 900 mg NaCl and 100 mg CaCl$_2$/100 ml.

Pepsin was added in the proportion of 10 μg/mg lyophilized powder. The mixture was incubated at 37°C for $2 \cdot 25$ hr in glycin buffer $0 \cdot 2$ M, pH $1 \cdot 9$.

Controls were incubated in the same conditions but without enzyme. Some materials in

solution with labelled B$_{12}$ were dialysed at room temperature for 24 hr against a solution of urea 8 M or guanidine 5 M, then for 24 hr against veronal buffer 0·025 M, pH 8·2. Controls consisted of materials dialysed for 48 hr against veronal buffer 0·025 M, pH 8·2.

RESULTS

Vitamin B$_{12}$-binders in various body fluids

Gastric juice. The autoradiographic pattern of gastric juice changed with the immune serum used. Immunoelectrophoretic analysis revealed a maximum of three radioactive lines, labelled 1, 2 and 3 (Fig. 1) and in the Ouchterlony technique a maximum of two radioactive lines was visible (Fig. 2).
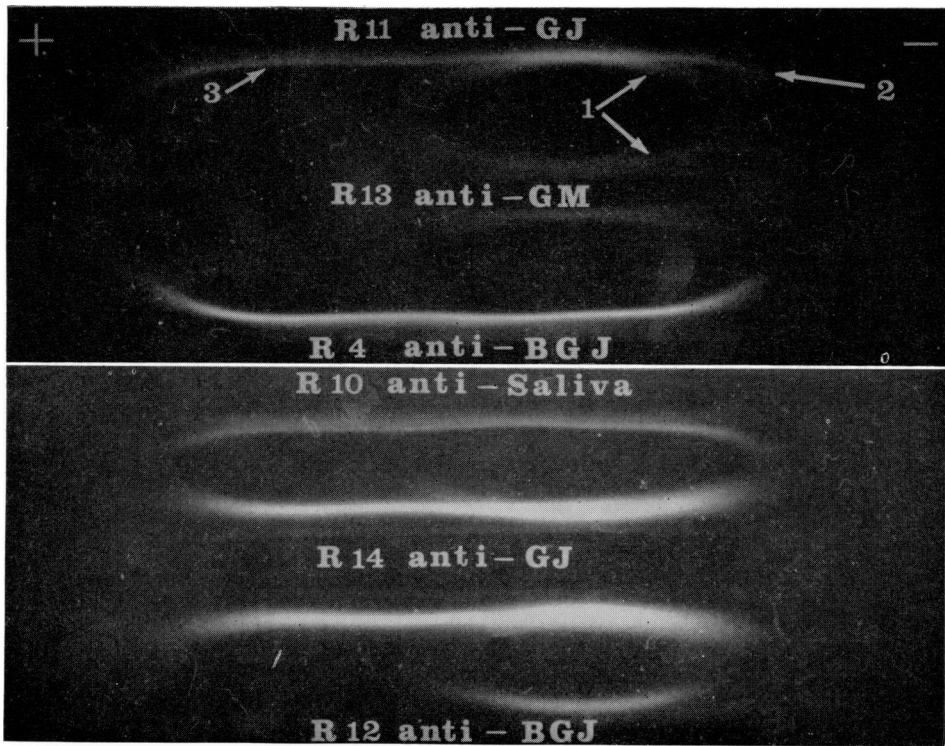

FIG. 1. Immunoelectrophoretic autoradiographs of buffered gastric juice developed by six different rabbit antisera (R 4, R 10, R 11, R 12, R 13 and R 14). Binders 1, 2 and 3 are indicated with arrows. GJ, Gastric juice; GM, gastric mucosa extract; BGJ, buffered gastric juice.

Three anti-gastric juice sera (3, 11 and 14), two anti-buffered gastric juice sera (4 and 12) and one anti-gastric mucosa serum (13) showed line 1. Two anti-saliva sera (10 and 22) never revealed this line regardless of the concentration of the antigen or antiserum. The curvature of line 1 was less symmetrical in gastric juice than in buffered gastric juice. Line 1 had no identity with lines 2 and 3, crossing these two lines in immunoelectrophoresis and Ouchterlony technique (Figs. 1 and 2a).

All the anti-gastric juice, anti-saliva and anti-gastric mucosa sera revealed line 2. With some antisera this line was superimposed on line 1 and could be separated only by varying the concentrations of antigen and antiserum. Line 2 had a β mobility with different lengths in buffered and non-buffered gastric juice, the first being long, spreading 14–17 mm from the antigen well, the second short, spreading 8 mm from the antigen well (Fig. 3b and c).

The two anti-saliva sera and only four anti-gastric juice sera showed line 3. This line had an $\alpha_1-\alpha_2$ mobility and the same curvature in non-buffered and buffered gastric juice.

The lines 2 and 3 in immunoelectrophoresis always presented a pattern of identity with a unique double curved line without a spur. In the Ouchterlony technique the components 2 and 3 could not be differentiated and formed one unique precipitation line.
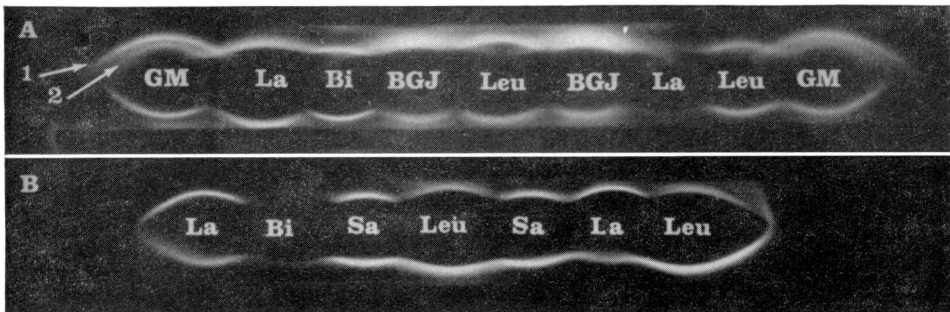

FIG. 2. Autoradiography of double diffusion test in agar. In (A) gastric mucosa extract (GM), lactoserum (La), bile (Bi), buffered gastric juice (BGJ) and leucocytic extract (Leu) are developed by rabbit serum against buffered gastric juice (on top) and by rabbit serum against gastric juice (below).

In (B) lactoserum (La), bile (Bi), saliva (Sa) and leucocytic extract (Leu) are developed by rabbit serum against buffered gastric juice (on top) and by rabbit serum against gastric juice (below).

Saliva. One radioactive double curved line was revealed with four anti-gastric juice sera and two anti-saliva sera (Fig. 3d). The line had the same position as the lines 2 and 3 in gastric juice. Line 2 was always long. The anti-gastric mucosa serum and one anti-buffered gastric juice serum (12) revealed only the line 2.

Isolation of components corresponding to precipitation lines 2 and 3 was attempted. Saliva was chromatographed on DEAE-cellulose according to Method 1. Three fractions were obtained (Fig. 4a). After lyophilization each of them was dissolved in a solution of radioactive vitamin B_{12} and analysed by autoradiographs. In immunoelectrophoresis, the first fraction showed no radioactive line. The second one showed one radioactive line of β-mobility corresponding to the line 2. The third one showed one line of $\alpha_1-\alpha_2$ mobility corresponding to line 3. These last two fractions analysed in the Ouchterlony technique with two anti-gastric juice sera (11 and 14) each showed one radioactive line with a pattern of complete identity between them (Fig. 5).

Bile, lactoserum, extract of leucocytes. They showed one radioactive line in immuno-electrophoresis with anti-gastric juice and anti-saliva sera, but no line with anti-gastric mucosa serum. The line was short beneath the antigen well (Fig. 3e, f and g).

Serum. Serum showed one radioactive line at the limit of visibility, but only in double diffusion test and concentrated eight-fold in a 14-μl well.
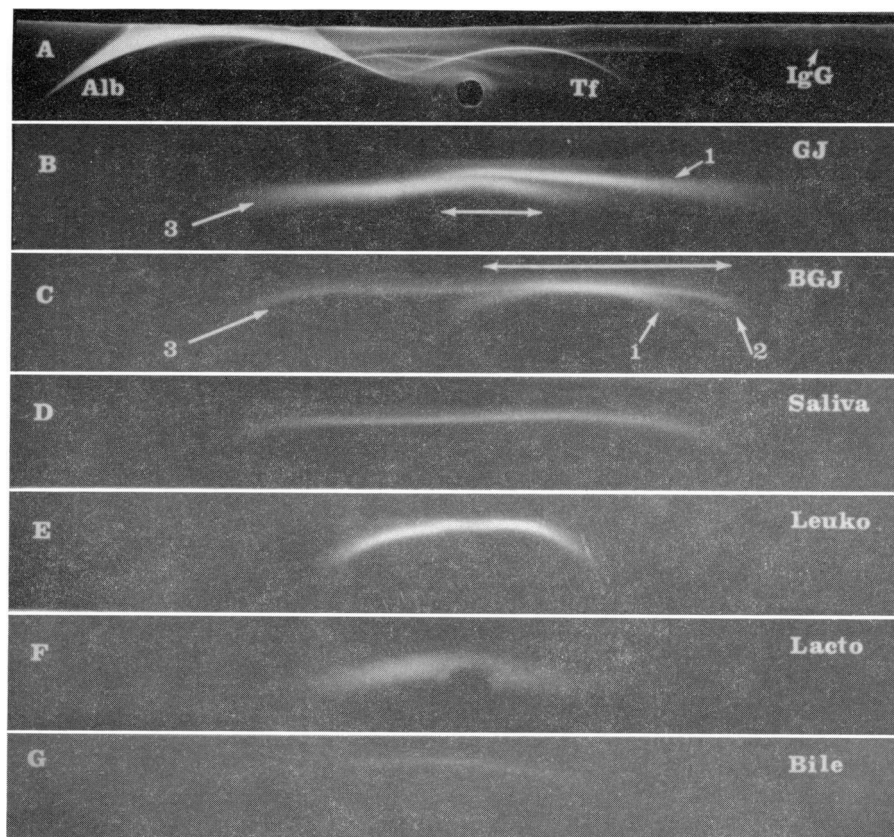

FIG. 3. Immunoelectrophoretic autoradiographs of gastric juice (GJ), buffered gastric juice (BGJ), saliva, leucocytic extract (Leuko), lactoserum (Lacto) and bile. In (B) and (C) the horizontal arrows indicate the different lengths of binder 2. In (A) Amido Black stained immunoelectrophoresis of serum for comparison of electrophoretic mobilities. Alb, Albumin; Tf, transferrin; IgG, immunoglobulin G.

Gastric mucosa extract. The radioactive lines 1 and 2 were visible. Extract of gastric mucosa was chromatographed on DEAE-cellulose, according to method 1 and 2, in an attempt to separate components 1 and 2 and to eventually discover a component 3.

With method 1, three fractions were obtained (Fig. 4b). The radioactive line 1 was found in the second fraction only. Radioactive line 2 was found in the first and second fraction and in one chromatography in the third fraction as well. With method 2, the elution pattern was as illustrated in the diagram of Fig. 4c. Five fractions were analysed. The radioactive lines 1 and 2 were found in the first fraction only. None of the fractions obtained with either of the two methods contained component 3.

The radioactive lines did not correspond to precipitation lines stained by Amido Black or with esterase activity in any of the analysed materials.

Comparison of the binders from various fluids

Gastric mucosa extract, saliva, bile, leucocytes extract, lactoserum and gastric juice were compared in double diffusion test in agar. Four different anti-gastric juice sera developed line 1 only in buffered gastric juice and gastric mucosa extract (Fig. 2a). In all the materials tested, they developed one line corresponding to components 2 and 3 with a pattern of complete identity between all these lines (Fig. 2b).
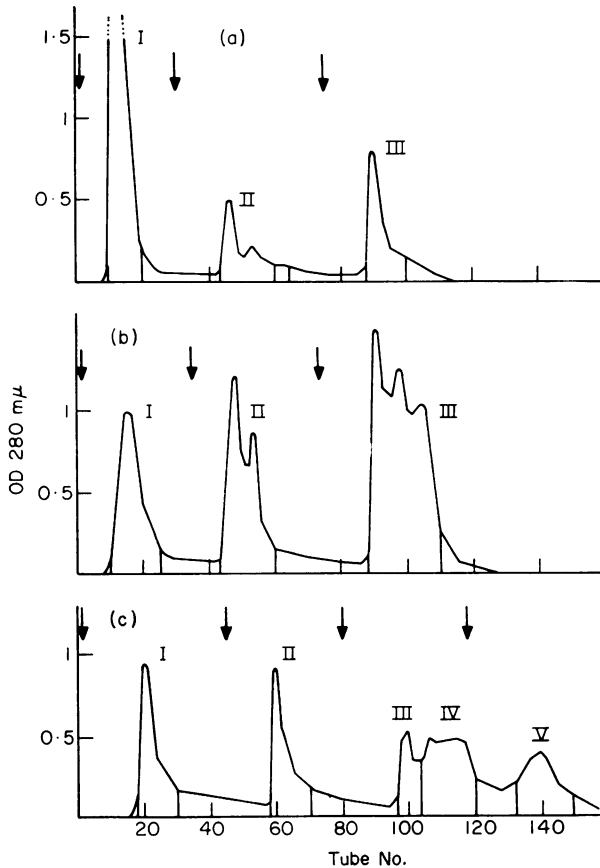

FIG. 4. DEAE-cellulose chromatography. (a) Elution diagram of saliva. (b) Elution diagram of gastric mucosa extract according to method 1. (c) Elution diagram of gastric mucosa extract according to method 2. Fractions were eluted by a stepwise gradient. Buffers were changed as indicated by the arrows (details in 'Materials and methods').

The component 2 isolated from gastric mucosa by DEAE-cellulose chromatography, and 2 and 3 isolated from saliva by DEAE-chromatography were compared in double diffusion test in agar. They showed a complete immunological identity (Fig. 6).

Quantitation of components 1, 2 and 3 was made by double diffusion test in agar. Each material was used in serial dilution with a constant concentration of antiserum. For each material, the concentration at which the radioactive lines were sharp and at an equal distance from the antigen well, and the last concentration at which the antigen was just

detectable were determined. The concentration of component 2 was the same in saliva and gastric juice, but 140 times lower in serum. In lactoserum the concentration of component 2 was sometimes identical and sometimes four times more than the concentration in saliva. In bile the concentration of component 2 was sometimes identical and sometimes four times less than the concentration in saliva.

Analogous comparisons of saliva and gastric juice were made by immunoelectrophoretic analysis which indicated that component 2 was two to four times more concentrated than component 3.
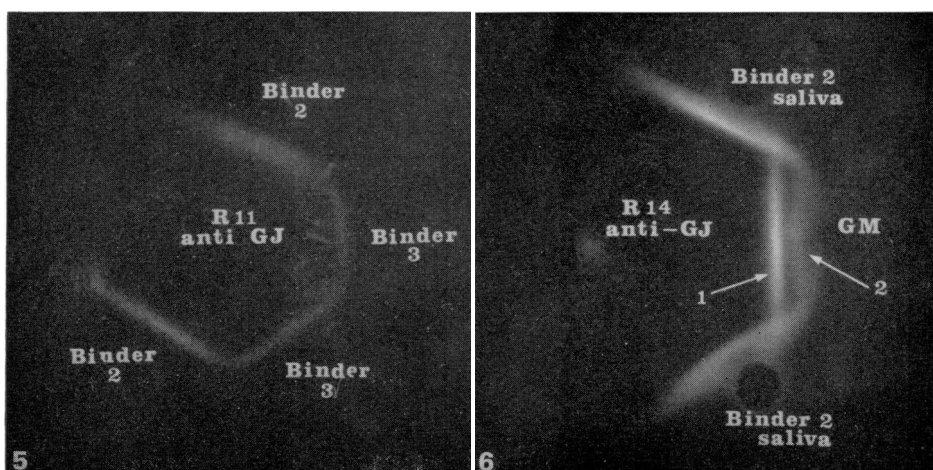

FIG. 5. Autoradiography of double diffusion test in agar. In central well rabbit serum 11 against gastric juice (R 11 anti GJ). In peripheral wells, fractions from DEAE-cellulose chromatography of saliva containing binder 2 (Binder 2) and binder 3 (Binder 3). The materials from two different chromatographies are compared.

FIG. 6. Autoradiography of double diffusion test in agar. In central well, rabbit serum 14 against gastric juice (R 14 anti-GJ). In peripheral wells, fractions from DEAE-cellulose chromatography of saliva containing binder 2 (Binder 2 saliva) and fraction from DEAE-chromatography of gastric mucosa extract containing binders 1 and 2 (GM).

Three anti-gastric juice sera were absorbed with various amounts of salvia, buffered and non-buffered gastric juice. These antisera were tested by immunoelectrophoresis with saliva. The radioactive lines 2 and 3 disappeared when antisera were absorbed with the same quantity of the three materials. Thus, these results confirmed previous findings.

The concentration of component 1 was estimated by the same techniques. It was the same in buffered and non-buffered gastric juice, zero in saliva. Four different anti-gastric juice sera were absorbed by sixty-five times the amount of saliva necessary to suppress lines 2 and 3. However, these antisera were still able to reveal line 1 in gastric juice (Fig. 7).

Some characteristics of the B$_{12}$-binders

Fixation of vitamin B$_{12}$. Samples of buffered gastric juice with radioactive vitamin B$_{12}$ were dialysed for 5, 24, 48 and 72 hr before analysis by immunoelectrophoretic autoradiography. The pattern obtained was compared with those of a non-dialysed gastric juice.

Whatever the duration of dialysis, lines 1, 2 and 3 were identical to the lines of the undialysed gastric juice: same curvature, same position and same intensity of labelling. Although these experiments were not conducted with various concentrations of vitamin and the average association constant could not be calculated, they suggest that components 1, 2 and 3 bind vitamin B_{12} with the same affinity.
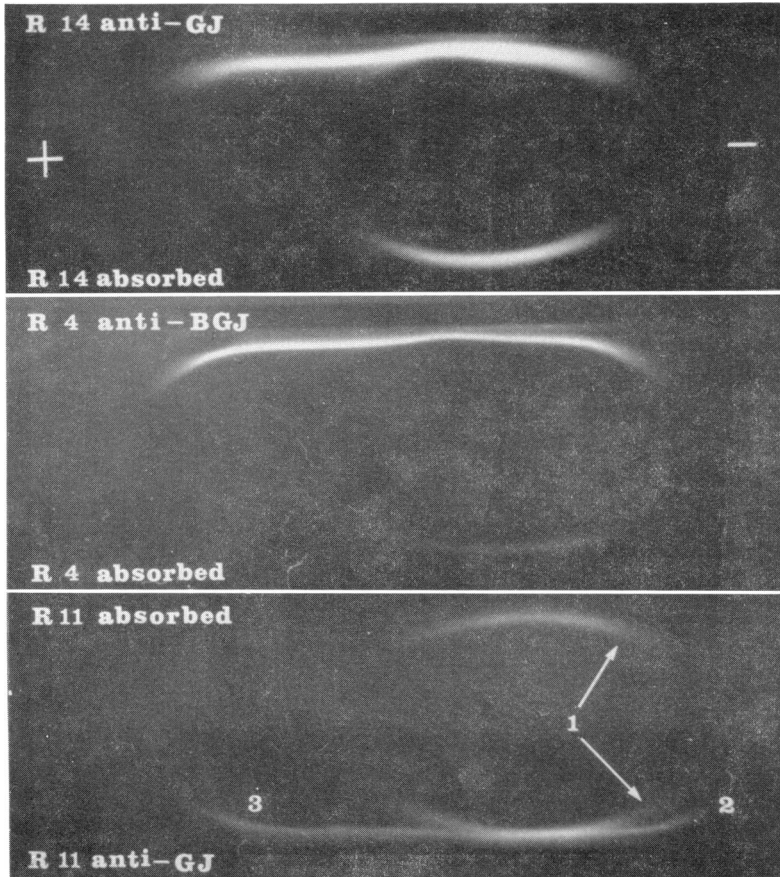

FIG. 7. Immunoelectrophoretic autoradiographs of buffered gastric juice. Buffered gastric juice is developed by three different antisera: rabbit serum 14 against gastric juice (R 14 anti-GJ), rabbit serum 4 against buffered gastric juice (R 4 anti-BGJ), rabbit serum 11 against gastric juice (R 11 anti-GJ) and by these three antisera absorbed with saliva (R 4, 11 and 14 absorbed).

Influence of pepsin. Comparison was made by immunoelectrophoretic autoradiography between gastric juice shielded from proteolysis by *in vivo* buffering and native gastric juice. Line 3 showed no difference, i.e. no spur and no mobility modification. Line 2 showed a difference in the length being long for buffered gastric juice and short for native gastric juice. Line 1 with a symmetrical curvature in buffered gastric juice, was asymmetrical in non-buffered gastric juice, spreading farther toward the anode and the cathode.

Similar comparison was made between control samples and samples treated *in vitro* with pepsin. Materials analysed were *in vivo* buffered gastric juice and saliva. One sample was first digested with pepsin then vitamin B_{12} was added, another sample was mixed first with vitamin B_{12} then digested with pepsin.
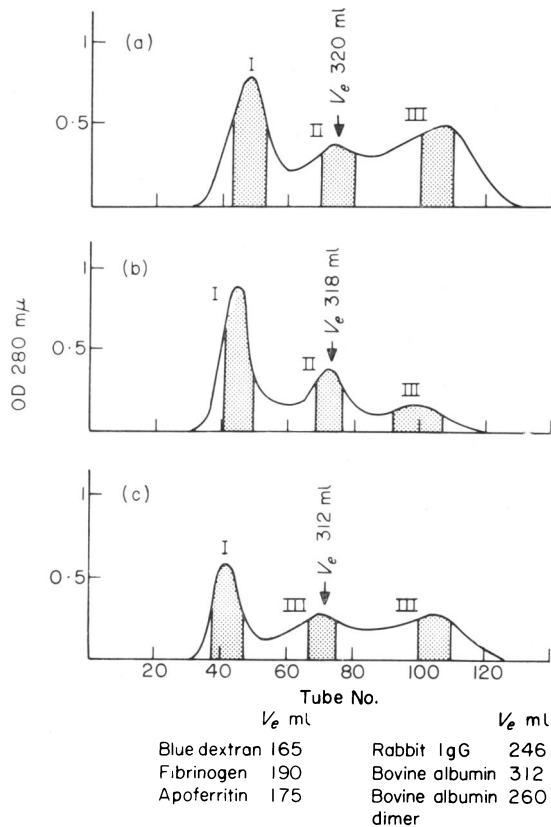
FIG. 8. Gel filtration on Sephadex G-200 (column size $2\cdot4 \times 110$ cm; flow rate 13 ml/hr; $4\cdot3$ ml/tube). (a) Filtration of peak III from a DEAE-cellulose chromatography of saliva (cf. Fig. 4a). (b) Filtration of peak II from a DEAE-cellulose chromatography of saliva (cf. Fig. 4a). (c) Filtration of peak I from a DEAE-cellulose chromatography of gastric mucosa extract according to method 2 (cf. Fig. 4(c)). Elution volumes (V_e) of products used for column calibration and of peaks containing vitamin B_{12}-binders are indicated. The dotted zones correspond to fractions which were lyophilized and to which vitamin B_{12}-labelled with ^{57}Co was added.

For saliva, there was no difference between the controls and treated materials: lines 2 and 3 had the same intensity of labelling, without any spur or modification of mobility in the treated samples. In the sample of buffered gastric juice mixed first with vitamin B_{12}, the lines 1, 2 and 3 were similar to those of control. In the sample digested first with pepsin, lines 2 and 3 were similar but line 1 was at the limit of visibility.

Influence of neuraminidase. Buffered gastric juice and gastric mucosa extract mixed with vitamin B_{12} were digested with neuraminidase. For gastric mucosa extract there was no

difference between treated sample and control: lines 1 and 2 had the same mobility and intensity of labelling. For buffered gastric juice line 1 was identical to those of control, but line 2 was labelled stronger and line 3 was not visible.

Influence of urea and guanidine. Samples of buffered gastric juice with radioactive vitamin B_{12} were dialysed against urea or guanidine solutions, then against veronal buffer. Immunoelectrophoretic autoradiographs were made with three different antisera. The labelled lines were identical in treated and control samples.

Molecular weight. Three samples isolated by DEAE-chromatography were filtered on Sephadex G-200. One contained binder 2 of saliva (peak II of the DEAE-chromatography of saliva), the second binder 3 (peak III of the DEAE-chromatography of saliva), the third one binders 1 and 2 of gastric mucosa (peak I of the chromatography of gastric mucosa, method 2). Each sample gave the same elution pattern: three protein peaks were obtained (Fig. 8). Each of them was dialysed, lyophilized, and after addition of a solution of radioactive vitamin B_{12} analysed by autoradiography of immunoelectrophoresis.

For the first sample, the radioactive line corresponding to binder 2 was found only in the second peak. In the second sample, the radioactive line corresponding to binder 3 was found only in the second peak. In the third sample, two radioactive lines corresponding to binders 1 and 2, were found in the second peak; no radioactive line was seen in the first or third peak. All these peaks containing B_{12}-binders had the same elution volume (respectively, 318, 320 and 312 ml) and corresponded, according to the calibration curve, to proteins of 60,000 molecular weight.

DISCUSSION

The immunological techniques used confirmed the multiplicity of human B_{12}-binders (Glass, Uchino & Schwartz, 1962; Glass, 1963; Simons, 1964). Three B_{12}-binders were revealed by various antisera.

Binder 1 was present in gastric mucosa and gastric juice, absent in serum, lactoserum, saliva, bile and leucocytic extract, and had a β_2 electrophoretic mobility. Moreover, it was always absent in gastric juice of patients with pernicious anaemia (to be published). Thus, this binder corresponds certainly to intrinsic factor and is similar to slow binder (S) described in immunoelectrophoresis by Gullberg & Kistner (1962), Simons & Gräsbeck (1963), Simons (1964) and Gullberg (1966).

Binders 2 and 3 were immunologically identical and formed a unique double curved radioactive line, one curve of β-mobility, the other of $\alpha_1-\alpha_2$ mobility. These binders were found in serum, saliva, bile, lactoserum, leucocytic extract and gastric juice. They were often present in gastric juice of patients with pernicious anaemia (to be published). Thus, they correspond certainly to B_{12}-binders without IF activity and to the rapid component (R) described by Simons & Gräsbeck (1963) and by Simons *et al.* (1964). Simons (1964) noticed the heterogeneity of component R. From the present results it seems constituted of at least two components, 2 and 3, immunologically identical but distinguished by their different electrophoretic mobility, elution on DEAE-cellulose, and concentration in various fluids. It was proved that these binders enhanced the uptake of vitamin B_{12} by rat liver homogenates (Simons, Kvist & Sauren-Linfors, 1966).

The presence of binder 2, but not of binder 3 in gastric mucosa; the appearance of binder 3, and often the modification of binder 2 in gastric juice not shielded from proteolysis

by buffer, could suggest that binder 3 is not synthesized as such, but is the result of a modification of binder 2 by some proteolytic enzymes. Peptic proteolysis of buffered gastric juice and saliva was made *in vitro*. It did not cause any modification of B_{12}-binder 2, as judged by fixation of vitamin B_{12} and immunological properties. Thus, although the difference in length of line 2 in buffered and native gastric juice indicated some action of peptic digestion on binder 2, it seems that more than just pepsin is needed for transformation of binder 2 into binder 3. Treatment with neuraminidase which suppressed binder 3 and re-inforced labelling of binder 2 could indicate that this transformation is due to attachment of sialic acid molecules on binder 2.

It was demonstrated that peptic digestion also altered intrinsic factor (Gräsbeck, 1956; Simons, 1964; Uchino *et al.*, 1964b). This factor was transformed into a material which still had an intrinsic factor activity (Simons *et al.*, 1966) but had a different electrophoretic mobility and was called intermediate binder by Gräsbeck (1956) or secondary binder by Glass *et al.* (1962). A comparison between native gastric juice and buffered gastric juice showed modifications of line 1 in native gastric juice which suggested peptic action on binder 1. In experiments of proteolysis *in vitro*, if the incubation with pepsin was made after mixing with vitamin B_{12}, binder 1 showed no modification. If the incubation with pepsin was made first, the binding of vitamin B_{12} was extremely diminished. From the present results, it is possible to assume that binder 1 is sensitive to peptic digestion, but not after previous saturation with vitamin B_{12}. This fact was also mentioned by Uchino *et al.* (1964b) and Gräsbeck (1967) who demonstrated that B_{12}–IF complexes are less susceptible to denaturation and peptic digestion than free intrinsic factor.

The immunological techniques used showed that binder 1 (IF) was susceptible to peptic digestion, however they did not show the result of this digestion, namely the intermediate binder. However, this binder exists without any doubt since it was demonstrated by chromatography (Gräsbeck *et al.*, 1965), immunoelectrophoresis (Simons, 1964) and paper (Glass *et al.*, 1962; Uchino *et al.*, 1964a) and starch block electrophoresis (Gräsbeck, 1956). It is possible that antigenic determinants of intrinsic factor revealed by antisera, are not present on the intermediate binder. The part of the molecule carrying these antigenic determinants would be small since there is not a great difference in molecular weight between intermediate binder and intrinsic factor (Gräsbeck *et al.*, 1966). Another explanation might be that the extension of line 1 in native gastric juice is already a sign of new molecules (intermediate binder) with a complete immunological identity with binder 1, but with a slight difference in electrophoretic mobility.

The three binders have the same molecular weight of about 60,000 as judged by the volume of exclusion on Sephadex G-200. This volume was calculated on the peak of optical density. The product in which B_{12}-binders were demonstrated contained materials on both sides of the peak and therefore corresponded to a molecular weight of $60,000 \pm 20,000$. This imprecision does not permit the detection of small differences, if they exist, between the binders. These results are in accordance with those of Chosy & Schilling (1963), Gräsbeck *et al.* (1966) and Garrido-Pinson *et al.* (1966). Values greater than 100,000 given by some authors (Kubo *et al.*, 1965; Rothenberg, 1966a) probably correspond to polymers.

None of the binders corresponded to a component with esterase activity, as reported by Burtin, Vendrely & Rapp (1964). This discrepancy can be explained; with some concentrations of antiserum the precipitation line of esterase is superposed in part with binder

1 or 2. It is only with the technique of interrupted troughs or by variation of the amount of antiserum that a dissociation of these lines can be shown.

With the antisera used, no immunological identity was demonstrated between intrinsic factor (binder 1) and the other binders. It was demonstrated by Roitt, Doniach & Shapland (1964) and Samloff & Turner (1967) that intrinsic factor can elicit two kinds of antibodies. One is directed against the non-binding part of the binder, the other directed against the binding site of the binder and able to block the vitamin B_{12}-binding (blocking antibodies). Autoradiographs of immunoelectrophoresis and Ouchterlony tests revealed the antibodies against the non-binding part of the binders. They also revealed blocking antibodies only if antiserum reacted with B_{12}-binders before the addition of [^{57}Co]vitamin B_{12} (Samloff & Barnett, 1965). However, in this instance the labelled vitamin B_{12} could not combine with the binder and thus there was no radioactive line. Therefore, the immunological techniques used revealed only the antigenic determinants of the nonbinding part of the binders and did not demonstrate similarities and differences of the binding part of the binders. Dialysis experiments did not prove a complete identity between the binding sites of the three binders; they suggested a similarity since binding affinity of the three components seemed equal. Moreover, urea or guanidine treatment was unable to release vitamin B_{12} from the binders. However, finer methods than dialysis or denaturation as binding competition between vitamin B_{12} and pseudovitamin (Bunge & Schilling, 1957) would perhaps show differences between binding sites of the three binders.

The large amount of non-IF binder in gastric juice or gastric musoca with same electrophoretic mobility, same molecular weight and same binding properties as the intrinsic factor is of practical interest. It would be necessary in a determination of antibodies against intrinsic factor (Samloff & Barnett, 1965; Abels *et al.*, 1963), or in a detection of intrinsic factor with antibodies (Jeffries & Sleisenger, 1963; Rothenberg, 1966b), to prove that antibodies are specific for intrinsic factor and not also directed against the other non-IF binders.

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