Comparative Studies on Intrinsic Factor and Cobalophilin in Different Parts of the Gastrointestinal Tract of the Pig

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The vitamin B₁₂ binders in the pig pyloric mucosa gastric and intestinal juice from the upper gastrointestinal tract were fractionated into only two molecular forms, classified as intrinsic factor and cobalophilin. The unsaturated vitamin B₁₂-binding power due to cobalophilin was lower in the intestinal than in the gastric juice. Electrofocusing revealed that intrinsic factor and cobalophilin in the intestinal juice contained more of the 'neutral'-type isoproteins, and the suggestion is made that this is due to enzyme activity. The gastric-juice intrinsic factor contained more acidic isoproteins, which supports the hypothesis that carbohydrate is added on to the polypeptide chain of this protein before it is secreted into gastric juice. The gastric- and intestinal-juice cobalophilins, studied also by electrofocusing, differed from that of pyloric mucosa and they appeared to be of salivary origin. With regard to molecular dimensions there was no significant difference between the intrinsic factors and cobalophilins from all sources studied. All cobalophilins had molecular weights by the formula of Svedberg of approx. 92 500, Stokes radii of 4.62 nm and sedimentation coefficients of 5.15 S. The corresponding values for the intrinsic factors were 63 600, 3.57 nm and 4.38 S. In addition, the intrinsic factors exhibited similar avidities for binding to the solubilized ileal intrinsic-factor receptor. Also the intrinsic factors and cobalophilins, irrespective of their source, bound to the analogous specific xenoantibodies with the same avidity. The present results demonstrate that intrinsic factor remains practically unaltered during its passage through the proximal intestine and render unlikely the speculations made about the presence of an endogenous binder for intrinsic factor as well as the existence of a 'pancreatic intrinsic factor'. In addition, they are compatible with the theory that the interference by undegraded cobalophilin may be the reason for the abnormal vitamin B₁₂ absorption observed in patients with pancreatic insufficiency.

The protein-bound vitamin B₁₂ is first liberated from foods by enzymic digestion (Schade & Schilling, 1967) in the gastrointestinal tract. Intrinsic factor, the apparent exclusive function of which is to mediate vitamin B₁₂ absorption by the ileum, is secreted by the parietal cell of the stomach mucosa (Hoedemaker *et al.*, 1964), and thereafter binds the vitamin competitively. Cobalophilin (Stenman, 1974a) (also called 'R protein', 'non-intrinsic factor' etc.) (Simons, 1964) is present in the gastric juice and it also binds the vitamin. The vitamin B₁₂-intrinsic-factor complex is very resistant to digestion (Gräsbeck & Salonen, 1976) and affords considerable protection against vitamin B₁₂ uptake by bacteria (Ternberg & Eakin, 1949), and it therefore survives the passage down to the ileum where the complex binds to

specific membrane receptors (Donaldson *et al.*, 1967). For this latter process, Ca²⁺ and a pH around neutrality are required (Donaldson *et al.*, 1967). It has been suggested that by binding the vitamin, cobalophilin also prevents the vitamin B₁₂ from being taken up by bacteria (Gullberg, 1973).

So far, very little is known about the chemistry, physiology and pathophysiology of the phenomena accompanying various discrepancies occurring during the vitamin B₁₂ transport and especially its absorption; e.g. it has been well documented that abnormal absorption of vitamin B₁₂ sometimes occurs in patients in pancreatic insufficiency (McIntyre *et al.*, 1956; Perman *et al.*, 1960; Veege *et al.*, 1962; Toskes *et al.*, 1971; von der Lippe *et al.*, 1976a). A number of explanations have been offered to account for this, including low pH because of insufficient

Abbreviation used: IgG, immunoglobulin G.

bicarbonate output from the pancreas (Veeger *et al.*, 1962), bacterial attack (Toskes & Deren, 1972) etc. However, each of these notions has not withstood the test of repetitive or experimental confirmation (Veeger *et al.*, 1962; Toskes *et al.*, 1971; Toskes & Deren, 1972). Furthermore, previous (Cheney, 1936) and more recent reports (Toskes *et al.*, 1971) speculated that the pancreatic juice is endowed with intrinsic-factor activity, or that an endogenic binder able to bind the intrinsic-factor-vitamin B₁₂ complex converts the complex into a non-absorbable form (Toskes & Deren, 1973) (see also the Discussion section).

To provide further evidence for the validity of the above and also other (von der Lippe *et al.*, 1976a) observations and especially to clarify the nature and the hypothetical interference or effect of other endogenic factors on the vitamin B₁₂ transport and absorption occurring in the gastrointestinal tract, the best approach would be the identification of structural, chemical or functional differences or similarities occurring with these proteins during their passage through the intestinal lumen. Such comparative studies on these proteins, including widely accepted criteria for protein comparison, are reported here for the first time. The results will rule out each one of the above-mentioned speculations, and provide evidence that intrinsic factor remains actually unaltered from the point of its synthesis up to the ileal absorptive surface, where this protein exerts its biological function. Finally, and in line with other more recent reports (Cotter *et al.*, 1977; Allen *et al.*, 1977), the suggestion will be made that the phenomena described above can be attributed to the uncontrolled interference of the other vitamin B₁₂-binding proteins lacking intrinsic-factor activity, i.e. cobalophilins, which may occur under non-physiological conditions.

Experimental

Materials

Extracts. Extracts of vitamin B₁₂ binders from pyloric mucosa and intrinsic-factor receptor from ileum were prepared as described (Marcoullis *et al.*, 1977; Marcoullis & Gräsbeck, 1977). Pig gastric juice was collected from several pigs by stimulation with either Histalog (0.5 mg per kg body weight), pentagastrin (6 µg per kg body weight) or carbamoylcholine chloride (12.5 µg per kg body weight) or by a combination of all three (Vatn *et al.*, 1975). The juice was collected through a nasogastric tube and immediately depepsinized by pH adjustments (Gräsbeck, 1956).

Gastric and intestinal juice were collected simultaneously from two pigs. An hour after a stimulation performed as described above, the pig was killed by a blow on the head. The abdomen was opened and within 3 min the gastric succus and the proximal

intestine starting from the pyloric sphincter and measuring 2.5 m were isolated by the placement of double silk ligatures at the appropriate intervals, i.e. gastric end of oesophagus, pyloric sphincter and the ligament of Treitz. The stomach was opened along the lesser curvature and the intestinal segment longitudinally. Their contents were then aspirated separately into bottles containing 25 ml of 0.9% NaCl, and depepsinized (Gräsbeck, 1956). The vitamin B₁₂-binding power was assayed and the two pools were stored at -20°C.

To decrease the possibilities of polymer formation, extracts containing high concentrations of intrinsic factor, e.g. the pyloric one (Marcoullis *et al.*, 1977), were routinely diluted with 0.1 M-phosphate buffer, pH 7.4, containing 0.15–0.3 M-NaCl. In the present case the final concentration of intrinsic factor was adjusted to correspond to 10–25 ng of vitamin B₁₂/ml of extract.

Antisera. Anti-(pig cobalophilin) and anti-(pig intrinsic factor) antibodies were produced in rabbits by using a leucocyte preparation (Gräsbeck & Aro, 1971) and purified pyloric intrinsic factor as antigens respectively (Marcoullis *et al.*, 1977; Marcoullis & Gräsbeck, 1977). Serum was also collected from a pernicious-anaemia patient known to have antibodies against human and pig intrinsic factor. It bound (per ml) 86 ng of vitamin B₁₂ bound to pig intrinsic factor. The unsaturated vitamin B₁₂-binding power of the sera was saturated with non-radioactive vitamin B₁₂.

Radioactive compounds. [⁵⁷Co]- and [⁵⁸Co]-Cyanocobalamin, [¹²⁵I]iodide and ³H₂O were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Purifications. Cobalophilin and intrinsic factor complexed to radioactive vitamin B₁₂ were purified (Marcoullis *et al.*, 1977; Marcoullis & Gräsbeck, 1977) by using specific immune sera and gel filtration. The vitamin B₁₂ binder not required in an experiment was removed as its immunocomplex with a specific antibody in the totally excluded volume (*V*₀) of Sephadex G-200, and the other, desirable, one was recovered as a well-defined peak in the included volume. When used for preparative purposes, the pyloric and gastric or intestinal juice from which intrinsic factor or cobalophilin was purified were not presaturated with radioactively labelled vitamin B₁₂. The free vitamin B₁₂-binding capacity was assayed in the fractions produced by gel filtration, and the peak of the free desirable vitamin B₁₂-binding protein was pooled and stored at -20°C. To remove a possible undesirable excess of anti-(intrinsic factor) or anti-cobalophilin antibodies added during incubation and which could be eluted together with the purified vitamin B₁₂-binding proteins from Sephadex G-200, the first incubation mixture was often incubated with anti-(rabbit IgG) serum (Orion, Helsinki, Finland) before gel filtration.

Electrofocusing. Sucrose gradients (0–50%, w/v) with 1.2% (w/v) Ampholine (LKB, Stockholm, Sweden) were prepared in 140-ml LKB standard columns thermostatically maintained at +4°C. Ampholines of pH range 3.5–7 and 2.5–6 were used for focusing intrinsic factors and cobalophilins respectively. A current was applied at 470 V for 4 days. Fractions (2 ml) were collected and the pH was measured at room temperature.

Gel-exclusion chromatography. Sephadex G-200 (Pharmacia, Uppsala, Sweden) was packed in columns 2.5 cm × 100 cm. The flow rate was 10 ml/h and fractions (1.2 ml) were collected at +4°C. The buffer was 0.1 M-phosphate (pH 7.4)/0.15 M-NaCl, except when the ileal intrinsic-factor-receptor extract was studied, where the buffer was 0.05 M-Tris/HCl (pH 7.4)/0.15 M-NaCl/0.02% NaN₃/1 mM-Ca²⁺/0.05% (v/v) Triton X-100/0.4 mM-phenylmethanesulphonyl fluoride (Sigma, St. Louis, MO, U.S.A.) as an anti-proteolytic agent (Marcoullis, 1977).

Density-gradient ultracentrifugation. The 12.2 ml 10–20% (w/v) sucrose gradients were prepared in 0.1 M-phosphate buffer (pH 7.4)/0.15 M-NaCl. The samples contained in a similar buffer were run in a Beckman-Spinco preparative ultracentrifuge with the SW 41 rotor at +3°C and 38 000 rev./min for 46 h. Human serum albumin and IgG were used as markers. Fractions (70–75) were collected.

Other procedures. Vitamin B₁₂-binding power was assayed by using haemoglobin-coated charcoal (Gottlieb *et al.*, 1965). Protein was measured by the method of Lowry *et al.* (1951), with human serum albumin as the standard. Human IgG was purified (Steinbuch & Audran, 1969) from ordinary serum. [¹²⁵I]Iodide labelling by the method of Greenwood *et al.* (1963) with chloramine-T was used to label marker proteins of human origin; purified IgG, serum albumin (Kabi, Stockholm, Sweden) and thyrotropin (kindly provided by the Pituitary Agency, National Institutes of Health, Bethesda, MD, U.S.A.). For calibrating the Sephadex G-200 columns, Blue Dextran 2000 (Pharmacia) and ³H₂O were used.

Calculations

Sedimentation coefficients were estimated by the method of Martin & Ames (1961). Preliminary runs were carried out indicating that, under the conditions of our experiments, ¹²⁵I-labelled and unlabelled human serum albumin or IgG, sedimented with unchanged rates in their monomeric form. Subsequently all experiments were carried out with the ¹²⁵I-labelled marker.

Stokes radii. Stokes radii were computed from the experimental K_D values by using the formula of Ackers (1964). As described previously (Marcoullis *et al.*, 1977), a desk computer was programmed to compute the K_D of any a/r value. Thus the effective

pore radius, r , of the column was first estimated by using the K_D of the marker protein, i.e. human serum albumin, and its known (Ackers, 1964) Stokes radius of 3.61 nm. Subsequently the Stokes radius of the unknown could be estimated, since the computed value of r was constant (the marker proteins, ¹²⁵I-labelled IgG, human serum albumin and thyrotropin were routinely used as marker proteins and gel-filtered simultaneously with the protein under study).

Other parameters. Diffusion coefficients were computed by the formula of Siegel & Monty (1966) by using the corresponding Stokes radii from the gel-filtration data. Subsequently the frictional ratios were computed (Marcoullis *et al.*, 1977; Marcoullis & Gräsbeck, 1977; Siegel & Monty, 1966) by using the $D_{20,w}$, $S_{20,w}$ and the known partial specific volumes (Allen & Mehlmán, 1973b) of pig intrinsic factor (0.714 ml/g) and cobalophilin (0.700 ml/g).

Molecular weights. First the 'molecular weights' by gel filtration were estimated by the method of Andrews (1962). The molecular weights were computed (Marcoullis *et al.*, 1978a) by a programmed desk computer from the slope of the line of the log(molecular weight) versus the experimental K_D values. Molecular weights were also computed by the formula of Svedberg by using the $D_{20,w}$, $S_{20,w}$ and the known partial specific volumes of the pig intrinsic factor and cobalophilin (Siegel & Monty, 1966; Allen & Mehlmán, 1973b).

Multiple-isotope counting. For comparative purposes the vitamin B₁₂-binding proteins from gastric and intestinal juice and pylorus were saturated with vitamin B₁₂ labelled with ⁵⁷Co and ⁵⁸Co respectively, and studied as mixtures of the two. In all experiments, ¹²⁵I-labelled marker proteins were simultaneously used. The spillover counts in the channels of the other isotopes were determined, and a desk computer was programmed to compute the true rates for each isotope.

Results

Vitamin B₁₂-binding power

A pool of gastric juice from five pigs bound 56.1 ng of vitamin B₁₂/ml of original juice. The intestinal juice bound 43.6 ng of vitamin B₁₂/ml.

Immunological identification

As described above, the vitamin B₁₂-binding proteins in pyloric extract were saturated with vitamin B₁₂ labelled with ⁵⁸Co, and the gastric and intestinal juice were saturated with vitamin B₁₂ labelled with ⁵⁷Co. Excess vitamin B₁₂ was removed with haemoglobin-coated charcoal. The proteins were then studied as mixtures of the pyloric extract and the intestinal or gastric juice. To identify the type of the vitamin B₁₂-binding protein present, the mixtures

were incubated for 1 h at room temperature with immune sera and then filtered through Sephadex G-200. Rabbit anti-(intrinsic factor) and the pernicious-anaemia sera removed in the V_0 47%, 8.0% and 17% of the vitamin B₁₂-binding power of the pyloric extract, gastric juice and intestinal juice respectively, and anti-cobalophilin removed the rest. None of the stimulants used produced a clear change in the relative content of these two vitamin B₁₂-binding proteins in the gastric and intestinal juices.

Binding to specific antibodies

Mixtures consisting of equal amounts (10–50 ng of vitamin B₁₂-binding power of each) of purified pyloric, gastric-juice or intestinal-juice radioactive intrinsic factors or cobalophilins were incubated with rabbit anti-(intrinsic factor) or anti-cobalophilin sera (binding 10–50 ng of vitamin B₁₂ complexed to the intrinsic factor or cobalophilin) respectively, and then filtered through Sephadex G-200. The relative proportion of each individual intrinsic-factor preparation eluted as immunocomplexes in the V_0 ranged from 8% (when the maximum antigen concentration of 50 ng of vitamin B₁₂ complexed to each intrinsic-factor preparation, and the minimum amount of antibody binding 10 ng of vitamin complexed to intrinsic factor, were used), to 99.9% (when the minimum antigen concentration and the maximum antibody amount were used). Approximately similar estimations were made when cobalophilin and its homologous antibodies were studied. Equal relative amounts of intrinsic factors and cobalophilins were eluted in the V_0 , indicating that intrinsic factors and cobalophilins from these three sources possess the same avidity for their homologous antibodies.

Binding to the solubilized ileal intrinsic-factor receptor

Similar radioactive mixtures to those in the antibody studies (each vitamin B₁₂-binding protein having 0.4–0.8 ng of vitamin B₁₂-binding power) were mixed with 2 ml of ileal intrinsic-factor-receptor extract (having a receptor activity corresponding to 0.3 ng of vitamin B₁₂ bound to intrinsic factor) (Marcoullis & Gräsbeck, 1977) at pH 7.4, in the presence of 1 mM-Ca²⁺ and 0.05% Triton X-100, and incubated for 1 h. The mixture was filtered through Sephadex G-200. Only the intrinsic factors formed radioactive complexes eluted in the V_0 . As in the immunological studies described above, the relative proportions of intrinsic factors eluted as receptor complexes were estimated; they ranged from 18 to 29%. It was observed that equal relative amounts of intrinsic factors from all sources used were eluted as such receptor complexes, indicating that they had the same avidity for the receptor.

Electrofocusing patterns

Radioactive mixtures similar to those described above, were also studied by electrofocusing (Fig. 1). The following main isoproteins were observed (Table 1); for the cobalophilins of pylorus pI 3.88, 4.12, 4.28 and 4.68; of gastric juice pI 4.30, 4.50 and 4.71; of intestinal juice pI 4.11, 4.28 and 4.58; for the intrinsic factors of pylorus pI 4.60, 4.79, 4.92, 5.03 and 5.10; of gastric juice pI 4.61, 4.80, 5.08 and 5.20; and of intestinal juice pI 4.78, 5.03 and 5.15.

In general, the isoelectric-focusing patterns revealed approximately similar isoproteins for the intrinsic factors irrespective of their source. The same applied to the cobalophilins. However, there were statistical differences in their relative content between gastric juice and pyloric proteins; gastric juice contained more of the acidic intrinsic factor and more of the neutral cobalophilin isoproteins (Figs. 1a and 1c) than pyloric extracts; the intestinal-juice cobalophilin differed also from the pyloric protein and it was even more neutral than the corresponding proteins in gastric juice (Figs. 1c and 1d). Surprisingly, the intestinal intrinsic factor resembled the pyloric rather than the corresponding gastric-juice protein.

Gel-filtration patterns

Mixtures containing either intrinsic factors or cobalophilins from gastric or intestinal juice and pylorus were examined. The intrinsic factors and the cobalophilins from all three sources were eluted approximately with similar K_D values respectively (Table 2). Similar series of experiments were carried out with as chromatographic eluent the Triton X-100-containing buffer (see the Experimental section). None of the proteins under study changed its elution characteristics.

Preparative ultracentrifugation

Intrinsic factors and cobalophilins from gastric or intestinal juice and pylorus sedimented in a similar way, the cobalophilins after IgG and before human serum albumin, and the intrinsic factors approximately with albumin (Table 2).

Molecular dimensions

The Stokes radii, the molecular weights calculated from gel-filtration data and by the formula of Svedberg, the diffusion and sedimentation coefficients and the frictional ratios, were computed for the six proteins studied here (Table 2). The molecular characteristics of the intrinsic factors and cobalophilins were approximately similar.

Free intrinsic factor

Free intrinsic factors from gastric or intestinal juice and pylorus were found to be eluted from

Fig. 1. Electrofocusing patterns

Examples of simultaneous electrofocusing of intrinsic factors (a,b) or cobalophilins (c,d) with the analogous proteins from pyloric mucosa (a, b, c, d, ●), gastric (a and c, ○) and intestinal (b and d, □) juices, all complexed with vitamin B₁₂. Gastric-juice intrinsic factor (a, ○) contains relatively more acidic isoproteins, whereas the intestinal-juice intrinsic factor (b, □) contains about equal concentrations of acidic and neutral isoproteins. In contrast, gastric juice contains more of the neutral isoproteins of cobalophilin (c, ○), and intestinal juice (d, □) gives approximately the same pattern. Each curve represents an individual run. For detailed results concerning isoelectric points, see Table 1. The unlinked dotted line shows the pH gradient.

Table 1. Mean isoelectric points of the isoproteins of pyloric-mucosal, gastric-juice and intestinal-juice vitamin B₁₂-binding proteins complexed to vitamin B₁₂

Values in *italics* represent the isoelectric points of the main isoproteins. The standard deviations of the mean were, on average, 0.03 pH unit. The values in parentheses indicate times observed/number of runs. Abbreviations used: B₁₂-IF and B₁₂-CP, vitamin B₁₂ complexed to intrinsic factor and cobalophilin respectively.

Pyloric B ₁₂ -IF	4.20 (16/33)	4.60 (28/33)	4.79 (28/33)	4.92 (27/33)	5.03 (20/33)	5.10 (15/33)	5.20 (20/33)
Gastric-juice B ₁₂ -IF	5.41 (30/33)	5.60 (28/33)	5.79 (11/33)	5.89 (10/33)	6.16 (4/33)		
Intestinal-juice B ₁₂ -IF	4.20 (5/8)	4.39 (6/8)	4.61 (8/8)	4.80 (8/8)	4.93 (5/8)	5.00 (4/8)	5.08 (7/8)
Pyloric B ₁₂ -CP	5.20 (7/8)	5.45 (6/8)	5.59 (4/8)	5.78 (2/8)	5.94 (2/8)	6.11 (2/8)	
Gastric-juice B ₁₂ -CP	4.21 (7/10)	4.39 (10/10)	4.59 (10/10)	4.78 (9/10)	4.90 (7/10)	5.03 (9/10)	5.15 (9/10)
Intestinal-juice B ₁₂ -CP	5.30 (8/10)	5.47 (8/10)	5.64 (5/10)	5.83 (5/10)			
Pyloric B ₁₂ -IF	3.31 (6/15)	3.50 (4/15)	3.63 (7/15)	3.88 (11/15)	4.12 (10/15)	4.28 (12/15)	4.46 (9/15)
Gastric-juice B ₁₂ -CP	4.68 (10/15)	5.02 (7/15)	5.20 (6/15)				
Intestinal-juice B ₁₂ -IF	3.52 (2/8)	3.63 (4/8)	3.83 (5/8)	4.10 (8/8)	4.30 (7/8)	4.50 (6/8)	4.71 (8/8)
Pyloric B ₁₂ -CP	4.98 (6/8)	5.41 (5/8)					
Gastric-juice B ₁₂ -CP	3.52 (3/7)	3.64 (3/7)	3.89 (7/7)	4.11 (6/7)	4.28 (7/7)	4.42 (6/7)	4.58 (6/7)
Intestinal-juice B ₁₂ -CP	4.77 (5/7)	5.02 (4/7)	5.22 (7/7)				

Table 2. *Molecular parameters*

Intrinsic factors (B₁₂-IF) and cobalophilins (B₁₂-CP) complexed to vitamin B₁₂ from pylorus, gastric juice (GJ) and intestinal juice (IJ) were in all respects approximately similar. Slight differences may reflect differences in the relative content of carbohydrate; free IF=intrinsic factor unsaturated with vitamin B₁₂; values are means $\pm$ s.d. for the numbers (*n*) of observations shown.

	Molecular weight		Stokes radius (nm)	Sedimentation coefficient (<i>s</i> _{20,w}) (S)	Partial specific volume* (ml/g)	10 ⁷ × Diffusion coefficient (cm ² ·s ⁻¹)	Frictional ratio
	By gel filtration	By the formula of Svedberg					
Pyloric B ₁₂ -IF	69 300 $\pm$ 900 (<i>n</i> =20)	63 450	3.57 $\pm$ 0.05 (<i>n</i> =15)	4.37 $\pm$ 0.06 (<i>n</i> =6)	0.714	5.84	1.39
GJ B ₁₂ -IF	70 000 $\pm$ 1000 (<i>n</i> =7)	63 100	3.59 $\pm$ 0.05 (<i>n</i> =7)	4.36 $\pm$ 0.02 (<i>n</i> =6)	0.714	5.86	1.39
IJ B ₁₂ -IF	69 500 $\pm$ 1000 (<i>n</i> =7)	64 350	3.55 $\pm$ 0.05 (<i>n</i> =7)	4.41 $\pm$ 0.07 (<i>n</i> =6)	0.714	5.81	1.39
Pyloric B ₁₂ -CP	120 000 $\pm$ 2000 (<i>n</i> =8)	91 200	4.59 $\pm$ 0.1 (<i>n</i> =15)	5.12 $\pm$ 0.07 (<i>n</i> =6)	0.700	4.54	1.59
GJ B ₁₂ -CP	121 000 $\pm$ 2000 (<i>n</i> =4)	93 800	4.66 $\pm$ 0.1 (<i>n</i> =7)	5.21 $\pm$ 0.05 (<i>n</i> =6)	0.700	4.49	1.60
IJ B ₁₂ -CP	121 000 $\pm$ 2000 (<i>n</i> =4)	91 950	4.62 $\pm$ 0.1 (<i>n</i> =7)	5.14 $\pm$ 0.03 (<i>n</i> =6)	0.700	4.52	1.59
Free IF			3.87 $\pm$ 0.07 (<i>n</i> =7)			5.39	

* From Allen & Mehlman (1973*a,b*).

Sephadex G-200 before the vitamin B₁₂-intrinsic factor complex, with a *K_D* value corresponding to a Stokes radius of 3.87 nm.

Discussion

Several reasons prompted the present studies; in general the fate and role of the early formed protein complexes of vitamin B₁₂ in the gastrointestinal tract are rather obscure [see reviews by Toskes & Deren (1973) and Gräsbeck & Salonen (1976)]. Even less is known about the free forms of the vitamin B₁₂-binding proteins present in this system. In addition, numerous misconceptions and misinterpretations about the molecular processes involving these proteins [see reviews by Glass (1963, 1974)] could be cited, e.g. because unexplained impairment of vitamin B₁₂ absorption occurs sometimes in patients with pancreatic insufficiency (McIntyre *et al.*, 1956; von der Lippe *et al.*, 1976*a*) the presence of a 'pancreatic intrinsic factor' was probably erroneously (see Glass, 1974) speculated (Cheney, 1936; Toskes *et al.*, 1971). In addition, the presence of an endogenous binder that can bind to the gastric intrinsic factor-vitamin B₁₂ complex, converting it from an absorbable into a non-absorbable form, was probably also erroneously (see Glass, 1974) speculated (Toskes & Deren, 1973). Furthermore, because the pig gastric mucosa (Allen & Mehlman, 1973*b*) and the human gastric juice (Allen & Mehlman, 1973*a*) intrinsic

factors isolated with almost identical procedures exhibited a difference in their mol.wts. of 10000, the question arose (Marcoullis & Gräsbeck, 1975) whether if in the same species, that is, in man or in the pig, the mucosal intrinsic factor would be a larger molecule than the corresponding molecule in gastric juice.

In general, the chemistry and the pathophysiology of the above-mentioned abnormalities and phenomena have not been definitely established, but the malabsorption in pancreatic insufficiency can be corrected in humans (Veeger *et al.*, 1962; Toskes *et al.*, 1973*a*) and partially pancreatectomized rats (Toskes & Deren, 1972) by the administration of pancreatic extract. The observation (Toskes *et al.*, 1973*b*) that the active principle in pancreatic extract correcting vitamin B₁₂ malabsorption in these patients resides with the tryptic activity would support this notion.

In the present study only one molecular species containing vitamin B₁₂ and possessing intrinsic-factor immunological identity was identified in the entire gastrointestinal tract (see also Marcoullis *et al.*, 1977; Nicolas *et al.*, 1978) and it displayed neither molecular nor functional differences with the analogous protein present in the pyloric mucosa. This protein in addition exhibited the known property of intrinsic factor to decrease its size after binding vitamin B₁₂ (Gräsbeck, 1967). These facts provide strong evidence that much of the gastric intrinsic

factor remains unaltered (see also Marcoullis *et al.*, 1977) during its passage from the gastric succus down to the small intestine. The results do not support the hypothesis about the existence of an additional intrinsic factor speculated to be secreted by the pancreas. On the other hand, it has been shown (von der Lippe *et al.*, 1976b) that pancreatic juice contains only one vitamin B₁₂-binding protein lacking intrinsic-factor activity. The fact that the other molecular form containing vitamin B₁₂ reacted only with specific anti-cobalophilin sera ruled out the hypothesis that an endogenic binder binds to the intrinsic-factor-vitamin B₁₂ complex (see above). Purified duodenal-juice and pyloric intrinsic-factor preparations saturated with vitamin B₁₂ exhibited the same capacity as the known pyloric intrinsic factor for binding to the solubilized ileal intrinsic-factor receptor. Thus the observation previously made by others that human duodenal juice actually decreased uptake of vitamin B₁₂ complexed to intrinsic factor by intestine and guinea-pig ileal brush borders (von der Lippe *et al.*, 1976a) is unlikely to be due to a direct effect on the intrinsic-factor molecule but rather to non-absorbable vitamin B₁₂ binders known (Simons, 1964) to be present in bile (see also below). Like the gastric juice, ileal mucosa was found to contain relatively more of the acidic-type intrinsic-factor isoproteins (Marcoullis *et al.*, 1977; Marcoullis, 1977). This could be interpreted as meaning that the latter corresponds to the secreted gastric-juice protein that has survived (Kapadia *et al.*, 1976) the passage down to the ileum. However, in the present studies, the intrinsic factor in the intestinal juice contained less of the acidic-type isoproteins, like that of pyloric mucosa. The most plausible explanation for this discrepancy could be the fact that, like other glycoproteins the sialic acid-rich isoproteins survive passage through the gastrointestinal tract because they are most resistant to proteolysis (Faillard *et al.*, 1962; Spiro, 1963).

The present study supplements information on the beneficial effect of pancreatic juice on the ileal absorption of vitamin B₁₂. As stated above, our results neither support the notion that pancreatic juice is endowed with intrinsic factor activity nor that an endogenic binder for intrinsic factor exists in the intestine. However, intestinal juice was found here to contain less of the acidic-type intrinsic factor and cobalophilin isoproteins than the gastric juice. It is well known that the isoprotein phenomena of these proteins are related to differences in the carbohydrate moiety, and especially to variations in sialic acid (Gräsbeck & Salonen, 1976). Thus it is plausible to interpret the changes as results of removal of carbohydrate from the proteins. Enzyme activity or hydrolysis at low pH may be responsible for this phenomenon. Cobalophilin may, in addition, be degraded (Gräsbeck, 1956) by proteolysis in the gastrointestinal

tract, which could explain the lower total unsaturated vitamin B₁₂-binding capacity of intestinal juice and the higher relative content of intrinsic factor in it observed during the present study. In line with this explanation are two reports (Cotter *et al.*, 1977; Allen *et al.*, 1977) suggesting that the hydrolysis of vitamin B₁₂-binding proteins in intestinal juice and especially proteolysis of cobalophilins can be promoted by pancreatic enzymes and lead to a transfer of vitamin B₁₂ from cobalophilin to intrinsic factor *in vitro*. Thus lack of enzymic breakdown of cobalophilin could explain the impaired vitamin B₁₂ absorption observed in cases of pancreatic insufficiency.

The present study also supports previous observations (Marcoullis & Gräsbeck, 1975) on human material that demonstrated that unsecreted mucosal intrinsic factor seems to lack carbohydrate residues, mainly sialic acid. Thus the mucosal protein from human and pig contains less of the acidic isoproteins which are considered (Marcoullis & Gräsbeck, 1975) to be somewhat more precursor- or sialic acid-deficient, whereas the intrinsic factor secreted in gastric juice consists of more of the acidic isoproteins containing more sialic acid. This is in line with Eylar's (1965) hypothesis, which suggests that extracellular proteins are generally glycoproteins and that the carbohydrate moiety is added to the protein molecule to allow it to escape through the cell membrane. The same concept cannot be applied for the cobalophilins found in the pyloric mucosa and gastric juice, since the latter material, contrary to the results with intrinsic factor, contained less of the acid-type cobalophilin isoproteins. The reason could be that they are of different origin. Indeed, compared with human cobalophilins, the gastric-juice protein had a pattern resembling that of saliva (Stenman, 1974a,b,c) rather than that of pyloric mucosa, and at least a great part of the gastric juice and, in turn, of the intestinal-juice cobalophilin content, could thus be of salivary origin. The same results further support the thesis (Stenman, 1974a) that variations in the relative content of the numerous isoproteins of cobalophilins from different sources do not imply the presence of more or less different proteins, but rather only one microheterogeneous protein or subfractions of such a protein with a variable carbohydrate composition. As previously mentioned, intrinsic factor appeared to decrease its size on binding the vitamin B₁₂. The reason could be that either intrinsic factor shrinks (Gräsbeck, 1967) or it loses some of its carbohydrate or other residues. However, pig and human intrinsic factors isolated by a combined affinity-chromatographic system (Marcoullis *et al.*, 1978a) including adsorption and desorption on vitamin B₁₂-Sephadex had the same molecular dimensions as the native analogous proteins present in crude extracts. Thus this phenomenon is actually due to shrinking only.

The sedimentation coefficient of the pyloric intrinsic factor was found to be lower than previously was reported (Marcoullis *et al.*, 1977). The tendency to formation of intrinsic-factor polymers (Allen & Mehlman, 1973a,b; Marcoullis *et al.*, 1977; Marcoullis & Gräsbeck, 1977) was (see the Experimental section) probably decreased in the present studies. The discrepancy in the mean isoelectric point of the same protein observed during the present and our previous studies (Marcoullis *et al.*, 1977) could also be attributed to the same phenomena. Such polymer-formation phenomena has been definitely shown to be the reason for some of the discrepancies occurring with transcobalamin II when studied by isoelectric focusing (Marcoullis *et al.*, 1978b).

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