Microheterogeneity of the Complex between Human Intrinsic Factor and Cyanocobalamin Demonstrated by Isoelectric Focusing

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Human intrinsic factor (IF) is a glycoprotein, which we have isolated from gastric juice as two cyanocobalamin complexes, termed Complexes S and I. The former was homogeneous in chromatography and gel filtration, analytical ultracentrifugation and disc and immuno electrophoresis.[1] Complex I had the same molecular weight as Complex S but was bound more firmly to DEAE-Sephadex. The fact that two IF-active components are observed rouses the suspicion that IF is microheterogeneous like many other glycoproteins. This has now been demonstrated.

Isoelectric focusing was performed in the 110 ml column described by Svensson.[2] In the early experiments mixtures of peptides, casein hydrolysate, *etc.* were used to produce the pH gradients, which, however, were so steep in the region of interest that only a rough estimate of the pI was obtained.[3] Smooth gradients were obtained with the synthetic ampholytes [4] (Ampholine, LKB), used in a concentration of 1 %. Usually the ampholytes covered two pH units, but for control purposes narrower pH ranges were used. The density gradient was produced with glycerol (maximum concentration 50 % w/v), because sucrose interfered with the binding of B_{12}.[5] Ethylene glycol of + 5°C was circulated through the cooling jacket. The final potential was about 500 V. Fractions 1 ml in volume were collected after 66 h. pH was measured at 23°C with a relative accuracy of 0.01 unit. The sample was introduced by adding it either to the light solution vessel in a gradient mixer or by mixing it with the dense solution in a test tube, if the gradient was built up stepwise. The results were not influenced by the mode of introduction of the sample. For control purposes, B_{12} was run alone or added to the sample; it was found not to migrate.

B_{12}—IF complexes labelled with $^{57}Co-B_{12}$ were purified from human gastric juice or stomachs obtained during operation as described earlier.[1] Recently, the author has developed a simplified two-step procedure to separate the complexes with and without IF activity: As before,[1] the complexes are first adsorbed to DEAE-cellulose from 5 mM phosphate of pH 7.5 and then a gradient of falling pH and rising concentration is applied. The single radioactivity peak eluted is passed through Sephadex G-150, which splits it into two, the R-type complex of higher molecular weight and the IF complexes of lower molecular weight. This procedure does not separate Complexes S and I. When stomach extracts were treated with DEAE-cellulose, part of the bound radioactivity was found not to be adsorbed. In gel filtration this non-adsorbed component "N" had the same elution volume as Complex S. The samples containing Complexes S and I were active in the Schilling test; component N has not been tested owing to lack of material. All complexes behaved immunologically as B_{12}—IF: When they were mixed with anti-B_{12}—IF serum from a pernicious anaemia patient and filtered through Sephadex G-150, the radioactivity was eluted in the void volume.

IF prepared from pooled human gastric juice always gave multiple peaks. To elucidate the cause of this heterogeneity B_{12}—IF complexes were prepared from individual gastric secretions neutralised *in vitro* or *in situ* and from stomachs. Irrespective of the source of the purified IF complex, multiple peaks and very similar patterns were observed (Fig. 1). The pattern was somewhat reminiscent of a Gaussian curve, the central peaks being the highest. The number of components was 7 in the best runs. Peaks were observed at the following pH values: 4.84, 4.94, 5.06, 5.18, 5.32, 5.44, and 5.73. The highest were from 5.06 to 5.32. All components reacted with the antiserum and all had the same molecular size as determined by gel filtration.

An attempt was made to identify Complexes S and I among the peaks. Purified I derived from one individual was found to consist of components with the pI values 4.94, 5.06, and 5.18. The pI 4.94 and 5.06 components, derived from a sample which had been purified by the two-step procedure, were chromatographed in DEAE-Sephadex, which separates S and I.[1] Most of the radioactivity was eluted as I. The peaks with higher pI values behaved chromatographically like S. It seems that I is more acidic than S, which is consistent with their behaviour in chromatography [1] and immunoelectrophoresis.[6]

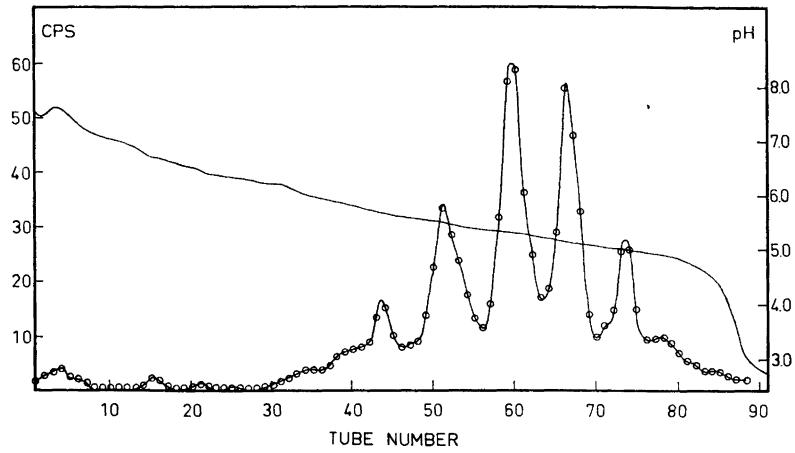

Fig. 1. Pattern obtained by isoelectric focusing of the radioactive cyanocobalamin-intrinsic factor complex. The material was isolated by the two-step procedure from gastric juice neutralized *in situ*.

To elucidate whether variation in sialic acid content was responsible for the microheterogeneity the B_{12}—IF complex was digested with neuraminidase (Behringwerke). Conditions: Incubation at 37°C up to 48 h, continuous dialysis against 0.05 M acetate buffer of pH 5.9 containing 0.1 % $CaCl_2$ and 0.9 % NaCl, 250—500 units of enzyme added before or in small portions during incubation. Free ^{57}Co—B_{12} was added in great excess, since B_{12} has a tendency to dissociate at 37°C and free IF is very labile. (If no free B_{12} was present during incubation, all B_{12}-binding power was destroyed).

During incubation the enzyme-containing samples showed a progressive decrease in content of the principal components, the small components at the alkaline end of the isoelectric pattern increased and finally the components with the following pI values remained: 6.35, 6.03, 5.73, and 5.44. These components were shown to react with the antiserum.

Component N was found to consist mostly of the pI 6.35 fraction; in addition the peak at pH 6.03 was present.

These findings are interpreted as follows: At least part of the microheterogeneity is due to variations in sialic acid content. Since the pI 6.35 fraction was the most alkaline component produced by neuraminidase treatment, it is possible that it represents IF devoid of sialic acid. This component occurs in stomach wall but not in gastric juice. It would appear that the sialic acid is added to the molecule just before it leaves the cell (*cf.* Eylar [7]). It is also likely that sialic acid spontaneously dissociates from IF preparations. For instance, when a pI 5.18 peak was rerun, it was found at pH 5.32. Another B_{12} transport protein, transcobalamin I, also seems to lose sialic acid during purification.[8]

The isolated complex S gave one single narrow line in disc electrophoresis,[1] which has very great resolving power. The salivary B_{12} complex, which we recently isolated in this laboratory,[9] was also homogeneous in disc electrophoresis. All these B_{12} complexes are heterogeneous in isoelectric focusing. It seems that this technique has greater resolving power than most other physicochemical methods of protein chemistry.

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Table 1. Experimental conditions for the preparation of heamatite from amorphous iron(III) hydroxide.

Expt. No.	Time of treatment h	pH of mother liquid after treatment	X-ray investigation of product
1	0.3	4.59	amorphous
2	0.5	3.20	»
3	0.5	3.15	»
4	1	2.70	α-Fe_2O_3
5	1	2.43	»
6	1.25	2.50	»
7	1.5	2.44	»
8	1.75	2.43	»
9	2	2.40	»
10	2	2.44	»
11	2	2.41	»
12	4	2.41	»
13	4	2.41	»
14	8	2.41	»
15	16	2.38	»

Hydrothermal Preparation of Haematite from Amorphous Iron(III) Hydroxide

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The hydrothermal preparation of haematite (α-Fe_2O_3) from amorphous iron(III) hydroxide was studied in acid solutions at 180°C. An investigation by Christensen [1] demonstrated that the pH of the mother liquid is decreased by a hydrothermal treatment of freshly pricipitated iron(III) hydroxide when the precipitation of ferric hydroxide is interrupted at pH = 5 or at lower pH values. The decrease in pH is explained by hydrolysis, resulting in a complete precipitation of the iron. In the present investigation the rate of the decrease in pH was investigated.

A solution of 0.1 M ferric nitrate was used in all the experiments. 5 ml of the solution was titrated with a solution of 0.25 M ammonia, as reported in Ref. 1. The titration was interrupted at pH = 4.50 and the precipitated ferric hydroxide with the mother liquid was transferred to a thick-walled pyrex ampoule. The sealed ampoule was heated in a thermostated oven kept at 180 ± 1.5°C. After the hydrothermal experiment the pH of the mother liquid was measured, and the reaction product was washed with water and dried at 25°C. The X-ray powder pattern was obtained with a Philips powder diffractometer. The results are given in Table 1.

The investigation shows that the decrease in the pH of the mother liquid from pH = 4.5 to 2.4 takes approximately 1.5 h. Further treatment does not result in a significant decrease in pH. The product obtained after heating for 1 h is crystalline α-Fe_2O_3. The diffractometer powder patterns show, that there is no detectable increase in the grain size of α-Fe_2O_3 for samples treated for a longer period than 2 h.

The hydrothermal formation of crystalline α-Fe_2O_3 from amorphous iron(III) hydroxides is in the present investigation completed within a period of 1 to 2 h. α-Fe_2O_3 prepared by the hydrothermal method is possibly a useful material in the preparation of γ-Fe_2O_3 for magnetic tapes.

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