Studies on Human Gastric Intrinsic Factor
I. Purification of Intrinsic Factor from Human Gastric Juice

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A method for the purification of intrinsic factor from normal human gastric juice was described. The pepsin-inactivated neutralized gastric juice was saturated or partially saturated with radioactive vitamin B12 and purified by a sequential column chromatography using Sephadex G-100 gel, DEAE-cellulose and Sephadex G-50 gel. The purified materials obtained by this purification proved intrinsic factor active by Schilling test in a total gastrectomy patient. This material had about 60-fold increase in vitamin B12 binding capacity as compared with the dialyzed gastric juice. The final product was homogeneous on ultracentrifugal analysis, and showed the sedimentation coefficient of 10.5 S with the estimated molecular weight of approximately 150,000. This was composed of 16 amino acids, of which threonine, serine, proline, glutamic acid, glycine, alanine and aspartic acid were relatively high in amount. The minimal molecular weight was considered to be approximately 10,000.

It has been well known that gastric intrinsic factor (IF) enhances vitamin B12 absorption, since Castle presented the hypothesis of intrinsic and extrinsic factors in 1929 (1). Ternberg and Eakin (2) found in 1949 that normal human gastric juice (NHGJ) contains a nondialyzable, heat-labile substance which combines with vitamin B12 (B12). Although no clinical evidence was given, their observation was epoch-making for the study of IF. Various new physicochemical techniques, including ultrafiltration and paper or zone electrophoresis, were introduced for the isolation of IF active material from NHGJ. Recently the column chromatographic techniques were introduced for the purification of IF (3-8). However, the purification of IF from NHGJ has not succeeded yet (9-12). This paper deals with the author's attempts to isolate IF from NHGJ. Radioactive vitamin B12 was added to pepsin-inactivated NHGJ and the major B12 binding component in NHGJ was pursued through a series of the purification procedures using Sephadex G-100, DEAE-

1 Presented in part at the 3rd World Congress of Gastroenterology held in Tokyo, September, 1966 [31].
2 森下玲児
cellulose and Sephadex G-50 column chromatography. The final product was tested for IF activity by Schilling test, and further the physicochemical properties were studied by ultracentrifugation and amino acid analysis. Thus an IF-active B₁₂ binder, homogeneous on ultracentrifugation, was separated from NHGJ.

MATERIALS AND METHODS

1. Gastric Juice

NHGJ was collected by Rehfuss tube from fasted patients without hematological abnormalities following histamine stimulation in a dose of 0.1 mg per 10 kg body weight. The patients were noticed to keep minimum salivary contamination. The fasting gastric residue was discarded as completely as possible. Gastric juice was carefully collected for 60 minutes at 10 minute intervals avoiding the contamination of blood and bile. The juice was immediately filtered through gauze into a beaker kept at ice-cold temperature. The pH was raised to 10 with N NaOH, left for 10 minutes, and finally brought down to 7.0 with N HCl at 0°. The gastric juice was centrifuged at 1,500 rpm for 15 minutes at 4° and removed of the mucus.

2. Dialysis and Concentration

The collected juice was placed in a Visking tube (Visking Co., Chicago, Illinois, U.S.A.) and dialysed at 4° for 48 hours against distilled water with several changes. Thereafter, the materials remaining in the tube were concentrated to about 1/5 of the original volume against Carbowax-6,000 and stored frozen until use. In some cases, the dialyzates were lyophilized and stored in a desiccator at 4°.

3. Radioactive Vitamin B₁₂

³⁵Co-cyanocobalamin with specific activity of 9.302 μCi/μg, ³⁵Co-hydroxocobalamin with specific activities of 0.869-1.192 μCi/μg were used. The radioactivity was counted in a well-type scintillation spectrometer (SP-I, Shimadzu Co. Ltd., Kyoto).

4. Sephadex G-100 and Sephadex G-50

These were individually suspended in borate buffer at pH 9.0 and packed by gravity into columns of 2.0×50 or 3.0×50 cm (for Sephadex G-100) and of 1.0×30 cm (for Sephadex G-50).

5. DEAE-cellulose

DEAE-cellulose (capacities of 0.62-0.84 mEq/g) was suspended in distilled water and washed four or five times by decanting the supernatant. The washed DEAE was treated alternatively with 0.1 N NaOH and 0.1 N HCl three times each and suspended in 0.005 M sodium phosphate buffer, pH 7.5. This cellulose was packed into a column of 1.0×30 cm and was equilibrated with the starting buffer before fractionation.

6. Protein Measurement

Protein content in each effluent was determined by absorbancy at 280 mμ in a Beckman type DU spectrophotometer (Shimadzu Co. Ltd., Kyoto) and in some preliminary experiments by the method of Lowry et al. (13). The measurement

³ Generously supplied from Merck Co. Sharp and Dohme Laboratory, New Jersey, U.S.A.
⁴ Pharmacia, Uppsala, Sweden.
⁵ Serva Entwicklungslabor., Heidelberg, Germany.
of protein concentration was made according to Warburg and Christian (14) and Kalekar (15), assuming that protein concentration (mg/ml) corresponds to 1.55 $E_{280}$
$-0.76 E_{260}$.

7. Ultracentrifugation

The purified materials were analysed in an ultracentrifuge (Spinco Model E, Beckman Instruments Co., Spinco Division, U.S.A.) at 59,780 rpm. The approximate molecular weight was calculated from the coefficient of ultracentrifugation.

8. Amino Acids Analysis

The purified materials obtained after dialysis against distilled water and lyophilization were hydrolyzed with 5 N HCl for 20 hours at 105°. After removal of HCl, the hydrolyzed materials were dissolved in a small volume of citrate buffer, pH 2.2. One aliquot was passed through a 150 cm column of Amberlite CG-120 for the separation of acidic and neutral amino acids, and the other through a 15 cm column of the same adsorbant for basic amino acids. The determination of amino acid composition was carried out in an automatic amino acid analyzer (Type III, Hitachi Manuf. Co. Ltd., Tokyo). Tryptophan was spectrophotometrically estimated on unhydrolyzed material by the method of Goodwin and Morton (16).

9. Evaluation of Intrinsic Factor Activity

The purified materials were assayed for intrinsic factor activity by Schilling test in patients with pernicious anemia (PA) or total gastrectomy (17), with an oral dose of 0.5 µg of radioactive B12. When the binding capacity of the purified material tested was lower than 0.5 µg of B12, more radioactive B12 was added so as to make the total amount of 0.5 µg.

All these procedures were made at 4° except for the optical density and radioactivity measurements.

RESULTS

1. Preliminary Experiments

Gel filtration through Sephadex column has been used for the fractionation of large molecular materials in gastric juice (3) since the introduction of the dextran gel Sephadex by Porath and Flodin (18). Ten milligrams of lyophilized NHGJ was dissolved in 1.0 ml of borate buffer, pH 9.0 and centrifuged at 3,000 rpm for 10 minutes. The supernatant was pipetted and $^{60}$Co-B12 of 0.878 µg was added in excess of the binding capacity. After standing at room temperature for 30 minutes, this mixture was filtered through a Sephadex G-100 gel column of $1.0 \times 30$ cm with borate buffer. Eluates were collected in 1.0 ml fractions by an automatic fraction collector. Each fraction was counted in a well-type scintillation counter. Two radioactive peaks were obtained. The first peak appeared immediately after a void volume, and the second one at the end of elution. After the dialysis against the running tap water for 24 hours, only the first peak showed to contain the bound form of B12. $^{60}$Co-B12, 1.32 µg, was added to 15 ml of freshly collected NHGJ and dialysed against distilled water for 48 hours at 4° and then lyophilized. Ten milligrams of the lyophilized materials was redissolved in 1.0 ml of borate buffer, pH 9.0. After centrifugation at 3,000 rpm for 10 minutes, the supernatant was filtered through a
Freshly collected NHGJ, 15 ml, was saturated with 1.32 μg of 60Co-B12 and dialysed against distilled water for 48 hr at 4°C and then lyophilized. The lyophilized materials, 10 mg, were redisolved in 1.0 ml of borate buffer, pH 9.0. After centrifugation at 3,000 rpm for 10 min., the supernatant was filtered through a column of Sephadex G-100 of 1.0×37 cm with the same buffer. The eluate was collected in 2.0 ml fraction. The radioactivity —, and the protein peaks estimated by absorbancy at 280 mμ; ---, and by the method of Lowry et al. (13) at 750 mμ; ----, were compared.

Sephadex G-100 column of 1.0×37 cm with the same buffer. The eluates were collected in 2.0 ml fractions by an automatic fraction collector. The radioactivity and its protein concentration in each eluate were measured and plotted as shown in Fig. 1. The curves of the optical density read at 280 mμ and at 750 mμ by the method of Lowry et al. (13) were associated with a single peak of radioactivity of the material eluted. As a result of these preliminary experiments, Sephadex G-100 gel was proved useful for the separation of B12 binders or IF from free B12 and other small molecular substances in NHGJ. Therefore, in the first step of the present purification the filtration through Sephadex G-100 column was adopted.

2. Isolation of Human Gastric Intrinsic Factor

The purification procedures were shown schematically in Fig. 2.

![Fig. 1 Elution Pattern of Lyophilized Normal Human Gastric Juice on Sephadex G-100 Column](image-url)
Sephadex G-100 gel filtration — Various amounts of dialysed and lyophilized NHGJ, ranging from 50 to 100 mg, were dissolved in 4.0 to 7.0 ml of borate buffer, pH 9.0. Then, 1.0 μg of $^{57}$Co-B$_{12}$ or 6.63 μg of $^{60}$Co-B$_{12}$ per 100 mg of lyophilized gastric juice was added. These mixtures were applied on top of the column (2.0×50 or 3.0×50 cm) and eluted with the same buffer. The flow rate were 5-15 ml per hour, and the collection volumes 3.0-5.0 ml, depending on the size of the columns used. When lyophilized NHGJ was oversaturated with radioactive B$_{12}$, two peaks of radioactivity appeared (Fig. 3). The first peak, containing bound B$_{12}$, was named Fraction I. The second one was only free B$_{12}$ as described in the preliminary experiment. B$_{12}$ binding capacity of Fraction I was increased to about 10 times as high as that of the starting gastric juice, when compared in terms of the amount of B$_{12}$ bound per mg of protein. Recovery of radioactive B$_{12}$ in this step of gel filtration was 95 to 100 per cent of the initially applied amounts.

DEAE-cellulose column chromatography — Pooled Fraction I, obtained from a single batch of pooled gastric juice, was transferred in Visking tube and concentrated against Carbowax-6,000. The concentrate was equilibrated with 0.005 M sodium phosphate buffer, pH 7.5 and applied to a DEAE-cellulose column (1.0×30 cm). The elution of the column was made with the same buffer, using a gradient of increasing ionic strength and descreasing pH. The final buffer was 0.5 M phosphate, pH 3.0. The flow rate was 7-10 ml per hour and 3.0 ml fractions were collected. As shown in Fig. 4, the first peak with high radioactivity was pooled and named Fraction II. After this peak, a very low radioactive peak with a large sharp protein peak was observed. The latter peak, however, was discarded because of its low B$_{12}$ binding capacity. The recovery of radioactive B$_{12}$ in this step of chromatography on DEAE-cellulose was 75 to 90 per cent of the amounts applied. B$_{12}$ binding
Pooled Fraction I concentrated against Carbowax-6,000, was equilibrated with 0.005 M sodium phosphate buffer, pH 7.5 and applied to a DEAE-cellulose column of 1.0×30 cm. The elution of the column was made with the same buffer, using a gradient of increasing ionic strength and decreasing pH. The final buffer was 0.5M phosphate, pH 3.0. The eluate was collected in 3.0 ml fraction. The first large radioactive peak was collected as Fraction II.

The capacity of Fraction II was increased 5 times as much as that of the Fraction I, when compared in terms of the amount of B12 bound per mg of protein. Fraction II was assayed by Schilling test on a total gastrectomy patient using 1.5 mg dose of peroral administration of this material. The urinary excretion of B12 radioactivity increased from 0.4 per cent with radioactive B12 alone to 9.3 per cent with this purified material, showing that Fraction II is fairly active as IF. Fraction II was also found to be homogeneous in ultracentrifugation as shown in Fig. 5. The sedimentation coefficient was 10.1 S and the molecular weight was calculated to be approximately 155,000, assuming the partial specific volume as 0.74. The carbohydrate and amino acid compositions were shown in Table 1. Fraction II had considerably high contents of threonine, proline, serine, glutamic acid, glycine, alanine and tryptophan in the protein moiety. The minimum molecular weight calculated from the basic compositions of the Fraction II was assumed to be 10,059.

Sephadex G-50 gel filtration—— Several batches of Fraction II were combined and reconcentrated against Carbowax-6,000 and equilibrated with borate buffer, pH 9.0. The concentrate was again filtered through Sephadex G-50 gel (1.0×30 cm) with the same buffer (Fig. 6). Fractions of 3.0 ml were collected at a flow rate of 15 ml per hour. All the bound-B12 appeared in a single peak just after the void volume. A sharp protein peak was associated with this radioactive peak. This was named Fraction III. Binding capacity of this protein for B12 was further increased up to 460.1 μg/mg of protein. The second protein peak was not associated with radioactivity. The increase in B12 binding capacity (μg/mg of protein) of Fraction...
Several batches of Fraction II were combined, reconcentrated against Carbowax-6,000 and equilibrated with borate buffer, pH 9.0. The concentrate was filtered through Sephadex G-50 column of 1.0 × 30 cm with the same borate buffer, pH 9.0. The eluate was collected in 3.0 ml fraction. The radioactive peak was named as Fraction III.

**TABLE 1**

*Amino Acid Composition of Purified Human Intrinsic Factor*

The calculated residues per mole were obtained by assuming the molar content of tyrosine equal to 1.

<table>
<thead>
<tr>
<th>Protein moiety</th>
<th>Found</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Residue/mole</td>
<td></td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.18</td>
<td>4</td>
</tr>
<tr>
<td>Threonine</td>
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<td>8</td>
</tr>
<tr>
<td>Serine</td>
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<td>7</td>
</tr>
<tr>
<td>Proline</td>
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<tr>
<td>Glutamic acid</td>
<td>0.25</td>
<td>5</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.24</td>
<td>5</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.26</td>
<td>5</td>
</tr>
<tr>
<td>Valine</td>
<td>0.13</td>
<td>3</td>
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<tr>
<td>Cystine</td>
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<td>—</td>
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<tr>
<td>Methionine</td>
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<td>—</td>
</tr>
<tr>
<td>Isoleucine</td>
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<tr>
<td>Leucine</td>
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<td>3</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.05</td>
<td>1</td>
</tr>
<tr>
<td>Phenylalanine</td>
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<td>1</td>
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<tr>
<td>Lysine</td>
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<td>1</td>
</tr>
<tr>
<td>Histidine</td>
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<td>1</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.07</td>
<td>1</td>
</tr>
<tr>
<td>Tryptophan*</td>
<td>0.23</td>
<td>5</td>
</tr>
<tr>
<td>Ammonia</td>
<td>0.97</td>
<td>19</td>
</tr>
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</table>

| Total amino acid residue | 78 | 99 |

<table>
<thead>
<tr>
<th>Carbohydrate moiety</th>
<th>Found</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucosamine</td>
<td>0.67</td>
<td>13</td>
</tr>
<tr>
<td>Minimal molecular weight</td>
<td>10,059</td>
<td>10,219</td>
</tr>
</tbody>
</table>

* Tryptophan was estimated spectrophotometrically by the method of Goodwin and Morton (16).
I, II and III was shown in Table 2. Unfortunately, Fraction III was proved inactive at 1.0 mg by Schilling test, showing the urinary excretion of B₁₂ radioactivity of 3.1 per cent. The ultracentrifugation of the Fraction III also revealed a single protein peak. The sedimentation coefficient was 10.5 S. The molecular weight of 160,000 was calculated from the sedimentation coefficient, assuming the partial specific volume as 0.74. The carbohydrate and amino acid compositions were almost the same as those of the Fraction II, except for the increase of aspartic acid, leucine and ammonia, and the decrease of proline and tryptophan in the Fraction III (Table 1). The minimum molecular weight of Fraction III was calculated to be 10,219.

**Discussion**

At present it is generally accepted that B₁₂ binding by IF-active materials is a prerequisite of IF activity (12) and the B₁₂ binding sites are protected from heating (19, 20) and purification procedures when they are preoccupied by B₁₂ (10, 5, 6). The saturated or unsaturated B₁₂ was added to NHGJ in order to trace and preserve IF. According to Kakei et al. (3) and Kubo et al. (21), the molecular weight of the B₁₂ binder in NHGJ was more than 50,000. As shown in the preliminary experiments, a good separation of B₁₂ binders in NHGJ from free B₁₂ was obtained by Sephadex G-100 gel filtration. Therefore, Sephadex G-100 gel is useful for the first step of the purification of IF. The anion exchanger, DEAE-cellulose, was suitable for the separation of proteins (22). Elution was achieved by applying a gradient of decreasing pH and increasing molarity of the buffer. As the final step of purification Sephadex G-50 gel was again used. The final product, Fraction III, had about 60-fold increase in B₁₂ binding capacity as compared with that of the initial lyophilized NHGJ (Table 2). Fraction II was IF-active at 1.5 mg by Schilling test, but with Fraction III, it was unable to ascertain IF activity by the same test because of the small amount obtained. However, only trace amounts of Fraction III proved IF-active by an in vitro assay using the everted sacs of guinea pig intestine (23). The second protein peak, which was obtained on the final step of the purification and not associated with radioactivity, was IF-inactive when checked by the in vitro assay (23). This might be the degradation products of IF formed during the storage and previous steps of the purification. The B₁₂ binding capacity of Fraction III, containing 460.1 mg B₁₂/mg of protein, was much lower than the values reported by other authors (6, 7). It might be due to the polymerization of IF during the purification. The purification of IF concentrates is usually followed by an increase of B₁₂-binding capacity. This is true only for IF materials processed from the same sources and obtained with the same techniques. Ellenbogen and Highley (24) discussed that IF which was not complexed with B₁₂ lost approximately 67 per cent of its binding capacity after one year storage, but did not lose its IF activity. Both of Fractions II and III were homogeneous in the ultracentrifugal

<table>
<thead>
<tr>
<th>Table 2</th>
<th>The Increase in B₁₂ Binding Capacity (mg B₁₂/mg of protein) during the Puri-</th>
<th>fication Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B₁₂-binding capacity</td>
<td>mg B₁₂/mg of protein</td>
</tr>
<tr>
<td>Normal human gastric juice</td>
<td>8.5</td>
<td></td>
</tr>
<tr>
<td>Fraction I</td>
<td>78.9</td>
<td></td>
</tr>
<tr>
<td>Fraction II</td>
<td>387.5</td>
<td></td>
</tr>
<tr>
<td>Fraction III</td>
<td>460.1</td>
<td></td>
</tr>
</tbody>
</table>
The sedimentation coefficient of Fraction II was 10.1S and that of Fraction III 10.5S. The molecular weight was calculated to be in the range of 155,000 to 160,000, assuming their partial specific volumes as 0.74. This figure, however, is a little higher than those reported by other authors (7, 8). Gräsbeck, Simons and Sinkkonen (7) reported that the sedimentation coefficient of IF-active Fraction S was 5.75S and the molecular weight 119,000. Fraction S, isolated by them, was considered as polymer, probably dimer of IF molecule. Garrido-Pinson, Turner, Miller and Segal (8) obtained an IF-active material from human gastric juice after passing through Sephadex G-100 gel. The molecular weight of the purified material was assumed to be 65,000-70,000, judging from its distribution coefficient determined by Sephadex G-100 gel filtration. But their pattern of gel filtration with Sephadex G-100 was quite different from those of the author or others (3, 21). They obtained two peaks of B12-binding materials, a small first one was composed of 15–20 per cent of the total B12 binders in NHGJ and the second one composed of 80–85 per cent. But it might be questionable to determine molecular weight from the distribution coefficient determined by the Sephadex G-100 gel filtration of such a viscous fluid as gastric juice. Chosy and Schilling (6) also obtained a purified IF preparation from human gastric juice, but they did not determine the molecular weight or the sedimentation coefficient. As to hog IF, it appears that the purification is more advanced. Bromer and Davison (25) purified a B12-IF complex from hog stomach with a sedimentation coefficient of 5.4 S. The molecular weight, calculated from the B12-binding capacity, was about 53,000 and had a potent IF activity by the urinary excretion test, as well as the classical clinical test. The sedimentation coefficient of 5.4 S, however, was too high for the supposed figure of the molecular weight (26). They suggested that association of IF had occurred or that the complex was unusually dense in the ultracentrifugal analysis. Holdsworth (27) obtained two B12 binders from hog pylorus, one of which possessed IF activity. The sedimentation coefficient of the IF active material purified by him was 3.8 S and the molecular weight was assumed to be about 55,000. More recently, Inada (28) purified a B12-IF complex from IF concentrates from hog stomach, using the column chromatographic fractionations with DEAE-cellulose, CM-cellulose and so on. It was found that the purified hog B12-IF complex had the sedimentation coefficient of 3.4 S or 3.8 S and that the molecular weight was in the range of 50,000-55,000. Highley, Davies and Ellenbogen (29) isolated a B12-binding glycoprotein from hog pyloric mucosa. The molecular weight of the IF-active material was about 50,000 in B12-noncomplexed form and the sedimentation coefficient was 3.7 S, but the molecular weight of IF-active material was about 100,000 in B12-complexed form and the sedimentation coefficient was 4.6 S. The B12 binding complex was interpreted as the reversible dimer. So far as hog IF is concerned, both the values of the sedimentation coefficient and the molecular weight are reasonably identical among the investigators. On the contrary, the informations on the molecular weight and the sedimentation coefficient of human IF or its B12-IF complex are still limited, because there are a lot of difficulties in the purification of human IF, one of which is the limited collection of starting materials and the other is relatively heat-labile even in the presence of coupled B12. The values of the sedimentation coefficient and the molecular weight of human IF are considerably different from investigator to
investigator. As a rule, both the sedimentation coefficient and the molecular weight are more larger than those of hog IF.

Amino acid and glucosamine compositions of the Fraction II and III were utilized to establish a minimal molecular weight by assuming the molar content of tyrosine to be unity (30). The values of the Fractions II and III were almost the same, approximately 10,000. In addition, the data from the amino acid analysis indicate that these purified materials, the Fractions II and III, contain almost the same composition except for high concentration of proline and tryptophane in the Fraction II and of aspartic acid, leucine and ammonia in the Fraction III. As there is no observation of amino acid composition in human IF, the author compared amino acid composition of human IF with that of hog IF (27-29). As the result, the contents of aspartic acid, glutamic acid, valine, methionine and histidine were decreased to one half and those of leucine, isoleucine and lysine to one third. However, the ammonia content was increased three times. As it is well known that cystine is apt to be destroyed during hydrolysis, no value is given for this compound. The total carbohydrate content of the Fraction III was calculated to be approximately 38 per cent including 19.6 per cent of glucosamine, when the protein moiety was subtracted from the total amount of the material assayed. Holdsworth (27) obtained 20 per cent of reducing substances in his purified hog IF. Bromer and Davission (25) demonstrated that their preparation EB-6 from hog gastric mucosa contained only 6.8 per cent total reducing sugars. Recently, Highley, Davies and Ellenbogen (29) obtained 39.2 per cent of carbohydrate in their hog IF-B12 complex, containing 10.4 per cent of glucosamine. On the other hand, Gräsbeck, Simons and Sinkkonen (7) obtained about 13 per cent of carbohydrate in the purified human IF, Fraction S. The carbohydrate content is different among the investigators. It may partly depend on the differences of the assay methods used. From the results obtained above, it is concluded that human IF may be different from hog IF. In this point further investigation will be necessary.

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