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Interferon: Evidence for Its Glycoprotein Nature

(sialic acid/galactose/phytoagglutinin/affinity chromatography)

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ABSTRACT **In an attempt to understand the structure of rabbit interferon, the possibility of carbohydrate being part of the molecule was tested. Interferon incubated with neuraminidase from *Vibrio cholera* is homogeneous in charge as revealed by isoelectric focusing. Treatment of "asialointerferon" with galactose oxidase (EC 1.1.3.9) from *Dactylium dendroides* and subsequent reduction with tritiated sodium borohydride yields labeled material with unimpaired antiviral activity. Enzymic incorporation of *N*-[^{14}C]acetylneuraminic acid into tritiated asialointerferon restores the original charge heterogeneity. The newly generated sialointerferon contains both ^{3}H and ^{14}C activity. Asialointerferon is retained by an affinity column containing phytohemagglutinin from *Phaseolus vulgaris* and can be displaced from the adsorbent by a glycoprotein of known structure. It is concluded that rabbit interferon is a glycoprotein containing the terminal oligosaccharide sequence sialic acid → galactose.**

One of the salient features of interferon seems to be the absence of reliable data on its chemical composition. This situation is due to the fact that, despite substantial efforts devoted to purification, no interferon preparation has reached a state of purity that would permit direct chemical analysis. Most of our present information is derived from experiments where interferon preparations were subjected to various enzymic and chemical treatments. The existence of specific structural features was then inferred from the loss or conservation of antiviral activity. It could thus be established that interferons are proteins or contain protein as an essential component.

Additional, although weaker, evidence suggested the presence of carbohydrate as part of the molecule. Early in the study of interferon it was reported by several investigators that mild periodate treatment destroys the biological activity of interferon preparations (1). Moreover, it has become apparent that most, if not all, interferon preparations exhibit a striking heterogeneity of electric charge when examined by electrophoresis, ion exchange chromatography, or isoelectric focusing (1). Polymorphism of this type has been observed in many glycoproteins and could be attributed to structural variations in the carbohydrate moiety (2). In fact, Schonne *et al.* (3) have found that incubation of rabbit interferon with neuraminidase from *Clostridium perfringens* markedly decreases the charge heterogeneity as revealed by electrofocusing. Consequently, these authors have proposed that rabbit interferon is a glycoprotein possessing a variable content of sialic acid. Interferon would thus join the ever-increasing list of glycoproteins exhibiting what has been described as peripheral heterogeneity, i.e., differences in the terminal saccharide residues (4).

The most attractive features of this hypothesis are that (*a*) it can be tested experimentally even with interferon preparations of low purity, and (*b*) it generates the possibility of several potentially important investigations.

Basically, only a few types of oligomeric units are found in glycoproteins. Thus, in many glycoproteins containing terminal sialic acid, this monosaccharide is preceded by galactose as the penultimate residue in the carbohydrate sequence (2). Since galactose can be specifically tritiated by enzymic oxidation at the primary hydroxyl group and subsequent reduction with tritiated $NaBH_4$ (5), it should be possible to label specifically the carbohydrate moiety of asialointerferon. In addition, if interferon of the highest purity available is used, such a preparation can be expected to contain much more radioactivity than interferon induced in the presence of labeled amino acids. The usefulness of highly labeled interferon for a wide range of studies, in the whole animal as well as in cell cultures, is evident.

Several glycoproteins, when injected into animals, are rapidly cleared from the circulation if a few sialic acid residues have been enzymically removed. Conversely, if the galactose residues becoming terminal are removed or enzymically oxidized, the half life of the glycoprotein is increased. Consequently, the idea has been put forward that the clearance of such glycoproteins is regulated by the exposure of penultimate galactose units and that the presence of sialic acid attached to galactose protects the glycoprotein from being catabolized (6).

It is tempting to speculate that the extremely high clearance of exogenous interferon is also due to a subcritical content of sialic acid, the half-life of interferon administered to animals being in the same order of magnitude as that of partially desialylated glycoproteins (7). In fact, if the charge heterogeneity of rabbit interferon, as shown by Schonne *et al.* (3), is merely the consequence of a variable content of sialic acid, then the large majority of interferon molecules are not fully sialylated, as judged from the distribution of interferon activity over a wide range of pH zones during electrofocusing. Considering the potential medical importance of exogenous interferon, it would seem compelling to investigate possibilities of modifying the terminal carbohydrate sequence and to study the fate of such modified interferon in the organism.

In view of these implications, we decided to test the glycoprotein hypothesis and to demonstrate that rabbit interferon contains a carbohydrate component that includes the terminal sequence sialic acid → galactose.

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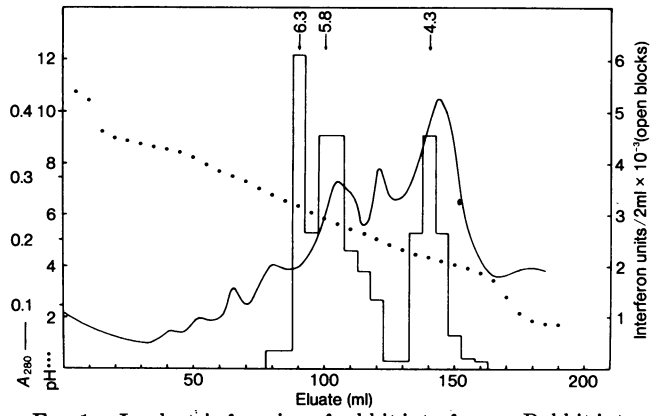

FIG. 1. Isoelectric focusing of rabbit interferon. Rabbit interferon (1.3 mg of protein, 65,000 units/mg) was focused on pH 3–10 ampholine gradients. After equilibration, 5-ml fractions were collected and 2 ml were assayed for interferon activity. ···, pH profile; ——, UV profile at 280 nm; *open blocks*, antiviral activity.

MATERIALS AND METHODS

Preparation and Assay of Interferon. Interferon was produced in primary rabbit kidney cells according to the method of Tan *et al.* (8) with the following modification: Monolayers were incubated with 200 μg/ml of poly(I)·poly(C) for 1 hr at 37°. After the inducer was removed, the cells were washed twice with phosphate-buffered saline (pH 7.2), and 10 μg/ml of cycloheximide in Eagle's minimum essential medium containing 2% fetal-calf serum was added. The cultures were incubated for 3.5 hr at 37°. Then 3 μg/ml of actinomycin D was added and the incubation was continued for 30 min. The antimetabolites were removed and the cells were washed five times with phosphate-buffered saline and covered with fresh medium without serum. After another 8- to 10-hr incubation, the supernates were harvested, centrifuged, and stored at −70° until use.

20 Liters of supernate were concentrated 200 times by ultrafiltration through Diaflo PM-10 membranes (Amicon), dialyzed against dilute acetic acid (pH 3.0), and freed from precipitated proteins by centrifugation.

For interferon assays, the plaque reduction test on primary rabbit kidney cells was used. Monolayers in 6-cm petri dishes were treated for about 18 hr with 2 ml of interferon dilutions and then challenged with 50–80 plaque-forming units of vesicular stomatitis virus. Titers are expressed as the interferon dilution causing a 50% plaque reduction. A laboratory standard was included in each series of assays. All results were corrected to this standard and are expressed as laboratory units per 2 ml.

Preparation of Asialointerferon. Neuraminidase from *Vibrio cholera* was obtained from Behringwerke AG, Marburg, Germany, and further purified by affinity chromatography (9). After this purification step, the enzyme was virtually free from proteases, as determined by the very sensitive dimethyl casein test (10). 40 ml of a concentrated interferon preparation were treated with neuraminidase over a period of 2 hr. The incubation mixture contained 0.7 mg of protein per ml, 2.8×10^6 units of interferon, 0.1 international unit of neuraminidase, and 0.1 ml of chloroform in Na acetate buffer (pH 5.5)–0.15 M NaCl–20 mM Ca Cl_2. After 2 hr, additional 0.1 unit of neuraminidase was added. Control samples were incubated without the enzyme under the same conditions. After a total of 4 hr, the material was dialyzed first against dilute acetic acid (pH 3.0) and then against distilled water, and subjected to electrofocusing.

Preparation of Tritiated Asialointerferon. 10 ml of asialointerferon (43 μg of protein per ml) containing 1.1×10^5 units of interferon were incubated at 37° with 100 units of galactose oxidase (EC 1.1.3.9; Miles Laboratories) in 0.05 M sodium phosphate buffer (pH 7.0), containing 0.05 M NaCl and 1% toluene. After 20 hr, the mixture was dialyzed against dilute acetic acid (pH 3.0) and subsequently against 0.05 M sodium phosphate buffer (pH 7.8)–0.05 M NaCl; 1.6 μmol of sodium [^{3}H]borohydride (7.2 Ci/mmol, The Radiochemical Center, Amersham, England) was added for each milligram of protein. After 10 min at room temperature (25°), the reaction was terminated by acidification with 4 N acetic acid to pH 4.0. After decomposition of excess sodium borohydride, the pH was brought to 6 with 1 N NaOH. The preparation was dialyzed against distilled water until the dialysate was free of radioactivity.

Incorporation of N-Acetylneuraminic Acid into Asialointerferon. Labeled CMP-*N*-acetylneuraminic acid was prepared with *N*-[^{14}C]acetylneuraminic acid (The Radiochemical Center, Amersham, England) and CMP-sialic acid synthetase from hog submaxillaris glands, as described by Kean and Roseman (11). Sialyl transferase was isolated from rat liver by the method of Hickman *et al.* (12). This enzyme was assayed with CMP-*N*-[^{14}C]acetylneuraminic acid and asialoceruloplasmin as acceptor glycoprotein. For the sialyl transfer reaction, essentially the procedure described by Hickman *et al.* (12) was used. 4 ml of a solution containing 0.02 mg of protein, 2×10^4 units of interferon, and a total of 2×10^5 dpm of tritium were incubated at 37° with 75 units of sialyltransferase and 2 μmol of CMP-*N*-[^{14}C]acetylneuraminic acid (5 Ci/mol) in 0.05 M Tris·HCl (pH 7.5)–10 mM EDTA–5 mM Mg acetate. After 2 hr, the solution was dialyzed against dilute acetic acid (pH 3.0), then against distilled water. The precipitate formed during dialysis was removed by centrifugation, and the labeled product was subjected to electrofocusing.

Affinity Chromatography. Crude phytoagglutinin from *Phaseolus vulgaris* was obtained as Bacto–Phythemagglutinin from Difco Laboratories Inc. and purified as described by Weber *et al.* (13).

The erythroagglutinating fraction (peak III of Weber *et al.*) was subjected to gel filtration on Sephadex G-150 and coupled to the *N*-hydroxysuccinimide ester of succinylated aminoalkyl agarose as described by Cuatrecasas and Parikh (14).

TABLE 1. *Tritium incorporation into interferon and asialointerferon preparations*

Preparation reduced with sodium [^{3}H]borohydride	Incubation with galactose oxidase	Total ^{3}H incorporated dpm/μg of protein	^{3}H in neutral sugar fraction dpm/μg of protein
Interferon	—	39,100	2,180
Interferon	+	24,800	6,220
Asialointerferon	—	26,600	4,080
Asialointerferon	+	113,000	77,700

A glycoprotein fragment released from the membrane of human erythrocytes after trypsin treatment that was capable of binding to the erythroagglutinating phytoagglutinin was prepared as described by Kornfeld and Kornfeld (15). 6 ml of a "prefocused" asialointerferon preparation with pI 6.3, containing a total of 2.35×10^5 dpm of tritium and 4.2×10^4 units of interferon in 0.1 N sodium acetate buffer (pH 6.5) were applied to a 0.4×2-cm column containing the adsorbent. The column was washed sequentially with 90 ml of buffer and 60 ml of 0.1 N galactose in sodium acetate buffer. 3-ml Fractions were collected and tested for tritium content and antiviral activity. Elution was achieved with a 0.1% solution of the glycoprotein from human erythrocytes in sodium acetate buffer (pH 6.5).

Isoelectric Focusing. The isoelectric focusing column (LKB 7900 Uniphor Column Electrophoresis System, Volume 220 ml) and pH 3–10 ampholine carrier ampholytes were obtained from LKB Producter AB, Stockholm, Bromma 1, Sweden. The isoelectric focusing procedure was performed according to the LKB Instruction Manual. All operations were performed at 2°. After equilibration, 5-ml fractions were collected and the pH was immediately recorded.

Protein was measured according to Lowry *et al.* (16).

RESULTS

The strategy of our experiments is outlined in the following steps: (*a*) neuraminidase treatment of interferon and isoelectric focusing of the resulting asialointerferon; (*b*) specific tritiation of the newly generated terminal galactose residues in asialointerferon; and (*c*) enzymatic transfer of [^{14}C]sialic acid into tritiated asialointerferon and analysis of the material containing the double label by isoelectric focusing.

Fig. 1 shows the typical distribution pattern of interferon on electrofocusing throughout the focusing column. The biological activity is characteristically divided into three discrete peaks with isoelectric points of 6.3, 5.8, and 4.3.

Similar results were obtained with more than 25 preparations, confirming the charge heterogeneity of interferon. The effect of neuraminidase treatment on the distribution of antiviral activity in the pH gradient is shown in Fig. 2. Desialylated interferon invariably focused as one peak around pH

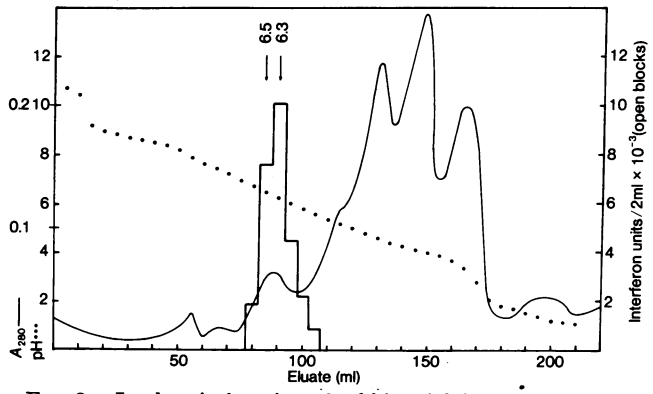

FIG. 2. Isoelectric focusing of rabbit asialointerferon. Rabbit asialointerferon (1.2 mg of protein, 57,000 units/mg) was focused on ampholine gradients as for Fig. 1. ···, pH profile; ——, UV profile at 280 nm; *open blocks*, antiviral activity.

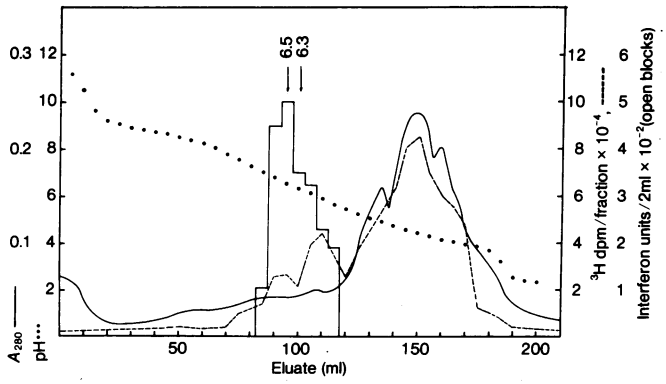

FIG. 3. Isoelectric focusing of tritiated asialointerferon. 12,000 units of asialointerferon containing 45 μg of protein were tritiated by oxidation with galactose oxidase and subsequent reduction with NaB^3H_4, as described under *Methods*. The resulting material was focused as for Fig. 2. ···, pH profile; ——, UV profile at 280 nm; - - - ^{3}H dpm/fraction; *open blocks*, antiviral activity.

6.3. Recovery of antiviral activity after incubation with neuraminidase and isoelectric focusing was almost 100%. Since the total activity after removal of sialic acid is concentrated in one peak that is well separated from the bulk of material absorbing at 280 nm, considerable purification is achieved. Compared to the biological activity of the starting tissue culture supernate, the simple steps of concentration, dialysis at pH 3.0, neuraminidase treatment, and isoelectric focusing yield a preparation with 5×10^7 units/mg of protein in the peak fractions, which represents a 1500-fold purification.

When a preparation containing asialointerferon was treated with galactose oxidase and subsequently reduced with sodium [^{3}H]borohydride, considerable incorporation of tritium activity was observed. Although various control samples of interferon and asialointerferon also showed incorporation of radioactivity, the uptake of radioactive label was 4-fold higher in the preparation of oxidized asialointerferon (Table 1). That tritiation of galactose residues had occured was confirmed by acid hydrolysis of the labeled asialointerferon and isolation of the neutral sugar fraction. The material was hydrolyzed in 2 N HCl at 100° for 2 hr, neutralized with silver carbonate, and passed successively through columns of Dowex 50W × 8 (H^+ form) and Dowex 1 × 2 (acetate form). The effluent was evaporated to dryness, and the residue was chromatographed on Whatman paper no. 1 in ethylacetate–pyridine–water (72:20:23, v/v). All the ^{3}H activity contained in the neutral sugar fraction was found in a spot identified as galactose by cochromatography. The data given in Table 1 indicate that appreciable incorporation of tritium is dependent on sequential treatment with neuraminidase, galactose oxidase, and tritiated sodium borohydride. If incubation with neuraminidase or galactose oxidase is omitted, only small amounts of tritium are incorporated.

Recovery of biological activity after the oxidation and reduction steps ranged from 70 to 90%.

It is obvious that the labeling of terminal galactose in a preparation containing asialointerferon is no proof for the existence of terminal galactose residues in asialointerferon itself. The crucial experiment is, therefore, the enzymic transfer of *N*-[^{14}C]acetylneuraminic acid into ^{3}H-labeled asialointerferon.

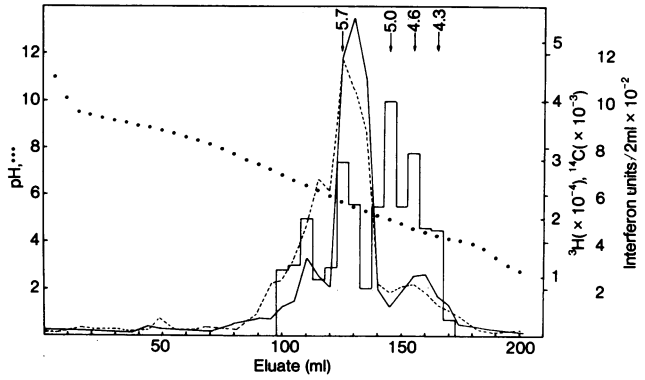

FIG. 4. Refocusing of tritiated intereron after incorporation of *N*-[^{14}C]acetylneuraminic acid. Tritiated focused asialointerferon, containing 20,000 units of interferon and 20 μg of protein, was treated with sialyltransferase and CMP-*N*-[^{14}C]acetylneuraminic acid and refocused as for Fig. 3. ···, pH profile; - - - ^{3}H dpm/fraction; ——, ^{14}C dpm/fraction; *open blocks*, biological activity.

Incorporation of sialic acid residues will shift the isoelectric point to lower values and can thus be detected by isoelectric focusing. Since sialic acid can only be transferred to a carbohydrate as acceptor, the appearance of interferon activity banding at pH < 6 will prove the glycoprotein nature of interferon. In addition, the presence of both ^{3}H and ^{14}C radioactivity in these fractions will be conclusive evidence for the terminal sequence sialic acid → galactose.

When tritium-labeled asialointerferon was focused, the pattern depicted in Fig. 3 was obtained. The biological activity is again located in a narrow zone between pH values 6.3 and 6.5. Tritium activity is present in this peak as well as in a broad zone at lower pH that corresponds probably to material introduced with the preparation of galactose oxidase and labeled during the tritiation procedure. In contrast, incubation of the radioactive fraction banding at pH 6.3–6.5 with CMP-*N*-[^{14}C]acetylneuraminic acid and sialyl transferase generates a strikingly different profile on isoelectric focusing (Fig. 4). Most of the interferon activity is now located in discrete peaks at pH 5.7, 5.0, and 4.6–4.3. The biological activity contained in these fractions represents about 75% of the total activity, indicating that sialyl transfer has occurred to a considerable extent. When the distribution of radioactivity throughout the pH gradient is correlated with the profile of biological activity, it becomes evident that the ^{3}H label has also shifted to lower pH values with a maximum at pH 5.7 and a shoulder at pH 4.3–4.6. Interestingly enough, the ^{14}C label follows almost exactly the same pattern, except in the zone of pH 6.4 where comparatively less ^{14}C than ^{3}H has been incorporated.

It is well known that glycoproteins can bind to specific agglutinins that occur in various plant seeds and invertebrates. This reaction is due to the interaction of specific saccharide-binding sites of the agglutinin with corresponding sugar residues in the carbohydrate moiety of glycoproteins. Consequently, agglutinins have been widely used for probing the structure of glycoproteins on cell surfaces and for the isolation and purification of carbohydrate-containing polymers.

It seemed interesting to test the possibility of adsorbing asialointerferon onto an immobilized agglutinin. Since the binding reaction is, in many instances, inhibited or reversed by simple sugars, agglutinins could be used to develop a simple affinity chromatography technique for purifying interferon. In our experiments, a phytoagglutinin isolated from the red kidney bean (*Phaseolus vulgaris*) was used. This agglutinin interacts specifically with the oligosaccharide sequence galactose → *N*-acetyl glucosamine → mannose (15), which is a common structural pattern to be found in many glycoproteins. As shown in Fig. 5, ^{3}H-labeled asialointerferon strongly adsorbs to the agglutinin coupled to agarose. A total of 10,000 units of interferon containing 120,000 dpm of ^{3}H activity was applied. About 50% of the radioactivity did not bind; additional 20% of the labeled material, but no biological activity, was eluted from the adsorbent with 0.1 M galactose. Complete release of interferon and elution of the remaining radioactivity was achieved in one sharp peak when a glycoprotein fragment from human erythrocytes was added to the eluent. This glycopeptide contains a branched carbohydrate structure (17) that seems to compete successfully with asialointerferon for the binding sites of the agglutinin.

DISCUSSION

The major result of the present study is that rabbit interferon is indeed a glycoprotein possessing at least one terminal carbohydrate sequence sialic acid → galactose. These findings have a wide range of implications for further studies on interferon, and it was this possibility that provided the stimulus for our investigation.

The first question raised concerns the specific properties that the carbohydrate component imparts to the molecule. It is evident from our experiments, as well as from those of Schonne *et al.*, that the charge heterogeneity in rabbit interferon is the consequence of variable amounts of sialic acid present as terminal monosaccharide. Since it is possible to remove most or all sialic acid residues without impairing the antiviral activity, one could conclude that this component is not involved in the biological function of interferon. However, with the foregoing discussion on the clearance of asialoglycoproteins as background, the possibility exists that the relative content of sialic acid in interferon controls its rate of clearance in the organism.

Until now, the administration of exogenous interferon for prophylaxis and treatment of viral diseases has been hampered by the need for large quantities. However, the quantitative

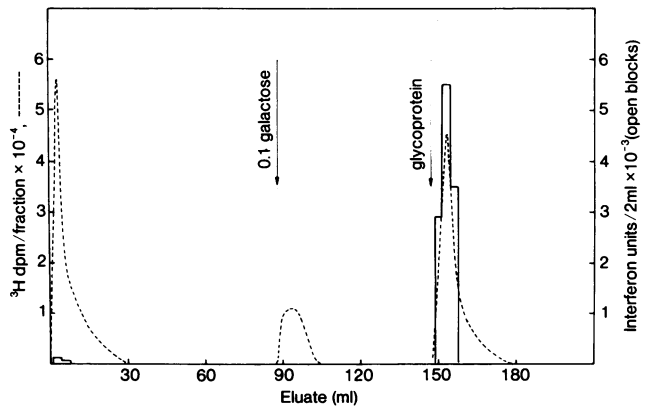

FIG. 5. Affinity chromatography of tritiated asialointerferon on *Phaseolus vulgaris* agglutinin coupled to agarose. Detailed data are summarized under *Methods*. - - -, ^{3}H dpm/fraction; *open blocks*, antiviral activity.

requirements may be due, at least partly, to the short half-life of interferon.

As a consequence, if the information obtained from work on serum glycoproteins is applicable to interferon, we anticipate that modification of the carbohydrate moiety could provide a novel approach to this problem. Enzymic oxidation or removal of terminal galactose in asialointerferon on the one hand and additional incorporation of sialic acid into interferon on the other hand would then allow a simple way of increasing the half-life and, hence, the therapeutic use of interferon. An extension of this work would be the sequential enzymic degradation of the carbohydrate moiety beyond the penultimate galactose residue. By analogy to several well-studied serum glycoproteins, one may expect the carbohydrate sequence to contain *N*-acetylglucosamine and mannose in the more internal part of the oligosaccharide unit. In addition, L-fucose residues may be present as terminal monosaccharides. Since glycosidases specific for these monosaccharides are available, the functional role of these components could be easily assessed.

The presence of carbohydrate may also account for another kind of polymorphism to be found in interferon preparations. It has been recognized for some time that, depending on the inducer and the tissue used for production, molecular species of different sizes are synthesized, ranging in molecular weight from 30,000 to 150,000. At present, the molecular basis of this polydispersity is not understood. It is possible that each molecular species represents a distinct entity with a different primary structure. On the other hand, it is conceivable that variations in the carbohydrate moiety contribute to the variations in size by influencing a state of equilibrium between associated subunits. For mouse and human interferon, Carter has recently presented data that suggest that the species with molecular weight around 30,000 is a dimer and that, under certain conditions, dissociation into the monomers occurs (18).

This process is accompanied by a shift of isoelectric points toward lower values. It seems, therefore, promising to investigate whether the changes in pI generated by removal or incorporation of sialic acid residues are also paralleled by changes in the molecular-weight distribution.

Of considerable interest is the finding that asialointerferon binds to the phytoagglutinin isolated from *Phaseolus vulgaris* and can be displaced from the adsorbent by a glycoprotein fragment prepared from human erythrocytes. Since the carbohydrate structure of this membrane component is known, it is tempting to assume a related saccharide sequence in interferon. This hypothesis can be tested by use of other agglutinins with different specificities. We hope that the relative affinity of interferon toward these agglutinins and especially the eventual competition with glycoproteins of known structure will provide additional information on the composition of the carbohydrate part. A further implication of this finding is the potential use of agglutinins for affinity chromatography of interferon. Our goal is to develop a simple method by which large amounts of tissue culture supernates can be processed and rapid concentration of interferon is achieved. It is clear that the feasability of this approach depends on two parameters: preferential adsorption of interferon in relation to other glycoproteins present and efficient elution from the adsorbent with low molecular weight saccharides. Preliminary experiments seem to indicate that the phytoagglutinin found in castor beans, which is specific for terminal galactose residues, fulfills these requirements, and we hope that affinity chromatography of interferon will add a novel tool to the existing armamentarium of purification procedures.

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