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Elimination of size and charge heterogeneities of human leukocyte interferons by chemical cleavage

(sodium dodecyl sulfate–polyacrylamide gel electrophoresis/isoelectric focusing/deglycosylation)

WILLIAM E. STEWART II*, LEO S. LIN*, MARZENNA WIRANOWSKA-STEWART*, AND KARI CANTELL†

* Interferon Laboratories, Memorial Sloan-Kettering Cancer Center, New York, New York 10021; and † Central Public Health Laboratories, Helsinki, Finland

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ABSTRACT **Human leukocyte interferon (HuLeIF) preparations contain distinct molecular forms of interferon exhibiting significant heterogeneities in sizes when analyzed by electrophoresis in sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gels, migrating in two broad bands of activity with peaks at about 21,000 and 15,000 daltons. HuLeIFs exhibit extensive charge heterogeneities when analyzed by isoelectric focusing, resolving into several major peaks of approximately equal activity distributed from pH 5.7 to 7.0. When HuLeIF preparations are treated with 0.01 M sodium periodate buffer, pH 4.5, at 4°, both the size and charge heterogeneities rapidly disappear: periodate-treated HuLeIF migrates as a single, narrow band at 15,000 daltons in $NaDodSO_4$/polyacrylamide gels and focuses as a single, narrow band at pH 5.7. Quantitative considerations suggest that either the larger, heterogeneously charged HuLeIFs are converted to the smaller, size- and charge-homogeneous interferon by extensive chemical deglycosylation, or, alternatively, the smaller, 15,000 dalton, pH 5.7 interferon is much more stable to periodate treatment than are the other interferon forms. However, the activity of each of the variously charged forms of HuLeIF isolated from focusing gels exhibited the same stability as the pH 5.7 component; similarly, the activity of each of the size-forms of HuLeIF isolated from $NaDodSO_4$/polyacrylamide gels exhibited the same stability as the 15,000-dalton interferon.**

Even as the important roles played by interferons in processes of natural recovery from viral infections are becoming increasingly evident (1, 2), it also becomes apparent that production potentials will drastically restrict the interferons' availability for the clinic. Several laboratories have, therefore, felt it important to attempt complete purification of interferons with the goal of determining their essential components for possible chemical synthesis.

However, many laboratories involved in attempting purification of human leukocyte interferons (HuLeIFs) to homogeneity have found their efforts thwarted by the realization that there are, in fact, distinct molecular forms of interferons in HuLeIF preparations, differing in size (3–6) and charge (7–9). These findings have prompted considerable speculation on the nature of these heterogeneities. Some workers have claimed that HuLeIFs are glycoproteins and that their size and charge heterogeneities can both be reduced by glycolytic enzymes (7). However, other investigators have reported that the charge heterogeneities are unaffected by treatment with glycosidases (9), and, on the basis of failure of HuLeIFs to bind to lectins, some authors have maintained that HuLeIF is not a glycoprotein (10).

Analysis of the broad distribution of interferon activities within the two constant peaks of activity isolated by sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis of HuLeIF preparations (3, 4) tempted us to speculate that the heterogeneities of HuLeIFs are attributable to varying degrees of extraneous, nonessential, carbohydrate embellishments of polypeptide backbone(s). We, therefore, undertook to reduce the heterogeneities of HuLeIFs by chemical cleavage of carbohydrate moieties by oxidation with sodium periodate. Our findings indicate that this treatment can be used to convert HuLeIF preparations to size and charge homogeneity.

MATERIALS AND METHODS

Interferons. Human leukocyte interferons were prepared from human leukocytes induced with Sendai virus and were purified as previously described (11). The two preparations used in these studies had a titer of approximately 6×10^6 units/ml and a specific activity of about 10^6 units/mg of protein, and a titer of approximately 120×10^6 units/ml and a specific activity of about 2×10^6 units/mg of protein; these samples are designated P-IF and P-IF concentrate, respectively. Interferon samples were assayed by a microtitration assay on human fibroblast cultures by protection of cytopathogenic effect of vesicular stomatitis virus. In this assay 1 unit is equivalent to approximately 1 NIH human leukocyte interferon reference unit when titrated against reference reagent G023-901-527.

Periodate Treatment. Sodium metaperiodate (0.02 M) was made up in 0.1 M sodium acetate buffer and the final pH was adjusted to 4.5 with glacial acetic acid (12).

This reagent was stored at 4° in a foil-wrapped bottle, and has given identical results over a period of several weeks.

Interferon samples were diluted with equal volumes of periodate buffer and were kept at 4° for the indicated times, when 0.1 ml aliquots were removed and diluted 1:10 with 50% (vol/vol) ethylene glycol solution to stop the reaction. Samples were then divided into two equal parts, one for $NaDodSO_4$/polyacrylamide gel electrophoresis, and one for isoelectric focusing. Samples for $NaDodSO_4$/polyacrylamide gels were dialyzed against 0.01 M sodium phosphate buffer, pH 7.1. Samples for isoelectric focusing were dialyzed against 0.03 M NH_4HCO_3 buffer, pH 7.6, lyophilized, and resuspended in 0.2 ml of focusing sample buffer which contained 9.5 M urea (Schwarz/Mann, Ultrapure), 2% (wt/vol) Nonidet P40 (NP-40; Shell Imperial), and 2% Ampholine (vol/vol) (LKB; 4:1 mixture of pH 5–7 and pH 3–10).

Isoelectric Focusing. A modification of the isoelectric focusing procedure of O'Farrell (13) was used to determine the various components of HuLeIF preparations. Briefly, these gels consisted of 4% acrylamide, 9 M urea, 2% Nonidet 40, and 2% Ampholine mix. Cylindrical gels (0.25 cm × 24 cm) were used. An aliquot of 0.02 ml of interferon sample in focusing buffer

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Abbreviations: HuLeIF, human leukocyte interferon; $NaDodSO_4$, sodium dodecyl sulfate; M_r, molecular weight.

Table 1. Sensitivity of human leukocyte interferon preparations to periodate

Interferon*	Residual activity at indicated times, $\log_{10}$ (units)/ml								
	0	15 min	30 min	45 min	1 hr	2 hr	4 hr	6 hr	24 hr
P-IF	5.0	5.0	5.0	4.8	4.8	4.5	4.5	4.0	3.0
	5.3	5.0	5.0	4.8	4.8	4.8	4.8	4.5	4.0
P-IF	5.0	4.9	4.6	4.6	4.6	4.6	4.5	4.0	3.3
concentrate	5.0	4.8	4.8	4.6	4.6	4.6	4.5	4.0	3.0

* Interferon preparations were diluted to contain about 2×10^6 units/ml and were mixed with equal volumes of periodate buffer. At indicated times, aliquots were diluted 1:10 with 50% ethylene glycol and assayed for interferon. Results from two experiments with each interferon are presented.

was applied to each gel. Isoelectric focusing was performed at room temperature with a potential of 400 V, for approximately 16 hr until the current reached a minimum of 0.3 mA. The potential was then raised to 800 V for 1 hr. Gels were fractionated into 2.2-mm segments, and each fraction was eluted into 1 ml of Eagle's minimal essential medium containing 10% fetal calf serum and was stored at 4° for interferon assays. The pH gradient was determined by eluting 1-cm fractions of a gel overnight in 1 ml of 0.0375 M NaCl and measuring the pH at room temperature.

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. Interferon samples in 0.01 M sodium phosphate buffer were constituted to contain 5 M urea and 1% NaDodSO$_4$. Bromphenol blue in sucrose was added to make samples 6% sucrose and samples were heated to 100° for 1 min. Aliquots of 0.1 ml of each sample were simultaneously electrophoresed on parallel 20-cm cylindrical NaDodSO$_4$/polyacrylamide gels, as previously described (14). When the dye front had migrated about 18 cm into gels, gels were sliced into 2.2-mm segments, and two adjacent slices were pooled into each fraction and were eluted overnight at 4° into 1 ml of minimal essential medium containing 10% fetal calf serum and stored at 4° for interferon assays.

RESULTS

Effect of Periodate Oxidation on Antiviral Activity of Human Leukocyte Interferon. Conflicting reports have appeared as to the sensitivity of HuLeIF to inactivation by periodate (15, 16). We found that HuLeIF preparations were relatively stable to mild treatment with periodate: 30–50% of the original activity was consistently recovered after 4-hr treatment (Table 1). However, approximately 90% of original activity level was lost by 6 hr and about 99% by 24 hr.

Elimination of Charge Heterogeneity of Human Leukocyte Interferon by Periodate Treatment. Human leukocyte interferon preparations were treated with periodate buffer for 15, 30, 45, 60, 120, 240, and 360 min and 24 hr and were analyzed by isoelectric focusing. The total activity recovered from the gels was, in each case, approximately 100% of the activity applied to the gels. Untreated HuLeIF was resolved into 12 sharply defined peaks, 7 of which were of about equal activity (Fig. 1*A*). The activity of these peaks was spread approximately evenly over about 50% (12 cm) of the total length of the 24 cm gel. (Initial experiments were performed using 11.5-cm focusing gels, which resolved four major peaks of activity; however, the breadth and structural irregularity of these peaks suggested these gels did not accurately reflect the true extent of charge heterogeneity of the HuLeIF preparations; we therefore changed to the 24-cm focusing gels.) Importantly, the peak at pH 5.7 contained about 2,500 units (16%) of the 19,000 units recovered from the gel (15,000 units applied; 125% recovery). HuLeIF treated for 4 hr with periodate buffer resolved into a single narrow band of activity at pH 5.7 containing about 4500 units spread over <2% of the total length of the gel Fig. 1E. This single peak contained about 30% of the total activity of the original interferon sample prior to periodate treatment. Of all the focusing gels of HuLeIF samples treated with periodate for 15, 30, 45, 60, 120, and 240 min., the total recovery

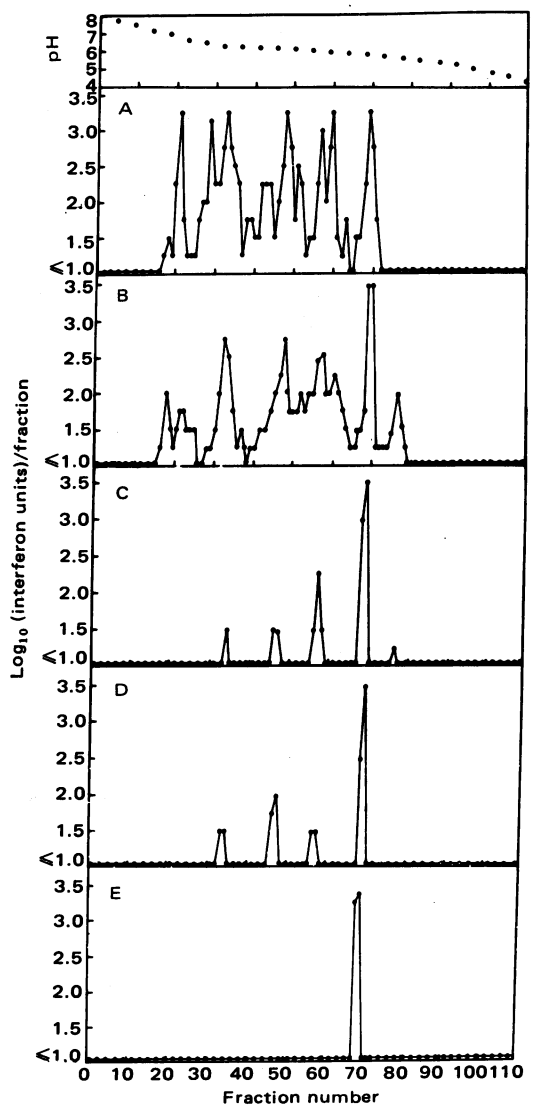

FIG. 1. Elimination of charge heterogeneities of human leukocyte interferons by periodate treatment as demonstrated by isoelectric focusing. (*A*) Isoelectric focusing pattern of normal HuLeIF; 15,000 units applied to the gel; approximately 19,000 units (125%) recovered. (*B*) HuLeIF treated with periodate buffer 30 min; 10,600 units (71% of original activity of HuLeIF preparation) recovered. (*C*) HuLeIF treated 1 hr; 4400 units (29%) recovered. (*D*) HuLeIF treated 2 hr; 3600 units (24%) recovered. (*E*) HuLeIF treated 4 hr; 4500 units (30%) recovered.

in the peak at pH 5.7 was always higher than the activity in "native" HuLeIF preparations at this position (treated samples: 3500–6000 units; untreated: 2500). Although the range of variability of the interferon assays (approximately 2-fold differences are subjective) prevents determination that the absolute activity increased at this position during disappearance of the other equal-sized peaks of original activity, the constancy of this slight increase certainly suggests this possibility. Indeed, while all the major peaks in the original HuLeIF preparation lost more than 99% of their original antiviral activities, the pH 5.7 peak apparently increased its activity. By 6 hr, the peak of activity remained at pH 5.7 but diminished to approximately $\frac{1}{3}$ and by 24 hr had decreased to about $\frac{1}{10}$ (data not shown). This suggests that elimination of extraneous carbohydrate moieties is initially achieved to shift the interferons to the pH 5.7 peak, and, once shifted to this position, the molecules are attacked further by the periodate at positions which are essential for biological activity. These sensitive targets could be either indispensible carbohydrate components or certain periodate-sensitive amino acids (17). It is important to emphasize that we have repeatedly obtained results essentially identical to those shown in Fig. 1 with different HuLeIF preparations on several occasions.

Elimination of Size Heterogeneity of Human Leukocyte Interferon by Periodate Treatment. Human leukocyte interferon preparations treated with periodate for 15, 30, 45, 60, 120, 240, and 360 min and 24 hr were electrophoresed in NaDodSO$_4$/polyacrylamide gels. The total activity recovered from the gels was in each case about 100% of the activity applied to the gels. As previously reported (3, 4, 18), the activity was isolated from two broad overlapping bands of activity covering about 25% of the gel length with peaks of activity at 21,000 and 15,000 daltons (Fig. 2*A*). However, after treatment with periodate for 4 hr, all the activity was recovered in a narrow band of activity at 15,000 daltons (Fig. 2*E*). Again, as with the focusing gels, the activity recovered in this size-homogeneous peak was about 30% of the original total activity of the HuLeIF sample prior to periodate treatment.

The total activity recovered in the 15,000-dalton peak after 4-hr periodate treatment was not significantly different from the activity at this position in the untreated HuLeIF preparation, but the material in the 21,000-dalton peak had lost all (more than 99%) of its detectable activity. However, even had all the activity of the 21,000-dalton peak been quantitatively converted to the smaller interferon form, its addition would not have detectably increased the total activity at that position, owing to the imprecision of the interferon assays. After 6 hr of treatment the activity at the 15,000-dalton position began to decline further (10% recovery) and by 24 hr (data not shown) had decreased to only about 1% of the total starting activity of the preparation. This suggests that the size-heterogeneous interferons are first converted to the smaller component by cleavage of nonessential carbohydrate components and once so stripped are inactivated by further attack, either on essential carbohydrate components or on periodate-sensitive amino acids. Results essentially identical to those shown in Fig. 2 have been obtained with different HuLeIF preparations.

We have also determined that the periodate treatment did not affect the mobility of non-carbohydrate-containing protein markers. Marker proteins treated for 24 hr with periodate were quantitatively and qualitatively electrophoretically identical to untreated marker proteins.

Relative Periodate Sensitivities of the Various Human Leukocyte Interferon Forms Isolated from Isoelectric Focusing Gels. The disappearance of HuLeIF activity at each of

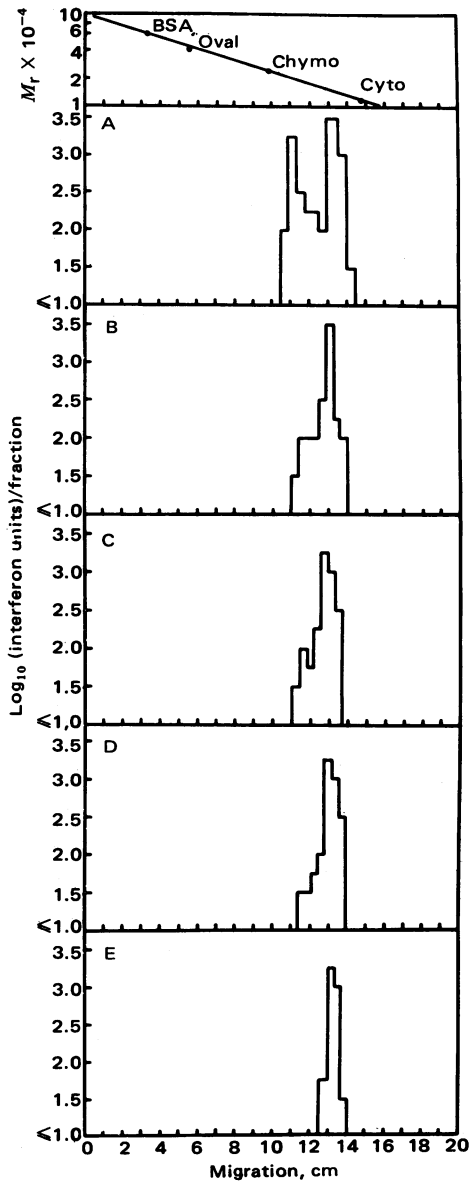

FIG. 2. Elimination of size heterogeneities of human leukocyte interferons by periodate treatment as demonstrated by NaDodSO$_4$/polyacrylamide gel electrophoresis. (*A*) Pattern of normal HuLeIF; 10,000 units applied to the gel; approximately 9900 units (99%) recovered. (*B*) HuLeIF treated with periodate buffer 30 min; approximately 4000 units (40% of the original activity of HuLeIF preparation) recovered. (*C*) HuLeIF treated for 1 hr; 3400 units (34%) recovered. (*D*) HuLeIF treated 2 hr; 3500 units (35%) recovered. (*E*) HuLeIF treated 4 hr; 3100 units (31%) recovered. Protein molecular weight (M_r) markers (●): BSA, bovine serum albumin; Oval, ovalbumin; Chymo, chymotrypsinogen; Cyto, cytochrome *c*.

the higher pH values prior to loss of activity at the pH 5.7 position tempts one to speculate that cleavage of carbohydrate moieties or periodate-sensitive amino acids shifts the interferons to the pH 5.7 peak. Alternatively, the activity of the pH 5.7 species of interferon might be relatively resistant to inactivation while all the other interferons are rapidly destroyed by the periodate treatment. To determine which of these possibilities is the more likely, we tested the periodate sensitivities of five of the major peaks of activity.

The materials in the activity peaks eluted from focusing gels at indicated pH values were treated with periodate for indicated times (Table 2), and reactions were terminated by addi-

Table 2. Relative periodate sensitivities of human leukocyte interferons isolated from isoelectric focusing gels

Gel fraction*	pI	Residual activity at indicated times, $\log_{10}$ (units)/ml								
		0	15 min	30 min	45 min	1 hr	2 hr	4 hr	6 hr	24 hr
21	6.7	2.5	2.5	2.3	2.3	2.2	2.2	2.0	2.0	1.3
33	6.4	2.3	2.0	2.0	2.0	2.0	1.8	1.8	1.5	1.0
48	6.2	2.8	2.5	2.3	2.3	2.3	2.0	2.0	1.8	1.5
60	6.0	2.5	2.5	2.3	2.3	2.3	2.0	2.0	1.8	1.5
69	5.7	2.8	2.5	2.5	2.5	2.3	2.3	2.3	1.8	1.5

* Gel fractions from isoelectric focusing gels, as indicated in Fig. 1, were eluted into 1 ml of minimal essential medium containing 10% fetal calf serum and treated with periodate.

tion of ethylene glycol. The activity of each of the interferons disappeared at similar rates. When total interferon was treated with periodate, the activities focusing at pH values greater than 5.7 declined to less than 1% of their original levels by 4 hr of treatment, while no activity was lost at pH 5.7 (Fig. 1). However, each of the interferons isolated at the various pH values, including the pH 5.7 peak, declined to about 10–30% of their original activities by 4 hr.

Thus, these data support the interpretation that the activity focusing at pH 5.7 is initially maintained by conversion of the variously charged interferons to this form.

Relative Periodate Sensitivities of the Large and Small Human Leukocyte Interferon Forms Isolated from NaDodSO$_4$/Polyacrylamide Gels. The disappearance of HuLeIF activity at the 21,000-dalton position prior to loss of activity of the 15,000-dalton form suggested that either the larger interferon was first cleaved to the smaller component or that the larger component was sensitive to destruction by periodate oxidation, while the smaller interferon was relatively resistant to such inactivation.

However, when each of these components was isolated from NaDodSO$_4$/polyacrylamide gels and treated with periodate, they were found to have similar stabilities (Table 3). Thus, when interferon was treated before application to gels, though activity of the interferon migrating at 21,000 daltons disappeared completely by 4 hr of periodate treatment, while the 15,000-dalton peak remained unchanged (Fig. 2), the interferons isolated from gels at these positions lost activity at the same rates, declining to about 20–30% by 4 hr.

These data thus support the interpretation that the activity at the 15,000-dalton peak is maintained by conversion of the larger interferon to the smaller form.

DISCUSSION

The heterogeneities of HuLeIFs have been demonstrated by a number of parameters, including size (3–6, 18) and charge (7–9). Some workers have attributed these heterogeneities to differences in carbohydrate composition, because treatment of HuLeIF preparations with glycosidases slightly reduced this charge heterogeneity and changed the apparent molecular weights as determined by gel filtration (7). However, other investigators have reported that neuraminidase treatment failed to change the isoelectric focusing pattern of HuLeIF preparations (9), and some authors have claimed that HuLeIFs are not glycoproteins (10). Because HuLeIFs are resolved into two major components on NaDodSO$_4$/polyacrylamide gels (3–6, 18) and can also be separated into two components by ion-exchange chromatography, it has been suggested that one size component possesses charge properties for binding to the ion-exchange resin and the other size component does not adsorb to the resin (8). However, analysis of the width of the two size-peaks isolated from HuLeIF preparations by NaDodSO$_4$/polyacrylamide gels suggested to us that each size component might be variously embellished by carbohydrate moieties or that the two peaks might vary entirely on the basis of major carbohydrate differences, while microheterogeneities within each major carbohydrate modification would broaden the two peaks.

Enzymatic removal of carbohydrate groups has been attempted in a number of laboratories to determine whether carbohydrate moieties are essential for interferon activities (7, 9). However, we decided against this approach because many glycolytic enzymes are difficult to separate from contaminating proteolytic enzymes, and, more importantly, quantitative considerations of the total protein applicable to either analytical NaDodSO$_4$/polyacrylamide gels or isoelectric focusing gels would necessitate separation of added enzyme proteins from the reaction mixtures before analyses. We, therefore, undertook to chemically cleave the purported nonessential carbohydrate components from HuLeIFs.

Our data suggest that the heterogeneity of HuLeIF preparation is eliminated by the conversion of the larger and charge-heterogeneous interferons to the small, charge-homogeneous interferon (pI 5.7; molecular weight M_r 15,000). This interpretation is suggested both by the constancy (and even slight increase) of activity at this position in both focusing and NaDodSO$_4$/polyacrylamide gels, while all the other components rapidly and completely disappear. Alternatively, the pI 5.7, M_r 15,000 HuLeIF could be completely stable in periodate while the other components are all destroyed by its oxidative activity. However, because the activity of the M_r 15,000, pI 5.7

Table 3. Relative periodate sensitivities of human leukocyte interferons isolated from NaDodSO$_4$/polyacrylamide gels

Sample*	Apparent molecular weight	Residual activity at indicated time, $\log_{10}$ (units)/ml								
		0	15 min	30 min	45 min	1 hr	2 hr	4 hr	6 hr	24 hr
Large interferon	21,000	2.3	1.8	1.8	1.5	1.5	1.5	1.5	1.3	<1.0
		2.0	1.8	1.5	1.5	1.5	1.3	1.3	1.3	<1.0
Small interferon	15,000	2.5	2.3	2.0	2.0	2.0	2.0	2.0	1.8	1.0
		2.5	2.0	2.0	2.0	1.8	1.8	1.8	1.5	1.0

* Gel fractions from NaDodSO$_4$/polyacrylamide gels, corresponding to the slower (large interferon) and faster (small interferon) peaks of activity indicated in Fig. 2*A*, and treated with periodate. Results from two experiments with each interferon are presented.

interferon is only stable until all the activity at other positions disappears in the gels and then itself begins to lose activity, it is tempting to speculate that activity at this position is initially maintained by conversion of activity to this form from the other components.

Further support for this interpretation was obtained when each of the interferons isolated from both focusing gels and $NaDodSO_4$/polyacrylamide gels at various pH values and sizes, respectively, were all found to have similar rates of inactivation by periodate treatment. Thus, though interferon activity focusing at pH 5.7 or migrating in $NaDodSO_4$/polyacrylamide gels at 15,000 daltons remained constant for 4 hr while all other positions were losing more than 99% of their original titers in the same period, the various peaks isolated from the gels all exhibited the same stability as the pH 5.7- and 15,000-dalton gel-isolated interferons.

The finding that after all the activity is resident in the small molecular weight interferon, it too begins to lose activity but stays at this size position suggests two possibilities: either all the carbohydrate is removed by the process of converting the interferons to this size and further oxidation attacks amino acids essential for activity, or further removal of carbohydrate reduces the size even further but the interferon is no longer active. Evidence has previously been presented that favors the latter possibility: the smaller HuLeIF component isolated in $NaDodSO_4$/polyacrylamide gels has a peak of activity at 15,000 daltons *when assayed on human cells;* however the same gel isolates *assayed on cat cells* have peak activity at 13,500 daltons, a position practically devoid of activity on human cells (4). We have confirmed this observation in bovine cells (W. E. Stewart II, M. Wiranowska-Stewart, and L. S. Lin, unpublished data). These data suggest, therefore, that further cleavage of carbohydrate from HuLeIF after its conversion to 15,000 daltons may eliminate its activity for homologous cells while converting it to a lower molecular weight interferon active on heterologous cells.

Unlike the HuLeIFs, human fibroblast interferons appear to be glycoproteins relatively homogeneous in size at about 20,000 daltons (5, 19,20). It has been reported that at least part of the carbohydrate moiety of this interferon is not necessary for activity (7), and Havell *et al.* (21) have shown that interferon preparations synthesized by human diploid fibroblasts treated with glycosylation inhibitors contain interferons that have a molecular weight of about 16,000 in $NaDodSO_4$/polyacrylamide gels. It should be possible to chemically eliminate the carbohydrate components from human fibroblast interferons. Native HuLeIFs and human fibroblast interferons have been shown to be distinguishable antigenically (22), biologically (4, 23), in stabilities (24, 25), and in "stickiness" (10, 26, 27). We hope that deglycosylation experiments similar to those described here will provide smaller interferon cores from both HuLeIFs and human fibroblast interferons which can be directly compared for determining the contribution of carbohydrates to each of these properties of the various human interferons.

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