The Isolation and Amino Acid/Sugar Composition of Human Fibroblastoid Interferon*

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Human fibroblastoid interferon produced from an established human cell line was purified by controlled-pore glass and concanavalin A-Sepharose column chromatography followed by preparative two-dimensional gel electrophoresis. The purification procedure provided a 10% recovery of pure interferon with good reproducibility. The purified protein was homogeneous with respect to its molecular weight of 20,000 and net electrical charge at pH 2.5. Interferon of high specific activity of $5 \times 10^6$ units/mg of protein was directly demonstrated in the polyacrylamide gel before staining with Coomassie brilliant blue. Parallel purification of a sham-induced interferon preparation did not yield an equivalent product indicating the purified interferon is not derived from uninduced cells or from the fetal calf serum of the tissue culture growth medium. Pure interferon was radiodiiodinated by Bolton-Hunter reagent. Amino acid analysis of the pure preparation shows interferon to be a leucine-rich glycoprotein containing a high percentage of glutamic/glutamine residues and that disulfide bridge(s) are important for its biological activity.

The large scale purification of interferon to homogeneity has eluded investigators for almost two decades. Initially, the difficulty in the purification of interferon was the scarcity of this protein. In recent years, concerted efforts have been made to produce adequate amounts of interferon for the development of procedures for its purification (1-10). One criticism of these procedures concerns the use of a single parameter to show the purified product is homogeneous, i.e. the demonstration of interferon activity in one or more protein fractions after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The problem with introducing another parameter such as the homogeneity of net electrical charge is that interferon usually does not migrate as a singular discrete fraction in electrophoresis (10). Furthermore, interferon is polydisperse during electrofocusing (8, 9). This polydispersity was attributed to differences in the degree of glycosylation, possible microheterogeneity in the peptide composition, and even differential removal of sodium dodecyl sulfate from interferon previously exposed to sodium dodecyl sulfate during electrophoresis (10). We now report the purification of interferon to homogeneity with respect to its molecular weight and with respect to its net electrical charge at pH 2.5 in the presence of 2.5 M urea. The recovery of pure interferon by this purification procedure is at least 10% and no equivalent product is obtained when appropriate sham-induced interferon preparations are subjected to the same purification procedure. Pure human interferon has now been obtained for (a) the preparation of biologically active $^{125}$I-labeled interferons, (b) analysis of the amino acid/sugar composition of interferon, and (c) chemical modification experiments to determine which residues are important for biological activity.

RESULTS AND DISCUSSION

Preliminary Purification to 10% Purity—Our approach was to find a method that would provide a preliminary purification of interferon to about 10% purity with nearly quantitative recovery and then to purify it to homogeneity by two-dimensional gel electrophoresis. The preliminary purification procedure consists of chromatography on controlled-pore glass column followed by chromatography on concanavalin A-Sepharose column. Interferon bound to the CPG column at neutral pH is eluted by lowering the pH of the eluting buffer to 3.0. The CPG-purified interferon is stable at pH 3.0, and if the pH is adjusted to neutrality most of the interferon is irreversibly lost in co-precipitation with other proteins. For this reason, it was necessary to maintain the pH of CPG-purified interferon at an acid pH not exceeding 4.5 to avoid the irreversible loss of interferon through precipitation. The CPG-purified interferon is applied to a Con A column to which it binds strongly at pH 4.5 but most of the non-interferon proteins do not. Interferon is not eluted from the column by buffers of high ionic concentrations and only a small percentage (5%) is eluted by 100 mM α-methylmannoside. However, most (80%) of the interferon activity applied to the Con A column is recovered in 50% ethylene glycol. The binding of interferon to CPG and to Con A is most probably due to a combination of hydrophobic and ionic interactions. This explanation is consistent with the analysis given in Table II, that interferon is rich in hydrophobic amino acid residues and contains charged amino acids as well as amino sugars. The impurities of the interferon preparation derived from this preliminary purification are shown in Fig. 1. The specific activity of the partially purified interferon is nearly $1 \times 10^6$ reference units, with a recovery of

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† The "Experimental Procedures" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 79M-355, cite author(s), and include a check or money order for $1.00 per set of photocopies.

‡ The abbreviations used are: CPG, controlled-pore glass; Con A, concanavalin A-Sepharose column.

§ Interferon activity was referenced to a human diploid fibroblast standard prepared and tested by Drs. W. Merk and G. Bodo. This standard was in turn referenced against the 69/19 MRC human leukocyte interferon.
60 to 70% (Table I). However, this preliminary procedure introduces concanavalin A and its peptides into the preparation. We had previously reported that phenyl-Sepharose column chromatography could remove these extraneous concanavalin A contaminants (10) but on closer examination a small amount of the $M_r = 20,000$ concanavalin A fragment may sometimes be present. Consequently, other procedures were investigated, and a method which removes the concanavalin A contaminants as well as separating interferon from the other proteins is the two-dimensional gel electrophoretic step described below.

**Two-Dimensional Gel Electrophoresis**—The first step consists of electrophoresis in a 15% polyacrylamide gel containing 2.5 M urea (acid-urea). The second step is polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate at pH 8.8. Proteins are separated in the first dimension according to their charge and in the second dimension according to their molecular weight. In the acid-urea gel, interferon migrates well ahead of concanavalin A and its peptides and most of the extraneous proteins remaining in the Con A-purified preparation (Fig. 2A). Interferon obtained from the acid-urea gel is already highly purified and the pure protein is isolated in the final step by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The purity of the final product in Figs. 2 and 3 was examined by Coomassie blue staining as well as by radioiodination with the Greenwood-Hunter or the Bolton-Hunter reagent (11, 12). In the case of radioiodination by the Bolton-Hunter reagent, it was possible to correlate the biological activity of interferon with its radioactive label.

**Homogeneity, Polydispersity, and Monodispersity**—The definition of pure interferon requires some discussion. A pure or homogeneous preparation usually is one which consists of identical molecules, but this is an uncommon situation even with highly pure native proteins. For example, several pure proteins possess more than one isoelectric point. Evidently, this is the rule rather than the exception for most glycoproteins (14). Consequently, a protein preparation can be pure and yet polydisperse, meaning the molecules in the preparation have the same overall chemical structure but vary in detail with respect to shape, size, or charge. Thus, in current glycoprotein purification literature, the term homogeneity refers to the monodispersity of a protein with respect to a specific parameter such as the molecular weight and a net charge under certain conditions. The purity of most interferon preparations until now has relied on the demonstration of interferon activity with a monodisperse population of molecules of the same molecular weight. In this study, we extend the criterion of homogeneity to its electrical charge in an acid-urea gel. In this system, the antiviral activity of interferon can be either eluted from a polyacrylamide gel column (Uniphor LKB, Sweden) with an $R_f$ of 0.44 or excised from a slab gel ($I$ in Fig. 2A). In either case, electrophoresis in the second dimension shows the fraction containing interferon activity to be a protein of 20,000 molecular weight (Figs. 2 and 3). A similar co-migration of interferon activity with protein can be demonstrated by gel electrophoresis at higher pH values (10) but the protein fraction is diffuse indicating a degree of polydispersity in its net charge at alkaline pH.

In these studies, we purified sham-induced interferon preparations to determine whether a protein of the same molecular weight as interferon is obtainable. We regard this to be an important control to eliminate the possibility that another protein may have co-purified with interferon. The control preparation was obtained by treating cells with 2.5 $\mu$g/ml of actinomycin D for 45 min in the absence of poly(rI)-poly(rC) and cycloheximide and harvesting the culture medium 24 h later. After this treatment, many endogenous cell proteins are released into the medium as also occurs during actual interferon production, but the amount of interferon produced is only 0.01% that of the induced cells. Purification of the sham-induced preparation did not yield an equivalent $M_r = 20,000$ component with the charge characteristics of the protein isolated from the medium of induced cultures (Fig. 4), indicating that the putative interferon is derived only from induced cells and is unlikely to be a serum or endogenous cell protein or a contaminant introduced during the purification.
Isolation and Composition of Human Fibroblastoid Interferon

FIG. 2. Two-dimensional gel electrophoresis of partially purified C-10 interferon. A, human fibroblast interferon obtained from C-10 cells was chromatographed on a CPG column and on a Con A column. The partially purified interferon was dialyzed to remove ethylene glycol and lyophilized. The freeze-dried interferon was solubilized in 50 µl of 8% acetic acid containing 4 M urea and electrophoresed in an acid-urea polyacrylamide gel. The gel was prepared by polymerizing 27 ml of 15% acrylamide (Bio-Rad; electrophoresis grade) with a cross-link of 40% acrylamide:bisacrylamide containing 12 µg of thiourea and 100 µl of H2O. The polymerized gel had a dimension of 12 cm × 14 cm × 0.75 cm and electrophoresis was at 20 mA constant current and 230 V for 3.5 h. The resulting gel was stained in 0.05% Coomassie brilliant blue in methanol:water:glacial acetic acid (5:5:1, v/v/v) and destained in 7% acetic acid. The proteins in this gel are indicated by the letters a to l. The protein fractions were sliced from the gel and extracted by first homogenizing the gel in 1% sodium dodecyl sulfate and incubating the extract at room temperature for 48 h. The extracts were then assayed for human interferon activity by the method of Tan (15). B, distribution of antiviral activity in the acid-urea gel shown in A is presented. Interferon activity is coincident with the stained protein(s) in l (arrow). About 1 × 10⁶ units of interferon were applied and 2 × 10⁹ units recovered from the stained gel. Higher recovery of activity was obtained when the extract was boiled in 1% sodium dodecyl sulfate. C, acid-urea-purified interferon was subjected to electrophoresis on a 12% (see D) and a 10 to 18% gradient (see E) polyacrylamide gel according to the method of Berthold et al. (10). After electrophoresis, the gel was sliced into 1-mm sections which were extracted in 1% sodium dodecyl sulfate and assayed for anti-viral activity. Interferon activity in the gels was observed to co-migrate with the stained protein (arrows). About 3.5 × 10⁷ units of interferon were applied and in both cases about 2.5 × 10⁶ units were recovered. D, acid-urea-purified interferon was subjected to electrophoresis in a 12% polyacrylamide gel in the presence of sodium dodecyl sulfate. A portion of the gel was cut out and the remainder of the gel was stained in Coomassie brilliant blue and then destained. The molecular weight of the stained protein is 20,000. E, acid-urea-purified interferon was subjected to electrophoresis in a 10 to 18% gradient polyacrylamide gel in the presence of sodium dodecyl sulfate and stained/destained as in D. The molecular weight of the stained protein is 20,000. F, human fibroblast interferon from an acid-urea gel was radioiodinated by the Greenwood-Hunter procedure described for interferon by Berthold et al. (10). The labeled protein was subjected to electrophoresis in a 12% polyacrylamide gel as in D. Kodak X-omatic film was exposed to the dried gel for 12 h and the resulting autoradiograph is presented. A parallel gel sample was analyzed for distribution of antiviral activity. The results indicated that interferon activity co-migrated with the labeled protein (arrow). The molecular weight of both biological activity and labeled protein is 21,000, slightly higher than the unlabeled and radioiodinated (Greenwood-Hunter) Mᵋ = 20,000 concanavalin A fragment used as a marker (not shown). An unassayed amount of ¹²⁵I-labeled interferon was applied on the gel. The specific radioactivity of the interferon extracted from the gel was 93.3 dpm/unit of antiviral activity.

FIG. 3. Polyacrylamide gel electrophoresis of C-10 interferon labeled by Bolton-Hunter reagent in the presence of sodium dodecyl sulfate. Acid-urea-purified interferon was radioiodinated using the method of Bolton-Hunter and subjected to electrophoresis on a 12% polyacrylamide gel in the presence of sodium dodecyl sulfate. Kodak X-omatic film was exposed to the dried gel for 12 h and the resulting autoradiograph is presented. A parallel gel was analyzed for distribution of antiviral activity. The results indicate that interferon activity co-migrated with the labeled protein (arrow). The molecular weight of both biological activity and labeled protein is 21,000, slightly higher than the unlabeled and radioiodinated (Greenwood-Hunter) Mᵋ = 20,000 concanavalin A fragment used as a marker (not shown). An unassayed amount of ¹²⁵I-labeled interferon was applied on the gel. The specific radioactivity of the interferon extracted from the gel was 93.3 dpm/unit of antiviral activity.

Amino Acid and Amino Sugar Composition of Interferon—Two different methods were used to prepare the pure interferon for acid hydrolysis (see miniprint). The results of these analyses are given in Table II. The amino acid compositions derived by either method of sample preparation are compa-
Isolation and Composition of Human Fibroblastoid Interferon

Fig. 4. Two-dimensional gel electrophoresis of partially purified human fibroblast interferon and a sham-induced interferon preparation. A, interferon purified through a CPG and then a Con A column was subjected to electrophoresis in an acid-urea gel as described in the legend for Fig. 2. The stained gel reveals proteins a to l. B, unstained proteins in a parallel acid-urea gel were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The stained proteins in the second dimension are identified alphabetically to correspond to the proteins resolved in the first dimension. C, unstained proteins in an acid-urea gel containing TABLE I Purification of C-10 human fibroblast interferon

| Step | Total activity | Specific activity |
|------|---------------|------------------|
| 100,000 X g supernatant | $2 \times 10^6$ | 1 to $2 \times 10^6$ |
| Ammonium sulfate precipitation (70%) | $2 \times 10^6$ | 1 to $2 \times 10^6$ |
| Controlled-pore glass column | $1.5 \times 10^6$ | $1 \times 10^6$ |
| Con A column | $1.2 \times 10^6$ | $1 \times 10^6$ |
| Acid-urea gel electrophoresis | $4 \times 10^5$ | 2 to $5 \times 10^5$ |
| Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate | $3 \times 10^5$ | 2 to $5 \times 10^5$ |

* Bovine albumin as standard.

Table I

rable (see Table II and miniprint). The analyses agree with previous observations on the physicochemical properties of fibroblast interferon. For example, hydrophobic amino acids (valine, phenylalanine, leucine, and isoleucine) are relatively abundant (30%) which is consistent with the well known hydrophobicity of the molecule. Fibroblast interferon also contains a moderate amount (15%) of the basic amino acids histidine, lysine, and arginine. These residues would provide a relatively strong net positive charge at low pH. This is what we observe since interferon migrates faster than other proteins in the acid-urea gel system (Fig. 2A).

Although the two-dimensional gel system should separate concanavalin A and its fragments from interferon, it is possible that a small amount of the $M_r = 20,000$ fragment is still present. To test this possibility, we isolated this protein from a preparation from sham-induced C-10 cells were subjected to electrophoresis under conditions similar to those for B. Examination of the resulting gel after staining/destaining reveals the absence of a protein(s) corresponding to that labeled i' in B. No antiviral activity was detectable from this gel in the vicinity of i'. D, distribution of antiviral activity of stained/destained gel in B. About $2.7 \times 10^8$ units of interferon activity were applied in the fast dimension. Recovery of antiviral activity from stained/destained gel was lower than from unstained gel.

Although human interferon was analyzed on the Beckman amino acid analyzer, a ninhydrin-positive fraction which did not correspond to an amino acid residue was eluted from the column shortly after tyrosine and phenylalanine. However, this peak did coincide with the galactosamine and the mannosamine standards which elute so close to each other that they overlap. This observation suggests that interferon contains galactosamine and/or mannosamine. These amino sugars are derived from either hexosamine and/or N-acetylhexosamine linked to interferon protein. These results are qualitative since the acid treatment carried out to hydrolyze the protein results in some degradation of amino sugars. Such degradation is known for other glycopeptides and glycoproteins hydrolyzed under conditions used for amino acid analysis of proteins (14). In order to quantitate the amino sugar content of interferon, it will be necessary to select conditions of acid hydrolysis conducive to the optimal release of amino sugars with minimum degradation. In addition, other analytical procedures must be tested to independently determine interferon's N-acetylhexosamine and sialic acid content (18).

Chemical Modification and Biological Activity—Purified human interferon was radiiodinated for the purpose of de-
**Table II**

Isolation and Composition of Human Fibroblastoid Interferon

Samples were hydrolyzed in 6 N HCl at 110°C for 20 h in evacuated tubes. The analyses were determined with a Beckman model 121 amino acid analyzer.

| Amino acid and sugar | Protein analyzed in gel | Protein eluted from gel |
|----------------------|-------------------------|------------------------|
|                      | Interferon(1)           | Concanavalin A fragment (M, = 20,000) | Control | Interferon(1) | Interferon(1) | Control |
|                      | nmole(2) (%)            | nmole(2) (%)            | nmole(2) (%) | nmole(2) (%) | nmole(2) (%) |
| Aspartic acid and asparagine | 0.34 (13.7) | 0.24 (16.7) | 0.02 | 0.35 (10.8) | 0.39 (11.8) | 0.10 |
| Threonine            | 0.12 (4.8) | 0.16 (11.1) | 0.00 | 0.15 (4.6) | 0.15 (4.6) | 0.02 |
| Serine               | 0.14 (5.7) | 0.19 (13.2) | 0.00 | 0.27 (8.3) | 0.22 (6.7) | 0.09 |
| Glutamic acid and glutamine | 0.42 (16.9) | 0.08 (5.6) | 0.00 | 0.54 (16.6) | 0.50 (15.2) | 0.13 |
| Proline              | 0.04 (1.6) | 0.01 (0.7) | 0.00 | 0.09 (2.8) | 0.11 (3.3) | 0.00 |
| Glycine              | 0.08 (3.2) | 0.09 (6.3) | 0.00 | N.I.(4) | N.I. | 0.52 |
| Alanine              | 0.12 (4.8) | 0.07 (4.9) | 0.00 | 0.18 (5.5) | 0.20 (6.1) | 0.05 |
| Valine               | 0.10 (4.0) | 0.10 (6.9) | 0.00 | 0.14 (4.3) | 0.18 (5.5) | 0.00 |
| Methionine(5)        | 0.02 Trace | 0.00 | 0.01 (0.3) | Trace | 0.00 |
| Isoleucine           | 0.18 (7.3) | 0.07 (4.9) | 0.00 | 0.17 (5.2) | 0.17 (5.2) | 0.00 |
| Leucine              | 0.42 (16.9) | 0.18 (12.5) | 0.00 | 0.51 (15.7) | 0.49 (14.9) | 0.02 |
| Tyrosine             | 0.02 (0.8) | 0.06 (4.2) | 0.00 | 0.11 (3.4) | 0.05 (1.5) | 0.00 |
| Phenylalanine        | 0.10 (4.0) | 0.04 (2.8) | 0.00 | 0.14 (4.3) | 0.17 (5.2) | 0.00 |
| Histidine            | 0.08 (3.2) | 0.03 (2.1) | 0.01 | 0.09 (2.8) | 0.07 (2.1) | 0.01 |
| Lysine               | 0.20 (8.1) | 0.10 (6.9) | 0.00 | 0.20 (6.2) | 0.24 (7.3) | 0.03 |
| Tryptophan(6)        | N.D. | N.D.(6) | N.D. | N.D. | (6) 0.00 | (6) N.D. |
| Arginine             | 0.12 (4.8) | 0.02 (1.4) | 0.00 | 0.15 (4.6) | 0.16 (5.8) | 0.00 |
| Cysteamine(7)        | N.D. | N.D. | N.D. | N.D. | N.D. | 0.16/0.56 |
| Galactosamine/mannosamine(8) | N.D. | N.D. | N.D. | N.D. | N.D. | 0.16/0.56 |

(1) Purified interferon derived from that in Fig. 2D.
(2) Purified interferon derived from that in Fig. 2E.
(3) All values were corrected for background as determined by analysis of a control gel or gel extract.
(4) N.I., not integrated; N.D., not done.
(5) The glycine residues are not integrated due to the high glycine background which originates from the incomplete removal of Tris/glycine by extensive dialysis.
(6) Methionine value includes that for methionine sulfoxide.

Acid hydrolysis of 6 N HCl at 110°C for 20 h in evacuated tubes. The analyses were determined with a Beckman model 121 amino acid analyzer. The glycine residues are not integrated due to the high glycine background which originates from the incomplete removal of Tris/glycine by extensive dialysis. Methionine value includes that for methionine sulfoxide. The glycine residues are not integrated due to the high glycine background which originates from the incomplete removal of Tris/glycine by extensive dialysis. Methionine value includes that for methionine sulfoxide.

Acid hydrolysis of 6 N HCl at 110°C for 20 h in evacuated tubes. The analyses were determined with a Beckman model 121 amino acid analyzer. The glycine residues are not integrated due to the high glycine background which originates from the incomplete removal of Tris/glycine by extensive dialysis. Methionine value includes that for methionine sulfoxide. The glycine residues are not integrated due to the high glycine background which originates from the incomplete removal of Tris/glycine by extensive dialysis. Methionine value includes that for methionine sulfoxide.
biologically active fragment would be an ideal solution to the supply problems of this substance. Many attempts have been made in this laboratory to chemically degrade interferon in the hope of isolating a small but biologically active fragment. Thus, preparations of pure as well as partially purified interferon were treated with cyanogen bromide as previously described to cleave the peptide bond adjacent to the methionyl residue (17). In our investigations with CNBr cleavage experiments, interferon activity was not grossly affected. Moreover, no change was observed in the molecular weight of the CNBr-treated interferon (Fig. 5). It is likely that the methionyl residue if present is located close to either terminus of the interferon molecule (Table II). Alternately it is located between an S-S bond. Not surprisingly, attempts to generate an active interferon fragment by cyanogen bromide have been futile. It may be necessary to use biological methods to degrade interferon for the production of active fragments.

In summary, using the present purification procedure and relying on a relatively small scale of interferon production (50 to 100 roller bottles, 490 cm² each, per week) we have already obtained enough pure interferon for its chemical analysis. With the accumulation of more interferon, it will be possible to perform experiments to determine its amino acid sequence. The amino acid composition (Table II) should be useful in determining the composition of a radioactive amino acid mixture to use in the labeling of interferon in vivo as well as in vitro. The preparation of a radioactive interferon with high radio specific activity will be most useful in the microsequencing of interferon.

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Isolation and Composition of Human Fibroblastoid Interferon

Supplemental Material to Isolation and Amino Acid/Stage Composition of Human Interferon

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EXPERIMENTAL PROCEDURES

Interferon Production Assay—Interferon was produced and concentrated from C-19 cell line as previously described (10) and quantified on human fibroblast according to a semi-micro-method (15) except that a human fibroblast preparation referenced to 69-19 MRC-H human embryonic fibroblasts was used as the interferon standard. The human fibroblast standard was kindly supplied by Drs. W. Merck and G. Reis.

Mock Interferon Preparation—Sham-induced interferon was prepared from C-19 cells of the same cell density as the culture used for interferon production. The cultures were treated with the same concentration (25 μg/ml) of actinomycin D ( gift of Merck, Sharpe and Dose, N. J.) as used during "superinduction" (19) for 60 min. A small amount of interferon is produced in 10 min units/ml by this treatment in the 6 x 10^6 actinomycin D treatment. The same amount of interferon was discarded and the cultures were reincubated with the same volume of harvesting medium so that 0 interferon producer is used during this time and cell damage resulting from actinomycin D treatment was the same as in interferon-induced cells at the end of the incubation.

Purification Procedures—Crude concentrated interferon preparation (1000 μg of protein) was applied on a controlled pore glass (CPG) column (4.5 cm x 8 cm) (Electro-Nerst AG, Switzerland), a mean rate of 120-200 and mean pore diameter of 340 Å. The CPG column was first described by Lea et al. (26) and adapted for use with the following modifications. Interferon was applied to the CPG column in sodium phosphate-buffered saline (0.15 mol sodium phosphate buffer, pH 7.2, containing 0.15 mol NaCl) at a flow rate of 35 ml/min and the column was eluted with 6 bed volumes of (1) phosphate-buffered saline, (2) 25 mM sodium acetate, pH 4.5, (3) 100 mM sodium acetate, pH 4.5, (4) 100 mM sodium acetate, pH 4.5, (5) 0.1 M sodium phosphate buffer, pH 7.2, (6) 20 mM sodium phosphate buffer, pH 7.2, containing 0.1 M sodium chloride, (7) 0.1 M sodium chloride, (8) 0.1 M sodium phosphate buffer, pH 7.2, (9) 0.1 M sodium phosphate buffer, pH 7.2, containing 0.1 M sodium chloride, (10) 0.1 M sodium chloride, (11) 0.1 M sodium phosphate-buffered saline, pH 7.2, containing 0.1 M sodium chloride, and 50% ethylene glycol. The eluates were collected in 1-ml fractions. Interferon was eluted in Solution A and was dialyzed against 1000-fold excess of 25 mM sodium acetate for 24 h with two changes of 25 mM sodium acetate and then removed ethylene glycol before lyophilizing it to dryness. The freeze-dried interferon appears as a dry white powder if all ethylene glycol is removed.

The freeze-dried CPG purified interferon preparation was resolved in 300 μl of 3% acetic acid containing 9 μl and the active portion (except for 5 μl, removed for assay) applied to a cylindrical gel column (Unigel, LKB, Sweden). The gel solution containing 10% acrylamide (40% bisacrylamide) in 3% acetic acid and 2.5% u was filtered before polymerization. The gel was subjected to 20 mA constant current and 220 V. The reservoir buffer as well as the elution buffer was 3% acetic acid in 2.5 μl. The gel was eluted at 10 ml/h with an elution pump. Fractions of about 3 ml were collected and aliquots were removed for assay. The interferon containing fractions (not more than five fractions were pooled and dialyzed to remove residual urea and glycerol. The freeze-dried interferon was readily solubilized and applied to electrophoresis in the second dimension in the presence of sodium dodecyl sulfate. This was performed either in a 12% polyacrylamide gel or the Unigel gel column electrophoresis apparatus (LKB, Sweden) or on a vertical slab gel apparatus (Bio-Rad, CA) as previously described (19).

Analytical slab polyacrylamide gel electrophoresis was performed in the presence of 5% urea at pH 6.5 as previously described (19). Radioiodination of Interferon—Radioiodination of the pure interferon preparation was performed after the method of Bolton and Hunter (11). Pure interferon preparation in 10 μl of 0.1 M borate buffer, pH 8.5, was added to 1 μl of "I Before-Hunter reagent in a V-vial and incubated for 45 min. Thereafter, the entire reaction mixture was extensively dialyzed against a 2000-fold volume of 1 mol acetic acid containing 0.01% sodium dodecyl sulfate for 5 days, changing the dialysis medium once a day to remove low molecular weight reagents and radioactive iodine. The dialyzed radioiodinated interferon was lyophilized to dryness. Radioiodometric measurements of the iodinated proteins were performed by autoradiography using Kodak X-OMAT paper. Radioiodination was also performed by the method of Greenwood and Hunter as previously described by Berthold et al. (10).

Staining of Proteins in Gel—Unless otherwise stated, the gels were stained and destained as previously described (18).

Amino Acid Analysis—Batches of purified interferon containing about 10 μg of protein were obtained from each batch of crude harvest. Two different methods were used to prepare samples for amino acid analysis. All reagents were used in analytical grade and were taken to prevent the possible contamination of the solutions by extraneous amino acids and/or proteins. One method was to extract interferon from the gel (575 μl) by homogenizing the gel in a Dounce homogenizer with 1 ml of 0.1 mol sodium dodecyl sulfate. (Bio-Rad, CA, electrophoresis grade). The extract was dialyzed against a 1000-fold volume of 0.1 mol sodium dodecyl sulfate for 9 days with a change of distilled water daily. The dialyzed tubing was preheated by boiling in 1 mol sodium bicarbonate and 1% EDTA, washing in 1 mol sodium dodecyl sulfate, and thoroughly rinsing in glass-distilled water to remove as much of the trace amino acids that may be present in this mixture. In spite of these precautions, analysis of control gel extracts showed that small amounts of amino acids and a significant amount of glycine persist (Table II). The high glycine background is most likely due to Tris/glycine buffer in the gel. The other amino acid residues are probably derived from a combination of sources such as the polyacrylamide gel, extraction buffer, and dialysis tubing. Alternatively, interferon was applied in the gel by methanol/glacial acetic acid/water, 5:5:4, and stained with Coomassie brilliant blue. The gel (575 μl) containing the stained protein was thoroughly washed in 7% acetic acid and then in glass-distilled water. The gel was then hydrolyzed in 6 N HCl. This procedure was found to yield an extremely clean background in control gel by containing protein, though a small amount of glycine persisted.

The amino acid composition of a protein of known sequence was determined by the above method using an equivalent amount of lysine that is the amino inhibitor. The resulting values are in close agreement with the previously reported composition of this protein (22) indicating that the method of analysis used here should be reliable for interferon as well.
The isolation and amino acid/sugar composition of human fibroblastoid interferon.
Y H Tan, F Barakat, W Berthold, H Smith-Johannsen and C Tan

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