Preparative Electrophoresis of Isotopically Labeled L-Cell Interferons

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The preparative method of polyacrylamide gel electrophoresis was adapted for purification and characterization of isotopically labeled L-cell interferons. Recovery of interferon activity was quantitative, and purification and resolution were comparable to those obtained by analytical polyacrylamide gel electrophoresis. Ultimate specific activities attainable ranged from $2 \times 10^4$ to $3 \times 10^6$ international units per mg of protein.

The purification of L-cell interferon by ion-exchange chromatography and disc electrophoresis in polyacrylamide gels, yielding materials which contained in excess of $10^4$ units per milligram of protein (10), was recently described. Although the electrophoretic step in the procedure permitted excellent recovery of interferon and significant separation of extraneous proteins (15), the amount of material ultimately collected was not sufficient for further study. To overcome this limitation, attempts were made to adapt gel electrophoresis as a preparative method to obtain adequate quantities of a highly purified material for biological experimentation. These efforts form the subject of the present report.

MATERIALS AND METHODS

Preparation and isotopic labeling of interferon. The procedure followed was essentially as described (10). L cells were grown as monolayers in round prescription bottles fitted on a drum revolving at 8 rev/hr. For stimulation of interferon, cultures were exposed either to ultraviolet-irradiated Newcastle disease virus (NDV-UV) at an input multiplicity of 300 EID$_{50}$ equivalents per cell for 1 hr or to the synthetic double-stranded ribonucleotide poly I:poly C (10 µg/ml) admixed with diethylaminomethyl-dextran (500 µg/ml) made up in serum-free Eagle's basal medium (Hanks base) containing 0.15% bicarbonate and antibiotics for 2 hr. Upon termination of the incubation period, the cells were extensively washed with Hanks solution and reincubated in a serum-free mixture of Scherer's maintenance medium (60%) and Parker's medium 199 (40%) for a total of 8 hr from the time of initial contact with the inducer. Then, the cultures were refed with the same medium and returned to the incubator, and interferon was collected during the period of maximal release, i.e., between 8 and 13 hr after stimulation.

Isotopic label in the form of $^3$H-protein hydrolysate (Schwarz BioResearch, Orangeburg, N.Y.) at a concentration of 10 µCi per ml was substituted for the amino acids normally present in the serum-free medium as indicated. In the case of poly I:poly C-interferon, the best labeling was achieved when the isotope was present during the 5-hr interval preceding stimulation. With NDV-UV-induced interferon, optimal incorporation of label occurred when tritiated amino acids were present during the time of maximal liberation and collection of interferon.

Partial purification of interferon. The labeled products were concentrated approximately 20- to 50-fold by ultrafiltration, acidified at pH 4.5 (11), and extensively dialyzed against 0.1 M phosphate buffer at pH 6 to remove free label (10). Chromatography on carboxymethyl or sulfoethyl Sephadex C-25 was carried out as described (10), and interferon was eluted at constant molarity in a rising pH gradient. To preserve biological activity, crystalline bovine plasma albumin (BPA) was present during concentration of the eluate by ultrafiltration to give a final concentration of 0.5% (w/v). The presence of BPA in the chromatographed material was previously shown not to interfere with the migration and distribution of interferon during electrophoresis in polyacrylamide gels (16). Radioactive measurements were carried out with 0.1-ml samples dispensed into vials containing Bray's solution (1) in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.). Purification was assessed in terms of reduction of radioactive counts per unit of interferon, since the presence of BPA precluded direct protein measurements.

Preparative electrophoresis. This procedure was carried out in a Canalco Prep-Disc electrophoresis apparatus (Canal Industrial Corp., Rockville, Md.). The composition of gels was slightly modified from the description given earlier for the analytical method (15, 16) as follows. Solution A consisted of 1 N KOH,
50 ml; glacial acetic acid, 17.2 ml; TEMED, 4.0 ml; and water, 32.8 ml; pH 4.5. Solution B contained acrylamide, 28.0 g, and methylenebisacrylamide, 0.73 g, diluted in water to 100 ml. As a catalyst, 0.1% ascorbic acid, 0.0025% ferrous sulfate, and 0.03% hydrogen peroxide were employed (9). The elution buffer consisted of one part of solution A and seven parts of water, pH 4.5. Electrode buffers were composed of beta-alanine, 15 g; glacial acetic acid, 4.0 ml; and water to make 1 liter (pH 4.5).

Separating gels were prepared by mixing one part of solution A, two parts of solution B, and three parts of water. Catalyst was added shortly before the gel solution was poured into the column. Polymerization was completed within 5 min at 2 to 5°C. Gels, usually 3 cm in length, were loaded with 0.5- to 2.5-ml amounts of sample. In all runs described, sample gels were replaced by chambers formed by placing an additional 3- to 5-mm gel layer on top of the interferon sample containing 20% sucrose and methyl green as tracking dye. The omission of sample and spacer gels did not alter the migration and elution profiles of interferon. A current of 10 ma was applied for 4 to 6 hr and then increased to 15 ma for an additional 1 to 9 hr. The temperature was kept at 6°C throughout the run. A flow rate of 1 ml/min was maintained up to the elution of the first tracking dye band and then reduced to 0.55 ml/min. Fractions of 3 to 5 ml were collected into BPA to give a final concentration of 0.05%.

Preparative disc electrophoresis was also attempted under acid conditions with riboflavin for polymerization (7) and at alkaline pH with persulfate as catalyst (2, 7). In either instance, recovery of interferon was poor and further technical details regarding these procedures are omitted.

Analytical electrophoresis. The procedure was carried out in a Canaco model 12 electrophoretic apparatus as described elsewhere (10, 15).

Isoelectric focusing. According to Vesterberg and Svensson (17), an LKB 8101, 110 V gel ampholine electofocusing column was filled with 24 fractions of 4.6 ml of 1%, carrier ampholites, covering a pH range from 3 to 10 in sucrose solutions of densities varying from 50 to 1%. Interferon was added to several fractions located in the center of the column. A current of 300 to 1,200 v was applied for 48 to 72 hr. Fractions of 3 ml were collected and stored in the presence of BPA at a final concentration of 0.5%.

Assay of interferon. Samples which had been sterilized by ultraviolet light were titrated on monolayers of L cells by measuring the reduction in the number of plaques of vesicular stomatitis virus (10). One unit of interferon as defined by this assay corresponds to 10 units of the international mouse interferon reference standard.

RESULTS

In preliminary experiments, a number of conditions were explored to achieve maximal recovery of interferon activity during preparative electrophoresis. These variables included pH of the buffers, type of catalyst, and composition of the gels. The optimal system which ultimately evolved from those studies is described in some detail above. An example of a run performed with a nonlabeled, concentrated NDV-UV-induced interferon preparation containing 5 × 10⁴ units per mg of protein is shown in Fig. 1. As demonstrated earlier by analytical electrophoresis (15), interferon presented a rather broad elution spectrum, suggesting the heterogeneous nature of this material. However, more than half of the recoverable interferon (61%) was eluted as two distinct major peaks in a zone which comprised 12 fractions with a total elution volume of about 50 ml. Recovery of interferon was quantitative.

In other experiments, purification of interferon by preparative electrophoresis was contrasted with that obtained by ion-exchange chromatography. Both methods were essentially comparable...
in removing extraneous proteins. Total purification (including all preliminary steps) ranged from 30- to 40-fold. However, better resolution of distinct interferon components was achieved by electrophoresis.

Preparative electrophoresis of previously chromatographed interferon gave variable results, depending on whether the chromatographic eluate was collected over a wider or narrower range of the pH gradient. When selection was confined to the peak of eluting interferon activity, only moderate additional purification was achieved during subsequent electrophoresis. On the other hand, a less discriminating selection of chromatographic fractions to include more interferon, but of lower specific activity, permitted up to 10-fold further purification on preparative electrophoresis. In that respect, the two procedures complemented each other and a final specific activity in the order of $2 \times 10^4$ to $3 \times 10^4$ international units per mg of protein could not be exceeded.

An electropherogram of a chromatographed $^3$H-labeled poly I:poly C interferon preparation is shown in Fig. 2. In this instance, purification in the peak region of interferon activity was approximately twofold (see legend for details). As in the case of the preceding preparation, some interferon activity and some isotopic label were detectable in most of the fractions. However, about one-third of the interferon eluted as a sharply defined radioactive peak distributed among only three fractions.

Comparable electrophoretic elution profiles were obtained with three different preparations of tritium-labeled NDV-UV-induced L-cell interferon. Inasmuch as the initial specific activities were approximately three times lower in the case of these chromatographed input materials than with poly I:poly C interferons and recovery of biological activities was somewhat better, purification was also improved, ranging from 230- to 250-fold totally.

To gauge the extent of association between radioactive label and interferon activity, two additional experiments were carried out with NDV-UV-induced interferons which had been subjected successively to ion-exchange chromatography and preparative electrophoresis. In the first, a single-peak fraction of interferon was concentrated and reelectrophoresed by the analytical method. More exacting conditions than usually employed were provided by longer gels (16.5 cm) and accordingly prolonged time of migration (4.5 hr at 5 ma per gel). As illustrated in Fig. 3, this fraction migrated again as a single radioactive peak. The minor separation between isoform and interferon in the lower portion of one of the limbs may be attributed either to some inactivation of interferon or denote some con-

![Fig. 2. Preparative electrophoresis of $^3$H-labeled chromatographed poly I:poly C-induced L-cell interferon. Input, $3.6 \times 10^4$ units, $2.38 \times 10^4$ counts/min; specific activity, $1.1 \times 10^4$ units per mg of protein. Total recovery, $3.78 \times 10^6$ units (105%), $1.67 \times 10^4$ counts/min (70%). Peak-associated recovery, $1.02 \times 10^6$ units (28%), $3.33 \times 10^6$ counts/min (15%); purification, 1.94-fold; specific activity, $2.1 \times 10^4$ units per mg of protein. Arrow indicates position of tracking dye (methyl green).](image1)

![Fig. 3. Analytical reelectrophoresis of single-peak fraction from preparative electrophoresis of $^3$H-labeled chromatographed L-cell interferon induced by ultraviolet-irradiated Newcastle disease virus. Input, $1.20 \times 10^5$ units, $2.20 \times 10^5$ counts/min. Recovery, $1.30 \times 10^5$ units (108%), $5.65 \times 10^5$ counts/min (26%).](image2)
FIG. 4. Electrophoretic profile of selected peak from preparative electrophoresis of chromatographed L-cell interferon induced by ultraviolet-irradiated Newcastle disease virus. Input, 9.0 x 10⁴ units, 6.67 x 10⁴ counts/min. Peak-associated recovery, 2.44 x 10⁴ units (27%), 1.53 x 10⁴ counts/min (23%); total purification, 27.1-fold; specific activity, 1.73 x 10⁴ units per mg of protein.

contamination with nonreactive proteins. This result indicates that preparative electrophoresis as a method achieves considerable resolution of distinct interferon components.

However, uniform migration in polyacrylamide gels does not necessarily imply homogeneity of composition by other criteria. This is evident from the second experiment where three adjacent peak fractions collected in eluant buffer were subsequently subjected to isoelectric focusing. The distribution of labeled proteins and interferon is shown in Fig. 4. Biological activity was found in fractions corresponding to a pH range from 6 to 10.5, as previously reported (13, 14), with a major peak at pH 9.5 to 10.2 and a minor one at pH 7.6. Tritiated proteins were closely associated with both peaks comprising a total recoverable interferon activity of 47%. Yet, approximately half of the labeled proteins (48%) were located in the region below pH 7 which ordinarily contains little or no interferon (14). Unless one assumed that the interferon lost through inactivation was acidic in nature, it would in fact appear that some further dissocia-

transmission between nonreactive proteins and interferon had occurred. Inasmuch as percentage recoveries, although apparently for different reasons, were similar for interferon and associated radioactivity, no additional purification by electrofocusing could be recorded. However, it is clear that a selected peak region of highly purified interferon displayed considerable heterogeneity of microelectric charge in spite of uniform behavior in polyacrylamide gels.

**DISCUSSION**

The analytical method for polyacrylamide gel electrophoresis has been used in the past with variable success in purification studies with interferons (4, 7). However, it was only recently that modifications in technique permitted significant purification without concomitant loss in activity of mouse (10, 13) and rabbit (19) interferons. To make adequate quantities of purified interferon available for biological studies, possibilities were explored to adapt for this purpose the preparative procedure used by others for the separation and purification of enzymes (20), proteinase inhibitors (8), nucleotides (3), and polysaccharides (18). Commonly employed catalysts for gel electrophoresis under acid or alkaline conditions, such as riboflavin (7) or persulfate (2, 7), permitted only low recovery. On the other hand, in the system described by Jordan and Raymond (9), interferon activity was fully retained and quantitative recovery became possible.

The purification of interferon by preparative electrophoresis was comparable to that obtained by means of the analytical method (13, 15) and was dependent upon the quality of the chromatographed input material. When sampling had been confined to the interferon peak which only contained the highest specific activity (poly I:poly C-induced interferon of Fig. 2), little additional separation of extraneous proteins was achieved on electrophoresis. With other chromatographed virus-induced materials, collected over a wider pH range and consequently less pure because of partial contamination with acidic proteins, additional purification by preparative electrophoresis varied from 5- to 10-fold.

The electrophoretic profile of electrophoresed interferon covered a narrower pH range than previously reported for the same material (14). Activity was confined mainly to a peak in the extreme alkaline region, the exact position of which can only be estimated. The lesser prominence of acidic interferon components can be explained by more restrictive sampling of adjacent interferon fractions of predominantly alkaline electric charge for isoelectric focusing.
Although the data indicate that even highly purified interferon appears to contain a multiplicity of molecular components both with respect to size and charge, as has also been shown for chick (5), human (6, 14), and rabbit (12, 14) interferons, the resolution achieved on electrophoretic migration would permit selection of individual species of interferon molecules with distinct physical characteristics for use in further biological studies. The advantages of preparative electrophoresis over the analytical method lie principally in the greater loading capacity of the system, which, in the case of the columns used in this study, would permit the application of at least 10 mg (i.e., $\geq 2 \times 10^4$ to $10 \times 10^4$ units) of partially purified interferon, the shorter time required for elution, and the ease of operation. Studies on interferon-host cell interaction with isotopically labeled materials obtained by this method are currently in progress.

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