Purification of Interferon from Mouse Ehrlich Ascites Tumor Cells

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Interferon production was induced in mouse Ehrlich ascites tumor cells by infection with Newcastle disease virus. The interferon produced was purified by precipitation with ammonium sulfate, chromatography on carboxymethyl-Sepharose, treatment with blue dextran and polyethylene glycol, gel filtration on Bio-Gel P-60 and Bio-Gel P-200, chromatography on phosphocellulose, isoelectric focusing, and chromatography on octyl-Sepharose. The specific activity of the product was $1.6 \times 10^7$ NIH mouse interferon reference standard units/mg of protein. Electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulfate indicated that the apparent molecular weight of the interferon-active material ranged from 25,000 to 35,000. As revealed by staining the gels with Coomassie brilliant blue, the interferon activity co-migrated with the major, broad protein band. Minor, stainable bands of proteins were free of interferon activity and their apparent molecular weight was smaller than 12,000.

Since the discovery of interferons as antiviral agents by Isaacs and Lindenmann in 1957, much effort was devoted to their purification from various sources. For reviews see Refs. 2 to 4.

There are several recent communications about the purification of mouse interferon from mouse L929 fibroblasts to a high specific activity. Using affinity chromatography on Sepharose-bound anti-interferon globulin Ogburn et al. (5) purified interferon to a specific activity of $2.7 \times 10^9$ units/mg of protein. Applying conventional techniques of protein purification Knight (6) obtained an interferon preparation with a specific activity of $2.5 \times 10^9$ units/mg of protein and Yamamoto and Kawade (7) a preparation with a specific activity of $3 \times 10^9$ units/mg of protein.

We report here a procedure for purifying mouse interferon from Ehrlich ascites tumor cells to a specific activity of $1.6 \times 10^9$ units/mg of protein. Gel electrophoresis in the presence of sodium dodecyl sulfate reveals that although the preparation contains small amounts of material with no interferon activity, the interferon-active material co-migrates with the major protein band.¹

RESULTS

Purification of Interferon

Mouse Ehrlich ascites tumor cells grown in monolayer cultures were infected with Newcastle disease virus to induce interferon formation. The cells released the interferon produced and the interferon was isolated from the culture medium. The medium was first stored at pH 2 overnight or longer to inactivate the Newcastle disease virus.

Ammonium Sulfate Fractionation—The purification was started by differential precipitation of proteins in the culture medium with ammonium sulfate. Most of the interferon-active material was found in the precipitate formed on increasing the saturation of ammonium sulfate from 50 to 80%.

Chromatography of CM Sephadex This precipitate was dissolved in 10 mM phosphate buffer (pH 6.2) and fractionated by chromatography on a CM-Sephadex column. Under the conditions used, only 25% of the applied protein was adsorbed to the column and after washing with 10 mM phosphate buffer (pH 8.5), only 5% of the protein remained bound together with 50% of the interferon activity. The interferon-active material was eluted by a NaCl gradient in the same buffer. Interferon was eluted in a rather broad peak between 0.5 and 0.8 M NaCl.
0.15 and 0.35 M NaCl and most of the protein was eluted at a somewhat lower salt concentration (Fig. 1). The salt gradient seemed to be superior to pH gradients (see Ref. 8) in giving a better purification of interferon (about 70-fold) and a good recovery.

The choice of a CM-Sephadex column of the proper size (2.5-ml volume for 4 to 5 × 10⁸ units of interferon) was important. Overtreatment caused poor recovery with only little improvement in the resolution of proteins. Overloading resulted in poor adsorption of interferon activity to the columns.

Treatment with Blue Dextran and Polyethylene Glycol—After concentration to 1/5 of the original volume and dialysis, the CM-Sephadex fraction was supplemented with blue dextran and the resulting protein-blue dextran complexes were precipitated by added polyethylene glycol. (It should be noted that not all proteins cause the formation of a precipitate under such conditions. Thus the presence of bovine serum albumin alone did not result in precipitate formation.) Although the purification factor in this step was rather small (see Table 1), it removed some proteins similar in size to interferon (Fig. 4) and also resulted in the concentration of the sample. Polyacrylamide gel electrophoresis in SDS of the interferon preparation at this stage of purification (specific activity close to 10⁴ units/mg of protein) revealed that it was still very heterogeneous.

In preliminary experiments, instead of the precipitation with blue dextran, chromatography on blue dextran/Phosphocellulose was used. In line with results of Jankowski et al. (9), this gave substantial purification. Unfortunately, however, the procedure had to be abandoned since the interferon preparations recovered from blue dextran/Phosphocellulose were unstable even in the presence of glycerol. The reason for this labilization of interferon is unknown to us. We noted a similar labilization after adsorption and elution of interferon from bovine serum albumin/Phosphocellulose (10).

Gel Filtration on Bio-Gel P-60 and P-200—The interferon fraction from the blue dextran treatment was further purified by gel filtration on Bio-Gel P-60. The interferon activity was separated in this process from the major protein peak resulting in an over 3-fold purification (Fig. 2). The recovery of interferon was improved when Tween 40 was included in the elution buffer. This might have been due to a decrease in nonspecific adsorption of interferon to the gel in the presence of the detergent.

Gel filtration on Bio-Gel P-200 was performed to remove further proteins from the interferon-active material. This step did not result in an increase in the specific activity of the interferon preparation. Although only 55% of the protein was recovered in the active fractions, there was significant loss of interferon activity mainly in consequence of low recovery during concentration.

Gel filtration on Bio-Gel P-60 and P-200 was replaced in the most recent purification by a single gel filtration on a larger Sephadex G-150 column (see also Ref. 6). This allowed the omission of the concentration step between the two gel filtrations and thereby improved the recovery of interferon during gel filtration from 32 to 100%.

Chromatography on Phosphocellulose—The interferon fraction from the Bio-Gel P-200 column was applied to a phosphocellulose column. Approximately 50% of the protein applied appeared in the flow-through fraction with less than 5% of the interferon activity. Over 95% of the interferon activity, together with most of the adsorbed proteins, was eluted with 0.4 M sodium phosphate. This step resulted in about a 2-fold purification.

A stepwise elution was preferred to gradient elution in this step to avoid diluting the sample unnecessarily. Gradient elution resulted in a poorer recovery with little, if any, improvement in purification (not shown).

Isoelectric Focusing—The interferon fraction from phosphocellulose was further fractionated by isoelectric focusing. The major peak of interferon activity appeared between pH 9.5 and 10 (Fig. 3). The sharpness of this peak was similar to that obtained by running a pure protein (cytochrome c) separately under the same conditions (not shown). This step resulted in 2.5-fold purification and a decrease in the amount of most of the low molecular weight proteins (compare slot 8 with slot 7 in Fig. 4). A long time period (as described under "Experimental Procedures") was needed to focus interferon into a sharp band. Similarly 60 h at 600 V was required to focus cytochrome c (which has an isoelectric point close to that of interferon) into a sharp band in the same buffer.

The sharp interferon peak (with only small satellite bands; see Fig. 3) was obtained from material not treated with neuraminidase. This was unexpected since mouse interferon (from L cells) not treated with neuraminidase was shown to be divided into several peaks in isoelectric focusing (11) for a discussion (see Ref. 12). It remains to be seen if this apparent discrepancy between published data and our observations is due to a difference in the source of interferon or in the conditions of isoelectric focusing.

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**Fig. 4.** Analysis of the interferon preparation after various steps of purification by polyacrylamide gel electrophoresis in the presence of SDS. The 7.5 to 15% acrylamide-SDS-Tris/Cl pH 8.8 system was used. Slots 1 to 7 were analyzed in one experiment, slot 8 in a second experiment, and slots 9 and 10 in a third experiment. Marker proteins were run in each experiment to allow the matching of corresponding bands. The direction of protein movement in the figure is downward. 1, marker proteins in order of increasing mobility: bovine serum albumin, carbonic anhydrase, and cytochrome c; 2, ammonium sulfate fraction; 3, CM-Sephadex fraction; 4, blue dextran fraction; 5, Bio-Gel P-60 fraction; 6, Bio-Gel P-200 fraction; 7, phosphocellulose fraction; 8, isoelectric focusing fraction; 9, octyl-Sepharose fraction; 10, octyl-Sepharose fraction after dialysis against water. For details see "Polyacrylamide Gel Electrophoresis in Presence of SDS."

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*The abbreviations used are: SDS, sodium dodecyl sulfate; L cells, mouse L929 fibroblasts.*
Chromatography on Octyl-Sepharose – The interferon fraction from isoelectric focusing was further purified by chromatography on an octyl-Sepharose column (13) (see also Ref. 14). Much of the protein in the sample was adsorbed to the column in 2 M ammonium sulfate. To decrease the loss of interferon, stepwise elution was used instead of gradient elution. Washing with 0.3 M ammonium sulfate resulted in the elution of about 33% of the protein and the recovery of 40% of the interferon activity. This step resulted in the removal of one contaminating protein (M, = 35,000) and in an about 25% increase in the specific activity (compare slot 9 with slot 8 in Fig. 4). Dialysis against water overnight resulted in the disappearance of some low molecular weight protein (compare slot 10 with slot 9 in Fig. 4).

A summary of the purification procedure is shown in Table 1.

Range of Apparent Molecular Weight Distribution of Interferon

The apparent molecular weight of interferon was determined by polyacrylamide gel electrophoresis in the presence of SDS. For this purpose, two identical samples of interferon were electrophoresed in two adjacent tracks of the gel. One gel track was stained with Coomassie brilliant blue to visualize the protein and the other was cut into slices and the interferon-active material was extracted and assayed for activity. It can be seen in Fig. 5 that the interferon activity was distributed over a 25,000 to 35,000 molecular weight range, with a peak at about 30,000. This apparently wide molecular weight range is in agreement with that reported by Knight (6).

We considered the possibility that the broadness of the range might be a consequence of nonuniform modification (e.g., by glycosylation) of a common polypeptide (12, 15). Since our mouse Ehrlich ascites tumor cell culture used for interferon production had not been cloned, it was conceivable that different cells in the culture differ in their capacity to modify the hypothetical common polypeptide. Consequently we cloned our culture and tested by polyacrylamide gel electrophoresis in SDS the size distribution of interferons made by three different clones with that made by the uncloned culture. (It should be noted that each of the three clones produced similar amounts of interferon as the uncloned culture.) The size distribution of interferon produced by one of the clones is shown in Fig. 6A and of interferon produced by the uncloned culture in Fig. 6B. It can be seen that the distributions are indistinguishable and wide. The distributions of interferons made by the other two clones (not shown) were also indistinguishable from those in Fig. 6.

In spite of the wide variety in apparent molecular weight the interferon molecules produced by mouse Ehrlich ascites tumor cells seem to share various chemical characteristics. This can be concluded from the data in Fig. 6, B and C. These facts reveal that the size distribution of highly purified interferon (specific activity 1.6 × 10^4 units/mg of protein) is wide and indistinguishable from that of crude interferon (specific activity 2.9 × 10^4 units/mg of protein). Thus the interferon molecules with different apparent molecular weights are copurified through a 5500-fold enrichment.

Relationship between Interferon-active Substance and Protein in Purified Interferon Preparation

The positions in the gel and the widths of the band of interferon-active material and of the major stained protein band were very similar if not identical (Fig. 5). These facts taken together with the high specific activity of the interferon preparation (1.6 × 10^4 interferon units/mg of protein) seem to indicate that the interferon-active material is identical with the major protein band. The gel pattern also revealed that the interferon-active substance and protein in the purified interferon preparation were in the same size range (See Table 1).

![Diagram](http://www.jbc.org/)

Table 1

<table>
<thead>
<tr>
<th>Interferon fraction</th>
<th>Total units</th>
<th>Recovery of activity</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>&lt;10^6</td>
<td>% mg</td>
<td>7920</td>
<td>2.9 × 10^8</td>
<td>5</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>22.7</td>
<td>80</td>
<td>1980</td>
<td>9.2 × 10^8</td>
<td></td>
</tr>
<tr>
<td>CM-Sephadex</td>
<td>18.2</td>
<td>59</td>
<td>22</td>
<td>6.1 × 10^8</td>
<td>210</td>
</tr>
<tr>
<td>Blue dextran</td>
<td>13.4</td>
<td>44</td>
<td>11.5</td>
<td>8.6 × 10^8</td>
<td>300</td>
</tr>
<tr>
<td>Bio-Gel P-60</td>
<td>9.9</td>
<td>27</td>
<td>2.2</td>
<td>2.8 × 10^8</td>
<td>970</td>
</tr>
<tr>
<td>Bio-Gel P-200</td>
<td>3.2</td>
<td>14</td>
<td>1.2</td>
<td>2.7 × 10^8</td>
<td>930</td>
</tr>
<tr>
<td>Phosphocellulose</td>
<td>3.1</td>
<td>14</td>
<td>0.6</td>
<td>5.2 × 10^8</td>
<td>1800</td>
</tr>
<tr>
<td>Isoelectric focusing</td>
<td>1.3</td>
<td>6</td>
<td>0.1</td>
<td>1.3 × 10^8</td>
<td>4500</td>
</tr>
<tr>
<td>Octyl-Sepharose²</td>
<td>0.5</td>
<td>2</td>
<td>0.032</td>
<td>1.6 × 10^8</td>
<td>5500</td>
</tr>
</tbody>
</table>

² Thirty per cent of the interferon fraction obtained by isoelectric focusing was taken for purification by chromatography on octyl-Sepharose. The values in the table were calculated by multiplying the experimental data by 3.3.

Fig. 5. Analysis by polyacrylamide gel electrophoresis in SDS of the interferon preparation purified through fractionation on octyl-Sepharose. Assay of the interferon activity of material recovered from the gel and determination of the apparent molecular weight of interferon. To determine the size distribution of interferon-active materials the octyl-Sepharose fraction was electrophoresed in duplicates in adjacent tracks of 10% acrylamide-SDS-Tris/Cl pH 8.8 gels. One of the duplicates was stained with Coomassie brilliant blue, the other was cut transversally into 3 mm thick slices in the portion of the gel in which interferon-active material was expected and 3 mm thick slices in other portions. Each slice was finely minced with a plastic rod and interferon was extracted into 1 ml of phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4containing 10% (w/v) glycerol, 0.1% SDS at room temperature overnight. The interferon activity in each eluate was determined by the cytopathic assay. The molecular weight of the marker proteins used was as follows (28): bovine serum albumin, 68,000; ovalbumin, 43,000; carbonic anhydrase, 29,000; cytochrome c, 11,700. For further details see "Polyacrylamide Gel Electrophoresis in the presence of SDS."
the interferon preparation contained small amounts of inert proteins. The most pronounced of this had a molecular weight smaller than 10,000.

The following finding is in line with the proposal that the protein(s) in the stained band (molecular weight between 25,000 and 35,000) is (are) identical with the interferon-activity material. When fractions 20 to 26 from isoelectric focusing (see Fig. 3) were compared for interferon activity and for darkness of staining of the above band on polyacrylamide gel electropherograms run in the presence of SDS, the fractions with higher interferon activity gave darker bands than fractions with lower interferon activity (data not shown).

**DISCUSSION**

**Potency of Interferon Preparation**—The specific activity of our most highly purified interferon preparation is $1.6 \times 10^6$ units/mg of protein. To obtain a 50% vesicular stomatitis viral plaque reduction, our L cells have to be treated with 13 units/ml of interferon. This corresponds to a protein concentration of about $8.2 \times 10^{-9}$ mg/ml. Assuming that the interferon preparation is free of contaminants (which is, however, not the case) and that the average molecular weight of our interferon is 30,000, this protein concentration would correspond to a $2.7 \times 10^{-13}$ M interferon concentration. Our L cells are less responsive to the antiviral effect of interferon than some other types of cells. Thus, to obtain a 50% vesicular stomatitis viral plaque reduction, L cells of strain CCL 1 (American Type Culture Collection, Rockville, Md.) have to be treated with only 1.6 unit/ml of interferon which corresponds to $3.4 \times 10^{-14}$ M interferon concentration.

**Comparison of Different Highly Purified Mouse Interferon Preparations**—Our purification procedure until the chromatography on phosphocellulose is similar to those used by Knight (6) and by Yamamoto and Kawade (7). The specific activity of our interferon preparation at this stage is close to that reported by these authors.

The specific activity of our best interferon preparation is about 5-fold higher than that of other preparations described in the literature (6, 7). It remains to be seen if this results from a more effective removal of noninterferon proteins in the course of purification or a better preservation of the activity of interferon during purification or both.

The apparent molecular weight range of our interferon preparation (25,000 to 35,000) is very broad. This is in agreement with the earlier findings of Knight (6). The molecular basis of this wide molecular weight range remains to be established. It might result, in principle, from having a single gene specifying a single interferon polypeptide, if different molecules of the polypeptide become differently modified. It is also conceivable, however, that we are dealing with a mixture of two or more interferon species specified by different genes (see Ref. 16). Either of these possibilities might be in line with the discovery of two distinct interferon species (with $M_\text{r} \approx 22,000$ and 38,000) from L cells by Stewart (17) and Yamamoto and Kawade (7).

If our interferon consists of more than one species, then these should be similar in chemical characteristics since they are co-purified through a 5500-fold enrichment and give a rather sharp peak in isoelectric focusing.

If our interferon is a glycoprotein, then the amounts of carbohydrate substitution of different interferon molecules might be unequal and this could account for or at least contribute to the heterogeneity (for discussion see Ref. 12). This heterogeneity in terms of apparent molecular weight is one of the most puzzling features of our interferon preparation. Attempts to characterize the molecular basis of this heterogeneity are hindered by the limited availability of highly purified material. However, several lines of experiments are underway in this laboratory to clarify this problem.

**Lack of Endonuclease Activity of Mouse Ehrlich Ascites Tumor Interferon Preparation**—Graziadei et al. (18) reported the finding of endonuclease activity in a purified interferon preparation from mouse Ehrlich ascites tumor cells in 1973. At that time, it was not known if the endonuclease was an activity of the interferon or of a contaminant in the preparation.

The process of interferon purification used in 1973 consisted of fewer steps than that described in this communication.

In the interferon preparation purified according to the new procedure, we failed to detect nuclease activity (19). Thus, the endonuclease activity in the preparations from 1973 was probably due to the presence of a contaminating protein.

**Pleiotropic Effect of Interferon**—The exposure of mouse or human cells to partially purified homologous interferon preparations is manifested in the cell extract in various ways. (For reviews see Refs. 20 and 21.) We tested for two of these manifestations: the double-stranded RNA-activated protein kinase (22-24) and the double-stranded RNA-activated endonuclease (19, 25) in an extract of mouse Ehrlich ascites tumor cells that were treated with 800 units/ml of interferon of $1.6 \times 10^6$ units/mg of protein specific activity. Both were clearly present (not shown).

Partially purified mouse interferon preparations inhibit the multiplication of mouse cells from various lines in cell culture (26). The lack of success of the attempts to separate the cell multiplication inhibitory activity from the antiviral activity in the preparations served as the basis for the proposal that it is interferon itself which blocks cell multiplication (26, 27).

In line with the above, we find that our interferon preparation with a specific activity of $1.6 \times 10^6$ units/mg of protein impairs the multiplication of L 1210 mouse leukemia cells in suspension culture in RPMI medium (GIBCO). In the absence of interferon, the number of cells increased in 4 days from $10^6$ cells/ml to $15.4 \times 10^6$ cells/ml, whereas in the presence of $1.5 \times 10^5$ units/ml of interferon it increased to only $8 \times 10^6$ cells/ml. The cell multiplication inhibitory activity in the interferon preparation migrates together with the antiviral activity in polyacrylamide gel electrophoresis in the presence of SDS (data not shown).

**REFERENCES**

The references appear on p. 602.
Purification of interferon from mouse Ehrlich ascites tumor cells.
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