Purification of Two Components of Mouse L Cell Interferon: Electrophoretic Demonstration of Interferon Proteins

By YOKO YAMAMOTO AND YOSHIMI KAWADE
Institute for Virus Research, Kyoto University, Kyoto, Japan

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SUMMARY

Mouse L cell interferon induced by Newcastle disease virus was purified by the procedure described previously (Yamamoto et al. 1974) followed by gel filtration. The two fractions obtained containing interferon species S (36,000 daltons) and F (24,000 daltons), respectively, were analysed electrophoretically at pH 4:3, or in the presence of sodium dodecyl sulphate (SDS) at pH 7:2. In both fractions, interferon activity was invariably associated with distinct protein bands. In the F-containing fraction there were essentially no other proteins, and in the S-containing fraction, impurity proteins were well separated from the interferon activity. The apparent mol. wt. determined by SDS-gel electrophoresis showed little or no dependence on gel concentration, suggesting that the interferons had low carbohydrate contents, and did not change after reduction with thiol reagents in SDS and urea.

INTRODUCTION

Complete purification of interferons, especially mouse and human interferons, is now being actively pursued in several laboratories. In our studies on purification of mouse L cell interferon (Kawade, 1973; Yamamoto et al. 1974), the presence of two components, F and S, was shown by electrophoresis at pH 4, and a preparation of high sp. act. revealed a protein band at the position of F among impurity proteins. We now report on further purification, including separation of the two components.

Thanks to the recent findings by Mogensen & Cantell (1974) and Stewart, De Clercq & De Somer (1974a) that various interferons are stable to treatment with SDS, we could add SDS-polyacrylamide gel electrophoresis to the methods for analysing purified samples.

Two fractions were obtained containing the S and F components, respectively, by the previously described procedure followed by gel filtration. Both revealed in electropherograms distinct protein bands which may be presumed to represent interferon itself. In particular, the F-containing fraction was found to contain no extraneous proteins outside the region of interferon activity.

METHODS

The cultivation of L cells and the preparation of crude interferon induced by Newcastle disease virus (NDV) have been described (Kawade, 1973; Yamamoto et al. 1974).

Assay of interferon. A micromodification of the method developed by Suzuki, Akaboshi & Kobayashi (1974) was used (Y. Kawade et al., in preparation), which is based on inhibition by interferon pre-treatment of $^3$H-uridine incorporation into virus RNA in one-step growth of vesicular stomatitis virus in L cells. The endpoint was defined as 50% reduction in uridine.
incorporation. The mouse research reference interferon (code G002-904-511, containing 12000 units of activity) had a titre of 2400 to 6000 endpoint units on different occasions. The titres in this paper are expressed in reference units.

**Purification of interferon.** Culture fluids of NDV-infected L cells amounting to 177 l in total were harvested in batches of several litres during a twelve-month period (Table I, step 1). They were concentrated by zinc acetate precipitation (Yamamoto et al. 1974) soon after harvest, and stored at -70 °C until they were pooled for the second zinc acetate precipitation (step 2). The concentrated material was then fractionated with ammonium sulphate, and a fraction which precipitated between 40 and 80 % saturation (step 3) was purified by chromatography on DEAE-Sephadex (Pharmacia, Uppsala, A-25, 3·2 mEq/g) and CM-Sephadex (Pharmacia, C-25, 4·5 mEq/g; steps 4 and 5), as described previously (Yamamoto et al. 1974). The material was concentrated by ultrafiltration using an Amicon Diaflo membrane PM-10 (Amicon Corp., Lexington, Mass., U.S.A.), dialysed against 0·05 M-NaCl–0·01 m-tris-HCl, pH 7·2 (step 6), and lyophilized (step 7). It was then redissolved and subjected to gel filtration through Bio-Gel P30, 400 mesh (Bio-Rad Labs., Richmond, Calif., U.S.A.). Two fractions expected to contain the high and low mol. wt. interferon components, respectively, were collected, concentrated by ultrafiltration using a collodion bag (Sartorius Membranfilter, Göttingen, no. SM 132 00, pore size 8 nm), and dialysed against 0·01 M-tris-HCl, pH 7·2 (step 8).

**Polyacrylamide gel electrophoresis (PAGE).** PAGE at pH 4·3 and determination of protein and interferon distributions were carried out as before (Yamamoto et al. 1974).

SDS-PAGE was done essentially according to Shapiro, Viñuela & Maizel (1967). In some experiments, samples were reduced before the run by heating at 70 °C for 30 min in 1 % SDS–5 M-urea–0·01 m-dithiothreitol or 0·014 M-mercaptoethanol (Stewart et al. 1974a). Marker proteins were added for mol. wt. determination: bovine serum albumin (BSA), ovalbumin, chymotrypsinogen A, whale myoglobin and cytochrome c (Schwartz/Mann). The gels (5 × 80 mm) contained 0·1 % SDS and 0·1 m-phosphate buffer, pH 7·2. A constant current of 5 mA/gel was used. Coomassie brilliant blue (CBB) served as the tracking dye. After the run, the gels were stained for protein with 0·4 % CBB in methanol–acetic acid–water (50:10:40 by vol.) for 1 h at 37 °C or overnight at room temperature, and destained in methanol–acetic acid–water (10:10:80 by vol.). Interferon activity was assayed on 2 mm gel slices extracted overnight in 0·1 % SDS–0·01 m-tris-HCl, pH 7·2, at room temperature unless otherwise stated.

**Protein determination.** Protein concentrations were determined by measurement of $E_{280}$. One $E_{280}$ unit is considered to correspond roughly to 1 mg protein.

**RESULTS**

**Mol. wt. determined by gel filtration**

The two components, F (fast) and S (slow), of L cell interferon which had been resolved in PAGE at pH 4·3 (the peak fractions of the run shown in Fig. 2 of Matsuzawa & Kawade, 1974), were separately subjected to gel filtration through a Bio-Gel P100 column (2·6 × 52 cm) in 0·1 M-NaCl–0·1 m-tris-HCl, pH 8·0, at a flow rate of 13 ml/h at 4 °C. To prevent inactivation during the run, a non-u.v.-absorbing weak stabilizer, Tween 80 (100 μg/ml; Yamamoto et al. 1974), was added to the effluent for the S component, but for the F component, a stronger stabilizer, BSA (100 μg/ml), was needed. Furthermore, BSA (1 mg/ml) was added to each eluted fraction after measurement of extinction. Recovery of activity was 70 % and 98 % for F and S, respectively. Greater instability of F compared to S was noted in our
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previous study (Matsuzawa & Kawade, 1974), and was often also found in the present work.

The mol. wt. thus determined by comparison with the admixed reference proteins were 20,000 and 32,000, respectively, for the F and S components (Fig. 1).

The pooled crude interferon preparation used in the subsequent purification work showed two activity peaks in a similar gel filtration experiment, with mol. wt. in good agreement with the above-mentioned values (20,000 and 34,000), indicating the feasibility of separating them from each other by means of gel filtration.

Purification and separation of the two components

Interferon was purified from 177 l of culture fluids from NDV-infected L cells as described in Methods and summarized in Table 1. The interferon and protein contents listed for the starting material (step 1) are calculated values based on the average of several batches examined among the many that were pooled.

The lyophilized material (step 7), when redissolved, was found to have lost about 50% of the activity at step 6 (this loss may have occurred before lyophilization, since several weeks elapsed between steps 6 and 7). Upon electrophoresis, the interferon and protein constituents were as shown in Fig. 2. In agreement with the previous results (Kawade, 1973; Yamamoto et al. 1974), but with better resolution, PAGE at pH 4.3 resolved the interferon into F and S components (Fig. 2a). The recovery of activity was quantitative, and the results indicated the presence in this preparation of roughly equal amounts of F and S (56:44). Some of the protein bands observed corresponded to the activity peaks. On SDS-PAGE (Fig. 2b), four to five distinct protein bands were detected, two of which coincided with the activity peaks. The activity of the faster peak (F) was markedly lower than the slower peak (S), and most probably this was due to its greater instability.

The material was then filtered through Bio-Gel P30, with the result illustrated in Fig. 3. Fractions I and II were collected as indicated, containing the S and F components, respectively. Only a portion of the lyophilized material from step 7 was, in fact, filtered, but the total interferon and protein contents shown for step 8 in Table 1 are those which would have been obtained if all the step 7 sample had been used. The interferon activity recovered in the first peak of Fig. 3 (the S component, tube numbers 103-110) was 20% of the total loaded, and that in the second (the F component, tube numbers 111-118) 13% (total recovery 33%). Since the material loaded consisted of 44% S and 56% F, the S and F components must have been inactivated to 1/2.2 and 1/4.3, respectively, during the gel filtration. The low recovery of interferon activity in fractions I and II (Table 1) is mainly due to this inactivation, and partly to omission of intermediate column fractions (from 108-112). Assuming that the 50% loss of activity from step 6 to step 7 affected equally the S and F interferons, then the sp. act. of fraction I must be 4.4 times, and that of fraction II 8.6 times, that given in Table 1, if the inactivation after step 6 could have been prevented.

Analysis by PAGE

The two fractions obtained after the gel filtration step were analysed for interferon and protein by PAGE in two systems, namely (i) PAGE at pH 4.3 as hitherto used, and (ii) SDS-PAGE.

PAGE at pH 4.3

Fraction I gave the electropherogram shown in Fig. 4(a), with S as the only interferon component. Staining for protein revealed two closely adjacent bands with similar intensities at the interferon peak, although the splitting of the bands is not so clear in the densitometer
Fig. 1. Estimation of mol. wt. of F and S interferon (If) components by gel filtration through a Bio-Gel P100 column together with reference proteins (BSA, 67,000; Ov, ovalbumin, 45,000; Myo, myoglobin, 17,000). ○--○, Interferon; ——, $E_{280}$; ----, $E_{406}$. 
Table 1. Purification of mouse L cell interferon

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Interferon activity</th>
<th>Protein</th>
<th>Sp. act.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vol. (ml)</td>
<td>Total (units x 10^-8)</td>
<td>Yield (%)</td>
</tr>
<tr>
<td>(1) Starting material</td>
<td>177000</td>
<td>35</td>
<td>100</td>
</tr>
<tr>
<td>(2) Zinc-precipitated (twice)</td>
<td>880</td>
<td>28</td>
<td>80</td>
</tr>
<tr>
<td>(3) (NH_4)_2SO_4-precipitated (between 40 and 80 % saturation)</td>
<td>81</td>
<td>24</td>
<td>69</td>
</tr>
<tr>
<td>(4) DEAE-Sephadex unadsorbed fraction*</td>
<td>230</td>
<td>20</td>
<td>57</td>
</tr>
<tr>
<td>(5) CM-Sephadex pH 7.9 eluate†</td>
<td>170</td>
<td>12</td>
<td>34</td>
</tr>
<tr>
<td>(6) Concentrated by ultrafiltration</td>
<td>60</td>
<td>10</td>
<td>29</td>
</tr>
<tr>
<td>(7) Lyophilization</td>
<td>5</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>(8) Gel filtration through Bio-Gel P300‡</td>
<td>Fraction I (S)</td>
<td>0.9</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>Fraction II (F)</td>
<td>1.5</td>
<td>0.38</td>
</tr>
</tbody>
</table>

* The fraction that passed a column of DEAE-Sephadex unadsorbed in 0.2 M-NaCl - 0.01 M-tris-HCl at pH 7.2 amounted to 83 %, and the remaining fraction eluted with 0.1 M-NaCl (not used for further purification) to 16 % of the total activity recovered (120 % of that charged).
† Interferon was charged to CM-Sephadex in 0.1 M-phosphate buffer at pH 5.9, and the unadsorbed fraction (3 % of charged interferon) was discarded. The activity eluted with 0.1 M-phosphate buffer at pH 7.9 was 97 %, and a main portion of it (two thirds) was taken for further purification (total yield 100 %).
‡ The actual volumes are shown, but values given for the total interferon and protein are those observed multiplied by 2.86, since only 1/2.86 of the step 7 material was used for step 8.

tracing presented as it is to the naked eye. In addition, impurity proteins were disclosed, but well resolved from the interferon, with about twice the intensity of the interferon bands.

Fraction II contained F as the only interferon component, and again two closely adjacent protein bands were observed at the activity peak (Fig. 4b), as seen in the unfractionated sample (Fig. 2a). It is noteworthy that no other significant protein bands were seen.

SDS-PAGE

Both fractions I and II showed a distinct protein band corresponding to the respective activity peak (Fig. 5). Impurity proteins were detectable in fraction I on the leading side of the interferon peak, clearly visible to the naked eye as a broad shoulder in the stained gel but inconspicuous in the densitometer tracing due to the background noise. Perhaps the resolution in this case was inferior to PAGE at pH 4.3. Fraction II had no detectable impurities. Correspondence of activity and protein was observed at all of four different gel concentrations tested (3.5, 5, 7.5 and 10 %; Fig. 6).

The mobility of the interferon in both the fractions did not change when the preparation was treated with 2-mercaptoethanol (ME) or dithiothreitol before the run, in agreement with Stewart (1974).

Glycoproteins are known to show mobilities in SDS-PAGE at low gel concentrations which are anomalous to simple proteins (Segrest & Jackson, 1972). Since many interferons appear to be glycoproteins (Weil & Dorner, 1974), the mobilities of the F and S components in SDS-PAGE at various gel concentrations were determined relative to marker proteins.
As seen in Fig. 6, the apparent mol. wt. of the S component in fraction I increased moderately at lower gel concentrations, suggesting a glycoprotein nature, perhaps with a low carbohydrate content for this interferon. On the other hand, that of the F component in fraction II did not show dependence on the gel concentration, suggesting that this interferon contains even less or no carbohydrate.

The mol. wt. of the F and S components are estimated to be 24 000 and 36 000, respectively, values which agree well with those obtained by gel filtration, and also with those obtained by Stewart (1974) for another L cell interferon preparation.
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Stability of interferon; stabilization by SDS

Preservation of the activity of highly purified preparations is excellent in the lyophilized state, but in solution is still a problem. Both S and F were inactivated strongly after cycles of freezing and thawing. During our experiments, the stability of F was considerably less than that of S, as already noted in connexion with the experiments shown in Fig. 1, 2(b), 3 and 4.

In agreement with Stewart et al. (1974a), our interferon was stabilized by the addition of SDS–urea–ME. This is shown in Table 2, in which the unstabilized control preparation was inactivated to 20% of the original activity after 24 h at room temperature, and to 2% after 1 min at 100°C. Thermally inactivated interferon could be reactivated by heating to high temperatures in SDS–urea–ME as they described, but when various samples which had been partially inactivated during storage were heated, the recovery of activity was variable (40 to 230%), indicating that not all forms of inactivated interferon can be rescued by this treatment. Our impression is that the F component may not be entirely stable at 100°C in SDS–urea–ME (Table 2).

Stewart, De Somer & De Clercq (1974b) observed partial inactivation of L cell interferon when SDS alone was added, and this was especially marked for the higher mol. wt. component (Stewart, 1974). In contrast, our preparation was as stable in SDS alone as in SDS–urea–ME (Table 2), in agreement with Knight (1975). That the isolated higher mol. wt. S

Fig. 3. Gel filtration of sample from step 7 on Bio-Gel P30. One ml of interferon containing 1.66 x 10⁸ units and 4.89 E₅₉₀ units was loaded on to a column (2.6 x 59 cm) and eluted with 1 M-NaCl-0.02 M-tris-HCl, pH 7.2, at a flow rate of 4 ml/h at 4°C. Recovery of interferon activity was 33% and of protein (E₅₉₀) 73%.
Fig. 4. PAGE at pH 4.3 of (a) fraction I and (b) fraction II (7.5% gel). ---, densitometer tracing of stained gel, 0.005 (Fraction I) and 0.007 (Fraction II) $E_{180}$ unit run. ○---○, interferon activity in 1 mm fractions, 0.030 (Fraction I) and 0.070 (Fraction II) $E_{180}$ unit run, extracted with 0.1% SDS-0.01 M-tris-HCl, pH 7.2, at room temperature overnight, 96% recovery for fraction I, and 28% for fraction II. (For fraction II, the extracted materials were heated at 70°C for 30 min in 1% SDS before assay; without heating, only 3% recovery).

Our main finding in this study is of distinct proteins associated with the biological activity of both of the two components of L cell interferon. This was found in PAGE at pH 4.3 as well as in SDS-PAGE at various gel concentrations. Since the two electrophoretic systems resolve proteins on a different basis, it may be reasonable to assume that we are now observing
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Fig. 5. SDS-PAGE of (a) fraction I and (b) fraction II in 7.5 % gel. ——, densitometer tracing of stained gel (upper part); O——O, interferon activity in 2 mm fractions (lower part). Samples heated before the run at 70 °C for 30 min in 1 % SDS-0.01 M-tris-HCl, pH 7.2. (a) 0.007 and 0.0015 Aeg unit run, respectively, for staining and interferon assay (70 % recovery). (b) 0.007 Aeg unit run; the gel was cut lengthwise, one half used for staining, and the other for interferon assay (71 % recovery). Mol. wt. marker proteins (as in Fig. 1; also Ch, chymotrypsinogen A, 25,000; Cy, cytochrome c, 12,400) were run in parallel gels.

Fig. 6. Apparent mol. wt. of S and F determined by SDS-PAGE at various gel concentrations. Fractions I and II were pre-treated at 70 °C for 30 min in 1 % SDS-5 M-urea-0.01 M-dithiothreitol-0.01 M-phosphate buffer, pH 7.2, and 0.004 to 0.009 Aeg unit was used per gel. Mol. wt. marker proteins were either co-electrophoresed, or run simultaneously in a separate gel where necessary for adequate resolution of the interferon protein. Peak interferon activity: ●, fraction I-S component (70 to 120 % recovery); ▲, fraction II-F component (6 to 70 % recovery). Peak interferon protein: ○, fraction I-S component; △, fraction II-F component.
Table 2. Stability of interferon to SDS and reduction

<table>
<thead>
<tr>
<th>Interferon</th>
<th>Reagents added</th>
<th>Residual activity (%) after 24 h at room temp.</th>
<th>Residual activity (%) after 1 min at 100 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated*</td>
<td>None</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>0.1% SDS</td>
<td>117</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>1% SDS</td>
<td>130</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>0.1% SDS—5 M-urea—0.014 M-ME</td>
<td>130</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>1% SDS—5 M-urea—0.014 M-ME</td>
<td>130</td>
<td>130</td>
</tr>
<tr>
<td>Fraction II (F)†</td>
<td>0.1% SDS—5 M-urea—0.014 M-ME</td>
<td>ND§</td>
<td>40</td>
</tr>
<tr>
<td>Fraction I (S)‡</td>
<td>0.1% SDS—5 M-urea—0.014 M-ME</td>
<td>ND§</td>
<td>83</td>
</tr>
</tbody>
</table>

* Material at step 7 in Table 1, 1.0 × 10⁶ interferon units and 0.028 E₂₈₀ unit/ml in 0.01 M-tris-HCl, pH 7.2.
† Fraction II in Table 1, 2.5 × 10⁶ interferon units and 0.1 E₂₈₀ unit/ml in the same buffer.
‡ Fraction I in Table 1, 1.0 × 10⁷ interferon units and 0.1 E₂₈₀ unit/ml in the same buffer.
§ Not done.

The interferon proteins themselves. However, further analyses are obviously necessary before this can be definitely concluded. It is especially noteworthy that in fraction II, obtained by gel filtration, there were protein bands only in the region of activity of the F interferon component. In the case of fraction I containing the S component, some impurity proteins were present, but they should in principle be readily removed after PAGE at pH 4.3, since they are well resolved from the interferon peak.

Two closely adjacent protein bands were observed in the interferon peak on PAGE at pH 4.3 for both F and S. This may possibly indicate a certain degree of microheterogeneity in the interferon proteins, due to non-uniformity in the carbohydrate moiety, in the amino acid side chains, or in peptide length. It may, however, also be possible that only one of the two bands is the interferon protein. The present resolution of our PAGE system does not allow us to decide between these alternatives, but preliminary isoelectric focusing experiments tend to favor the former case.

Assuming that the proteins in the interferon peak of the PAGE at pH 4.3 (Fig. 4) are interferon itself, we can estimate the sp. act. of the S interferon to be about three times that of fraction I, i.e. 3 × 10⁸ units/mg protein, since the protein under the peak represents about one-third of the total protein in the electropherogram. That of F is 2.5 × 10⁷ units/mg protein, equal to that of fraction II. This latter value was unexpectedly low, especially in view of our previous estimate (Yamamoto et al. 1974) for F of at least 3 × 10⁸ units/mg protein, obtained with a less pure preparation. It undoubtedly resulted from extensive inactivation of the F component during purification and storage. Some inactivation of the S component also occurred, although it was considerably more stable than F. If, as argued under Results, the losses in activity observed after step 6 had been prevented, and so the sp. act. of fractions I and II were 4.4 and 8.6 times higher than given in Table 1, then the S and F interferons would have 1.3 × 10⁹ and 2.2 × 10⁸ units/E₂₈₀, respectively. Determination of the sp. act. of native interferons must await further investigations.

Ogburn, Berg & Paucker (1973) obtained a value of 1 to 2.7 × 10⁸ units/mg protein for L cell interferon after affinity chromatography using anti-interferon antibody, although there were indications that their preparations were still impure. Knight (1975) purified L cell interferon by a procedure fairly similar to ours, to a sp. act. of 2.5 × 10⁸ units/mg protein, but he gave no estimate of purity, although single protein bands were observed in electrophoretically separated fractions upon re-electrophoresis.
Our mol. wt. values determined by gel filtration in neutral buffer solution agreed well with those determined by SDS-PAGE, indicating the absence in both F and S of molecular aggregation in the undenatured state, which Carter (1970) claimed to have found. Absence of intermolecular S-S bonds, reported for L cell interferon by Stewart (1974), was confirmed here. Our finding of two interferon components and the mol. wt. values assigned to them also agree well with the results of Stewart (1974). Knight (1975), on the other hand, resolved L cell interferon into 10 or more components which differed slightly from one another in mol. wt. It remains to be seen whether such heterogeneity exists in our preparation too.

We found that a sample intermediate between fractions I and II in Fig. 3 was essentially a mixture of fractions I and II both in SDS-PAGE and PAGE at pH 4.3 (data not shown), but this does not necessarily preclude the presence of small amounts of heterogeneous components of intermediate mol. wt.

Apart from mol. wt., F and S were found to differ in their cell growth suppressing activity (Ohwaki & Kawade, 1972; Matsuzawa & Kawade, 1974), in agreement with Borecky et al. (1972), but at variance with Gresser et al. (1973). Further studies are now in progress to find out if there are differences between F and S in antigenicity, lectin-binding and isoelectric point.

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REFERENCES


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