

# Purification, Characterization, and Attempts at Isotopic Labeling of Mouse Interferon

K. PAUCKER, B. J. BERMAN, R. R. GOLGHER,<sup>1</sup> AND D. STANČEK<sup>2</sup>

*Virus Laboratory of Children's Hospital of Philadelphia, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19146*

Received for publication 18 August 1969

Under optimal conditions which minimized the accumulation of extraneous proteins, interferon preparations were obtained in L cells containing from  $2 \times 10^4$  to  $5 \times 10^4$  units/mg of protein. The radiolabeled proteins were liberated simultaneously with interferon from cultures exposed to tritiated amino acids after viral stimulation and from corresponding controls, and were subsequently purified with the following results. Chromatography of interferon on carboxymethyl-Sephadex C-25 eliminated selectively unlabeled or poorly labeled proteins, resulting in a greater than sixfold increase in counts per minute per milligram of protein. Similarly purified control material harbored at least 12 times less tritium per milligram of protein than interferon, and the label was more diversely distributed among proteins of different molecular weights. On electrophoresis of interferon in polyacrylamide gels, labeled proteins were reduced further by a factor of at least 10 without loss in titer. Final purification was estimated at greater than 280-fold, representing a calculated specific activity of at least  $1.4 \times 10^7$  units of interferon per milligram of protein.

Most data on the purification and properties of interferon have been obtained from studies with chick interferon (5), presumably because of its ready availability. A few attempts at purifying mouse interferon have been made, but the degree of purification attained has either been low, or experimental details were not given. In part, the lack of success with mouse interferon may have been due to a starting material of poor quality, heavily contaminated with extraneous proteins (15, 16, 23), or to the necessity of securing sufficiently large quantities of crude interferon. We thought that some of the aforementioned difficulties might be overcome in a tissue culture system used in this laboratory, which yields substantial quantities of a potent mouse interferon low in protein content. These expectations were to a large extent fulfilled, and the way was paved for attempting to incorporate an isotopic label into interferon in the course of its synthesis. The present report describes procedural details used in the preparation of such a radioactive material.

<sup>1</sup> Fellow of the Rockefeller Foundation. Present address: Microbiologia, Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, Belo Horizonte, M. G., Brazil.

<sup>2</sup> On leave of absence from the Institute of Virology, Czechoslovak academy of Sciences, Bratislava, Czechoslovakia.

## MATERIALS AND METHODS

**Cell cultures.** For production of interferon, L cells were propagated in suspension as previously described (17, 22), except that Eagle basal medium in Spinner salt solution was used, supplemented with 10% calf or horse serum and antibiotics. For attachment to glass surfaces, this medium was replaced by Eagle basal medium (Hanks base, HBME) containing 10% calf serum, 0.15% bicarbonate, and antibiotics.

Assay of interferon was carried out in monolayers of L (MCN) cells (20) grown in the presence of 60% Scherer maintenance solution, 30% medium 199, and 10% inactivated (56 C, 30 min) horse serum.

Human amnion (FL) cells were grown in HBME as described above for L cells.

**Viruses.** The Victoria strain of Newcastle disease virus (NDV), propagated for an unknown number of passages in chick embryos, was utilized for induction of interferon. For challenge in interferon titrations, the chick-embryo-adapted Indiana strain of vesicular stomatitis virus (VSV), carried subsequently through 30 to 40 dilute passages in L cells, was employed.

**Virus titration.** Infectivity assay for NDV was carried out on monolayers of FL cells in 30-ml Falcon tissue culture flasks. Serial 10-fold dilutions of virus in 0.4-ml amounts were permitted to adsorb for 1 hr at 37 C. The final overlay contained HBME, supplemented with 2% calf serum and neutral red 1:40,000, as well as 1.2% Difco Agar. Plaques were read after 48 to 72 hr and recorded as plaque-forming units (PFU) per ml.

**Ultraviolet irradiation of virus.** Allantoic fluid preparations of NDV were dialyzed overnight against 20 volumes of 0.1 M phosphate-buffered saline (PBS) at pH 7.2. Subsequently, 15- to 20-ml amounts of virus were exposed to a 15-w germicidal lamp at a distance of 18 cm (7 inches) for 10 to 20 sec, with an emission of about 5,000 ergs per sec. The plates were rocked mechanically at a rate of 90 times per min with excursions of 2.54 cm (1 inch). Irradiated virus is referred to in this report as NDV<sub>uv</sub>.

**Production and labeling of interferon.** Approximately  $10^7$  cells, contained in 50 ml of medium, were seeded into 500-ml round prescription bottles fitted on a roller drum revolving at a speed of 8 rev/hr (18). Three days later, the bottles were refed and incubated for one additional day. Confluent monolayers contained from  $5 \times 10^7$  to  $10 \times 10^7$  cells. The medium was then discarded, and the flasks were inoculated with NDV<sub>uv</sub> at an input multiplicity of 300 PFU (before irradiation). One hour later, the virus was removed and the cells were refed with 10 ml of serum-free medium. After a total incubation time of 8 hr, this medium was replaced by one of the same composition, except that <sup>3</sup>H-protein hydrolysate (Schwarz Bio-Research, Orangeburg, N.Y.) at a concentration of about 10 μc/ml was substituted for the amino acids normally present. Interferon was collected into this medium during the period of its maximal release, i.e., between 8 and 12 hr after initial contact with virus.

A 5-ml sample of the crude interferon was dialyzed in the cold against four changes of 4 liters each of 0.1 M phosphate buffer at pH 8, changed daily to eliminate free tritium, as well as ingredients which give positive readings in the procedure of Lowry et al. (14) (vitamins, amino acids, glutamine, antibiotics, etc.) and to avoid precipitation of proteins. Initial protein and radioactive determinations were carried out with this material. A separate sample of the crude product was dialyzed against pH 2 buffer and brought back to neutrality, to measure interferon activity of the starting material. The rest of the crude interferon was concentrated about 20-fold by ultrafiltration, dialyzed against 0.01 M sodium acetate buffer (pH 4.5), and subsequently against four changes of 4 liters each of 0.1 M phosphate buffer, pH 6.

For measuring radioactivity, 0.1-ml samples were dispensed into vials containing Bray solution (2) and counted in a Tri-Carb Liquid Scintillation Spectrometer (Packard Instrument Co., Downers Grove, Ill.).

Controls were handled in an identical manner except that ultraviolet-irradiated dialyzed allantoic fluid from uninfected eggs was substituted for NDV<sub>uv</sub>.

**Assay of interferon.** Serial fourfold dilutions of interferon were inoculated in 0.5-ml amounts into 30 ml of tissue culture flasks (Falcon Plastics Co., Los Angeles, Calif.) containing  $6 \times 10^6$  L (MCN) cells bathed in 5 ml of medium. The flasks were incubated overnight at 37 C. On the following day, the cultures were drained and inoculated with 0.4 ml of VSV containing approximately 100 PFU. Virus was permitted to adsorb for 1 hr, and during this time the bottles were rocked intermittently. They were then overlaid with a mixture of 2.4% Difco Agar, and regular growth medium at twice the usual concentra-

tion, containing neutral red 1:20,000. Plaques were counted on the 2nd or 3rd day, and the 50% plaque reduction end point was computed from a standard regression curve. One unit of interferon was defined as the reciprocal of the highest dilution of interferon contained in 0.5 ml of inoculum which reduces the number of plaques found in controls by one-half. Twice that amount expresses the number of units per milliliter.

Interferon samples not destined for purification were dialyzed against 100 volumes of HCl-KCl buffer at pH 2 to eliminate residual interfering virus (3). The materials were brought back to neutrality by exhaustive dialysis against 0.1 M PBS.

**Proteins.** Determinations were carried out in a Beckman DB spectrophotometer by the method of Lowry et al. (14), with crystalline bovine plasma albumin used as a standard.

**Concentration of interferon.** Tissue culture fluids were syphoned into U-shaped bags of Visking tubing (diameter 0.25 inch, inflated), and subjected to a vacuum of approximately 20 lb/in<sup>2</sup>. This resulted in a flow rate of 5 ml/hr, and the biological activity was quantitatively recovered after 20-fold concentration (21).

**Chromatography.** Interferon was purified in a refrigerated column (Pharmacia, K25/45) of carboxymethyl-Sephadex C-25 (CMS). The loading ratio was 1 mg of protein to 10 ml of gel volume. The flow rate was adjusted to approximately 60 ml/hr, and fractions were collected in a refrigerated cabinet (Beckman model 133A). Passage through flow-cell assemblies permitted continuous recording of pH and optical density at 280 nm.

**Sephadex gel filtration.** For determination of molecular weights, a Sephadex G-100 column (1.5 by 90 cm) was operated at room temperature with a flow rate of 20 ml/hr. The following markers, of known average molecular weights, were supplied by Mann Research Laboratories, New York, N.Y.: cytochrome c (12,400), myoglobin (17,800), chymotrypsinogen A (25,000), ovalbumin (45,000), and bovine albumin (67,000). Elution was carried out in 0.1 M PBS at pH 7.4.

**Equilibrium density gradient centrifugations.** Gradients ranging from 0 to 20% sucrose in PBS were prepared in 4.5-ml amounts. The solutions were overlaid with 0.5 ml of sample followed by 0.3 ml of paraffin oil. Centrifugation was carried out in a Spinco SW39 rotor at 35,000 rev/min for 17 hr. Fractions of 20 drops were collected for assay. Markers were the same as those used for Sephadex gel filtration.

Cesium chloride equilibrium sedimentation was performed by adjusting the starting specific gravity of 5-ml samples with cesium chloride powder to 1.32 (12). The solutions were overlaid with 0.3 ml of paraffin oil and centrifuged in a Spinco SW50 rotor at 48,000 rev/min, for 72 hr. Samples of 20 drops were collected. Refractive indices were determined in an Abbe-3L refractometer.

**Polyacrylamide gel electrophoresis.** Chromatographed interferon samples were subdivided into two portions, one of which was concentrated 10-fold by pressure dialysis in the presence of 0.05% bovine

plasma albumin (BPA) to preserve biological activity, and the other 100-fold for staining of proteins. Samples of 0.4 to 0.8 ml of these materials in 0.1 M phosphate buffer (pH 6 to 7) were enclosed between two gels to permit sampling of residual activities (Stanček and Paucker, *Ann. N.Y. Acad. Sci., in press*) upon termination of the run. The gel columns (87 by 5 mm) consisted of 7.5% gel (w/v), composed of 28% acrylamide and 0.73% methylene-bisacrylamide, in an acid (pH 4.3) buffer system. Polymerization proceeded in the presence of 0.005% riboflavin (8). Electrophoresis was carried out in a Canalco model 12 electrophoretic apparatus, using  $\beta$ -alanine electrode buffers at pH 4.3, and 5 ma per gel was applied for 60 to 120 min. Methyl green, at a concentration of 0.001%, was added as tracking dye to the anode buffer.

Upon completion of the run, gels intended for interferon and isotope determinations were cut into 1-mm discs, and from two to three of these were pooled. Elution was carried out for several days in the cold, into 0.5-ml volumes of PBS containing from 0.05 to 0.5% BPA. Non-elutable radioactivity was extracted from the gels by dissolving the discs in 0.2 to 0.5 ml of 30% hydrogen peroxide at 60 C for 20 hr. Portions of 0.1 ml were then transferred to scintillation vials containing Bray solution for counting.

**Enzyme digestion.** Samples of nonpurified radioactive interferon preparations in 0.1 M phosphate buffer at pH 8 were exposed in 1-ml amounts for 3 and 20 hr at 37 C to each of the following enzymes, at concentrations listed in Table 3. Pronase (Calbiochem, Los Angeles, Calif.). Deoxyribonuclease 1 (Worthington Biochemical Corp., Freehold, N.J.) and ribonuclease (C. F. Boehringer & Söhne GmbH, Mannheim, Germany). After incubation, 400  $\mu$ g of BPA (Armour Pharmaceutical Co., Chicago, Ill.) was added to all samples as a carrier, and proteins were precipitated in the cold by an equal volume of 40% trichloroacetic acid. Supernatant fluids were saved for counting, and sediments were washed three times with 20% trichloroacetic acid. The final precipitates were dissolved in 0.5 ml of NCS reagent (Amersham/Searle, Des Plaines, Ill.), transferred to scintillation vials, and counted. Enzyme activities were controlled by incubation with their corresponding substrates for 2 hr at 37 C. This step was followed by precipitation in the cold with trichloroacetic acid as above. Optical densities of the supernatant fluids were then determined at 260 nm for ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), and at 280 nm for BPA.

**Sterilization of interferon samples.** All materials obtained after nonsterile handling were irradiated for 2 min by ultraviolet light in the same set-up as that used for inactivation of NDV. Interferon titers were not diminished by this procedure.

## RESULTS

### Optimal conditions for interferon production.

Previous experiments had shown that NDV subjected to ultraviolet irradiation often promoted higher yields of interferon from L cells than non-inactivated virus. In view of securing adequate quantities of interferon for purification, this

question was studied more systematically in the following experiment. Samples of NDV were dialyzed and irradiated for time intervals ranging from 5 to 120 sec. Suspension cultures numbering  $4 \times 10^6$  cells in 9 ml of medium were inoculated with 1-ml amounts of NDV<sub>uv</sub> at an input multiplicity of 500. Twenty-four hours later, media of all cultures were harvested, and interfering virus was eliminated by dialysis against low pH. Figure 1 illustrates the inactivation kinetics of NDV under these conditions, as well as the interferon titers generated by virus irradiated for periods ranging from 0 to 40 sec. Inactivation of virus proceeded linearly with increasing length of ultraviolet treatment; and after 40 sec no viable virus could be recovered. The amount of interferon produced rose approximately 16-fold after 5 sec of irradiation, and this level persisted for an interval of at least 20 sec. After exposure to 40 sec of ultraviolet light, interferon titers were slightly reduced, and they declined further with progressively longer ultraviolet treatment (not shown). Optimally irradiated virus proved, therefore, superior as an inducer of interferon in this system, and an interval of 10 to 20 sec for irradiation of NDV was adopted. Comparable yields of interferon were elicited from L cells with multiplicities of NDV<sub>uv</sub> ranging from 500 to 100. Below that concentration the amounts of interferon produced were less than those obtained with viable NDV.

Additional experiments disclosed that by restricting the time interval for collection of interferon to the period of maximal liberation from

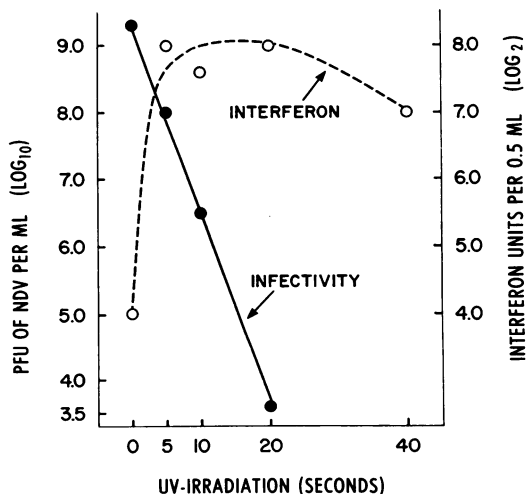


FIG. 1. Effect of ultraviolet (UV) irradiation on interferon production by Newcastle disease virus. Emission of UV was 5,000 ergs per sec.

the cultures, i.e., from 8 to 12 hr after viral stimulation, the accumulation of extraneous proteins from damaged cells was further minimized. As a result of these studies, crude interferon materials became available which had specific activities in the range of 20,000 to 40,000 units/mg of protein.

**Purification.** In preliminary attempts, non-labeled materials prepared as described above were treated with perchloric acid and concentrated by zinc acetate by the method of Lampson et al. (13). However, recovery of interferon was extremely variable, ranging from 67 to 3% on five separate occasions. Concentration by ultra-filtration (21) resulted in quantitative recovery of interferon. Controlled lowering of the pH of concentrated interferon and removal of the precipitates which formed by intermittent centrifugations (4) occasioned parallel losses of interferon and protein. Dialysis of the concentrated interferon against 0.01 M sodium acetate at pH 4.5 (21) did not diminish interferon potency, but nonreactive proteins were precipitated in the process. The resulting purification was approximately fourfold. Passage through diethylaminoethyl-Sephadex equilibrated at the same pH (pH 4.5) caused partial retention of interferon in the column but no selective removal of extraneous proteins. The acidified concentrated interferon was, therefore, dialyzed against 0.1 M phosphate buffer at pH 6.0, and was loaded on a CMS column equilibrated against the same buffer (16). Interferon was eluted in a continuous gradient between pH 6.8 and 7.7. In 10 experiments, the average recovery of interferon during this step was 26%. This procedure was, therefore, adopted for further studies with isotopically labeled preparations.

Interferon and control materials were prepared as described and were subjected to the purification steps outlined in the preceding paragraph. Interferon, protein, and isotope levels were sur-

veyed at different stages during this procedure; the values found are given in Table 1.

Three stages are compared, namely, the initial material (crude interferon), the concentrated product before chromatography on CMS (pre-CMS interferon), and the pool of the peak fractions of interferon eluted from the column by the pH gradient (post-CMS interferon). Loss of interferon activity between the first two stages amounted to 67%, and the overall recovery of interferon was about 13%. On the other hand, protein was reduced from 22 mg to <0.1 mg, i.e., at least 200 times. Comparing these values with the changes in radioactivity, it can be seen that tritium counts per minute declined only about 34-fold. Accordingly, the counts per minute per milligram of protein rose from 7.9 for the starting material, to more than 51 in the final product. The high specific activity of crude interferon, amounting to 50,500, made it possible to obtain with a limited degree of purification a preparation which contained in excess of  $1.4 \times 10^6$  units/mg of protein.

The crude control harbored about the same amount of tritium per milligram of protein than the corresponding interferon preparation. However, in the course of purification, using comparable amounts of protein for both groups, the counts per minute per milligram of protein declined 1.85-fold, whereas they increased more than 6.4 times with interferon. Therefore, the chromatographed control material contained about 12 times less tritium per milligram of protein than purified interferon.

These relationships were confirmed in a number of experiments by using different amounts of isotope in the collection fluid. Table 2 lists the values recorded for the purified (post-CMS) preparations. With increasing quantities of input label, the ratios between tritium and interferon and between tritium and protein were likewise improved.

TABLE 1. Purification of  $^3\text{H}$ -labeled NDV<sub>uv</sub>-induced L-cell interferon and of control material by chromatography on carboxymethyl-Sephadex

Material	Stage <sup>a</sup>	Vol (ml)	IFU <sup>b</sup>	Protein (mg)	Counts/min	Counts per min per IFU	Counts per min per mg of protein	Specific activity <sup>c</sup>
Interferon	Crude	176	$11.1 \times 10^5$	22	$174 \times 10^5$	15.7	$7.9 \times 10^5$	50,500
	Pre-CMS	15.5	$3.7 \times 10^5$	3.8	$41 \times 10^5$	11.1	$10.8 \times 10^5$	97,000
	Post-CMS	90	$1.4 \times 10^5$	<0.1	$5.1 \times 10^5$	3.6	$>5.1 \times 10^6$	>1,400,000
Control	Crude	100		5.4	$41 \times 10^5$		$7.6 \times 10^5$	
	Pre-CMS	7.5		0.6	$14 \times 10^5$		$23.4 \times 10^5$	
	Post-CMS	22		0.04	$0.18 \times 10^5$		$4.1 \times 10^5$	

<sup>a</sup> Representing unconcentrated, nonpurified preparations, and the steps before and after chromatography on carboxymethyl-Sephadex C-25.

<sup>b</sup> Interferon units.

<sup>c</sup> Interferon units per milligram of protein.

Especially noteworthy is the comparison between interferon and control in the last experiment given in the table. Incorporation of the isotope was eight times higher in the interferon pool than in the control.

**Properties.** Purified mouse interferon is extremely unstable under a variety of conditions which include storage in glass, Visking tubing, and 30% sucrose. Preservation of activity in polypropylene vessels (13) was intermediate. However, full interferon potency was preserved for more than 24 weeks at 4 C by the addition of BPA at a final concentration of 0.5%. Lesser amounts were partially or not at all effective. BPA-containing pools of purified interferon were stable to ultraviolet light for more than 15 min under the standard conditions described. This property made it possible to inactivate selectively any traces of interfering NDV which might still have been present in the materials. In fact, viral interference was totally abolished after 2 min of irradiation, i.e., the time used routinely for sterilization of samples.

In parallel experiments, interferon preparations purified to the extent described and concentrated 10 times in the presence of 0.05% BPA (one-tenth the usual amount) were examined by Sephadex filtration and in equilibrium sucrose density gradients together with markers of known molecular weights (1). On the basis of these studies, a molecular weight of 23,000, slightly less than that of Chymotrypsinogen A, was assigned to NDV<sub>uv</sub>-induced L-cell interferon. Comparison with crude interferon showed no evidence that a heavier component might have been selectively eliminated during the purification procedure.

Examination of chromatographed interferon on equilibrium sedimentation in cesium chloride gradients disclosed an effective buoyant density of 1.3 gm/cm<sup>3</sup>.

**Characterization.** To make certain that incorporation of the isotope into protein constitu-

ents had actually occurred, samples of a non-purified, tritium-labeled interferon preparation and of a control material were subjected to digestion with Pronase, deoxyribonuclease, and ribonuclease at concentrations listed in Table 3. Since the results were comparable for both periods of treatment, presentation is confined to data obtained after 3 hr of incubation. All enzymes, at the levels tested, were active on their respective substrates.

The results were essentially similar for both interferon and control materials. Digestion with Pronase was effective in reducing the amount of tritium in the trichloroacetic acid precipitate to 13.5 and 23%, respectively, of that found in the absence of treatment. In contrast, on exposure to the nucleases, more than 90%, or virtually all of the isotope, remained associated with the fraction precipitable by trichloroacetic acid. These data indicate that the tritium label was essentially associated with protein components in the preparations under test.

To determine the distribution of the isotopic label in the two types of preparations, <sup>3</sup>H-labeled interferon and similarly purified <sup>3</sup>H-labeled control material, adjusted to comparable levels of protein, were subjected to equilibrium density gradient centrifugations in sucrose. The results (Fig. 2) show that the elution curve of interferon was broadly covered by a displaced isotope profile, and it is apparent that a variety of light and heavy proteins dissimilar from interferon had incorporated the label. The control, on the other hand, as mentioned before, contained significantly less radioactivity than interferon, and the label displayed a more or less uniform distribution pattern.

Similar discrepancies between interferon and isotope were noted when chromatographed <sup>3</sup>H-labeled interferon was subjected to filtration on Sephadex G-100. The results (Fig. 3) reveal that the juxtaposition of the two activities was imperfect. In addition, there is a minor radioactive

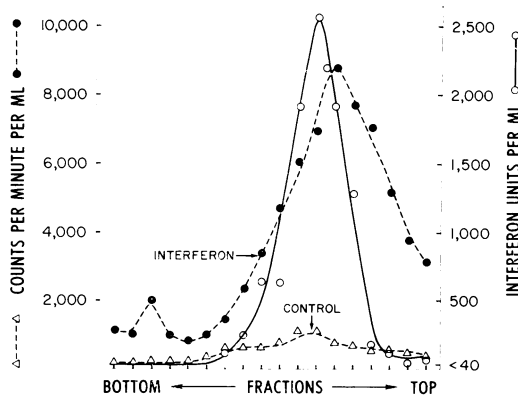
TABLE 2. Association between <sup>3</sup>H-label, interferon, and protein in various purified preparations

Material	<sup>3</sup> H-Amino acids (μc/ml)	Carboxymethyl-Sephadex pools			
		Interferon units per ml	Specific activity <sup>a</sup>	Counts per min per interferon unit	Counts per min per mg of protein
Interferon.....	3.4	330	165,000	0.9	1.5 × 10 <sup>6</sup>
Interferon.....	9.1	1,600	>800,000	3.6	>2.85 × 10 <sup>6</sup>
Interferon.....	15	1,400	700,000	9.3	6.50 × 10 <sup>6</sup>
Interferon.....	9.5	1,400	700,000	4.9	3.42 × 10 <sup>6</sup>
Control.....	9.5				4.1 × 10 <sup>6</sup>

<sup>a</sup> Interferon units per milligram of protein.

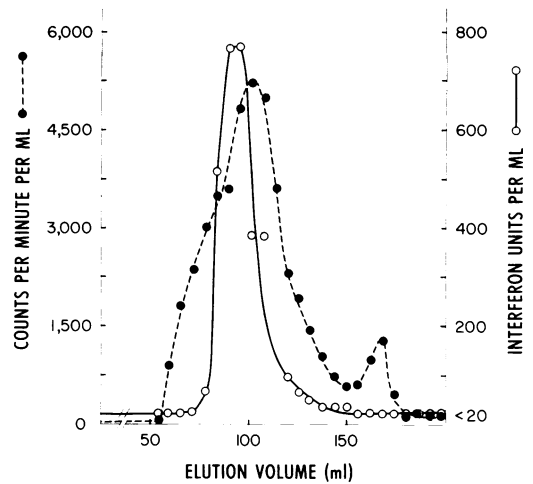
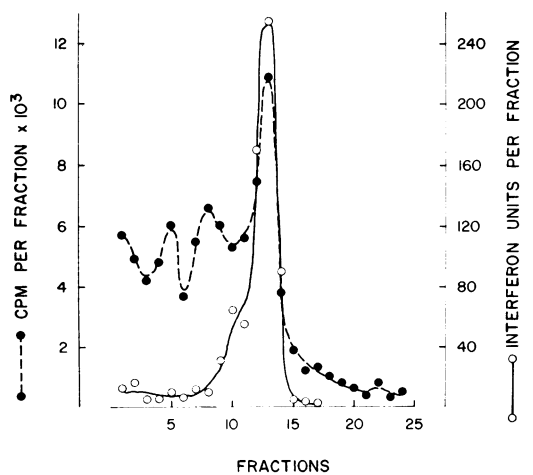
TABLE 3. Enzyme treatment of nonpurified  $^3\text{H}$ -labeled interferon

Material	Treatment <sup>a</sup>		Counts per min		
	Enzyme	Amount (g/ml)	Total	Tri-chloro-acetic acid (ppt)	Per cent
Interferon	None		2,600	2,600	100
	Pronase	100	2,600	350	13.5
	DNAase <sup>b</sup>	20	2,610	2,350	90.5
	RNAase <sup>c</sup>	10	2,100	2,100	100
Control	None		2,430	2,400	99
	Pronase	100	2,430	550	23
	DNAase <sup>b</sup>	20	2,860	2,700	94
	RNAase <sup>c</sup>	10	2,250	2,200	98

<sup>a</sup> Three hours at 37 C.<sup>b</sup> Deoxyribonuclease.<sup>c</sup> Ribonuclease.FIG. 2. Sucrose gradient centrifugation of chromatographed  $^3\text{H}$ -labeled L-cell interferon induced by  $\text{NDV}_{uv}$ . Control signifies a comparable material obtained from cells exposed to dialyzed, ultraviolet-irradiated normal allantoic fluid.

peak to the right of the interferon region, which corresponds to an entity with a molecular weight of less than 1,000.

To achieve further separation of nonreactive proteins from interferon, a tritium-labeled chromatographed preparation was subjected to electrophoresis in polyacrylamide gels. The gel destined for detection of proteins, by means of staining with amido black, was loaded with approximately 80 to 100  $\mu\text{g}$  of protein, corresponding to the interferon region eluting from CMS. The parallel gel received 558 units of interferon with which  $4.88 \times 10^5$  radiocounts were associated. Electrophoresis of this portion proceeded

FIG. 3. Filtration on Sephadex G-100 of chromatographed  $^3\text{H}$ -labeled L-cell interferon induced by  $\text{NDV}_{uv}$ .FIG. 4. Migration and distribution in polyacrylamide gels of chromatographed  $^3\text{H}$ -labeled,  $\text{NDV}_{uv}$ -induced L-cell interferon.

in the presence of 0.5% BPA to prevent inactivation of interferon activity. As described elsewhere (Stanček and Paucker, *Acta Virol.*, *in press*), the addition of BPA did not materially affect the pattern of migration of interferon in polyacrylamide gels. The results of a representative experiment are shown in Fig. 4. Interferon activity, which was recovered more than quantitatively, was spread throughout a considerable portion of the gel, but most of it was confined to a relatively narrow zone located in the center. The elutable radioactivity, representing 18.4% of the input, was distributed over a wider area of the gel, but

one third (6.6%) of the tritium counts paralleled the peak region of interferon activity. Most of the remaining isotope migrated more slowly than interferon, and either little or no antiviral activity was recorded in the eluates from this portion of the gel. A small amount of the label preceded interferon in its migration toward the anode. No stainable proteins were visible, except for a vaguely diffuse zone in the area of interferon activity which permitted no quantitative estimation.

### DISCUSSION

Past attempts at purifying mouse interferon were greatly impeded by the presence in crude materials of considerable amounts of extraneous proteins. In the present study, methods for the elicitation and collection of interferon were devised to minimize as much as possible the accumulation of these contaminating factors. The steps consisted of the selection of optimally ultraviolet-inactivated inducer virus (10, 11), the use of slowly rotating monolayers of cells bathed in a minimal amount of fluid (18), the omission of serum from the collecting medium during release of interferon, and restriction of the harvest interval to a 4-hr period, during which maximal liberation of interferon from the cells took place. As a result of these manipulations, starting materials with specific activities ranging from 20,000 to 40,000 units of interferon per mg of protein were regularly obtained, which represented an approximately 400- to 800-fold improvement over crude chick interferon (5).

Subsequent procedures, based primarily on previously published reports of Merigan et al. (16) and Schonke (21), yielded a product of considerable purity containing in excess of  $1.4 \times 10^6$  units per mg of protein. No distinction between the degree of incorporation of tritium into interferon and control was found when crude materials were compared, although less total protein was released from noninduced cells. However, subsequent to chromatography on CMS, which resulted in a greater than 100-fold elimination of proteins as compared to the starting products, the counts per minute per milligram of protein increased severalfold for interferon, whereas they declined less than twofold in the control preparation. These changes are interpreted to indicate that the label was preferentially incorporated into proteins synthesized at the same time as interferon and presenting similar configurational characteristics. Labeled proteins liberated from nonstimulated cells appeared to display more diversified properties, as shown by their greater elimination during chromatography and distribution in sucrose density gradients.

Molecular weights, determined for chromatographed L-cell interferon in Sephadex G-100 and sucrose gradients, gave values in the range of 23,000, which compares well with the results obtained by others with crude mouse interferons derived from tissue cultures (9, 13, 15, 19; T. C. Merigan, *Bacteriol. Proc.*, 1964, p. 115). The buoyant density value of  $1.3 \text{ g/cm}^3$  found on equilibrium centrifugation in cesium chloride also parallels the specific gravity of chick interferon reported by Kreuz and Levy (12).

The distribution patterns of isotope and interferon titers on Sephadex filtration and in sucrose gradients indicated that a majority of the labeled proteins eluted with interferon from CMS may be sufficiently different to warrant further separation attempts. Electrophoresis in polyacrylamide gels, when carried out under carefully predetermined conditions (Stanček and Paucker, *Ann. N.Y. Acad. Sci.*, *in press*, and *Acta Virol.*, *in press*), permitted more than quantitative recovery of interferon from disc eluates. On the other hand, only about 6.6% of the input radioactive label remained intimately associated with the peak region of interferon activity. Therefore, without taking into account the enhanced recovery of interferon, the chromatographed material underwent a 15-fold or greater purification during electrophoresis in polyacrylamide gels. In a number of experiments, total purification during the entire procedure ranged from 280- to 850-fold.

However, the relatively broad migration profiles of L and chick interferons (6, 8) in polyacrylamide gels, and the diversity of electric charge described for chick interferons (7), point to a heterogeneous composition of even highly purified materials. Whether further separation of nonreactive labeled proteins from interferon can be achieved by electrofocusing is at present under investigation. Studies are also in progress to determine whether the more poorly labeled proteins released from noninduced cells and those liberated concomitantly with interferon present comparable or dissimilar radioactive distribution patterns during electrophoresis in polyacrylamide gels and isoelectric focusing.

### ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants AI-02405 from the National Institute of Allergy and Infectious Diseases and 2T1-GM-694 from the National Institute of General Medical Sciences.

We thank Boaz Moav for many helpful discussions. The excellent technical assistance of Marilyn J. Mundy, Patricia Davis, and Tina Dabrow is gratefully acknowledged.

## ADDENDUM IN PROOF

One unit of interferon as defined in this study corresponds to approximately 10 units of the international reference standard for mouse interferon.

## LITERATURE CITED

1. Andrews, P. 1964. Estimation of the molecular weights of proteins by sephadex gel-filtration. *Biochem. J.* 91:222-233.
2. Bray, G. A. 1960. A simple, efficient liquid scintillator for counting aqueous solution in a liquid scintillation counter. *Anal. Biochem.* 1:279-285.
3. Cantell, K., and K. Paucker. 1963. Quantitative studies on viral interference in suspended L cells. IV. Production and assay of interferon. *Virology* 21:11-21.
4. Fantes, K. H. 1965. Further purification of chick interferon. *Nature* 207:1298.
5. Fantes, K. H. 1966. p. 119-180. *In* N. B. Finter (ed.), *Interferons*. North-Holland Publishing Co., Amsterdam.
6. Fantes, K. H. 1968. p. 223-229. *In* M. Sanders and E. H. Lennette (ed.), *Medical and applied virology*. Warren H. Green, Inc. St. Louis, Missouri.
7. Fantes, K. H. 1969. Chick interferon: heterogeneity of electric charge. *Science* 163:1198-1199.
8. Fantes, K. H., and I. G. S. Furringer. 1967. Polyacrylamide gel electrophoresis of highly purified chick interferon. *Nature* 216:71-72.
9. Hallum, J. V., J. S. Youngner, and W. R. Stinebring. 1965. Interferon activity associated with high molecular weight proteins in the circulation of mice infected with endotoxin or bacteria. *Virology* 27:429-431.
10. Ho, M. 1964. Identification and "induction" of interferon. *Bacteriol. Rev.* 28:367-381.
11. Ho, M. 1966. p. 21-54. N. B. Finter (ed.), *Interferons*. North-Holland Publishing Co., Amsterdam.
12. Kreuz, L. E., and A. H. Levy. 1965. Physical properties of chick interferon. *J. Bacteriol.* 89:462-469.
13. Lampson, G. P., A. A. Tytell, M. M. Nemes, and M. R. Hilleman. 1963. Purification and characterization of chick embryo interferon. *Proc. Soc. Exp. Biol. Med.* 112:468-478.
14. Lowry, O. H., N. J. Rosebrough, L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265.
15. Merigan, T. C. 1964. Purified interferons; physical properties and species specificity. *Science* 145:811-813.
16. Merigan, T. C., C. A. Winget, and C. B. Dixon. 1965. Purification of vertebrate interferons. *J. Mol. Biol.* 13:679-691.
17. Paucker, K., Z. Skurska, and W. Henle. 1962. Quantitative studies on viral interference in suspended L cells. I. Growth characteristics and interfering activities of vesicular stomatitis, Newcastle disease, and influenza A viruses. *Virology* 17:301-311.
18. Paucker, K., K. Cantell, and W. Henle. 1962. Quantitative studies on viral interference in suspended L cells. III. Effect of interfering viruses and interferon on the growth rate of cells. *Virology* 17:324-334.
19. Rotem, Z., and Charlwood, P. A. 1963. Molecular weights of interferons from different animal species. *Nature* 198:1066-1068.
20. Rothfels, K. H., A. A. Axelrad, L. Siminovitch, E. A. McCulloch, and R. C. Parker. 1959. The origin of altered cell lines from mouse, monkey, and man as indicated by chromosome and transplantation studies. *Can. Cancer Conf.* 3:189-214.
21. Schonke, E. 1966. Properties of rat-tumor interferon. *Biochim. Biophys. Acta* 115:429-439.
22. Siminovitch, L., A. F. Graham, S. M. Lesley, and A. Nevill. 1957. Propagation of L strain mouse cells in suspension. *Exp. Cell Res.* 12:299-308.
23. Vilček, J., J. Tomisova, F. Sokol, and L. Hana. 1964. Concentration and partial purification of interferon from mouse brains. *Acta Virol.* 8:76-79.