HUMAN LYMPHOBLASTOID INTERFERON: PURIFICATION, AMINO ACID COMPOSITION, AND AMINO-TERMINAL SEQUENCE

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INTRODUCTION

The purification and characterization of human interferons (IFs), type I and type II (immune), are currently under investigation because of the clinical potential of these molecules as antiviral, antitumor, and immunomodulatory agents.[1] Recently, the purification and initial characterization of several mouse[2,3] and human[4-7] IFs have been described. Major advances in the large-scale production of IFs[7-12] and improvements in the detection and analysis of picomole quantities of amino acids, peptides, and proteins[4,13-15] have contributed to this success. In spite of developments in production, only minute quantities of these purified interferons are currently available for sequencing studies.

Purified human lymphoblastoid interferon has been obtained in nanomole quantities from Namalwa cells induced with Newcastle disease virus (NDV).[16] The major component, with an apparent molecular weight of 18,500, has been purified to homogeneity.[16] This report describes the isolation of two components of human lymphoblastoid IF with the apparent molecular weights of 21,500 and 18,500 by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The amino acid composition and the amino-terminal sequence of the 18,500 dalton component is presented.

MATERIALS AND METHODS

Production and Concentration of Crude Interferon

Human lymphoblastoid interferon was obtained from Namalwa cultures induced with NDV, strain B1, as previously described.[10] The crude IF was concentrated by precipitation with 5% (wt/vol) trichloroacetic acid (TCA).[11] A major portion of the IF was supplied by Litton Bionetics under National Cancer Institute contract NO1-CO-75380 as the redissolved TCA precipitate. Newcastle disease virus was prepared by Jack Campbell, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, MD.[10]

0077-8923/80/0350-0390 $01.75/0

Interferon Assay

IF was assayed on low passage human foreskin fibroblast cells (HSF-4) with vesicular stomatitis virus as the challenge virus, using a modification of the semi-micro method described by Armstrong.[17] All IF titers are expressed as reference units based on the human leukocyte standard G-023-901-527 obtained from the Research Resources Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD.

Protein Determination and Amino Acid Analyses

Protein concentrations were determined by the method of Lowry *et al.*[18] with bovine serum albumin as a standard, or by amino acid analyses. The latter analyses were done in the laboratory of P. E. Hare, Geophysical Laboratory, Carnegie Institute of Washington, D.C., using high-performance liquid chromatography (HPLC), and fluorescence detection of *o*-phthalaldehyde amino acid derivatives.[13,14] Samples were analyzed directly or were dialyzed prior to analysis twice against 6-liter volumes of phosphate buffered saline, pH 7.4, 0.05% NaN_3 (PBS) containing 0.1% sodium dodecyl sulfate (SDS) and then twice against 1-liter volumes of 0.01% SDS prepared with Baker HPLC reagent water (J. T. Baker Chemical Co., Phillipsburg, NJ) at 25°C over a 24-hr period. The analyses were done with samples containing 1-3 μg protein. Contaminating amino acids were monitored by analyzing duplicate unhydrolyzed samples. The quantity of each residue in the unhydrolyzed sample was less than 5% of that found in the hydrolyzed sample for all the amino acids with the exception of Ser and Gly, which were less than 10 percent. These values were subtracted from the values observed with the hydrolyzed samples. Proline was measured by gas chromatography as described,[19] except that trifluoroacetic anhydride was used rather than pentafluoropropionic anhydride to form the derivative. Cysteic acid and methionine sulfone were measured after performic acid oxidation.[20-21] Tryptophan was determined after hydrolysis in 6 N HCl/4% (vol/vol) thioglycolic acid.[22]

Immunoabsorbant Affinity Chromatography

Sheep anti-lymphoblastoid IF was prepared, purified, and coupled to Sepharose 4B as described.[16] Columns were successively eluted with PBS, McIlvaine's citrate/phosphate buffer, pH 5.0, McIlvaine's citrate/phosphate buffer, pH 2.4-2.6, PBS, PBS containing 4M Guanidine · HCl and PBS, respectively.[16] Components of lymphoblastoid IF, antigenically identical to human leukocyte IF, were eluted by the pH 2.6 buffer and were stored at −70°C until a sufficient quantity ($2-5 \times 10^8$ units) was accumulated for the next purification step.

Sephadex G-150 Chromatography

The pH 2.6 eluates from the immunoabsorbant affinity columns were thawed and concentrated on a Pellicon cassette ultrafiltration system (Millipore) PT series, (Millipore, Bedford, MA) to a volume of 75-100 ml.[16] The concentrate was adjusted to pH 3.5 with 1N NaOH and applied to a Sephadex G-150 column (46.5

cm in height, 6200 ml in volume) and developed with McIlvaine's citrate/phosphate buffer pH 3.5 containing 0.05% NaN_3 at 4-6°C. Interferon was eluted, at a flow rate of 60 ml/hr, between the effluent volumes 3800-5000 ml.

SP-Sephadex Chromatography and Treatment of Interferon with Glycosidase Mixture

The Sephadex G-150 fractions containing the major antiviral activity peak were concentrated by ultrafiltration to 300-450 ml and applied to an SP-Sephadex C-25 column (1.5 cm in diameter, 5 cm in height) and equilibrated with McIlvaine's citrate/phosphate buffer pH 3.5. The column was developed with a step pH gradient at a flow rate of 1.3 ml/min as previously described.[16] An average of 90% of the recovered IF was eluted at pH 5.5. This fraction was adjusted to pH 6.0 with 1N NaOH and incubated at 37°C for 4-5 hr with a mixture of glycosidases from *Streptococcus pneumoniae*, Type 1, prepared by S. Bose,[23] which was shown to remove most of the carbohydrate moiety of the leukocyte IF.[23]

L-Tryptophyl-L-Tryptophan-Affi-Gel 10 Chromatography

After the glycosidase mixture treatment, the IF preparation was applied to an L-tryptophyl-L-tryptophan-Affi-Gel 10 column (11.3 cm in height, 22.7 ml in volume) at a flow rate of 1.3 ml/min. It was developed with PBS, PBS containing 1.0 M NaCl, and then PBS containing 1.0 M NaCl and 75% (vol/vol) ethylene glycol as described.[16] Interferon was eluted with the buffer containing 75% ethylene glycol and collected in an equal volume of 0.1% SDS. The eluate was then dialyzed three times against 6-liter volumes of 0.05% SDS over a 24-hr period at 23°C.

Preparative and Analytical SDS Polyacrylamide Gel Electrophoresis

After dialysis, the ethylene glycol eluate was concentrated by ultrafiltration to 0.5-1.0 ml.[16] The samples were adjusted to 0.05 M Tris HCl pH 6.8, 10% (vol/vol) glycerol, 1.0% SDS, and 0.002% bromophenol blue, and incubated 2-3 hr at 25°C. Polyacrylamide gel electrophoresis was performed using a modification of the Laemmli system.[24-25] Preparative gels were 1.5 mm in thickness and 24-27 cm in length. Analytical gels were 0.75 mm in thickness and 9 cm in length. A 3.7% acrylamide stacking gel and a 16% acrylamide separating gel were used in both preparative and analytical procedures. Details of the electrophoresis, staining and destaining of the slab gels, and excision and elution of the IF components from the gels have been reported.[16]

Amino-terminal Sequence Determination

Samples to be sequenced were dialyzed twice against 6-liter volumes of PBS containing 0.1% SDS and then three times against 1-liter volumes of 0.01% SDS prepared with Baker HPLC reagent water at 25°C. The SDS was purchased from

Bio-Rad Laboratories, Inc., Richmond, CA. The amino-terminal sequence was primarily determined using a Caltech spinning cup sequencer, the nonprotein carrier Polybrene, and slight modification of the Edman Quadrol program as previously described.[15] A portion of the amino-terminal sequence was also obtained using a LKB solid phase sequencer as described by Bridgen.[26] The amino acid phenylthiohydantoins (PTHs) from the spinning cup sequencer were identified by high-performance liquid chromatography using a Du Pont Zorbax CN column developed by gradient elution employing methanol/acetonitrile (17:3) in sodium acetate buffer, pH 5.4.[27] Radioactive PTHs from the solid-phase sequencer were identified by adding a mixture of unlabeled standards, running on HPLC on a Zorbax ODS column, and collecting and counting each peak.

TABLE 1

PURIFICATION OF HUMAN LYMPHOBLASTOID INTERFERON

Step	Interferon Units $\times 10^{-6}$	Protein (mg)	Specific Activity (interferon units $\times 10^{-6}$/mg protein)	Cumulative Recovery (%)
Trichloroacetic Acid precipitation	800			
Sephadex G-25	690	334,756	.0021	86
Anti-lymphoblastoid interferon-Sepharose 4B	640	878	.73	80
Storage at −70°C	400			50
Ultrafiltration	220	700		28
Sephadex G-150	190	61.9	3.1	24
Ultrafiltration	140			18
SP-Sephadex*	110	31.9	3.4	14
Glycosidase treatment	101	32.3	3.1	13
L-tryptophyl-L-tryptophan Affi-gel 10	83	7.6	11	10
Dialysis and ultrafiltration	48			6
SDS PAGE				
Total†	48			6
A	5	.065	77	0.6
B	23	.104	220	3
C	9	.101	89	1

*pH 5.5 eluate only.
†The total quantity of interferon eluted from the gels.

Solvent A was 21mM sodium acetate pH 4.5, solvent B was acetonitrile. A step gradient of 19% B for 4 min followed by 34% B at a flow rate of 2 ml/min was used.

RESULTS

A summary of the purification of human lymphoblastoid interferon is shown in TABLE 1. As previously described, the IF present in the redissolved trichloroa-

cetic acid precipitate was chromatographed on Sephadex G-25 to remove the trichloroacetate ions and to equilibrate the sample in PBS prior to immunoabsorbant affinity chromatography.[11] The eluate of the Sephadex G-25 column containing 35.2–88.2 g protein and 0.5–2 × 10^8 units of IF was then applied to a sheep anti-lymphoblastoid interferon-Sepharose 4B column. In this preparation an average of 93% of the antiviral activity was eluted by McIlvaine's citrate/phosphate buffer pH 2.6. This step yielded the largest purification, a 350-fold increase in the specific activity over the Sephadex G-25 eluate. The pH 2.6 fractions were then stored at −70°C until sufficient IF (1–5 × 10^8 units) was accumulated for the next purification step. As shown in TABLE 1, a 37% loss in the antiviral activity was observed after the pH 2.6 fractions were stored at −70°C for 2 months and thawed. Recent studies in this laboratory have indicated that partially purified IF having protein concentrations below 100 μg/ml lose up to 60% of their antiviral activity after brief storage at 6°C or −70°C in plastic bottles and tubes. Collection in siliconized glass bottles and concentration of the dilute protein solutions by ultrafiltration prior to storage have reduced losses. The next step in the purification is the concentration of the partially purified IF by the Pellicon cassette ultrafiltration system. In this preparation, 55% of the antiviral activity was recovered in the concentrate; less than 1% was found in the filtrate. Two previous preparations did not exhibit this loss (i.e. 44% of the antiviral activity); 90% of the antiviral activity was recovered in the concentrates. Gel filtration on Sephadex G-150 followed. Approximately 5% of the antiviral activity eluted at the void volume and 86% eluted between 3800–5000 ml. The major peak of antiviral activity obtained from Sephadex G-150 column was then purified by an SP-Sephadex column. Approximately 90% of the applied IF was eluted with pH 5.5 buffer and 10% with pH 7.0 buffer. Although the increase in specific activity of the IF preparation appears slight, as shown in TABLE 1, analysis by analytical SDS gel electrophoresis showed that several polypeptide species migrating in the region of the antiviral activity were removed by this step. The pH 5.5 eluate was then incubated with a mixture of glycosidases, which removes a major portion of the carbohydrate moiety.[23] Examination of the glycosidase preparation by analytical SDS polyacrylamide gel electrophoresis showed that no detectable Coomassie brilliant blue R-250 staining components were present in the region of the antiviral activity.

Amino acids and dipeptides bound to solid phase supports have been used previously to purify a variety of IFs.[28] The dipeptide, L-tryptophyl-L-tryptophan coupled to Affi-gel 10 was effective in the purification of human lymphoblastoid IF. A 75% (vol/vol) ethylene glycol solution was required to elute the IF. Extended exposure of lymphoblastoid IF to this high concentration of ethylene glycol resulted in large losses of antiviral activity. These losses were virtually eliminated by collecting the eluate in an equal volume of 0.1% SDS. This procedure immediately reduced the concentration of ethylene glycol and added SDS which stabilizes the interferon. This stabilization by low concentration of SDS has been shown with several other IFs.[6,29,30] FIGURE 1 shows a typical preparative SDS gel. The Coomassie brilliant blue R-250-stained components, A and B, with the apparent molecular weights of 21,500 and 18,500 respectively, comigrated with the peaks of antiviral activities recovered from the gel. As shown in TABLE 1 and FIGURE 1, B is the major IF species. A third component migrating slightly faster than the major 18,500 dalton species (C) is also observed and appears as a shoulder on B. Recovery of the IF activity from the stained gels ranges from 50 to 100 percent. The B component was then analyzed by analytical

gel electrophoresis as shown in FIGURE 2. Only one Coomassie brilliant blue R-250-stained polypeptide was detected. The amino acid composition of B is shown in TABLE 2. The combination of Leu, Ile, Val, Ala, and Phe accounted for 31% of the amino acid residues; Leu alone was present at a level of 11%. The high content of these residues is consistent with the hydrophobicity of IF. Approximately 12% of the molecule consisted of the basic amino acids, Arg and

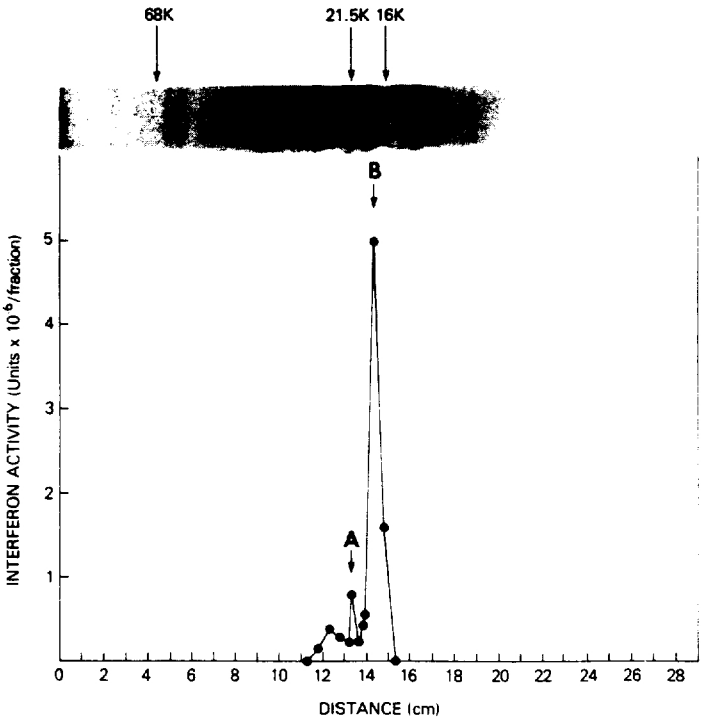

FIGURE 1. Preparative SDS polyacrylamide gel electrophoresis of human lymphoblastoid interferon (IF). Partially purified IF (1.9×10^7 units, 1.2 mg protein) was applied to the gel. Total IF recovered was 9.7×10^6 units; 8×10^5 units comigrated with the 21,500 dalton species (A); 5.0×10^6 units comigrated with the 18,500 dalton species (B); 1.6×10^6 units comigrated with C (defined in text). The Coomassie brilliant blue R-250 polypeptide pattern shown at the top of the figure represents one lane of the preparative gel. In this experiment the total recovery of antiviral activity was 51 percent.

Lys. The value obtained for met, shown in TABLE 2, represents a minimum value because of losses incurred during the oxidation.[21]

The amino acid sequence of the amino-terminal 20 residues of B is presented in FIGURE 3. Multiple analyses (6 determinations) using the spinning cup sequencer on three independent preparations of interferon (20-500 pmol protein) yielded the sequence shown in FIGURE 3. A portion of the amino-terminal

sequence, to residue 10, was obtained with the solid-phase sequencer (500 pmol protein, one determination). These results were in essential agreement with those obtained with the spinning cup sequencer. A second sequence (approximately 10%) was found in an earlier preparation of B. Fraction C had equal amounts of both sequences and one-half the specific activity of B (TABLE 1), indicating that the second sequence is probably a contaminant. Improved fractionation of B from the preparative SDS polyacrylamide gels essentially eliminated this sequence. Two recent preparations have yielded the sequence shown in FIGURE 3 with undetectable quantities (<3%) of the contaminant.

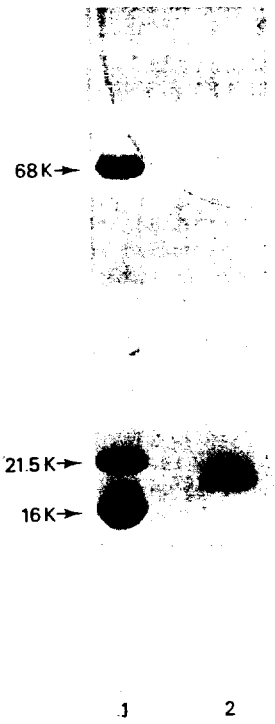

FIGURE 2. Analytical SDS polyacrylamide gel electrophoresis of the purified 18,500 dalton component of human lymphoblastoid interferon. *Lane 1*, molecular weight standards: 2.5 μg bovine serum albumin (68,000) and 5.0 μg each of soybean trypsin inhibitor (21,500) and myoglobin (16,000). *Lane 2*, 2.2 μg B from the preparative SDS polyacrylamide gel shown in FIGURE 1.

DISCUSSION

A human lymphoblastoid interferon with an apparent molecular weight of 18,500[8] has been purified to homogeneity and has a specific activity of 2.2–2.5 × 10^8 units/mg protein. Major advances in the large scale production of lymphoblastoid IF[10-12] coupled with improvements in amino acid analysis[13,14] and microsequencing[15] have permitted the purification and initial characterization of this IF species.

A preparation of IF from uninduced Namalwa cells,which produce low levels of IF constitutively,[16,31] was purified by the steps described in TABLE 1. Analysis of

TABLE 2

AMINO ACID COMPOSITION OF THE MAJOR 18,500 DALTON COMPONENT OF HUMAN LYMPHOBLASTOID INTERFERON

Amino Acid	Residues*
Asx	14.9
Thr	8.0
Ser	10.7
Glx	27.3
Pro	10.9
Gly	10.7
Ala	11.0
Cys	1.8
Val	7.7
Met	1.2
Ile	6.9
Leu	17.8
Tyr	3.8
Phe	7.1
His	4.4
Lys	10.4
Arg	9.6
Trp	0.6

*The number of amino acid residues is 165, based on the apparent molecular weight of 18,500 determined by SDS polyacrylamide gel electrophoresis.

the ethylene glycol eluate from the L-tryptophyl-L-tryptophan-Affi-gel 10 column by SDS polyacrylamide gel electrophoresis showed A and B in greatly diminished amounts.[16] This provides additional evidence that we are indeed purifying proteins produced during the induction of IF.

The amino acid composition of B showed an abundance of hydrophobic amino acid residues. Similarly, several human[4,7] and mouse[32] interferons have been characterized as being enriched in hydrophobic amino acids by amino acid analysis. In addition, 8 of the first 20 amino acid residues of B consisted of Leu, Ile, and Ala, and contribute a very hydrophobic character to the amino-terminal portion of the IF molecule.

Preliminary sequencing of the amino-terminus of B to approximately residue 40 is currently being reexamined using larger quantities (0.5–2 nmol) of protein. Fragment production using cyanogen bromide and trypsin, and subsequent peptide purification and sequence determination are in progress.

Amino-terminal sequencing studies of A and C yielded one sequence similar to B (the major 18,500 dalton component) plus a second sequence (preliminary

Ser[1]-Asp-Leu-Pro-Gln[5]-Thr-His-Ser-Leu-Gly[10]-
Asn-Arg-Arg-Ala-Leu[15]-Ile-Leu-Leu-Ala-Gln[20]

FIGURE 3. The sequence of the 20 amino-terminal residues of the 18,500 dalton component of human lymphoblastoid interferon.

amino-terminal sequence data are available on the second protein). It is of interest that the second amino-terminal sequence observed in A appears to be the same as that observed in C. At this point it is not clear whether this second protein is a modification of B or if it is a contaminant of the IF preparation. The observation that the specific activities of A and C are approximately twofold less than that of B and that both A and C contain 25-50% of the second protein suggests that the second sequence is a contaminant. Purification of this other protein and the determination of its antiviral activity should distinguish between these two possibilities.

REFERENCES

1. BARON, S. & F. DIANZANI, Ed. 1977. Tex. Rep. Biol. Med. **35:** 394-541.
2. KAWAKITA, M., B. CABRER, H. TAIRA, M. REBELLO, E. SLATTERY, H. WEIDELI & P. LENGYEL. 1978. J. Biol. Chem. **253:** 598-602.
3. IWAKURA, Y., S. YONEHARA & Y. KAWADE. 1978. J. Biol. Chem. **253:** 5074-5079.
4. RUBENSTEIN, M., S. RUBINSTEIN, P. FAMILLETTI, R. MILLER, A. WALDMAN & S. PESTKA. 1979. Proc. Nat. Acad. Sci. USA **76:** 640-644.
5. LIN, L. S. & W. E. STEWART II. 1978. Fed. Proc. Fed. Am. Soc. Exp. Biol. **37:** 1441.
6. KNIGHT, E., JR. 1976. Proc. Nat. Acad. Sci. USA **73:** 520-523.
7. TAN, Y. H., F. BARAKAT, W. BERTHOLD, H. SMITH-JOHANNSEN & C. TAN. 1979. **254:** 8067-8073.
8. CANTELL, K. & S. HIRVONEN. 1978. J. Gen. Virol. **39:** 541-543.
9. EDY, V. G. 1977. Tex. Rep. Biol. Med. **35:** 132-137.
10. ZOON, K. C., C. E. BUCKLER, P. J. BRIDGEN & D. GURARI-ROTMAN. 1978. J. Clin. Microbiol. **7:** 44-51.
11. BRIDGEN, P. J., C. B. ANFINSEN, L. CORLEY, S. BOSE, K. C. ZOON, U. T. RÜEGG & C. E. BUCKLER. 1977. J. Biol. Chem. **252:** 6585-6587.
12. KLEIN, F., R. T. RICKETTS, W. I. JONES, I. A. DE ARMON, M. J. TEMPLE, K. C. ZOON & P. J. BRIDGEN. 1979. Antimicrob. Agents Chemother. **15:** 420-427.
13. HARE, P. E. 1977. Methods. Enzymol. **47:** 3-18.
14. BENSON, J. R. & P. E. HARE. 1975. Proc. Nat. Acad. Sci. USA **72:** 619-622.
15. HUNKAPILLER, M. W. & L. E. HOOD. 1978. Biochemistry **17:** 2124-2133.
16. ZOON, K. C., M. E. SMITH, P. J. BRIDGEN, D. ZUR NEDDEN & C. B. ANFINSEN. 1979. Proc. Nat. Acad. Sci. USA **76:** 5601-5605.
17. ARMSTRONG, J. A. 1971. Appl. Microbiol. **21:** 723-725.
18. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL. 1951. J. Biol. Chem. **193:** 265-275.
19. FRANK, H., G. J. NICHOLSON & E. BAYER. 1977. J. Chromatogr. Sci. **15:** 174-176.
20. HIRS, C. H. W. 1967. Methods. Enzymol. **11:** 197-199.
21. MOORE, S. 1963. J. Biol. Chem. **238:** 235-237.
22. MATSUBARA, H. & R. M. SASAKI. 1969. Biochem. Biophys. Res. Commun. **35:** 175-181.
23. BOSE, S., D. GURARI-ROTMAN, U. T. RÜEGG, L. CORLEY & C. B. ANFINSEN. 1976. J. Biol. Chem. **251:** 1659-1662.
24. LAEMMLI, U. K. 1970. Nature (London) **227:** 680-685.
25. WEINTRAUB, H., K. PALTER & F. VAN LENTE. 1975. Cell **6:** 85-110.
26. BRIDGEN, J. 1976. Biochemistry. **15:** 3600-3604.
27. JOHNSON, N. D., M. W. HUNKAPILLER & L. E. HOOD. 1979. Anal. Biochem. **100:** 335-338.
28. SULKOWSKI, E., M. W. DAVEY & W. A. CARTER. 1976. J. Biol. Chem. **251:** 5381-5385.
29. TÖRMA, E. T. & K. PAUCKER. 1976. J. Biol. Chem. **251:** 4810-4186.
30. STEWART, W. E., II, P. DESOMER, V. G. EDY, K. PAUCKER, K. BERG & C. A. OGBURN. 1975. J. Gen. Virol. **26:** 327-331.
31. ADAMS, A., B. LIDIN, H. STRANDER & K. CANTELL. 1975. J. Gen. Virol. **28:** 219-223.
32. CABRER, B., H. TAIRA, R. J. BROEZE, T. D. KEMPE, K. WILLIAMS, E. SLATTERY, W. H. KONIGSBERG & P. LENGYEL. 1979. J. Biol. Chem. **254:** 3681-3684.