

UNCLASSIFIED

AD NUMBER

ADB134571

LIMITATION CHANGES

TO:

Approved for public release; distribution is unlimited.

FROM:

Distribution authorized to U.S. Gov't. agencies only; Premature Dissemination; 01 APR 1989. Other requests shall be referred to Army Medical Research and Development Command, Attn: SGRD-RMI-S, Fort Detrick, Frederick, MD 21701-5012.

AUTHORITY

USAMRDC dtd 5 Aug 1994

THIS PAGE IS UNCLASSIFIED



AD NUMBER

AD-B134 571

LIMITATION CHANGES

TO

Approved for Public Release;
Distribution Unlimited.

- Distr Stmt: A
- Limitation Code: 1

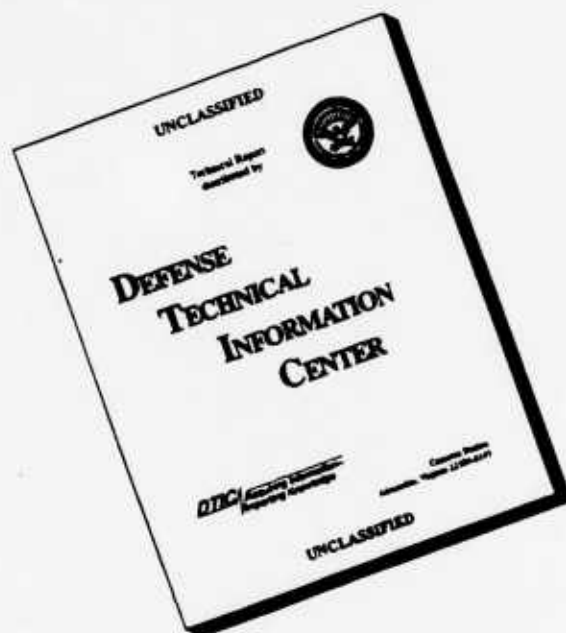
FROM

N/A

AUTHORITY

Lt Col C.O. Laverett, SGRD-RMI-S(70-1y), DCS/Information Mgt,
Ft Detrick, MD., via Memo dtd 5 Aug 94...

DISCLAIMER NOTICE



**THIS DOCUMENT IS BEST
QUALITY AVAILABLE. THE
COPY FURNISHED TO DTIC
CONTAINED A SIGNIFICANT
NUMBER OF PAGES WHICH DO
NOT REPRODUCE LEGIBLY.**

AD-B134 571

AD _____

INTERFERON INDUCERS AGAINST INFECTIOUS DISEASES

ANNUAL REPORT

JAKE BELLO AND JUDITH O'MALLEY

APRIL 1, 1989

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-87-C-7111

Roswell Park Memorial Institute
Buffalo, New York 14263

DTIC
ELECTE
JUL 25 1989
S B D
CL

Distribution authorized to U.S. Government agencies only;
premature dissemination, 1 April 1989. Other requests shall
be referred to Commander, US Army Medical Research and
Development Command, Attn: SGRD-RMI-S, Fort Detrick,
Frederick, Maryland 21701-5012.

The findings in this report are not to be construed as an
official Department of the Army position unless so designated
by other authorized documents.

89 7 25 039

REPORT DOCUMENTATION PAGE

Form Approved
OAH No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified		1b. RESTRICTIVE MARKINGS	
2a. SECURITY CLASSIFICATION AUTHORITY		3. DISTRIBUTION/AVAILABILITY OF REPORT Distribution authorized to U.S. Government agencies only; premature dissemination, 1 April 1989.	
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE		5. MONITORING ORGANIZATION REPORT NUMBER(S)	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)		7a. NAME OF MONITORING ORGANIZATION	
6a. NAME OF PERFORMING ORGANIZATION Roswell Park Memorial Institute	6b. OFFICE SYMBOL (If applicable)	7b. ADDRESS (City, State, and ZIP Code)	
6c. ADDRESS (City, State, and ZIP Code) Buffalo, New York 14263	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-87-C-7111		
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command	8b. OFFICE SYMBOL (If applicable) SGRD-RMI-S	10. SOURCE OF FUNDING NUMBERS	
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21701-5012	PROGRAM ELEMENT NO. 62770A	PROJECT NO. 3M1-6270A871	WORK UNIT ACCESSION NO. 394
11. TITLE (Include Security Classification) (U) Interferon Inducers Against Infectious Diseases			
12. PERSONAL AUTHOR(S) Jake Bello and Judith O'Malley			
13a. TYPE OF REPORT Annual	13b. TIME COVERED FROM 3/23/88 TO 3/22/89	14. DATE OF REPORT (Year, Month, Day) 1989 April 1	15. PAGE COUNT 48
16. SUPPLEMENTARY NOTATION			
17. COSATI CODES		18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)	
FIELD	GROUP	SUB-GROUP	
06	01		
06	13		
19. ABSTRACT (Continue on reverse if necessary and identify by block number) We have been able to formulate effective IFN inducers without CMC. Most of these inducers are less toxic than is ICLC, and some are about as toxic as IC itself. Therefore, the other components are essentially non-toxic. Preliminary data showed that one formulation may be less toxic than is IC. All but one of the formulations so far tested for immunogenicity have given negative results; the one exception is to be examined further. We have a number of formulations which are effective IFN inducers, and some which are effective against Rift Valley Fever virus in mice. Some show both activities. cont. on back of pg.			
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT ☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTC USERS		21. ABSTRACT SECURITY CLASSIFICATION Unclassified	
22a. NAME OF RESPONSIBLE INDIVIDUAL Mary Frances Bostian		22b. TELEPHONE (Include Area Code) 301-663-7325	22c. OFFICE SYMBOL SGRD-RMI-S

20. ABSTRACT, cont.

The effective IFN inducers are:

ICL-CM-dextran

ICL-CM-amylose

ICL-SO₂-Gel

ICL-Gel

ICL-CM-cyclodextrin (IFN titer apparently dependent on slow arrangement of structure)

IC-(PLL-dextran) (weaker than the first four)

The effective anti-RVSV agents are:

ICL-CM-amylose

ICL-Gel

IC-(PLL-dextran)

ICL-SO₂-Gel (moderate)

Exploratory chromatographic data suggest that: 1) ICLC, as made by the standard procedure, may contain excess CMC; 2) chromatography may be useful to separate components which may have different biological properties; 3) dissociation of inducer complexes may affect biological properties.

Results of melting profile experiments with ICL-Gel suggest that changes in the order of mixing components may alter the nature of the inducer complexes. And work with ICL-CM-cyclodextrin suggests that aging of a complex may improve it.

Light scattering melting profiles suggest a correlation between IFN induction and increased polarization of scattering above T_m, and an inverse correlation between IFN induction and the magnitude of polarization at room temperature.

Foreword.

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).



Accession For	
NTIS GRA&I	☐
DTIC TAB	☒
Unannounced	☐
Justification	
By	
Distribution/	
Availability Codes	
Dist	Avail and/or Special
B-3	

Table of Contents

Foreword.....	1
1. Problem Under Study.....	3
2. Background.....	3
3. Rationale of the Research.....	3
4. Experimental.....	6
5. Results.....	12
6. Conclusions.....	13
7. Bibliography.....	44
8. Glossary.....	45

Tables

1. Table 1. IFN Induction by ICL-CMdextran.....	14
2. Table 2. IFN Induction in Mice by ICL-C(open)....	16
3. Table 3. IFN Induction by ICL-CMamylose.....	18
4. Table 4. IFN Induction by ICL-SO ₄ Gel, and ICL-Gel.....	20
5. Table 5. IFN Induction in Mice by IC-(PLL-Dextran).....	23
6. Table 6. Polarization of Scattered Light.....	35
7. Table 7. Substances Tested Immunologically.....	37
8. Table 8. Substances Against Which the Various Sera Were Tested.....	38
9. Table 9. LD ₅₀ Values of Inducer Formulations....	39
10. Table 10. Toxicity of Some Inducers.....	40
11. Table 11. Antiviral (Rift Valley Fever) Action in Mice.....	42

Figures

1. Figure 1. Molecular sieve chromatography of poly I·poly C on Bio-gel A5M, elution with standard saline.....	28
2. Figure 2. Molecular sieve chromatography of poly I·poly C·poly(L-lysine) with fluorescent label on poly(L-lysine)....	29
3. Figure 3. Molecular sieve chromatography of ICLC with fluorescent label on poly(L-lysine).....	30
4. Figure 4. Molecular sieve chromatography of ICLC with fluorescent label on CMC.....	31
5. Figure 5. Molecular sieve chromatography of ICL-SO ₄ Gel with fluorescent label on gelatin.....	32
6. Figure 6. Molecular sieve chromatography of ICL-CMD with fluorescent label on poly(L-lysine).....	33

1. Problem Under Study.

The double-stranded synthetic polynucleotide poly I·poly C (i.e., poly-inosinic-poly-cytidylic acid, also to be called poly IC or IC) is an interferon (IFN) inducer. In primates it is not effective, presumably because of circulating nucleases which quickly degrade it. When complexed with cationic poly(L-lysine), PLL, and anionic carboxymethylcellulose, CMC, it is effective in humans. But there are reservations about CMC. It may not be excreted or metabolized, and it is suspected as a carcinogen (1).

The problem being addressed in this research is the preparation of effective, safe IFN inducers devoid of CMC. In addition to seeking formulations which are effective IFN inducers, we are also seeking formulations which are effective antiviral agents. Substances which are effective antiviral agents may not necessarily be effective IFN inducers, and vice versa. The formulations should be less toxic than ICLC, non-immunogenic, and metabolizable or excretable. To these ends our main efforts have been to develop anionic polymers to replace CMC. We are also seeking to replace CMC by modifying the PLL with engrafted polysaccharides.

2. Background.

Our expectation that replacements for CMC can be found is based on earlier work from this laboratory (2). We found that a complex of ICL, PLL and carboxymethyl dextran (CMdextran) could be prepared (called ICL-CMdextran), which was as effective an inducer in mice as ICLC, and of lower toxicity than ICLC. ICL-CMdextran was also as effective an IFN inducer in rhesus monkeys as ICLC.

3. Rationale of the Research.

Since CMC has undesirable features, including non-excretion, non-metabolization, and possible carcinogenicity, we are seeking formulations without CMC. We are exploring two approaches to this goal. One is the replacement of CMC with other anionic biopolymers, selected on the basis of known or expected safety, and being excretable or metabolizable. Most of the CMC replacements were selected on the basis of a history of safe use as blood volume expanders, or being closely related to such. These include gelatin, anionically-modified gelatin, carboxymethyl polysaccharides and carboxymethyl cyclodextrin. The second approach is the use of poly(L-lysine), PLL, covalently grafted to a saccharide (without anionic groups). The PLL would bind to the poly IC, through its cationic groups, while the grafted saccharide would provide a solubilizing and hydrating effect. PLL-dextran grafts are being studied. Another reason for studying these graft polymers is that dextran is more readily cleared from the circulation of dogs than is CMdextran (3). We would

graft saccharide to only a fraction of the PLL residues. It may be expected that a graft polymer will be cleaved by trypsin-like enzymes and the fragments produced will be dextran bearing terminal oligolysine.

A. Chemical Studies: CMC Replacements.

1. Carboxymethyl Polysaccharides as CMC Replacements

Carboxymethyl polysaccharides are anionic polymers in which the ionized carboxymethyl group has been introduced:



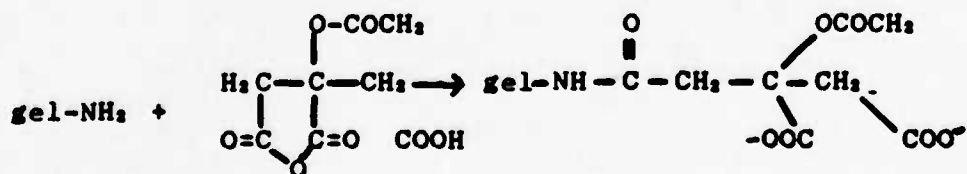
The carboxymethyl polysaccharides investigated as CMC replacements are the following:

a. CMdextran. During the first year we chiefly examined CMdextran of 10 kDa molecular weight, and with a degree of substitution (DS) of about 0.5 (i.e., 0.5 CM group per average glucose residue). During the second year we also studied CMdextran of 40 kDa, and with DS from 0.2 to 2. (The maximum possible DS = 3). The work of Chang et al. (3) showed that CMdextran is about 50-60% cleared from the circulation in dogs in about 1/2 hour, with no difference as to molecular weight of the CMdextran. We shall also examine higher molecular weights, as these appear to provide more readily soluble formulations (2).

b. CMamylose. This was selected in expectation of low toxicity, and because of a report from China (4) (on its use as a blood volume expander) that it is non-immunogenic and is rapidly cleared from the body. In the work done so far, molecular weight has not been a concern. If results warrant it, we shall study the use of different molecular weights.

2. Anionically Modified Gelatin

Acetylcitryl gelatin. This is a modified gelatin made by reaction of amino groups of gelatin with acetylcitric anhydride in water.



(and/or isomer with gelatin on the middle carboxyl)

The product is purified by dialysis, and is freeze-dried for storage.

Gelatin and succinyl gelatin have a long history of use as blood volume expanders. AcCitgelatin is closely related to succinyl gelatin, but with twice as many carboxylate groups introduced per amino group acylated. Metabolism of AcCitgel should lead to small fragments which will be excreted or further metabolized to amino acids and to citrate and acetate.

During the first year we extensively examined AcCitgel. While some formulations with effective IFN induction were prepared, solubility problems of the ICL-AcCitgel formulations have blocked progress along this line. A single experiment with succinyl gelatin gave moderate IFN induction; this may be worth further study, since high IFN titers are not required for effective antiviral action.

During the second year of the contract, we have extensively investigated inducer formulations containing sulfated gelatin. This derivative is prepared by reacting gelatin with sulfuric acid or chlorosulfonic acid. The numerous hydroxyl groups of gelatin (about 15 moles per 100 residues, or 9300 grams) react as follows:



The reactions are carried out with gelatin in cold sulfuric acid, or with gelatin dissolved in cold trifluoroacetic acid-sulfuric acid, or with chlorosulfonic acid on gelatin in H_2SO_4 .

3. Anionically Modified Serum Albumin (HSA)

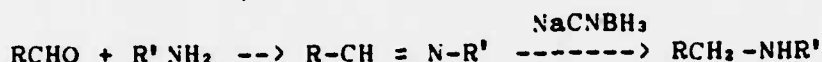
HSA was acylated with acetylcitric anhydride. This introduces a large amount of negative charge since HSA contains about 65 amino groups. The AcCit-HSA was not immunogenic in mice, although the starting HSA was strongly immunogenic. It was difficult to prepare complexes from AcCit-HSA because of solubility difficulty with the modified albumin itself. This has been put aside for the moment.

4. Anionically Modified Poly(L-lysine):

AcCit-PLL. An acylation reaction similar to that shown for the reaction with gelatin, converts the positive ammonium groups of PLL to negative acetylcitryl groups. If AcCitPLL is metabolized, it will be converted to acetic and citric acids and to small oligolysines or to lysine. If not metabolized, it may be excreted, if the molecular weight is not too large. Molecular weights of PLL from about 8 kDa to 55 kDa have been studied.

5. PLL-Saccharide graft.

We are grafting dextran to PLL. The reaction is a reductive alkylation of the amino groups of PLL with the terminal aldehyde groups of dextran. In the presence of sodium cyanoborohydride the intermediate Schiff base is reduced to a stable secondary amine:



Future work might involve polysaccharides other than dextran.

Formulations for biological testing are made from IC and the graft. No polyanion is used (although this would be another route to IFN inducers), and no unmodified PLL is incorporated.

B. Physical Studies.

The experimental inducers are examined by a variety of procedures, to seek predictive correlations with biological action, and, ultimately, to try to understand the physical characteristics which are associated with IFN induction and antiviral action. Melting profiles are routinely measured by spectrophotometry. In some cases the profiles are also measured by fluorescence and/or light scattering. Gel filtration chromatography has been used for initial screening of the possibility of isolating valuable fractions and characterizing the complexes.

4. Experimental

A. Preparation of Inducer Formulations.

a. General Procedure

ICLC was prepared according to Levy et al. (5). Experimental formulations were prepared in the same way, with modification, where necessary, in the ratios of components and/or the strength of saline or buffered saline.

b. Sterilization of Components.

IC was obtained sterile from the vendor (Pharmacia). Solutions of PLL (Sigma), modified PLLs and modified gelatins were filtered through a 0.45 μ filter. The sterile components were mixed under a sterile hood and sealed in sterile serum vials.

B. Carboxymethylation of Polysaccharides.

The carboxymethylation is done by the procedure of Chang et al.(3), with modification. A typical experiment is carried out as follows: To a solution of 4 g of polysaccharide in 35 mL of NaOH solution (15 g of NaOH in 100 mL of water) at 60°C, is added 5.8 g of chloroacetic acid over 4 minutes. After 90 minutes the pH is brought to about 4.8 with glacial acetic acid, and dialyzed against water and freeze-dried. The dialysis procedure is used for CMamylose.

An alternative purification procedure for CMdextran (following Chang et al.) was to bring the reaction mixture to pH 4.5, followed by precipitation with 100 mL methanol, decantation of methanol, redissolution in water followed by a second and a third precipitation, centrifugation, dissolution in water and freeze-drying. The dialysis procedure appears not to be as satisfactory for CMdextran as for CMamylose.

C. Preparation of Carboxymethyl- β -Cyclodextrin.

This reaction was carried out by a modification of the foregoing carboxymethylation procedure. To 2.2 g of β -cyclodextrin in 5 mL water was added 2.34 g of sodium chloroacetate. NaOH (10 M) was added in small portions over 2 hr., until 2 mL had been added. After standing overnight at room temperature, the solution was heated at 50° for 4 hours. Methanol, 20 mL, was added to precipitate the CM- β -cyclodextrin, followed by filtration and washing with 30 mL methanol, and vacuum-drying. The product was dissolved in 2 mL H₂O and passed through a column of Sephadex G-10, to separate it from NaCl and sodium glycolate (byproducts of the reaction). The fractions were tested for CM-cyclodextrin and cyclodextrin with iodine. These gave brown and purplish spots, respectively, on paper with iodine. NaCl-containing fractions were detected with AgNO₃.

D. Quality of Poly(L-lysine) Lots.

The poly(L-lysine), PLL, used must meet several criteria for suitability: absence of residual carbobenzyloxy (CBZ) protecting groups, absence of excessive light scattering, adequate molecular weight and, in general, having the characteristic conformational properties of PLL. Every batch of PLL is tested by spectrophotometry and circular dichroism. The substantial absence of residual CBZ groups is demonstrated from the absorption spectrum in the benzene range, 280-250 nm, using a concentration of 5 or 10 mg/mL, which permits detection of one CBZ group per 1000 lysine residues. CBZ below about 2 per 1000 is considered satisfactory. The same spectrum also gives information on the presence of light scattering, which would be manifested as a rising slope at 350-300 nm. Light scattering would be evidence of aggregation

or cross-linking. The spectrum also gives notice of the presence of other absorbing impurities.

We also measure the circular dichroism, CD, of PLL in 1 M NaClO₄ at 0°. When the molecular weight is sufficiently high, about 30,000, the CD spectrum will show 100% α -helix. Less than 100% helix indicates a substantial fraction of low molecular weight, or of other factors interfering with helix formation (e.g., chemical damage, presence of helix-breaking amino acids in the chain). PLL of molecular weight below 30,000 does not give 100% helix; and the lower the molecular weight the lower the helix content. Suitability of low molecular weight PLL is judged by conformity to calibration curves which have been amassed over some years.

Preparation of Acetylcitryl PLL.

Acetylcitric anhydride was made by the procedure of Klingemann (6), by reacting acetyl chloride and citric acid.

To 0.4 g of PLL in 30 ml water maintained at pH 9, was added 1.9 g of acetylcitric anhydride. After 20 minutes the solution was dialyzed against water and freeze-dried.

F. Titration of carboxymethyl groups.

The sample is dried in vacuo over silica gel. A portion of about 50 mg is dissolved in 50 mL of water. For the titration of starting polysaccharide 100-150 mg is dissolved in 50 mL water. A volume of 15 mL is made 0.1 M in KCl with 2 M KCl, and titrated with 0.1 M HCl to about pH 2.25. A blank of the same volume of 0.1 M KCl is titrated, and a difference titration curve is drawn. Below pH 2.4 the difference curve is constant on the volume axis, indicating that the titration is complete.

G. PLL-Dextran Graft.

To 0.06 g of PLL-HBr (M_r 6000) in 5 mL H₂O was added 1 g of dextran T-10 and 0.063 g (1 mmole) NaCNBH₃, at room temperature. After two weeks stirring the reaction mixture was dialyzed against water and freeze-dried to yield 0.69 g of product. The crude material is purified on a 0.7 X 40 cm column of AG-500-X2 (protonated form). Unreacted dextran is eluted first with 200 mL H₂O, and the PLL-dextran graft is eluted with 400 mL 3 N NH₄OH, followed by evaporation to a small volume and freeze-drying (yield 0.2 g). Then the product is put through Sephadex G-100, eluting with 0.2 M NaCl. Four peaks are obtained, representing unreacted PLL and three products. The latter three are not well resolved, as expected for a series of species of varying degrees of substitution.

H. Ring-opening of CMC.

To 2 g of CMC in 0.1 M acetate buffer, pH 5, was added NaIO_4 (1 mole per two glucose residues or 1 mole per 10 glucose residues) at room temperature. After standing overnight in the dark, the pH was brought to 8, and a fourfold ratio of NaBH_4 was added (based on NaIO_4). After 4 hr., 4 mL of ethylene glycol was added to consume excess NaBH_4 and the solution was dialyzed against 0.1 M acetic acid, followed by extensive dialysis against water.

I. Fluorescent-labelled Polymers.

PLL and SO_4 gelatin were labelled by reaction with dansyl chloride, followed by dialysis and freeze-drying. About 1-2 dansyl groups were introduced per 100 lysine residues. CMC was labelled by first reacting CMC with NaIO_4 to oxidize one glucose residue in 80 to a dialdehyde, then reacting with fluorescein thiosemicarbazide, followed by dialysis and gel filtration on Sephadex G-75.

J. Melting Profiles.

Inducer complexes were diluted to contain 50 $\mu\text{g/mL}$ of IC. Absorption spectra were recorded at room temperature from 325 nm to about 240 nm, using cells of 1 cm optical path and a Cary 219 spectrophotometer. Melting profiles were recorded at 246 nm with the Cary 219 Tm accessory and a Neslab circulating bath driven by a Neslab temperature programmer at 1° per minute.

Fluorescence melting profiles were carried out on complexes containing one component bearing a fluorescent label, dansyl on PLL or SO_4 gelatin, and fluorescein on CMC. Temperature was raised stepwise and fluorescence was measured at each step. Using vertically polarized illumination, the fluorescence intensity was measured with the emitted light passing through a vertically and then a horizontally oriented analyzer.

Light scattering profiles were measured in the same instrument, and usually on the same sample, as for fluorescence. The incident light was vertically oriented, and the scattered light intensity was read after passage through vertical and horizontal analyzers.

Fluorescence and scattered light measurements were made with an Aminco-Bowman spectrophotofluorometer fitted with a photon counter. For fluorescence illumination was at 375 nm for dansyl groups and 450 nm for fluoresceinyl groups. Emitted light was measured at 550 nm, with a 550 nm interference filter to supplement the emission monochromator. Light scattering was done at 550 nm for both illumination and emission. Fluorescence intensities were read with the

analyzer Polaroid vertical and horizontal, and the ratio of intensities was taken. (Strictly, this is not polarization or anisotropy; but it is suitable for our purposes).

K. Chromatography.

Gel filtration chromatography of inducer formulations was done on several types of column packings: Sephadex G200 and G25; Sephacryl S-400; Biogel A, 5 m; Biogel A, 15 m; Biogel A, 150 m; and Fractogel. In some cases fluorescent labels were incorporated into PLL or into the polyanion. Elution was followed by absorbance at 260 nm (for IC), and in some cases also by fluorescence and light scattering.

L. Biological Studies

1. Interferon Induction in Mice.

The interferon inducers were evaluated for interferon production in BALB/c mice. Each inducer was compared to a standard poly ICLC preparation. Twenty gram mice were given a single i.v. injection of inducer containing 10 µg of poly IC. Blood was obtained by orbital bleeding 3 hours after injection. Usually there were 8 treated mice per group plus 2 mice that received placebo. Serum was assayed for interferon.

2. Interferon Assays.

Three-fold dilutions of serum were made in Minimal Essential Medium (MEM) containing 5% fetal bovine serum (FBS). The dilutions were done in 96-well microtiter plates, followed by addition of 20,000 murine L-cells/well. The cultures were incubated for 18-20 hours, the trays inverted to remove the medium, and vesicular stomatitis virus, at a multiplicity of infection of 0.15 plaque-forming units per cell, in MEM containing 2% FCS, was added to the cells. The cultures were incubated until virus controls showed marked cytopathic effects (24-48 hours). The medium was removed and antiviral activity determined by a standard colorimetric procedure which measures uptake of a vital dye, neutral red. Interferon titers are expressed in international reference units based upon standards received from the Research Resources Branch, National Institute of Allergy and Infectious Diseases, NIH.

3. Antiviral Effect.

Formulations were submitted to Dr. Meir Kende, Fort Detrick, for testing against Rift Valley Fever virus in mice.

4. Toxicity.

Method A: Mice were injected with a dose of formulation containing 150 µg of IC on each of four consecutive days. The

percentage surviving was compared with the result of a similar experiment with ICLC.

Method B: LD₅₀ was measured by injecting mice with doses of formulation containing 100, 200, 400, 600, 800 and sometimes 1000 µg of IC and observing the number surviving after 12 days.

5. Testing for Immunogenicity.

Immunization protocol.

Ten groups of 6-8 week old female BALB/c mice, 5-6 per group, were used. The mice in each group were injected subcutaneously with one of the test antigens. The first injection consisted of 100 µg of the material in 0.1 or 0.2 mL of a 1:1 emulsion of Freund's complete adjuvant and the PBS solution of the material. Injections were with 1 mL disposable syringes, and although the emulsion is very viscous 25-gauge needles were successfully used. After 3-week and 6-week periods a similar injection, but using Freund's incomplete adjuvant, was given.

Collection of serum.

About two weeks later the mice were bled by cutting off about 1/2 inch of the tail and letting the blood drip into a 1 1/2 ml plastic centrifuge tube. This method of bleeding consistently produces a better yield, is faster and easier than bleeding from the heart as in the previous report. Furthermore, the mice survive and can be again bled after a few days by removing 1/8-1/4" more of the tail; this can be done a number of times.

The tail-bleeding is facilitated and only really successful if the mice are warmed-up under an infra-red light for 4-5 minutes before bleeding. The whole group of mice is initially warmed-up and kept warm as individual mice are bled. Warming should be to a substantial degree and can be monitored by holding one's hand near the mice and by observing the activity of the mice. It is well to place a paper towel on the cage bottom (no sawdust) since the bare cage bottom may get uncomfortably hot. An individual mouse is removed from the cage by the tail and grasped firmly and gently in a crumpled paper towel with the tail held projecting from the towel by the thumb and forefinger. The tip of the tail (on a firm surface) is cut off with a razor blade and the drops of blood then permitted to fall into the centrifuge tube. With experience, about 15 good sized drops can be collected in less than a minute. The blood flow can be stopped by squeezing the tail for a half minute or so. A little bleeding may follow briefly when the mouse is returned to a clean cage but all of the mice have always survived.

Following over at clotting of the blood, the tubes are centrifuged (Microfuge) for a good recovery of clear serum. As much of the serum as can be withdrawn (along with some red blood cells) using a disposable pipet with a control, not a rubber bulb, is transferred to a second 1 1/2 ml centrifuge tube and this is centrifuged. From this tube a good yield of clear, cell-free serum can be withdrawn from the very small red cell pellet, and stored in the freezer.

Testing sera for antibodies

To determine whether or not the sera contained antibody, a precipitation-in-gel procedure was used. In this procedure, a commercially available apparatus (LKB) permits one to pour an approximately 1.3 mm thick layer of molten 1.5% agar solution in buffer onto labelled 1 inch X 3 inch microscope slides, after which a hexagonal array of 3 mm holes, 8 mm between holes, and around a single central hole, is cut in the agar on each half of the slide.

The 5-6 sera obtained from each of the ten groups of mice were tested against one or more of the injected materials. Thus, 8-10 μ L of each serum from the five individual mice injected with an antigen (say, carboxymethyl dextran) was placed in five individual wells of each of four of the hexagonal arrays on two labelled slides; the center well of each of the four arrays was then filled (8-10 μ L) with one of the four solutions which the serum is to be tested against. Diffusion was allowed to proceed in a closed, moist chamber for 24 hours at room temperature; under these conditions, even the finest precipitin arcs ordinarily appear and little or no increase in the density of the arcs occurs with longer periods of time. In the present case, careful examination of the slides after 24, 48 and 72 hours revealed no precipitin arcs in any experimental case, but one, and in the case of bovine serum albumin. Repetition of the procedure using an agar solution containing 0.5% polyethylene glycol which enhances the precipitin reaction in gel still did not result in any precipitin arcs.

5. Results.

A. Introductory Notes to the Results.

Each type of inducer candidate has three polymeric components, which means that many variations in composition are possible. In addition to the proportions of the components, other variables are molecular weight and degree of modification. In addition, reproducibility must be determined, using a biological assay subject to variability in mouse response. Promising inducers are to be tested sufficiently to provide adequate statistics. The exploration of the possible combinations is limited by our biological testing capacity.

1. IFN Induction vs. Antiviral Action.

We test our experimental agents for their ability to induce IFN in mice. IFN induction is measured by the ability of mouse blood serum to protect cells in culture against vesicular stomatitis virus. Direct protection of mice against a virus is not measured here. Since there is no clear understanding yet of the optimum IFN blood titers, we do not reject agents which induce modest IFN titers. As will be seen, modest IFN titers can accompany effective antiviral action. The latter is measured at Fort Detrick by Dr. Meir Kende, using Rift Valley Fever virus in mice.

2. Tables of IFN Induction.

All inducer tests are done with a single dose containing 10 μ g of IC per mouse. Each IFN titer for an experimental formulation shown is the average of the titers of 7-8 mice, and is compared with the average titer for 7-8 mice obtained with ICLC as the standard.

In the tables of IFN induction the composition of inducers is given in actual mg/mL, for each component (in the order shown), and, where needed, normalized to 2 mg/mL of IC.

B. Summary of Results of Interferon Induction Experiments:

1. Formulations with CMdextran.

During the first year of the contract we prepared and tested a series of ICL-CMdextran formulations, using a variety of proportions of components. The CMdextran was made from dextran of 10 kDa molecular weight, and several procedures were studied for the preparation and purification of CMdextran. Some formulations gave good IFN induction, but many did not.

During the second year we extended these results, using a standardized procedure for isolation and purification of CMdextran. This procedure (Experimental section) had given the best results. We extended the range of CM-substitution (DS = degree of substitution) from 0.2 to 2 CM groups per glucose residue. (Earlier we had used a DS of 0.5), and also used CMdextran of 40 kDa molecular weight. In the new series all formulations, except one, induced substantial titers of IFN, Table 1 shows results for a series ICL-CMdextran formulations with 10 kDa CMdextrans. The titers for all but batch II-278 were good to high, and even II-278, the lowest, gave a titer of 245 units. Two batches were tested for toxicity; and were more toxic than was ICLC.

When CMdextran of 40 kDa molecular weight was used, we also obtained high titers with DS of 0.46 and 1.1. One of these, III-59 with DS = 1.1 was much less toxic than ICLC. III-59 was tested for anti-RVSV effect and gave no protection (Table 11), despite its high IFN titer. We plan to do additional studies to see if 40 kDa or higher CMdextran uniformly provides less toxic agents, and if antiviral action will be obtained.

Table 1. IFN Induction by ICL-CMdextran

Lot Number	Composition	Exptl. Formulation	IFN Titers	
	IC, PLL, CMdextran		ICLC Standard	% of ICLC Standard
10 kDa ^a				
II-238 ^b DS = 0.4	0.5/1.5/5 (2/6/20)	1302	643	202
II-266 DS = 0.22	0.5/0.33/1.25 (2/1.3/.5)	669	1450	46
II-278 DS = 0.22	0.25/0.75/1.25 (2/2.8/20)	245	1450	17
III-45A ^c DS = 2.0	2/1.5/5	1020	1669	61
III-45B DS = 2.0	0.5/0.38/1.25 (2/1.5/5)	496 1247	808 1669	61 75
III-90 DS = 2.0	0.5/0.38/1.25 (2/1.5/5)	618	800	76
40 kDa ^a				
III-59 ^e DS = 0.46	0.5/0.38/1.25 (2/1.5/5)	830 479	1280 808	65 59
III-79 DS = 1.1	2/1.5/5	994 856	1280 808	78 106

*. CMdextran made from 10 kDa dextran.

^b. Toxicity by Method A: 0% survival vs 28% for ICLC.

^c. Toxicity by Method A: 0% survival vs 75% for ICLC.

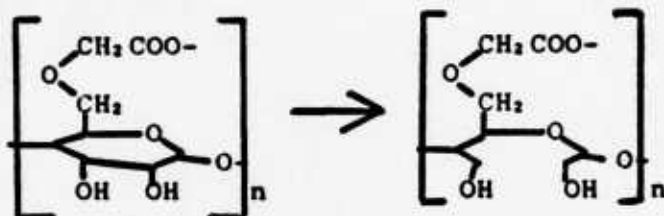
*. CMdextran made from 40 kDa dextran.

^e. Toxicity by Method A: 87% survival vs 0% for ICLC.

2. Ring-Opened CM-Polysaccharides.

A polysaccharide chain is a string of fairly rigid beads on a string, i.e. the cyclic sugar residues linked through the ether linkages. Rotation may occur around C-O bonds of the ether linkages, but not around the ring bonds (except for the limited conformational flexibility permitted to a ring). If greater flexibility were permitted perhaps improved properties would result. We have begun to study this possibility, beginning with CMC, because of its availability.

CMC glucose rings were opened by reaction with NaIO_4 , followed by reduction of the newly-formed aldehyde groups to alcohols with NaBH_4 . The resulting polymer is chemically very similar to the original polysaccharide, but has greater flexibility.



Two levels of NaIO_4 treatment were done, 1 per 10 glucose residues, and 1 per 2 residues. These were formulated into inducer preparations with different ratios of PLL, and are designated ICL-C (open).

The 1/2 type was formulated in the proportions of 2:1.5:5 (III-228Ia), 2:0.75:5 (III-228Ib) and 2:0.375:5 (III-228Ic), for the components in the order IC:PLL:CMC(open). The first of these was not adequately soluble. The other two gave clear solutions. III-228Ib gave a T_m of 79.5, while III-228Ic gave a three-step melting profile with T_m 's of: 57° (30% of total ΔA), 67° (10% of total ΔA) and 79.5° (60% total of ΔA). The lowest T_m for III-228Ic probably represents uncomplexed IC, arising from there being insufficient PLL to cover all of the IC. Sample III-228Ib induced an IFN titer of 666 units or 54% of the standard (Table 4).

With the 1/10-ring opened CMC, complexes of 2:1.5:5 III-228IIa, 2:0.75:5 III-228IIb, and 2:0.375:5 III-228IIc were soluble. Melting profiles of these gave T_m values of 75.4° for III-228IIa, 80° for III-228IIb, and two transitions at 63° and 80.5° for III-228IIc. For the last the ratio of ΔA of the 63° transition to ΔA of the 80.5° transition was 30% to 70%, and the low T_m transition of 63° was similar to that of IC alone. The IFN titers of III-228IIa and III-228IIb were 430 units and 1021 units, or 35% and 83% of the standard ICLC. All three experimental formulations of Table 2 were resistant to pancreatic ribonuclease, essentially similar to standard ICLC in this respect.

Further studies on reproducibility, toxicity and anti-viral action are planned. Also, chemical or nmr analysis is planned to see what fractions of rings were actually opened.

Table 2. IFN Induction In Mice by ICL-C(open)

Batch #	Composition IC:PLL:X [X = CMC or CMC (open)]	IFN Titers	
		Mean Titer	% of ICLC-STD.
ICLC III-201 (Standard)	2:1.5:5	1235	(100)
ICL-C(open) ^a III-228Ib	2:0.75:5	666	54
ICL-C(open) ^b III-228IIa	2:1.5:5	430	35
ICL-C(open) ^b III-228IIb	2:0.75:5	1021	83

^a. CMC reacted with NaIO₄ to open 1/2 of the glucose rings.

^b. CMC reacted with NaIO₄ to open 1/10 of the glucose rings.

3. Formulations of Carboxymethyl- β -Cyclodextrin Complex of ICL.

A soluble complex of IC, PLL and CM- β -cyclodextrin was made. This is our first IFN inducer complex in which the anionic solubilizer is not a polymer. β -cyclodextrin is a cyclic oligosaccharide of seven glucose residues. This was carboxymethylated to a degree of substitution of 0.59 CM group per glucose residue (or 4.1 per β -cyclodextrin). The complex, ICL-CM- β -cyclodextrin, showed two T_m values, 83° and 87° in the ratio of 7:1 for two ΔA 's. There was no free IC. Either there are two types of complete complexes, or the 87° transition arises from some ICL not complexed with CM- β -cyclodextrin. The 83° transition probably is not from ICL, because the quantity involved would be far beyond the solubility limit of ICL, and would have precipitated.

On the first trial in mice this complex induces an IFN titer of 123 units or 9% of that of the ICLC standard. On cold storage for several months III-62 showed an increase in IFN titer to 443 units, or 55% of the ICLC standard. The

melting profile also changed to a single transition at 78.5°. If reproducible, it would appear that ICL-CM- β -cdextrin improved on aging. If so, we would try to accelerate the aging process, perhaps by storage at room temperature or above. Complexes of CM-cyclodextrin (α , β , and γ) may be worth further testing, as cyclodextrins may have advantages over polymeric anions in respect to purity and reproducibility. The complex of ICL-CM- β -cdextrin will be tested for antiviral effects and toxicity, and complexes as will be made with cyclodextrin of different degrees of substitution.

The potential advantages of CM- β -cyclodextrin are:

- 1) Cyclodextrins have been described in many pharmaceutical preparations, and appear to be essentially non-toxic;
- 2) CM-Cyclodextrins are expected to be non-immunogenic;
- 3) As small organic compounds of definite size and structure they should be amenable to purification by common techniques, free of microorganisms, pyrogens, etc.

4. Formulations of ICL-CMamylose.

In the first year we prepared half a dozen ICL-CMamylose formulations which gave good IFN titers in mice. These were made with two proportions of IC:PLL:CMamylose, namely, 2:1.5:5 and 2:0.75:2.5. All had CMamylose of DS = 0.5.

We have extended this work to three new batches (Table 3) all of which are moderate to good inducers. The data for II-166 are for a repeat test of an earlier batch. This gave 119% of the IFN of ICLC, compared with 183% the first time. III-167A uses a CMamylose of low DS (0.22), and is a good inducer. III-107 is a good inducer, and also has low toxicity, LD₅₀ = 25 mg/Kg, compared with 13 for ICLC. Both II-166 and III-107 showed good antiviral action against RVFV (Table 11). The use of 0.75 and 2.5 mg/mL of PLL and CMamylose are preferred to the 1.5 and 5 mg/mL concentrations, since it is difficult to sterilize by filtration the viscous solutions needed for the higher concentrations of CMamylose.

Table 3. IFN Induction by ICL-CMamylose

Lot Number	Composition IC, PLL, CMdextran mg/mL	IFN Titer		
		Exptl. Formulation	ICLC Standard	% of ICLC Standard
III-107 DS = 0.5	2/0.75/2.1	787	756	104
II-166 DS = 0.5	2/0.75/5	764	643	119
III-167A DS = 0.22	2/0.75/2.5	256	351	73
III-167B DS = 0.43	2/0.75/5	123	351	35

5. Single-Stranded RNA in Formulations.

We have begun to study the use of single-stranded polyribonucleotides as polyanionic replacements for CMC. Some are antiviral agents in themselves, which may provide improved antiviral activity. Poly I complexed with IC and PLL was insoluble. Poly C gave a soluble complex, designated ICL-Cyt (where Cyt stands for poly C, to avoid confusion with the use of C for CMC). One complex of composition 2:1.5:5, for IC:PLL:Cyt, gave 271 units of IFN, or 36% of that of the ICLC standard. This is a substantial titer, and indicates that further work should be done. Also, other single-stranded RNA's will be tried.

6. Formulations with Sulfated Gelatin (ICL-SO₃Gel).

Three methods of sulfation of gelatin were used: 1) cold concentrated sulfuric acid; 2) cold trifluoroacetic acid with cold concentrated sulfuric acid; 3) cold chlorosulfonic acid in H₂SO₄. The last is not as satisfactory as the first two because of extensive foaming. (A fourth method, the use of the mild reagent trimethylamine-SO₃, failed to sulfate gelatin).

Gelatin, unmodified or modified in several ways, has a long history of use as a plasma volume expander. As a replacement for CMC, gelatin, per se, would appear to be an unlikely candidate since its net charge at pH 7 is slightly positive or only slightly negative (depending on whether the gelatin was extracted from tissue by acid or alkali). To increase the negative charge, we tried sulfation of the hydroxyl groups of gelatin, of which there are about fifteen per hundred residues:



Formulations of IC, PLL and SO₄Gel were prepared and tested in mice for IFN induction (Table 4). The earliest preparations, III-31A, III-31B and III-43 gave 47-72% of the IFN titers of ICLC standards. III-111 gave a substantial titer (427 units), but no ICLC standard was run. III-31A, III-31B, and III-111 were of lower toxicity than ICLC (Tables 9,10).

A series of formulations (III-106, III-155, III-164 and III-175B) gave very little IFN. All of these showed two melting transitions, one of which corresponded to uncomplexed IC and accounted for 50-85% of the total .C. The reasons for these failures are not known. Ostensibly they contained too little PLL.

New batches of SO₄Gel were synthesized and incorporated into three inducer formulations III-198, III-199 and III-200. They were all effective inducers, the variation in titers between two tests of III-198 and of III-199 is not unusual as can be seen by the results with ICLC. III-198 and 199 had high melting temperatures and were resistant to hydrolysis by ribonuclease, both being hydrolyzed at 9% of the rate for IC.

III-198 and III-199 were tested by Dr. Kende and were found to be effective agents against Rift Valley Fever virus (Table 11). This is in contrast to an earlier ICL-SO₄Gel III-106, which was a moderately effective anti-RVSV agent. III-106 was a weak inducer (IFN titer 5% of standard ICLC), while III-198 and III-199 are effective inducers. III-106, by melting profile, had only 50% of its IC covered; III-198 and III-199 were completely covered.

While ICL-SO₄Gel is a promising IFN inducer, of lower toxicity than ICLC, two batches (III-31A and III-31B) were found to be immunogenic when administered under conditions designed to promote immunogenicity. This is discussed in the section on immunogenicity, below.

Table 4. IFN Induction by ICL-SO₄Gel, and ICL-Gel.

Lot #	Composition IC:PLL:X ^a	IFN Titer	IFN titer of ICLC Std.	% of ICLC Std.
ICL-SO ₄ Gel				
III-31A	2/0.375/5	952	2046	47
III-31B	2/0.375/5	1345	2046	66
III-43	2/0.75/6	1203	1669	72
III-111	2/0.75/5	427	-	-
III-155	2/0.375/5	38	357	11
III-164	2/0.75/5	14	357	4
III-106	2/0.375/6	37	756	5
III-175B	2/0.375/2.5	23	351	7
III-198 ^b	2/0.75/6	1263	394	321 ^c
III-199 ^d	2/0.75/6	344	394	87
III-200 ^e	2/0.75/6	122	394	31
III-198	2/0.75/6	289	670	43
III-199	2/0.75/6	373	670	56
ICL-Gel				
III-210	2/0.75/6	340	670	51
IC-Gel				
III-222 ^f	2/0/6	<6	670	<1

^a. X = SO₄Gel or Gel. Composition is in mg/mL.

^b. Gelatin sulfated with H₂SO₄.

^c. 172% if one mouse with very high titer is deleted.

^d. Gelatin sulfated with trifluoroacetic acid-H₂SO₄.

^e. Gelatin sulfated with H₂SO₄-ClSO₃H.

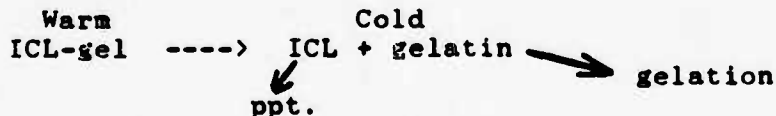
^f. Control IC-Gel without PLL.

7. Formulation with ICL-Gelatin.

We also prepared an ICL-gel complex, i.e., a complex of IC, PLL and unmodified gelatin, as an intended control for ICL-SO₄Gel. It was not expected that gelatin would complex with PLL, since at pH 7 the gelatin would have a net positive charge, nor was it expected to complex with IC, since the small net positive charge on gelatin was not expected to compete with the large charge and high charge density of PLL. However, a complex of all three components was formed, as shown by the fact that a soluble complex was formed, whereas without gelatin the ICL precipitates. The ICL-gelatin (III-210) was an effective inducer (Table 4).

IC-gel is a moderately effective protector against RVFV (Table 11) at 10 µg per mouse, better at 2.5 µg; but these should be repeated. This is a promising complex in the light of the extensive experience with gelatin as a safe plasma volume expander. Gelatin is usually immunogenic; the gelatin we are using is claimed to be non-immunogenic, and was found to be so by our screening procedure. ICL-gel had a high T_m, and was hydrolyzed by ribonuclease at 5% of the rate of uncomplexed IC. A control of IC-gel (without PLL) was not an inducer, and was not a complex, as shown by the T_m of 63°, the normal T_m of uncomplexed IC.

ICL-gel on storage in the refrigerator sets to a white, opaque gel. On warming to room temperature, a clear solution is regenerated, and the ICL-gel shows a T_m of 79.5°, near to the original T_m of 80°. The chilled-then-warmed solution is to be retested for IFN induction. A possible explanation of the above observations is the following: Because of its low charge density gelatin binds rather weakly to ICL. At low temperature, gelatin-gelatin interactions (i.e., the interactions which lead to gelatin) become stronger, shifting the equilibrium, as shown, leading to gelatin of the gelatin and precipitation of the ICL.



There is a difference between the melting profiles before and after cold storage. Before, the absorbance decreases above T_m; after cold gelatin and reliquefaction, the melting profile is normal with a flat plateau above T_m. Aside from the obvious appearance and disappearance of the opaque gel, there is another difference, namely, the order of mixing the components. The original ICL-gel is prepared by mixing PLL and gelatin, after which IC is added. When the opaque gel is thawed, the mixing procedure is that the ICL precipitate interacts with gelatin, i.e., the mixing procedure is essentially reversed. The original mixing procedure is based

on Levy's procedure for making ICLC. We have chosen, so far, to use the same procedure for the inducer formulations involving IC + PLL + polyanion, to avoid doubling all of the formulations and their biological tests. However, it may be desirable to try the reverse procedure, since this may result in different particle structures and biological properties.

8. Poly(lysine)-Dextran Grafts.

An approach being studied is the replacement of both PLL and the anionic polymer by a PLL-polysaccharide graft. The rationale is that the positive charges would result in binding to the IC, with protection against serum nucleases, while the grafted polysaccharide would enhance solubility. (Since the graft polymer retains its positive charge, it could be used in place of PLL along with a polyanion. This more complex assembly has not yet been tried.)

The graft is made by the reductive alkylation method, in which aldehyde groups at the termini of the dextran chains condense with amino groups of PLL, in the presence of NaCNBH_3 , to generate dextran-substituted amino groups. Reactions have been run with a large excess of dextran to PLL, since the alkylation reaction is not efficient. A number of grafts have been made, mostly with PLL of 21.5 kDa, but also 38 kDa PLL, and with 10 kDa dextran. Some grafts using higher molecular weights of both components have been, or are being prepared; these have not reached the stage of formulation and testing.

Separation of the graft from the polymeric reagents is achieved chromatographically (after dialysis to remove low molecular weight reagents and by-products). On a column of cation exchanger, the unreacted dextran is not retained and is washed out with water. The retained PLL and PLL-dextran are eluted with NH_4OH , and these are separated by gel filtration on Sephadex, according to molecular weight. The PLL is eluted last, being of the lowest molecular weight. The graft appears as several overlapping peaks, as expected, since there must be a series of species with different numbers of dextran chains per PLL chain.

^1H nmr of a graft of 21.5 kDa PLL with 10 kDa dextran, indicates a glucose:lysine ratio of about 33:1. If the engrafted dextran is of the same molecular weight distribution as the starting dextran, then about one-half of the lysines would be substituted. However, as the shorter dextran chains may be more reactive than the longer ones, the proportion of substituted lysines may be greater than 50%. A different method of analysis is required to settle this question. Both the dextranated and unmodified amino groups are protonated at pH 7. However, electrostatic binding to the IC would be expected to be stronger for unmodified lysine than for modified, because of steric constraints in the latter.

(We shall return to this point in connection with melting temperatures of IC-(PLL-dextran)). It may be desirable to have a substantial fraction of lysine amino groups unmodified to permit enzymic cleavage to promote excretion. Enzymic digestion of the grafts is planned.

Several formulations of IC-(PLL-dextran) have been made with PLL of 21.5 kDa and dextran of 10 kDa, and one with 38 kDa PLL with 10 kDa dextran. IFN induction by these are shown in Table 5. The ratios, by weight, of IC to PLL-dextran are 2:10, 2:7.5 and 2:5. All of these are IFN inducers of modest to moderate ability, ranging from 6 to 21% of the ICLC standard. Lots III-135A and III-190 were found by Dr. M. Kende at Fort Detrick to be effective antiviral agents (Table 11), at least as good as ICLC. In one test, III-190, at 2.5 µg per mouse protected 10/10 mice, compared with 9/10 for ICLC. In a second test at 10 µg/mouse, 7/10 were protected against 250 pfu of virus, and after an additional 500 pfu of virus at day 22, 6/10 were still protected at day 30. (However, ICLC was not run in this set.) III-190 had a high T_m and was resistant to ribonuclease. III-190 is less toxic than is ICLC, having an LD_{50} of 25 mg/Kg compared with 13 mg/Kg for the latter.

Table 5. IFN Induction in Mice by IC-(PLL-Dextran)

Batch #	Composition IC:(PLL-dextran)	Exptl. Batch	IFN Titer	
			ICLC Standard	% of ICL-standard
III-190 ^a	2:10 mg/mL	151	710	21
III-230 ^a	2:5 mg/mL	173	1235	14
III-232 ^a	2:7.5 mg/mL	77	1235	6
III-135A ^a	2:10 mg/mL	45	357	13
III-135B ^b	2:10 mg/mL	28	357	8

^a. 21.5 kDa PLL, 10 kDa dextran.

^b. 38 kDa PLL, 10 kDa dextran.

The T_m of the IC-(PLL-dextran) in the 2:10 (III-135A or III-190) complex is quite high, 87° , well above that of ICLC or most other complexes. This is unexpected on the basis of electrostatic binding between cationic lysine residues and anionic IC, as it would be expected that electrostatic interactions would be hindered by the large dextran chains, and would appear to require an important contribution from dextran-IC interactions. In principle, the high T_m might be explained by relatively weak binding to the denatured IC. That is, binding of PLL-dextran to the denatured IC may be weaker than to native IC, as compared with binding of PLL to IC in other formulation. Since the polyanion (CMC for example) in other formulations binds to PLL, the binding of PLL to IC must be weakened. Since the dextran in PLL-dextran is not anionic, this effect would not occur, and the ammonium groups of PLL might bind to IC strongly despite the steric restraints of the dextran chains. Plainly, the question of strength of binding (strictly, the free energy of binding) is very complex. On the hypothesis that a high T_m arises from tight binding to IC in the native state, the high T_m suggests that dissociation of IC-(PLL-dextran) may be slower or less extensive than for ICLC or some other inducer complexes. If dissociation is required in order to permit cellular recognition of the IC, then slow or less complete dissociation could result in: a) less induction of IFN; b) delayed induction; or c) lower levels of IFN over longer periods (i.e., a sort of built-in time-release). Possibilities b and c are testable by kinetic studies. This line of reasoning opens a new avenue of study, namely, the study of the dissociation equilibrium and kinetics, and their correlation with biological activity. Some preliminary gel chromatographic studies on ICLC, diluted at different times before chromatography, show time effects (as noted elsewhere in this report).

We are investigating the best proportions of IC and (PLL-dextran) in the complex. The complexes III-135A, III-135B and III-190 had weight ratios of 2:10 (i.e., 2 mg of IC and 10 mg of grafts per mL). Two new batches of IC-(PLL-dextran) have been prepared, one, III-230, with a ratio of IC to (PLL-dextran) of 2:5, and III-232 with a ratio 2:7.5. The IFN titers were 173 for III-230, and 77 for III-232, or 14 and 6%, respectively, of the ICLC standard (Table 5). These values are consistent with those found for III-135A, III-135B and III-190. For III-230 (ratio 2:5) the melting profile showed two transitions, at 66° and 76° . The lower T_m may be a complex of only slightly greater stability than free IC. RNase hydrolysis proceeds to 35% of the extent of free IC (corresponding to the ratio of the first transition ΔA to the total ΔA), but the rate of digestion by RNase is about one half of that expected if 35% of the IC is free. Either about 35% of the IC is weakly complexed, or IC double helix is only partially covered by (PLL-dextran), and the intervening IC is

somewhat stabilized by the adjacent covered segments. This is to be studied by other methods. The 2:5 (III-230) complex has a second transition at 76°, or 8° lower than the single transition of the 2:10 (III-190). Thus, a higher ratio of (PLL-dextran) to IC raises T_m . This suggests a cooperative interaction in the latter.

At an IC to (PLL-dextran) ratio of 2:7.5, the low melting transition vanishes, and only the transition at high T_m is seen, at 83°. Thus, the stoichiometry has been narrowed to a ratio somewhere near 2:7.5.

We may ask if the low-melting fraction in the 2:5 complex is an IFN inducer and if it is an anti-viral agent. The answer may require fractionation to separate the complexes if they are separable and stable, i.e., do not interconvert.

Batches III-230 and III-232 had LD_{50} values of 33 and 30 mg/Kg, respectively. Tentatively, these (especially the LD_{50} for III-230) appear to be better than for any other formulation of ours, and is about 2.5 times greater than for ICLC. This is to be followed up. On the basis of low toxicity and good antiviral action the grafts look promising. The low IFN titers should be checked by doing kinetic studies. We have begun to work on PLL-amylose grafts, but, so far, have been stopped by the insolubility of amylose under the reaction conditions. Various strategies are being considered.

9. Molecular Sieve Chromatography of Inducer Formulations.

A question concerning all of the inducer formulations is whether or not some of the polyanion (CMC, etc.) is not bound, i.e., is some of the polyanion free? Also, is any of the PLL present as a binary complex with the third component, i.e., without IC, and does more than one type of ternary complex form? To answer these questions we turned to molecular sieve chromatography, to see if all components move together, or if some move separately from the ternary complex. It is emphasized that these experiments are introductory, to see if the method is suitable, to learn the difficulties, and to try to find the best procedures.

In order to follow the elution of the components conveniently it was necessary to label the PLL and polyanion with fluorescent labels. The IC can be monitored by its absorbance. The PLL was lightly labelled with fluorescent dansyl groups, as was sulfated gelatin. CMC was labelled with fluorescein thiosemicarbazide, after oxidation of about 1% of glucose residues with sodium periodate.

The ternary complexes were passed through Biogel A5m, exclusion limit 5×10^6 daltons, and the fractions were measured for absorbance at 260 nm (for IC), fluorescence (excitation at 340 for dansyl, 440 for fluorescein; emission at 550 for

both), and for light scattering. The last is sensitive to aggregates.

Fig. 1 shows the elution of IC alone, which emerges at the void volume. This result shows that some future work should be done with a gel of greater exclusion limit. The emergence of IC at the void volume does not mean that its molecular weight is 5×10^6 or more. The elongated double helix results in behavior similar to a larger, more compact polymer.

Fig. 2 shows chromatography of ICL^s (L^s indicates fluorescent dansyl-labelled poly(lysine)). Most of the L^s emerges with the IC. The nature of the small fluorescent peak at fraction 8 is not yet established. Light scattering shows a peak corresponding to the A_{2,22} and dansyl fluorescence, as well as a second peak at fraction 8. Since poly(lysine) is a very weak scatterer, the meaning of this peak is not known. It does not contain IC. For solubility reasons the ratio of L^s to IC is lower than in the complexes to follow, and no uncomplexed L^s is expected. This must be further studied.

Fig. 3 shows chromatography of ICL^sC. A_{2,22}, dansyl fluorescence and light scattering all peak at fraction 4. Since this experiment tells us nothing about the CMC, we also made ICLC^s (CMC labelled with fluorescein), and chromatographed it, with the results of Fig. 4. The IC (A_{2,22}) appears essentially in one peak (fraction 6). A very small peak is at fraction 3; this has not yet been identified. Also, we do not yet know why the main peak emerges at fraction 6 instead of 3. A second large fluorescent peak appears at fractions 8-9, showing that the CMC is not all bound in the complex. It will be necessary to rechromatograph fraction 6 in order to see if it emerges as a single peak, or if some ICLC^s dissociates to give, again, the fraction 8-9 peak. If, on rechromatography of fraction 6, a single peak is obtained, this would mean that ICLC uses excess CMC, and that it may be possible to use less of it, with a reduction in toxicity.

However, if rechromatography of the fraction 6 peak should show a second peak at fraction 8-9, this would indicate that ICLC dissociates when diluted on passage through the column. This may be important, since it would be expected that inducer complexes dissociate in-vivo, and such dissociation may be required in order to expose IC for cellular interaction. The rate and extent of dissociation may determine the kinetics of IFN appearance, the magnitude of the titer, and duration of IFN induction, the duration of antiviral effects, and toxicity. (We shall return to this point.)

We also chromatographed ICL-SO₄Gel^s (dansyl-label on sulfated gelatin), with the results seen in Fig. 5. IC peaks at fraction 4 (A_{2,22}), as do fluorescence and light scattering. Excess fluorescence emerges, as a shoulder, at

fractions 6-7. Thus, some excess SO₄Gel is present. The fraction 4 peak should be rechromatographed for the reasons given above in connection with ICLC.

Chromatography of ICL³CMDextran (dansyl fluorophore on poly-lysine) is shown in Fig. 6. Two peaks were observed, fractions 3 and 7-8, both of which show the presence of IC and of L³, and both of which show light scattering, which probably indicates the presence of all three components. Labelled CMDextran would be needed to verify this. The fact that light scattering of fraction 3 is smaller than that of fraction 7-8 cannot yet be explained, since light scattering is a complex phenomenon affected by the size, shape, refractive index, anisotropy and hydration of the particles, and is dependent on the angle of observation. All of our scattering experiments were done at an observation angle of 90°.

The polarization of fluorescence and of scattering was measured for the two peaks, and with both of these methods the polarization was greater for fractions 7-8 than for 4. For fluorescence the polarizations were 1.0 and 4.2 for the first and second peaks, and for scattering the polarizations were 13 and 22, indicative of structural differences between the two fractions. It would be interesting to see if each of the two peaks rechromatograph as a single peak, and if they differ in biological properties, chemical constitution and physical properties. (On the basis of the data of Table 6 for ICL-CMDextran we predict that the material of the second peak would be the better inducer.)

Returning to the question of dissociation of complexes, we have chromatographed ICLC and ICL-SO₄gel at different times after dilution, from a few minutes to 7 days. Chromatograms differ with the appearance of late-eluting (smaller size?) fractions, indicating that these complexes undergo dissociation. These experiments are quite preliminary; more extensive work is required. One problem to be examined is the possibility that the column packing may interact preferentially with one or more component of the complex, thereby producing a dissociation artefact. Nevertheless, chromatography of formulations may be useful to permit the preparations of formulations without excess components (which may reduce toxicity) or to isolate complexes of superior properties.

In the Figures to follow, light scattering and fluorescence intensities are drawn to arbitrary scales.

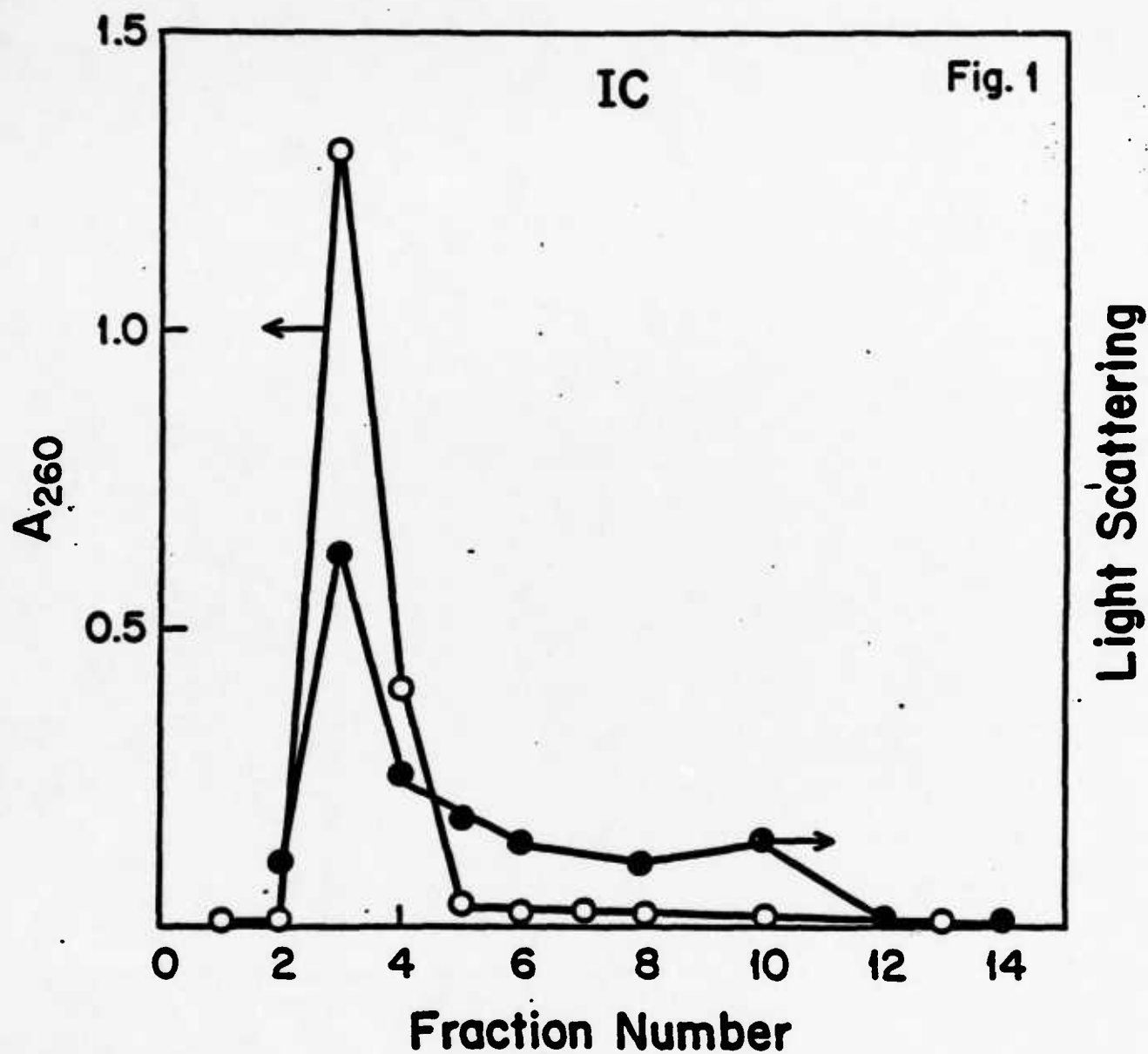


Figure 1. Molecular sieve chromatography of poly I-poly C on Biogel A5m, elution with standard saline.

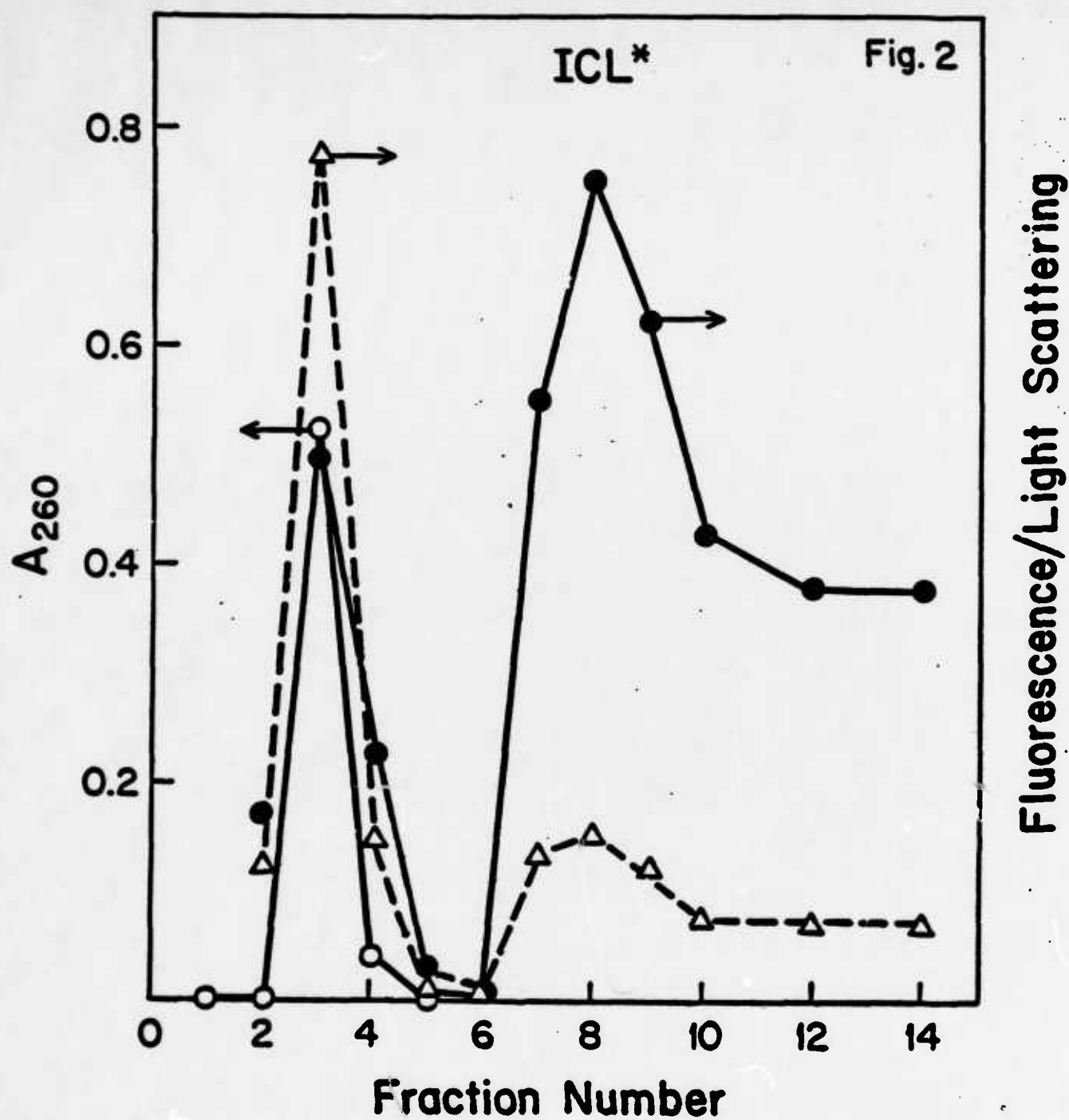


Figure 2. Molecular sieve chromatography of poly I-poly C-poly(L-lysine) with fluorescent label on poly(L-lysine); method as for Figure 1.

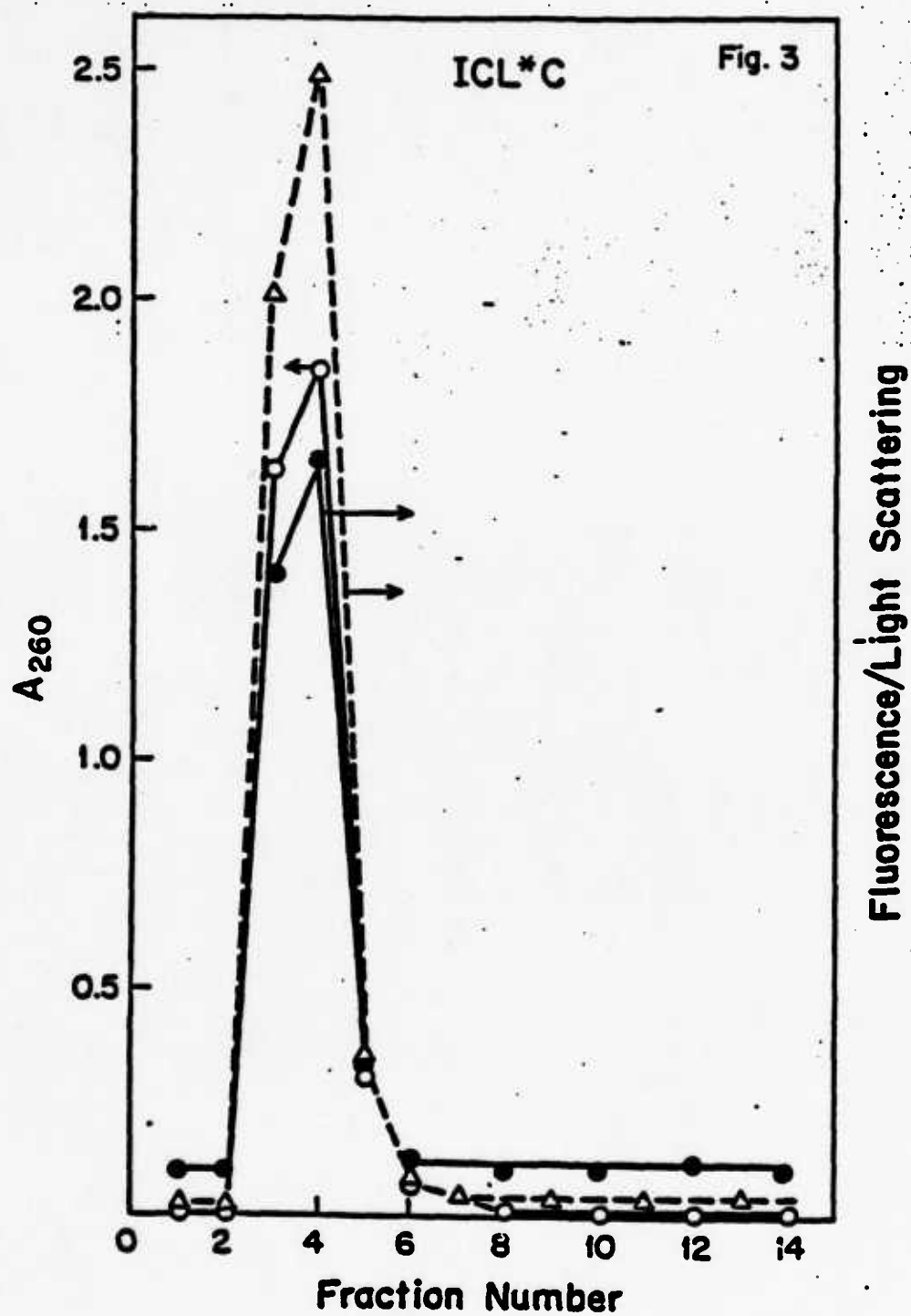


Figure 3. Molecular sieve chromatography of ICLC with fluorescent label on poly(L-lysine); method as for Figure 1.

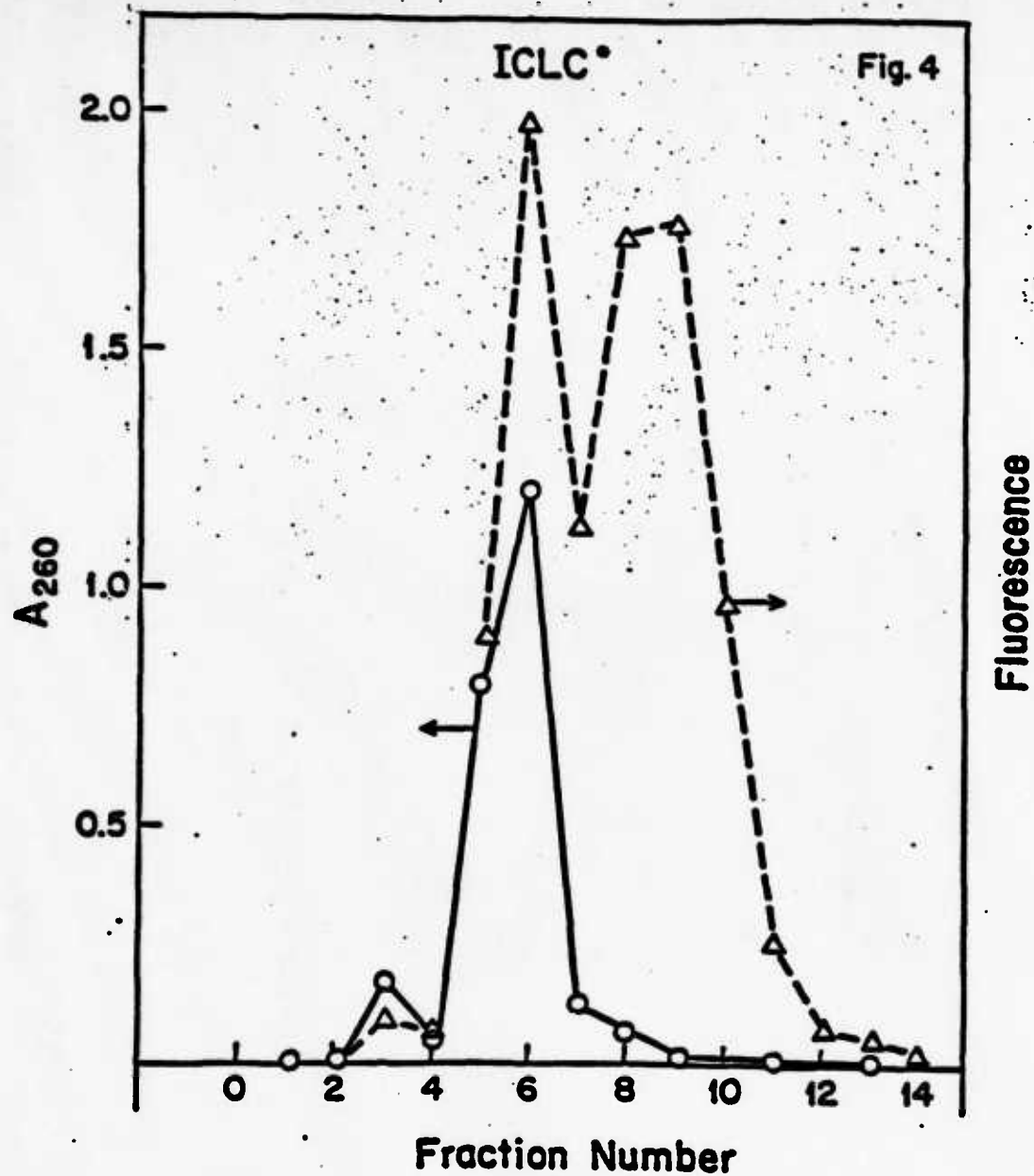


Figure 4. Molecular sieve chromatography of ICLC with fluorescent label on CMC; method as for Figure 1.

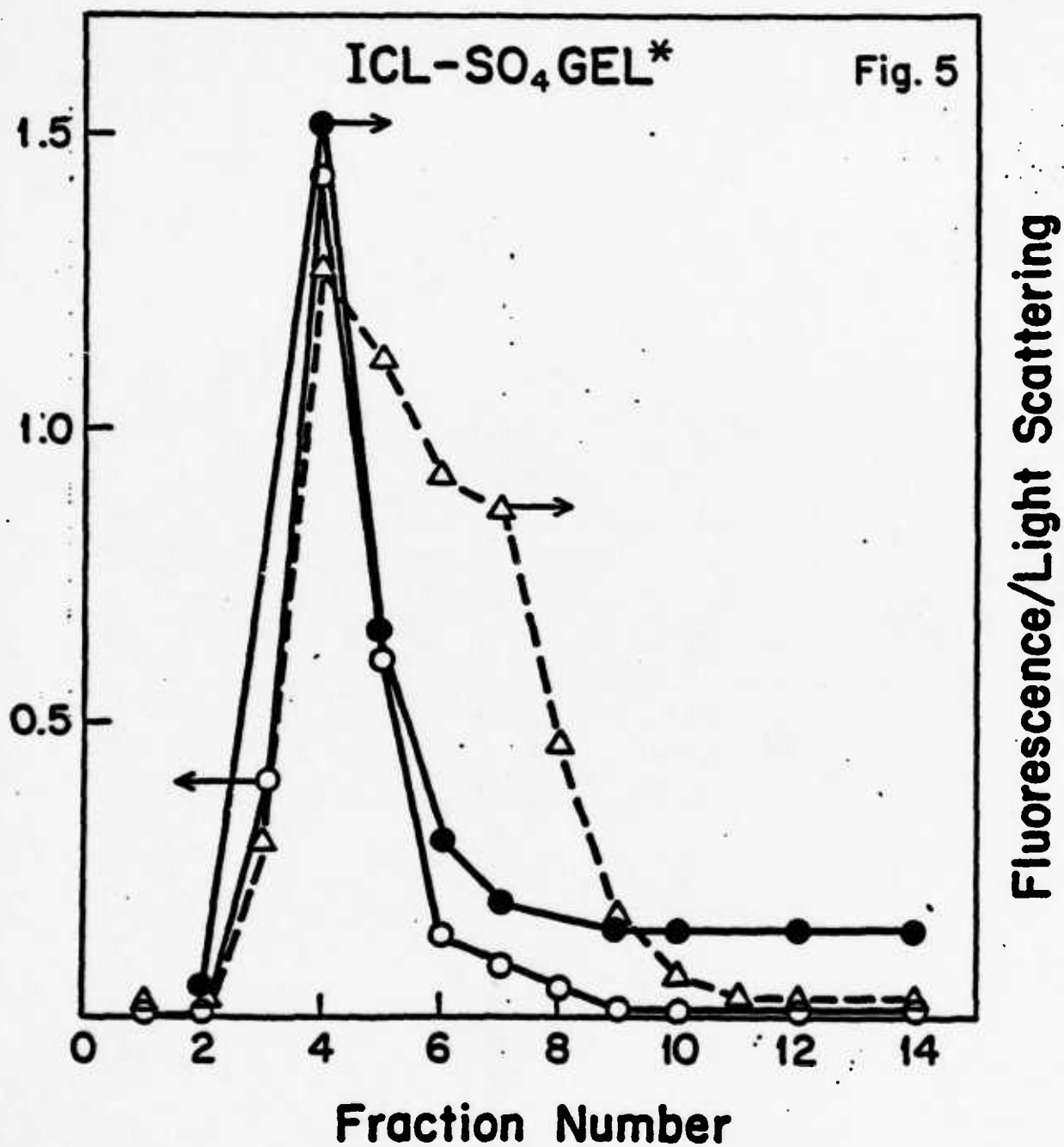


Figure 5. Molecular sieve chromatography of ICL-SO₄Gel with fluorescent label on gelatin; method as for Figure 1.

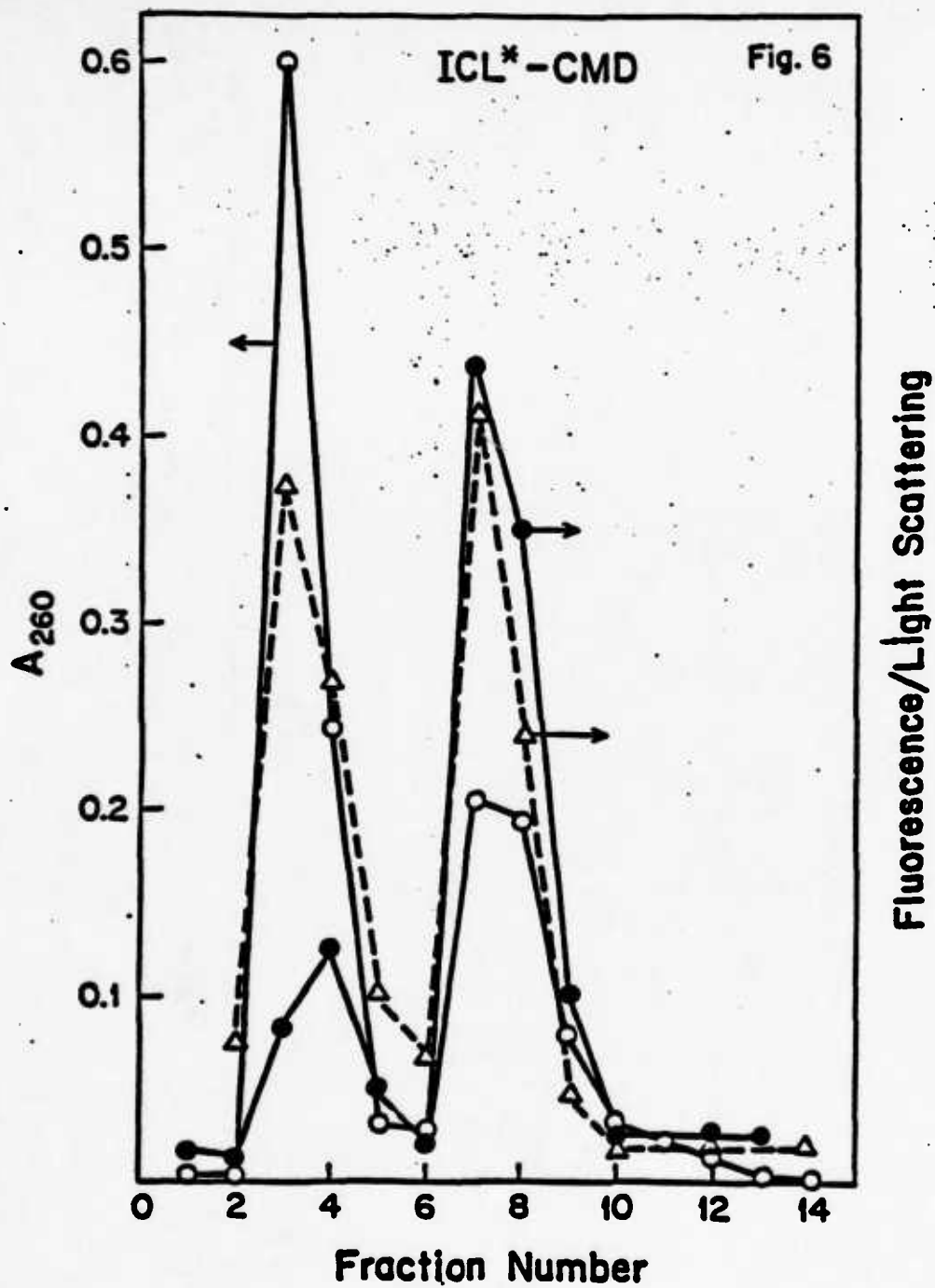


Figure 6. Molecular sieve chromatography of ICL-CMD with fluorescent label on poly(L-lysine).

10. Melting Profiles of Complexes; Light Scattering and Fluorescence.

The inducer formulations which were chromatographed (see above) were also subjected to melting transitions, monitored by absorbance at 260 nm (for IC), and, where appropriate, by fluorescence (for dansyl-PLL, dansyl-SO₄Gel and fluoresceinyl-CMC) and by light scattering. Both intensity and polarization were measured for fluorescence and scattering.

In all cases the intensity of light scattering increased on melting. But the polarization of scattered light decreased for ICLC and ICL-CMamylose (Table 6). Two batches of ICL-CMDextran were measured. One (III-238) which was a good IFN inducer, showed a decrease in polarization, and one (III-191), essentially a non-inducer, showed an increase in polarization of scattering. Thus, on the basis of a few experiments, and only one with a poor inducer, we tentatively suggest the possibility of a correlation between IFN titer and polarization of light scattering. Also, at room temperature the good inducers have polarizations of 20 or more, while the poor inducer has a value of 9. This is the basis of our prediction that the second peak of Fig. 6 would be a better inducer than the first. This requires further study; it may be possible to use light scattering to learn about particle characteristics (size, shape, hydration, etc.) associated with IFN induction. As noted earlier, light scattering is a complex phenomenon, and the change in scattering intensity may arise from several causes, one of which is a change in the degree of anisotropy, which can change the relative scattering in the forward direction vs 90°. A scattering polarization decrease may indicate a greater degree of anisotropy.

Furthermore, when the complex is prepared with dansyl-PLL, the melting transition is accompanied by an increase in fluorescence intensity and an increase in fluorescence polarization, indicative of a tighter binding of the PLL. This was unexpected. When the complex contained dansyl-SO₄Gel or fluoresceinyl-CMC, the magnitude of fluorescence increase was small, compared with dansyl-PLL. This indicated a relatively smaller structural change for these components; these may be on the outside of the complex, relatively less rigidly bound on the complex than is PLL, or presumably, IC.

Table 6. Polarization of Scattered Light.

Complex	Polarization	
	25°	Above T _m
ICLC III-95	22	4
ICLC III-94	26	12
ICL-CMamylose III-97	27	7
ICL-CMdextran III-238	23	9 (1300 units of IFN)
ICL-CMdextran III-191	9	13 (18 units of IFN)

11. Immunogenicity Studies.

The investigation of the immunogenicity in mice of compounds and complexes under study in this project, as well as some control substances, was continued. An additional twenty-two of these, not previously examined, were included (Table 7).

Of the twenty two substances tested, two different preparations of ICL-SO₄Gel (Table 7, BB, CC) showed weak to strong immunogenicity among the five mice in each of the two test groups. The five strongest antisera of the ten obtained against the ICL-SO₄Gel were tested against gelatin and sulfated gelatin or complexes (Table 8, BB, CC). None of these had previously been found to be immunogenic and none of them showed any reaction with sera BB or CC; substances BB and CC did, of course, react. Further work will be necessary to determine what structure(s) on the ICL-SO₄Gel are responsible for the immunogenicity. The immunogenicity may arise from the SO₄Gel component in a different conformation not present in free SO₄Gel, or from some combination of groups in two or all three components.

Bovine plasma albumin (Table 7, N), used as a positive immunization and test control, was, of course, strongly immunogenic.

None of the remaining substances of the twenty two showed any immunogenicity; each serum was tested against the injected material and in most cases against one or more related substances (Table 8).

These tests were done under conditions designed to enhance a positive response, in order to increase the probability of detecting such a response if the test substance is antigenic. Thus, 100 µg of substance (i.e., 100 µg of the

IC component) is injected with Freund's adjuvant. We shall retest ICL-SO₄Gel with 10 µg of IC (the quantity used for IFN induction) and without Freund's adjuvant. Also, other lots of ICL-SO₄Gel will be tested under both the stringent and relaxed conditions.

A substantial portion of each of the sera collected in the present work is frozen away and should be examined for antibody by a more sensitive method. Also, the immunogenicity of these and other compounds involved in this project should be tested in other species. The more sensitive and involved procedures will be reserved for inducers of the greatest promise.

Table 7. Substances Tested Immunologically.

K.	IC (Pharmacia Lot #292947)
L.	ICLC
M.	CMC
N.	Bovine Plasma Albumin
O.	PLL 32 kDa
P.	PLL 37 kDa
Q.	PLL 38 kDa
R.	SO ₄ Gel #1
S.	SO ₄ Gel #2
T.	Gel
U.	PLL (52 kDa)
V.	Ac-Cit-Gel (BGI-3)
W.	CM-Amylose (BGII-127; DS = 0.50)
X.	CM-Dextran (BGII-256; T-10, DS = 0.22)
Y.	ICL-CM-Amylose (BGII-193)
Z.	ICL-CM-Dextran (BGII-238)
AA.	ICL-CM-Dextran (BGII-266)
BB.	ICL-SO ₄ Gel (BGIII-31A)
CC.	ICL-SO ₄ Gel (BGIII-31B)
DD.	ICL-AcCitPLL (14kDa, BGIII-22)
EE.	CM-Dextran (D.S. = 2.02)
FF.	ICL-CMDextran (BGIII-45A; CMDextran is sample EE, above)

Table 8. Substances Against Which the Various Sera Were Tested

Sera	Tested Against
K	K, L
L	L, M
M	K, M
N	N
O	O
P	P, Q
Q	Q, P
R	R
S	S
T	R, S, T
U	U, V
V	V, U
W	W, Y
X	X, Z, AA
Y	W, Y
Z	X, Z
AA	AA, X
BB	BB, CC, R, T, V, succinyl-Gel, ICL-AcCitGel
CC	CC, BB, R, T, V, succinyl-Gel, ICL-Ac-Cit-Gel
DD	DD
EE	EE, FF
FF	FF, EE

12. Toxicity LD₅₀.

Several complexes were evaluated for LD₅₀ values in mice. The results (Table 9) show that several of our inducer formulations were less toxic than ICLC, and, in particular, that an ICL-CMamylose and an IC-(PLL-dextran), III-190, had LD₅₀ values twice that of ICLC (average of two samples), and about equal to the LD₅₀ of IC alone. Thus, the CM-amylose and the PLL-dextran graft components of the complexes appear to be non-toxic. The LD₅₀ values of 33 and 30 for III-230 and III-232, especially the former, require confirmation, and also repetition of LD₅₀ for IC, to see if III-230 is really less toxic than IC alone.

Table 9. LD₅₀ Values of Inducer Formulations.

Inducer	Batch No.	LD ₅₀ mg/Kg
ICLC	III-46	15
ICLC	III-201	11
		} Average 13 mg/Kg
IC	Pharmacia 00114729	26
ICL-CMamylose	III-107	25
ICL-SO ₄ Gel	III-186	21
ICL-SO ₄ Gel	III-106	25
ICL-SO ₄ Gel	III-111	25
IC-(PLL-dextran) ^a	III-190	25
IC-(PLL-Dextran) ^b	III-230	33
IC-(PLL-DExtran) ^c	III-232	30

^a. Ratio of IC to (PLL-dextran), 2:10.

^b. Ratio of IC to (PLL-dextran), 2:5.

^c. Ratio of IC to (PLL-dextran), 2:7.5.

Some toxicities were also done by method A (see experimental), and several formulations were found by this procedure to be less toxic than ICLC (Table 10). Method A has the advantage of requiring one-sixth as many mice as the LD₅₀ method, but we are now using LD₅₀ only, for consistency with published work.

Table 10. Toxicity of Some Inducers*

Inducer	Lot #	Survivors*
ICL-AcCitPLL ² 2:1.5:2.5	III-22	100%
ICL-SO ₄ Gel 2:0.375:5	III-31A	62
ICL-SO ₄ Gel 2:0.375:5	III-31B	62
ICL-SO ₄ Gel 2:0.75:6	III-43	50
ICLC 2:1.5:5	II-172	0

* MW PLL = 14,000

* Method A (See Experimental).

13. Antiviral Action of IFN Inducers.

Table 11 shows the results of Dr. M. Kende on the protection of mice against Rift Valley Fever virus. It will be apparent that there is no necessary connection between antiviral action and ability to induce a high IFN titer, (not a new finding).

ICL-SO₄Gel. The first batches tested (III-106) was a poor inducer (about 37 IFN units), and was a moderate anti-RVFPV agent. The next batch (III-111) was a moderately good inducer (427 units) and was rather like III-106 in anti-RVFPV action. Both of these had about half of their IC uncomplexed. III-106 and III-111 were less toxic than ICLC. Newer batches of ICL-SO₄Gel (III-198 and III-199) were good inducers (see Table 4), and were better antiviral agents than III-106 and III-111, but the usual variations of animal responses indicates that further testing is required.

ICL-Gel. One batch has been tested (III-210), a good inducer (Table 4), which had anti-RVFPV action comparable to that of ICLC.

ICL-CMdextran. One lot has been tested, III-59, which is a good IFN inducer (830 units, Table 1), but showed no anti-RVFPV protection. (Protection against other viruses is not ruled out.)

ICL-CMamylose. Two lots, II-166 and III-107 were tested. Both are effective IFN inducers (1052 units and 787 units, respectively), and both showed anti-RVSV action about equal to that of ICLC. III-107 was less toxic than ICLC (Table 9).

ICL-(PLL-dextran) (Graft polymer of PLL and dextran). Two lots were tested (III-135A and III-190), and were comparable to ICLC in antiviral activity, but III-190 is less toxic than is ICLC. Both III-135A and III-190 are weak inducers of IFN.

The antiviral tests show that some of our formulations are as good as ICLC; some of these are less toxic than ICLC, making them promising candidates.

Table 11.

Antiviral (Rift Valley Fever Action in Mice)^a

Type of Agent	Lot No.	Dose μ g	Survivors		ICLC ^b
			Exptl. Agent		
ICL-SO ⁴ Gel	III-106	2.5	IV	5/10	9/10
					9/10 (NIH)
ICL-SO ⁴ Gel	III-111	10	IP	5/10	10/10
ICL-SO ⁴ Gel	III-111	2.5	IP	4/10	9/10
ICL-SO ⁴ Gel	III-111	10	IV	5/10	6/10
ICL-SO ⁴ Gel	III-111	2.5	IV	2/10	3/10
ICL-SO ⁴ Gel	III-198	2.5	IP	8/10	9/10 (NIH)
ICL-SO ⁴ Gel	III-198	10	IP	6/10 (1/10)	-
ICL-SO ⁴ Gel	III-199	10	IP	8/10	10/10
		2.5	IP	4/10	9/10
ICL-Gel	III-210	10	IP	6/10 (3/10) ^c	9/10 (NIH)
ICL-Gel	III-210	2.5	IP	9/10	9/10
ICL-CMamylose	II-166	10	IV	8/10	9/10
		5	IV	9/10	9/10 (NIH)
		2.5	IV	9/10	9/10
		1.2	IV	9/10	6/10
ICL-CMamylose	III-107	10	IV	13/15	10/10 (NIH)
		2.5	IV	19/20	13/15
					15/15 (NIH)
		10	IP	10/10 (5/10) ^d	10/10
		2.5	IP	9/10 (8/10) ^d	9/10
		10	IV	10/10 (9/10) ^d	4/10
		2.5	IV	2/10 (2/10) ^d	0/10
		2.5	IV	3/10	10/10
					7/10 (NIH)
ICL-CMdextran	III-59	2.5	IV	0/10	7/10 (NIH)
IC-(PLL-dextran)	III-135A	2.5	IV	15/15	13/15
					15/15 (NIH)
		2.5	IV	8/10	10/10
					7/10 (NIH)
IC-(PLL-dextran)	III-190	2.5	IP	10/10	9/10 (NIH)
IC-(PLL-dextran)	III-190	10	IP	7/10 (6/10) ^d	

^a. Mice challenged with 250 pfu of virus, followed by drug at 0, 3 and 7, or 0, 2, 4 and 6 days. In some cases a second viral challenge was done at day 22 with 500 pfu, as noted. The surviving fraction is for day 19-21, except where noted after a second challenge.

^b. NIH denotes that the ICLC was obtained from the NIH (per Dr. Hilton Levy). Otherwise the ICLC was of our own making.

^c. The first fraction is at day 21; the second at day 37, after 500 pfu of virus on day 22.

^d. The first fraction is at day 21; the second (at day 30), after 500 pfu of virus on day 22.

6. Conclusions.

The results which we have obtained show that we have been able to formulate effective IFN inducers without CMC. Most of these inducers are less toxic than is ICLC, and some are about as toxic as IC itself. Therefore, the other components are essentially non-toxic. Presumably, one cannot expect to develop inducer complexes less toxic than the active ingredient. But preliminary data showed that one formulation may be less toxic than is IC. All but one of the formulations so far tested for immunogenicity have given negative results; the one exception is to be examined further.

We have a number of formulations which are effective IFN inducers, and some which are effective against Rift Valley Fever virus in mice. Some show both activities.

The effective IFN inducers are:

ICL-CMdextran
ICL-CMamylose
ICL-SO₄Gel
ICL-Gel
ICL-CM-cyclodextrin (IFN titer apparently dependent on
slow rearrangement of structure)
IC-(PLL-dextran) (weaker than the first four)

The effective anti-RVFP agents are:

ICL-CMamylose
ICL-Gel
IC-(PLL-dextran)
ICL-SO₄Gel (moderate)

Exploratory chromatographic data suggest that: 1) ICLC, as made by the standard procedure, may contain excess CMC; 2) chromatography may be useful to separate components which may have different biological properties; 3) dissociation of inducer complexes may affect biological properties.

Results of melting profile experiments with ICL-Gel suggest that changes in the order of mixing components may alter the nature of the inducer complexes. And work with ICL-CM-cyclodextrin suggests that aging of a complex may improve it.

Light scattering melting profiles suggest a correlation between IFN induction and increased polarization of scattering above T_m , and an inverse correlation between IFN induction and the magnitude of polarization at room temperature.

Further work is required along the following lines:

1. Test reproducibility, stability/aging of formulations in respect to IFN induction and antiviral effects.

2. Find the optimum proportions of components, molecular weights and degrees of chemical modification.

3. Test toxicity, pyrogenicity, and immunogenicity.

4. Pharmacokinetics.

5. Prepare larger quantities of components for scaling up the preparation of the most promising formulations.

6. Characterize inducers in respect to resistance to nucleases, and in respect to physical properties.

7. Bibliography.

1. U.S. Environmental Protection Agency, Office of Toxic Substances, March 1976.

2. "Poly ICL-CM Dextran: An Interferon Inducer of Reduced Toxicity", Granados, E.N., Dawidzik, J., O'Malley, J., McGarry, M. and Bello, J. (1984) J. Interferon Res. 4, 155-160.

3. "An Assessment of the Potential Use of Anionic Dextrans as a Plasma Substitute", Chang, L.S., Crawford, M.P. and West, M.D. (1980) J. Biomed. Eng. 2, 41-44.

4.a. "Polymers for Blood Replacement", Calvert, P. (1979) Nature 280, 108.

b. "Carboxymethylamylose as a New Plasma Substitute" Plasma Substitute Joint Research Group, (1978) Acta Chimica Sinica, 36, 49-76.

5. "A Modified Polyriboinosinic-polyribocytidylic Acid Complex that Induces Interferon in Primates", Levy, H.B., Zaer, G., Baron, S., Buckler, C.E., Gibbs, C.J., Iadorola, M.J., London, W.T. and Rice, J.A. (1975) J. Infect. Dis. 132, 437-439.

6. "Ueber die Einwirkung von Aromatischen Aminen auf acetylcitronsaureanhydrid", Klingemann, G. (1894) Chemische Ber. 22, 983-987.

8. Glossary.

IFN:	Interferon
IC:	PolyI·PolyC, Poly IC
PLL:	Poly(L-lysine)
ICL:	Complex of IC with PLL
ICLC:	Complex of IC with PLL and CMC
CM:	Carboxymethyl
CMC:	Carboxymethylcellulose
AcCitPLL	Acetylcitryl derivative of PLL
AcCitGel:	Acetylcitryl derivative of gelatin
T _m :	Melting, or transition, temperature
CMamylose:	Carboxymethyl amylose
ICL-CMamylose:	Complex of IC, PLL and CMamylose
CMdextran:	Carboxymethyl dextran
ICL-CMdextran:	Complex of IC, PLL and CMdextran
ICL-AcCitPLL:	Complex of IC, PLL and AcCitPLL
ICL-AcCitGel:	Complex of IC, PLL and AcCitGel
U:	Units of interferon
CBZ:	Carbobenzyloxy
A:	Absorbance (spectrophotometer)
RNase:	Ribonuclease
RVFV	Rift Valley Fever virus
kDa:	Molecular weight in thousands

DISTRIBUTION LIST

4 copy Commander
US Army Medical Research Institute of
Infectious Diseases
ATTN: SGRD-UIZ-M
Fort Detrick, Frederick, MD 21701-5011

1 copy Commander
US Army Medical Research and Development Command
ATTN: SGRD-RMI-S
Fort Detrick, Frederick, Maryland 21701-5012

2 copies Defense Technical Information Center (DTIC)
ATTN: DTIC-DDAC
Cameron Station
Alexandria, VA 22304-6145

1 copy Dean
School of Medicine
Uniformed Services University of the
Health Sciences
4301 Jones Bridge Road
Bethesda, MD 20814-4799

1 copy Commandant
Academy of Health Sciences, US Army
ATTN: AHS-CDM
Fort Sam Houston, TX 78234-6100

SUPPLEMENTARY

INFORMATION



DEPARTMENT OF THE ARMY
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
FORT DETRICK, FREDERICK, MD 21702-5012



REPLY TO
ATTENTION OF
SGRD-RMI-S (70-1y)

ERRATA

05 AUG 1994

MEMORANDUM FOR Administrator, Defense Technical Information
Center, ATTN: DTIC-HDS/William Bush,
Cameron Station, Building 5, Alexandria, VA
22304-6145

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research, Development, Acquisition and Logistics Command (USAMRDALC) (Provisional), has reexamined the need for the limited distribution statement on technical reports for Contract No. DAMD17-87-C-7111. Request the limited distribution statement for AD No. [REDACTED] ADB134571 be changed to "Approved for public release; distribution unlimited," and a copy of these reports be released to the National Technical Information Service.

2. Point of contact for this request is Mrs. Judy Pawlus,
DSN 343-7322.

ERRATA -

Pamela C. Leil MS, MS
for CAREY O. LEVERETT
LTC, MS

Deputy Chief of Staff for
Information Management

AD-B/34571

151.20/14