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Biochemical Characterization Of Molluscum Contagiosum Virus.

Henry Bernard Bradford Jr
University of Alabama at Birmingham

BIOCHEMICAL CHARACTERIZATION OF MOLLUSCUM CONTAGIOSUM VIRUS

by

HENRY BERNARD BRADFORD, JR.

A DISSERTATION

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I. INTRODUCTION

Molluscum contagiosum is a disease of the epidermis of man. Most lesions are localized raised areas of hypertrophied infected cells. The generally accepted causative agent is a virus with a seemingly complex structure. In the current classification scheme of animal viruses, molluscum contagiosum has been placed in the "ungrouped" or tumor subgroup of the poxviruses. Among the characteristics prompting this placement are the relatively large size (230-275 nm), the complex structure, cytoplasmic inclusion formation, and hypertrophy of the cells in naturally occuring infections. The virus contains DNA as indicated by chemical analysis and positive Feulgen staining of mature inclusions (Postlethwaite, 1970).

Detailed studies of molluscum contagiosum virus have not been possible in the past because of the inability to successfully propagate the virus in a non-human host system. However, the recent isolation and serial propagation of several strains of virus in cell culture should enable the undertaking of basic studies of biophysical and biochemical characteristics. A more complete understanding of the properties of the virus would enable a more valid judgment to be made on the classification of molluscum virus with the poxvirus group.

The purpose of this study was the initiation of the basic characterization of molluscum contagiosum virus. Initial experimentation was designed to establish a growth kinetics pattern and to investigate the most efficient means of concentrating large volumes of virus by

means of protein precipitating agents. Experiments were conducted revealing the molecular weights of resolved viral polypeptides and establishing the presence of glycoproteins and lipid in the virus structure. Attempts to solubilize outer portions of the virus aided in the location of some of the polypeptides. Finally, the sequential synthesis of polypeptides in infected cells was examined.

II. HISTORICAL

Molluscum contagiosum has a long history dating back to the initial recognition of the disease by Bateman in 1817. Henderson (1841) and Patterson (1841) (from van Rooyen and Rhodes 1940) emphasized the infectious nature of molluscum lesions and drew attention to the large, oval inclusions in infected cells. The first indication of the viral etiology of the disease was supplied by Juliusberg in 1905 (from van Rooyen and Rhodes 1940), who demonstrated that Chamberland filtrates of human lesion suspensions were capable of producing typical skin lesions in volunteers. Similar volunteer studies later undertaken by Wile and Kingery (1919) and Findlay (1930) confirmed the infectiousness of Chamberland and Berkefeld filtrates of lesion extracts.

The large cytoplasmic inclusions characteristically present in infected human epithelial cells were found by Lipschutz in 1911 (from van Rooyen and Rhodes 1940) to contain granular elements, which now are accepted as the etiologic virus. In the two classic microdissection experiments of Goodpasture and Woodruff (1931) and van Rooyen (1938), it was demonstrated that digestion of molluscum inclusion with trypsin resulted in the liberation of a sticky gelatinous matrix in which were embedded the granules described by Lipschutz. The removal of inclusions from cells by microdissection created a large cytoplasmic cavity suggesting that the intracytoplasmic area had been walled off. Rake and Blake (1950) undertook a histochemical study of human material and traced development of the inclusion body. They found that

strands of RNA appeared in the cytoplasm early in infection and seemed to coalesce to form trabeculae which eventually constituted demarcated areas of the developing inclusions. Large amounts of DNA appeared in these areas as the inclusions matured. Melnick et al. (1952) confirmed these observations by means of electron microscopy and extended the information by demonstrating that the cytoplasms differentiated into viral factories having a "provirus" and later containing the mature virion. Banfield and Brindley (1959) clearly showed the demarcation between the nucleus and the inclusion body. Charles (1960), Williams et al. (1962), Middlekamp and Munger (1964), and Noyes (1965) provided additional electron microscopy data on the replication stages of molluscum virus in human epidermal lesions, and confirmed that the virus has a complex structure and is approximately 230-275 nm in diameter.

Over a period of nearly fifty years many unsuccessful attempts were made to infect a wide variety of animals, embryonated hen's eggs, and human skin grafted onto the chorioallantois of hen's eggs. However, the increasing usefulness of cell cultures in virus experimentation during the past two decades has provided considerable encouragement in the search for an experimental host system susceptible to molluscum virus. While the results of reported studies on the use of cell culture have failed to satisfy the required criteria for assessing continued serial propagation of the agent, they have revealed interesting aspects of virus-cell interaction. Dourmashkin and Febver (1958) exposed HeLa cells to suspensions of molluscum lesions and obtained electron photomicrographs of cytoplasmic inclusions containing virus like particles. In later experiments by Dourmashkin and

Bernard (1959) electron dense bodies also were observed in the nuclei of exposed HeLa cells and human epithelial cells naturally infected with molluscum contagiosum virus. It is noteworthy, from the standpoint of relationship to the poxviruses, that similar structures have not been detected in nuclei of cells infected with vaccinia, fowlpox, ectromelia or fibroma virus (Morgan et al., 1954). Several subsequent reports (Chang and Weinstein, 1961; Neva, 1962; Raskin, 1963; LaPlaca et al., 1967) have confirmed that molluscum suspensions indeed are capable of inducing cytopathic changes in a variety of cell cultures with the most prominent alterations occurring in primary human amnion cells. The cytopathic changes were manifest by the appearance of large cytoplasmic inclusions but were not reproducible beyond the fifth or sixth serial passage. The finding by Neva (1962) that the activity of molluscum suspensions is destroyed by heating at 56°C for 30 minutes and by exposure to human anti-molluscum serum or rabbit anti-molluscum serum suggests that the cytopathic effects induced in cell culture were not entirely the result of a simple viropexic phenomenon.

The mechanism of the cytopathic effects of molluscum virus in cell culture has been studied by several investigators, and Burnett and Neva (1966) postulated that the activity may be mediated by a phenomenon acting at the cell surface. However, in electron microscopy studies by Robinson et al. (1969) and Prose et al. (1969) with "infected" chick embryo cell cultures and explants of human skin naturally or experimentally infected with molluscum virus, considerable support was found for the argument that the cytopathic changes may be related to an incomplete virus uncoating process. On the basis of excellent electron photomicrographs which showed the intracellular

removal of only a portion of the viral coat, these workers considered that molluscum virus may undergo a two-stage uncoating process resembling that discussed by Woodson (1968) for certain of the poxviruses. Thus, failure of the second stage of the uncoating process to occur would prevent the release of the active viral genome and account for its inability to code for the synthesis of new virus. This concept, if substantiated in a permissive cell line, might explain the inability of LaPlaca et al. (1967) to demonstrate cross reactivation of vaccinia virus in cells exposed to molluscum virus suspensions.

In consideration of these data, it would appear that some degree of biochemical activity is associated with the cytoplasmic changes in cells exposed to molluscum virus. This also is suggested by the work of Postlethwaite (1964) in which molluscum lesion suspensions were found to induce resistance in mouse embryo cells to subsequent infection with vaccinia, herpes simplex, and encephalomyocarditis viruses. It remains to be determined whether this resistance is attributable to an interferon-like material or is an example of intrinsic type of interference.

The inability to successfully propagate molluscum contagiosum virus in an experimental host system has been largely responsible for the paucity of information regarding the biophysical and biochemical characteristics. Only two such studies have been reported in the literature. Employing purified preparations derived from human skin lesions, Pirie et al. (1971) found that the virus has buoyant density of 1.288 gm/cm^3 and a sedimentation coefficient of 5250 $S_{20,w}$. It was calculated that the individual viral particle contains approximately 4×10^{-15} gram of protein and DNA accounts for 6.7% of the total viral

mass. In an attempt to study the polypeptides synthesized during infection, Bernardini et al. (1973) exposed cultures of human embryonic fibroblastic cells to lesion virus in the presence of ^{14}C labelled amino acids. He was unable to demonstrate any difference between "infected" and uninfected cells.

III. MATERIALS AND METHODS

Cell Culture and Media

The strain FL cell employed in this study originally was derived from a primary culture of human amnion cells (Fogh and Lund, 1957). The cells were grown in Eagle's basal medium containing 10% heated calf serum, 2X concentration of vitamins and glutamine, and 0.5% lactalbumin enzymatic hydrolysate. Penicillin and streptomycin were added to give a final concentration of 200 units of 100 μg per ml, respectively. Serum content of the medium was reduced to 5% for maintenance of monolayer cell cultures. Unless otherwise dictated by experimental requirements, the medium was replaced every three of four days in order to maintain cells in an optimum physiological condition.

Stock cell cultures were propagated in 200 ml milk dilution bottles at 36°C. When a confluent monolayer cell growth was obtained, usually on the fourth or fifth day of incubation, the medium was removed and 5.0 ml of either 0.05% trypsin (Difco 1:250) in Hanks' balanced salt solution or 0.05% disodium salt of ethylenediaminetetraacetic acid in phosphate-buffered saline (PBS) (0.16M NaCl, 0.01M PO_4, 0.003M KCl, pH 7.4) was added to each bottle. After incubation at 36°C for a few minutes for detachment of cells from the glass surface, the cells were sedimented by low speed centrifugation and resuspended in an appropriate volume of growth medium for dispensing into culture bottles. In the transferring of cells each monolayer bottle culture usually could be subdivided at a ratio of three to one.

During the course of this investigation stock cell cultures were tested at regular intervals for the presence of mycoplasma by methods recommended by the National Communicable Disease Center (Crawford, 1966). When contamination was detected by culture or suspected on the basis of changes in microscopic appearance, the cells were not used in experimental work.

Virus

The 876 strain of molluscum contagiosum virus chosen for study was one of five similar strains isolated in this laboratory by Dr. Robert D. Francis from human skin lesions. These isolations were made in WI-38 strain of human embryonic lung cells. After only one passage in WI-38 cells, the 876 virus strain proved capable of replication in FL cells. Virus preparations used here were from FL cell passages 28 through 57. During the period of these passages it was observed that the virus replication cycle decreased by approximately three hours with no significant alteration in virus yield.

Standard virus pools were prepared by inoculation of monolayer bottle cultures with 0.5 ml quantities of virus diluted to contain approximately 2.5×10^5 plaque forming units (PFU). After incubation at room temperature for one hour for adsorption of virus to cells, 10 ml of maintenance medium were added to each bottle and incubation continued at 36°C. When at least 75% of the infected cell monolayer had undergone degeneration, in from fifteen to eighteen hours, the cultures were harvested by freezing at -62°C. The cultures then were thawed, centrifuged at 500xg for ten minutes to remove cell debris, and the virus containing supernatant dispensed in 0.5 ml aliquots into

rubber stoppered tubes and stored at -62°C. Once a tube of standard virus was removed from storage, it was never refrozen and used in quantitative work.

Virus Titration

The relative amount of virus in standard pools and other experimental materials was determined by infectivity titrations performed by the plaque assay method. Increasing serial ten-fold dilutions of virus were made in cold PBS, and 0.25 ml of each dilution was added to each of two plastic petri dishes (60 x 15 mm) containing a confluent monolayer of FL cells from which the growth medium had been removed. After incubation at room temperature for one hour for adsorption of virus to cells, 7 to 10 ml of overlay maintenance medium containing 1.5% methylcellulose (4000 centipoise) were added to each dish. Several uninfected cultures were included with each series of titrations and served as cell controls. All cultures were incubated in a 5.0% CO_2 atmosphere at 36°C and examined daily for the development of plaques. Maximum plaque size (0.5mm) was reached on the fifth day, at which time the plaques were fixed and stained overnight with 7.0% buffered formalin (pH 7.4) containing 1.0% methylene blue before removal of the overlay and counting. Virus titers were expressed as plaque forming units (PFU) per ml.

Concentration and Purification of Virus

When the volume of virus containing tissue culture fluid was 100 ml or less, the virus was concentrated by sedimentation in a 40.3 fixed angle Spinco rotor at 44,680xg for one hour. However, for larger quantities of virus a more practical and convenient method of

concentration was desired. The reported usefulness of polyethylene glycol (McSharry, 1970) and zinc acetate (Smolen et al. 1955; Metcalf, 1957) in the concentration of other DNA and RNA viruses without significant loss of infectivity suggested their possible application in the present investigation. A comparison of the two chemicals revealed that, while both were capable of precipitating molluscum virus, certain advantages were associated with the use of zinc acetate.

To pooled infected tissue culture fluid, freed of cell debris by centrifugation at 500xg for ten minutes, was added a solution of 30% polyethylene glycol (PEG) (molecular weight 6000-7500) in PBS to give a final concentration of 6.0% PEG. The material was divided into six equal portions and the respective sodium chloride concentrations adjusted to 0.15, 0.20, 0.40, 0.50, 0.60 and 0.80M. After incubation overnight at 7°C the precipitated mixtures were centrifuged at 1500xg for thirty minutes, supernatants removed, and the sediments resuspended to original volume in distilled water. The relative amount of infectious virus present in the untreated culture fluid, supernatants, and resuspended sediments was determined by the plaque assay method.

Clarified infected culture fluid also was used in zinc acetate precipitation experiments. In certain instances, the sedimented cell debris was washed one or more times with PBS in an attempt to increase virus recovery. These washings were added to the original clarified material and virus then precipitated by the addition of zinc acetate to a final concentration of 0.02M. During precipitation the virus preparation was maintained at pH 7.0-7.2 by the addition of 2.0 M sodium hydroxide solution. After incubation overnight at 7°C the precipitated virus was sedimented at 1500xg for thirty minutes, super-

natant removed, and the sediment dissolved in a minimum volume of saturated solution of disodium salt of ethylenediaminetetraacetic acid in water buffered at pH 8.0 by addition of crystalline Tris base. Final pH of the concentrated virus was adjusted to 7.2 with 0.1N HCl whenever necessary.

Crude virus concentrates prepared by ultracentrifugation or zinc acetate precipitation were centrifuged at 44,680xg for one hour and the sedimented virus resuspended in 0.01M Tris-HCl buffer, pH 7.2, containing 0.001M disodium salt of ethylenediaminetetraacetic acid and 5% sucrose. The virus was centrifuged through a discontinuous sucrose gradient (20%, 30%, 40% sucrose in 0.01M Tris-HCl buffer) using a SW-39 rotor at 68,500xg for one hour. This step was repeated using a gradient containing 20%, 30%, 40% and 45% sucrose. Purified virus pellets were resuspended in 2-3 ml distilled water and stored at -62°C. The relative protein content of purified virus preparations and other experimental materials was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Preparation of Isotope Labelled Virus

Confluent monolayer bottle cultures of FL cells were washed three times with Hanks' balanced salt solution and incubated at 37°C for thirty minutes with freshly added Hanks' solution to deplete the intracellular pool of amino acids. To each washed culture was added 7.5 ml of isotope medium and, after further incubation at 36°C for thirty minutes, approximately 10^6 PFU of standard virus were inoculated. The isotope medium consisted of 25% Eagle's basal medium, 65% Hanks' solution, and 10% dialyzed fetal calf serum and contained either 0.5

microcurie per ml of ^{14}C amino acids or 5.0 microcuries per ml of ^{3}H amino acids from protein hydrolysate.

In the labelling of virus with radioactive sugars or amino sugar, cell cultures were treated in the same manner as described for amino acid labelling, except glucose-free PBS was substituted for Hanks' solution. Isotope medium contained either 1.1 microcuries per ml (UL^{14}C) D-galactose (Specific Activity 32 mCi/mM), 5.0 microcuries per ml (1, 5, 6-^{3}H) L-fucose (Specific Activity 3.73 Ci/mM, 1.0 microcurie per ml (1-^{14}C) D-mannose (Specific Activity 52.7 mCi/mM), or 3.0 microcuries per ml (1-^{3}H) D-glucosamine (Specific Activity 12 Ci/mM).

Infected cultures were harvested and isotope labelled virus concentrated and purified as described in previous sections.

Isotope Labelling of FL Cell Proteins

Cultures were prepared by seeding each culture with approximately 4.0×10^5 cells previously washed in Hanks' balanced salt solution. The growth medium employed consisted of 50% Eagle's basal medium, 40% Hanks' solution, 10% dialyzed fetal calf serum, and 3.5 microcuries per ml of ^{14}C amino acids from protein hydrolysate. Confluent monolayer cultures were washed in four changes of Hanks' solution and the isotope labelled cells harvested by treatment with disodium salt of ethylenediaminetetraacetic acid. After additional washing in Hanks' solution by three cycles of low speed centrifugation, the pooled cells were resuspended in 0.01M Tris-phosphate buffer (pH 7.2) to a concentration of 4.0×10^6 per ml and ruptured in a Tenbroeck type cell grinder. Macrocellular components were sedimented by centrifugation at 1500xg for ten minutes, and the supernatant containing isotope

labelled proteins removed and stored at -20°C. Hereafter, this material will be referred to as "labelled cell sap" for purposes of clarity.

Pulse Labelling of Virus

Two types of pulse labelling experiments were performed in which the replicating virus was exposed to radioactive isotope for short periods of time. The method of washing cell cultures and composition of isotope medium containing ^{3}H amino acids from reconstituted protein hydrolysate were the same as described for the isotope labelling of virus.

In the first experiment chilled cell cultures were infected and incubation continued for one hour at 7°C for adsorption of virus. The cultures then were placed at 36°C and the infection allowed to proceed until the end of the pulse period. At this time the isotope medium was removed, the cells thoroughly washed with Hanks' balanced salt solution, and prewarmed maintenance medium added. Incubation was continued at 36°C until complete degeneration of infected cell monolayers had occurred. The progeny virus was harvested, concentrated, and purified as previously described.

In the second experiment the method of pulse labelling virus was a modification of that used by Esteban and Metz (1973) for vaccinia virus. Monolayer cell cultures were infected and incubated for one hour at 7°C, and then transferred to 36°C. At zero, twenty, thirty or sixty minute intervals at 36°C, three to four cultures were pulsed with prewarmed isotope medium for periods ranging from twenty to sixty minutes. Infected cells from each serial pulse period were chilled, washed in

cold PBS, detached from the glass surface, and processed as described for the isotope labelling of FL cell proteins.

Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate-discontinuous polyacrylamide gel electrophoresis (SDS-PAGE) was used for the characterization of polypeptides of molluscum contagiosum virus. This approach has proved to be of considerable value in elucidation of the major groups of animal viruses. A modification of the method described by Maizel (1971) was employed in this investigation.

Stock solutions of reagents were prepared in the following manner: highly purified acrylamide and N,N' methylene-bis-acrylamide were dissolved in double distilled water at a concentration of 30% and 1%, respectively; gel buffer was 3.0 M Tris-HCl at pH 8.0; purified sodium dodecyl sulfate (SDS) and ammonium persulfate were made to 10% solutions in double distilled water with the latter solution being replaced every two weeks; N,N,N',N'' tetramethylethylenediamine (TEMED) was stored as a concentrated solution at room temperature in the dark. The electrode buffer consisted of 0.381M glycine, 0.049M Tris base, and 0.1% SDS in double distilled water and was replaced after each electrophoretic run. Dissociating solution contained 4% SDS and 40% sucrose in 0.02M Tris-phosphate buffer, pH 7.1.

The resolving gels were prepared in glass tubes 28 cm long and 4 mm inside diameter. Before use the clean tubes were treated with a dilute solution of Siliclad, rinsed in Photo Flo and dried. A solution of acrylamide with a final concentration of 3.5%, 7.5%, 9.0% or 12.0% in 0.375M Tris-HCl buffer, 0.2% SDS, 0.005% TEMED and 0.05%

ammonium persulfate was dispensed into the tubes to a height of 20 cm and overlaid with distilled water to a depth of 2 cm. The acrylamide solution was allowed to polymerize at room temperature for approximately twenty minutes until a sharp boundary developed between the water layer and gel. After polymerization the gels were sealed in parafilm and stored at 7°C for no longer than two weeks.

Aqueous preparations of materials to be electrophoresed were mixed with an equal volume of dissociating solution and sufficient 2-mercaptoethanol added to give a final concentration of 0.02%. The mixtures were incubated at room temperature for thirty minutes with gentle agitation and then heated in a boiling water bath for 2.5 minutes. After cooling the pH was adjusted to 7-8 by the addition of crystalline Tris base, and a trace of phenol red added as an internal marker for visually determining the maximum allowable period of electrophoresis. The water overlay on gels was replaced with electrode buffer and from twenty to one hundred microliters of dissociated sample layered on top of each gel by means of a small glass pipette. In each instance the volume of sample used contained approximately 100 μg of protein.

Electrophoresis of dissociated samples was conducted in two stages. The initial stage was performed with 0.5 milliamp constant current per gel for thirty minutes to permit uniform entry of sample. In the final stage the current was increased to 1.5 milliamps per gel for 4.5 hours.

Processing of Electrophoresed Gels

Gels were removed from the glass tube holders and stained or

sectioned for radioactivity determination. In locating the position of migrated polypeptides by tinctorial means the gels were fixed overnight in 15% trichloroacetic acid, washed for twenty-four hours in several changes of a solution containing 10% acetic acid and 25% isopropyl alcohol in distilled water, and stained for eighteen to twenty-four hours in acetic acid-isopropyl alcohol washing solution containing 0.04% Coomassie Blue. Gels were destained with several changes of a solution of 10% acetic acid and 10% isopropyl alcohol in water over a forty-eight hour period and then stored in 10% acetic acid (Fairbanks et al. 1971).

Lipid material of dissociated virus was demonstrated by incubating 3.5% electrophoresed gels for eighteen hours at 36°C in a solution containing equal parts of saturated Oil Red O in methanol and 10% trichloroacetic acid. Stained gels were washed, rehydrated in distilled water, and stored in 10% acetic acid (Beaton et al., 1961). Acrylamide gels of the same concentration in which dissociated bovine albumin and human plasma had been simultaneously electrophoresed were included as negative and positive controls for lipid staining.

In radioactivity determinations, the electrophoresed gels were placed at -62°C for twenty minutes and then sectioned while frozen with an apparatus equipped with razor blades spaced approximately 1.5 mm apart. Each section was placed in a separate glass scintillation vial and completely digested at 60°C for two hours with 0.2 ml of 30% hydrogen peroxide and 0.1 ml of 72% perchloric acid. After neutralizing the residual perchloric acid with 0.12 ml of 30% potassium hydroxide solution, 5 ml of ethanol were added to maintain miscibility of the sample, and 10 ml of scintillation fluid (7.45 gms 2,5-diphenyl-

oxazole, 0.299 gm 1,4-bis-[2-(5 methyl-5-phenyloxazolyl)]-benzene/liter of toluene) added. Vials were incubated overnight at 7°C and the radioactivity determined on a Packard liquid scintillation spectrophotometer.

Estimation of Molecular Weights

The relative molecular weights of viral polypeptides were determined by comparing their mobilities with those of proteins of known molecular weight using acrylamide gel concentrations of 7.5%, 9.0%, and 12.0% as described by Shapiro et al. (1967). The purified proteins employed were human gammaglobulin (167,000 daltons), bovine albumin (67,000 daltons), ovalbumin (45,000 daltons), chymotrypsinogen A (25,000 daltons), and cytochrome C (12,500 daltons). Gels containing a mixture of purified proteins (100 µg total protein/gel) were electrophoresed in parallel with gels containing dissociated virus. The relative mobility (Rf) of each of the known molecular weight proteins present in the mixture was determined by using the position of the migrated phenol red internal marker as a reference point. When the Rf was plotted against the log of the molecular weight, a straight line function graph was obtained as shown in Figures 1, 2 and 3. Molecular weights of viral polypeptides were estimated by calculating the Rf values and applying these values to the graphs prepared with purified proteins. It has been well documented that most polypeptide migrations are inversely proportional to the molecular weight.

Figure 1. Plot of the relative migration versus the logarithmic scale of known molecular weight proteins electrophoresed in 7.5% acrylamide gel.

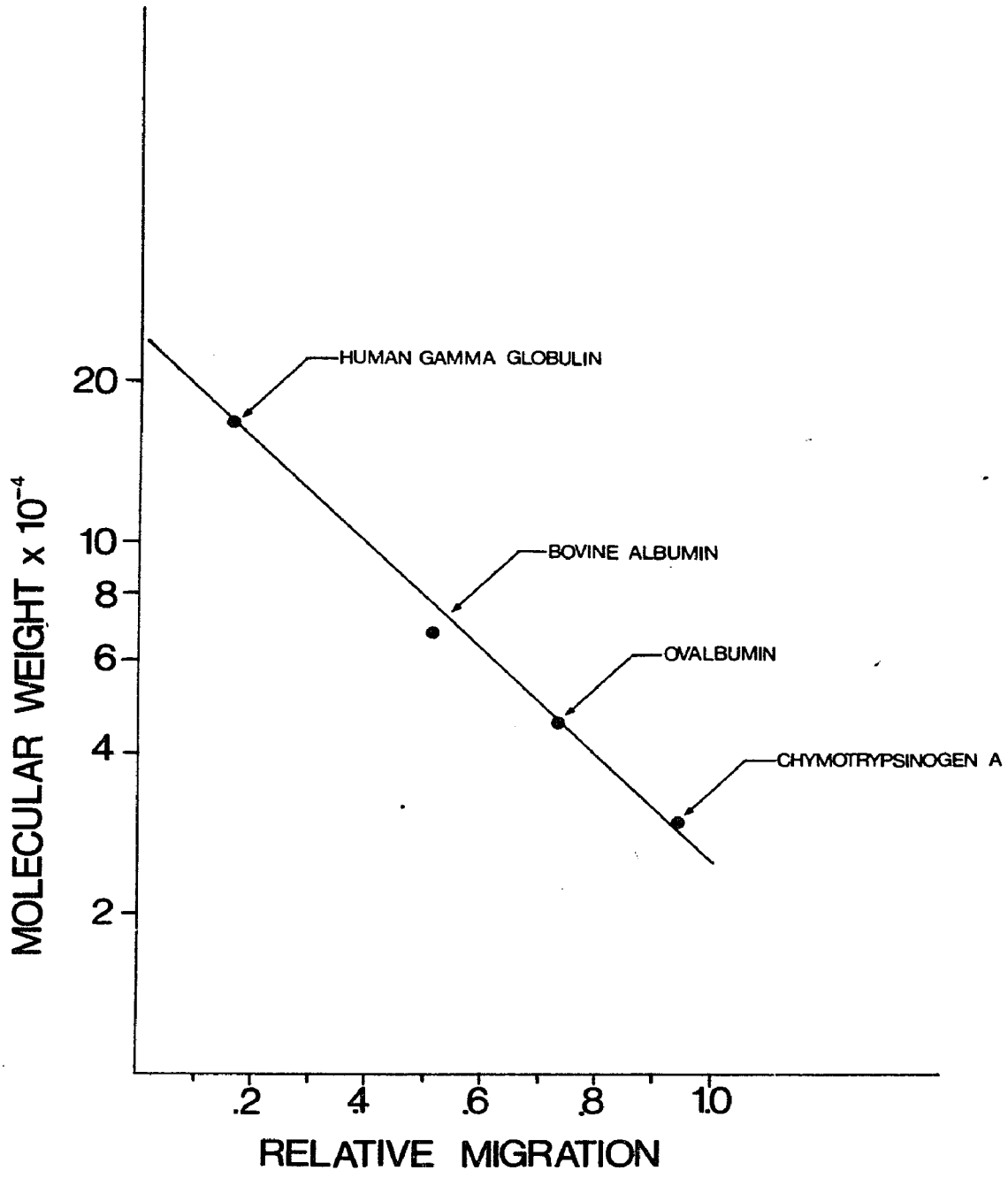

HUMAN GAMMA GLOBULIN
BOVINE ALBUMIN
OVALBUMIN
CHYMOTRYPSINOGEN A
MOLECULAR WEIGHT x 10^{-4}
20
10
8
6
4
2
.2
.4
.6
.8
1.0
RELATIVE MIGRATION

Figure 2. Plot of the relative migration versus the logarithmic scale of known molecular weight proteins electrophoresed in 9.0% acrylamide gel.

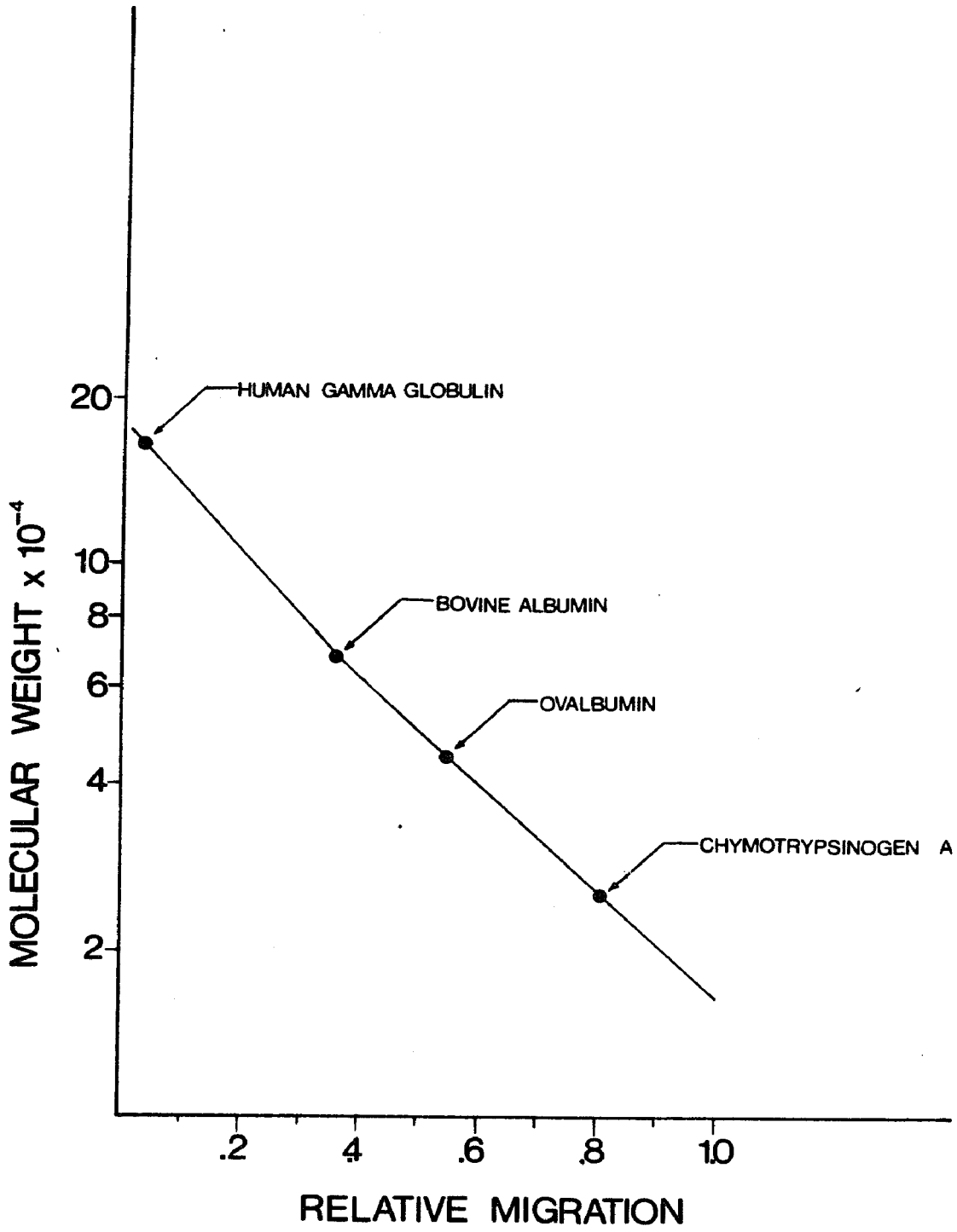
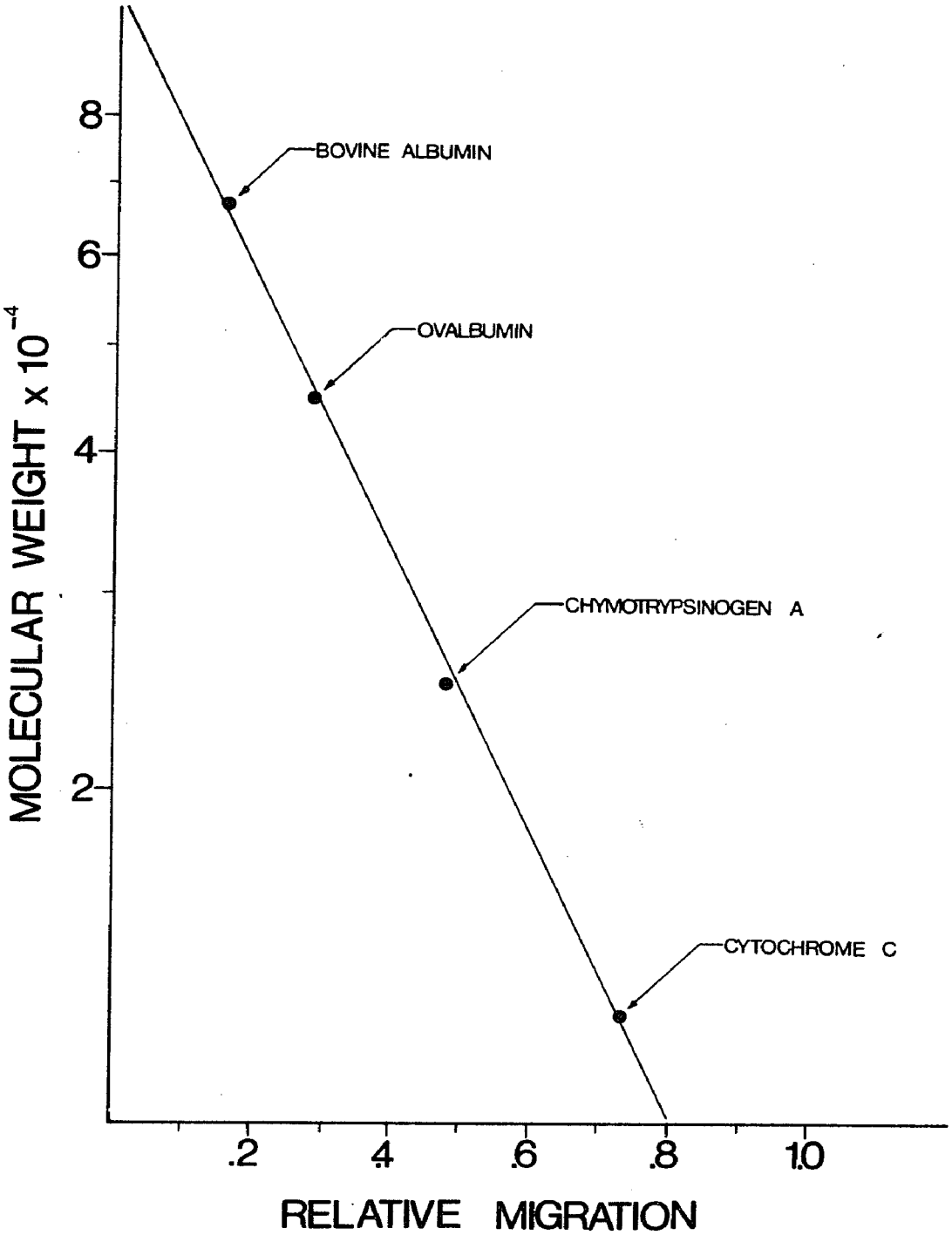

HUMAN GAMMA GLOBULIN
BOVINE ALBUMIN
OVALBUMIN
CHYMOTRYPSINOGEN A
MOLECULAR WEIGHT x 10^{-4}
20
10
8
6
4
2
.2
.4
.6
.8
1.0
RELATIVE MIGRATION

Figure 3. Plot of the relative migration versus the logarithmic scale of known molecular weight proteins electrophoresed in 12.0% acrylamide gel.


MOLECULAR WEIGHT x 10^{-4}
8
6
4
2
BOVINE ALBUMIN
OVALBUMIN
CHYMOTRYPSINOGEN A
CYTOCHROME C
.2
.4
.6
.8
1.0
RELATIVE MIGRATION

Extraction of Virus with Non-Ionic Detergents and Cesium Chloride

Purified ^{3}H amino acids and ^{14}C D-galactose labelled virus preparations were treated with Nonidet P-40 (NP-40), Triton X-100, and cesium chloride in a effort to selectively remove some of the outer viral structural components.

A solution of 1.0% NP-40 in 0.002 M Tris-HCl buffer, pH 8.0, was mixed with an equal volume of isotope labelled virus and incubated for two hours at 37°C. Crystalline dithiothreitol then was added to a final concentration of 0.01 M and incubation continued at room temperature for one additional hour. Treated virus was centrifuged in a SW-39 Spinco bucket rotor at 68,500xg for one hour and the supernatant was removed and dialyzed against several changes of distilled water at 7°C for forty-eight hours. The dialysate was concentrated by packing the dialyzing tubing in powdered polyethylene glycol 6000.

Labelled virus was mixed with an equal volume of Triton X-100 in 0.02 M Tris-HCl buffer, pH 7.9, containing 0.02 M disodium salt of ethylenediaminetetraacetic acid. After incubation at room temperature for two hours, the material was centrifuged at 68,500xg for one hour.

A solution of cesium chloride having a density of 1.70 gms/cc, as determined by refractometry, was prepared in 0.01 M Tris HCl buffer, pH 7.4, containing 0.001 M disodium salt of ethylenediaminetetraacetic acid. To this solution was added a sufficient volume of labelled virus to reduce the concentration of cesium chloride to 1.39 gms/cc. The mixture was incubated at 7°C for forty eight hours, diluted ten-fold with distilled water, and centrifuged at 68,500xg for one hour.

The supernatant containing extracted structural components and the sediment consisting of extracted viral particles, obtained by ultracentrifugation of labelled virus after treatment with the detergents or cesium chloride, were dissociated and analyzed by acrylamide gel electrophoresis.

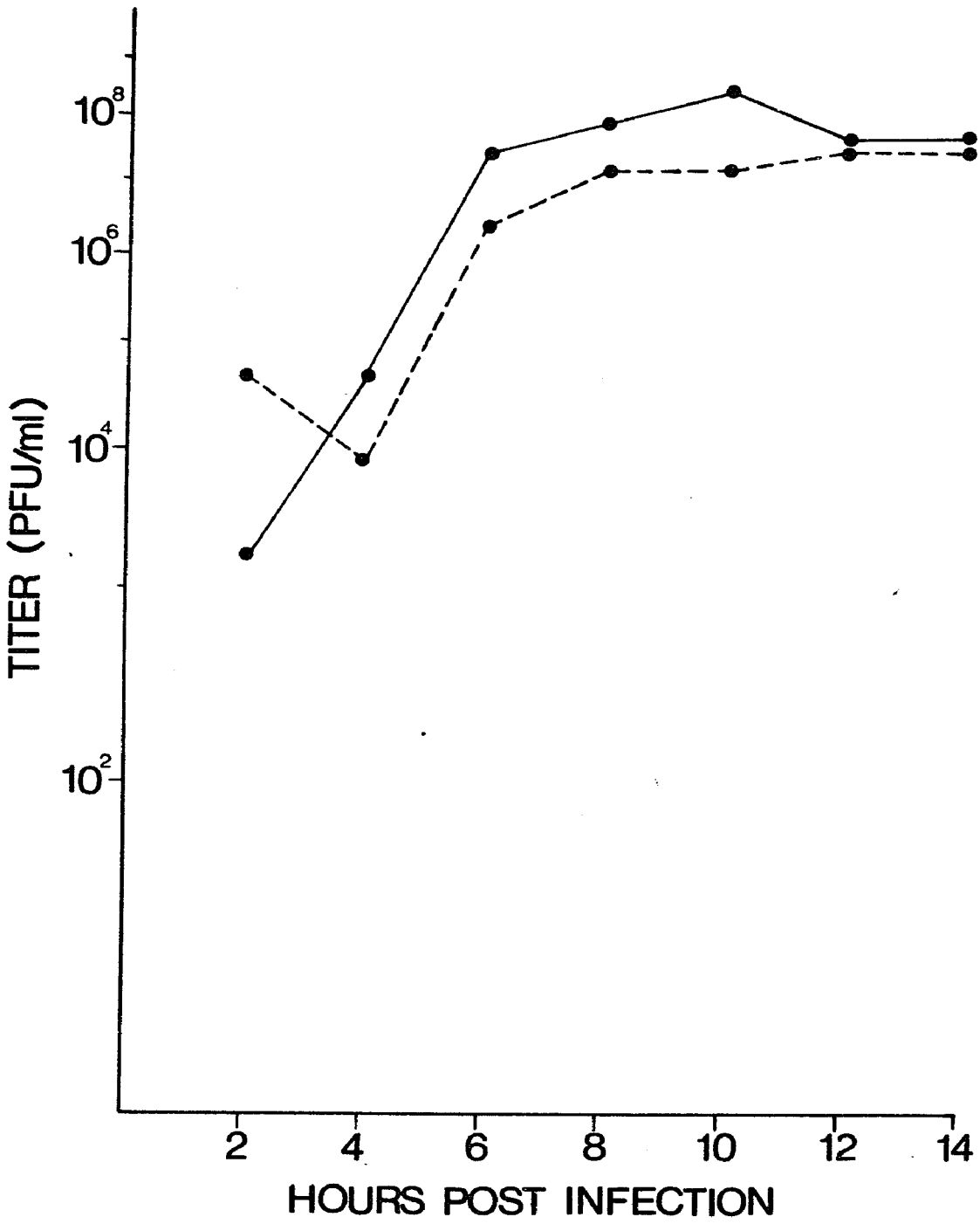
IV. RESULTS

Growth Kinetics of Virus

At the onset of this investigation the only information available regarding the growth characteristics of molluscum contagiosum virus was the quantity of infectious virus progeny released in degenerated infected FL cell cultures. It was considered necessary, therefore, to study in greater detail the kinetics of virus replication before proceeding to other planned phases of this project.

A series of replicate FL cell cultures were prepared and inoculated with a quantity of standard virus calculated to give a multiplicity of infection of approximately two. After incubation for one hour at 37°C the unadsorbed virus was removed by repeated washings with PBS and incubation continued in the presence of fresh medium. Cultures were harvested at two-hour intervals post-infection and the amount of infectious virus present in culture fluid and in cells was determined after separation by centrifugation at 700xg. The results presented in Figure 4 represent a composite of four growth curve experiments. It will be noted that the appearance or increase of intracellular virus preceded the release of extracellular virus by approximately two hours. Newly formed intracellular virus was detected as early as two hours and synthesis proceeded at an exponential rate for six hours post infection. Although not shown, approximately 75% of the infected cells were exhibiting cytopathic changes by ten hours, a period after which no further increase in the titer of

Figure 4. Growth kinetics of molluscum contagiosum virus in FL cells. (---) extracellular virus, (——) intracellular virus.


TITER (PFU/ml)
10^8
10^6
10^4
10^2
2
4
6
8
10
12
14
HOURS POST INFECTION

intracellular virus occurred. Between two and four hours there was nearly a ten-fold decrease in titratable extracellular virus and probably represents delayed adsorption of residual infecting virus not removed by washing. There was a marked increase in extracellular virus between four and six hours but, thereafter, the rate of release gradually plateaued until peak titer was reached at ten hours post-infection. The demonstration of comparable titers of extracellular and intracellular virus at the end of the twelve to fourteen hour replication cycle clearly indicates that a substantial amount of molluscum virus remained associated with degenerated cells.

Concentration and Purification of Virus

In preliminary experiments 0.6M sodium chloride was determined to be the optimum concentration for maximum virus precipitation with 6.0% polyethylene glycol. When the effect of serum on precipitation of molluscum virus was investigated (Table 1), 2.2% of the original infectious virus remained in the supernatants of virus grown in the presence and absence of serum. In the resuspended sediments 39% and 22%, respectively, of the original virus propagated in the presence and absence of serum was recovered.

It seemed possible that the low percentage recovery of virus in the resuspended sediment might be due to inactivation or clumping of the virus by polyethylene glycol. To determine if this was the case, the sediment was resuspended in 1.0 M $MgCl_2$. In unpublished work, Dr. Francis has shown that this chemical has a dispersing effect on molluscum virus suspensions. A 24% increase in titratable virus was found with serum propagated virus but not with virus propagated in

Table 1

Polyethylene Glycol (PEG) Precipitation of Molluscum Contagiosum Virus Grown in the Presence and Absence of Serum

	Serum		No Serum	
Material	PFU/ml[a]	Percent Recovery[b]	PFU/ml	Percent Recovery
Tissue culture fluid after removal of cell debris	2.62×10^7		2.62×10^8	
Supernatant after PEG precipitation	4.66×10^5	2.2	4.66×10^6	2.2
PEG precipitated virus	2.29×10^6	39	4.66×10^7	22

[a] Plaque forming units.
[b] Calculated from original volume.

Table 2

Dispersion with Magnesium Chloride of Polyethylene Glycol Precipitated Virus Grown in the Presence and Absence of Serum

	Serum		No Serum	
Material	PFU/ml[a]	Percent Recovery[b]	PFU/ml	Percent Recovery
Tissue culture fluid after removal of cell debris	2.62×10^7		2.62×10^7	
PEG precipitated virus	2.29×10^6	32	4.66×10^7	18
PEG precipitated virus + 1.0M $MgCl_2$	1.47×10^7	56	4.66×10^7	18

[a] Plaque forming units.
[b] Calculated from original volume.

the absence of serum (Table 2). In another experiment not shown, 90% of the polyethylene glycol precipitated virus grown in the presence of serum was removed by filtration through a 0.8 μ Millipore filter. Only 2.0% loss occurred when virus propagated in the absence of serum was employed. These results indicate that the loss of virus was due to clumping and not inactivation.

When crude cell culture fluid containing virus was centrifuged at 700xg to remove the cell debris, over 50% of the initial titratable virus was lost (Table 3). Precipitation of the virus remaining in the clarified supernatant resulted in a 45% recovery. Further concentration by ultracentrifugation resulted in only a slight loss of titratable virus. To increase the quantity of available precipitable virus, the cell debris was washed with PBS, resedimented, and the supernatant combined with the original clarified culture fluid. The results are shown in Table 4. The precipitation of the pooled material enabled a 27% recovery of initial virus and a similar recovery was noted after ultracentrifugation.

In an effort to substantiate the purity of virus preparations destined for use in biochemical studies, radioactive cell sap from uninfected cells was added to virus suspensions prior to zinc acetate precipitation. The total plaque forming units, protein content, and radioactivity at the various purification stages are shown in Table 5. A drastic reduction in the total protein content and radioactivity of the final purified viral preparation was obtained.

Table 3

Zinc Acetate Precipitation of Molluscum Contagiosum Virus

Material	Volume ml	PFU/ml[a]	Percent Recovery[b]
Crude tissue culture fluid	400	2.98×10^9	
Supernatant after removal of cell debris, cell debris not washed	400	1.49×10^9	47
Zinc acetate precipitated virus	52	4.9×10^9	21.4
44,680xg for one hour pelleted virus resuspended in Tris-HCl EDTA buffer	5.2	4.41×10^{10}	19.2

[a] Plaque forming units.
[b] Calculated from original volume.

Table 4

Zinc Acetate Precipitation of Molluscum Contagiosum Virus after Addition of Cell Debris Washing

Material	Volume ml	PFU/ml[a]	Percent Recovery[b]
Crude tissue culture fluid	750	5.6×10^8	
Supernatant after removal of cell debris, cell debris washed	848	4.4×10^8	88
Zinc acetate precipitated virus	68.5	1.64×10^9	26.7
44,680xg for one hour pelleted virus resuspended in 0.5 M $MgCl_2$	8.0	1.4×10^{10}	26.7

[a] Plaque forming units.
[b] Calculated from original volume.

Table 5

Use of ^{14}C Amino Acid Labelled Cell Sap as an Indicator of Virus Purification

Sample	Total PFU[a]	Total Protein[b]	Total C.P.M.[c]	Percent Recovery		
				PFU	Protein	C.P.M.
Starting material	1.83×10^{11}	1.6×10^{7}				
Original supernatant	7.9×10^{10}	1.15×10^{7}		43.1	72.2	
Pooled supernatant	9.6×10^{10}	1.54×10^{7}		52.5	96.8	
Supernatant with labelled cell sap	2.2×10^{11}	1.2×10^{7}	44,000	123.7	76.4	
Precipitated virus	8.4×10^{10}	3.3×10^{6}	20,672	46.1	20.9	46.3
Highly purified virus		4.0×10^{4}	3,467		.25	7.8

[a] Plaque forming units.
[b] Micrograms of protein.
[c] Counts per minute.

Electrophoresis of Dissociated Viral Polypeptides

Demonstration of Polypeptides by Tinctorial Means

Staining of electrophoresed polypeptides in acrylamide gel with Coomassie Brilliant Blue is both a rapid and relatively sensitive means of detection, and also enables the estimation of molecular weights by comparing the relative migration with that of proteins of known molecular weight. To facilitate the separation of most of the polypeptides, three gel concentrations, 7.5%, 9.0% and 12.0% were used.

The electrophoretic pattern obtained when dissociated virus was electrophoresed in 7.5% acrylamide gel is shown in Figure 5. The migration pattern of the polypeptide bands enables the segregation of the resolved polypeptides into three major groups. Group one was resolved in the first 34 mm of gel and contains eight polypeptides ranging in molecular weight from greater than 218,000 daltons to approximately 160,000 daltons. The second group located in the 42 to 77 mm region of the gel also consists of eight polypeptides with molecular weights ranging from 143,000 to 89,000 daltons. Nine bands, ranging in molecular weight from 71,000 to 29,000 daltons, comprise the third group found in the 97 to 160 region. It was noted in the terminal portion (177 mm) of the gel that a very intense staining band was located in the refractory sieving area just behind the phenol red marker, suggesting the presence of unresolved polypeptides. There appears to be at least one darker staining band in each group and most probably represent major polypeptide constituents.

The results of electrophoresis of dissociated virus in 9% acrylamide gel (Figure 5), while similar to the pattern developed in 7.5%

Figure 5. Photograph of dissociated purified virus electrophoresed in 7.5%, 9.0%, and 12.0% acrylamide gels and stained with Coomassie Brilliant Blue. Left gel (7.5%), Middle gel (9.0%), Right gel (12.0%). Direction of migration from top to bottom.

acrylamide, contain some notable differences. A noticeable amount of dye was retained at the point of origin of migration, verifying the presence of polypeptides too large in molecular weight to enter the gel. The first 30 mm of gel contain six bands of Group I ranging in molecular weight in excess of 132,000 to 122,000 daltons. In the 30 to 62 mm region of the gel are seven polypeptides of Group II with molecular weights ranging between 109,000 to 77,000 daltons. Between 75 to 145 mm of gel fourteen polypeptides were resolved with molecular weights from 69,000 to 33,000 daltons. A lighter staining band, 210 mm from the point of origin, was found just behind a broad intense staining band in the unresolved region and constituted a fourth group in which only one polypeptide (18,000 daltons) was sieved. One band in Group II and four bands in Group III seemed to predominate in stain retention and probably represent major constituents.

The further resolution of Group IV was achieved in 12.0% acrylamide gel as shown in Figure 5. The larger molecular weight polypeptides in excess of 100,000 daltons and assignable to Group I did not enter the gel, as evidenced by the deep staining at the point of origin. Polypeptides in Group II were resolved in the first 20 mm of gel and were estimated to have molecular weights in excess of 79,000 daltons. Sixteen polypeptides of Group III, with molecular weights from 77,000 to 27,000 daltons, were resolved in the 26 to 111 mm region of gel. Four polypeptides with molecular weights ranging from 19,000 to 15,000 daltons were contained in the 140 to 165 mm of gel and constitute Group IV. There was no deep staining band in the area of the phenol red internal marker, suggesting that all polypeptides were

sieved. On the basis of stain retention, three major bands were found in Group III and two major bands in Group IV.

A representative comparison of the electrophoretic patterns of dissociated virus and proteins of known molecular weight is made in Figure 6. By careful measurement and plotting of their relative migration, the molecular weights of the various polypeptides of molluscum virus were calculated, as shown in Table 6.

To ascertain if the previously described dissociating procedure resulted in complete solubilization of molluscum virus, the virus was solubilized with 2% SDS containing 8M urea and electrophoresed in a 9% acrylamide gel containing 6M urea. The close correlation in the electrophoretic patterns and the estimated molecular weights obtained with the two methods of dissociation evidenced that 2% SDS was adequate for complete dissociation.

Determination of Polypeptides of Isotope Labelled Virus

Although a large number of viral polypeptides was revealed in gels stained with Coomassie Brilliant Blue, the possibility existed that minor polypeptides present in low concentration might not be detected by tinctorial means. Therefore, a series of experiments were undertaken in which ^{14}C amino acid labelled virus was dissociated and electrophoresed in 7.5%, 9%, and 12% acrylamide gels.

It will be noted in Figure 7 that at least thirty polypeptide peaks were resolved in 7.5% gel. The distribution pattern of these peaks permits the separation of represented polypeptides into three general molecular groups. Group I is demarcated by fractions 1 through 40 and contains ten or more large molecular weight species,

Figure 6. Photograph of dissociated purified virus and known proteins electrophoresed in 9.0% acrylamide gel and stained with Coomassie Brilliant Blue. Left gel (dissociated virus), Right gel (known proteins).

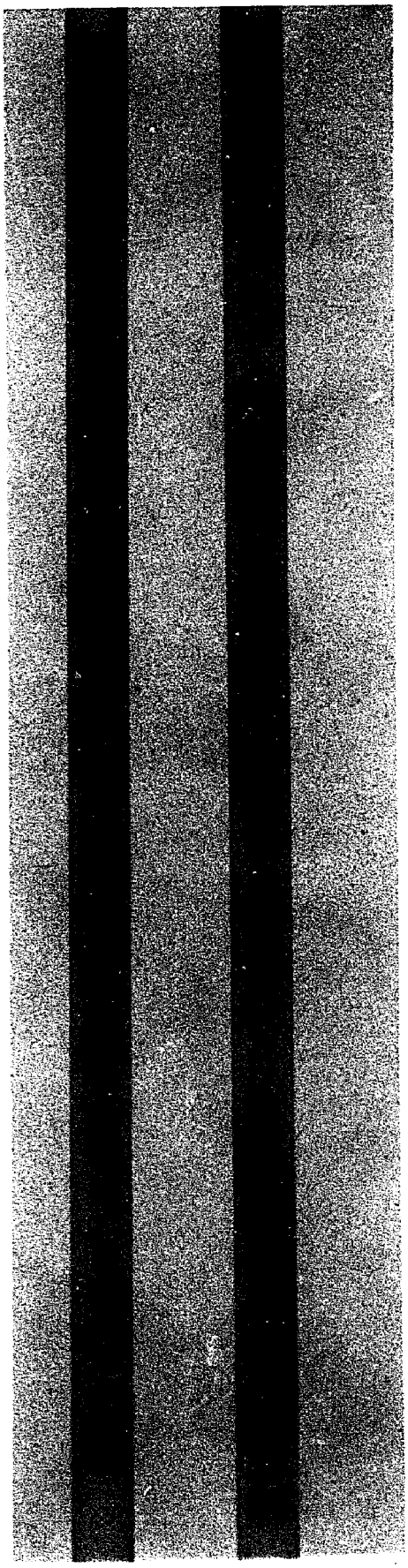
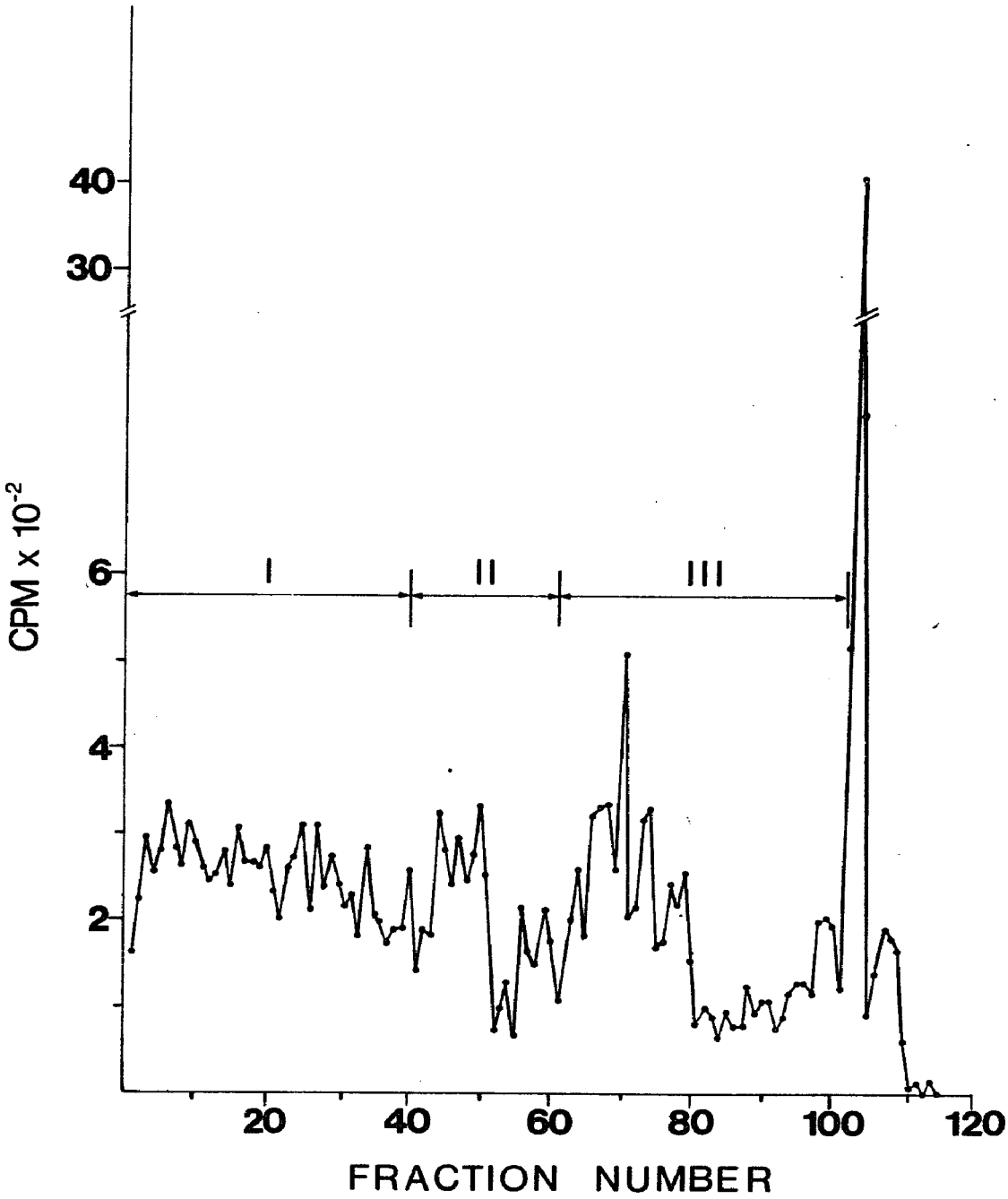


Table 6

Molecular Weights of Molluscum Contagiosum Polypeptides Estimated from Electrophoretic Mobilities

Percent Acrylamide Concentration					
7.5		9.0		12.0	
Protein Number	Molecular Weight x 10^3	Protein Number	Molecular Weight x 10^3	Protein Number	Molecular Weight x 10^3
1	>218	1	>132	1	>90
2	>218	2	>132	2	>90
3	218	3	>132	3	88
4	210	4	>132	4	84
5	170	5	132	5	83
6	165	6	127	6	79
7	163	7	122	7	77
8	160	8	109	8	73
9	143	9	102	9	71
10	137	10	99	10	68
11	133	11	96	11	65
12	122	12	88	12	64
13	120	13	83	13	60
14	105	14	77	14	58
15	96	15	69	15	54
16	89	16	63	16	45
17	71	17	61	17	43
18	65	18	59	18	42
19	57	19	57	19	40
20	47	20	55	20	37
21	42	21	52	21	31
22	35	22	48	22	27
23	29	23	46	23	20
		24	41	24	19
		25	39	25	18
		26	37	26	16
		27	33	27	15
		28	18		

Figure 7. Electropherogram of dissociated ^{14}C amino acid labelled virus in 7.5% acrylamide gel.


40
30
6
4
2
CPM x 10-2
I
II
III
20
40
60
80
100
120
FRACTION NUMBER

Group II by fractions 41 through 61, and Group III by fractions 62 through 101. The higher level of radioactivity exhibited by three of the seven polypeptides in Group II and by three of twelve polypeptides in Group III suggests that they probably are major virus constituents. Two peaks, one large and one small, at the end of the electropherogram are located in the region of migrated phenol red internal marker and presumably represent low molecular weight polypeptides that have not been sieved.

The results of electrophoresis of virus in 9% acrylamide gel are shown in Figure 8. While the basic pattern of the electropherogram is similar to that obtained with 7.5% gel, certain differences due to increased sieving are evident. Several of the large molecular weight polypeptides failed to migrate into the gel, as indicated by the elevated radioactivity in fraction 1. However, the six polypeptides that were resolved in fractions 2 through 24 would appear to represent the lowest molecular weight species in Group I. Position of the eight polypeptides in fractions 25 through 49 is consistent with Group II designation, and the polypeptides in fractions 50 through 92 correspond to Group III. Evidence for a fourth group was present in fractions 93 through 102, but only partial resolution of low molecular weight components was obtained.

Electropherogram obtained with 12% gel is shown in Figure 9. It more clearly defines the polypeptides of Groups II and III and provides confirmation of Group IV. Fractions 62 through 103 encompass Group IV and show at least eight species of polypeptides and a highly radioactive fraction of unresolved polypeptides. No attempt was made to resolve these low molecular weight constituents in gels great-

Figure 8. Electropherogram of dissociated ^{14}C amino acid labelled virus in 9.0% acrylamide gel.

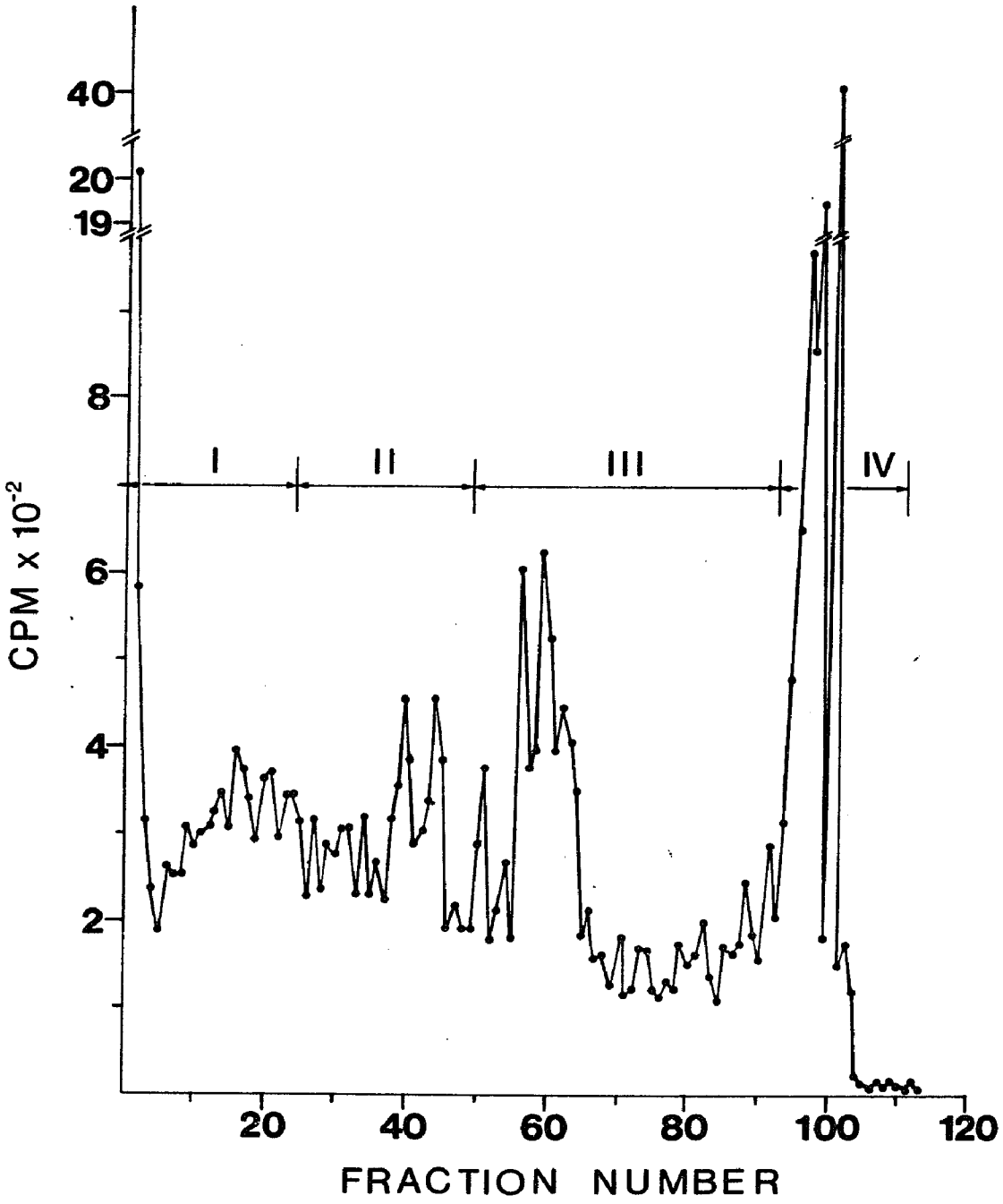
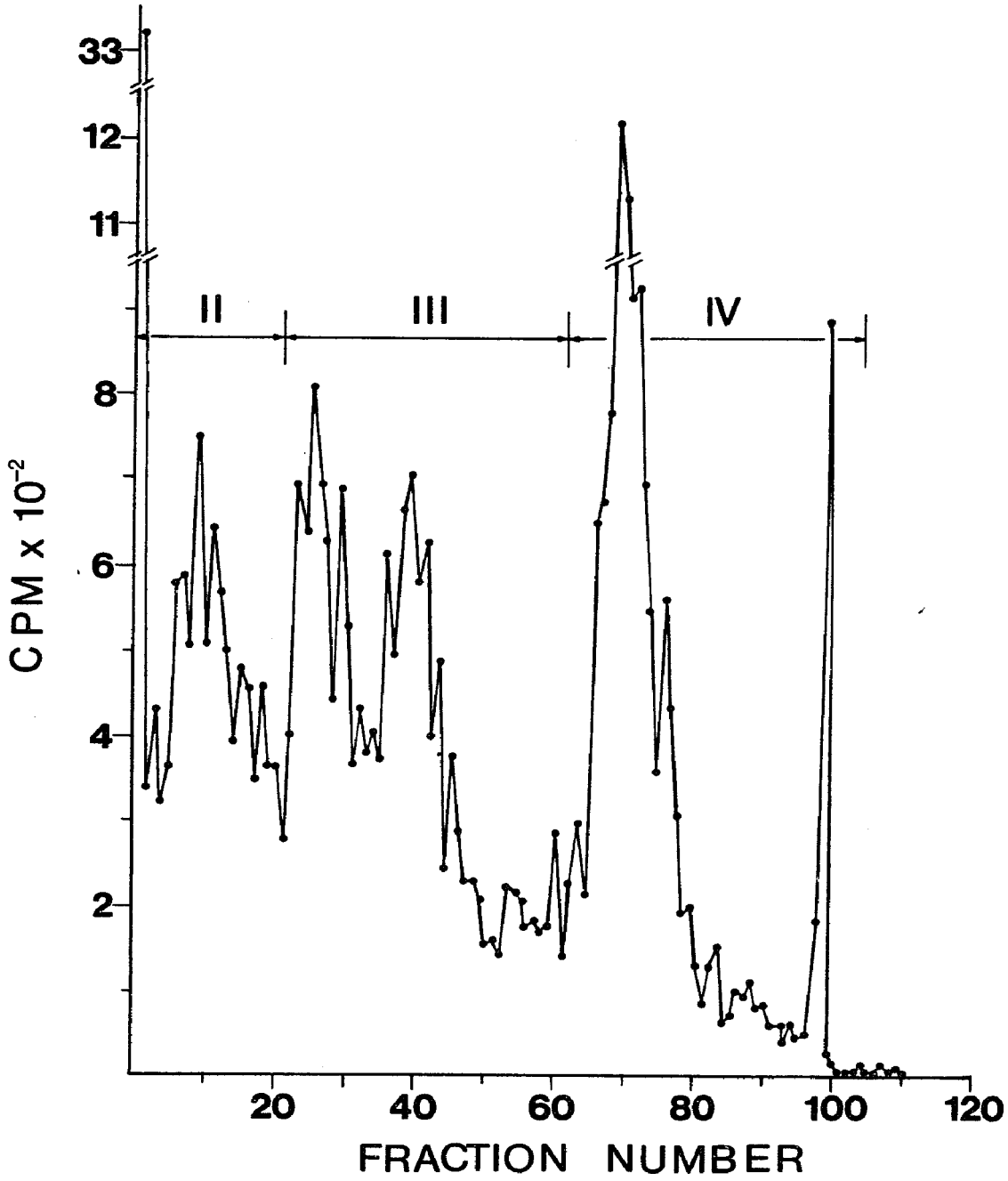

40
20
19
8
6
4
2
I
II
III
IV
CPM x 10⁻²
20
40
60
80
100
120
FRACTION NUMBER

Figure 9. Electropherogram of dissociated ^{14}C amino acid labelled virus in 12.0% acrylamide gel.


33
12
11
8
6
4
2
II
III
IV
CPM x 10⁻²
20
40
60
80
100
120
FRACTION NUMBER

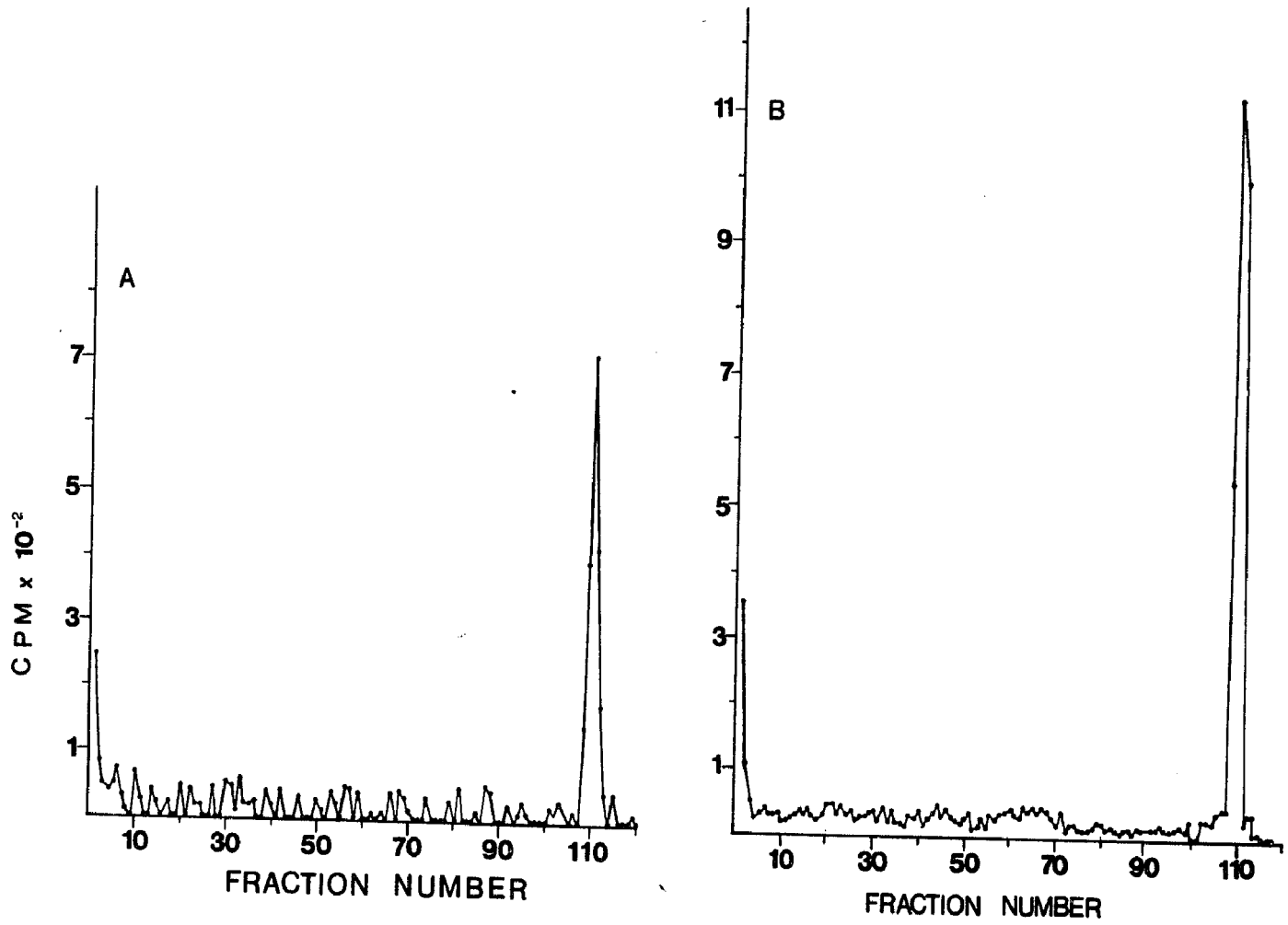
er than 12% concentration, because of limitations imposed by the electrophoresis equipment available for this investigation.

Determination and Composition of Viral Glycoproteins

In a preliminary search for glycosylated proteins in molluscum virus it was found that high concentrations of purified virus, attached to glass slides by gentle warming or to cellulose acetate strips by immersion in 10% acetic acid, gave a positive test for polysaccharide when stained by the Periodic Acid-Schiff procedure. If this were indeed an indication of the presence of glycosylated protein and not free polysaccharide, then it would follow that a similarly positive reaction should be demonstrable with gels on which dissociated virus has been electrophoresed. However, repeated attempts to obtain a positive stain with gels of concentrations used in this investigation were completely unsuccessful. The reason for this circumstance is not known, but earlier experiences in this laboratory with Coomassie Brilliant Blue stain suggest that residual sodium dodecyl sulfate in gels may be at least partially responsible.

Investigation of glycosylated proteins was extended by propagating virus in the presence of radioactive sugars or amino sugar and analyzing the labelled virus progeny by acrylamide gel electrophoresis. Results obtained in experiments with ^{3}H glucosamine and ^{14}C galactose are presented in Figure 10. The absence of a relatively uniform level of polypeptide labelling provides reasonable evidence not only that infected FL cells do not convert significant amounts of exogenous labelled glucosamine and galactose into amino acid precursors, but that the two areas of highest activity in each electro-

Figure 10. Electropherogram of dissociated ^{3}H glucosamine and ^{14}C galactose labelled virus in 9.0% acrylamide gel. A (^{3}H glucosamine), B (^{14}C galactose).


A
B
CPM x 10⁻²
1
3
5
7
9
11
10
30
50
70
90
110
FRACTION NUMBER
FRACTION NUMBER

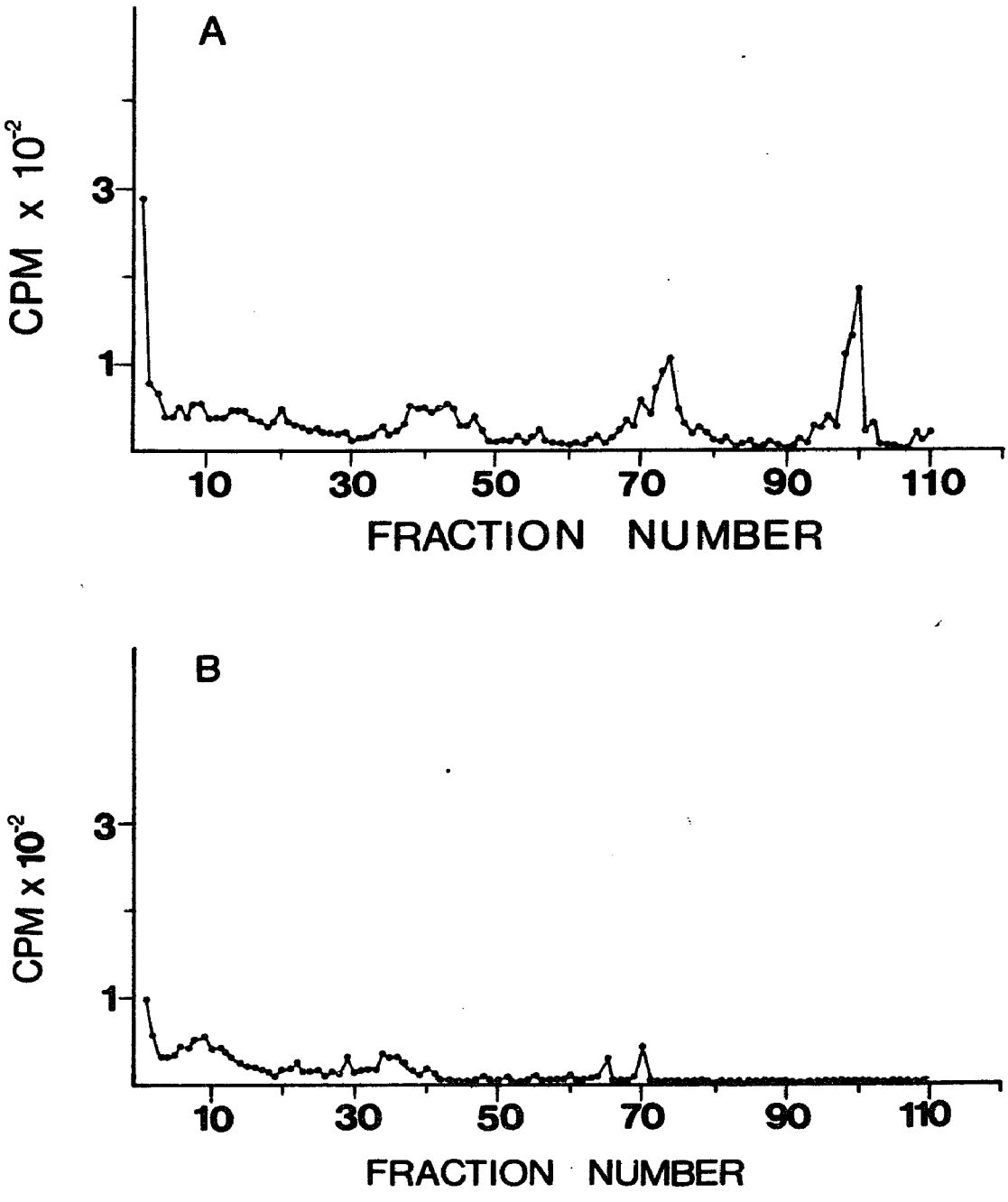
pherogram most probably represent glycosylated polypeptides. The largest peaks of glucosamine- and galactose-labelled polypeptides are located in the area where sieving has not occurred.

Electropherograms of virus propagated in the presence of ^{14}C mannose and ^{3}H fucose are shown in Figure 11. It is apparent that the level of mannose incorporation was much lower than obtained with glucosamine or galactose. Since infected cells removed approximately 41% of available ^{14}C mannose from the medium as compared to 45% for ^{14}C galactose, a lower mannose content of glycosylated polypeptides is suspected. In addition to larger molecular weight glycosylated polypeptides excluded from the 12% gel, the electropherogram shows two significant peaks. The peak at fraction 100 is in the same unresolved region as the polypeptides labelled with glucosamine and galactose (Figure 11). A second peak at fraction 75 previously was undetected (Figure 11) and presumably the represented glycosylated polypeptides were not labelled with glucosamine or galactose.

Little if any labelling of glycosylated polypeptides was obtained with ^{3}H fucose (Figure 11). This does not appear to be due to the failure of infected cells to incorporate the sugar, for approximately 21% of the labelled sugar was removed from the medium as compared to 23% for ^{3}H glucosamine.

Co-electrophoresis of ^{14}C galactose and ^{3}H amino acid labelled virus preparation in 9% and 12% gels further demonstrated that the radioactivity of sugar labelled virus was due to its incorporation into glycoproteins and not conversion to amino acid precursors. In preparing the electropherograms corrections were made for channel spillover of the two isotopes (Kobazahi and Maudsley, 1970). The 9%

Figure 11. Electropherogram of dissociated ^{14}C mannose and ^{3}H fucose labelled virus in 12.0% acrylamide gel. A (^{14}C mannose) B (^{3}H fucose).


A
CPM x 10^{-2}
3
1
10
30
50
70
90
110
FRACTION NUMBER
B
CPM x 10^{-2}
3
1
10
30
50
70
90
110
FRACTION NUMBER

gel electropherogram is not shown, since it resembled, as might be expected, a composite of electropherograms presented earlier in Figures 8 and 10b. However, the 12% gel electropherogram (Figure 12) revealed at least three coinciding peaks of different activity in fractions 90 through 104 and several unresolved peaks in the area of fraction 110.

Demonstration of Lipid or Lipid Complex in Virus

In his earlier investigation of molluscum virus propagated in FL cells, Dr. Francis observed that infectivity titers were not significantly reduced after treatment of virus with diethyl ether or chloroform. This finding was interpreted as an indication of the absence of extractable surface lipids, but did not definitively eliminate the possible presence of lipid material. It therefore was of interest to attempt the demonstration of lipid material by tinctorial means.

Dissociated virus was electrophoresed in 3.5% acrylamide gel and the migrated structural components stained with Oil Red O. A single bright orange staining band, approximately 4mm wide and located 180mm from the point of entry, was the only lipid containing component detected (Figure 13). Stain control gels on which dissociated human plasma and bovine albumin were electrophoresed gave positive and negative tests, respectively, for lipid.

Selective Degradation of Virus

Recent molecular studies of the more complex viruses and mammalian cells have shown that non-ionic detergents are useful for investigating the spacial position of structural components. Easterbrook (1966)

Figure 12. Co-electrophoresis of dissociated ^{14}C galactose and ^{3}H amino acid labelled virus in 12% acrylamide gel.

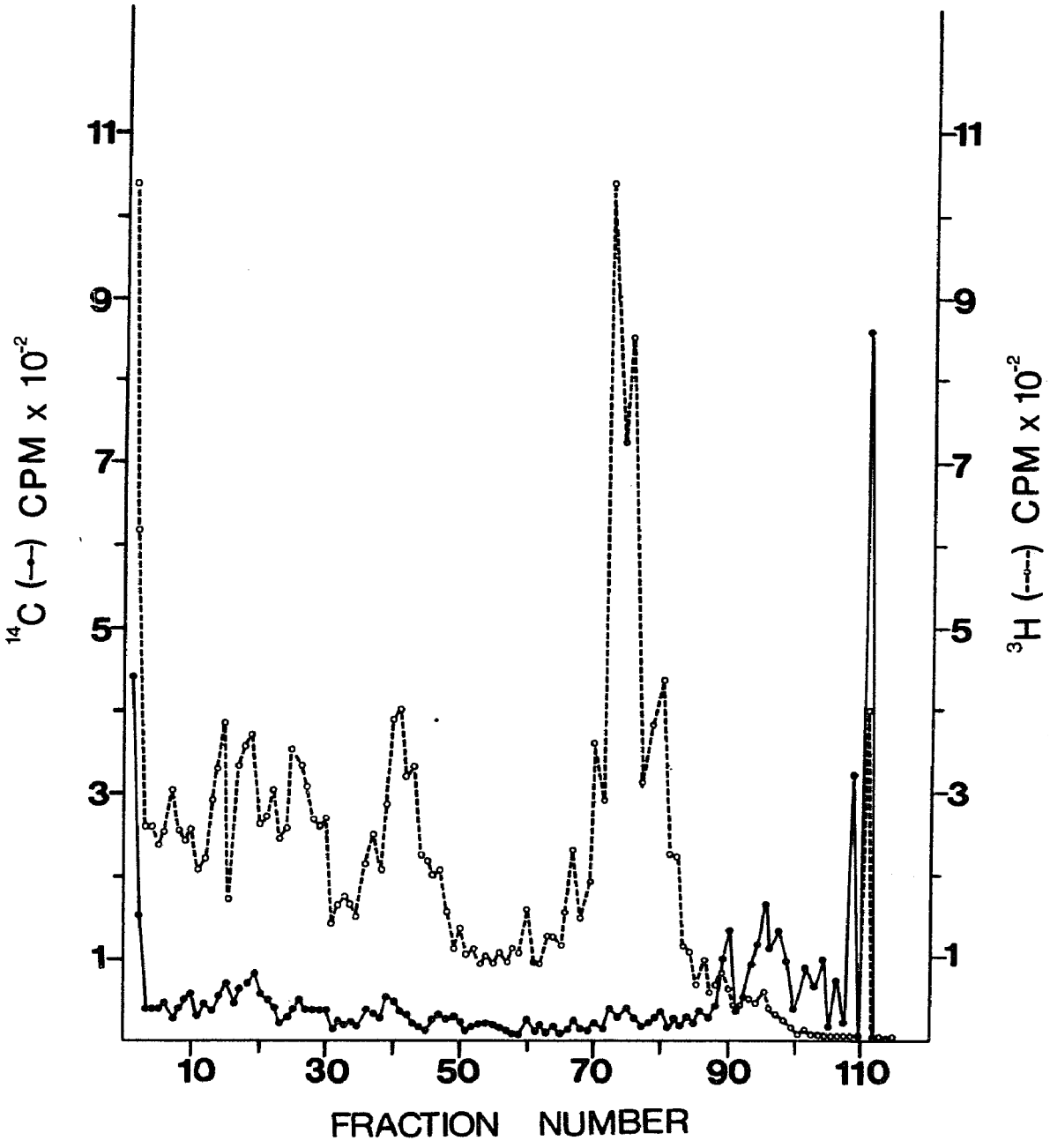
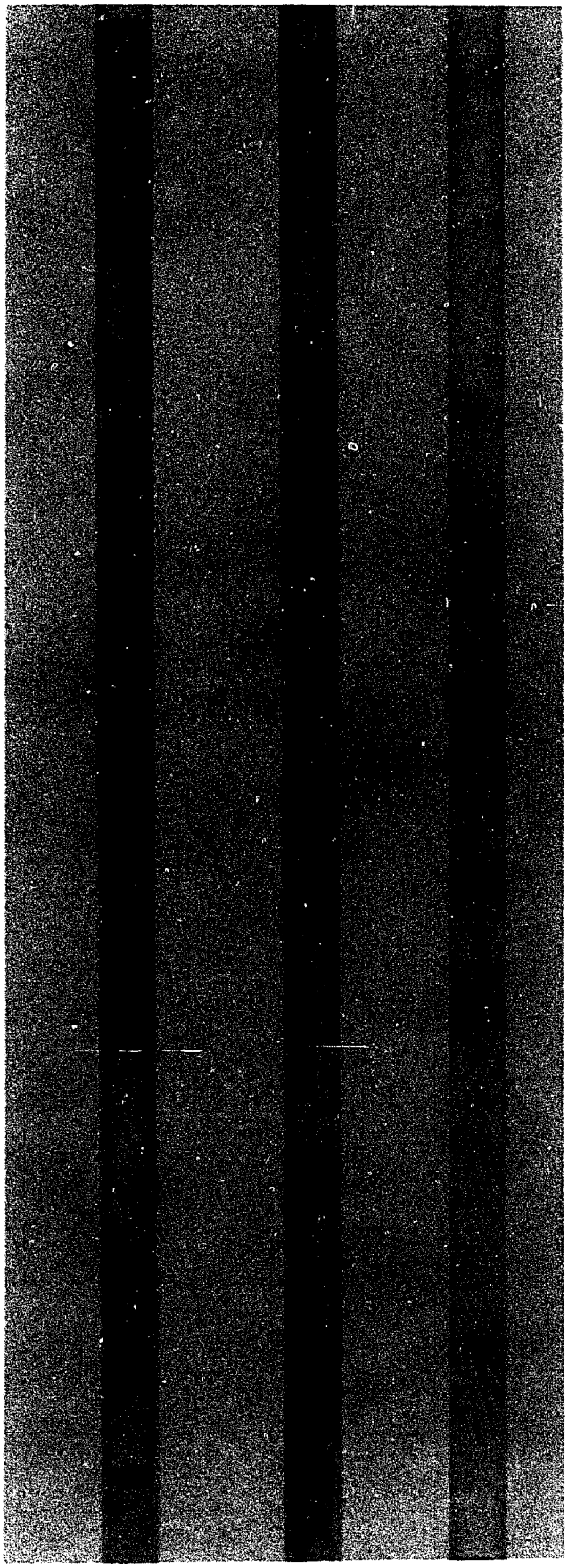

11
9
7
5
3
1
^{14}C (—•—) CPM x 10^{-2}
^{3}H (---○---) CPM x 10^{-2}
10
30
50
70
90
110
FRACTION NUMBER

Figure 13. Photograph of dissociated virus, human plasma, and bovine albumin electrophoresed in 3.5% acrylamide gel and stained with Oil Red O. Left gel (virus), middle gel (human plasma), right gel (bovine albumin).



the spacial position of structural components. Easterbrook (1966) found that treatment of vaccinia virus with NP-40 removed a portion of the outer envelope, and Tzagoloff and Penefsky (1971) exploited the solubilizing action of Triton X-100 for extracting various components from cell membrane. In the present investigation the demonstration of a large number of polypeptides, glycosylated polypeptides, and lipid complex clearly indicates a complex structure for molluscum virus. It was envisioned that experiments on selective degradation of the virus might yield information on the location and spatial association of some of these structural constituents.

^{14}C Galactose and ^{3}H amino acid labelled virus preparations were treated with NP-40, Triton X-100, or cesium chloride and processed as previously described. It will be noted in Figure 14 that NP-40 extracted only a small amount of labelled structural material. The supernatant contained two separate groups of polypeptides, a small molecular weight fraction and a large molecular weight fraction. The latter appears to be a lipoprotein on the basis of a positive lipid stain obtained when the supernatant was electrophoresed in 3.5% acrylamide gel. Extracted virus contains all of the major polypeptides previously demonstrated. NP-40 removed all of the low molecular weight glycosylated polypeptides from ^{14}C galactose labelled virus, as shown in Figure 15. Inasmuch as the same labelled virus preparation was used in all glycosylated polypeptide experiments, a comparison of the electropherogram in Figure 14 with that in Figure 12 clearly reveals that not all of the NP-40 extracted constituents were detected in the supernatant. It was necessary to reduce the volume of the supernatant with polyethylene glycol prior to electrophoresis and some of the

Figure 14. Electrophoresis of NP-40 treated ^{3}H amino acid labelled virus in 9% acrylamide gel. A (supernatant), B (residual virus).

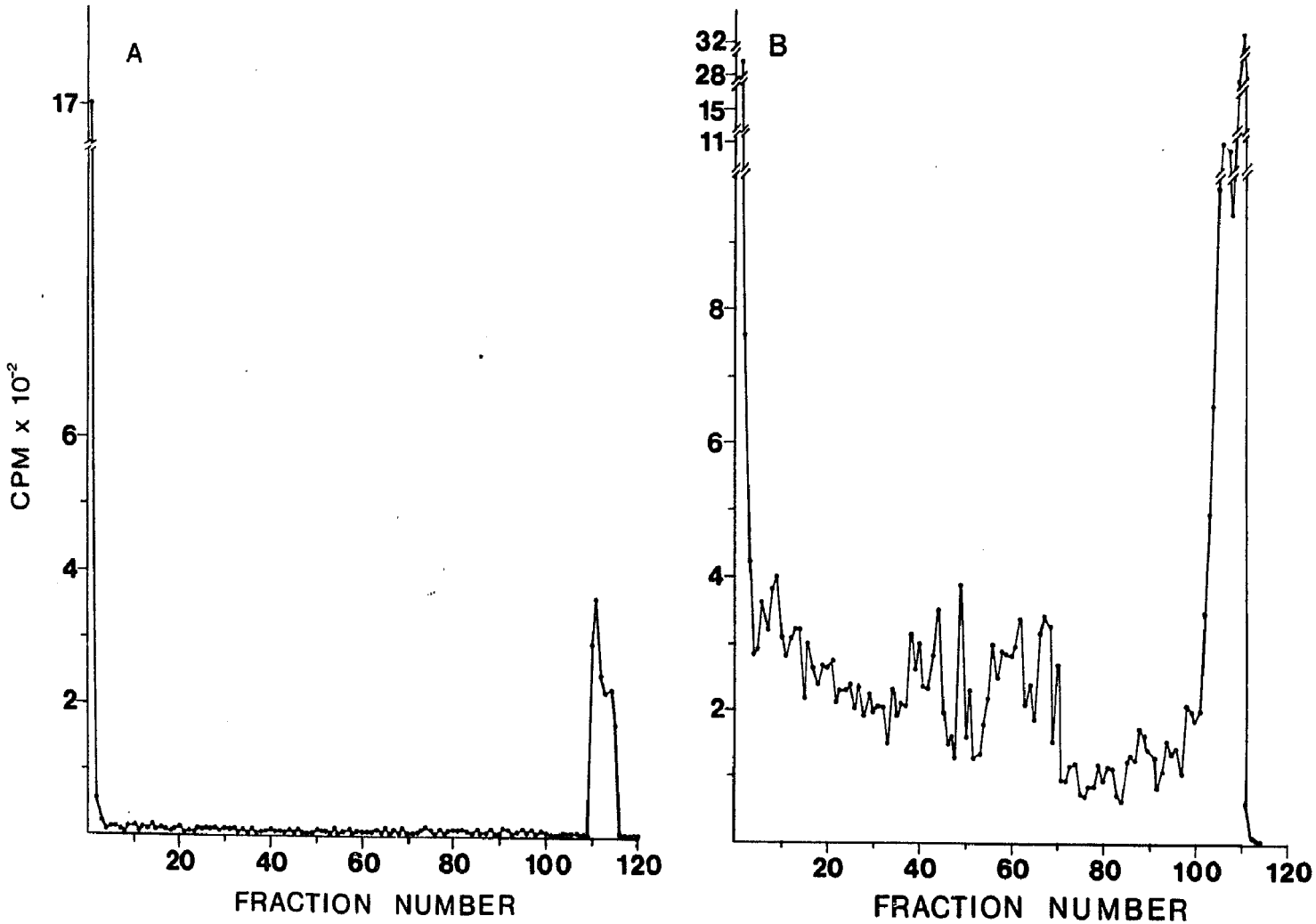
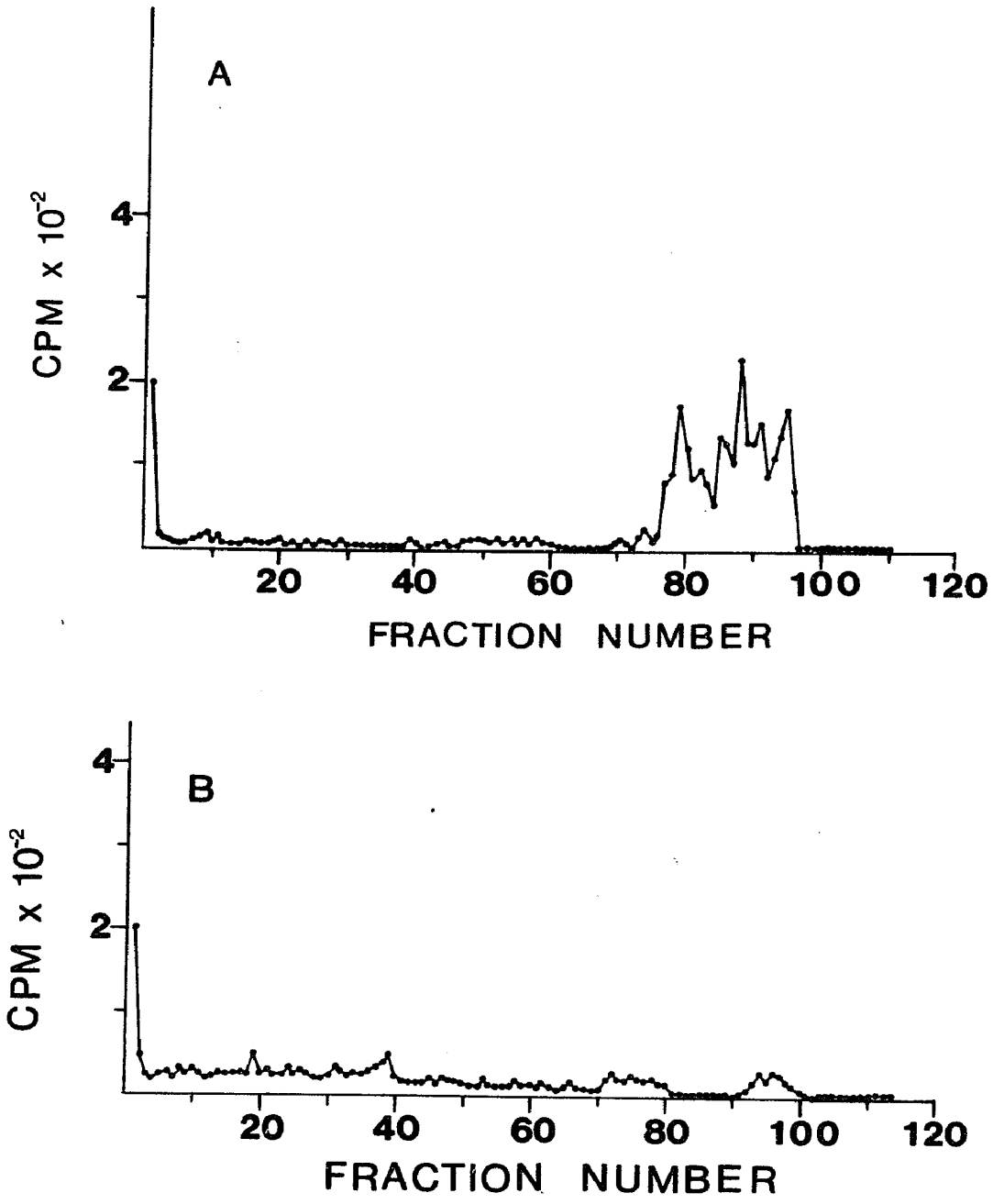

A
17
6
4
2
CPM x 10^{-2}
20
40
60
80
100
120
FRACTION NUMBER
B
32
28
15
11
8
6
4
2
20
40
60
80
100
120
FRACTION NUMBER

Figure 15. Electrophoresis of NP-40 treated ^{14}C galactose labelled virus in 12.0% acrylamide gel. A (supernatant). B (residual virus).


A
CPM x 10^{-2}
4
2
20
40
60
80
100
120
FRACTION NUMBER
B
CPM x 10^{-2}
4
2
20
40
60
80
100
120
FRACTION NUMBER

lower molecular weight glycosylated polypeptides probably were lost through dialysis.

Treatment with Triton X-100 released a large number of polypeptides from ^{3}H amino acid labelled virus, as shown in Figure 16. Although the entire molecular weight range of the polypeptides is not evident in the supernatant electropherogram of the 12% acrylamide gel used, most of the lower molecular weight polypeptides extracted can be assigned to Group IV. Triton X-100 removed some lipid material as evidenced by staining of electrophoresed dissociated virus in 3.5% acrylamide gel. The low molecular weight glycoproteins labelled with ^{14}C galactose also were removed as shown in Figure 17.

Numerous investigations have established that cesium chloride in relatively high concentrations does not appreciably alter the structural integrity of non-envelope viruses. In contrast, isopycnic centrifugation analysis of enveloped viruses has revealed instances where the salt produced marked structural changes. Spring and Roizman (1967) observed variations in the buoyant density of herpes simplex as great as 0.013 gm/cm^3 in cesium chloride gradients when the virus was not stabilized with formaldehyde. Related studies in this laboratory have indicated that a similar phenomenon also occurs with molluscum virus if the period of isopycnic centrifugation is thirty hours or longer. The fact that up to 85% of the input virus could be recovered in an infectious form in the cesium chloride gradient fraction seemed to imply that virion dehydration rather than structural degradation was responsible for variations in buoyant density. In the present work this conjecturable point was investigated and it was surprising to

Figure 16. Electrophoresis of Triton X-100 treated ^{3}H amino acid labelled virus in 12.0% acrylamide gel. A (supernatant) B (residual virus).

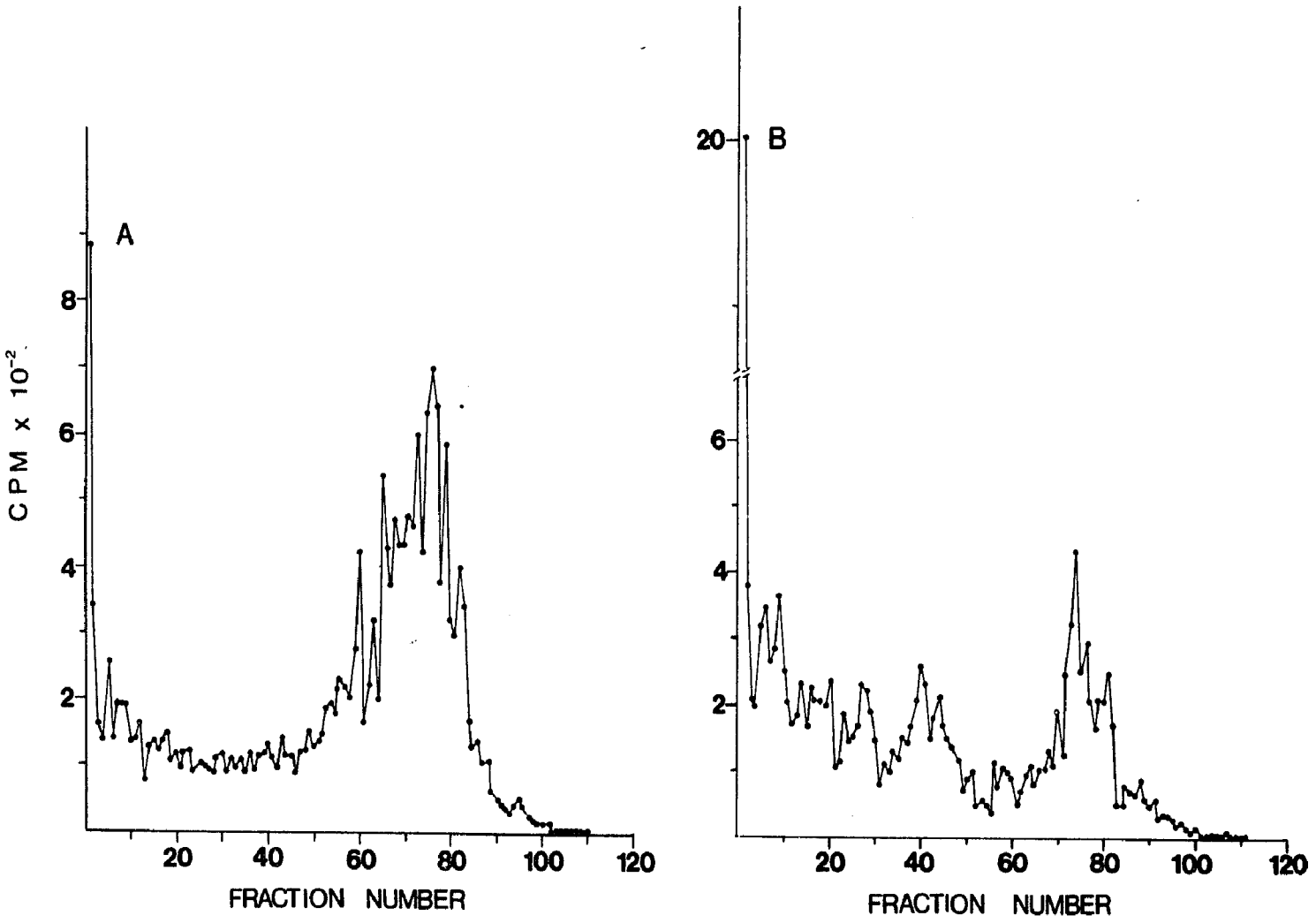
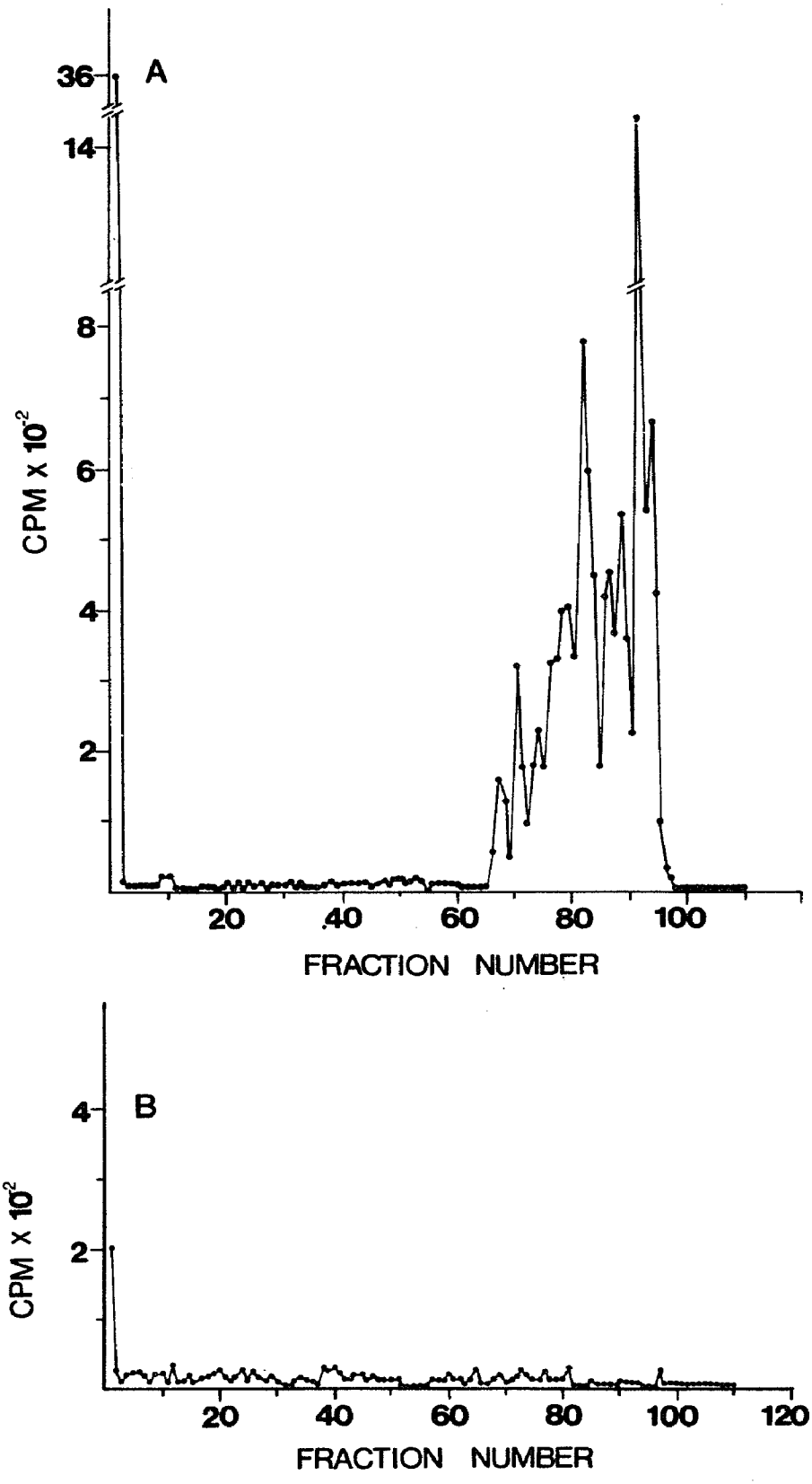

A
B
CPM x 10^{-2}
2
4
6
8
20
20
40
60
80
100
120
FRACTION NUMBER
FRACTION NUMBER

Figure 17. Electrophoresis of Triton X-100 treated ^{14}C galactose labelled virus in 12.0% acrylamide gel. A (supernatant) B (residual virus).


A
36
14
8
6
4
2
CPM X 10⁻²
20
40
60
80
100
FRACTION NUMBER
B
4
2
CPM X 10⁻²
20
40
60
80
100
120
FRACTION NUMBER

find that cesium chloride does indeed partially degrade molluscum virus structure.

It will be noted in Figure 18 that cesium chloride treatment of ^{3}H amino acid labelled virus resulted in the release of several structural polypeptides. One of the polypeptides, fraction 21 in the supernatant electropherogram, occupies a position which is consistant for molecular weight of approximately 60,000 daltons. The other polypeptides are positioned in the region of the gel containing the end of Group III and the beginning of Group IV. No structural glycosylated polypeptides were released by cesium chloride treatment.

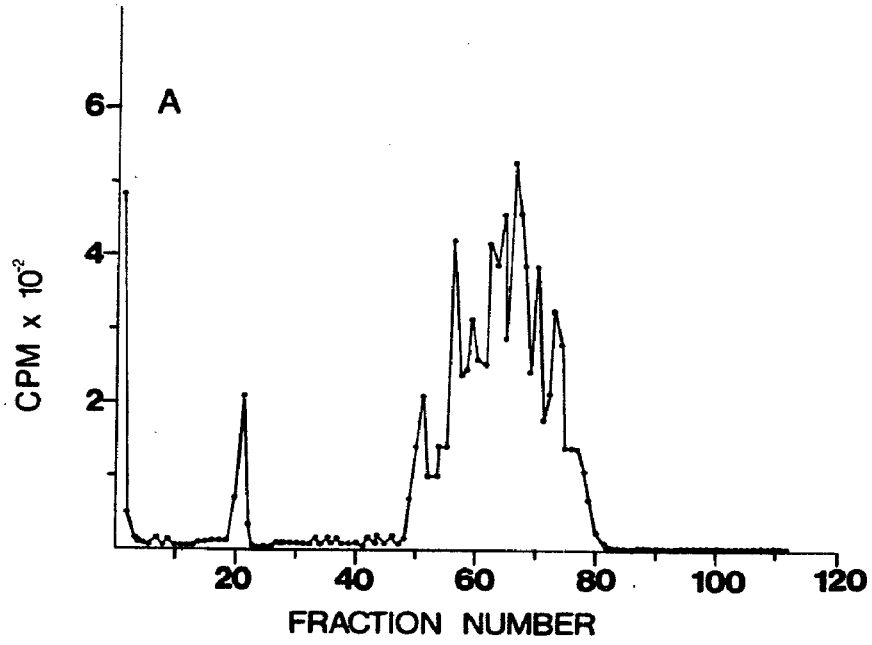
Plaque titrations performed on virus preparations before and after treatment with NP-40, Triton X-100, and cesium chloride revealed no loss in infectivity.

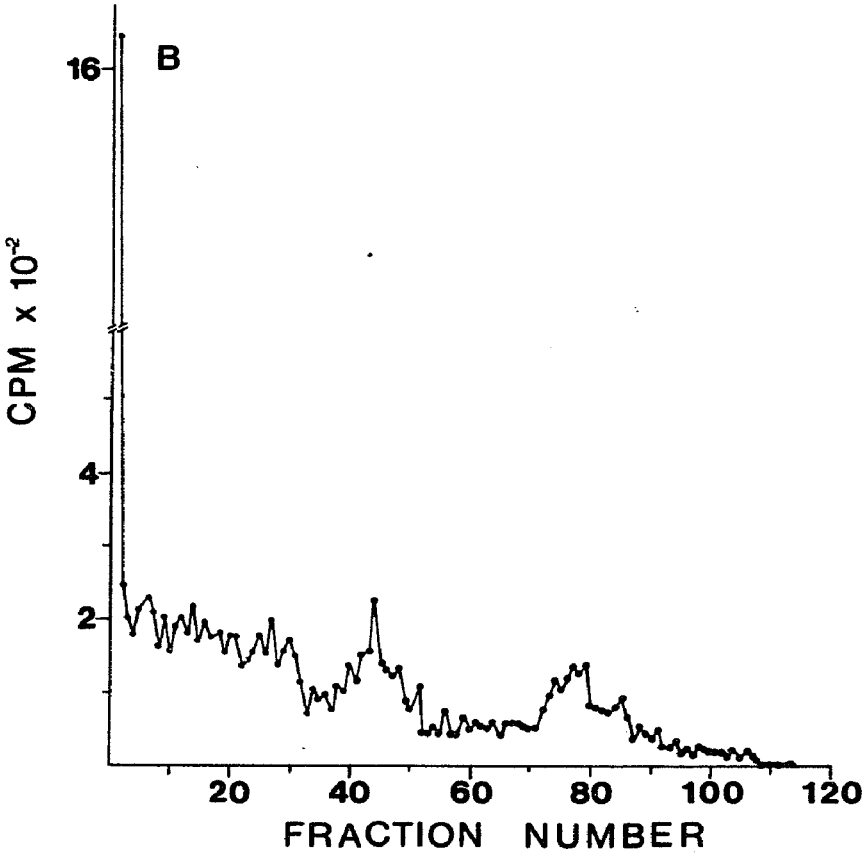
Morphogenesis of Viral Proteins

In general two methods of approach have been employed for studying the morphogenesis of genetically specified proteins of animal viruses. One involves isotope labelling of all polypeptides produced from the moment of infection and their harvesting at intervals thereafter, and the other makes use of various pulse labelling techniques in which the virus is exposed at intervals to short pulses of isotope throughout the replication cycle. A preliminary experiment therefore was undertaken in an attempt to determine if a modification of the first method might be applicable in the study of molluscum virus.

Several cell cultures were infected and simultaneously exposed to ^{3}H amino acid containing medium. At the end of three hours at 36°C the cultures were washed several times with Hanks' solution and divided

Figure 18. Electrophoresis of cesium chloride treated ^{3}H amino acid labelled virus in 12.0% acrylamide gel. A (supernatant), B (residual virus).


A
6
4
2
CPM x 10^{-2}
20
40
60
80
100
120
FRACTION NUMBER


B
16
4
2
CPM x 10^{-2}
20
40
60
80
100
120
FRACTION NUMBER

into two groups. One group was harvested and the cell sap prepared as earlier described. The other group of cultures was placed on isotope-free maintenance medium, incubated at 36°C until infected cells were destroyed, and the progeny virus then harvested and purified. After dissociation the cell sap and purified progeny virus were electrophoresed in separate 9% acrylamide gels.

The electropherogram in Figure 19 show the polypeptides resolved from cell sap harvested at three hours post-infection. While the profile of labelled polypeptides in fractions 15 through 97 is remarkably similar to that of virus structural polypeptides presented in Figure 8, fractions one through six contain additional polypeptides not previously detected. A large peak at fraction 99 represents unresolved constituents, and the peak at fraction 112 probably is due to free amino acids as evidenced by the concomitant migration with the phenol red internal marker. Undoubtably some of the polypeptides, particularly those in fraction 1 through 6, are not structural constituents. However, if one compares them with the polypeptides resolved from ^{14}C amino acid labelled cell sap from uninfected cells (Figure 20), it would appear that a majority were virus specified. Electropherogram in Figure 21 shows the distribution of polypeptides from purified progeny virus labelled for three hours post-infection. With one exception, all of the polypeptides previously resolved from virus labelled during the entire replication cycle (Figure 8) are present. In fractions 55 through 68 there appears to be incomplete labelling of polypeptides belonging to Group III.

On initial analysis of results obtained with infected cell sap

Figure 19. Electrophoresis of infected cell sap labelled with ^{3}H amino acids for three hours post-infection in 9% acrylamide gel.

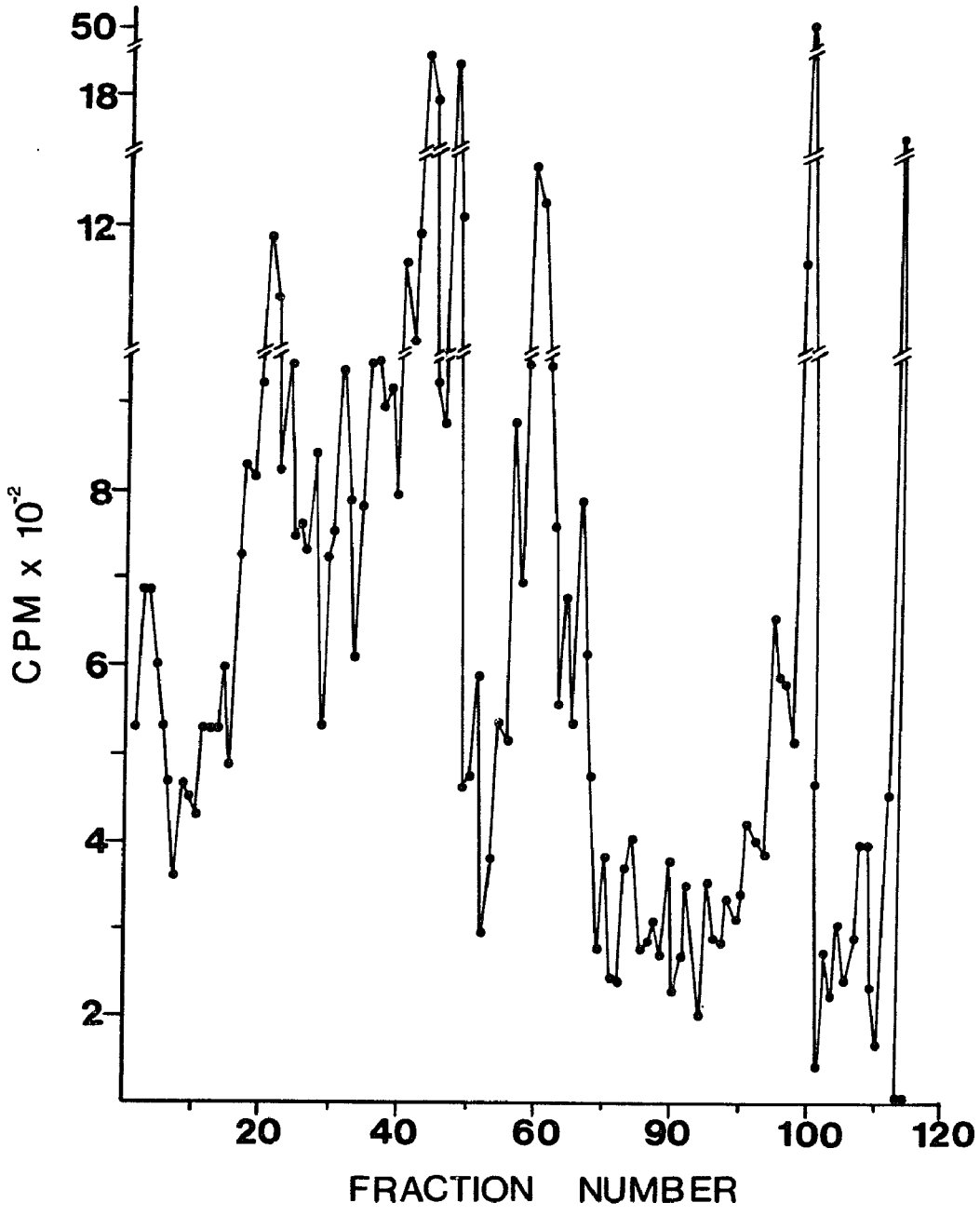

50
18
12
8
6
4
2
CPM x 10⁻²
20
40
60
90
100
120
FRACTION NUMBER

Figure 20. Electrophoresis of ^{14}C amino acid labelled uninfected cell sap in 9.0% acrylamide gel.

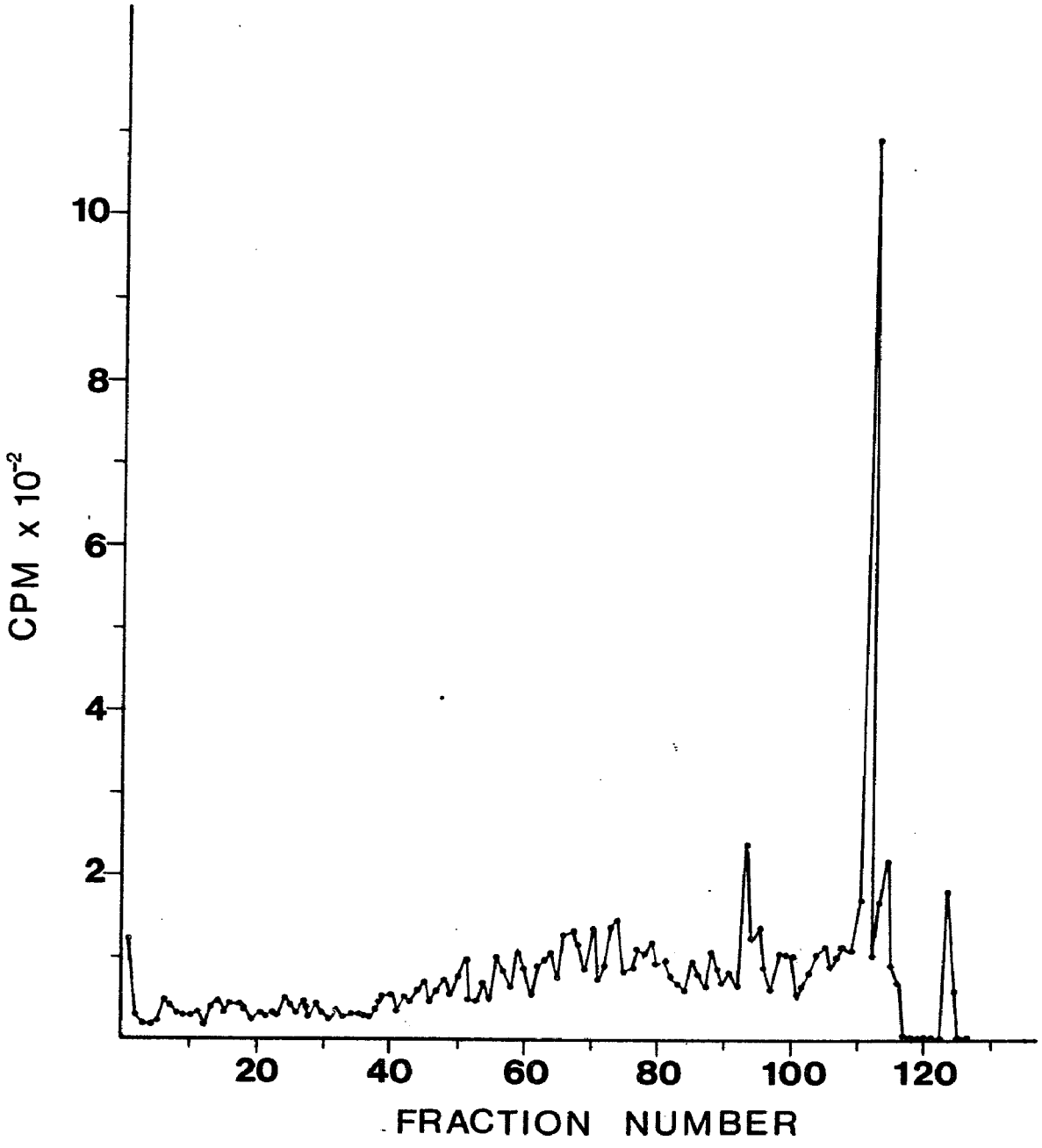
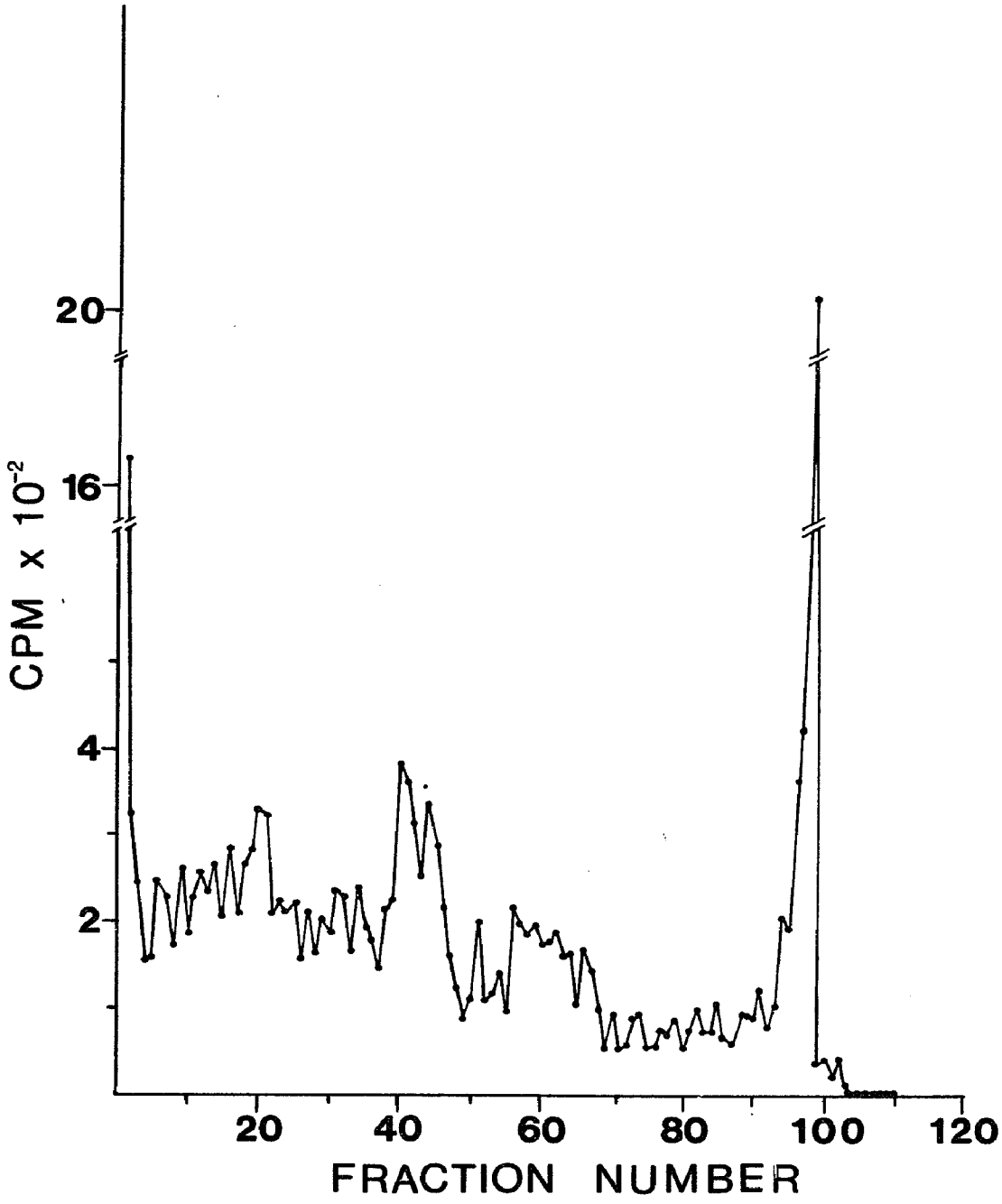

CPM x 10^{-2}
10
8
6
4
2
20
40
60
80
100
120
FRACTION NUMBER

Figure 21. Electrophoresis of pulse labelled virus for three hours post-infection with ^{3}H amino acid in 9% acrylamide gel.


20
16
4
2
CPM x 10^{-2}
20
40
60
80
100
120
FRACTION NUMBER

and progeny virus, it would seem that a majority of the structural polypeptides destined for incorporation into mature virus are synthesized during the first three hours of the 12-14 hour replication cycle. However, in view of the intracellular pool of labelled free amino acid electrophoresed from the cell sap, consideration must be given to the possibility that some of the polypeptides present in progeny virus may have been synthesized from this pool after the initial three hour labelling period.

An attempt was made to clarify further the sequential synthesis of structural polypeptides by pulse labelling infected and un-infected cells with ^{14}C amino acids for 20 to 30 minutes at intervals of 20 to 60 minutes for the first five hours of the replication cycle. Harvested cell sap from each pulsing was analyzed as in the previous experiment. The electropherograms (not shown) of both infected and uninfected cell sap were similar and revealed only a few small molecular weight polypeptide species. The extremely low level of labelling exhibited by most of the samples (average of 10 counts per minute above background) indicated that FL cells were unable to take up sufficient isotope during the short pulsing period employed.

V. DISCUSSION

The growth kinetics experiment showed the replication pattern of molluscum contagiosum virus to be similar to that of other members of the poxvirus group (Appleyard et al. 1962; Yohn et al. 1966). Molluscum virus adsorbed and initiated the infection process within two hours with a significant increase in infectious virus occuring after this period. A relatively short eclipse period was evident. The virus was synthesized and released at an exponential rate for several hours with peak titers of intracellular virus always higher than extracellular virus. Synthesis and release of progeny virus was completed from twelve to fourteen hours post-infection.

In concentrating virus from large volumes of infected tissue culture fluid, comparable results were obtained with polyethylene glycol and zinc acetate procedures. Both apparently depend upon the non-specific precipitation of protein. Experiments designed to investigate the degree of virus purification demonstrated the removal of more than 92% of isotope labelled cell sap used as a marker during the purification procedure.

Complete solubilization of the virus by SDS dissociating fluid was indicated by the rapid clearing of the turbid virus suspension and a concomitant increase in the viscosity due to the release of DNA. The addition of urea to the SDS dissociating fluid did not increase the number of polypeptides resolved or alter the basic electrophoretic pattern; a finding which further indicates that complete

solubilization of virus was obtained.

Use of three gel concentrations enabled the resolution of many polypeptides with estimated molecular weights ranging from greater than 218,000 daltons to 15,000 daltons or less. Even though the exact number of polypeptides comprising the virus is difficult to establish due to slight overlapping of position in gels of different concentrations, a reasonable estimate would be at least 38. This exceeds the number of polypeptides resolved for other members of the poxvirus group and one other DNA virus. Thirty-one polypeptides have been reported for vaccinia (Obijeski et al. 1972; Sarov and Joklik, 1973), 28 for fowlpox (Obijeski et al. 1972), and a minimum of 15 for cowpox (Joklik, 1966). Herpesvirus hominis, a smaller DNA virus, has at least 24 polypeptides (Spear and Roizman, 1972). In view of the large number of polypeptides resolved, the question arises as to whether cell polypeptides are involved in the structural makeup of the molluscum virus. A definitive answer is not possible at this time but studies on the size of the nucleic acid (Francis, unpublished data) suggest that the genome is sufficiently large to direct the synthesis of all the structural polypeptides.

In determining the molecular weights of these polypeptides by the comparison procedure used by other investigators (Shapiro et al. 1967; Weber and Osborn, 1969; and Neville, 1971) certain limitations were encountered. Usually the unknown proteins are co-electrophoresed with the proteins of known molecular weights. This approach was not applicable here due to the multitude of polypeptides comprising molluscum, virus and it was necessary to make the calculations from separate gels simultaneously electrophoresed. No attempt was made to estimate the

molecular weight of glycoproteins because these moieties do not retain sufficient stain for tinctorial detection. Nevertheless, incomplete sieving of radioactive glycoproteins in 12% acrylamide gel suggests that these components are less than 15,000 daltons. It has been reported (Segrest et al. 1971) that glycoproteins do not migrate inversely proportional to their molecular weight. Apparently this is due to the low level of SDS binding to glycoproteins. The relative migration is reduced which, in turn, results in an erroneously high molecular weight value.

The electropherograms of ^{14}C and ^{3}H amino acid labelled virus revealed similar migration patterns. On the basis of distribution the polypeptides were arbitrarily separated into four groups. If one assumes uniform labelling of all polypeptides, then Group I contained 27%, Group II 19%, Group III 27% and Group IV 27% of the total radioactivity detected. In contrast to the finding of two saccharides in vaccinia virus (Holowczak, 1970; Garon and Moss, 1971; and Sarov and Joklik, 1973), at least three different saccharides were demonstrated for molluscum virus. Glucosamine, galactose, and mannose were associated with small molecular weight glycoproteins. In addition, ^{14}C mannose labelled virus revealed the presence of a glycosylated moiety which was not evidenced with ^{3}H glucosamine or ^{14}C galactose. In all of the radioactive saccharide labelling studies,the dissociated material remaining at or near the entry to the gel exhibited high radioactivity suggesting the presence of a large molecular weight glycoprotein or a lipid complex. The presence of lipid in this material was verified by tinctorial means and it may be in the form of lipoprotein and/or glycolipid. Fucose was not found to be a constituent

of the small molecular weight glycoproteins, but it may be a minor component of the putative glycolipid.

Using non-ionic detergents and cesium chloride, the outer portions of the virus were removed. The liberation of some polypeptides after treatment with cesium chloride would seem to indicate that some of the surface polypeptides have a low ionic or electrostatic binding force. To complicate this picture, the polypeptides released after treatment with NP-40 were not the same as those released with cesium chloride. In fact NP-40 solubilized only the small molecular weight glycoproteins and lipid material. Thus these compounds also appear to be on the surface. When the virus was treated with Triton X-100 all of the polypeptides of Group IV were released along with several polypeptides from Groups II and III. These data suggest that the virus may consist of two structural layers, an inner layer resistant to Triton X-100 and an outer layer susceptible to Triton X-100. Three distinct classes of constituents are evidenced, those released by NP-40, by cesium chloride, and those not released by either of the two degrading agents. The data prompt further suggestion that the constituents released by NP-40 and cesium chloride are not layered in a continuous fashion, but may alternate or reside in clusters over the virus. To provide more definitive evidence for the structure of the outer surface double extraction studies of radioactive amino acid and sugar labelled virus preparations are indicated. It should be noted that in none of the extractions was the infectivity of the virus reduced. This clearly indicates that the released polypeptides and glycosylated polypeptides are not major constituents of the virus receptor.

Experiments on the morphogenesis of the polypeptides, while not entirely successful, did furnish some valuable information. In the preliminary experiment evidence was obtained for a profusion of structural and non-structural polypeptides in three hours post-infection cultures. When using very short pulse labelling or short pulse labelling after infected cells have been incubated with medium containing 25% of the normal amino acid concentration, the larger molecular weight polypeptides were not labelled. In the second experiment the initial 20 minute sample of infected cells was not incubated with unlabelled amino acid. Since no labelled polypeptides were detected in this harvest, it would appear that virus coded proteins were synthesized later than twenty minutes post-infection. In subsequent samples the unlabelled amino acid pools in cells were not sufficiently depleted to allow labelling of the coded polypeptides. The results prompt consideration that pulse labelling for a relatively short period in the molluscum virus-FL cell system is not an effective method of studying the morphogenesis of structural polypeptides.

In this study some of the biochemical aspects of molluscum virus have been investigated. The data obtained have revealed structural aspects of the virus which resemble those of other members of the poxvirus group. However, other studies pursued in this laboratory with biological inhibitors and antigen-antibody reaction suggest that molluscum virus may not be related to the poxviruses. Further studies are necessary to clarify the classification position of molluscum contagiosum virus.

VI. SUMMARY

In this study, virus progeny was shown to be synthesized at an exponential rate after two hours of infection, yet virus was not released from the FL cells until after four hours post-infection and then at an exponential rate until the sixth hour. With the rate of viral synthesis declining, the peak virus titer was reached by the tenth hour. The titer of intracellular virus declined during the later stages of infection but remained higher than the titer of extracellular virus. A replication cycle of twelve to fourteen hours was found.

Both polyethylene glycol and zinc acetate were capable of precipitating molluscum virus and the percent of recovery of virus was approximately the same for each. In both procedures the wide variation in recoverable infectious virus was probably due to clumping.

The polypeptide composition of the virus was analyzed by electrophoresis of SDS dissociated virus in 7.5%, 9.0%, 12.0% polyacrylamide gels. The molecular weights of the stained polypeptides were estimated to range from greater than 218,000 daltons to less than 15,000 daltons. Electropherograms of isotope labelled virus revealed similar electrophoretic patterns, and also demonstrated the existence of small molecular weight polypeptides not detected by staining. The resolved polypeptides were arbitrarily divided into four major groups on a size basis.

Labelling of the virus with radioactive amino sugar or sugars

established glucosamine, galactose, and mannose as components of the glycoproteins. Small molecular weight glycoproteins were not labelled with fucose. Electrophoresis of glucosamine or galactose labelled virus in 9.0% acrylamide gel revealed the existence of at least one glycoprotein. The electropherogram of mannose labelled virus in 12.0% acrylamide gel suggested the presence of two glycoproteins, one low molecular weight group and a larger glycoprotein not labelled by glucosamine or galactose. Co-electrophoresis of amino acid and galactose labelled virus in 12.0% acrylamide confirmed the presence of several low molecular weight glycoproteins.

The existence of lipid or lipid complex was verified by staining dissociated virus electrophoresed in 3.5% acrylamide gel with Oil Red O.

Some outer polypeptide components of molluscum virus were solubilized with NP-40, cesium chloride, and Triton X-100. NP-40 released the low molecular weight glycoproteins and some of the lipid material and cesium chloride liberated several low molecular weight polypeptides from Groups III and IV and a 60,000 dalton polypeptide but did not release the glycoprotein moieties. When molluscum virus was treated with Triton X-100 many polypeptides, small molecular weight glycoproteins and lipid material were removed. The release of these components suggested that their location is on or near the surface of the virion.

In experiments on sequential synthesis of structural constituents, a large number of polypeptides were resolved at three hours post-infection. When short periods of isotope labelling were used for infected cells, no labelled polypeptides were demonstrated.

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GRADUATE SCHOOL
UNIVERSITY OF ALABAMA IN BIRMINGHAM
DISSERTATION APPROVAL FORM

Name of Candidate Henry Bernard Bradford, Jr.

Major Subject Microbiology

Title of Dissertation Biochemical Characterization of Molluscum Contagiosum Virus

Dissertation Committee:

Robert M. Francis, Chairman

Gerald L. Carlson

Thomas W Feary

James C. Lacey Jr.

Barnie J. Johnson

Director of Graduate Program Ray Cooley

Dean, UAB Graduate School J B Barker

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