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A RADIOIMMUNOASSAY FOR THE DETECTION
OF ROUS SARCOMA VIRUS PROTEINS

by

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B.A., University of California Berkeley, 1968

THESIS

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INTRODUCTION

It was first established by Peyton Rous in 1911 that a virus was the cause of a spontaneous chicken sarcoma, and he was able to passage the sarcoma through closely related chickens (Rous, 1911). Now each virus of the group which causes chicken sarcomas is referred to as Rous sarcoma virus (RSV), or more generally avian sarcoma virus (ASV). These viruses are part of the large group of RNA tumor viruses, viruses known to cause tumors in a variety of animals.

The susceptibility of chicken cells to infection by different strains of Rous sarcoma virus is determined genetically depending on the presence or absence of receptor sites on the host cell membrane (references in Payne et al., 1973). Many other avian species are also susceptible to specific strains of RSV, as was first shown in 1928 (Fujinami and Suzue, 1928). It is now known that in cell culture, RSV can even infect many different mammalian cells (Svoboda, 1967).

Infection of chick embryo fibroblasts in culture by RSV is a complex event. The virus is absorbed to cell surface receptors and penetrates the membrane. Inside the cell the virus is uncoated, and in accord with the provirus hypothesis (Temin, 1964), the virion RNA dependent DNA polymerase directs the synthesis of a double-stranded DNA copy of the RNA genome of the virus. Viral DNA probably becomes incorporated into the genome of the host cell (Varmus et al., 1973). Events leading to the production of virion RNA and protein and to the packaging and assembly of these into mature virions are not understood. The virion particles are transported to the cell surface and then bud from the surface, thereby acquiring a lipo-protein envelope. During the entire process, the host cell is neither killed, nor are cellular processes curtailed.

The mature virion of Rous sarcoma virus is composed of an electron-dense nucleoid, made up of ribonucleoprotein, surrounded by a shell which is covered by an envelope (Stromberg et al., 1973). The shell and nucleoid are the components of the core. Protein comprises about 60—70% of the virion, lipid about 20-30%, carbohydrate 2%, and RNA about 1% (Beard, 1973). The protein consists of seven polypeptides which have been studied in some detail. The two largest polypeptides, representing the type-specific (TS) antigenicity of the virus, are glycoproteins (containing glucosamine, galactose, and fructose) which form the spikes on the outer envelope and account for 10-20% of the total protein (Duesberg et al., 1970; Bolognesi et al., 1972). In polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS), the larger of these polypeptides migrates with an apparent molecular weight of about 98,000 daltons and is designated gp85. The other has an apparent molecular weight of about 35,000 daltons and is designated gp37 (Duesberg et al., 1970; Fleissner, 1971). The designations are in accord with the recommendations of the Oncornavirus Discussion Group held in New York City in June 1973, which suggests that viral polypeptides larger than 30,000 daltons be named according to their apparent molecular weight (in thousands) in SDS-PAGE. Viral polypeptides smaller than 30,000 daltons are named according to their apparent molecular weight by agarose gel filtration chromatography in 6M guanidine hydrochloride.

The five other polypeptides reside within the envelope of the virion and comprise the group specific (gs) antigens of the virus. Their sizes, designations, and locations within the virion, according to the best evidence presently available, are given below (Fleissner, 1971; Stromberg et al., 1974). The largest gs antigen, p27, which apparently is the major component of the core shell of the virion, is seen in SDS-PAGE as a single sharp peak. p27

has been found in all tested avian viruses.

p19 is completely absent from preparations of virion cores, yet disruption of the viral envelope is necessary for p19 to be recognized antigenically by appropriate antibodies (Bolognesi and Bauer, 1970). Thus it is thought that p19 either is associated with the envelope or resides between the envelope and the core shell. p15 is partially lost in core preparations and it is thought that it is a component of the core shell which is partially stripped off in the core preparation procedure. Another possibility is that it, like p19, lies intermediate between the envelope and the core shell.

p12 is the most abundant polypeptide in the preparations of ribonucleoprotein (RNP) from the virion, and most of the total virion p12 can be accounted for in the RNP. Therefore p12 is thought to be associated with the RNA in the intact virion. p10, the most rapidly migrating polypeptide in agarose gel filtration chromatography, is not always clearly distinguished in SDS-PAGE, and might possibly be broken fragments of larger polypeptides.

In SDS-PAGE, p19 and p12 migrate as a broad peak intermediate between p27 and p15. p15 and p10 also migrate as a broad peak in SDS-PAGE, with p10 sometimes forming an shoulder in front of p15. Under the conditions of SDS-PAGE, p12 has an apparent molecular weight of 14,000 daltons, whereas p15 has an apparent molecular weight of 12,000 daltons.

Two polypeptides of importance which are difficult to detect in most experiments using SDS-PAGE (presumably because of the very low concentrations of these polypeptides in each virion) are p64 and p91. These polypeptides are present in preparations of fractions of the virions which contain the RNA-dependent DNA polymerase activity, and they are absent from the fractions without DNA polymerase activity. p64 and p91 also co-electrophorese with purified avian myeloblastosis virus DNA polymerase. Thus it is thought that p64 and p91 represent the subunits of the DNA polymerase of the virus

(Stromberg et al., 1974).

The polypeptides described above are integral components of mature virions which bud from the surface of susceptible chicken cells infected with RSV. Many mammalian cells in culture, as mentioned, are also susceptible to infection by RSV. However, there is a major difference in the infection by RSV of chicken cells and mammalian cells. Whereas in both, the cells are stably transformed and DNA copies of the viral genome are probably incorporated into the host genome, in most transformed mammalian cells no virus particles are produced. Some of the viral gs antigens are still produced and can be detected by the COFAL test (a complement fixation test using serum from a RSV tumor-bearing hamster) (Sarma et al., 1964), and virus can be rescued by fusion of the transformed cell with chick fibroblasts (Svoboda et al., 1967). Since some RSV-specific protein is made in RSV-transformed mammalian cells but no virus particles are produced, it is obvious that complex control mechanisms are at work which are probably different from the control mechanisms in the avian cell. Knowledge about the production of the virus polypeptides could help elucidate the control processes involved. For example, it would be interesting to know the sequence of production of the polypeptides in cells shedding mature virions and in what proportions the polypeptides are made. In contrast, in mammalian cells transformed by RSV but not producing virus, it would be of interest to know which specific viral polypeptides are produced. In order to examine these and related questions concerning the production of viral polypeptides, a method of detecting small quantities of specific proteins in cells must be used. The method should be very sensitive to small differences in quantity and should be able to distinguish individual proteins. Thus it must be a major improvement over the COFAL test which is limited by its insensitivity to very small quanties of proteins

and its inability to distinguish individual proteins. The method currently used which best fits these criteria is the double-antibody radioimmunoassay procedure as developed in several laboratories for the detection of ASV proteins and murine sarcoma virus proteins (Weber and Yohn, 1972; Parks and Scolnick, 1972; Orozlan et al., 1972; Srini et al., 1973; Fritz and Qualtiere, 1973; Stephenson et al., 1973; Parks et al., 1973; Strand and August, 1973; Chen and Hanafusa, 1974).

In this investigation a double-antibody radioimmunoassay method is described which offers some refinements and is more rapid than other double-antibody radioimmunoassay systems thus far described. The radioimmunoassay is used to detect the time of production of some of the gs antigens in duck cells newly infected with the B77 strain of ASV. Several mammalian cells transformed by RSV but not producing virus are tested for the presence of a specific avian gs antigen and compared to infected and uninfected avian cells. As this methods continues to be developed and improved, it is hoped that the information it provides can be of help in answering basic questions about the control processes of cells.

<div align="center">EXPERIMENTAL PROCEDURES</div>

Buffers and Reagents

PO_4 buffer: phosphate buffer, equimolar solution of NaH_2PO_4 and Na_2HPO_4, pH 7.4, diluted to desired molarity.

PBS: phosphate buffered saline, 0.02M PO_4 buffer, 0.15M NaCl, pH 7.4.

PBSTD: PBS with 0.5% Triton X 100 (Rohm and Haas) and 0.5% deoxycholate.

SMTD: 0.5M sucrose, 0.01M $MgCl_2$, 0.5% Triton X100, 0.5% deoxycholate.

Tris: Tris (hydroxymethyl) aminomethane.

Sample buffer: 0.0625 M Tris HCl pH 6.8, 2% sodium dodecylsulfate (SDS), 10% glycerol, 5% β-mercaptoethanol (BME).

Electrophoresis buffer: 0.025M Tris, 0.02M glycine, 0.1% SDS.

TRGS: one liter contained 8 g NaCl, 0.38 g KCl, 1g glucose, 3 g Tris,

20 ml penicillin-streptomycin (GIBCO), 1 ml phenol red,

and HCl to give pH 7.4.

Growth medium: one liter contained 77.4 ml of 10X 199 (GIBCO), 690.6

ml of H_2O, 50 ml of calf serum (Pacific Biological), 10

ml of chick serum (GIBCO), 100 ml of tryptose phosphate

broth, 68 ml of 2% $NaHCO_3$, 4 ml of penicillin-streptomycin

(GIBCO).

Infecting medium: medium harvested from B77-transformed chick embryo

fibroblasts, centrifuged to remove cellular debris, then

filtered through a 0.2 μm Nalgene filter, with polybrene

added to a final concentration of 2 μg/ml.

Methods

1. Cell culture: Primary monolayer cultures of chick embryo fibroblasts
(CEF) and duck embryo fibroblasts (DEF) were prepared by trypsinization of
the body wall of 11 day old embryos (Kimber Farms, Palo Alto, Ca.) and seeding
onto plastic petri dishes in growth media. Secondary cultures were obtained
from confluent primary cultures by washing the primary cultures with TRGS,
trypsinizing, centrifuging, and resuspending the cells in growth media. The
resuspended cells were seeded at a one to four or a one to five dilution.
Tertiary cultures were obtained in a similar manner from secondary cultures.
The following mammalian cell lines were kindly supplied by Dr. H.E. Varmus:
3T3 BALB/c mouse cell line), 3T3/B77 (3T3 cells transformed by the B77 strain
of ASV), XC (cells from a rat tumor induced by the Prague strain of RSV),
SR-3 (BHK-21 hamster cell lines transformed by SR with a high frequency of
reversion to normal morphology), and SR-3/1a (a transformed subclone of SR-3).

2. Virus: Purified SR (Schmidt-Ruppin subgroup A) and B77 (Bratislava

77 subgroup C) strains of RSV were kindly supplied by Dr. J.M. Bishop. ^{3}H-amino acid-labeled and ^{14}C-amino acid labeled SR and B77 strains of RSV were kindly supplied by Dr. L. Levintow.

3. <u>Preparation of antibodies</u>: Antiserum to RSV proteins was obtained from male New Zealand white rabbits immunized with three injections of RSV at monthly intervals. Purified virus for the first immunization was divided into two pools. One pool was combined in suspension with an equal volume of Freund's adjuvant. To the other pool was added about five volumes of ether, followed by vigorous mixing and removal of the ether phase. This pool was also combined with Freund's adjuvant before injection. Each rabbit received about 1 mg of total viral protein injected into the foot pads, with 1/2 mg coming from each pool. In the subsequent injections, the viral protein was treated as before except that incomplete Freund's adjuvant replaced the complete Freund's adjuvant and the injections were given intramuscularly in the thighs. The blood from the immunized rabbits was obtained by heart puncture and allowed to clot. The serum was kept and used diluted 1:8 in PBS. It was stored at -20°C. Blood from each rabbit before immunization was obtained initially from the ear vein and treated as just described.

4. <u>Iodination</u>:

a. <u>Chloramine-T method</u>: This is basically the method of Greenwood et al. (1963). To a solution of 10 µg of B77 virus in 100 to 200 µl of 0.05 M PO$_4$ buffer was added 10% NP-40 (Shell) to a final concentration of 1% NP-40. About 2 mCI of ^{125}I (Na^{125}I from ICN Pharmaceutical) was added, followed by 25 µl of a 4 mg/ml solution of chloramine-T in 0.05 M PO$_4$ buffer. The solution was agitated and followed immediately by the addition of 100 µl of 2.4 mg/ml solution of sodium metabisulfite in 0.05 M PO$_4$ buffer. About 200

μg KI in 0.05 M PO_4 buffer was added as carrier. The solution was then

chromatographed on a pasteur pipette column of G-50 fine Sephadex (Pharmacia)

which had been equilibrated with PBS containing 0.25% (w/v) gelatin. The

PBS-gelatin solution was also used as the elution buffer. Five-drop fractions

were collected and counted directly in a Gamma Spectrometer (Packard). The

fractions containing the radioactivity in the void volume were pooled and

used diluted 1:10 in PBSTD. Specific activities obtained by this method

ranged from 3 to 6 x 10^6 cpm/μg protein. Preparations of ^{125}I-labeled B77

were 50-75% precipitable by rabbit anti-B77 serum and about 50-75% acid

precipitable (10% trichloracetic acid).

　　　　b. <u>Lactoperoxidase method</u>: This method employs an enzyme, lacto-

peroxidase, to effect the iodination of viral proteins. Published reports

of similar procedures can be found in David and Reisfeld, 1974; Miyachi

<u>et al</u>., 1972; Morrison <u>et al</u>., 1971; Morrison and Bayse, 1970; and Marchalonis,

1969. B77 virus was treated with NP-40 in 0.05 M PO_4 buffer as described in

the chloramine-T method. To a solution of about 20 μg B77 were added about

2 mCi of $Na^{125}I$ (ICN) and about 3 μg of lactoperoxidase (Calbiochem Co.). Then

5μl of a 1:20,000 solution of hydrogen peroxide to water was added followed

by a 5 minute incubation at room temperature. Again 5 μl of the H_2O_2 solution

was added followed by another 5 minute interval and another 5 μl addition of

the H_2O_2 solution. After 5 minutes, 2 μg of additional lactoperoxidase was

added followed by 5 μl of the H_2O_2 solution. Again at each of two successive

5 minute intervals, 5 μl of the H_2O_2 solution was added, followed by 5

successive additions of 5 μl of the H_2O_2 solution at 30 second intervals.

The final reaction mixture was chromatographed on a G-50 fine Sephadex

column as described in the chloramine-T method. Specific activities, immune

precipitability, and acid precipitability of the iodinated viral proteins

obtained with the lactoperoxidase method were approximately the same as those obtained using the chloramine-T method.

c. "Ester" method [3-(4-hydroxyphenyl) propionic acid N-hydroxy-succinimide ester method]: This method follows Bolton and Hunter, 1973. 3-(4-hydroxyphenyl) propionic acid N-hydroxysuccinimide ester was kindly prepared by Dr. Wolfgang Rohde using the method in the appendix of Bolton and Hunter, 1973. In a conical tube, about 0.22 µg of the ester in 1.7 µl of dimethyl formamide (DMF) was mixed with about 4 mCi $Na^{125}I$ (ICN) (about 5-15 µl). 50 µg of chloramine-T in 10 µl of 0.25 M PO_4 buffer was added, followed by agitation of the mixture and the immediate addition of 120 µg of sodium metabisulfite in 10 µl of 0.05 M PO_4 buffer. Then 200 µg of KI in 10 µl of 0.05 M PO_4 buffer was added. Rapidly, 5 µl of DMF and 250 µl of benzene were added, the mixture agitated, and the benzene phase transferred to a second tube. An additional 250 µl of benzene was added to the original tube, the mixture agitated, and the benzene phase transferred to the second tube. This second tube was placed under vacuum and the benzene evaporated. Then about 5 µg of NP-40 treated B77 in 10 µl of 0.1 M borate buffer, pH 8.5, was added to the tube from which the benzene had been evaporated. The virus solution was agitated in the tube and incubated in ice for 30 minutes. Then 90 µl of 0.2M glycine in 0.1 M borate buffer, pH 8.4, was added, the mixture agitated, and incubated for 5 minutes in ice. The final reaction mixture was chromatographed on G-50 fine Sephadex as described in the other iodination procedures. Specific activities obtained with this method ranged from 6-20 x 10^6 cpm/µg protein. Acid precipitability and immune precipitability of the iodinated protein ranged from 65 to 90%.

5. Preparation of cellular extracts: Six to ten petri dishes of con-fluent monolayer cells were washed twice with TRGS and treated with 0.05%

trypsin in TRGS for 5 minutes at 41°C. The cells were scraped from the dishes, pelleted, and twice resuspended in PBS and repelleted. The final pellet was resuspended in a volume of PBSTD approximately equal to the volume of the pellet. The concentration of the cells was determined by counting an aliquot in a hemocytometer. The resuspended cells were sonicated twice on a Branson Sonifier at mode 2 for 15 seconds (in ice), followed by centrifugation at 2000 rpm for 10 minutes to remove cellular debris. The supernatant was clarified by centrifugation at 30,000 rpm for 30 minutes (Type 40 rotor, Spinco). The clarified supernatant was stored at 4°C for short periods of time or at -20°C for longer periods of time. Protein concentrations of the extracts were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

6. Polyacrylamide gel electrophoresis: Protein samples to be electrophoresed were precipitated in 10% trichloracetic acid (TCA), incubated in ice for twenty minutes, and centrifuged at 2000 rpm. The precipitate was washed once with acetone and centrifuged again. The acetone was removed and 0.2 ml of the sample buffer was added to the precipitate. The precipitate was resuspended and dissolved in the sample buffer and put in boiling water for 1-1/2 minutes. The mixture was cooled to room temperature and one micro-liter of bromphenol blue was added as a tracking dye. The sample was now ready to be layered onto the upper gel for electrophoresis.

For analytical purposes, the lower gels were 10 cm long and .9 cm in diameter made of 13% acrylamide (acrylamide-bis acrylamide, Maizel, 1971) with a 1 cm long upper gel of 3% acrylamide on top. For preparative purposes, the length of the 13% gel was increased to 15 cm with the same upper gel as before. The gels were electrophoresed at a constant current of 4 m amps/gel. Upon completion of the run, the gels were removed from the tubes by breaking

the glass and washing in tap water. The gels were frozen and sliced into

1 mm slices, and ^{125}I-labeled samples counted directly in a gamma spectro-

meter (Packard). ^{3}H-amino acid labeled and ^{14}C-amino acid labeled RSV

samples, after slicing, were placed in scintillation vials containing 10

ml of a toluene, Liquifluor (New England Nuclear), and 3% Protosol (New

England Nuclear) solution. The samples were incubated overnight at 60°C,

then cooled to room temperature prior to counting in a Tricarb Spectrometer

(Packard).

7. <u>Separation and purification of viral gs proteins</u>:

a. <u>Iodinated virus</u>: About 5 μg of iodinated viral protein plus

about 50 μg of BSA as a carrier were prepared for electrophoresis and run

as described above. The locations of the gs proteins in the sliced gel

were determined from the peaks of the gamma counts. The appropriate slices

containing each protein were taken together, minced into very small pieces,

and put into about 4 ml of PBSTD overnight with continual stirring. The

acrylamide pieces were removed by low speed centrifugation (2000 rpm for 10

minutes) and the supernatant clarified by high speed centrifugation (30,000

rpm for 30 minutes in a Type 40 fixed angle rotor, Spinco).

b. <u>"Cold" virus</u>: About 100 μg of total B77 viral protein plus

about 20,000 cpm of ^{14}C-amino acid labeled B77 were electrophoresed as

described above. The gel slices were counted in a gas flow beta counter

(Packard), and the peaks of ^{14}C activity treated as described for the

^{125}I-labeled virus.

8. <u>Radioimmunoassay procedure</u>:

a. <u>Determinations of antiserum concentrations to use in the assay</u>:

The ratio of sheep antiserum (against rabbit immunoglobulins) and rabbit

serum used was determined by reacting increasing amounts of sheep antiserum

with each of several different amounts of rabbit serum in equal final
reaction volumes. After a thirty minute incubation, the mixture was cen-
trifuged, the supernatant removed, and the pelleted material dissolved in
1 ml of 1/4 N acetic acid. The optical density of the solution at 280 nm
was determined in a Beckman spectrophotometer. The amount of sheep anti-
serum which gave the highest optical density with a given amount of rabbit
serum was chosen as the standard for the subsequent radioimmunoassay.

For each preparation of iodinated viral proteins it was necessary to
determine the optimal concentration of rabbit antiserum to use for a given
amount of iodinated protein. In most cases, 10,000 acid precipitable counts
of ^{125}I-labeled protein were chosen as the standard. The labeled protein
was mixed with increasing amounts of rabbit antiserum and sufficient PBSTD
to give a final reaction volume of 150 μl. The reaction was incubated at
room temperature for 30 minutes. Then the appropriate amount of sheep
antirabbit serum was added, followed by an additional 30 minute incubation
at room temperature. This mixture was then layered on top of 100 μl of
SMTD in a Beckman microfuge tube (450 μl volume) and centrifuged at 10,000
rpm (HB-4 rotor, Sorvall) for 30 minutes. This procedure allows the bound
antigen to be separated from the unbound antigen since the antigen-antibody
complex moves through the SMTD while the unbound antigen remains in the
supernatant. The microfuge tubes were then placed in a container of powdered
frozen CO_2 until the entire mixture was frozen. The tips containing the
antigen-antibody complex were cut off, and either the tips were counted
directly in a gamma spectrometer (Packard Instruments) or the complexes were
dissolved in 1 ml of 1/4 N acetic acid and prepared for electrophoresis.
The amount of rabbit antiserum used for subsequent assays was chosen as that
amount which would precipitate with 50% of the total precipitable counts

obtained by this procedure.

b. <u>Competition experiments</u>: Before reliable unlabeled isolated gs antigens were obtained, the competition experiments used unlabeled disrupted B77 RSV for calibration. Later, the isolated antigens were used separately as competitors. The competition experiments were performed as follows. To a set of tubes with a given quantity of rabbit antiserum and sufficient PBSTD to give a constant reaction volume (150 or 235 μl) were added increasing quantities of known concentrations of unlabeled RSV or unlabeled isolated gs antigen from RSV. The mixture was incubated for 30 minutes at room temperature, followed by the addition of labeled virus (or labeled isolated gs antigen) and another incubation of 30 minutes at room temperature. The appropriate amount of sheep antiserum was added, followed by another 30 minute incubation at room temperature. Centrifugation of the mixture on SMTD, the counting of the radioactivity, and the preparation for electrophoresis were described above. Cell extracts to be tested for the presence of RSV-like protein were used in the competition experiments in place of the unlabeled RSV or unlabeled isolated gs antigen.

In the early competition experiments using whole labeled virus and no competitor, the antigen-antibody complex which precipitated was electrophoresed and the assay was calibrated as follows. From the electrophoretic pattern of the radioactivity, the total number of ^{125}I counts in each of the three peaks were determined after subtracting the background. Then, in experiments using known quantities of unlabeled virus as competitor, there was a decrease in the total radioactivity in each peak, and this decrease could be related to the amount of unlabeled competitor added. When cell extracts were added as competitors, any decrease in total radioactivity in the peaks could be related to the competition experiments in which known

concentrations of unlabeled virus were added. This method only allowed

an estimation of individual gs antigens present as related to the amount of

whole virus used as competitor. The later experiments used labeled isolated

gs antigens, and unlabeled isolated gs antigens were used in known concen-

trations as competitors. These experiments allowed more precise determinations

of the amount of each gs antigen present in different cell extracts used

as competitors.

RESULTS

It is important to know that the iodination process does not sub-

stantially alter the viral protein with respect to its immune reactivity

and its migration in SDS-PAGE. ^{14}C-amino acid labeled B77 and ^{125}I-

labeled B77 were prepared for electrophoresis, as described, with the

addition of 50-100 μg of bovine serum albumin to each sample as carrier

for the precipitation of protein by TCA. The electrophoretic pattern of

the two virus preparations, as shown in Fig. 1, demonstrates that there is

no noticeable alteration in mobility of RSV on SDS-PAGE due to iodination

(in this case, by the "ester" method). The usual electrophoretic pattern

as observed in this investigation is seen in Fig. 1. Three peaks are clearly

defined, representing, in the order of slowest to fastest migrating protein,

p27 in the first peak, p19 and p12 in the second peak, and p15 and p10 in

the third peak. An additional peak, which migrates faster than p10, is

often seen when the chloramine-T method is used for iodination and is

occasionally seen when the lactoperoxidase method is used. It has not been

observed when the ester method is used or when virus is labeled with ^{3}H-

or ^{14}C-amino acids. The most likely explanation for this extra peak seems

to be that it represents fragments of larger polypeptides broken by the

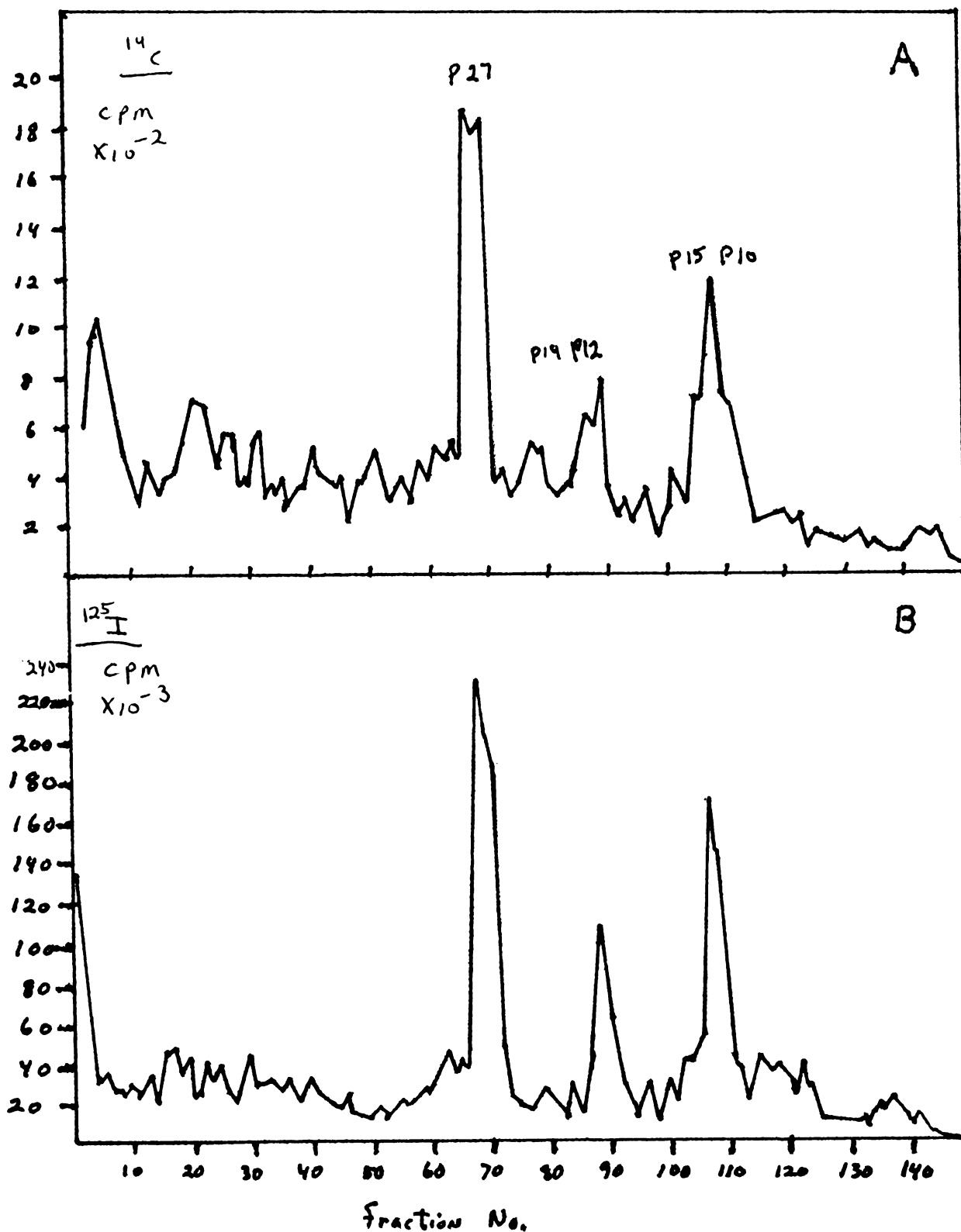relatively harsh oxidation reactions of the chloramine-T method (and, to a

Fig. 1. Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE) of preparative amounts of the B77 strain of RSV. About 50 g of BSA was added as carrier and the protein precipitated by TCA. A.) Virus grown in the presence of ^{14}C-amino acids. B.) Virus labeled with ^{125}I by the "ester" method (see Methods for details).

lesser degree, of the lactoperoxidase method). This explanation is further

supported by the electrophoretic patterns of ^{125}I-labeled virus precipitated

by immune serum (see Fig. 2). The additional peak is never seen in SDS-PAGE

of immune precipitations of virus labeled by any of the three methods used

for iodination.

Fig. 2 shows the electrophoretic patterns of ^{14}C-amino acid labeled

B77 and ^{125}I-labeled B77 precipitated by the double antibody method and

prepared for SDS-PAGE (see Methods). A comparison with Fig. 1 will show

that the three major protein peaks seen in electrophroesis of whole virus

are still seen in the electrophoresis of protein specifically precipitated

by antiserum to RSV. Fig. 2a and 2b shows that the iodination procedure

does not appear to alter the immune reactivity of the viral proteins in

comparison to virus labeled with ^{14}C-amino acids. And of particular importance

is that iodination can provide protein labeled to much higher specific

activities in comparison to labeling with ^{14}C-amino acids.

After obtaining ^{125}I-labeled virus proteins of high specific activity,

competition experiments were tried to see the change in the total radio-

activity preciptiated by the antiserum when increasing amounts of unlabeled

antigen were added as competitor. It should be noted that in the early

experiments, two refinements used in later experiments were not yet tried.

Thus, in these experiments, too much antiserum was used (the amount which

precipitated about 90% of the radioactivity instead of 50%) and the unlabeled

antigen was added at the same time as the labeled antigen (instead of pre-

incubating the unlabeled antigen with the antiserum and then adding the

labeled antigen). Consequently, the antiserum was not in limiting concen-

trations, causing the sensitivity of these experiments to be lowered (compared

to more recent experiments). Competition still occurred and could be related

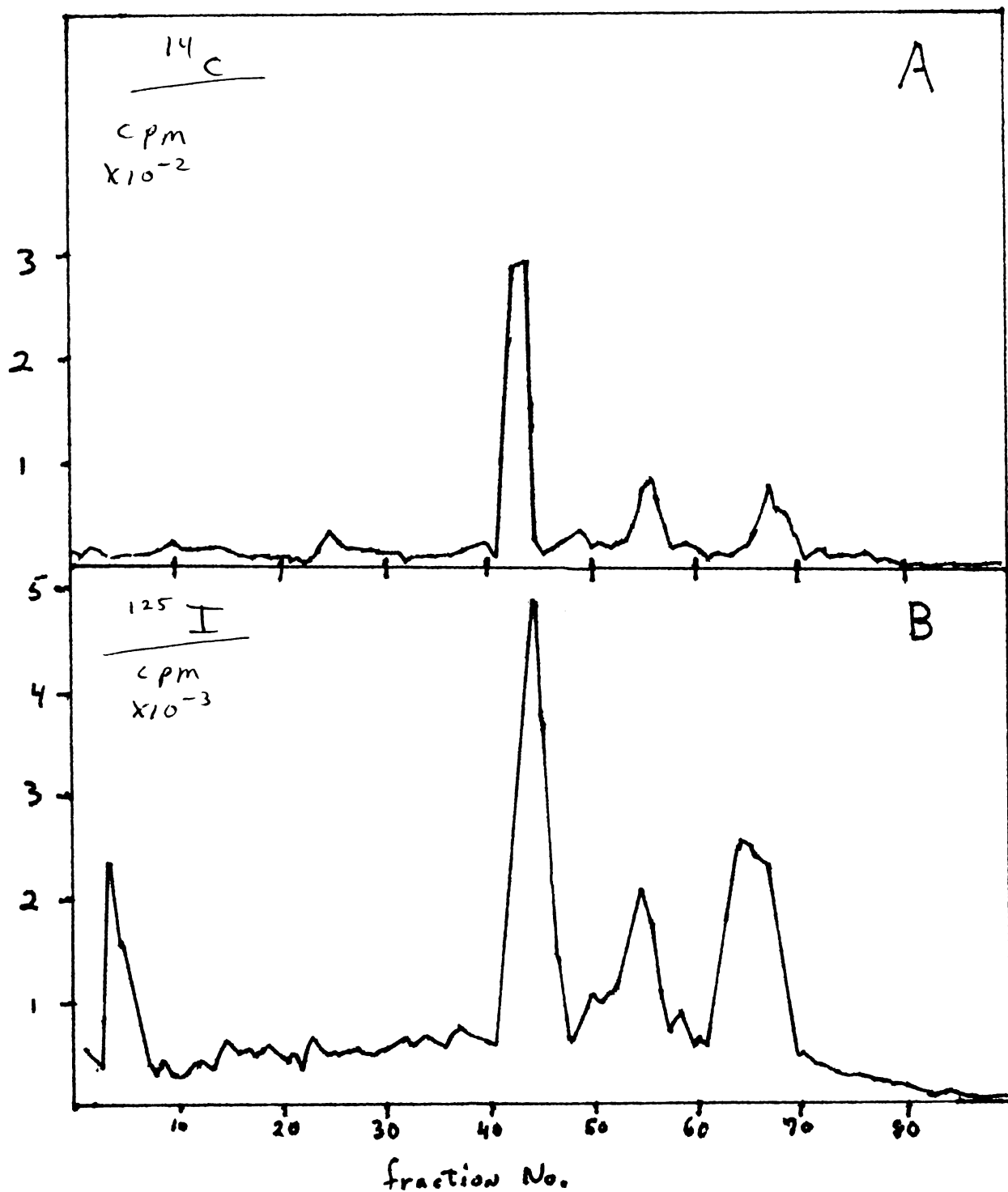to concentrations of unlabeled competitor added.

Fig. 2. SDS-PAGE of B77 virus specifically precipitated by antiserum and separated from unbound virus as described in Methods. A.) B77 grown in the presence of ^{14}C-amino acids. B.) B77 laceled with ^{125}I by the lactoperoxidase method.

Fig. 3 shows the competition curves obtained when increasing amounts
of disrupted unlabeled virus were used as competitor. The precipitated
immune complex from each experiment was electrophoresed as described.
From the electrophoretic pattern of each experiment, the total radio-
activity in each of the three major peaks was determined. The total radio-
activity in each peak is plotted against the amount of unlabeled competitor
added in that experiment. As can be seen, with the addition of 30 ng of
unlabeled virus protein as competitor, there is a decrease of between 15
and 20% in the total radioactivity in eack peak. From this information,
competition which is seen in extracts with unknown amounts of RSV protein
can be related to the amount of viral protein actually present.

To study the production of gs antigens in newly infected avian cells,
infecting medium was added to thoroughly washed nearly confluent cultures
of duck embryo fibroblasts (3 ml per mm plate). The cells with infecting
medium were incubated for 2-3/4 hours at 41°C. The infecting medium was
removed and replaced with growth medium. At this time, 6 plates were used
for the preparation of cellular extracts (see Methods). This was called
the zero-hour extract. At each of various time intervals after infection,
6 more plates were used for the preparation of extracts. (Once each day,
the growth medium on the remaining plates was removed and replaced with
fresh medium). The extracts were then used in the competition experiments,
followed by SDS-PAGE of the immune precipitates. The data were treated as
described above. Controls in these experiments included using normal rabbit
serum (obtained from a pre-immunization bleeding of the rabbit) with each
extract and using immune serum without competition. The control using normal
rabbit serum showed only background radioactivity in the immune precipitate.
The results from the control using immune serum without competition were in
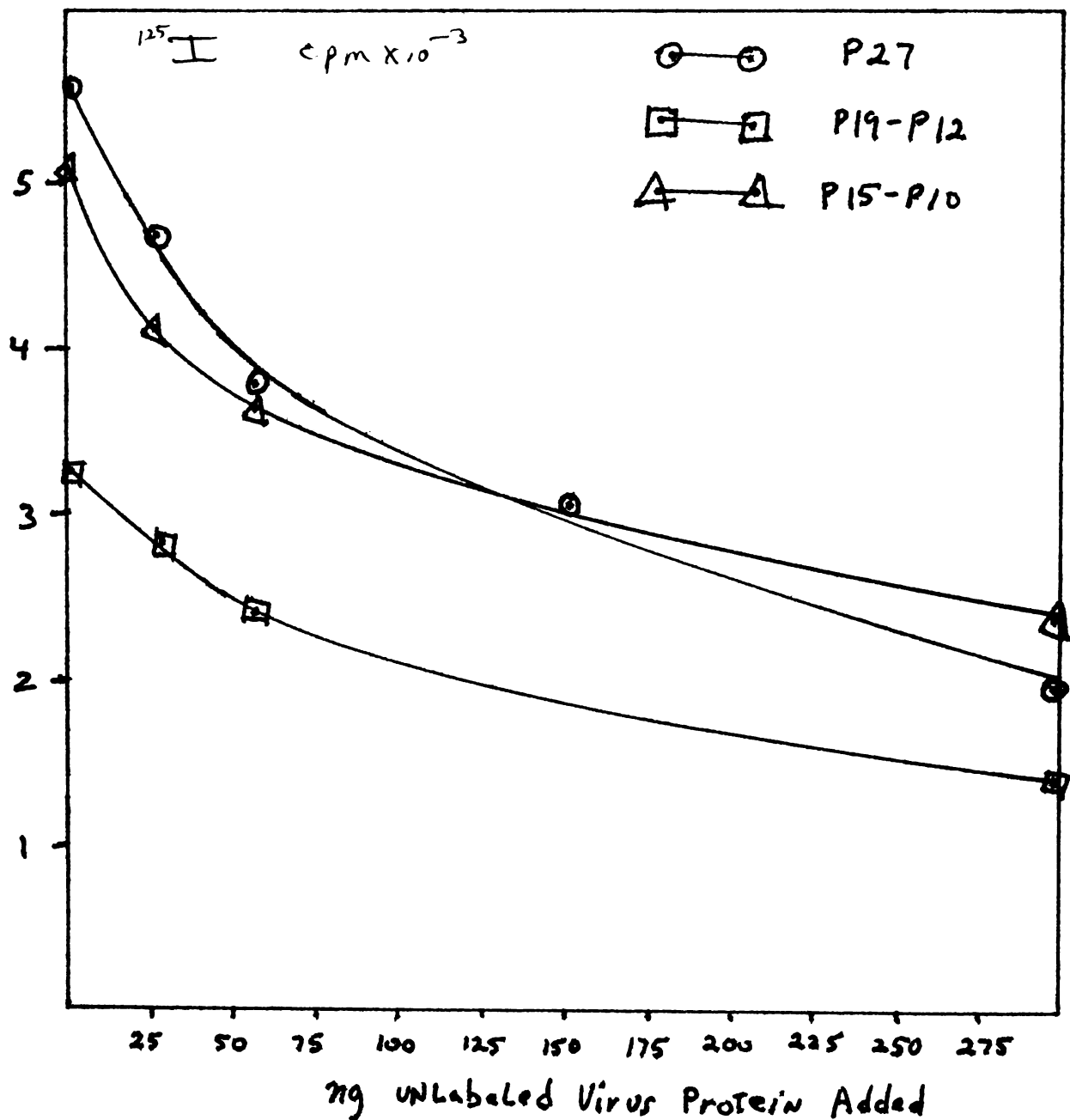
Fig. 3. Competition curves (or dose response curves) for each of the three main peaks seen on SDS-PAGE when increasing amounts of unlabeled virus are added as competitor. The number of counts at each point represents the total radioactivity in the peak after electrophoresis of the immune precipitate (see Methods for details).

agreement with the results with the zero-hour time point.

Fig. 4 shows, as an example, the electrophoretic patterns of the competition experiments using the zero-hour extract and the 2-day extract. Of the proteins in the three major peaks, p27 appears to be the most sensitive to competition, and, as shown in Fig. 4, is particularly decreased by the second day after infection. Fig. 5 shows the results of the competition experiments using the extracts obtained at each of the different time periods after infection. The results are plotted for each of the three major peaks as the total number of ^{125}I counts in each peak with respect to the number of days after infection. By the twenty-first hour after infection, there is a definite decrease in the total radioactivity in each peak with a continual decrease in the p27 peak. The apparent leveling off in the radioactivity in the other two peaks with increasing time is unexplained at this time. Also listed in Fig. 5 is the total protein content of the sample sued for the competition experiments. Comparing Fig. 5 with Fig. 3, it can be seen that 21 hours after infection, there is in 1 mg of cell extract an amount of p27 (and p19 and p12) equal to the amount of p27 (and p19 and p12) in about 60 ng of whole virus. This same comparison can be made for each of the proteins at each time point, except that the accuracy is questionable. For proper comparisons, the virus titers at each time point should be obtained. Future experiments will contain them. The refinements of the competition experiments discussed below in which the isolated ^{125}I-labeled gs antigens are used, improve the accuracy of the quantitation of the results. It should be remembered that the experiments as performed are measuring the intracellular concentration of RSV-specific protein and virus still budding from the cell. Mature virus particles which have budded from the cell will not be included in the extract.
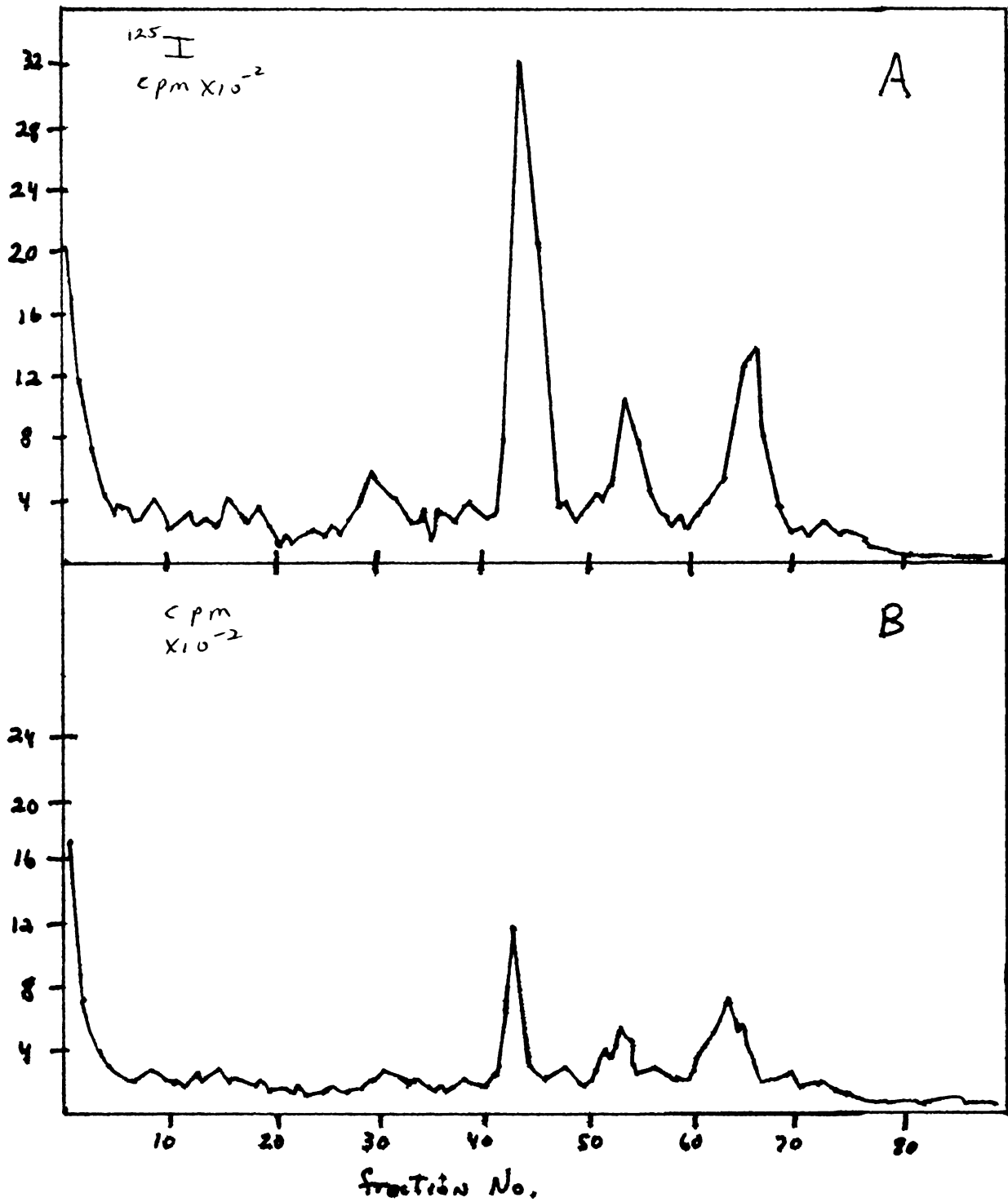
Fig. 4. SDS-PAGE of the immune precipitates from competition experiments using cellular extracts from two different time points after infection of duck embryo fibroblasts by B77.   A.) Zero-hour extract.   B.) Two-day extract.
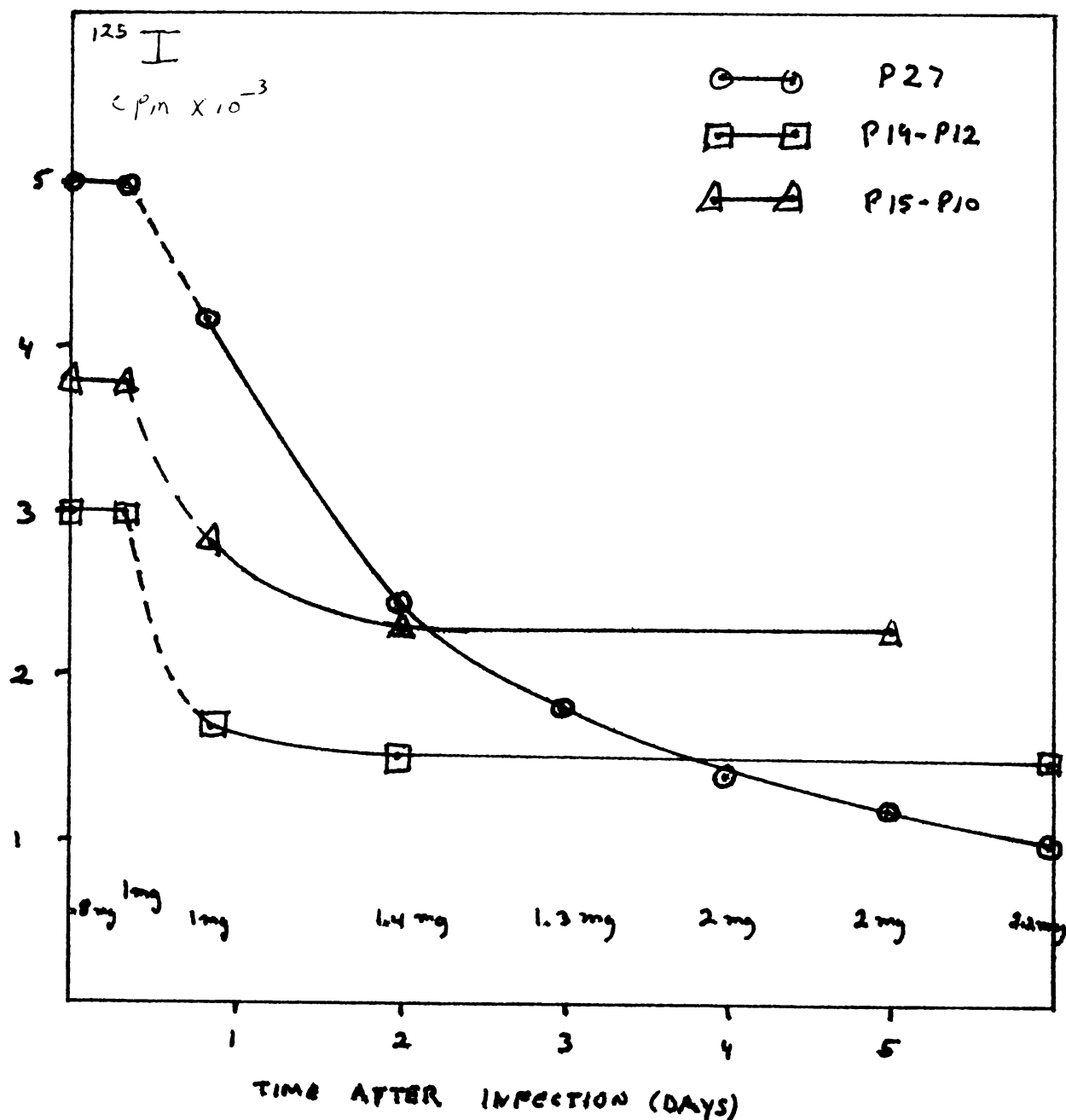
Fig. 5. Competition curves for each of the three major peaks seen on
SDS-PAGE. Duck embryo fibroblasts in culture were infected with B77,
and at various times after infection, cellular extracts were prepared
from some of the cells. The extracts were used in competition experiments,
and the immune precipitates which formed were subjected to SDS-PAGE.
The total radioactivity in each peak for each experiment was determined
as described. Below each experimental point is listed the total protein
content in the sample of extract used for that experiment.

An improvement in the assay can be achieved by obtaining the isolated gs-antigens, both unlabeled and labeled with ^{125}I. Using the procedure described in Methods, ^{125}I-labeled p27 was obtained and subjected to SDS-PAGE to determine its purity. Also, the ^{125}I-p27 was precipitated with immune serum (using enough rabbit antiserum to precipitate half of the radioactivity) and then subjected to SDS-PAGE. These results are shown in Fig. 6. The majority of the radioactivity of the ^{125}I-p27 fraction resides in the single peak at the location of p27 (as seen when compared to a parallel gel of ^{125}I-B77 or when compared with Fig. 2), with a portion of the radio-activity in a peak which has a faster mobility than the third major peak (p15 and p10). This faster moving material most likely represents broken fragments of p27, since none of that material was seen on the gel of ^{125}I-whole virus and none of it is preciptiated by the immune serum (Fig. 6b).

Unlabeled p27 was isolated in the same way as the ^{125}I-p27, except that ^{14}C-amino acid labeled B77 was used as a marker along with the unlabeled B77 in the electrophoresis. The unlabeled (and ^{14}C-labeled) p27 was subjected to SDS-PAGE, with results similar to those shown for ^{125}I-p27. The protein concentration of the unlabeled p27 was determined by the method of Lowry et al. (1951), and aliquots of known concentrations of p27 were used as competitors with ^{125}I-p27 in the radioimmunoassay. (The ^{14}C label will not affect the results, since ^{14}C is not detected by the gamma counter). The competition curve (or dose-response curve) obtained is shown in Fig. 7 in which the percent of maximum counts bound by the antiserum is plotted against the amount of unlabeled p27 (in ng) added as competitor. Using this competition curve, a more exact determination of the amoutn of p27 present in cell extracts can be obtained.

Table I lists the results of competition experiments using ^{125}I-P27
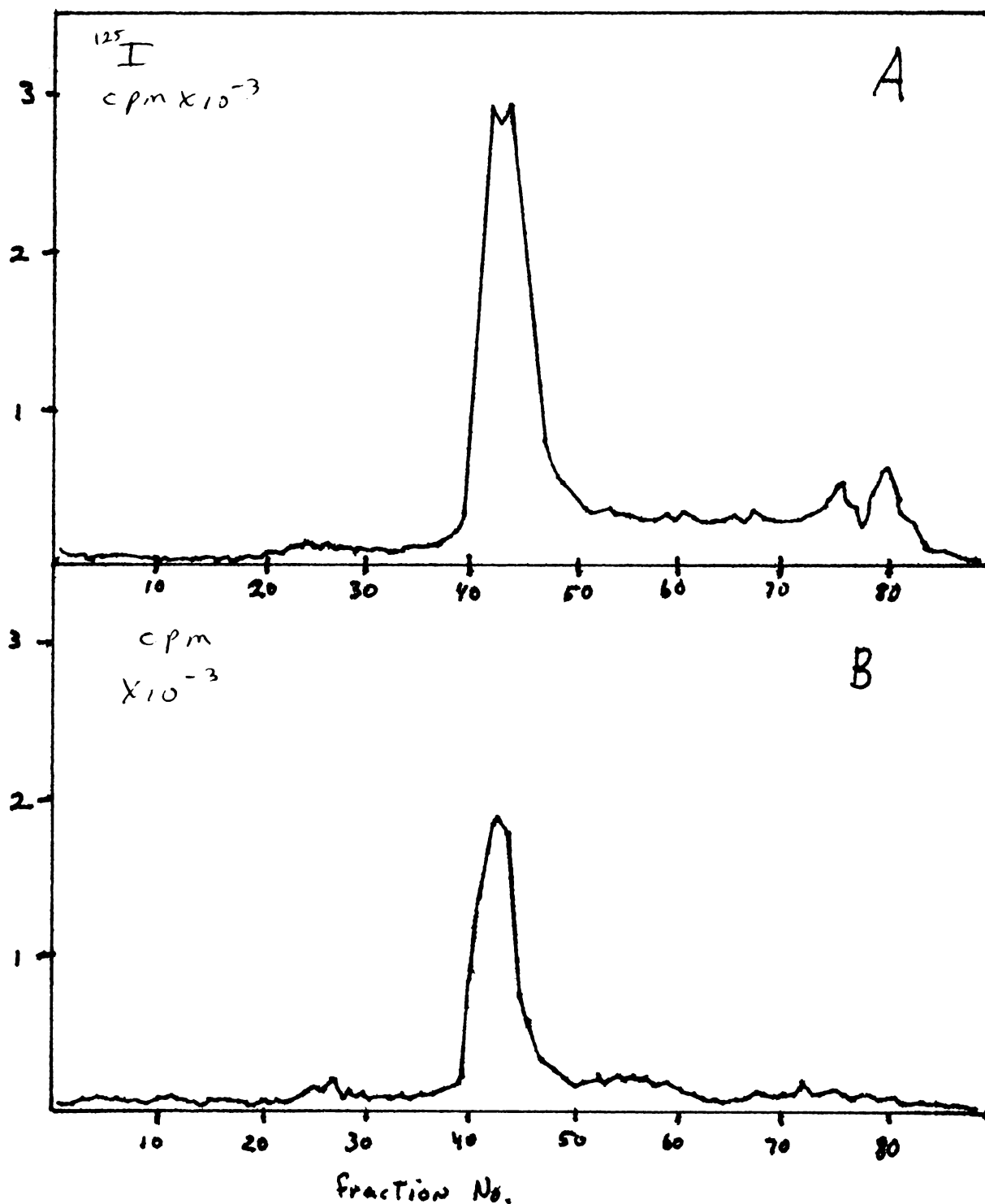
Fig. 6. SDS-PAGE of the isolated ^{125}I - labeled P27. A.)^{125}I - P27 precipitated by TCA using BSA as a carrier. B.)^{125}I - P27 specifically precipitated by antiserum to B77. The samples were prepared for electrophoresis as described.

and several dilutions of extracts from different cell lines (see Methods).
These experiments used as controls extracts from normal duck embryo fibro-
blasts, HeLa cells, and BALB/3T3 cells (uninfected). No detectable p27-
like protein was detected in the controls. Also, preliminary results have
shown that there is no detectable cross-reaction between p27 and the other
two major protein peaks. The results in Table I are presented as ng of
p27 per mg of total cell protein in the extract used in the competition
experiment. For comparison, Chen and Hanafusa (1974) have found 1430 ng
of p27 per mg of cell protein for B77-infected chick cells.

Also listed in Table I for comparison are data from Deng et al. (1974)
and Varmus et al. (1974) for the $C_r t_{1/2}$ and the number of viral genome
equivalents of the RNA in the cells. The $C_r t_{1/2}$ was obtained by hybrid-
izing ^{3}H- or ^{32}P-labeled virus-specific single-stranded DNA with large
quantities of cellular RNA. In general, the smaller the $C_r t_{1/2}$ value,
the greater the amount of RSV-specific sequences in the cellular RNA.
The number of viral 35S RNA genome equivalents is calculated from the $C_r t_{1/2}$.

Table I

| Cell extract	ng of p27 mg total cell protein	$C_r t_{1/2}$ (from Deng et al., 1971)	Viral 35S RNA equivalents per cell (from Varmus et al., 1974)
Normal duck embryo fibroblast	0	–	0
HeLa cells	0	–	–
BALB/3T3	0	–	–
B77-infected chick embryo fibroblasts	20,000	5	60,000
XC	2,500	3×10^3	100
3T3/B77	380	$7\text{-}8 \times 10^3$	40
SR-3	60	3.5×10^4	8
SR-3/1a	30	7×10^3	40

DISCUSSION

The reliable detection of small amounts of RNA tumor virus-specific proteins has been attempted for several years. The COFAL test (Sarma et al., 1964) was the first reliable method, but it did not have sufficient sensitivity to detect less than about 100 ng of viral protein. Additionally, it could not detect individual proteins, but rather could indicate only if RSV-specific protein was or was not present. Thus there was a search for more specific and more sensitive methods. The double antibody radio-immunoassay has proved to be more sensitive than COFAL, and can be used to detect one or more proteins, depending on the available antiserum. The two important features which determine the sensitivity and specificity of the method are the specific activity of the labeled RSV protein and the titre and specificity of the antiserum to RSV proteins. In the course of this investigation, various attempts have been made to improve both features.

Originally, labeled RSV protein was obtained by growing B77-infected chick cells in medium containing ^{3}H- or ^{14}C-labeled amino acids. The virus was purified from the growth medium and then used in the immunoassay after treatment with suitable detergents which disrupt the envelope. Although this method of labeling worked, it could not provide protein with high enough specific activity to give the desired sensitivity (about 5000 cpm per µg of protein). Labeling with ^{125}I provided a method of obtaining specific activities greater than 10^6 cpm per µg of protein. The most common method of labeling with ^{125}I uses chloramine-T as an oxidizing agent, and one or two atoms of ^{125}I can be covalently linked to the phenol ring of tyrosine residues. This method of labeling is very rapid and effective, but is limited by the number of tyrosine residues in each protein molecule. An additional disadvantage is that chloramine-T is a relatively harsh oxi-

dizing agent and tends to cause some breakage of the protein exposed to

it. Thus a more gentle method of labeling was sought. The enzyme lacto-

peroxidase in the presence of very dilute solutions of H_2O_2 will also

effect the covalent linkage of one or two atoms of ^{125}I onto tyrosine.

Apparently in the process of iodination, a complex is formed involving

the lactoperoxidase, the peroxide, the iodine, and the phenolic compound

(Morrison and Bayse, 1970). The use of lactoperoxidase provides a more

gentle method for iodination than chloramine-T, but still has the limitation

of the number of tyrosine molecules which are available to the enzyme. An

additional drawback is the self-iodination of the enzyme and the subsequent

separation of the iodinated enzyme from the iodinated virus proteins. In

this investigation, the only difficulty with the presence of iodinated

enzyme might be "trapping" during the immune precipitation, giving an increase

in the background radioactivity. The use of lactoperoxidase does cause some

breakage in the polypeptides, but it is not as severe as in the chloramine-T

method. The "ester" method has two features which offer advantages over

the other two methods. First, the virus protein is never directly exposed

to an enzyme or to a strong oxidizing agent, and second, the iodinated ester

attaches by chemical reaction to terminal amine groups (mainly in lysines).

Presumably there are more lysine residues than tyrosine residues in the virus

proteins. The ester is formed between 3-(4-hydroxyphenyl) propionic acid

and N-hydroxysuccinimide. The ester is then reacted with chloramine T in

the presence of $Na^{125}I$, causing the iodination of the phenolic group. The

iodinated ester is extracted with benzene and dried, after which it can be

reacted with protein solutions. The €-amino group of lysine residues can

attack the ester bond, replacing the N-hydroxysuccinimide and leaving the

iodinated hydroxyphenyl propionic acid attached to the lysine. This method

has been used with good success to give virus proteins labeled to high
specific activities while retaining their immune reactivity (greater than
20×10^6 cpm per μg protein, with up to 90% of the radioactivity precipitable
by antiserum). Recent results indicate that even higher specific activities
can be obtained.

3 - (4 - hydroxy phenyl)
Propionic Acid

N - hydroxy succinimide
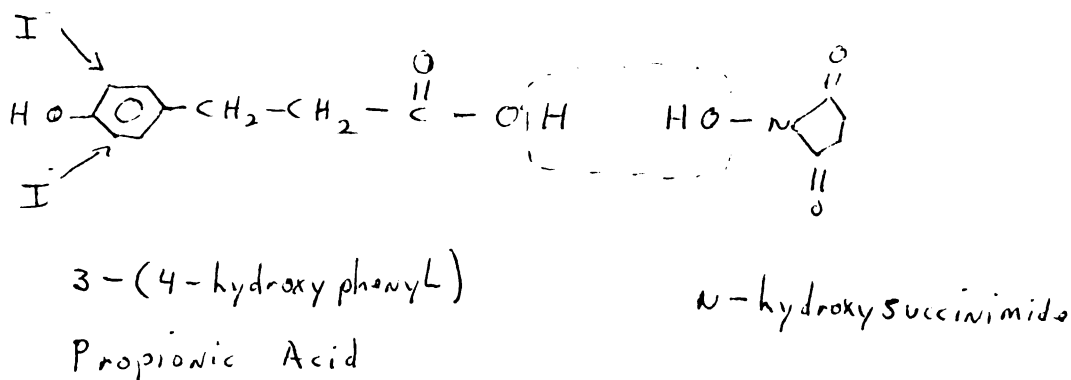
Greater specificity and sensitivity in the radioimmunoassay can be
obtained using the isolated gs proteins. To separate the gs proteins, two
methods were tried with only slight success, agarose gel filtration in 6M
guanidine hydrochloride (Fish et al., 1969; Fleissner, 1971; Nowinski et al.,
1972; Fleissner and Tress, 1973) and isoelectric focusing (Svensson, 1962;
Oroszlan et al., 1970; Hung et al., 1971). The separation of the viral poly-
peptides using agarose gel chromatography was never as successful in our
hands as the published results of Fleissner (1971), but some separation did
occur. The eluates from the gel filtraion were dialyzed and injected into
rabbits to obtain monospecific antiserum. Unfortunately, the viral polypeptide,
after exposure to guanidine hydrochloride, apparently did not regain their
original antigenic properties, since the antiserum obtained would not react
with iodinated virus. Isoelectric focusing was also used to attempt the

separation of viral proteins. Again, the results were not as successful

as those published by Hung et al. (1971), and each isolated gs antigen was

contaminated with the other gs antigens, as was observed on SDS-PAGE analysis.

Both methods of separation of viral proteins will be tried again in the

future, hopefully with more success, since the isoalted gs antigens can

be used to obtain monospecific antiserum and can be used for iodination.

The method used in this investigation which did successfully separate the

gs proteins was SDS-PAGE and the subsequent elution of the proteins from the

appropriate gel slices as described in Methods. The separated labeled proteins

retain their antigenicity when reacted with antiserum (Fig. 7) and thus can be

used in the radioimmunoassay. Attempts are currently underway to obtain the

separated unlabeled gs proteins and then label them with ^{125}I, hopefully to

an even higher specific activity than that obtained by labeling whole virus

and then separating the proteins.

One diversion from the radioimmunoassay method used in this investigation

was an unsuccessful attempt to use a solid phase radioimmunoassay procedure

(Catt and Tregear, 1967; Ball, 1973). In this method, antiserum was coated

onto plastic tubes and then reacted with iodinated antigen. The tubes are

rinsed with saline, so that unbound antigen is removed, and then counted directly

in a gamma counter. Unfortunately, the background radioactivity was very high

in these experiments and sufficient amounts of labeled antigen were never

sufficiently bound onto the antibody-coated tubes to allow reasonable com-

petition experiments. Presumably, inadequate amounts of antiserum were bound

onto the tubes, in spite of attempts to bind the antiserum in different

buffers at different pH levels and in different types of tubes. Because of

the ease and convenience of this method, it might have promise for the future

if the various technical problems can be solved.

The techniques successfully employed in this investigation have been described in Methods. The preferred method of iodination was the "ester" method, although the chloramine-T and lactoperoxidase methods were both used successfully before the "ester" method was perfected. The individual gs proteins were separated with SDS-PAGE followed by elution of the proteins from the gel slices. In the radioimmunoassay, it was necessary to separate the bound antigen from the unbound antigen. The method used allows the antigen-antibody complex to be centrifuged through a 0.5M sucrose solution (SMTD) while the unbound antigen remains in the supernatant. This is probably a better method than merely removing the supernatant from a pellet of the antigen-antibody complex, because of possible residual unbound antigen reamining on or about the pellet, although washing the pellet should remove most of the residual unbound antigen. Stephenson et al. (1971) and Chen and Hanafusa (1974) use the method of removing the supernatant from the antigen-antibody complex and determining the amount of radioactivity remaining in the supernatant (that is, not bound to antibody). The method used in this investigation directly determines the amount of labeled antigen bound to antibody.

The radioimmunoassay in this investigation was used to study the course of infection by the B77 strain of ASV. Duck embryo fibroblasts were chosen since they contain no detectable endogenous RSV-like DNA sequences in their genome (Varmus et al., 1973) and are susceptible to infection and transformation by B77 with the subsequent production of whole virus. The competition experiments were performed with whole virus labeled with ^{125}I as described in Methods section. Without having the isolated labeled gs antigens to use in the experiments, it is difficult to make precise quantitative judgments about the production of each antigen, but qualitative statements can be made. As

shown in Results, sufficient amounts of the gs antigens are made by 21 hours after infection to be detected. Each of the antigens continues to be made, although the amount of intracellular p27 appears to increase continually. The other gs antigens seem less sensitive to the assay and the data are not as easy to interpret. The difference may be partly because p27 is one protein whereas the other two peaks seen on electrophoresis are each made up of two proteins. Much more information from these experiments will be obtained when (1) the isolated labeled antigens are used in the competition experiments, (2) time points between 8 and 21 hours after infection are tested, and (3) the growth medium from each time point is tested to determine the virus titre. Then the intracellular production of each gs antigen can be correlated with actual virus production.

The separation of labeled gs antigens can be accomplished by electrophoresis as described. The separated proteins retain their antigenicity and electrophoretic mobility (Fig. 6), and can be used in the competition experiments to determine the quantities of the individual gs antigens produced in different cells. This has worked well for p27, and Table I lists the amount of p27 produced in several different cell cultures. Chick cells are permissive for the B77 strain of RSV, and if infected with B77, the chick cells are transformed and shed virus. Thus a large quantity of intracellular p27 would be expected and was found. XC cells are from a rat tumor induced by Prague strain of ASV and contain in the genome about 20 copies of the ASV genome (Varmus et al., 1973b). Whole virus is not shed from XC, although as seen in Table I, a substantial amount of p27 was found. 3T3/B77 is a mouse cell line, which was also transformed by B77 but does not shed whole virus. 3T3/B77 contains in its genome about 1-2 copies of the B77 genome (Varmus et al., 1973a) and it also produces some p27, though only about 15% as much as XC cells per mg of cell protein. BHK 21 is a cell line from

Syrian hamsters, which, when transformed by the SR strain of RSV, gives a cell line called SR-3 which has a high frequency of reversion to normal morphology. SR-3/1a is a transformed subclone from SR-3 cells. Both SR-3 and SR-3/1a cells contain small but detectable quantities of p27, as seen in Table I. Experiments are presently under way to test for the presence of the other gs antigens, p19-p12 and p15-p10, in the above extracts. Until those tests are completed, the only firm conclusion which can be stated is that in the above mentioned mammalian cells transformed by RSV, whole virus is not produced but p27, the largest gs antigen, is made.

It is of interest to compare the amount of intracellular p27 with the amount of RNA genome equivalents present in the cells (see Table I). The trend is that the greater the number of viral RNA genome equivalents, the greater the amount of p27 produced, although two different cell lines can have about the same number of viral genome equivalents and about a tenfold difference in p27 concentration (compare 3T3/B77 and SR-3/1a). This difference might be partially explained by different rates of normal protein synthesis in different cells (since the concentration of p27 is given in relation to the amount of total cell protein). Another explanation for the difference is that the control of translation in cells is a complex phenomenon which is somewhat different in each cell.

As seen by the results, the radioimmunoassay as developed can detect amounts of RSV proteins as small as 20 ng of p27 (and recent results indicate 2 ng can be detected). Other published radioimmunoassay methods can detect about 1-10 ng of p27, although Chen and Hanafusa (1974) detect 0.3 ng p27. The method is reliable using the criterion of SDS-PAGE analysis. It is also rapid once the system is established. Thus it is a convenient and rapid way to detect the presence of RSV proteins in cells which compares favorably with other published radioimmunoassay methods. The published methods have

been used to detect the presence of the ASV gs antigens in cells previously determined to be gs negative by the COFAL test and in transformed mammalian cells (see in particular, Chen and Hanafusa, 1974; Stephenson et al., 1973; Weber and Yohn, 1972).

Attempts to improve the radioimmunoassay are currently under way. One attempt is to obtain the isolated gs antigens labeled to higher specific activities. Increasing the incubation time of antigen with antiserum has had some promising results in increasing the sensitivity of the competition experiments. Antiserum which reacts only to one gs antigen will be obtained in the future, and this should improve the sensitivity of the immune reaction. It is hoped that with improvements and refinements of the immunoassay, the ability to detect RSV proteins will be increased several-fold. It is then that the more important questions of the mechanisms for control of protein syntehsis can be examined with respect to infection by RSV. It is hoped that when sufficient sensitivity of the assay is attained, polyribosomes from cells can be isoalted and nascent RSV-protein synthesis can be studied. Then the course of infection by RSV and the production of whole virus can be closely studied. Hopefully it can be discovered why RSV-transformed mammalian cells produce some gs protein (in particular, p27) but no virus is made.

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