Incorporation of Radioactive Seleno-\(^{75}\text{Se}\)-Methionine into Mumps Virus

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Mumps virus was grown in embryonated chicken eggs in the presence of radioactive seleno-\(^{75}\text{Se}\)-methionine. Virus in the allantoic and amniotic fluids was concentrated in a sucrose density gradient, and a peak of viral material coincided with a significant peak of \(^{75}\text{Se}\)-radioactivity. The radioactivity was acid-insoluble and remained associated with the virus after purification by erythrocyte adsorption and elution and centrifugation on a second sucrose density gradient. After amino-acid hydrolysis of the radioactive virus, only \(^{75}\text{Se}\)-methionine was recovered by chromatographic analysis. These results demonstrate that the radioactive \(^{75}\text{Se}\)-methionine was incorporated into protein of infectious mumps virus.

High-energy gamma-emitting isotopes have had little application in virus and tissue culture research, perhaps because of the potential damage to the virus and host cells by the radiation and decay of free or incorporated radioisotopes (3). To date these isotopes have primarily been employed to label biologically active macromolecules in vitro, such as iodination of insulin and antibodies with \(^{131}\text{I}\) (2). The work of Tuve and Williams (6) and of Cowie and Cohen (1), however, has shown that incubation of \textit{Escherichia coli} in the presence of the gamma-emitting selenium compound \(\text{H}_3\text{SeO}_3\) resulted in the incorporation of the \(^{75}\text{Se}\) into bacterial proteins, and more recently radioactive \(^{75}\text{Se}\)-insulin has been produced in rats by injection of \(^{75}\text{Se}\)-selenocysteine (5). The experiments presented in this report demonstrate that the radioactive \(^{75}\text{Se}\)-methionine is incorporated into the protein of mumps virus grown in embryonated eggs.

**MATERIALS AND METHODS**

**Virus production.** The Enders strain of mumps virus, with an infectivity titer of \(10^{8.5} \text{ (EID}_9\text{)} /0.5 \text{ ml} \), was inoculated into the allantoic cavity of 7-day-old leukosis-free embryonated chicken eggs (SPAFAS, Norwich, Conn.). The eggs were incubated at 37 C for 6 days and then chilled overnight at 4 C, and both the amniotic and allantoic fluids were harvested.

**Hemagglutination and infectivity titrations.** Hemagglutination titrations were performed by the microtiter method (4) and were expressed in hemagglutinating units (HA). Infectivity titrations were performed in 7-day-old embryonated chicken eggs.

**Neuraminidase.** Neuraminidase (\textit{Vibrio cholera}), 100 units/ml (Calbiochem, Los Angeles, California), was used to elute virus adsorbed to erythrocytes for virus purification.

**\(^{75}\text{Se-methionine.}** Radioactive \(^{75}\text{Se-methionine} \) (Sothotope, E. R. Squibb and Sons, Inc., New Brunswick, N. J.) with specific activity of 250 mCi/mg was used. Radioactivity was assayed in a Packard Auto-Gamma model 410-A counter and is expressed as counts/minute.

**Density gradient centrifugation.** Infected and control egg fluids were layered onto 10 to 60\% sucrose gradients prepared in TMK buffer \([0.05 \text{ m tris(}\text{H}^+\text{)}\text{aminomethane-hydrochloride with }0.05 \text{ m KCl and }0.0015 \text{ m MgCl}_2\) \]. Preformed gradients were prepared by layering, in succession, 4 ml of 60, 50, 40, 30, 20, and 10\% (w/w) sucrose-TMK solutions into cellulose nitrate centrifuge tubes. The gradients were kept for 16 hr at 4 C to allow diffusion to form linear sucrose density gradients. Nine milliliters of egg fluid was carefully layered on top of each gradient, and the gradients were centrifuged in the SW25.1 rotor of an L2-65 centrifuge (Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.) for 9 hr at 40,000 \(\times \) g at 4 C. After centrifugation, the absorbancy at 254 nm was recorded as the gradients were fractionated into 1-ml fractions by an ISCO (Instrumentation Specialties Co., Lincoln, Neb.) fractionator. The individual fractions were assayed for HA and radioactivity as described above.

**Chromatography.** Virus recovered from the particular gradient fractions was concentrated by centrifugation at 55,000 \(\times \) g for 90 min in a type 30 rotor. The pellet was suspended in 6 N HCl and hydrolyzed at 100 C for 12 hr in a sealed vial under a nitrogen atmosphere to prevent oxidation. The hydrochloride was removed by rotary evaporation, and the hydrolysate was rinsed several times with distilled water. The residue was finally suspended in approximately 0.1-ml volume of water and spotted.
on Whatman no. 1 chromatography paper. Two-dimensional chromatography was then performed; the solvent for the first dimension was a mixture of glacial acetic acid-butanol-water (120:30:50) and for the second, phenol-saturated water.

Radiography. The chromatograms were dried and exposed to high-speed X-ray film (Eastman Kodak, Rochester, N. Y.) while still in the envelope. After one week of exposure, the films were developed in the usual manner.

RESULTS

Leukosis-free eggs were inoculated allantoically with 10⁵ EID₅₀ of mumps virus and 10 μCi of ⁷⁵Se-methionine. Control eggs were inoculated with comparable concentrations of ⁷⁵Se-methionine but without virus. After 6 days of incubation, the allantoic and amniotic fluids were harvested and centrifuged at 1,000 × g for 10 min to remove tissue debris. The supernatants were then layered on sucrose density gradients and the gradients were centrifuged as described above. The profiles of absorbancy at 254 nm, HA, and ⁷⁵Se-radioactivity are shown in Fig. 1A. A relatively narrow peak of 254-nm absorbing material was observed in fractions 21 to 25 in the gradients of infected egg fluids; this peak contained most of the HA and a significant portion of the ⁷⁵Se radioactivity. The gradients of the uninfected control egg fluids do not contain this peak (Fig. 1B). An infectivity titration of fraction 23, i.e., the fraction containing the maximum HA activity, was performed in embryonated chicken eggs. This peak had an EID₅₀/HA ratio of 10⁻⁴.₅ to 10⁻⁵.₅. Because the ultraviolet light used to monitor the absorbency profile of the gradient fractions markedly reduced infectivity, the infectivity titer of virus in fraction 23 was determined in gradients fractionated without the ultraviolet light analysis.

It was found that lower concentrations of ⁷⁵Se-methionine were less effective to label mumps virus. Embryonated eggs were inoculated and incubated as described above, except that

![Fig. 1. Correlation of 254-nm absorbancy, HA, and ⁷⁵Se-radioactivity of radioactively labeled mumps virus. Nine milliliters of clarified egg fluid from virus-infected (A) or uninfected (B) embryonated eggs incubated in the presence of ⁷⁵Se-methionine was layered on top of a preformed 10 to 60% (w/w) sucrose gradient and centrifuged in an SW25.1 rotor at 40,000 × g for 16 hr at 4 C. The gradients were fractionated into 1-ml fractions while monitoring of 254-nm absorbancy continued. The individual fractions were assayed for HA and gamma radioactivity.](image-url)
only 1.0 or 0.1 μCi of 75Se-methionine was injected into each egg. The fluids were collected and analyzed on sucrose density gradients as above. The ratio of radioactivity to HA in fraction 23 was used to compare the degree of 75Se-methionine incorporated into virus. Eggs infected and simultaneously injected with 10 μCi of 75Se-methionine resulted in a counts/minute to HA ratio of approximately 100 (Fig. 1A), whereas similarly infected eggs injected with 1.0 or 0.1 μCi yielded a counts/minute to HA ratio in fraction 23 of 4 and less than 1, respectively.

To demonstrate that the 75Se-methionine was actually incorporated into virus, a more purified virus preparation was analyzed in the same manner. The fractions of sucrose gradients with the highest HA were pooled and dialyzed overnight at 4°C in TMK buffer to remove the sucrose. The viral dialysate was made isotonic, and a sufficient volume of packed chicken erythrocytes was added to give a final concentration of approximately 33% chicken red cells. This suspension was incubated for 4 hr at 4°C with frequent stirring, and the erythrocytes were collected by centrifugation at 1,200 × g for 10 min. The erythrocytes were resuspended in cold isotonic saline solution and centrifuged three times to wash the virus-erythrocyte aggregates. Neuraminidase (5 units/ml) was added to the suspension, and the mixture was incubated at 37°C for 30 min. The erythrocytes were removed by centrifugation, and the supernatant which contained virus was layered onto another 10 to 60% sucrose gradient. Analysis after centrifugation of the second gradient revealed that the virus again sedimented in fractions 21 to 25 in which the peaks of the 254 nm absorbancy, HA, and 75Se radioactivity coincided. Moreover, the counts/minute to HA ratio in the peak fraction of this gradient was of the same order of magnitude as in the original gradients; values in fraction 23 of the second gradient varied from 50 to 110% of the counts/minute to HA ratio in fraction 23 of the first gradient. These results suggested that the radioactivity was indeed associated with the virus particles.

Further evidence that 75Se-methionine was incorporated into the virus was obtained by comparing the total radioactivity with the acid-insoluble radioactivity of each sucrose fraction. To achieve this, embryonated eggs were inoculated simultaneously with mumps virus and 10 μCi of 75Se-methionine; after 6 days of incubation, the infected allantoic and amniotic egg fluids were centrifuged on sucrose gradients as before. The radioactivity in each 1-ml fraction of the gradient was determined by placing each fraction into the auto-gamma counter without further processing. Then to each fraction was added 100 μg of bovine serum albumin and sufficient trichloroacetic acid to give a final concentration of 5%. The precipitate that formed at 4°C in 24 hr was collected on cellulose acetate membranes (0.45-μm pore size) and washed with 5% trichloroacetic acid and distilled water. The radioactivity of the acid-insoluble portion of each fraction was then determined and the results are graphically presented in Fig. 2. In the

Fig. 2. Acid-insoluble radioactivity of mumps virus. Mumps virus was produced in embryonated eggs in the presence of 75Se-methionine, and the fluids were centrifuged on a sucrose density gradient as described in Fig. 1. Each fraction was assayed for the total radioactivity directly in the auto-gamma counter. Then the acid-insoluble radioactivity in each fraction was measured after precipitation with 5% trichloroacetic acid in the presence of human serum albumin.
FIG. 3. Two-dimensional paper chromatography of the amino-acid hydrolysate of purified labeled mumps virus (A) and free $^{75}$Se-methionine (B). Fractions 21 to 25 of sucrose gradients (Fig. 1) which contain mumps virus were pooled and centrifuged in a type 30 rotor at 55,000 $\times$ g for 30 min. The virus pellet was suspended in 6 $\times$ HCl and hydrolyzed at 100°C for 12 hr in an N$_2$ atmosphere. Hydrochloride was removed from the hydrolysate by rotary evaporation and washing several times with water and applied to Whatman no. 1 chromatography paper. Free $^{75}$Se-methionine was also chromatographed on a separate Whatman paper for comparison (B). The solvent for the first chromatographic phase was a glacial acetic acid-butanol-water mixture (120:30:50), and for the second phase phenol-saturated water was used.

region of the gradient containing less-dense egg material, only 10% of the radioactivity was acid-insoluble, whereas in fractions 21 to 25 where the virus sediments over 90% of the radioactivity was acid-precipitable.

In other sucrose gradients of radioactive mumps virus, the fractions of the gradients containing the peak of absorbency, radioactivity, and HA were pooled, freed of sucrose by dialysis, and hydrolyzed for amino acid analysis by paper chromatography. As a control, 100 $\mu$Ci of $^{75}$Se-methionine as obtained from E. R. Squibb and Sons, Inc., was also chromatographed separately. It can be seen in Fig. 3A and 3B that the radioactive component in the virus hydrolysate was chromatographically indistinguishable from the original $^{75}$Se-methionine. This finding and the correlation of radioactivity, HA, absorbency, and infectivity indicated that the isotopically labeled amino acid is incorporated into protein of infectious mumps virus.

To determine whether virus infectivity would be reduced by the irradiation or decay of the incorporated gamma isotope, infectivity of labeled and unlabeled virus preparations were compared. Freshly harvested egg fluids containing either $^{75}$Se-methionine-labeled virus or unlabeled control virus were centrifuged on sucrose density gradients and fractionated. The infectivity titers of both the control and radioactive virus in fraction 23 were $10^{6.0}$ to $10^{6.5}$ EID$_{50}$/ml. Both samples had an EID$_{50}$ to HA ratio of approximately $10^{4.5}$. These results indicated that gamma isotope incorporated into viral proteins has no immediate deleterious effect on viral infectivity.

To rule out that the incorporated $^{75}$Se-methionine did not exert a suicide effect on viral infectivity after storage, freshly harvested egg fluids containing virus labeled with $^{75}$Se-methionine were pooled and dispensed into 0.5-ml portions. These portions were frozen at $-70°C$ with 0.5% bovine serum albumin added as a preservative. At weekly intervals, sample was withdrawn and titrated in embryonated eggs for infectivity. These results (Table 1) demonstrate that the incorporated $^{75}$Se-methionine does not cause any reduction of infectivity during storage.

**DISCUSSION**

Many workers have succeeded in producing virus labeled with the comparatively low-energy beta-emitting isotopes such as $^3$H, $^{14}$C, $^{32}$P, and $^{35}$S (3), but to date there have been no reports on the use of high-energy gamma-labeled compounds to label virus proteins although incorporation of gamma isotopes into proteins has been reported in bacterial and mammalian systems.

**TABLE 1. Survival of $^{75}$Se-methionine labelled virus stored at $-70°C$**

| Time of storage (weeks) | Infectivity (EID$_{50}$/HA)$^a$ |
|-------------------------|-------------------------------|
| 0                       | $10^{6.5}$                    |
| 1                       | $10^{4.0}$                    |
| 2                       | $10^{4.3}$                    |
| 3                       | $10^{6.7}$                    |
| 4                       | $10^{5.1}$                    |

$^a$ Egg infective doses per hemagglutination unit.
MUMPS VIRUS UPTAKE OF $^{75}$Se-METHIONINE

(1, 5, 6). The data in the experiments reported here indicate that mumps virus can be produced in the presence of the gamma-emitting $^{75}$Se-methionine without markedly affecting the production of infectious virus. Sucrose density gradient centrifugation revealed a well-defined peak of material from virus-infected egg fluid with excellent correlation between absorbancy, HA, infectivity, and $^{75}$Se-methionine radioactivity. The radioactivity remained associated with mumps virus after adsorption and elution from chicken erythrocytes and a subsequent sucrose gradient centrifugation. Additional evidence for the $^{75}$Se-methionine incorporation into viral protein was provided by the finding that radioactivity was precipitated by trichloroacetic acid and was recovered from the virus after amino acid hydrolysis as the original $^{75}$Se-methionine. Under present experimental conditions, the injection of 10 $\mu$Ci of $^{75}$Se per egg is sufficient to label virus with a counts/minute to HA ratio of approximately 100.

This method of labeling virus protein with high-energy gamma isotopes has several advantages over the use of $^3$H- or $^14$C-labeled protein precursors. One advantage is that the amount of radioactivity incorporated into virus can be easily measured directly in an autogamma counter without elaborate processing of the material necessary in the case of beta isotopes. This is particularly important where the availability of material is limited and radioactivity is to be monitored throughout many experimental steps. Moreover, the possibility of using radioautography provides more rapid and sensitive detection of radioactivity in chromatographic and electrophoretic analysis. Despite the high energy of gamma isotopes, the amount of $^{75}$Se-methionine used here was not lethal to embryonated eggs, and there was no marked effect upon virus multiplication or infectivity after storage. The technical advantages of using $^{75}$Se-methionine to label viral proteins as shown here may be applicable to other animal virus studies.

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