Virus (Herpes Simplex, Vaccinia) Studies in Embryonated Eggs with Radioactive Phosphorus.* (19394)

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A direct method for detecting the presence of virus within multicellular animals would offer many advantages over the several indirect methods now in use. The incorporation of radioactive elements within the virus offer one method of immediately detecting virus as it is disseminated in the organism and as it is absorbed onto and enters into cells. In addition radioactive "tracers" facilitate the study of the chemical changes which occur with infection. Although radioactive isotope methods have been used profitably with plant (1) and bacterial viruses(2), the study of animal viruses with "tracers" has presented difficulties. This paper and a succeeding one report on the technical problems involved and the methods used in this laboratory to prepare animal viruses with incorporated radioactive phosphorus (P^{32}). In addition other methods of using P^{32} to study virus infections in embryonated eggs are described. Taylor and Saenz demonstrated that the concentration of P^{32} inoculated into the allantoic sac of influenza infected eggs was reduced to half that found in non-infected allantoic fluids(3). Ward reported an abnormal phosphorus metabolism as demonstrated with P^{32} in embryonated eggs infected with influenza virus and with an agent isolated from the common cold(4). Rafelson, Winzler, and Pearson, using P^{32} and C^{14} glucose, found changes in the metabolism of mouse brain infected with Theiler's GD VII virus(5). Graham and McClelland reported the incorporation of P^{32} in influenza virus in chick embryos and performed chemical analyses on purified virus to demonstrate that the P^{32} was in both the phospholipid and nucleic acid fractions of the virus. Although their careful study is the first definitive one of its type, the low specific activity of P^{32} in their virus made analytical work difficult(6).

Investigation of materials and methods. Adaptation and evaluation of existing technics for handling and determining radioactive phosphorus in biological material was essential to the study. Although some of this information is known to biologists working with radioactive isotopes, it is not readily accessible, and we have been advised to report in considerable detail our basic studies which may be of help in a variety of biological problems.

Radioactivity determinations. Radioactive phosphorus was obtained from the Isotope Division, A.E.C., Oak Ridge, and one batch from the Canadian A.E.C., Chalk River. It was calibrated against a simulated P^{32} reference source (Tracerlab No. 129) and found to agree with the values supplied with each shipment. Most of the determinations of radioactivity were performed by evaporating 0.1 ml of the solution on a copper planchet and counting the activity with a mica end window Geiger Müller tube and scaler‡ It was desirable in some cases, however, to count small amounts of radioactivity in large volumes of fluid such as allantoic fluid or tissues digested with fuming nitric acid. Experiments were performed to evaluate various methods of measuring radioactivity in these biological materials in order to increase the flexibility of radioactivity determinations in tracer experiments with small amounts of P^{32}. For this study 3 ways of using Geiger-Müller tubes were compared (Fig. 1). All measurements were made with the same scaler (El-Tronics Decimal Scaler) and aliquots were prepared from the same solution of P^{32}. Wet counting of solutions was performed with a glass en-

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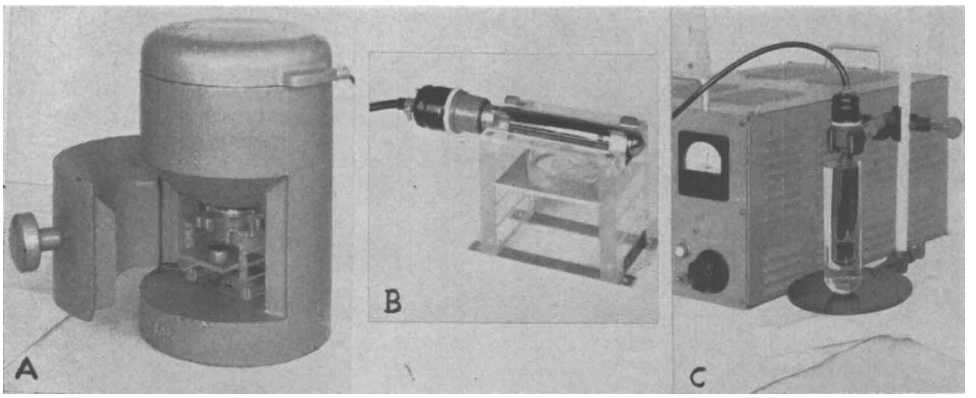

FIG. 1. Methods of measuring radioactivity with Geiger-Muller tubes. a. Mica end-window G-M tube over a sample evaporated on a copper planchet. b. Glass walled G-M tube over a fluid sample for "wet" counting. c. Glass walled G-M tube immersed in a fluid sample for "dip" counting.

velope G.-M. tube (Tracerlab TGC-5) with a window thickness of 30 mg/cm^2 held horizontally above the dish. It was found by experiment that 5 mm of solution constituted "infinite thickness" in the dish if the distance from the surface of the fluid to the tube was kept constant. In dip counting as well, with the glass G.-M. tube immersed directly into the radioactive solution a radial thickness of 5 mm of fluid constituted "infinite thickness." Radioactive contamination of the dip tube is no problem if the tube is rinsed immediately after the determination, with water containing a few drops of phosphoric acid, then dipped in 10% nitric acid and rinsed again. A "background" count must be performed before each determination to make certain that the tube is not contaminated from the previous determination.

From the results of this comparison (Table I) it is apparent that "dip counting" will detect minimal amounts of radioactive phosphorus in dilute solution which may be impossible to measure readily with the other methods.

Decontamination of glassware. In virus or tissue culture work many cleaning agents such as sulfuric acid-dichromate solution and certain detergents are undesirable because of residual toxic films adhering to the glassware(7). Studies were conducted, therefore, with strong warm solutions of sodium metaphosphate (Calgonite^R) as a cleaning agent for virus laboratory glassware and needles. This detergent seemed suitable because it does not leave toxic films on glassware, does not damage needles, and because it contains a great excess of phosphate ions which would tend to remove adhering radioactive phosphate ions by "exchange" as well as by its detergent action. Several experiments indicate that this solution will remove P^{32} satisfactorily, either in inorganic form or when contaminated with proteins, from glassware used for solutions containing as much as 10 millicuries per ml. Glassware requires approximately 48 hours and needles approximately 96 hours of immersion in this solution to be cleaned to "background."

Sterility of P^{32} solution. Review of the technic used at Oak Ridge for the extraction of radioactive phosphorus indicates that the solution as received contains little but water, phosphate ions and very dilute hydrochloric acid(8). Because of the possibility of contamination of the material with microorganisms, the solutions as received from the U. S. Atomic Energy Commission at Oak Ridge, Tenn., and one sample prepared in the reactor at Chalk River, Ontario, Canada, were repeatedly cultured on blood agar plates, on glucose agar, and in thioglycollate media. No growth was noted in any medium after 3 weeks incubation. Although the solutions were sterile, penicillin (500 units per ml) and streptomycin (100 μg per ml) were added to obviate bacterial contamination in subsequent procedures in the laboratory.

TABLE I. Relative Sensitivity of Various Methods of Detecting P^{32} Activity in Solution. For the results below, a specific activity of 10^{-3} u C/ml was used.

Method of counting	Counter tube	Vol of solution, ml	Distance from sample surface to counter window, mm	Counts/min (corrected)	Relative sensitivity
Copper planchet with end window counter (Fig. 1a)	Type TGC-1 2.4 mg/cm² mica window	.1* evaporated	1	247	.046
5 cm glass dish with end window counter (not illustrated)	"	10†	4.3	1260	.236
5 cm glass dish with glass envelope counter horizontal above dish (Fig. 1b)	Type TGC-5 30 mg/cm² glass envelope	10‡	7.3	1171	.220
3 cm diam. cylinder, tube immersed in sol. (Fig. 1c)	"	40§	0	5340	1

* The sensitivity of the planchet method of counting can be increased by evaporation of larger volumes of solution, but the presence of solids introduces "self absorption" errors.

† This corresponds to a depth of 5 mm in a 5 cm diameter dish; greater depth does not increase the counting rate. It is not practical to get the liquid surface closer than about 4 mm to the tube window. An increase in diameter of the dish will not increase counting rate substantially because 5 cm already exceeds the window diameter.

‡ This corresponds to a depth of 5 mm in a 5 cm diameter dish. Greater depth will not increase counting rate. Some improvement will result from putting tube closer to liquid surface, and from using a larger diameter dish. An increase by a factor of 2 might result.

§ This volume corresponds to a radial thickness of liquid surrounding counting tube of 5 mm in a cylindrical container 11 cm long and 28 mm inside diameter. If a larger container is used, more solution will of course be required, but no increase in counting rate occurs after the liquid layer reaches 5 mm.

Safety precautions with P^{32}. The important precautions to prevent injury to laboratory workers handling P^{32} are defined in many Atomic Energy Commission publications and other texts. These of course must be followed. In addition, methods of handling certain problems in this project may be of interest to other virus workers. Eggs containing radioactivity were deposited in large, wide mouthed, screw capped bottles and sufficient concentrated formalin was added to prevent decomposition. They were dated and stored in a special "radioactivity decay closet" until they could be incinerated without danger. Candling of eggs containing 100 or more μc of P^{32} per egg was performed with lead rubber gloves with a glass shield between the operator and the candling box. Large volumes of dialysing fluid containing P^{32} were treated inexpensively with alkali and with calcium chloride to precipitate most of the P^{32} as calcium phosphate. The precipitate was allowed to sediment and the supernate decanted into the sink. The precipitate was poured into smaller containers and stored until decayed.

Toxicity of P^{32} for embryonated hens' eggs. To incorporate as much radioactivity as possible within the virus it was desirable to obtain a high concentration of P^{32} in embryonated eggs. Graham has reported that amounts in excess of 5640 microrutherfords (0.152 μc) per egg caused an appreciable mortality in his eggs(6). We found, however, that we could inoculate more than 100 μc into an embryonated egg without significantly increasing the embryo mortality. In an attempt to explain this discrepancy we compared the toxicity of P^{32} obtained from Oak Ridge, Tenn., with that prepared at Chalk River, Ontario, Canada, Graham's source of material. Table II indicates that there is no appreciable difference in the material obtained in 1951 from the two sources, and that in embryonated eggs from White Leghorn hens in the Philadelphia area as much as 1000 μc can be used per egg without killing a majority of the eggs during

TABLE II. Toxicity of P^{32} for Embryonated Eggs 96 Hours Following Inoculation.

	Amt of P^{32} in microcuries	Inoculated into allantoic sac, 11-day-old eggs 10	100	1000	Inoculated into yolk sac, 9-day-old eggs 10	100	1000
American P^{32} (Oak Ridge) 1951	No. surviving	10	10	7	9	9	
	No. inoculated	10	10	10	10	10	
Canadian P^{32} (Chalk River) 1951	,,	9	9	7	10	8	3
		10	10	8	10	10	6

the time of the experiment. (In a personal communication, October 1951, Graham reported that he was able to confirm our findings.)

Distribution of P^{32} inoculated into normal embryonated eggs. An extensive study was made of the fate of P^{32} inoculated as a dilute phosphate solution into normal embryonated eggs as a basis for comparison with infected eggs. The P^{32} solution was inoculated into the yolk sac via a hole in the air sac end of the eggs which were subsequently incubated at 37°C. The allantoic and amniotic fluids were harvested separately. Bloody fluid was discarded. The radioactivity of 2 planchets, each containing 0.1 ml of fluid, was determined separately for each fluid from each egg. A minimum of 12 eggs of each age and for each time interval were used. For many points as many as 60 determinations were available from the controls of other experiments. The results which have been reproducible in many experiments are indicated in Fig. 2 and 3.

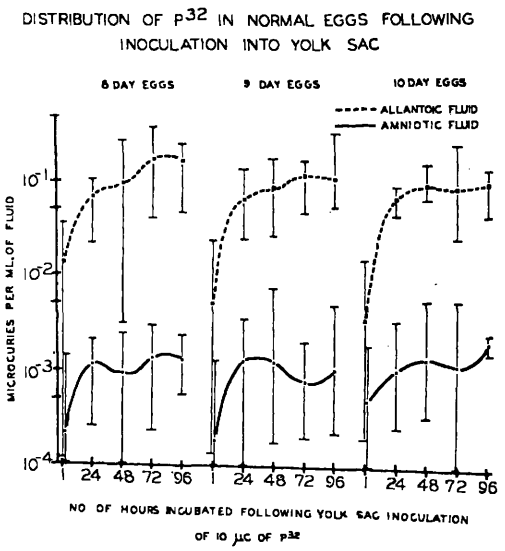

FIG. 2. Distribution of P^{32} in normal eggs following inoculation into yolk sac. A minimum of 12 eggs were used to determine each point on the curves.

Fig. 2 demonstrates that there is no significant difference within 96 hours incubation in the distribution of P^{32} injected into 8- or 9- or 10-day-old eggs. Although there is wide variation from egg to egg as indicated by the vertical lines, it is apparent that within 24 hours, there is at least a 100-fold increase in allantoic fluid P^{32} over the amniotic fluid P^{32}. Approximately 1% of the P^{32} injected into the yolk sac appears in the allantoic fluid but less than 0.01% can be found in the amniotic fluid of non-infected eggs. Additional experiments (Liu, Henle, Blank, and Kaneda, to be published), indicated that practically all of the allantoic fluid P^{32} in normal eggs can be removed by dialysis and probably is present as inorganic phosphate.

Distribution of P^{32} in virus infected embryonated eggs. The presence of radioactivity in infected tissues was determined by means of sensitive silver halide emulsions (autoradiography) as well as by counting with a Geiger-Müller tube.

Autoradiography of the chorioallantoic membrane. Autoradiograms were prepared of whole membranes and of histologic sections of membranes. The gross autograms were made by applying with pressure "No-Screen" x-ray film to the membranes which had been spread and dried on a glass lantern slide. Fig. 4 demonstrates radioactivity throughout the membrane with a concentration along blood vessels. Control exposures with non-radioactive membranes showed no "chem-

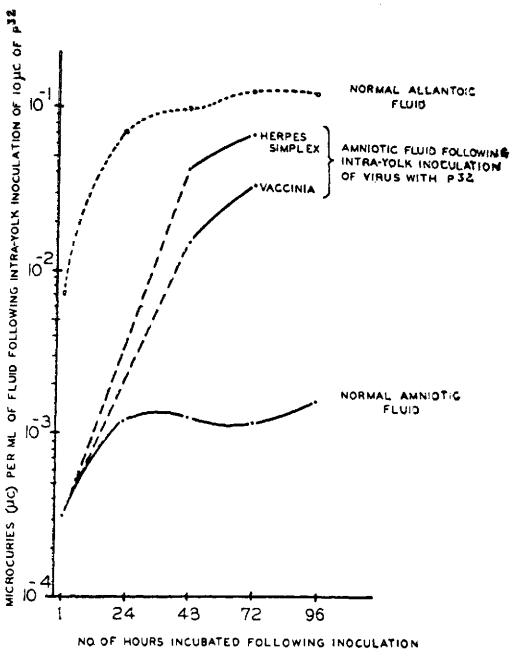

FIG. 3. Distribution of P^{32} in the allantoic and amniotic fluids of virus infected eggs as compared with non-infected eggs.

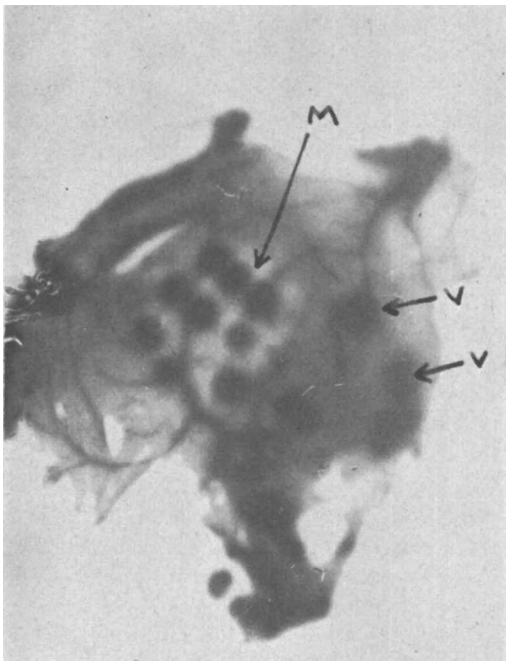

FIG. 4. Gross autoradiogram of a chorioallantoic membrane indicating areas of localization of P^{32}. In addition to the blood vessels, the P^{32} is concentrated in the "plaques." V were induced by infection with vaccinia virus and M were induced by mechanical trauma.

ical fogging" with this emulsion(9). Plaques were induced in membranes by infection with dilute vaccinia virus suspensions. Control plaques were induced mechanically by drilling small holes through the shell in a special fashion directly over the membrane. P^{32} was inoculated into the yolk sac just before infection or mechanical trauma and the eggs were incubated for 72 hours. It can be seen from Fig. 4, in which both artificial and virus induced plaques have been produced on the same membrane that a high concentration of radioactivity appears equally in both types of lesions. This probably is a non-specific phenomenon indicating merely that the rapidly growing cells which form the plaques incorporate large amounts of available phosphorus. In addition, autoradiograms were prepared of histologic sections of radioactive membranes with the method developed by the senior author which precludes the loss of P^{32} during histologic procedures(10), and these, too, show a non-specific but high concentration of P^{32} in the plaques (Fig. 5).

Partition of P^{32} in egg fluids with infection. Fig. 3 is the compilation of many experiments in which either vaccinia virus or herpes simplex virus was inoculated simultaneously with the P^{32} into the yolk sac. The vaccinia virus was a commercial calf lymph strain egg adapted in our laboratory to produce titers of 10^6 in amniotic fluid following yolk sac inoculation. The herpes simplex strain (W) was isolated from a patient, identified serologically and histologically, and egg adapted to produce titers of 10^8 in amniotic fluid following yolk sac inoculation(11). It is apparent that 48 hours after infection, at which time virus is regularly present in the amniotic fluid, there

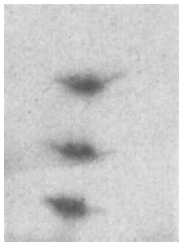

FIG. 5. Gross autoradiogram of histologic sections of chorioallantoic membrane with vaccinia induced plaque demonstrating concentration of P^{32} in the area of the plaque.

is a striking increase in the amount of P^{32} in the amniotic fluid. In practice this unmistakable change is observed as the difference, for example, between 150 counts/minute for 0.1 ml of normal fluid and 2000 counts/minute for 0.1 ml of infected fluid. In Fig. 3 the counts obtained experimentally have been converted into absolute amounts of P^{32} so that they may be compared with data obtained with other counting methods in other laboratories.

Conclusions. 1. The following observations were made on basic methods of using P^{32} in biological studies: a. P^{32} as received from Oak Ridge is usually sterile but penicillin and streptomycin can be added before direct inoculation into eggs to obviate autoclaving procedures potentially dangerous to laboratory personnel. b. Glassware and needles used for virus work can be decontaminated of P^{32} in 2-4 days with hot sodium metaphosphate (Calgonite®) solutions. c. Simple methods of handling and storing millicurie amounts of P^{32} in biological materials are described. 2. Radioactive phosphorus is of relatively low toxicity for chick embryos; 500-1000 μc per egg is not lethal in 72 hours. 3. P^{32} injected into the yolk sac appears in a 100-fold greater concentration in the allantoic fluid than in the amniotic fluid in 24-96 hours. 4. The partition of P^{32} in the egg fluids is the same for 8- or 9- or 10-day-old embryos with periods of incubation of 24-96 hours following inoculation. 5. Following infection with herpes simplex or vaccinia virus there is a 10-20-fold increase in the amount of P^{32} appearing in the amniotic fluid. 6. Chorioallantoic membrane plaques, whether virus or mechanically induced, concentrate P^{32} in their cells.

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Effects of Certain Amino Acids and Related Compounds on Propagation of Mouse Encephalomyelitis Virus.* (19395)

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Various factors which affect the propagation of Theiler's GDVII strain of murine encephalomyelitis virus in tissue cultures have been reported(1). The experiments described below were concerned with the effects of certain amino acids, amino acid analogues and other agents on this system; preliminary data were presented elsewhere(2).

Materials and methods. The methods used have been described in detail(1). The virus used was obtained from pooled tissue culture material after 50 to 60 tissue culture passages. It was added to cultures in final dilution of 1:1,000. Cultures were made in 50 ml Erlenmeyer flasks closed with rubber stoppers and containing 3 ml of Simms' X7 solution and

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