carcass, indicating that the presence of supplemental fluoride (Group III) did not alter the levels of molybdenum retained. When molybdenum was present in the drinking water (Groups II and IV) comparable concentrations of molybdenum were also found in the carcass, again indicating that the presence of supplemental fluoride (Group IV) did not alter the skeletal retention of molybdenum. These data indicate that the increased anticariogenic effectiveness and increased skeletal retention of fluoride in the presence of molybdenum may be due to the ability of molybdenum to increase the metabolic availability of the fluoride ion.

Summary. A study was conducted to determine the effect of molybdenum and fluoride upon dental caries in the albino rat. Addition of 25 ppm molybdenum to the drinking water decreased the incidence of dental caries by 18% while addition of 25 ppm fluoride decreased the caries incidence by 32%. Addition of both 25 ppm molybdenum and 25 ppm fluoride resulted in a greater effect, with the caries incidence reduced by 52%. Fluoride analysis performed upon the femurs, incisors, and whole carcasses confirmed earlier reports that the presence of molybdenum is associated with an increased skeletal retention of fluoride. The analysis of the carcasses for molybdenum indicated comparable concentrations of molybdenum in the presence and absence of fluoride suggesting that the increased anticariogenic effectiveness and skeletal retention of fluoride in the presence of molybdenum may be due to the ability of molybdenum to increase the metabolic availability of the fluoride ion.

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A Cell Culture System on Agar Permitting Direct Investigation of Viral Antigens by Immunodiffusion.* (27314)

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Since the report of Lewis and Lewis(1), investigators have used agar in studies on cell migration *in vitro* and in the field of organ culture. Zinsser *et al.*(2) obtained multiplication of rickettsiae in surviving tissue fragments on an agar substrate, and subsequently several viruses have been so propagated. Recently, Wallace and Hanks(3) and Keefe *et al.*(4) reported growth of cell lines on agar. The recognized usefulness of the gel-diffusion method for the demonstration of antigen-antibody reactions suggested its direct application to the problem of detection and identification of viruses replicating in cells grown on agar, provided that an appropriate agar-tissue culture system could be devised. The present report describes a technic that permits growth of cells in wells made in nutrient agar, the propagation of viruses therein, and the demonstration of specific diffusable viral components or products by addition of appropriate antisera to adjacent wells.

Materials and methods. Maintenance of cell line. Chang's conjunctival cells were grown in "milk dilution bottles" in Eagle's basal medium (Microbiological Assoc., Inc.) plus 5% inactivated horse serum, 0.042% so-

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dium bicarbonate, phenol red and antibiotics (100 U penicillin and 100 μg streptomycin per ml), adjusted to a pH of 7.2-7.4 with N NaOH. The fluid was changed every 3 or 4 days and subcultures were made every 2 to 3 weeks by scraping or by trypsinization of the cell sheet.

Nutrient agar. This was prepared by melting a 2% suspension of Noble (Difco) agar in twice distilled water, cooling to 45°C, and then combining with an equal volume of double strength fluid medium (described above) previously warmed to 38-40°C.

Diffusion dishes. The nutrient agar was dispensed in 3.5 ml amounts in petri dishes (50 mm in diameter), yielding a layer 1.25-1.5 mm thick. With sterile precautions, diffusion plates were prepared by using a #2 cork borer to cut symmetrically arranged holes with the aid of a plastic pattern; the blocks of agar were removed with a needle. Each hole measured 5 mm in diameter and wall-to-wall separation of the holes was 7.5 mm. To prepare the central hole for cell cultivation, a drop of nutrient agar was added to seal the bottom; the peripheral wells were not so treated.

Outline of technic. The "cell culture well" was seeded with approximately 500,000 cells in the form of a heavy suspension, and the dishes incubated at 35.5 ± 0.5°C in 2 to 3% CO_2 in humidified air. The suspending fluid was rapidly absorbed by the agar and the cells then appeared as a homogeneous light-cream colored, opalescent mass partially filling the cup. After incubation, microscopic examination revealed closely packed, sharply-defined round cells which stained when exposed to 1:50,000 neutral red. Histologic examination showed a mass several cells thick which contained scattered mitotic figures. It is to be emphasized that seeding with a heavy cell suspension in a small volume was essential to avoid the persistence of fluid in the culture well. Further, the initial pH of the system should be in the neutral or slightly acidic range as judged by the color of the agar.

Following incubation for 2 days the cell cultures were inoculated with virus and returned to the CO_2-humid air incubator for a period that varied with the agent under investigation. The agar-cell culture-precipitation test was then initiated by adding serum (0.025-0.03 ml) to appropriate circumferential wells as in a conventional Ouchterlony test. In addition a drop of Eagle's medium was added to each cell culture well to promote diffusion of the viral components. The dishes were reincubated and examined daily with oblique illumination for precipitation lines.

Viruses. Vaccinia. Calf pulp virus (Mass. Public Health Biologic Laboratories) was passaged twice in cultures of Chang's conjunctival cells to provide a working pool. *Adenovirus type 3,* strain supplied by F. A. Neva(5), was used as a pool derived from the second conjunctival cell passage.

Antisera. Vaccinia immune sera were obtained from a rabbit first vaccinated by abdominal scarification; 10 days later a series of 4 weekly injections was started, each incorporating the contents of one capillary tube of smallpox vaccine (Mass. Public Health Biologic Laboratories). Bleedings were done before the last injection (VaR2) and 15 days later (VaR3). The pre-vaccination specimen served as the control (VaR1). Samples of human vaccinia immune globulin (Cutter Laboratories, supplied by Dr. C. H. Kempe) and pre- and 11 days post- smallpox vaccination human sera were also available. *Adenovirus 3 antiserum* was prepared in guinea pigs by an initial 1-ml intramuscular injection containing equal parts of virus suspension (from conjunctival cell cultures; 10^6 $TCID_{50}$/0.1 ml) and Arlacel-Bayol adjuvant mixture followed 7 days later by 4 1-ml intraperitoneal inoculations of virus-adjuvant mixture over a 7-week period. Bleeding was done 9 days after last injection (AvGP2). Control guinea pig sera were the pre-vaccination serum specimen (AvGP1) and sera from animals receiving an identical series of injections of non-infected tissue culture-adjuvant mixture (TcGP). A sample of Adenovirus Group CF Human Reference Serum (Microbiological Assoc., Inc.) was also tested. Additional control material consisted of rabbit anti-Herpes simplex serum prepared by giving 2 intraperitoneal injections, one month apart, of infectious tissue culture fluid, with bleeding 6 days after last injection (HsR2).

TABLE I. Agar-cell culture-precipitation test with vaccinia virus and adenovirus type 3.

Description of diffusion dishes	No. of dishes	Day of addition of sera*	Presence or absence of precipitation lines 24 hr after addition of the following sera: VaR1†	VaR3	AvGP1	AvGP2	TcGP	HsR2
Cell culture inoculated with vaccinia virus	1	4	—	—	—	—	—	—
	3	5	—	+	—	—	—	—
	5	9	—	+	—	—	—	—
Cell culture inoculated with adenovirus 3	1	4	—	—	—	—	—	—
	1	5	—	—	—	—	—	—
	2	7	—	—	—	—	—	—
	3	9	—	—	—	+	—	—
Controls:								
Cell culture alone	1	5	—	—	—	—	—	—
	3	9	—	—	—	—	—	—
Vaccinia virus inoculum alone	2	5	—	—	—	—	—	—
	2	9	—	—	—	—	—	—
Adenovirus 3 inoculum alone	2	9	—	—	—	—	—	—

* Time expressed after inoculation with virus.

† VaR1 = pre-vaccination rabbit; VaR3 = anti-vaccinia rabbit; AvGP1 = normal guinea pig; AvGP2 = anti-adenovirus 3 guinea pig; TcGP = anti-tissue culture guinea pig; HsR2 = anti-herpes simplex rabbit.

Experimental. In a representative experiment, 2 groups of diffusion dishes were prepared consisting of: (A) a set with cell-cultures in central wells, and (B) identical dishes without cells. All were incubated for 2 days and the central wells of several of the dishes of A and B groups were inoculated with vaccinia virus (approx. $10^{2.75}$ $TCID_{50}$) or with adenovirus 3 (approx. $10^{2.25}$ $TCID_{50}$) contained in a volume of about 0.01 ml. Thus the controls were uninoculated cell cultures, and dishes without cells but with a virus inoculum in the central well. Sera not inactivated or diluted were added to peripheral wells of each dish as follows: pre-vaccination rabbit (VaR1), anti-vaccinia rabbit (VaR3), anti-Herpes simplex rabbit (HsR2), anti-cell culture guinea pig (TcGP), anti-adenovirus 3 guinea pig (AvGP2) and pre-vaccination guinea pig (AvGP1). Experience had indicated that detectable antigen appears more rapidly in the case of vaccinia than with adenovirus 3. Sera were added only once to each dish as follows: to vaccinia dishes at 4, 5, or 9 days and to adenovirus dishes at 4, 5, 7, or 9 days after inoculation with virus. The results are summarized in Table I.

Precipitation of vaccinial antigens was observed 5 days after virus inoculation as a sharp line which appeared within 24 hours of serum addition, followed by a second line 24 hours later, separated from the former by 0.25 to 0.5 mm. Further incubation resulted in the appearance of a third faint line nearer the cell culture well. A minimum of 5 days was required for elaboration of vaccinia antigens in amounts necessary for visible precipitation, but thereafter the reaction could be obtained for at least 4 more days.

Adenovirus 3 antigen was not detected earlier than 9 days after inoculation. The virus inocula alone and the non-inoculated cell cultures failed to react with the sera tested.

In a similar experiment with vaccinia inoculated cultures, 2 rabbit immune sera were used (VaR2 and VaR3) and serum TcGP was omitted; precipitation occurred only with the homologous antisera forming a 2-line "identity reaction" in 48 hours followed by a third line days later (Fig. 1). Similar results were obtained when human post-vaccination serum and human vaccinia immune globulin were used. With the latter, formation of 3 precipitation lines was consistently observed within 24 hours.

Cultures inoculated with adenovirus 3 were similarly tested with the human adenovirus reference serum and immune guinea pig serum (AvGP2); with both sera one precipitation line was obtained which showed a reaction of identity suggesting detection of a common group antigenic component. On further

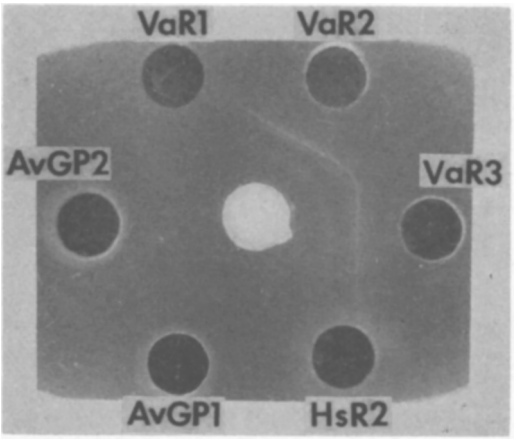

FIG. 1. Test with vaccinia virus. Peripheral wells containing sera as follows: VaR1 = pre-vaccination rabbit; VaR2 and VaR3 = anti-vaccinia rabbit; HsR2 = anti-herpes simplex rabbit; AvGP1 = normal guinea pig; AvGP2 = anti-adenovirus 3 guinea pig. Central well contained the infected cell culture. Note three fusing lines related to the homologous antibodies. Precipitates around AVGP2 serum well indicate the reaction of antibodies produced against non-viral antigens of the immunizing pool.

incubation, additional lines appeared, one of which was related to the type specific serum. This finding is under investigation.

Discussion. Immunodiffusion of antigens of vaccinia was first reported by Gispen(6) and Datt and Orlans(7), and of adenovirus antigens by Tanaka(8) and Pereira *et al.*(9). Although adequate concentrations of vaccinial antigen are usually present in infected materials (*i.e.*, calf lymph or culture fluid), adenovirus materials usually require concentration and purification of the antigens before the gel-diffusion precipitation test is feasible. The system herein described of infecting relatively large numbers of intimately associated cells growing in small wells permits the production of considerable amounts of adenovirus antigenic material. Observations on the dynamics of the system, including demonstration of replication of virus in the culture system, will be reported elsewhere. The technic as applied to viruses is comparable to the methods of Elek(10) and Ouchterlony(11) used for detection of bacterial products.

It is to be noted that this method minimizes problems associated with specific precipitation due to the presence of other antigen antibody complexes. Incorporation in the agar medium of some of the antigens present in tissue culture systems eliminates confusing reactions with the corresponding antibodies produced concomitantly in preparation of viral immune sera. For example, in the present tests the diffusion substrate contained 5% horse serum, and precipitation of anti-horse serum antibodies occurred around the wells containing the guinea pig anti-adenovirus sera appearing as one or more circular precipitates. (See Fig. 1—ring around AvGP2). This type of reaction has been described by Björklund(12). The formation of the circular precipitate apparently does not interfere with the diffusion of other antibodies.

We have observed that the agar-cell culture-precipitation test is also applicable to the study of the virus of herpes simplex, but to date have obtained negative results with the poliomyelitis viruses. The findings suggest that the method could profitably be explored in investigations on antigenically active materials elaborated by a variety of obligate intracellular parasites. Possible applications in the field of diagnostic virology are obvious.

Summary. Development of a method for the cultivation of viruses in cell cultures prepared in small wells in an agar medium has permitted detection by gel-diffusion procedures of specific antigenic materials elaborated by vaccinia virus and adenovirus type 3. The technic, termed the agar-cell culture-precipitation test, permits study of viral components without preliminary concentration thereof, and may find application in investigation of a variety of agents that are obligate intracellular parasites.

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Interference of Niagara Blue 2B with the Teratogenic Action of Trypan Blue.* (27315)

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The fact that the disazo dye trypan blue is a potent teratogen to the developing mammalian embryo is now well documented(1). Available evidence suggests a causal relationship between altered physiological states of the mother and embryo and the teratogenicity of trypan blue. It has been clearly demonstrated that injection of trypan blue results in an alteration in the electrophoretic pattern of the maternal serum proteins and the proteins of the yolk sac fluid(2,3,4). It now is of interest to learn whether or not it is possible to interfere with this teratogenic action. Assuming that trypan blue (or a metabolic by-product) reacts at a specific site(s), it is possible that a non-teratogenic substance of similar structure might also react at these sites and thereby interfere with the action of trypan blue. The non-teratogenic disazo dye Niagara blue 2B was selected to test this hypothesis because it differs structurally from trypan blue only in the absence of 2 methyl groups and because it apparently has the same fate following injection; appearing in cells of the maternal macrophage system, kidney tubules, and visceral epithelial cells of the yolk sac placenta(5).

Materials and methods. All experiments were carried out using the same sample of specially purified Niagara blue 2B and trypan blue.‡ The dyes were prepared as 2% solutions in distilled water and injected intraperitoneally into Sherman rats on the 6th and 7th, or 8th day of gestation, considering the morning on which sperm was found in the vaginal smear as day zero. Fetuses were recovered on the 20th day, weighed, examined for gross malformations, and fixed in Bouin's fluid for subsequent free-hand serial sectioning of the head. The trunk was not sectioned.

Results. Table I illustrates the results obtained when equivalent amounts of Niagara blue 2B and trypan blue were injected. At the highest dose used there was no protective or sparing effect. However, at each lower concentration the injection of Niagara blue 2B prior to injection of trypan blue afforded some measure of protection to the embryo with respect to number of resorptions and malformations produced.

Table II indicates the results obtained after varying the relative amounts of the 2 dyes administered. In each group the prior injection of Niagara blue 2B reduced the number of resorptions and malformed survivors. On the other hand, when trypan blue was injected prior to Niagara blue 2B there was no significant reduction in its teratogenic activity. These figures also suggest a direct correlation between number of implantation sites affected and amount of trypan blue administered, irrespective of the amount of Niagara blue 2B administered.

Discussion. These results indicate that Niagara blue 2B can effectively interfere with the teratogenic and lethal action of trypan blue in Sherman rats, over a selected range in dye concentration. This interference is

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