On periodate oxidation in bicarbonate buffer it liberated formaldehyde only in traces.

The data strongly indicate that fraction I is galactose-6-sulfate and that fraction II is *N*-acetylglucosamine-6-sulfate. Paper chromatography and electrophoresis of a similarly hydrolyzed sample of keratosulfate from cartilage of human rib likewise yielded galactose-6-sulfate. Judged from the intensity of the spots, the content of galactose-6-sulfate in this fraction appears to be higher than that of keratosulfate of cornea. The isolation of *N*-acetylglucosamine-6-sulfate is in agreement with the structure previously proposed.

The presence of galactose-6-sulfate is another instance of the variations in the mucopolysaccharides in general and in keratosulfate in particular, and is another example of the apparent randomness of the biosynthesis of these compounds.

The isolation of galactose-6-sulfate from keratosulfate of cornea is noteworthy in that the ratio of sulfate to hexosamine in cornea is always close to one, while keratosulfate of senile human cartilage, as well as that of elasmobranch cartilage, is oversulfated; that is, the ratio of sulfate to hexosamine is greater than one (*1*). In these sources the extra sulfate can be expected to be in the galactosyl moiety. Galactose sulfate in mammalian tissue occurs in the sulfatides of brain and other tissues (*8*), and recently galactose-6-sulfate was demonstrated in neuraminlactose sulfate from the mammary glands of rats (*9*).

The finding of sulfate ester groups in both galactosyl and hexosaminyl moieties of keratosulfate may explain the anomalous behavior of this polymer toward quaternary ammonium and pyridinium salts (*10*), which may be caused by the proximity of sulfate groups in neighboring instead of in alternating glycosyl groups, so that the compound behaves similarly to heparin.

In view of our finding, we should not assume that the keratosulfate fractions that are isolated from different normal and abnormal tissues, such as degenerating cartilage, nucleus pulposus, and tissue in Marfan's syndrome, are identical.

V. P. Bhavanandan
Karl Meyer
Department of Medicine, Columbia University College of Physicians and Surgeons, New York 10032

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Rubella Virus: Growth and Cytopathic Effect in Primary Cultures of Cells of Rabbit Embryos

Abstract. *Primary cultures of rabbit (New Zealand white) embryo cells support growth of rubella virus. Distinct cytopathic changes are discernible within 6 to 8 days after inoculation. This cell system has been successful for the recovery of rubella virus from clinical materials and the demonstration of neutralizing antibody in patient serum.*

We have found that primary cultures of rabbit embryo cells not only support rubella virus growth, but also develop marked specific cytopathic changes. These changes develop on primary isolation of virus from clinical materials and also are produced by virus previously adapted to other cell lines. In our hands, this cell system has provided a technique which is direct, sensitive, and reproducible for demonstration of rubella virus.

Weller and Neva first described subtle cytopathic effects of rubella virus in primary human amnion cell cultures (*1*). Other investigators have since reported cytopathic effects in several cell systems. McCarthy *et al.* observed cytopathic effects in a transformed line of rabbit kidney (RK-13) and, later, reported cytopathic effects in primary cultures of rabbit kidney (*2*). Günalp observed destructive changes in monolayers of a line of green monkey kidney (GMK-AH1) (*3*). These cytopathic changes have been described as subtle and may be difficult to detect on primary isolation of rubella virus, but they become more manifest after serial passage of the virus in the cell line under investigation. Leerhøy recently reported a cytopathic effect of strains of RK-13–adapted rubella virus in an established line of rabbit cornea cells (*4*). The effects described by Leerhøy are distinct and usually discernible within 4 days after inoculation; no reference was made to the utilization of this cell line for primary isolations from clinical materials.

Because of the difficulty in detecting cytopathic effects in early passages of rubella virus in primary human amnion and RK-13 cells, the indirect method described by Parkman *et al.* is most often used for diagnostic work (*5*). Although this method has proved successful for isolation and identification of rubella virus, it is somewhat laborious.

In the study now reported, four strains of rubella virus were used. The human amnion–adapted Bell strain was supplied by F. A. Neva (*6*); the green monkey kidney–adapted M33 strain was supplied by E. L. Buescher (*6*). Two additional strains (AE and LB) that we recovered from patients during the 1964 rubella epidemic were isolated in rabbit embryo cell cultures from original clinical materials. These strains were also isolated and identified by the indirect technique in green monkey kidney cells. Identification of the isolates was made with specific rubella antiserum to the Bell strain and antiserum supplied by J. L. Sever (*6*).

Rabbit embryos (New Zealand white), aged 17 to 21 days, were used for preparation of monolayer cultures. Whole embryos were minced to a fine pulp, and cells were dispersed from the tissue fragments with 0.25 percent trypsin (Difco 1:250) in Hanks balanced salt solution at *p*H 7.4. The cell concentration for planting was adjusted with growth medium to 10^6 cells per milliliter and 1-ml portions were planted in screw-cap Leighton tubes (16 by 120 mm). Growth medium consisted of Medium 199 (*7*) supplemented with 13 percent heat-inactivated horse serum, antibiotics (100 μg of streptomycin, 30 units of mycostatin, and 100 units of penicillin per milliliter), and sodium bicarbonate (0.35 g/liter). For maintenance medium, the serum concentration was reduced to 2 percent. All cultures were allowed to grow in the stationary position at 35°C for 3 to 4 days prior to inoculation. Twenty-four hours before inoculation, growth

medium was replaced by maintenance medium.

After being washed with Hanks balanced salt solution, cultures were inoculated with 0.25 ml of virus preparations. After incubation at 25°C for 1 hour to allow for virus adsorption, the inoculum was removed and cultures were fed with maintenance medium. Incubation was then continued at 35°C.

After inoculation, *p*H 7.2 was maintained with 1.4 percent $NaHCO_3$. Every 5 to 7 days, one-half of the fluid phase was replaced with fresh maintenance medium to prevent cell damage resulting from nutrient depletion.

Four to six cultures were used for each of the four virus strains studied in each experiment. Equal numbers of uninoculated cultures served as cell controls. Control cultures were processed in the same manner as those used for inoculation.

Uninoculated rabbit embryo cells form monolayers of densely packed elongated cells with a fibroblastic appearance. In cultures inoculated with rubella virus, the first indication of cytopathic effect is the loss of the dense arrangement of the monolayer in discrete areas. Infected cells lose their characteristic, elongated, spindle shape and become more rounded, and cytoplasmic threads become obvious. Finally the infected cells become condensed, degenerate, and detach from the surface, with discrete plaques remaining in the monolayer. On primary isolation, strains AE and LB produced foci by the 6th day after inoculation. Foci were noted by the 9th day after inoculation with the human amnion–adapted Bell strain and the monkey kidney–adapted M33 strain.

The cytopathic process progresses to involve 75 to 80 percent of the monolayer within 10 to 12 days after inoculation. Control monolayer cultures remain as sheets of densely packed elongated cells.

The four virus strains have been passaged in primary rabbit embryo cells four times without any loss of cytopathogenicity. The cytopathic changes produced by all four strains are comparable. Titers of virus in the supernatant fluids by the 8th to 9th day after inoculatioin reach 10^5 to 10^6 $TCID_{50}$ (tissue culture infective dose, 50 percent effective) per milliliter.

Neutralization studies were performed in rabbit embryo cell cultures with paired serums from three patients and with rabbit antiserum to the Bell strain. Previously titrated virus was diluted in Hanks balanced salt solution containing 0.1 percent peptone. Twofold serial dilutions of inactivated serum were prepared in Hanks balanced salt solution. Equal volumes of virus preparation and serum dilutions were mixed. Serum-virus mixtures and control virus preparations were held at 37°C for 1 hour before inoculation of 0.25-ml amounts into each of four cultures. Virus dilutions were prepared to contain 100 $TCID_{50}$ of virus per 0.25 ml of final virus-serum mixture. Inoculated cultures and serum, virus, and cell controls were held at 25°C for 1 hour, then 1 ml of maintenance medium was added to the inoculum, and incubation was continued at 35°C. Serums from the convalescent, but not acute, phase and rabbit antiserum to the Bell strain neutralized the cytopathic effects of all four virus strains. Cross-neutralization of our isolates with antiserum to the Bell strain indicated antigenic similarity. Neutralization studies have been performed with and without incorporation of fresh normal rabbit serum in the virus-antiserum mixture. The addition of fresh normal rabbit serum has not been a necessary requirement for consistent and reproducible neutralization of the cytopathic effects of rubella virus in this cell system.

Hemagglutination and hemadsorption tests, and also tests for the presence of bacteria and *Mycoplasma*, were negative.

Indications of the sensitivity of this technique for recovery of rubella virus from clinical materials are given by preliminary comparative studies of throat washings from 14 selected rubella patients. On primary isolation attempts, we recovered the virus from all of the 14 specimens tested in rabbit embryo cell cultures in contrast to recovery of the virus from 10 of 12 specimens tested in primary green monkey kidney cell cultures (*5*) and from 7 of 14 tested in primary human amnion cell cultures (*1*).

Definite and marked cytopathic effect of rubella virus in rabbit embryo cell cultures makes possible an efficient and reproducible direct technique for demonstration of virus and of specific neutralizing antibodies. In addition, growth of the virus in high titer in rabbit embryo tissue provides a convenient source of virus for other studies.

ANNE REDDICK
CATHERINE E. ROESEL
Department of Medical Microbiology and Public Health, Medical College of Georgia, Augusta

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7. Medium 199 was obtained from Microbiological Associates, Bethesda, as 10-times concentrated stock and was diluted to proper concentration with water distilled in glass twice.

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Hemoglobin Sphakiá: A Delta-Chain Variant of Hemoglobin A_2 from Crete

Abstract. *A new variant of the normal minor component Hb A_2 has been detected in a family that lives in Sphakiá, Crete. Chemical studies of this abnormal hemoglobin, designated Hb A_2 δ Sphakiá, indicates a substitution of the histidyl residue number two of the δ-chain by an arginyl residue.*

Hemoglobin A_2 (Hb A_2), which was first described by Künkel and Wallenius (*1*), is a minor red-cell component which is present in all normal humans. It constitutes about 2.5 percent of the total hemoglobin of the red cell. In electrophoresis on paper, starch-gel, or starch-block, at alkaline *p*H, it migrates considerably more slowly towards the anode than Hb A and at approximately the same speed as Hb E. It consists of two α-chains of the same composition as those in Hb A (*2*) combined with two δ-chains which differ from the β-chain of Hb A in ten amino acid substitutions (*3*, *4*). Its structure can be written $\alpha_2\delta_2$.

Several electrophoretically separable, inherited variants of Hb A_2 have been found. Some of them are due to changes in the α-chain and occur in association with major α-chain variants. The first example of this type (*5*) was in a subject carrying the α-chain variant Hb G Ibadan only. This subject had no Hb A_2 but his red cells showed a slower minor component which had the constitution $\alpha_2^G\delta_2$ and a number of hemoglobins of this type have since been described (*6*). Two variants of the Hb A_2 due to