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Method for Detecting Antiviral Agents on Paper Chromatograms. (25817)

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The procedure of placing paper chromatograms on the surface of agar seeded with bacteria to locate zones of antibiotic activity was first used to distinguish various components of a penicillin mixture(1,2). This method, now universally used in identification and purification of naturally occurring antibacterial agents, can also be used in a similar manner for characterization of antiviral agents. The following discussion describes an adaptation of the Dulbecco virus-plaque technic(3) to this purpose.

Materials and methods. Preparation of agar overlay, chick embryo cell cultures in Pyrex baking dishes, infection of these cultures with West Nile and vaccinia viruses, subsequent staining of cells to observe plaques, and detection of zones of virus inhibition have been described(4). *Antiviral test materials* used in our experiments include antibiotic W-112, extracted with ether from an unclassified streptomyces species culture and isatin-monothiosemicarbazone synthesized in our laboratories by Dr. Frank Villani. Antibiotic W-112 at concentrations as low as 0.1 μg produced significant inhibition of plaques formed by W. Nile, vaccinia, herpes simplex and Newcastle disease viruses. Isatin-monothiosemicarbazone inhibited only vaccinia virus plaque formation. Since this compound is also active in preventing death of mice infected with many lethal doses of vaccinia virus(5), it represents a model of an antiviral agent active both *in vitro* and *in vivo*. *Paper chromatographic* migration of antibiotic W-112 was obtained with partition systems using aqueous methanol as a stationary phase(6) on No. 1 Whatman paper at 20°C. Development time ranged from 1.1 to 3.2 hours. Solvent systems included system 6, a mixture of 300 ml toluene, 100 ml light petroleum, 300 ml methanol and 700 ml of water. Solvent system 9 was a mixture of 200 ml toluene, 800 ml light petroleum, 200 ml methanol and 800 ml of water. Analysis of isatin-monothiosemicarbazone was carried out on No. 1 Whatman paper at 20°C, using solvent system 16, a mixture of 120 ml n-butanol, 30 ml acetic acid and 50 ml of water, and solvent system 17 made up of 100 ml n-butanol, 100 ml pyridine and 100 ml water. After drying, the ethylene oxide sterilized paper chromatograms (10 x 34 cm) were cut in half and top and bottom portions placed for 5 min on separate agar overlays of virus infected cultures. This time period was adequate for diffusion of the antiviral agent into the agar. Longer time periods, however, frequently resulted in the cell sheet being destroyed, apparently by toxic residues left by chromatographic solvent systems. Position and orientation of the paper were indicated on bottom of baking dish. After removal of paper, baking dishes were sealed with Saran Wrap (Dow Chemical Co.), then incubated for 4 days at 36°C. The cell sheet was then

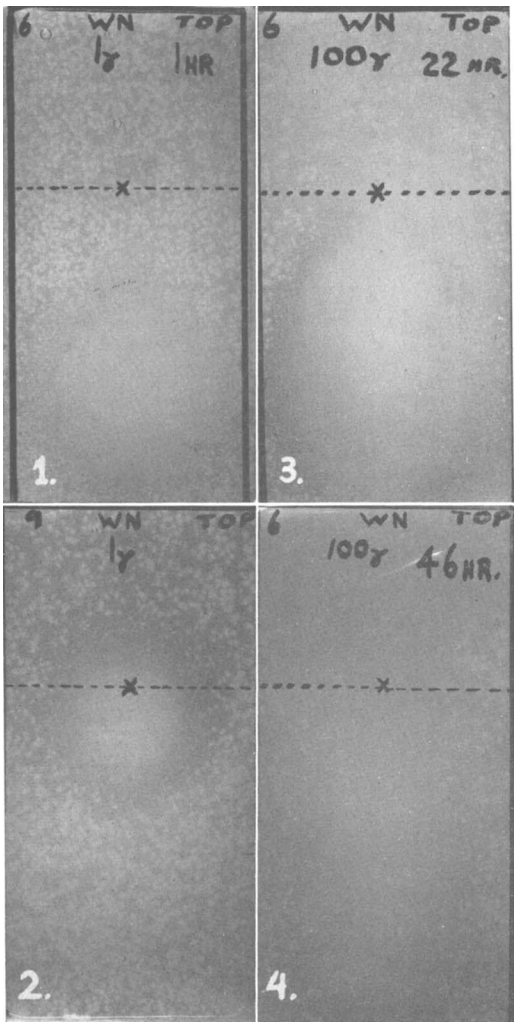

FIG. 1. A zone free of W. Nile virus plaques resulting from application of paper chromatogram to agar overlay 1 hr after virus infection. One μg of antibiotic W-112 was applied at $\times$. Migration of active component occurred after treatment with solvent system 6.

FIG. 2. Same procedure as for Fig. 1 except this paper chromatogram was treated with solvent system 9.

FIG. 3. Same procedure as for Fig. 1 except 100 μg of W-112 was used and paper applied to agar overlay culture 22 hr after virus infection.

FIG. 4. Same procedure as for Fig. 3 except paper applied 46 hr after virus infection.

stained with a second agar overlay containing indonitrotetrazolium chloride(4) and within a few hours plaques were readily observed.

Results. One μg of antibiotic W-112 was applied to chromatography paper and developed with solvent system 6 in a descending fashion for 1.6 hrs. Location of this antiviral agent could be observed by the resulting large, plaque-free zone in a baking dish culture infected with W. Nile virus (Fig. 1). When solvent system 9 was used, however, as a developing agent, the antibiotic migrated only very slightly (Fig. 2). In both cases the 4-day incubation period permitted the antibiotic to diffuse widely and, therefore, resulted in a plaque-free zone probably much larger than the original zone on the paper. Since W. Nile virus multiplies slowly, this no doubt also permits the antibiotic to diffuse for a considerable time prior to plaque formation. When 100 μg of W-112 was present in a paper chromatogram and this chromatogram was placed in contact with an agar overlay only 1 hr after virus infection, the resulting plaque-free zone covered the entire baking dish. It was found, however, that somewhat smaller zones were produced if the paper was applied to agar overlay culture 22 hours after virus infection (Fig. 3) and still smaller zones resulted if the paper was applied 46 hr after virus infection (Fig. 4). This suggests that size of zones of plaque inhibition can be controlled by proper choice of application time of paper chromatograms. When a very sensitive test is needed, the paper can be applied very soon after virus infection of overlay cultures. When sensitivity is not important but a more accurate determination of the area of antiviral activity on paper is needed, then application of the paper can be delayed. Each system would, no doubt, have to be worked out separately, depending on virus used and diffusion rate of antiviral agent.

Isatin-monothiosemicarbazone was chromatographed using previously described solvent systems. Migration of the active component was followed by using tissue cultures infected with vaccinia virus. With either solvent system 16 or 17 the active moiety migrated very rapidly and could be readily located on paper because of its bright yellow color. Subsequent development in agar overlay cultures showed that zones of plaque inhibition coincided with areas of yellow color on the paper. The plaque-free zones were, however, considerably larger than the color zones. It required

50 μg of isatin-monothiosemicarbazone to produce significant plaque-free areas. Smaller zones were observed with as little as 20 μg but none were seen when 10 μg was used. In no case were these zones equal in size to those produced by just 1 μg of antibiotic W-112; even though paper chromatograms of the synthetic compound were applied to the overlay cultures as soon after virus infection as possible.

Discussion. By adaptation of the Dulbecco plaque technic it is now possible to detect, assay and chromatograph antiviral antibiotics in a manner similar to antibacterial antibiotics. The same method is equally useful in chromatography of synthetic compounds such as isatin-monothiosemicarbazone. This synthetic antiviral agent was chosen for study because it is an agent unquestionably active both in virus infected animals and tissue cultures. Since this compound only inhibits plaque formation of vaccinia virus and not of W. Nile, herpes simplex and Newcastle viruses, this indicates that plaque-free zones are in this case a result of specific influences on the course of virus infection and not due to a subtle, general toxic effect on chick embryo cells.

Summary. A procedure has been developed whereby paper chromatograms of antiviral agents can be developed by applying them to virus infected, agar overlay, chick embryo cell tissue cultures. Plaque-free zones are produced, thus indicating the area on paper chromatogram where the active material can be located. Using antiviral substances antibiotic W-112 and isatin-monothiosemicarbazone, W. Nile and vaccinia viruses, and 4 chromatographic solvent systems, it was possible to localize the biological activity of these antiviral agents. As little as 1 μg of antibiotic W-112 produced large plaque-free zones when paper chromatogram was applied to the overlay culture 1 hr after virus infection. When 100 μg of same material was used, large plaque-free areas resulted even when the paper was applied 46 hr after virus infection. Isatin-monothiosemicarbazone at 50 μg level not only produced a plaque-free zone but also produced a yellow spot on paper chromatogram that coincided exactly with the zone of antiviral activity.

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Reserpine and Thyroid Activity in Fowls.* (25818)

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Tranquilizers have been suggested for use with poultry because of their stress ameliorating properties with possible beneficial effect on egg production, growth and feed efficiency(1, 2). Data are available, however, indicating that reserpine has an anti-thyroid effect(3,4). If this were true of poultry, administration of reserpine would then be contraindicated for purposes of sustained tranquilization where normal thyroid function is desired. Since data are extremely limited, it was of interest to study effects of reserpine in fowls using direct measurements of thyroid function *viz.*, thyroidal-I^{131} release rate and thyroxine secretion rate.

Materials and Methods. Adult New Hamp-

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