pigs, using the untreated stock, and stocks heated in water and in $MgCl_2$. When the virus stock was heated at 50°C for 30 minutes in water, the adenovirus component lost all of its infectivity and a large part of its antigenicity, while the SV40 component retained both infectivity and antigenicity. When the virus stock was heated in M $MgCl_2$ at 50°C for 30 minutes, the adenovirus component again lost all of its infectivity but it retained all of its antigenicity. However the SV40 component was reduced both in infectivity and in antigenicity.

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A Medium Free of Agar, Serum and Peptone for Plaque Assay of Herpes Simplex Virus.* (28362)

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The plaque technique developed by Dulbecco(1) has been applied to many animal viruses and several such techniques have been developed for plaquing of herpes simplex virus. Kaplan(2) developed a plaque assay in primary rabbit kidney cells utilizing an overlay which contained a peptone and serum. Farnham(3), however, was unable to plaque herpes simplex virus under agar in HeLa cells and developed, accordingly, a microscopic plaque method in HeLa cell cultures. Russell(4), employing various cell lines, developed a sensitive method involving the use of carboxymethyl cellulose and antiserum for restricting formation of secondary plaques. The medium utilized by Russell, like that of Kaplan, contained peptones and human serum. Liebhaber and Takemoto(5) reported inhibition of plaque formation of some viruses by an acidic polysaccharide in agar and reversal of the inhibition by protamine sulfate.

To test the direct effects of various drugs on plaque formation by viruses, it is often necessary to exclude from the medium the interfering substances introduced by addition of peptones or serum. In the present report,

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advantage is taken of certain of the preceding observations for development of an overlay medium free of agar, peptone and serum, which is effective for plaquing of herpes simplex virus in 3 cell culture systems.

Materials and methods. Cell cultures. Primary grivet monkey *(Cercopithecus aethiops)* kidney cell cultures were prepared in Smith bottles by methods previously described(6, 7). Primary rabbit kidney cell cultures were prepared similarly. Cultures of a stable subdiploid grivet monkey kidney cell line were prepared by seeding 100,000 cells per ml in Smith bottles in Eagle's minimal medium supplemented by the nonessential amino acids (6,7) and 10% agamma calf serum.† The stable grivet monkey kidney culture (BSC) was obtained from Miss Hope Hopps, Bureau of Biological Standards, Nat. Inst. of Health, Bethesda, Md.

Herpes simplex virus. A routine laboratory strain of herpes simplex virus (designated as strain McIntyre) was used in these studies. The virus was propagated in primary rabbit kidney and primary grivet monkey kidney cell cultures.

Agar overlay medium. The agar overlay medium employed in the preliminary studies was prepared as described earlier(6,7). It consisted of Eagle's medium in modified Hanks' balanced salt solution and 1.5% agar and contained no serum. The overlay was used with and without addition of protamine sulfate.‡

Methyl cellulose overlay medium. The methyl cellulose overlay medium was prepared similarly to overlay medium A described previously(6) except that methyl cellulose (Dow Chemical Co., 4,000 cps, USP) was substituted for the agar, and protamine sulfate was omitted. The overlay medium consisted of 1.5% methyl cellulose with Eagle's medium prepared in modified Hanks' balanced salt solution and contained no serum. The methyl cellulose was brought into solution by autoclaving the fine powder in the proper concentration in water at 18 lb for 20 minutes. The methyl cellulose preparation was then removed from the autoclave and allowed to cool at 5°C with stirring (magnetic bar) until solution was effected. Appropriate concentrations of Eagle's medium, glucose and salts were then added and homogenous solution obtained by further stirring.

Virus seeding. The seeding procedure for plaquing of herpes simplex virus followed an established procedure(2,6). A period of 1.5 to 2 hours, at 36°C, was allowed for virus adsorption. Bottles overlaid with agar medium were incubated in an inverted position, but bottles were not inverted when overlaid with methyl cellulose medium. Incubation also was at 36°C. Plaque formation was usually maximum at 5 to 6 days. At that time, the bottles overlaid with agar received a second overlay (10 ml) which contained neutral red to yield a final concentration of 40 μg/ml. To bottles with methyl cellulose overlay, an equal volume of sterile saline was added and the overlay was gently rocked to dilute the methyl cellulose. When suitable mixing was obtained, the overlay was decanted. At this point, the plaques were plainly visible since the cells of the infected plaques were carried away from the cell sheet with the methyl cellulose. However, for ease in counting, the cell sheet was stained with neutral red (80 μg/ml) in balanced salt solution for 2 hours at 36°C.

Use of Leighton tube cultures for assay. For convenience of handling and economy of material, principles evolved from use of the above materials finally were applied to Leighton tube cultures. Following adsorption of virus, an overlay containing 0.75% methyl cellulose and medium devoid of serum was added.

After 48 hours (BSC cells) or 60 hours (primary green monkey kidney or rabbit kidney cells) the cultures were stained with carbolfuchsin (Ziehl-Neelsen stain)(8).

Results and discussion. Promotion of plaque formation by protamine in agar overlay. Initial attempts to obtain plaques from herpes simplex virus under an agar overlay yielded small, very poorly discernible plaques and a low sensitivity as revealed by later experiment. Addition of serum to this medium did not improve plaque formation. Addi-

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‡ Mann Research Laboratories, New York.

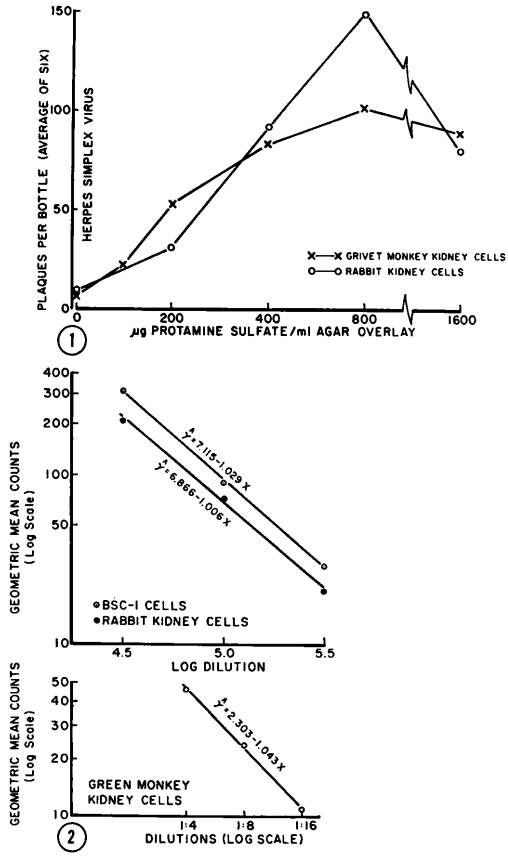

FIG. 1. Effect of protamine on plaque formation of herpes simplex virus.

FIG. 2. Proportionality evaluation of herpes simplex plaque assay.

tion of protamine sulfate did increase significantly the number of plaques formed in both primary grivet monkey kidney and primary rabbit kidney cells as detailed in Fig. 1. At the same time the plaques were enlarged and formed a greater contrast with the background. Concentrations of protamine sulfate above 800 μg/ml depressed plaque formation. It was apparent, as in the studies of Liebhaber and Takemoto(5), that agar, or possibly an acidic constituent of agar, inhibited the major portion of the adsorbed herpes simplex virus population and that this inhibition could be relieved by addition of protamine sulfate.

TABLE I. Plaque Titration of Herpes Simplex Virus in Grivet Monkey Kidney by Two Methods.

Overlay	Virus dilution	No. plaques in replicate bottles
Protamine—agar	10^{-3}	50, 29, 47, 25
Methyl cellulose	10^{-3}	331, 371, 325, 355
Protamine—agar	10^{-4}	2, 5, 6, 2
Methyl cellulose	10^{-4}	38, 40, 31, 39

Methyl cellulose overlay. Since the protamine and agar comprised a complex mixture, and since the possible variation of acidic polysaccharide content in different lots of agar could require repeated monitoring to determine the proper level of protamine sulfate, it seemed advisable to attempt to substitute a nontoxic, relatively inert, high viscosity material for the agar. Methyl cellulose, at 1.5% concentration, was found satisfactory for this purpose. Use of methyl cellulose overlay generally yielded about 10 times the number of plaques obtained with the agar overlay in presence of 800 μg of protamine sulfate/ml (Table I).

Plaque formation on various cell cultures. Satisfactory plaque formation with herpes simplex virus was obtained on primary grivet monkey kidney, primary rabbit kidney and the stable subdiploid cell line of grivet monkey kidney. Titers obtained on the 3 types of cell cultures were all of the same order of magnitude and, in all cases, plaque formation was much more sensitive with the methyl cellulose overlay than in the protamine-supplemented agar overlay.

Proportionality with dilution.§ An evaluation of the proportionality obtained with dilutions of virus samples was analyzed statistically and the regression curves for all 3 cell culture systems were plotted. In each case, there was no significant deviation from the expected slope of 1.0 (Fig. 2).

Assay with Leighton tube cultures. Application of the above principles to an assay utilizing Leighton tube cultures yielded comparable results with high precision and reproducibility. This modification is now routinely employed in this laboratory for viral assay and appraisal of antiviral agents. Typical appearance of the Leighton tubes is shown in Fig. 3. A plaque diameter of approximately 1 mm was obtained in only 48 hours on BSC cells and 1.5 mm on primary green monkey kidney or rabbit kidney cell cultures in 60

§ We are indebted to Mr. Arthur G. Itkin for statistical analysis of our data.

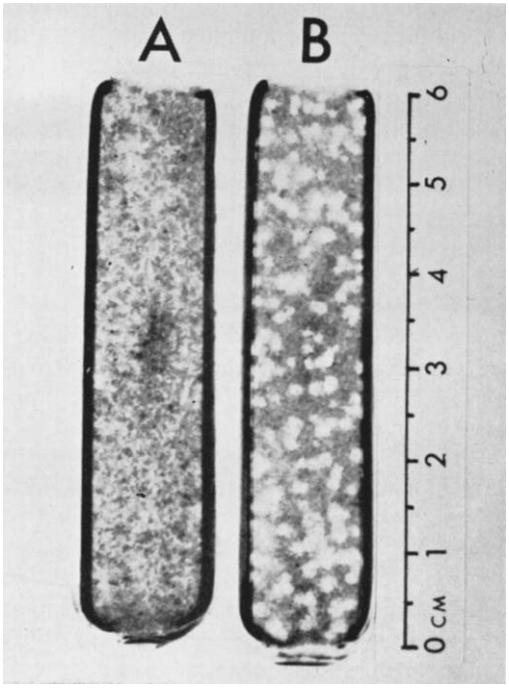

FIG. 3. Control culture and plaques obtained with herpes simplex virus grown 50 hr on BSC cell cultures in Leighton tubes. Carbolfuchsin (Ziehl-Neelsen stain).

hours. In our studies, the methyl cellulose was essential for circumscribing the viral growth and providing sharply outlined plaques, and thus afforded a distinct advantage over similar procedures in which methyl cellulose was not employed.

Summary. Methods have been described for quantitative plaque assay of herpes simplex virus either in bottle culture or in Leighton tubes. The overlay employed was devoid of agar, peptones and serum, which are viewed as undesirable constituents. Use of agar overlay results in production of plaques from only a small fraction of the potential plaque-forming population. The inhibition of plaque formation by agar can be reversed by addition of protamine sulfate at a level of approximately 800 μg/ml. Possible uncertainties associated with the use of mixtures of agar and protamine can be avoided by substitution of methyl cellulose with which a further 10-fold increase in sensitivity of the assay was obtained.

While this manuscript was in preparation, a paper was published in *Virology*, 1963, v19, 40, by I. T. Schulze and R. W. Schlesinger describing a methyl cellulose overlay for plaquing of arthropod-borne viruses.

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Studies on Fibrinogen Turnover Before and After Whole Body X-Irradiation in the Rat.* (28363)

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Various authors have reported the normal biological half-life of fibrinogen in several species to be between 1 and 5 days(3,5,7,8, 11,12). The biological half-life of fibrinogen following whole-body irradiation has not been reported. The plasma level of fibrinogen in dogs rises above normal concentrations to a peak 3 to 5 days after X-irradiation(4).

Fibrinogen survival in animals shows an

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