A STUDY OF THE FACTORS WHICH AFFECT THE PLAQUE ASSAY FOR ADENOVIRUS

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ABSTRACT

A STUDY OF THE FACTORS WHICH AFFECT THE PLAQUE ASSAY FOR ADENOVIRUS

The factors that affect the plaque assay of adenovirus type 5 in KB cells have been elucidated and the optimum features were combined to form an optimum plaque assay system.

In the agar overlay, horse serum, at 10%, was found to be optimum for plaque formation. Neutral red inactivated adenovirus type 5 photodynamically and also inhibited virus multiplication in the dark. Noble agar at 0.6% allowed the formation of the most plaques and autoclaved agar, which contains sulphated polysaccharides, did not inhibit plaque formation by adenovirus, compared to agarose. Proteose peptone was found to be an essential supplement to Eagle's minimum essential medium (MEM) for plaque formation by adenovirus type 5 on KB cells. The pH of the agar overlay was found to be critical and maximum plaque numbers were obtained at pH values between 7.1 and 7.2.

Adsorption of virus to cell monolayers was not influenced by pH in the range 6.6 and 7.5. The efficiency of virus adsorption was apparently increased by culturing KB cells in the presence of kanamycin, but decreased in the presence of DEAE-dextran.

The optimum conditions for assay of type 5 adenovirus allowed the formation of plaques by adenovirus types 3, 8 and 12.

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INTRODUCTION

INTRODUCTION

It is generally agreed that plaque formation is the most accurate method for assaying virus. However, plaque formation by adenoviruses is relatively difficult to achieve. The plaques require a minimum of 8 days to form and may take up to 28 days before the maximum plaque numbers are reached. This phenomenon is due to the inherent slow growth cycle of the adenovirus. Kjellen (1961) showed that the newly formed virions are not released from the host cells until very late in the growth cycle, thereby delaying the infection of the surrounding cells. This presents the problem of keeping the cell monolayer cultures alive under the agar overlay for the long period required for plaque formation. Due to this difficulty, many researchers have been unable to obtain plaques with adenovirus and therefore have resorted to the less accurate but more easily performed quantal end-point technique for virus assay.

Although some workers have utilized the plaque technique for assaying adenovirus, little work has been done to investigate the factors that affect plaque formation. Due to the necessity for having an accurate assay system for adenovirus, this study was undertaken to define the conditions under which consistent and reproducible results can be obtained, and to elucidate some problems relating to plaque formation by adenoviruses. Type 5 adenovirus was chosen for the study because it is one of the small number of serotypes which forms relatively large plaques in serial heteroploid human cell lines (e.g. Hela, KB).

LITERATURE REVIEW

LITERATURE REVIEW

Dulbecco (1952) introduced the plaque assay for animal viruses as a direct analogy with the plaque assay of bacteriophages (Ellis and Delbruck, 1939). The aims are to assay virus infectivity more accurately and to express virus concentrations in quantitative terms rather than quantal units.

A typical plaque assay consists of the inoculation of a statistically adequate yet easily countable number of infectious virus (say 30-100) particles onto a complete cell monolayer. Each particle is allowed to replicate under conditions where the resulting lesion remains local, and the lesions are finally counted. It has been shown for poliomyelitis viruses (Dulbecco and Vogt, 1954) that each lesion is caused by a single infective particle (or aggregate of particles not divisible by dilution) and this allows the calculation of the infective particle content of the original inoculum. Using this fact, the plaque technique could be used for the isolation of clones of virus from single infective particles for genetic studies and the plaque character used as a genetic marker. The recovery of virus from single plaques provides another way for virus purification.

I. PLAQUE FORMATION BY ADENOVIRUS

Adenovirus plaques were first produced by Bonifas and Schlesinger in 1959. They studied the nutrient requirements for plaque formation by adenovirus and discovered that the plating efficiency was enhanced by increasing the concentration of arginine in the overlay

medium. Larger clearer plaques and a ten-fold or greater increase in plating efficiency resulted when 0.4 to 1.0 mM arginine replaced the 0.1 mM concentration of Eagle's basal medium. There was insufficient arginine in Eagle's basal medium for optimum plaque formation by adenovirus.

Rouse, Bonifas and Schlesinger (1963) found that under conditions of Mycoplasma (henceforth referred to as PPLO) contamination, arginine depletion was greatly accelerated in KB cell cultures. The early exhaustion of arginine was accompanied by a low plating efficiency for adenovirus type 2 plaques. In the presence of increased arginine, PPLO contaminated cells yielded plaque titers as high as those on uncontaminated cells. Therefore, PPLO contamination interfered with the viral infectious process by depriving the cell-virus complex of a single essential nutrient, arginine.

In a later paper, Rouse and Schlesinger (1967) discovered that of all amino acids in Eagle's basal medium, only arginine is essential for the synthesis of infectious progeny virus in KB cells infected with type 2 adenovirus. Their results show that when arginine was restored to the infected KB cell cultures after 28-32 hours of deprivation, infectious virus was made without detectable lag and increased exponentially at the "normal" rate. Using the techniques of immunofluorescence and hemagglutination, they demonstrated that "early" antigen and proteins associated with the hexon and penton fiber subunits were synthesized during the arginine free period. They therefore proposed that arginine plays a specific role in a late synthetic step essential for adenovirus maturation.

Consequently, the depletion of arginine by PPLO in cell

cultures would certainly affect the plaquing efficiency of the adenovirus. Therefore, it is necessary to control the PPLO growth in contaminated cell cultures.

Most PPLO are resistant to the commonly used antibiotics in cell culture e.g. Penicillin and streptomycin. However, in the search for effective control of PPLO, Brown and Officer (1968) found that kanamycin was one of the best antibiotics for the control of PPLO contamination, due to the fact that it was the least toxic of the antibiotics tested to animal cells in culture and consequently could be used at high concentrations without killing the cells in the process.

In an attempt to isolate an early/large plaque variant from the wild type 5 adenovirus, Kjellen (1963) obtained a genetically stable mutant. The first plaques of the genetically stable variant could be seen on the 4th day after infection and the numbers increased up to 8 or 9 days, after which there was no further increase in plaque numbers. He also reported that DEAE-D, when incorporated into the agar overlay, did not enhance plaque formation.

Green et al. (1967) studied the plaquing efficiencies of purified human adenoviruses in a KB clonal subline. He found that the adenoviruses have an efficiency of plating (eop) smaller than one i.e. the virion:PFU ratio ranged from 11:1 for adenovirus type 3 to 2,300:1 for adenovirus type 25. The ratios were based on particle number derived from chemical analysis rather than the more commonly employed electron microscope counts. The results obtained were not corrected for adsorption efficiency variations which do exist e.g. the ratios for types 2 and 5 were 33/1 and 20/1 respectively. For adenovirus type 2 (Ad 2) 85-95%

of the PFU are adsorbed to KB cell monolayers in 60-120 minutes (Philipson, 1967); for adenovirus type 5 (Ad 5) 40-50% are adsorbed in 2 hours (Lawrence and Ginsberg, 1967). Nevertheless, the adsorption efficiency of most of the viruses studied is unknown.

Different serotypes of adenovirus produce plaques of varying sizes. This may be due to the inherent genetic character of the virus. Consequently, the plaque technique provides another method for the characterization and classification of the adenoviruses. Accordingly, Green (1967) tabulated the adenoviruses according to their plaque characteristics. Under the conditions used, the human adenoviruses formed three groups according to plaque size. Adenovirus types 1, 2 and 5 produced large plaques (5 mm.) visible 8-11 days after infection: Ad 12, 18, 31, 11, 14, 16 and 21 formed small plaques (0.5 mm.) 12 to 15 days after infection; and the remaining serotypes formed intermediate-sized plaques (1 to 2 mm.).

Since the plaque is the outcome of an initial encounter of single cells with single virions (Schlesinger, 1969), viral replication is highly asynchronous (Strohl and Schlesinger, 1965 a,b). Moreover, the release of the infectious adenovirus form the host cells is extremely slow. This may explain the observations made by Kjellen (1961) that the plaque numbers increased at a regular rate until about 21 days, after the first plaques were seen on day 8 with adenovirus types 4 and 5.

II. FACTORS AFFECTING PLAQUE ASSAY

1. Factors affecting virus adsorption

In the study of the rate of Ad 5 adsorption to MAS-A or MAS-A1

cells (human bone marrow), Kjellen (1961) found that about 50% of the viruses which form plaques attached after 30-60 minutes, using a 0.3 ml. inoculum on 60 mm. petri plates with 10^6 cells each. For adenovirus type 2, Philipson (1967) reported that 85-95% of the PFU were adsorbed to KB cell monolayers in a period of 60-120 minutes. Their results showed that under the respective conditions, the adsorption rates for adenovirus types 5 and 2 were approximately exponential over the 1 hour adsorption period.

The rate of adsorption also depends to a large extent on the volume of the inoculum. It has been found, for poliovirus, that the rate of adsorption is markedly affected by the volume of the virus inoculum, the virus adsorption rate being inversely proportional to the volume of virus inoculum (Taylor and Graham, 1961). However, they found that a small volume of virus inoculum, e.g., 0.1 ml., would not readily permit uniform distribution of virus over the plate; but when a larger volume of virus containing fluid was used over the monolayer to improve the distribution of virus, the rate of virus adsorption was markedly decreased. Consequently, the efficiency of plating increased with decreasing adsorption volumes. This phenomenon was also described by Bachrach et al. (1957) for foot-and-mouth disease virus.

As a result, a compromise has to be made between the application of small volumes of virus inocula for maximum virus adsorption and the use of larger volumes to permit uniform distribution of virus over the entire cell monolayer.

The effects of DEAE-D on virus adsorption

The plaque titre of certain infectious preparations of nucleic acid, e.g. poliovirus RNA, from animal viruses has been greatly enhanced (up to 100,000-fold) by diethylaminoethyl-dextran (DEAE-D)(Pagano and Vaheri, 1965). However, the addition of DEAE-D to inocula of whole particles either enhances or reduces the plaque titre depending on the virus (Kim and Sharp, 1969). They found that for poxviruses (rabbitpox and vaccinia) the dextran made insignificant improvement in adsorption of the virus particles to the cells, as determined by direct electron microscopic counts of unadsorbed virus, though there was a 12-fold increase in the plaque numbers of rabbitpox virus. This observation implies that in the absence of dextran, some of the virus that adsorbed to the cells were unable to initiate plaques. The DEAE-D particles were not aggregated with the virus particles nor were the virus particles induced to aggregate when observed in the electron microscope. Therefore, they proposed that plaque enhancement by dextran may be a protective action of the dextran particles upon freshly uncoated DNA within the cell vesicle, since the size of the dextran particles is comparable with the virus particles and the particles are probably present at the time of uncoating of the virus in the cell vesicles. However, no evidence was presented to support the proposal. The actual mode of action of DEAE-D on virus adsorption is still dubious.

2. Effects of variations in the composition of the overlay medium on plaque formation

a) Sera

Reports by different researchers have established the fact that

the ability of viruses to form plaques is dependent on both the type and amount of serum in the agar overlay.

Philipson (1961) investigated the effects of different types of sera (chick, calf and rabbit), in varying concentrations, on the production of Ad 2 plaques. He found that an overlay medium in which the serum components consisted of 1% calf and 2% chicken serum appeared to produce the largest number of plaques. No explanation was given for his finding. However, the results show that with increasing concentrations of each serum (with the exception of chicken serum alone), there was a corresponding reduction in plaque numbers. This may indicate the presence of virus inhibitors in the sera used.

Green et al. (1967) modified this overlay medium to contain 6% chicken serum and 6% horse serum instead of calf serum, which was used in the growth medium. This modification was needed because calf serum prevented the plaque formation of adenovirus types 12 and 18. This may be due to a number of reasons. Firstly, there may be some type-specific inhibitors to adenovirus types 12 and 18 in the calf serum, which were absent or present in smaller quantities in the chicken and horse sera. Secondly, there may be additional nutrients in the chicken and horse sera which were absent in the calf serum, though this is less likely.

Lawrence and Ginsberg (1967) used 15% chicken serum and Herman (1968) used 20% fetal bovine serum for their adenovirus plaque assay systems.

In addition to heat-stable virus inhibitors, Smull and Ludwig (1969) discovered a heat-labile factor in fresh bovine serum which inhibited plaque formation by poliovirus in HeLa cell monolayers when the host cells were subcultured several times in growth medium containing the

fresh bovine serum prior to their inoculation with virus.

Due to this unpredictable presence of non-specific virus inhibitors in sera from different sources, some workers have preferred to eliminate serum from the overlay altogether and instead have substituted 0.5% gelatin (Simpson and Hirst, 1961), or 0.25% skim milk (Stinebaugh and Melnick, 1962), or 0.1% serum albumin (Cooper, 1955).

The foregoing review indicates that it is necessary to test different batches of sera for the presence of virus inhibitors before using them for plaque assay, due to the fact that some batches of sera may contain inhibitors against the viruses.

b) Volume of agar overlay

The volume of the agar overlay strongly influences plaque formation by animal viruses. For example, Vogt et al. (1957) showed that an increase in the thickness of the overlay from 0.8 mm. to 1.6 mm resulted in a marked decrease in plaque numbers of poliomyelitis viruses. A further increase in the thickness from 1.6 mm. to 3.2 mm. had a similar inhibitory effect. He, therefore, proposed that a constant volume of agar-overlay and an accurate leveling of the plates during the overlaying were necessary to avoid a variability in the efficiency of plating (eop) both from plate to plate and within the same plate.

Along similar lines, Baron et al. (1961) studied the influence of the thickness of the agar overlay on plaque formation. He demonstrated that the size and number of plaques of Newcastle disease virus diminished as the depth of the agar overlay increased. This may be due to a reduction in the oxidative metabolism of the host cells. To determine whether this effect was due to the diminishing concentration of oxygen in the

deeper portions of the tube, the oxygen concentration was increased by maintaining infected and overlaid cultures in an atmosphere of oxygen. As a result, plaques developed at a much greater depth in the presence of oxygen than in air. Moreover, different viruses were found to require different amounts of oxygen for their growth. Therefore, a thick agar overlay reduces the oxygen available to the cells, which in turn inhibits the multiplication of the virus.

Plaque development may also be inhibited if the overlay is too thin (Dougherty, 1964). The lack of nutrients for the virus and cells was suggested as a possible cause. The optimal thickness of the agar overlay was found to be between 4-8 mm. in a 50 mm. petri dish.

c) Inhibitors in agar

Autoclaved agar is known to contain sulphated polysaccharides which inhibit the formation of some viruses, for example, arboviruses (Liebhaber and Takemoto, 1961), and encephalomyocarditis virus (Takemoto and Nomura, 1960). However, they do not affect the wild type poliovirus (Nomura and Takemori, 1960). Although several postulates have been made as to the mechanism of inhibition by sulphated polysaccharides on viral growth, none of them are universally accepted yet.

Consequently, some workers have attempted to use other solidifying agents, for example, clotted plasma (Mandel, 1958; Underwood, 1959) and methyl cellulose (Schulze and Schlesinger, 1963; Scherer, 1964; Salim, 1968).

Scherer (1964) obtained more plaques, with Japanese B encephalitis virus, when 1.6% methyl cellulose was used instead of 1.5% agar. He concluded that methyl cellulose was the enhancing agent compared to

agar. His conclusion, however, may be erroneous because the high concentration of agar used (1.5%) may be inhibitory to plaque formation by the virus compared to lower concentrations of agar, e.g., 0.6%. This was shown to be true for influenza virus (Sugiura and Kilbourne, 1965). Since Scherer used only one concentration of agar, his result is subject to possible misinterpretation.

Autoclaved agarose, which is free of sulphated polysaccharides has been widely used in plaque assay work. Bergold and Mazzali (1968) replaced Noble agar (1.2%) and DEAE-D with 0.5% agarose for the plaque assay of 52 arboviruses. They found that agarose had the same enhancing effects as agar with DEAE-D on plaque formation. Moreover, the standard overlay prepared with 0.5% agarose was much clearer, which allowed plaque counting without any staining of the BHK21 (baby hamster kidney), Vero (green monkey kidney) and W1-38 (human diploid lung) cell sheets. However, Ventura (1968) found no significant differences, either in plaque numbers or in size, between the use of agar and agarose, for plaque formation by Venezuelan encephalitis virus.

The plaque sizes of mengo virus variants under agar, agarose, methyl cellulose and agar with polyanions were examined by Campbell and Colter (1965). The plaque size of EMC virus was greatly enhanced when methyl cellulose was used instead of agar; but the results concurrently showed that methyl cellulose was not a good medium for use with the mengo virus-L (mouse) cell system due to the small sizes of plaques obtained with it. Both M-(medium plaque) and S-(small plaque) mengo virus variants produced much larger plaques under agarose than they do under the same concentration of agar. However, L-(large plaque) mengo

produced smaller plaques under agarose than under agar. This contradictory result was explained as being due to the stiffer gels produced by agarose compared to agar at similar concentrations. If this greater gelling capacity affected L-mengo virus unfavourably, it is difficult to understand why it has an opposite effect on the M- and S- mengo viruses.

The incorporation of several polyanions in agar overlays was shown to have little or no effect on the sizes of plaques produced in L-cell monolayers by L- and S- mengo, whereas M- mengo plaque size was greatly affected. There appears to be no simple relationship between the sulfate content of the polysaccharides tested and their inhibitory or enhancing effects on M-mengo plaque size. This variability could tentatively be explained as the result of differing sensitivities of each variant to an agar inhibitor.

Liebhaber and Takemoto (1961) first showed that polycations, e.g. DEAE-D, could neutralize the inhibitory effect of sulphated agar polysaccharides on plaque formation by encephalomyocarditis virus. The plaque size enhancement was accomplished with a concentration as low as 50 µg./ml. of the DEAE-D, with no further increase in plaque size at higher concentrations. On the other hand, Sugiura and Kilbourne (1965) demonstrated that concentrations of more than 400 µg./ml. of dextran were toxic to cells (clone 1-5C-4 derived from a variant line of Chang's human conjunctival cell). The DEAE-D had a markedly favorable effect on development of plaques with A1/CAM and Swine/S-15 influenza viruses, both of which failed to produce visible plaques without DEAE-D.

It was soon found that these polycations could also remove the inhibitory effects of agar polysaccharides on m-(plaque size) mutants of

poliomyelitis, coxsackie A9 and B4, ECHO 5, 6, 8, 11, 26, 32, foot-and-mouth disease, mengo, group B arbo and herpes viruses (Miles and Austin, 1963; Brown and Packer, 1964).

However, the use of polycationic substances does not always result in improvement of plaque formation by viruses under an agar overlay. Kjellen (1963) found that DEAE-D had no effect on plaque formation by Ad 5.

Wallis and Melnick (1968) claimed that the enhancement of plaque number and size by DEAE-D and protamine is not due to their binding of sulfated polysaccharides in agar as has been assumed before. In the case of simian adenovirus, enhancement by protamine is due to the presence of arginine in digested protamine. Herpes (JES) and echovirus types 3, 4, 5 and 6 are known to be susceptible to agar inhibitors. However, they were found to be enhanced by cationic polymers even under starch gel and methyl cellulose overlays, which are free of polyanions, compared to the respective overlays without cationic polymers.

In virus diffusion experiments, Wallis and Melnick found that cationic polymers enhance the diffusion of virus through agar or starch gel. Therefore, plaque enhancement seems to be the result of the gel becoming positively charged so that viruses can move effectively through it. Where starch gel and methyl cellulose enhanced plaque formation with viruses known to be inhibited under agar, it was because of higher viscosity of the agar gel. When the consistency of the agar gel was reduced from 1.5% to 0.4%, the same plaque counts and sizes were observed under all three overlays. However, the data does not eliminate the possibility that the lowering of the agar concentration also lowers some

other undefined inhibitors present in the agar.

Therefore, DEAE-D, when incorporated into the agar overlay, may either enhance plaque formation, or inhibit it, or have no effect at all, depending on the virus type, and the concentration of DEAE-D dextran.

d) Neutral red

The dye most generally used to aid the visual observation of infected dead cells and non-infected metabolising cells is neutral red. Live cells take up the dye while dead cells lose their ability to retain the dye. The plaque is a focus of unstained dead cells.

Neutral red is known to inactivate viruses and cells photodynamically. That is to say, the dye inactivates viruses and cells only in the presence of visible light, and not in its absence, though white light filtered through neutral red solutions does not make neutral red toxic (Green and Opton, 1959; Gochenour and Baron, 1959). Gochenour and Baron (1959) found that the cells themselves could change and would develop resistance to fresh neutral red after 24 hours in the dark. Therefore, plates should be darkened immediately on pouring and kept away from all light for 24 hours.

The inhibitory effects have been demonstrated by the reduction in the number and size of the plaques formed by many viruses, for example, adenovirus (Hiatt, 1960; Bonifas and Schlesinger, 1959); poliomyelitis (Cooper, 1961); and arboviruses (Tomita and Prince, 1963). When neutral red is incorporated into the overlay, the effect is minimized by using dilute stain, e.g. 1/20,000, and withholding the stain from the overlay

until plaques have formed (Pledger, 1960).

e) pH of the overlay

The optimum pH for plaque formation varies between 6.8 and 7.8 depending on the particular assay system (Cooper, 1961). Plaque formation by the d-mutants of poliovirus is markedly inhibited by low pH, e.g. pH values less than 6.8 (Vogt et al. 1957). Since only the d-mutants of poliovirus are inhibited by the low pH, the pH sensitivity of the poliovirus is then genetically controlled and is characteristic of each individual virus, depending on the genetic content of the virus. It should be noted that the true pH of incubation is rarely known. The reason is that cells under the agar overlay may produce a locally low pH. Acidic and basic components in the medium will affect the true bicarbonate ion content; the true CO_2 concentration in the agar overlay can rarely be estimated from the concentration in the gas mixture being passed (Cooper, 1961).

The pH of bicarbonate-buffered media may be maintained either by incubating petri dishes in an appropriate gaseous environment (Dulbecco and Vogt, 1954) or plaque assays can be done in sealed flasks or bottles (Hsiung and Melnick, 1955). Need for control of the gaseous environment can be eliminated by substituting tris-(hydroxymethyl)-aminomethane for bicarbonate (Cooper, 1955). Plating efficiency with some virus-cell systems, e.g. herpes and vaccinia virus in chick cells, may be higher with tris buffer than with bicarbonate buffer (Porterfield and Allison, 1960). However, tris may be toxic for some cells and is generally inadequate for cell multiplication (Cooper, 1961).

f) Nutritional requirements for plaque formation

From a perusal of literature on plaque assay, it appears that Eagle's minimal essential medium (MEM), without additional supplements, is very often inadequate for plaque assay, especially when plaque formation is delayed, as in the case of adenovirus.

Bonifas and Schlesinger (1959) found that the plating efficiency of adenoviruses was enhanced by increasing the concentration of arginine in the overlay medium, from 0.1 mM to 0.4 mM. In a later experiment, Rouse, Bonifas and Schlesinger (1963) demonstrated that free arginine is rapidly depleted from the overlay covering PPLO-contaminated cultures. These organisms have the capacity to rapidly deplete the available arginine, thereby lowering the efficiency of plating of various adenoviruses. In the presence of increased arginine, PPLO-contaminated cells yield plaque titers as high as those on uncontaminated cells. Therefore, the enhancement in plating efficiency of adenovirus with higher concentrations of arginine in the overlay can be attributed to the presence of PPLO in the cell cultures.

To maintain the cells in a viable state under the overlay, Kjellen (1961) supplemented his overlays with lactalbumin hydrolysate and yeast extract. Lawrence (1967) incorporated lactalbumin hydrolysate and peptone; and Hermann (1968) added tryptose phosphate broth to the agar overlay.

Another device for the prolonged maintenance of cells under agar is the use of multiple overlays (Temin and Rubin, 1958; Winocour and Sachs, 1959; and Stinebaugh and Melnick, 1962). Cultures are overlaid, as usual, at the time of infection, then additional nutrients are supplied

by a second overlay 3 to 6 days later; sometimes, a third overlay is added after an additional interval.

In spite of the many drawbacks, plaques for adenoviruses have been produced by many researchers by the use of nutritional supplements and multiple overlays.

In a plaque assay, the plaque numbers represent the apparent infectivity under the conditions used. Up to date, it has not been possible to measure the inherent infectivity of animal viruses using the plaque technique. However, the eop of the virus depends on the conditions of the assay. Therefore, by examining the factors that affect plaque formation and utilizing the optimum of these conditions, it is possible to improve the eop of the virus. Under such optimum conditions the apparent infectivity is a closer measure of the inherent infectivity. Therefore, it was the objective of this research to obtain a measure of the apparent infectivity that is as close as possible to the inherent infectivity for adenoviruses.

MATERIALS AND METHODS

MATERIALS AND METHODS

VIRUS STRAINS

The adenovirus serotypes 5, 3 and 8 are prototype strains and were obtained from the American Type Culture Collection, Viral and Rickettsial Registry and Distribution Centre (ATCC). The type 5 adenovirus, strain Ad 75, had undergone two passages in primary human amnion cells and several (more than ten) subsequent passages in KB cells in this laboratory. The adenovirus type 3, strain G.B., had been passaged several times in primary human amnion cells and KB cells. The adenovirus type 8, strain "Trim" had undergone two passages in primary human amnion cells.

The adenovirus type 12, strain "Huie"[1] was passaged 5 times in primary human amnion cells and once in KB cells. It was demonstrated to be oncogenic for newborn hamsters.[2]

VIRUS PRODUCTION

Sixteen ounce prescription bottles containing complete monolayers of KB cells (approximately 1.6×10^7 cells) were infected with about 10^6 plaque forming units (PFU) of virus in 3 ml. volumes for 3 hours before the maintenance medium was added. The maintenance medium consisted of

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[1] Obtained from W. Stackiw, Manitoba Provincial Virology Laboratory.

[2] Dr. Hannan, personal communication.

Eagle's MEM supplemented with 2% horse serum,[3] which was heat inactivated at 56°C. for 30 minutes before use. The cells were examined daily for cytopathic effect. The medium was changed twice, once after 3 days and again after the first sign of cytopathic effect (CPE) was observed. When the CPE was maximum, usually 7 to 10 days after infection, the virus was harvested by three cycles of alternate freezing at -20°C., and thawing at room temperature.

The virus material harvested was pooled, centrifuged at 220 g to remove the KB cell debris, and the virus in the supernate was stored at -20°C. in small aliquots.

CELL CULTURES

Two separate strains of KB cells were used. The KB cell line (strain-1) was obtained from Dr. A.C. Laing, Defence Research Laboratories, Shirley Bay, Ontario and had undergone more than 100 subcultures in this laboratory. This strain was tested[4] and found to be contaminated with PPLO. The PPLO-free KB cell line (strain-2) was obtained from ATCC and had undergone about 12 subcultures in this laboratory. These cultures were tested and found to be free from PPLO. The KB cells were calf-serum adapted. The KB cells (strain-1) were used for most of the

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[3] Obtained from National Biological Laboratory, Winnipeg.

[4] The tests for the presence of PPLO were kindly performed by Mrs. Helen Russell, Department of Medical Microbiology, University of Manitoba.

experiments in this research. However, the PPLO-free KB cells (strain-2) became available near the conclusion of this project. Consequently, the latter strain was used for some concluding experiments.

KB cells were grown in sixteen-ounce prescription bottles. Complete cell monolayer cultures were treated with two ml. of 0.25% trypsin[5] in Hanks balanced salts solution (HBSS), for approximately 5 to 10 minutes at room temperature, in order to detach the cells from the glass and to disperse the cells.

The KB cell suspension in trypsin was centrifuged at 140 g for 5 minutes to pellet the cells. Trypsin was removed and the cells were resuspended in growth medium. Clean bottles, each containing 30 ml. of growth medium were seeded to contain about 6×10^6 cells per bottle. The growth medium used was Eagle's MEM supplemented with 10% heat-inactivated calf serum.[6] Penicillin (100 I.U./ml.) and streptomycin (50 µg/ml.) were added to the growth medium before it was adjusted to pH 7.4 (approximately), with 7.5% sodium bicarbonate just prior to use.

MEDIUM FOR PLAQUE ASSAY

In the initial experiments, the agar overlay consisted of Eagle's Minimum Essential Medium, supplemented with 15% horse serum,

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[5]Trypsin (1:250) was obtained from Nutritional Biochemicals Company, Cleveland, Ohio.

[6]Calf serum was obtained from the National Biological Laboratory Winnipeg, Man.

penicillin, streptomycin, lactalbumin hydrolysate,[7] tryptose phosphate broth (Difco) and 1% noble agar. Full details are provided in Appendices C and D. In order to obtain an optimal medium for plaque assay of adenovirus, some of the components in the overlay were varied where indicated. In some experiments, L-arginine-HCl[7] was used as an added supplement.

The optimal agar overlay medium consisted of Eagle's MEM, supplemented with 10% horse serum, proteose peptone (Difco), 0.6% Noble agar and kanamycin.[8] The composition is listed in Appendix B.

To prepare the agar overlay, the nutrients and agar were made up separately. The fluid medium contained twice the final concentration of nutrients and was warmed to 45°C. Twice the final concentration of melted Noble agar (Difco) was cooled to 45°C. Equal volumes of nutrient medium and melted agar were mixed, after which sodium bicarbonate was added to obtain a pH of 7.1. It was determined that 4 ml. of sodium bicarbonate (0.5M) per 100 ml. agar overlay was needed to obtain a pH of 7.1. The agar was sterilized by autoclaving for 10 minutes at 115°C. and stored at 4°C. until used. It should be noted that the agar was not used again after it was remelted once. Where indicated in the text, Agarose[9]

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[7]Obtained from Nutritional Biochemical Corp.

[8]Kanamycin (Kantrex) obtained from Bristol Laboratories of Canada Ltd.

[9]Agarose (Seakem) made by Marine Colloid Ind., and distributed by Bausch and Lomb.

was used instead of agar and fetal calf serum[10] was used in place of horse serum.

The neutral red,[11] used for staining the cells, was prepared as a 0.4% stock solution in distilled water and was sterilized by filtration through a millipore filter (HA grade). It was added to the second agar overlay to make a concentration of 1/31,250, i.e., 0.8 ml. neutral red per 100 ml medium. Five ml. of the second agar overlay was added to 5 ml. of the first overlay which made a final neutral red concentration of 1/62,500, when taking the combined volumes of both overlays into account.

THE PLAQUE ASSAY PROCEDURE

Plastic tissue culture dishes[12] (60 mm. diameter) were used for all the plaque assay experiments. The dishes were seeded with approximately 4×10^6 cells in 5 ml. of growth medium which was adjusted to pH 7.1 with 0.5M sodium bicarbonate. The cells were allowed to attach to the dishes to form a confluent monolayer overnight in a 37°C. humidified incubator in an atmosphere of 6% CO_2 in air.

The growth medium was then removed and the KB cell monolayers

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[10] From Microbiological Associates Inc., Bethesda, Maryland.

[11] Neutral red (Toluylene Red) obtained from Fisher Scientific Company.

[12] Falcon Plastics, Los Angeles, California; tissue culture grade.

were washed once with sterile PBS[13] buffered at pH 7.2. Each dish was then inoculated with the appropriate virus dilution (0.5 ml./plate) in a specially prepared virus diluent which consisted of PBS supplemented with Eagle's working stock (Appendix D) to make a final concentration of amino acids and vitamins similar to that in the growth medium. The infected plates were incubated at 37°C. for 1½ hours with gentle agitation every 15 minutes. The excess unadsorbed virus was removed and discarded, with or without washing of the monolayer, as required by the individual experiment. In experiments where comparison of variables affecting plaque assay were performed, only a single virus dilution, which contained about 50 PFU per 0.5 ml., was used. After removal of virus, 5 ml. of the agar overlay medium at 45°C. was carefully added to each plate.

The agar overlay was permitted to solidify at room temperature (21°C. to 23°C.) for 15 to 20 minutes. The cultures were then placed in a 37°C. humidified incubator in an atmosphere of 6% CO_2 in air. When KB cell strain-1 was used, the plates were overlaid with an additional 5 ml. of neutral red overlay on day 10. Ad 5 plaques usually appear on the 13th day. However, when KB cell strain-2 was used, the neutral red agar overlay was added on day 7 and the Ad 5 plaques appeared on day 8. Where indicated, the plaque diameter was measured in one dimension with a ruler graduated in mm. Where the plaque size was smaller than 1 mm. an estimate was made as to the approximate size. Ten plaques were measured and the average diameter recorded.

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[13] PBS prepared according to the Dulbecco and Vogt (1954) formulation.

EXPERIMENTAL PROCEDURES AND RESULTS

EXPERIMENTAL PROCEDURES AND RESULTS

I. FORMATION OF TYPE 5 ADENOVIRUS PLAQUES

Initial experiments using Eagle's MEM supplemented with 15% horse serum in the agar overlay, did not allow the formation of Ad 5 plaques; the KB cells were dead by the time neutral red was added on day 10. As shown by later experiments this was probably due to lack of some essential nutrients. In the plaque assay of adenovirus, Lawrence (1967) used lactalbumin hydrolysate and peptone in the agar overlay. Hermann (1968) added tryptose phosphate broth to the agar overlay in the plaque assay of adenovirus.

Hence, in the subsequent experiments, the above mentioned three supplements to Eagle's MEM were added to the overlay medium with concentrations as shown in Appendix C. As a result, plaques of Ad 5 were formed.

1. Plaque Characteristics

The following observations were obtained using the agar overlay prepared according to Appendix B. The Ad 5 plaques obtained with KB cell (strain-1) monolayers were first seen 13 days after infection, if the neutral red overlay was added on day 10. The plaque numbers increased very rapidly till about day 25. The size of the plaques were pin-point initially, but they slowly enlarged in size to an average maximum diameter of 3.5 mm. on day 29. Microscopically, a plaque is a focus of unstained cells surrounded by stained cells, as shown in Figure 1. To the naked eye, a plaque shows up as an unstained spot in a red background, as shown in Figure 2.

On the other hand, the plaques obtained with KB cell (strain-2) monolayers were first seen eight days after infection if the neutral red was added on day 7. On day 29, the average diameter of the plaques formed by Ad 5 is 8 mm., as shown in Figure 7. The maximum plaque numbers of Ad 5 were attained after 15 days for KB cell (strain-2) monolayers. In addition, the titre of the same pool of Ad 5 is higher when KB cell strain-2 was used (3.8×10^7 PFU/ml.) compared with KB cell strain-1 (1.08×10^7 PFU/ml.) based on the plaque counts on day 15. However, the titre from the day 25 counts with strain-1 approaches that obtained with strain-2 (2.92×10^7 PFU/ml.). Therefore, the maximum plaque counts can be obtained much sooner with KB cell strain-2 than with strain-1.

2. Neutralization of Ad 5 with antiserum to Ad 5

Since most of the experiments were done with Ad 5, its purity was confirmed by neutralization with specific antiserum to adenovirus type 5.

Two ml. portions of specific rabbit antiserum to Ad 5[14] (diluted to 1/100, 1/1,000 and 1/4,000) were mixed with equal volumes of virus suspension diluted to contain about 100 PFU/0.5 ml. Rabbit antiserum to Ad 3[15] (diluted to 1/100, 1/1,000 and 1/4,000) and PBS were used as controls. The reaction mixture was incubated for one hour at room temperature

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[14] Type 5 antiserum was provided by T.R. Fargey and has a neutralizing titre of 1/4,000 against 100 $TCID_{50}$ of homologous virus.

[15] Type 3 antiserum was provided by Dr. C.K. Hannan and has a neutralizing titre of 1/12,000 against 100 $TCID_{50}$ of homologous virus.

(21°C), and then assayed by the plaque technique as described in Materials and Methods. The agar overlay was prepared according to Appendix C and the neutral red overlay was incorporated on day 10.

Antiserum to Ad 3 did not significantly reduce the titre of the Ad 5 compared to PBS, but there was a highly significant reduction in the titre by all the three dilutions of antiserum to Ad 5. However, one or two plaques always appeared even at the lowest antiserum dilution.

In order to determine whether the plaques represent un-neutralized type 5 adenovirus or contamination with another serotype, one of the plaques was picked and a pool of virus was grown. A neutralization test similar to the one described above, was performed and the results showed that there was a similar decrease of plaque numbers by the antiserum to Ad 5 but not by antiserum to Ad 3. Therefore, these plaques represent un-neutralized Ad 5, and the result confirms the identity of the virus.

3. Statistical analysis

The following experiment was carried out in order to ensure that plaque formation conformed to expectations regarding reproducibility, statistical reliability and that the plaque counts are proportional to the virus dose.

Four dilutions of the Ad 5 virus stock, namely 1/200, 1/400, 1/800 and 1/1,600, were made in virus diluent. KB cell (strain-1) monolayers were used for the plaque assay of the virus samples. The overlay medium was prepared according to Appendix C and the neutral red overlay was added on day 10. Other details of the plaque assay procedure are described in Materials and Methods.

The results of this experiment are presented in Table I and in Figures 3 and 4. Table I shows the relationship between virus dose and plaque numbers of Ad 5, Figure 3 illustrates the increase of plaque numbers of Ad 5 with time and Figure 4 shows that the virus dose is directly proportional to the plaque numbers. The latter is in agreement with the concept that a plaque is initiated by a single infectious particle.

For a reproducible plaque assay, plaques should be spread among cultures of one batch according to a Poisson distribution, i.e., randomly distributed. The Chi-squared (X^2) test was used to test for compatibility with Poisson distribution as described by Schefler (1969). Since the X^2 value is dependent on the magnitude of the differences between the observed and expected plaque frequencies, it is a measure of the goodness-of-fit of the observed means and the expected means, in this case, with a total population of 417 PFU (See Table II). The X^2 value for the day 17 plaque counts of Table I are tabulated in Table II. The day 17 counts were selected because the plaque counts for all the virus dilutions are available.

For a known Poisson distribution, the relative plaque counts should be in the ratio of 1:2:4:8, i.e. the relative virus concentrations. Our data fitted the Poisson distribution on basis of the Chi-squared test as shown in Table II. Since the X^2 values are not significant at the 0.95 level, the experimental results are therefore highly compatible with the assumption of a Poisson distribution.

The coefficients of variation were calculated as a means of determining the degree of dispersion of the counts around the mean. The degree of accuracy of the count based on each dilution is compared as

shown in Table II. The values for the coefficient of variation show that the plaque counts which average 56 are least dispersed around the mean compared to the counts of 26, 109 and 226. Because of this fact, the virus samples were diluted to contain about 56 PFU for the plaque assays in all subsequent experiments.

It should be noted that the accuracy depends, among other factors, on the area of the individual plaque relative to the area of the culture dish. That is to say, when plaques are approximately 2 mm. in diameter, using 60 mm. petri dishes, the plaque counts around 56 are expected to be least in error in a plaque assay. However, if a different cell-virus system or a different dish size is used, a change in the counts where there is least dispersion about the mean is to be expected.

Utilizing the results obtained in Table I, a graph is plotted showing the log PFU as a function of log virus dose (the day 17 values are used as the example). Figure 4 shows that the PFU is directly proportional to virus concentration. Therefore, the result indicates that plaques are initiated by single infectious particles (Dulbecco and Vogt, 1954). Assuming a direct linear relationship between virus concentration and numbers of plaques a theoretical plot is also made with the lowest dose (1/1,600 dilution) serving as the starting point. It is shown that the experimental plot of the actual plaque counts almost coincides with the theoretical expected plaque counts, i.e., there is excellent agreement between the experiment results and the theoretical result.

In order to demonstrate whether the plaque assay is reproducible, an analysis of variance was performed on the counts obtained from four separate assays of the same pool of virus carried out under identical con-

ditions but at different times. The overlay media were prepared according to Appendix B and the neutral red overlays were added on day 10.

When analysing all four assays, an F-value of 7.295 was obtained as shown in Table III, which is significant at the 0.05 level, indicating that the plaque assay is not reproducible. It should be noted that it is not significant at the 0.01 level. However, when analysing the first three assays only, an F-value of 3.98 was obtained, which is not significant at the 0.05 level, indicating that the plaque assay is reproducible. Since the F-value of 7.296 is not highly significant and considering the non-significance of the differences of the first three assays, conclusions should obviously be drawn cautiously and may be subject to error. This may be attributed to inherent errors of the plaque assay system, e.g., slight changes in the pH of the overlay (see Table XIX), possible variations in the susceptibility of the KB cells to virus through successive passages, or gradual inactivation of virus through storage.

From the foregoing statistical analysis of the results, it is shown that the PFU are distributed in a Poisson manner; the plaques are initiated by single infectious particles and that the plaque assay is reproducible if there is rigid control of the assay conditions.

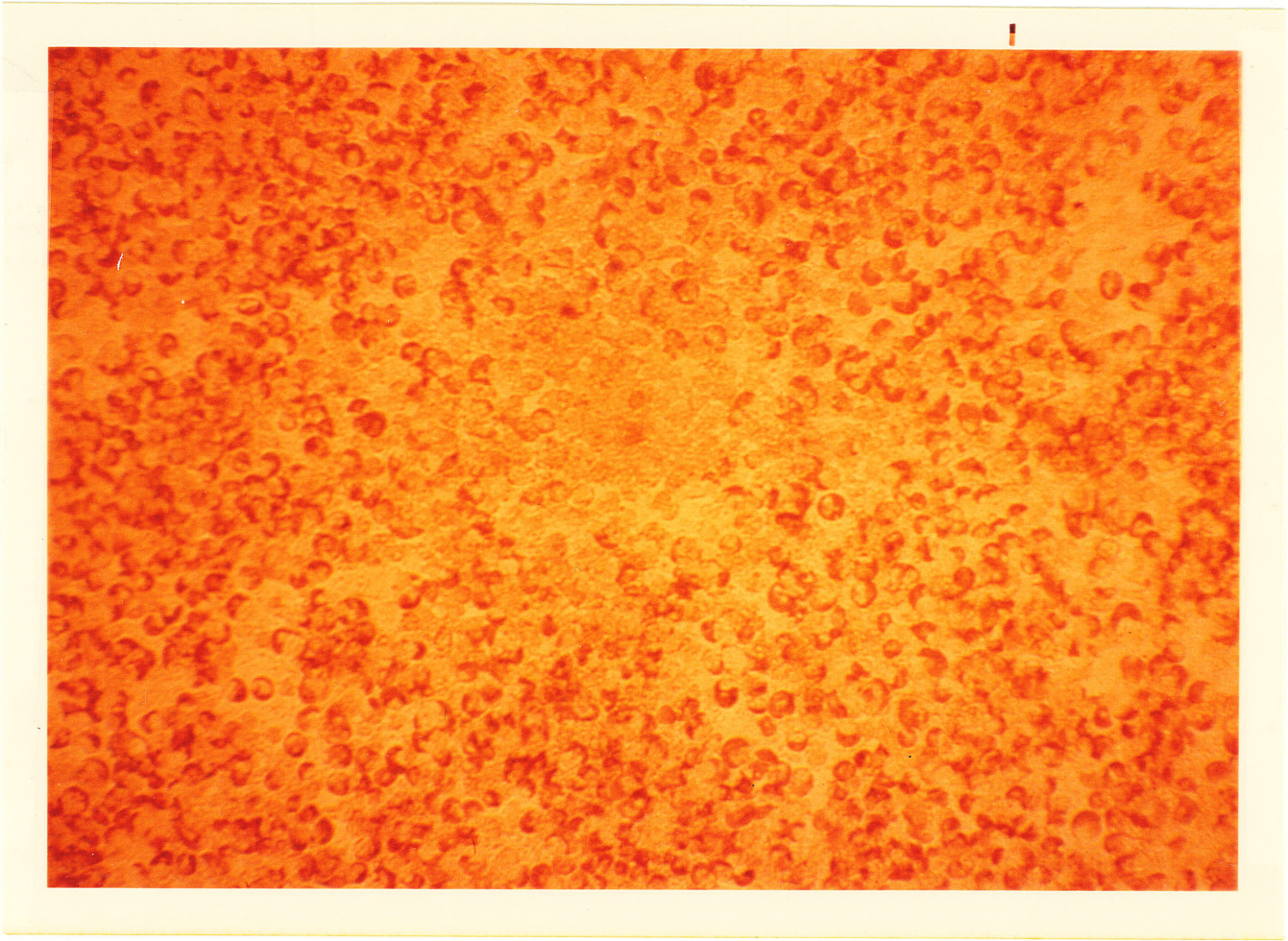


Figure 1. Photomicrograph of a type 5 adenovirus plaque. The plaque is the central area of unstained cells. (Total magnification: 350X)

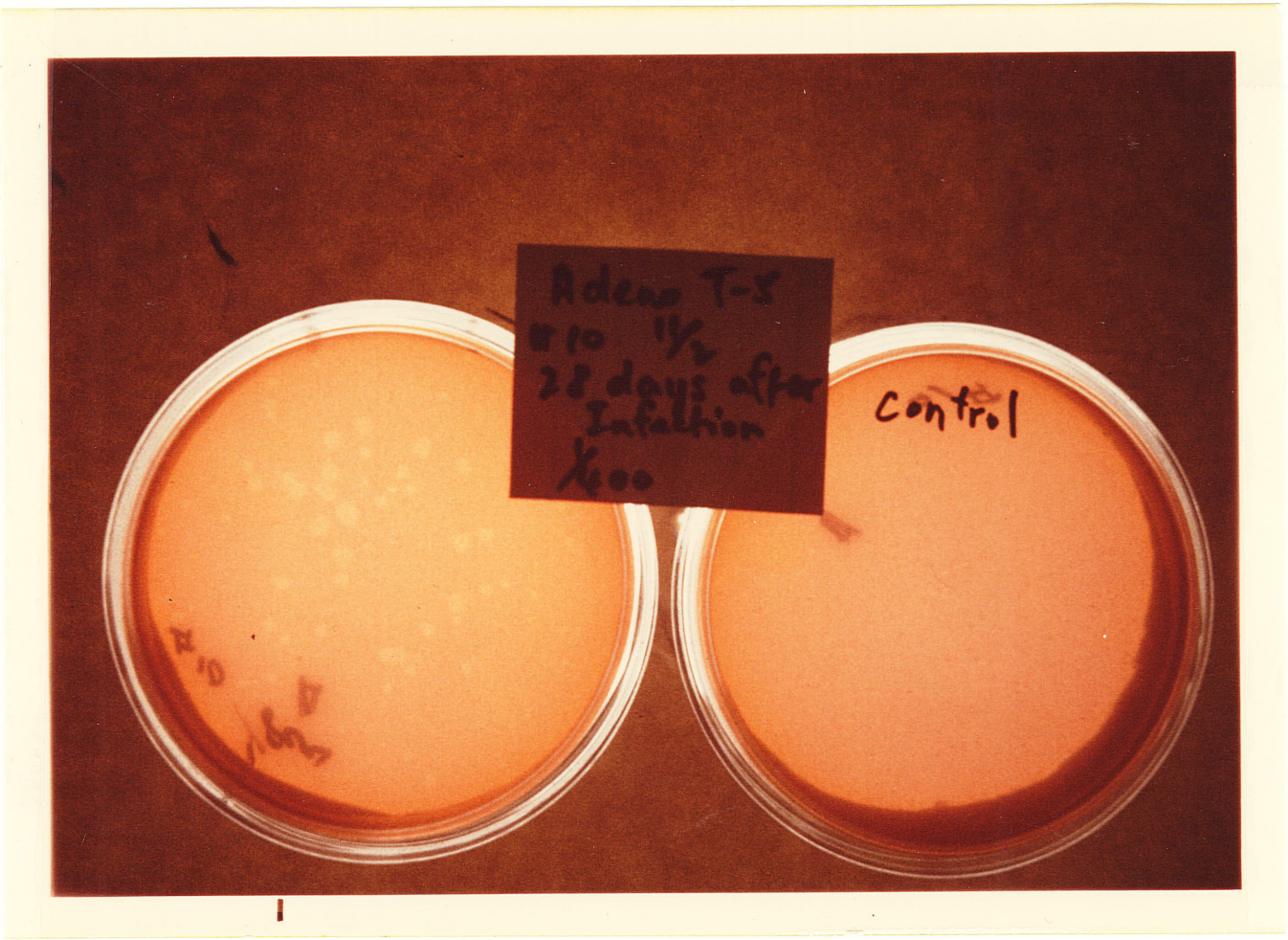


Figure 2. Plaque characteristics of Ad 5 in KB cell (strain-1) monolayers.

TABLE I

RELATIONSHIP BETWEEN VIRUS DOSE AND PLAQUE NUMBERS

Days after Infection	Virus dilutions			
	1/200	1/400	1/800	1/1,600
13	46*	23	12	6
15	156	69	34	15
17	226	109	56	26
19	OP	153	77	34
21	OP	OP	86	43
23	OP	OP	91	48
25	OP	OP	94	49

*Average plaque counts of 5 replicates
OP: Overlapping plaques

TABLE II

THE X^2 AND VARIANCE VALUES OF THE DAY 17 PLAQUE COUNTS

Relative virus concentration	Plaque counts	Mean (Observed)	Expected mean	X^2	Coefficient of variation
1	26,19,23,35,25	26	27.8	0.2208* (NS)	1.27
2	59,51,61,58,50	56	55.58		0.45
4	120,109,102,118, 98	109	111.17		0.85
8	242,240,216,217, 216	226	222.38		1.23

Total of the means = 417 PFU.

$X^2_{0.95}(3) = 7.815$

*Value of X^2 between the observed means and expected means, which is not significant (NS) at the 0.95 level.

TABLE III

THE F-TEST FOR FOUR PLAQUE ASSAYS OF THE SAME VIRUS STOCK

Date of Assay	Individual counts*	Average	F-values	
1. 4th April, 1969	62, 54, 62	59.3		
2. 19th May, 1969	65, 72, 58	65.0	3.98	7.295
3. 21st May, 1969	55, 52, 54	53.7		
4. 31st May, 1969	48, 46, 52	48.7		

*Plaque counts were made on day 21.

Samples 1 - 4: $F = 7.295$

$F_{0.05}\ (3,8) = 4.07$

$F_{0.01}\ (3,8) = 7.59$

Samples 1 - 3: $F = 3.98$

$F_{0.05}\ (2,6) = 5.14$

Figure 3. Increase of plaque numbers of adenovirus type 5 with time.

Each point represents the average plaque count of five replicates of the 1/1,600 virus dilution.

The bars represent the range.

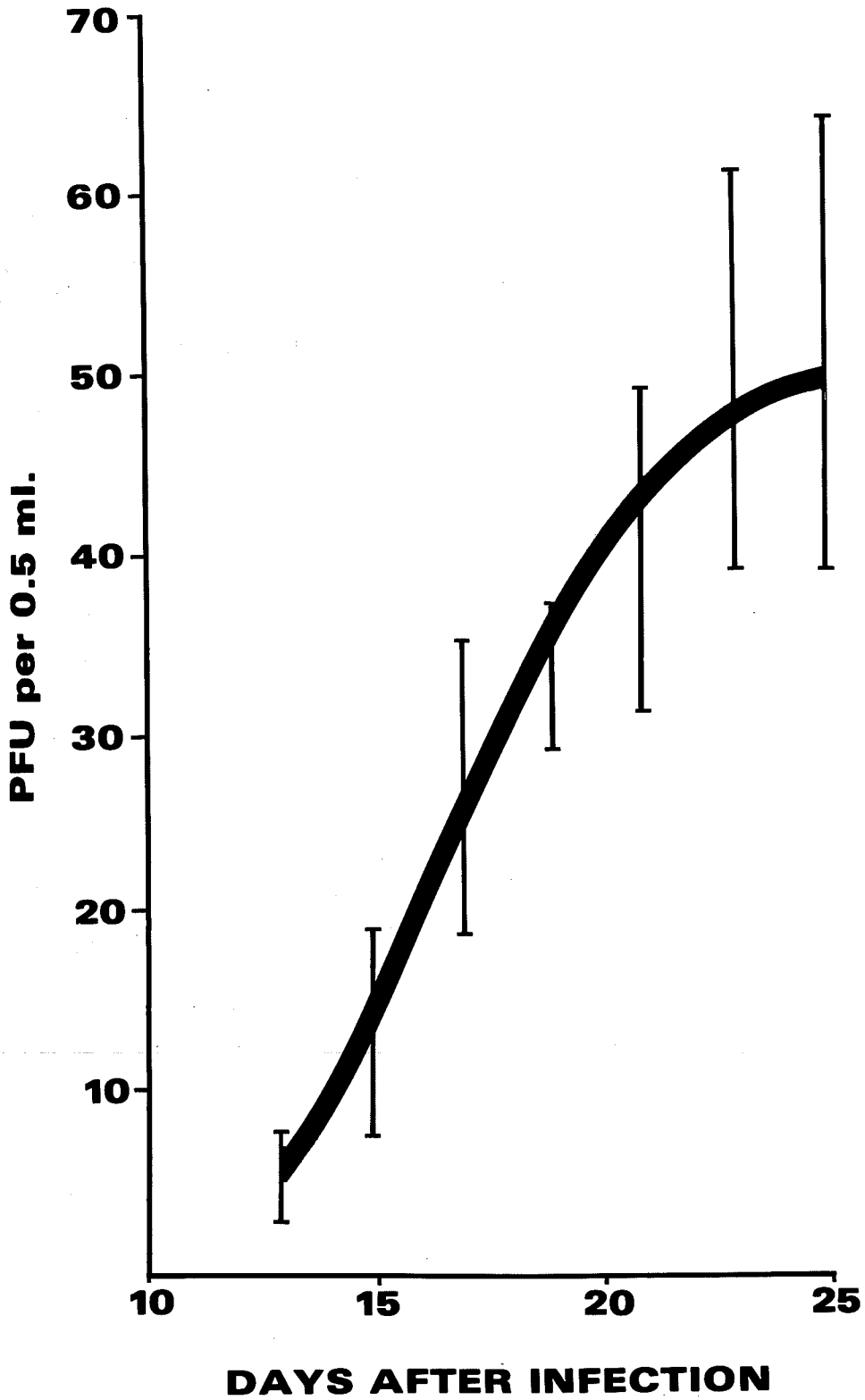
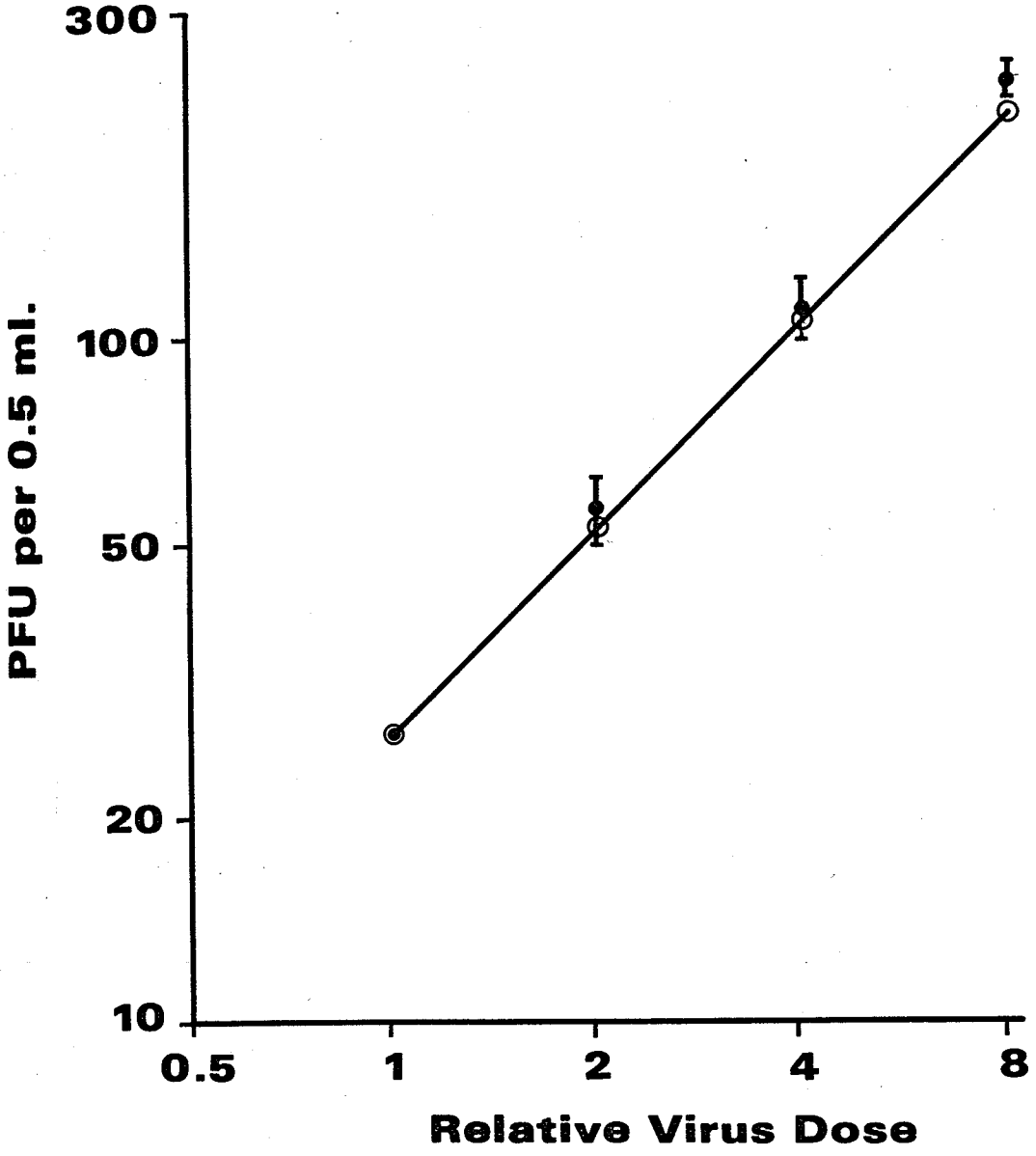

70
60
50
40
30
20
10
PFU per 0.5 ml.
10
15
20
25
DAYS AFTER INFECTION

Figure 4. Relationship between plaque numbers and virus dose of adenovirus type 5.

The open circles represent the theoretical plot with the lowest dose as the starting point, assuming a direct linear relationship between virus concentration and plaque numbers.

The closed circles represent the experimental plot (means of five replicates).

The bars represent the range.


300
100
50
20
10
PFU per 0.5 ml.
0.5
1
2
4
8
Relative Virus Dose

II. FACTORS AFFECTING PLAQUE ASSAY OF AD 5

1. Volume of overlay

Vogt et al. (1957) showed that an increase in the thickness of the agar overlay resulted in a marked decrease in the plaque numbers of poliomyelitis viruses. However, it has not been shown whether adenoviruses are sensitive to changes in the volume of overlay. Therefore, it was the purpose of the following experiment to determine the effects of changes in the volume of the agar overlay on the plaque formation of type 5 adenovirus.

Under one condition, the procedure as described in Materials and Methods was used, i.e., a total overlay volume of 10 ml. was added, 5 ml. immediately after infection of the cell monolayers, and an additional 5 ml., with neutral red, 10 days after infection. Under the other condition, the first overlay was 10 ml. in volume followed by a second overlay of 5 ml., with neutral red, making a total of 15 ml. The agar overlay was prepared according to Appendix C. The experiment was repeated twice.

Under the first condition, clear plaques were seen on day 13. However, when a total overlay volume of 15 ml. was used, plaques were not always formed and when formed they were streaky in appearance.

Therefore, under the conditions used, a total overlay volume of 15 ml. was too thick for plaque formation by Ad 5 in KB cell strain-1. This may be due to a reduction in the oxidative metabolism of the cells caused by the thick overlay, which may, in turn, have affected virus growth, (Baron et al. 1961).

These experiments were carried out before the plaque assay procedure as described in Materials and Methods was adopted. Hence, in all future work, a total volume of 10 ml. was used.

2. The influence of medium composition on plaque formation

a) The influence of the nutritional supplements

After obtaining plaques with the aid of the three supplements, proteose peptone, lactalbumin hydrolysate and tryptose phosphate broth, the need for each of them was investigated.

Fifteen plates of KB cell (strain-1) monolayers were infected with identical aliquots of the same virus dilution, as described in Materials and Methods. They were divided into five groups and overlaid with five different agar overlays. These overlays were prepared according to Appendix C, but with the following variations, as shown in Table IV: three of the overlays contained combinations of two of the three supplements; the fourth overlay contained all the three supplements and the fifth overlay did not contain any supplement. Neutral red overlays containing the same supplements were added on day 10.

The results (Table IV) show that in the presence of proteose peptone, the plaques were seen on day 14. Without proteose peptone, but with lactalbumin hydrolysate and tryptose phosphate broth, the plaques did not appear until 22 days after infection. In the absence of the three supplements, i.e., with Eagle's MEM alone, the KB cells were no longer viable after day 14. This indicates that proteose peptone is required for early plaque formation and for maximum eop.

A further experiment, similar to the one above, was performed

to elucidate the effects of the individual supplements on plaque formation. After infection of the twelve plates of KB cell (strain-1) monolayers, they were divided into four groups and overlaid with different media. One of the overlays was prepared according to Appendix C, which contained all the three supplements. The other three overlays were prepared similarly, but each of them contained only one of the three supplements, as shown in Table V.

The results clearly demonstrate the necessity for proteose peptone, for in its absence, cells did not take up the neutral red dye, i.e., they were dead. Presumably there was insufficient nutrient in the absence of proteose peptone, for the survival of the KB cells under the agar overlay. However, the plaques which were produced with one supplement, proteose peptone, appeared on the same day as those produced with all the three supplements, and their numbers are almost identical. Therefore, it is concluded that only one supplement, proteose peptone is needed for the plaque formation by Ad 5 in the KB cell strain-1.

b) The requirement for proteose peptone

Rouse and Schlesinger (1963) demonstrated that PPLO rapidly depleted the available arginine, an essential amino acid for adenovirus replication, in PPLO-contaminated KB cell cultures.

Since it was known that the KB cell strain-1 was contaminated with PPLO, the following experiment was attempted in order to study if the need for proteose peptone was in fact the need for additional arginine in the plaque assay of adenovirus.

The proteose peptone was replaced by L-arginine-HCl to a final

TABLE IV

EFFECTS OF MEDIUM SUPPLEMENTS ON
PLAQUE FORMATION (Experiment 1)

Supplements			Days after infection			
P.P.	L.H.	T.P.B.	14	16	22	24
-	+	+	0	0	77*	79
+	-	+	30	71	147	OP
+	+	-	32	73	142	OP
+	+	+	49	89	C	C
-	-	-	cd	cd	cd	cd

*Average plaque count of 3 replicates.

OP - overlapping plaques
C - contamination
P.P. - proteose peptone
L.H. - lactalbumin hydrolysate
T.P.B. - tryptose phosphate broth
cd - cells dead

TABLE V

EFFECTS OF MEDIUM SUPPLEMENTS ON
PLAQUE FORMATION (Experiment 2)

Supplements			Days after infection		
P.P.	L.H.	T.P.B.	15	18	21
+	-	-	16*	28	40
-	-	+	cd	cd	cd
-	+	-	cd	cd	cd
+	+	+	16	29	33

*Average plaque count of 3 replicates.

cd - cells dead

concentration of 2 mM. This concentration was chosen because Rouse et al. 1963 demonstrated that for PPLO contaminated KB cells, a minimum concentration of 1 mM arginine is required for maximum eop. Eagle's MEM contains 0.6 mM of arginine. To bring the final concentration of arginine to 2 mM, 14.7 mg. of arginine in 8.2 ml. solution was added to 50 ml. of agar overlay prepared according to Appendix B. A set of control plates contained the proteose peptone supplement alone.

Plaques were formed in the control plates but not in the plates with the added arginine. The KB cells were dead by day 12 in the latter case. Therefore, arginine in 2 mM concentration was unable to replace proteose peptone, at the concentration used, for plaque formation by adenovirus.

In another attempt to study the usefulness of arginine, the agar overlay with the proteose peptone was supplemented further with arginine-HCl to a final concentration of 1.5 mM. The results showed that there was no difference in either the plaque numbers or plaque size between the control plates and the arginine-supplemented plates.

In an attempt to elucidate the nature of the plaque promoting factors in proteose peptone, it was dialyzed overnight against two changes of distilled water (50 volumes), to remove small peptides and amino acids. The dialyzed peptone, which contains the large molecular weight materials, was used in the plaque assay of adenovirus. The purpose was to determine whether the large molecular weight materials or the small ones are necessary for plaque formation. The usual plaque assay procedure as described in Materials and Methods was performed. The infected KB cell (strain-2) monolayers were divided into two groups and overlaid with media

prepared according to Appendix B, with either dialyzed or undialyzed proteose peptone. Neutral red overlays were added on day 7.

The results demonstrated that there was a significant reduction in plaque numbers when dialyzed proteose peptone was used (mean of 6 plaques) compared to undialyzed proteose peptone (mean of 19 plaques). This implies that the small peptides and amino acids are at least partly responsible for the enhancement in plaque numbers. On the other hand, the results indicate that the proteose peptone component which is necessary for maintaining the viability of the KB cells under an agar overlay is in the undialysable fraction. Although cells of strain-2 were used in this latter experiment it is assumed that they behave the same as strain-1 cells.

c) Serum

Earlier work done in this laboratory (Aldcorn, 1966) demonstrated that inhibitors to adenoviruses are present in calf sera but absent from horse sera. Therefore, horse serum was chosen for the plaque assay work.

Experiments were carried out in order to determine the optimum concentration of serum to be used in the plaque assay of Ad 5. KB cell (strain-1) monolayers were infected with identical aliquots of the same virus dilution, as described in Materials and Methods. After adsorption, the infected plates were divided into three groups and overlaid with medium prepared according to Appendix C and containing either 15% or 10% or 5% horse serum. Neutral red overlays containing the same concentrations of serum were added on day 10 and plaques were first seen on day 13.

The results in Table VI show that there were more plaques in the presence of 10% serum; the plaques were also bigger. Plaques were not formed when 5% serum was used because the KB cells were dead by day 12. Therefore, 10% horse serum was the optimum concentration for use in the plaque assay compared to 5% and 15% horse serum.

In experiments not reported here, it was observed that the plaques formed by adenovirus type 5 with fetal calf serum in the overlay were smaller and less clearly defined than those obtained with horse serum. Therefore, the type of serum may be a factor which affects plaque formation. In addition, the type of serum used for growing the KB cell monolayers may affect their sensitivity to virus (Hannan, personal communication). Hence, in the following experiment, the effects of different types of serum in both the growth medium and in the overlay medium on the plaque characteristics of Ad 5 were examined.

The KB cell strain-1 was grown in the presence of 10% calf serum (the usual mode) in one set of plates and in 10% fetal calf serum in the other set. After infection, each set of plates was divided into two groups. Horse serum was used in the overlay for one group and fetal calf serum was used for the other, as shown in Table VII. The plaque assay procedure as described in Materials and Methods was used and the overlay medium was prepared according to Appendix B with the respective sera as outlined above.

The results are shown in Table VII and the most significant finding is that fetal calf serum appears to be inhibiting virus development relative to horse serum. This is manifested both in the reduced number and reduced size of plaques. The serum in the growth medium

appears to have some influence, but since the difference in the plaque numbers are not highly significant, further study on the effects of serum in the growth medium was not done.

The apparent inhibitory character of fetal calf serum was unexpected because Aldcorn (1966) reported that none of several fetal calf serum samples tested were inhibitory to adenovirus growth. The following experiment was therefore designed to compare the effects of different batches of fetal calf sera and horse serum on Ad 5 plaque formation. A batch of fetal calf serum, known to be non-inhibitory to adenovirus growth,[17] which is referred to as the reference serum in Table VIII, horse serum and bovine serum albumin (0.1%) were used to compare with the fetal calf serum in question (referred to in Table VIII as fetal calf "test"). This experiment was done when KB cell strain-2 became available. Calf serum was used in the growth medium. The procedure was similar to the above experiment, but the neutral red overlay was added on day 7 and plaques were first seen on day 8.

The results in Table VIII show that fewer plaques were formed when fetal calf ("test") serum was used, compared to the fetal calf (reference) serum. The plaque numbers are small, but the difference between the means is nevertheless found to be significant ($t=2.332$; $P_{0.90}(4)=2.132$). In addition the horse serum and bovine serum albumin allowed the formation of almost identical plaque numbers compared to the fetal calf (reference) serum.

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[17] Dr. Hannan, personal communication.

Consequently, horse serum was used for the routine plaque assay of adenovirus. However, this does not imply that every batch of horse serum is non-inhibitory to adenovirus plaque formation. Before it was used for the plaque assay, each new batch of horse serum was tested for the presence of adenovirus inhibitors by comparison with a batch of horse serum that was known to be non-inhibitory.

d) Effects of antibiotics on plaque formation

The KB cell cultures (strain-1) in this laboratory were tested and found to be contaminated with PPLO. Since PPLO are resistant to penicillin or streptomycin, it was necessary to control the growth of PPLO by other antibiotics. Brown and Officer (1968) recommended the use of kanamycin, in high but non-toxic doses, for the control of PPLO growth.

Since PPLO rapidly depletes the available arginine, which is an essential amino acid for the multiplication of adenovirus (Rouse and Schlesinger, 1967), it was the purpose of this experiment to determine whether kanamycin affects the eop of Ad 5.

KB cell (strain-1) monolayers were grown in the medium prepared according to Appendix A containing either 500 µg/ml. kanamycin or penicillin and streptomycin at 100 IU/ml. and 50 µg/ml., respectively. After adsorption of the virus, the infected plates were overlaid with medium prepared according to Appendix C. The neutral red overlay was added on day 10 and plaques appeared on day 13.

The results in Table IX (Experiment 1) show that kanamycin allowed the production of more plaques than penicillin and streptomycin

TABLE VI

THE EFFECTS OF HORSE SERUM CONCENTRATION IN OVERLAY MEDIUM ON PLAQUE FORMATION

Concentration of serum	Plaque numbers	Plaque diameter in mm.	
		Average	Range
5%	cd	-	-
10%	68*	2.0@	1-3
15%	50	1.3	0.5-2

*Average plaque counts, on day 26, of three replicates.

@Average diameter of ten plaques measured on day 26.

cd - cells dead

TABLE VII

EFFECTS OF DIFFERENT SERA ON PLAQUE FORMATION

Serum in growth medium	Serum in overlay	Plaque numbers	Plaque diameter in mm.	
			Average	Range
calf	horse	85*	2@	0.5-2.5
calf	fetal calf	55	1	0.5-2
fetal calf	horse	93	2.5	1-3
fetal calf	fetal calf	41	1	0.5-2

*Average plaque counts of three replicates on day 19.

@Average plaque diameter of 10 measurements.

TABLE VIII

EFFECTS OF SERA IN THE OVERLAY ON PLAQUE FORMATION

Serum in overlay	Plaque counts on day 9	Average counts
fetal calf (reference)	19, 20, 14	18
fetal calf ("test")	11, 9, 15	12
horse	19, 20, 17	19
bovine serum albumin 0.1%	16, 19, 15	17

Note: Calf serum was used in all the growth medium.

in combination.

The experiment was repeated but with a set of plates containing no antibiotics in the growth medium, as an additional control. This was done so as to determine if the difference in the plaque numbers was caused by the enhancing effects of kanamycin or by possible inhibitory effects of penicillin and streptomycin.

The results presented in Table IX (Experiment 2) confirm that kanamycin was the factor responsible for the increase in plaque numbers. Since there was no significant difference between the plaque numbers obtained with penicillin and streptomycin and the plates without antibiotics, these antibiotics are without effect.

If kanamycin was able to enhance the plaque numbers when KB cell monolayers were exposed to it for only 24 hours, it may further enhance the plaque numbers when incorporated into the overlay medium. Hence, the following experiment was designed to determine the effects of kanamycin in the overlay medium.

KB cell (strain-1) monolayers were grown in the medium prepared according to Appendix A. After adsorption of virus, the infected plates were overlaid with medium prepared according to Appendix B containing either 100 µg/ml. or 500 µg/ml. of kanamycin, or 100 IU/ml. and 50 µg/ml. of penicillin and streptomycin, respectively. Neutral red overlays containing the same antibiotics were added on day 10 and plaques were first seen on day 13.

The results in Table X show that the presence of 100 µg/ml. of kanamycin in the overlay allowed yet a further increase in plaque numbers. However, at 500 µg/ml., kanamycin showed inhibitory effects on plaque

formation.

The addition of kanamycin to the growth medium was originally intended to control the growth of PPLO in KB cell strain-1. However, from the foregoing observations, it was considered possible that kanamycin may have directly affected the response of the KB cells to virus, in addition to its effects on the growth of PPLO.

Consequently, the next experiment was designed to test the hypothesis that kanamycin, when added to the growth medium, alters the virus adsorption sites on the KB cell surface, which brings about a more efficient adsorption of adenovirus to the KB cells. In order to perform this experiment, a further variable, PPLO, had to be eliminated. Hence, the KB cell strain-2 (PPLO free) was used, when it became available. The KB cells were tested just prior to use and it was confirmed that they were not contaminated with PPLO. The virus suspension at the dilutions used for the experiment was also tested and found to be free of PPLO. The overlay medium was prepared according to Appendix B, but the neutral red overlay was added on day 7 instead of day 10. The plaque assay procedure as described in Materials and Methods was used.

As shown in Table XI, kanamycin in the growth medium, at 500 ug/ml., was able to enhance plaque numbers in the absence of PPLO. In spite of the small difference between the means of the plaque numbers obtained with and without kanamycin, it is found to be significant ($t = 3.2$; $0.05 > p > 0.02$).

Therefore, pretreatment of the KB cell cultures by kanamycin prior to virus infection enhances plaque formation by adenovirus type 5. This implies that the kanamycin may have opened up new adsorption sites

for the virus, thereby increasing the eop of the virus.

Consequently, in all further work, kanamycin was incorporated into both the growth and overlay media at 500 µg/ml. and 100 µg/ml. respectively, in the plaque assay of adenovirus, for both KB cell strains 1 and 2.

e) The influence of the solidifying agents on plaque formation

Autoclaved agar is known to contain sulphated polysaccharides which inhibit plaque formation by poliovirus for example (Liebhaber and Takemoto, 1964), but apparently not adenovirus (Kjellen, 1963). Agarose, on the other hand, does not contain the sulphated polysaccharides (Hjerten, 1968), and thus it is used in place of agar for the assay of viruses which are sensitive to sulphated polysaccharides (Bergold and Mazzali, 1968).

Since Kjellen (1963) provided no evidence to support his claim that adenoviruses are not sensitive to agar inhibitors, experiments were carried out to investigate the problem. In the first experiment, autoclaved Noble agar was compared with autoclaved agarose as the solidifying agent in the overlay.

KB cell (strain-1) monolayers were infected with virus as described in Materials and Methods. They were divided into four groups which were layered with four different overlays. One was the medium prepared according to Appendix B, which contained 0.6% Noble agar. In the other three media, the 0.6% agar was replaced with either 1% Noble agar or 0.4% agarose or 0.7% agarose.

The results in Table XII show that the 0.6% Noble agar allowed

TABLE IX

THE EFFECTS OF ANTIBIOTICS IN THE GROWTH MEDIUM ON PLAQUE FORMATION BY AD 5 IN KB CELLS (STRAIN-1)

Experiment No.	Days after infection	Kanamycin 500 µg/ml.	Penicillin (100 IU/ml.) Streptomycin (50 µg/ml.)	No antibiotics
1	16	49*	18	nd
	18	71	41	nd
2	20	102	79	70
	27	112	82	76

*Average plaque numbers of three replicates.

nd - not done

TABLE X

THE EFFECTS OF KANAMYCIN IN THE OVERLAY ON PLAQUE FORMATION BY AD 5 ON KB CELL STRAIN-1

Days after infection	Kanamycin (µg/ml.)		Penicillin (100 IU/ml.) Streptomycin (50 µg/ml.)
	100	500	
15	116*	94	87
23	157	132	133

*Average plaque counts of four replicates.

TABLE XI

THE EFFECTS OF KANAMYCIN IN THE GROWTH MEDIUM ON THE PLAQUE FORMATION OF AD 5 ON KB CELL STRAIN-2

Antibiotic in growth medium	Plaque counts	Average plaque counts on day 10
Kanamycin*	18, 18, 20	19
Penicillin - Streptomycin	13, 10, 16	13

*500 µg/ml.

the formation of most plaques. On the other hand, 1% Noble agar, 0.4% agarose and 0.7% agarose demonstrated inhibitory effects on plaque formation when compared to 0.6% Noble agar.

In another attempt to show the influence of the inhibitory substance in agar on adenovirus plaque formation, DEAE-dextran, which has a neutralizing action on sulphated polysaccharides (Takemoto and Fabisch, 1963), was added to the Noble agar to make a final concentration of 10.7 mg./ml. This was one of the effective concentrations used by Takemoto and Fabisch for neutralizing the sulphated polysaccharides in the study of plaque formation by influenza A2 and B viruses. The experiment was performed with KB cell (strain-2) monolayers. The results showed that there was no difference between the plaque numbers obtained in media with DEAE-dextran and those without. This is in agreement with the finding of Kjellen (1963).

f) The cumulative influence of the optimum features for the plaque assay of adenovirus.

In the attempt to obtain an optimum overlay for the plaque assay of adenovirus, the foregoing results have demonstrated the facts that the incorporation, into the agar overlay, of 10% horse serum, 100 µg/ml. kanamycin, and the use of 0.6% Noble agar allowed the formation of either more or larger plaques or both, than the other conditions tested. In addition, one supplement to Eagle's MEM, proteose peptone, allowed the production of almost identical plaque numbers as that obtained with all the three supplements.

In the following experiment, the cumulative effects of the optimum features in the plaque formation of Ad 5 were examined. Ten plates

TABLE XII

THE EFFECTS OF THE SOLIDIFYING AGENTS ON PLAQUE FORMATION

Solidifying agents	Average plaque counts on day 16 of three replicates
0.6% Noble-agar	31
1.0% Noble-agar	7
0.4% Agarose	10
0.7% Agarose	11

of KB cell (strain-1) monolayers were infected with a single dilution of virus and divided into two groups. One group was overlaid with the optimal medium which was prepared according to Appendix B, and the other group was overlaid with the initial medium prepared according to Appendix C. The initial overlay contained proteose peptone, lactalbumin hydrolysate, tryptose phosphate broth, 15% horse serum, penicillin-streptomycin, and 1% Noble agar; whereas the optimal overlay contained proteose peptone, 10% horse serum, kanamycin, and 0.6% Noble agar. Kanamycin was used in the growth medium for all the plates. The results in Table XIII show that there was a 3-fold increase in plaque numbers when the optimal overlay medium was used.

The increase in sensitivity can be attributed then to a combination of the effects of reducing the serum concentration, and the agar concentration, and to the incorporation of kanamycin in the overly medium.

3. The influence of neutral red on plaque assay

Neutral red is known to inactivate both virus and cells photodynamically (Klein and Goodgal, 1959), i.e. the dye inactivates virus and cells only in the presence of visible light and not in its absence. This is the reason for the common practice of withholding neutral red until maximum plaque numbers can be expected. The effects of neutral red on the adenovirus-KB cell system have not previously been studied in depth. Therefore, the following experiments were designed in order to elucidate the photodynamic inactivating effects of neutral red on the host cells, on the virus and on the host cells' ability to support virus multiplication.

In the first experiment, the effects of different concentrations

TABLE XIII

EFFECTS OF THE OPTIMAL OVERLAY AND THE INITIAL OVERLAY ON PLAQUE FORMATION

Overlay medium	Plaque numbers	Plaque diameter (mm.)	
		Average	Range
Appendix C (Initial)	65*	0.3@	0.1-0.5
Appendix B (Optimal)	185	1.0	0.5-1.2

*Average plaque numbers of five replicates on day 16.

@Average plaque diameter of ten measurements on day 16.

of neutral red on plaque formation were examined. Fifteen plates of KB cell (strain-1) monolayers were infected with a single dilution of virus and divided into five groups. Each plate was overlaid with the optimal medium which was prepared according to Appendix B. Ten days later, the neutral red overlay was added to each plate, but each of the five groups received a different concentration of neutral red as shown in Table XIV.

Since Gochenour and Baron (1959) reported the development of resistance of cell cultures to photodynamic inactivation if the cells were kept in total darkness for 24 hours before being exposed to light, the cell cultures, with neutral red incorporated, were kept in the CO_2 incubator, i.e. in the dark, for a minimum of 24 hours before being examined in the light for plaques.

The results in Table XIV show that the different concentrations of neutral red, from 1/125,000 to 1/50,000, did not cause any significant differences in the plaque numbers or in the rate of development of the plaques. However, when the neutral red concentration was increased to 1/31,000, the KB cells were killed; and at the lower concentrations of 1/125,000 and 1/84,000, the KB cells were very faintly stained and consequently the plaques were not as prominent as those with neutral red concentrations of 1/62,500 and 1/50,000.

Therefore, the optimum neutral red concentrations are 1/62,500 and 1/50,000, for the plaque assay of adenovirus in the KB cell strain-1.

The second experiment was designed to determine the optimum time for addition of the neutral red overlay, in terms of plaque numbers and cell survival. Since neutral red inhibits plaque formation when incorporated in the first overlay (Hiatt, 1960), it is proposed that the optimum

time for the addition of neutral red should be as close as possible to the time of plaque appearance. After adsorption of virus, the infected KB cell (strain-1) monolayers were overlaid with medium prepared according to Appendix B and divided into three groups. On day 7, 5 ml. of neutral red overlay was added to one group of plates; on day 10, neutral red overlays were added to another group of plates; and on day 13, neutral red overlays were added to the third group of plates.

The results in Table XV show that more plaques were present in the plates when neutral red was added on day 10. Also the plaques were still clearly visible 30 days after infection. When neutral red was added on day 7 or day 13, fewer plaques were formed. These plaques faded by 30 days, through failure of the uninfected cells to retain the dye. Since only one concentration of neutral red (1/62,500) was used, factors other than the effects of neutral red are involved here.

In the 13th day plates, the agar overlay, with nutrients, may have been added too late, i.e., the nutrients in the first overlay may have become depleted before fresh medium was added. This may have deleterious effects on the cells. In the day 7 plates, neutral red may be exhibiting its lethal action.

Work done by Hiatt in 1960 showed that adenoviruses are susceptible to photodynamic inactivation by neutral red. The foregoing experiment suggests that additional fresh medium is required 10 days after the first overlay, with the KB cell strain-1. Therefore, the application of both these findings may allow the formation of either more plaques or larger plaques or both.

Plaques formed by Ad 5 on KB cell (strain-1) monolayers were

first seen on day 13 when the neutral red overlay was added on day 10. The purpose of the following experiment was to determine whether the addition of neutral red after plaques have formed allows the formation of either larger plaques or more plaques.

Nine plates of KB cell (strain-1) monolayers were infected with a single dilution of virus and divided into three groups. After infection of the KB cell monolayers, they were overlaid with medium prepared according to Appendix B. On day 10, 5 ml. of fresh overlay, similar to the first and without neutral red, was added to each plate as a source of nutrients.

Immediately after the agar had solidified, 1 ml. of overlay containing neutral red at 10 times the usual concentration (i.e., 1/6,250)was added to each plate of one set of plates. This was repeated for the second set on day 16. The extra concentration of neutral red was required in order to compensate for the smaller volume.

The results in Table XVI show that the delayed incorporation of neutral red allowed the formation of both more plaques and larger plaques. Therefore, neutral red inhibits plaque formation if added before plaques are formed, i.e., on day 10 instead of on day 16.

The foregoing experiment raises two significant problems relating to the action of neutral red on virus multiplication. The neutral red may either have inactivated the virus or it may have reduced the ability of the KB cells to support the multiplication of the virus. In either case, one would expect a reduction in both plaque size and numbers.

The direct inactivating effect of neutral red on purified Ad 5 was examined. The virus was purified by differential centrifugation

TABLE XIV

EFFECTS OF DIFFERENT CONCENTRATIONS OF NEUTRAL RED ON ADENOVIRUS PLAQUE FORMATION

Days after infection	Neutral red concentration				
	1/125,000	1/84,000	1/62,500	1/50,000	1/31,000
18	34*	39	40	40	cd
21	45	50	49	56	cd
23	48	52	50	57	cd

*Average plaque counts of three replicates.

cd - cells dead.

TABLE XV

THE EFFECTS OF NEUTRAL RED ON PLAQUE FORMATION:
THE TIME OF ADDITION OF THE SECOND OVERLAY

Days after infection	Neutral red (1/62,500) overlay added after		
	7 days	10 days	13 days
16	0	31*	0
17	11	46	25
21	11	64	58
30	faded	65	faded

*Average plaque counts of 3 replicates.

using the Beckman Ultracentrifuge Model L2-65B. Eleven ml. of the stock virus was pipetted aseptically into a sterile cellulose nitrate tube. The tube was placed into a type 65 fixed-angle rotor and was centrifuged at 57,405 g for 1½ hours. The supernatant was discarded. The pellet was resuspended in 12 ml of Eagle's MEM with 2% horse serum. An hour was allowed for the fluid to absorb into the pellet and so make resuspension easier. The suspension was mixed thoroughly and centrifuged at 1,590 g for a half-hour to pellet particulate debris. The sediment was discarded. The supernatant was transferred to another sterile tube and centrifuged a second time at 57,405 g for 1½ hours to pellet the purified virus. The pellet of pure virus was resuspended in ¼ of the original volume, i.e., approximately 3 ml. of Eagle's MEM with 2% horse serum. The purified virus suspension was stored frozen at -20°C. until used in the following experiment.

Neutral red, at the two concentrations of 1/30,000 and 1/15,600 was mixed with the stock virus in equal proportions. One set was kept in darkened test tubes placed in a light tight box and the other set was exposed to light, six inches away from a 15 watt cool white fluorescent lamp (General Electric). The tubes were illuminated from one side only. Both mixtures were incubated for one hour at the measured room temperature of 23°C., after which they were placed in a light tight box and frozen at -20°C. until they were titrated. Each of the virus-neutral red mixtures was titrated using the procedure as described in Materials and Methods. After infection, the KB cell (strain-1) monolayers were overlaid with medium prepared according to Appendix B and neutral red was incorporated on day 16. The entire experiment was performed in a darkened

TABLE XVI

THE EFFECTS OF THE TIME OF INCORPORATION OF NEUTRAL RED ON PLAQUE FORMATION

Days after Infection	Neutral red (1/62,500) incorporated after			
	10 days		16 days	
	Plaque numbers	Plaque diameter in mm.	Plaque numbers	Plaque diameter in mm.
17	109*	nd	137	nd
21	129	0.8[@] (0.5-1.0)	147	2.8 (2-3.5)

*Average plaque counts of 3 replicates.

[@]Average plaque diameter of ten measurements.

room to minimize the effects of light.

The results showed that Ad 5 was inactivated by both concentrations of neutral red, but only when exposed to light. The virus-neutral red mixtures that were not exposed to light did not show any sign of virus inactivation when compared to the controls without neutral red.

The object of the following experiment was to show the kinetics of photodynamic inactivation by neutral red at 1/60,000 concentration. Five separate mixtures of equal parts of virus and 1/30,000 neutral red were made. They were exposed to light as described in the above experiment. At each interval of exposure as indicated in Figure 5., a mixture was placed in a darkened box and frozen at -20°C. After all the five mixtures were frozen, they were then thawed, diluted and assayed in duplicate. No control, i.e., without neutral red, was included. The entire assay procedure was performed in a darkened room to minimize photodynamic inactivation during the period. The plaques were counted on day 20. As shown in Figure 5., the adenovirus was inactivated photodynamically by the neutral red very rapidly.

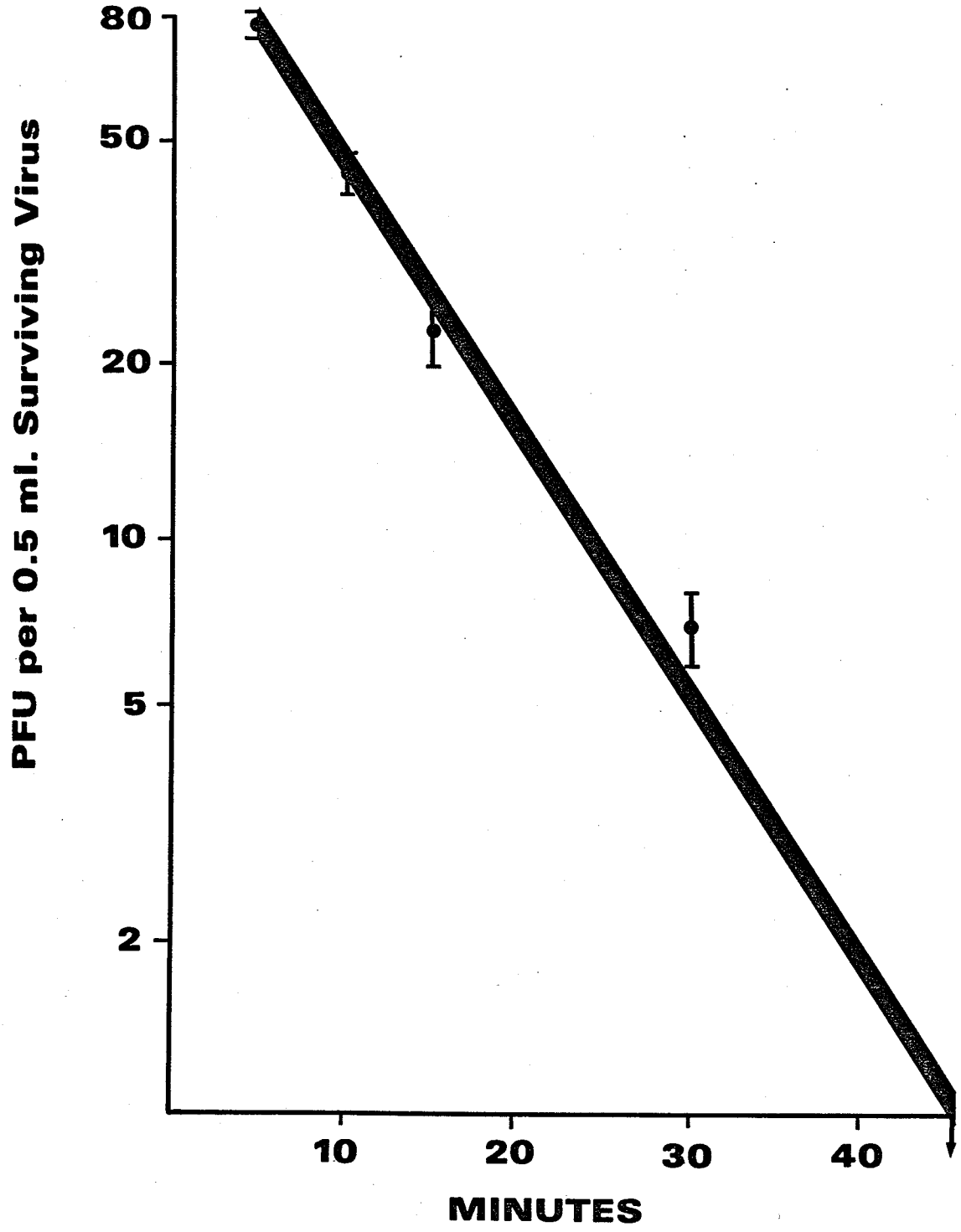
The experiments above demonstrate that Ad 5 is not inactivated by neutral red in the dark but it is very rapidly inactivated in the light. However, it is not known if the KB cells support the multiplication of the virus in the presence of neutral red, even if kept in the dark. Therefore, the objective of the next experiment was to elucidate the effects of neutral red on adenovirus multiplication in KB cells in the dark.

Petri dishes were seeded with 5×10^5 KB cells (strain-2). They were incubated overnight to allow for cell attachment to the plates. The

Figure 5. The photodynamic inactivation of type 5 adenovirus by neutral red (1/60,000) as a function of time.

Each point represents the average plaque count of two replicates.

The bars represent the range.


80
50
20
10
5
2
PFU per 0.5 ml. Surviving Virus
10
20
30
40
MINUTES

virus was diluted to contain $5x10^6$ PFU/ml. Each of the KB cell cultures was infected with 1 ml. of the virus for 1 hour at 37°C., i.e. the input multiplicity was approximately 10 PFU/cell.

Growth medium prepared according to Appendix A, containing 10% horse serum instead of calf serum, was added to two plates; and the same medium preparation with neutral red at 1/62,500 concentration was added to the other two plates. They were incubated at 37°C. in a CO_2 incubator, in the dark.

After 32 hours, two plates, one with neutral red and the other without, were placed in a light-tight box and frozen at -20°C. After 60 hours, the other two plates were also frozen. All plates were subjected to three cycles of freezing and thawing in order to release the virus from the KB cells. The suspensions were centrifuged in separate tubes at 140 g for a half-hour to pellet the cell debris. The supernatants containing the virus were assayed as described in Materials and Methods using KB cell strain-2. Note that all the work for this experiment was done in the dark to prevent photodynamic inactivation of the virus by the neutral red.

The results in Table XVII demonstrate that the yield of virus in the presence of neutral red was reduced by a factor of 10^4, in comparison with the control. Since the maximum titers of intracellular virus growth for adenovirus are attained at 28-40 hours after infection (Strohl and Schlesinger, 1965a), the possibility that the neutral red may delay instead of inhibit virus multiplication was ruled out since the 60 hour samples also show a wide difference in virus yield from the KB cell cultures between the two conditions.

TABLE XVII

THE EFFECTS OF NEUTRAL RED ON VIRUS MULTIPLICATION

Time for virus growth	Virus Yield (PFU/ml.)	
	With neutral red (1/62,500)	No neutral red
32 hours	$1x10^4$*	$1.5x10^8$
60 hours	$4.8x10^4$	$2.8x10^9$

*The mean of two assay plates.

The foregoing experiments demonstrate that neutral red inhibits plaque formation by two mechanisms, namely, photodynamic inactivation of virus and the inhibition of virus growth. Therefore, to achieve the highest possible eop, neutral red is best added after plaques have formed.

4. The influence of pH of the overlay on plaque formation.

Cooper (1961) stated that the pH of the overlay for plaque formation by polioviruses is generally not critical between the range of 6.8 and 7.8, but it is well known that certain attenuated poliovirus mutants plaque with reduced efficiency under an acid overlay (Vogt et al. 1957). However, there have been no reports on the sensitivity of the adenovirus type 5 to pH variations in the agar overlay. Therefore, the following experiments were designed to study the effects of variations in the pH of the agar overlay on the plaque formation by adenovirus.

The pH of the overlay was buffered by sodium bicarbonate and a continuous flow of CO_2. Two experiments of similar design were done, one with KB cell strain-1 and the other with KB cell strain-2.

Eagle's MEM prepared according to Appendix B, but without the agar, was made. Sodium bicarbonate (0.5M) was used to adjust the pH of the media electrometrically to the values listed in Tables XVIII and XIX. The agar overlays for the following two experiments were prepared by adding the predetermined quantities of sodium bicarbonate in order to obtain the pH values listed in the Tables. The agar portion was mixed with the fluid portion of the overlay medium just prior to use.

KB cell (strain-1) monolayers were infected with identical aliquots of the same virus dilution. After adsorption, the infected

plates were overlaid with medium adjusted to the pH values shown in Tables XVIII and XIX.

The results of the first experiment (Table XVIII) show that most plaques were formed at pH 7.1. At pH 7.3, fewer plaques were formed, but the plaque size was almost three times larger than at pH 7.1

When the experiment was repeated with KB cells of strain-2, and a wider range of pH values (Table XIX), the optimum plaque numbers were obtained with an initial pH of 7.2, with a gradual decrease in both plaque numbers and plaque size at lower pH values. However, plaques were largest when the initial pH was 7.5. The KB cells were killed at pH 7.8 and so no plaques could be detected.

The two experiments show one common feature, viz., the pH optimum for plaque numbers does not coincide with the pH optimum for plaque size.

Therefore, it is important, in the plaque assay of adenovirus, to standardize the pH of the agar overlay in order to obtain repeatable results, and to ensure that the initial pH of the overlay is at 7.1 or 7.2, in order to obtain the highest eop under these conditions.

5. The influence of adsorption conditions on plaque formation.

a) Kinetic aspects of virus adsorption to KB cells.

The rate of adenovirus adsorption to KB cells in culture has been determined by Kjellen (1961) and Philipson (1961). However, the strains of adenovirus and KB cell cultures in this laboratory may behave differently due to differences in the susceptibility of the cells to virus. Therefore, it was the purpose of the following experiment to

TABLE XVIII

THE EFFECTS OF pH OF THE OVERLAY MEDIUM ON PLAQUE FORMATION (Experiment-1)

pH	Plaque numbers	Plaque diameters in mm.	
		Average	Range
7.1	60*	1.2@	0.5-2
7.2	44	nd	nd
7.3	31	3	2-4

*Average plaque numbers of three replicates on day 23.

@Average plaque diameter of ten measurements on day 23. KB cell stain-1

TABLE XIX

THE EFFECTS OF pH OF THE OVERLAY ON PLAQUE FORMATION (Experiment-2)

pH of overlay	Plaque numbers	Plaque size	Range
6.7	6*	0.9 mm.@	0.5-1.2 mm.
6.9	10	nd	nd
7.0	13	1.2 mm.	0.5-1.5 mm.
7.1	15	nd	nd
7.2	22	2 mm.	1-2.5 mm.
7.5	16	3 mm.	2-3.5 mm.

*Average plaque numbers of three replicates counted on day 14.

@Average plaque diameters of 10 measurements on day 14.

nd - not done
KB cell strain-2

study the rate of adsorption of Ad 5 to KB cell (strain-1) monolayers.

The virus suspension was diluted, in virus diluent at pH 7.1, to a predetermined concentration that was known to contain between 20-100 PFU/0.5 ml. A virus inoculum of 0.5 ml. was added to each of several plates of preformed KB cell (strain-1) monolayers containing approximately $4x10^6$ cells. The time of adsorption was varied from a ½ hour to 3 hours in one-half hour intervals. The monolayers were washed twice with 3 ml. PBS after removal of the virus inoculum. The agar overlay was prepared according to Appendix B and the plaque assay was done as described in Materials and Methods. Neutral red was added on day 10.

The results in Figure 6 show that the maximum virus adsorption was achieved after about 3 hours and that more than half of the virus was absorbed in 1½ hours.

For the comparison of infectivity of two or more virus samples, the maximum adsorption time of 3 hours may not be necessary, but if the maximum eop is desired, the results of this experiment indicate that it is essential to allow an adsorption time of at least 3 hours.

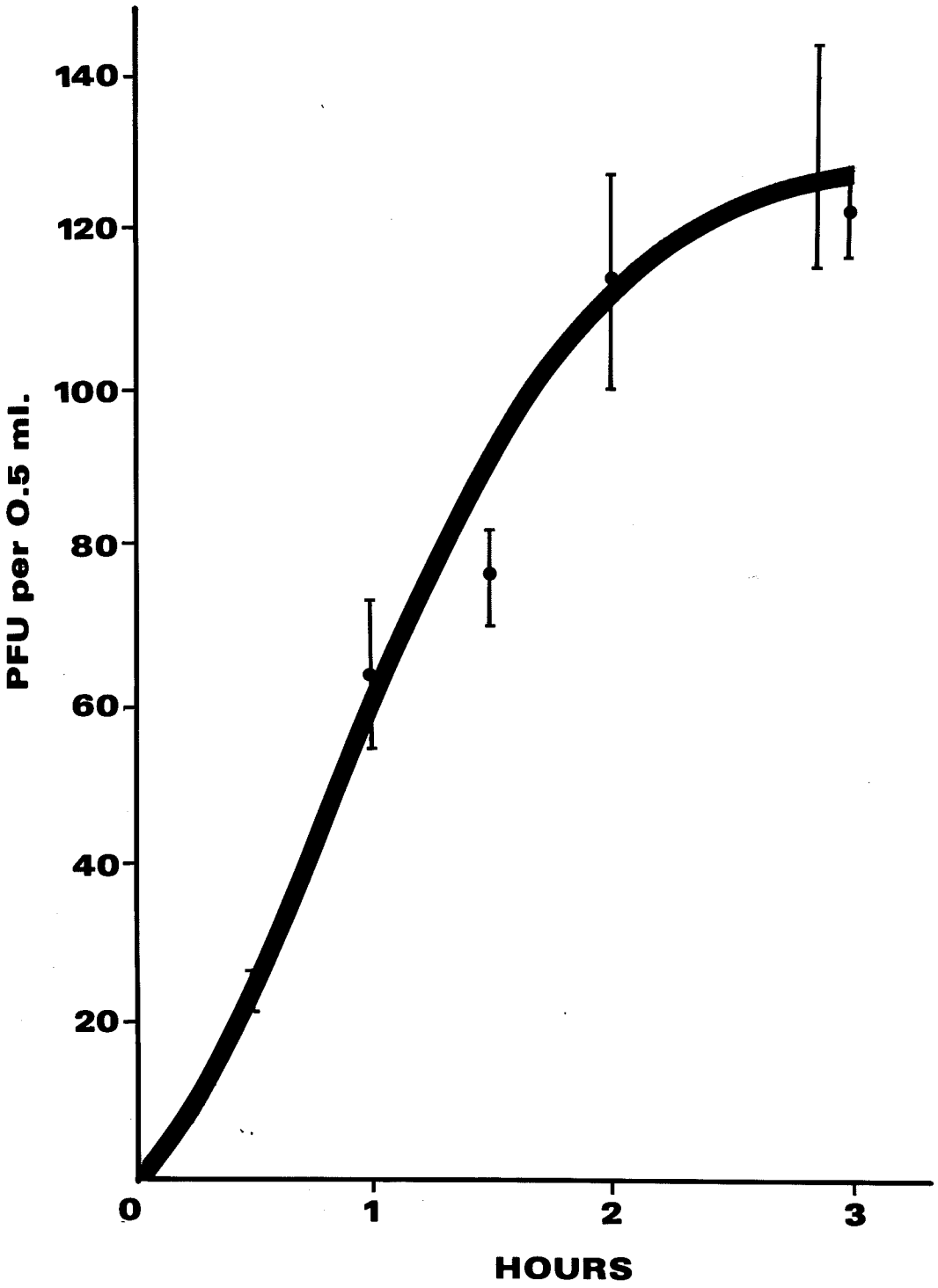
b) The effects of pH of the virus diluent on virus adsorption.

The purpose of the following experiment was to determine whether pH of the adsorption fluid affects the adsorption of adenovirus type 5 to KB cells. The virus was diluted in virus diluent which was adjusted to pH 6.6, 6.8, 7.0, 7.2 and 7.5. The virus diluent normally used was buffered at pH 7.1. To obtain these pH values, virus diluent at pH 7.0 was titrated with either KH_2PO_4 (2 mg./ml.) or Na_2HPO_4 (11.5 mg./ml.) to the desired pH, electrometrically. The KB cell (strain-2) cultures

Figure 6. Adsorption of adenovirus type 5 to KB cells as a function of time.

Each point represents the average plaque count of three replicates.

The bars represent the range.


140
120
100
80
60
40
20
PFU per O.5 ml.
0
1
2
3
HOURS

were washed with the respective buffers before they were infected with the virus. An adsorption time of 1½ hours was used.

The results in Table XX show that the pH of the adsorption fluid, within the pH range of 6.6 and 7.5, had no significant effect on the adsorption efficiency of Ad 5 in terms of plaque numbers. The difference in plaque numbers at pH values of 6.6 and 6.8 may be due to experimental error but since no further work was done, a definite conclusion cannot be made.

c) The effects of DEAE-dextran on virus adsorption.

DEAE-dextran has been shown to enhance the adsorption to cells not only of infectious nucleic acids, e.g. poliovirus RNA (Pagano and Vaheri, 1965), but also of virions, e.g. poxvirus (Kim and Sharp, 1969).

The aim of this experiment was to determine if DEAE-dextran affects the adsorption of adenovirus to KB cells in culture. DEAE-dextran, dissolved in PBS and sterilized by autoclaving, was mixed with an equal volume of diluted virus suspension, to make a final concentration of 25 mg./ml. (Kim and Sharp, 1969). The control consisted of virus suspension diluted similarly in an equal volume of sterile PBS. The virus preparations were inoculated onto KB cell (strain-2) monolayers and allowed to adsorb for 1½ hours. The neutral red overlay was added 7 days after infection and the overlay medium was prepared according to Appendix B.

The DEAE-dextran was found to inhibit virus adsorption as shown by the results (Table XXI).

Therefore, in contrast to the effects on poxvirus the adsorp-

TABLE XX

THE EFFECTS OF pH OF THE ADSORPTION FLUID ON VIRUS ADSORPTION

pH of adsorption fluid	Average plaque numbers of 2 replicates on day 10
6.6	22
6.8	15
7.0	19
7.2	19
7.5	17

tion of adenovirus type 5 is inhibited by DEAE-dextran at the concentration used. However, the mechanism of the inhibitory action of DEAE-dextran on adenovirus adsorption is unknown and no attempt was made to elucidate the mechanism.

III. A COMPARISON OF PLAQUE FORMATION BY DIFFERENT ADENOVIRUS SEROTYPES

Adenoviruses are divided into four subgroups according to the hemagglutination properties (Rosen, 1958). This classification shows a close correlation to the classification according to the plaque size of adenoviruses (Green, 1967) and also to many other biological properties (Schlesinger, 1969). With the exception of the highly oncogenic serotypes 12, 18 and 31, adenoviruses of the HA subgroups I, II and III form small, intermediate and large plaques, respectively.

The purpose of the following experiment was to compare the characteristics of the plaques formed by different adenovirus serotypes. Adenoviruses of types 3, 8 and 12, representative of subgroups I, II and IV, respectively, were selected for comparison with type 5 (subgroup III).

Ten-fold dilutions of the adenovirus types 8, 3, 5 and 12 were assayed using KB cell (strain-2) monolayers as described in Materials and Methods. The agar overlay was prepared according to Appendix B and neutral red was added on day 14. The time of neutral red addition was varied from the usual mode because the plaques were bigger and more numerous if neutral red was added shortly before the maximum plaque numbers were reached, i.e., day 15. The plates with discrete plaques were used for the measurement of plaque diameters. Plaques for adenovirus

TABLE XXI

THE EFFECTS OF DEAE-DEXTRAN ON VIRUS ADSORPTION

Days after infection	Virus+DEAE-dextran	Virus+PBS
8	6*	14
10	10	18

*Average plaque numbers of 2 replicates.

types 5, 3 and 12 were seen on day 15 and the plaques of type 8 were seen, pin-point in size, on day 20, as shown in Table XXII. The plaque numbers of type 5 did not increase after they were seen on day 15, but the plaques gradually increased in diameter from an average of 3.2 mm. on day 15, to 8 mm. on day 29. The plaques of type 3 increased in diameter from an average of 1 mm. on day 15 to 3 mm. on day 29. Figure 7 shows the plaque characteristics of adenovirus types 3 and 5 as seen on day 29. The plaques of types 8 and 12 were pin-point in size on day 29 and good photographs were not obtainable. It was noted that the plaques of types 8 and 12 were clearer on day 29 than on day 19, but measurable increase in plaque diameter was not observed. The plaque numbers for Ad 3, 8 and 12 were not recorded.

A similar experiment was repeated for KB cell strain-1, but comparing types 5, 3 and 12 only. The neutral red overlay was added on day 16. The plaques of types 5 and 3 appeared on day 15, but those of type 12 did not appear until day 19. However, there were two significant differences observed with this strain of KB cells. The number of plaques of type 5 adenovirus increased from 10 on day 15 to 73 on day 26, whereas there was no increase in plaque numbers after day 15 when KB cell strain -2 was used, as noted above. The average plaque diameters of the Ad 5, 3 and 12 were 2.8 mm., 0.9 mm. and 0.5 mm. respectively, on day 26.

From the results of these two experiments, it is obvious that the plaque characteristics of any given virus are significantly different in different KB cell strains. Therefore, it is important, when comparing the plaque characteristics of viruses to use the same strain of cultured cells. Otherwise, any given adenovirus may be deemed different due to

the differences in the plaque characteristics obtained. Also, these conditions for plaque assay allow detection of each of the four adenoserotypes examined. Though optimal for type 5 virus, they may not be so for the other three serotypes.

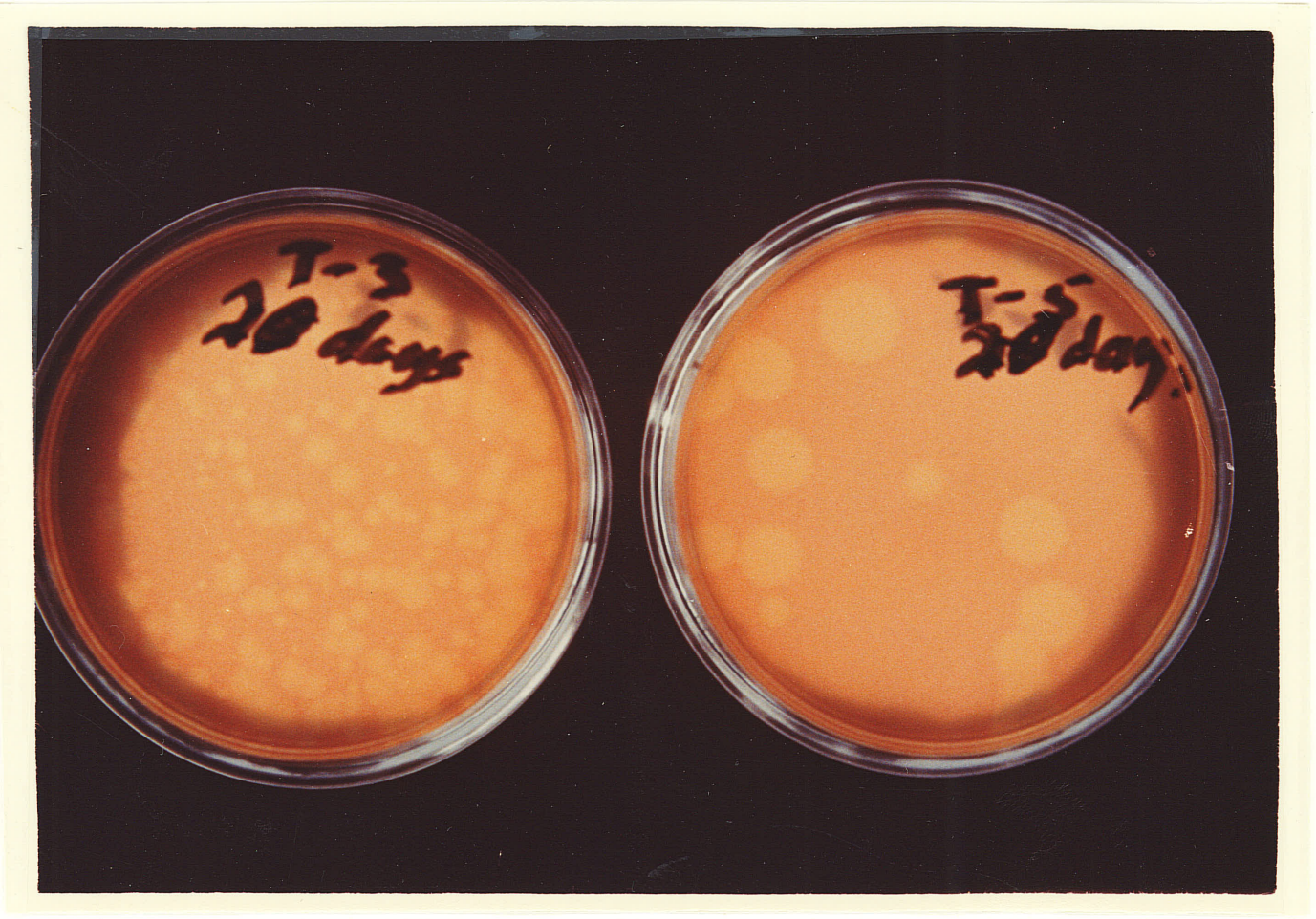


Figure 7. The plaque characteristics of adenovirus types 3 (left) and 5 (right) in KB cell (strain-2) monolayers, 29 days after infection.

TABLE XXII

COMPARISON OF THE PLAQUE CHARACTERISTICS OF ADENOVIRUS TYPES 5, 3, 8 and 12

Days after infection	Average plaque diameters in mm.			
	Type 5	Type 3	Type 8	Type 12
15	3.2* (1-5)	1 (0.5-1.2)	no plaques	pin-point
20	5 (2-8)	2 (0.5-2.5)	pin-point	pin-point
29	8 (5-11)	3 (1.5-3.5)	pin-point	pin-point

*Average of ten measurements.

KB cell strain-2 used.

DISCUSSION

The main theme of this research concerns the elucidation of some of the problems related to the plaque assay of adenovirus and the development of a plaque assay procedure that allows the production of consistent and reproducible results.

As shown by the experiments with proteose peptone, the initial failure to obtain plaques with Eagle MEM appeared to be chiefly due to nutritional factors. As a result of systematic investigation of several factors, a comparatively sensitive system has been developed for the assay of type 5 adenovirus.

Cooper (1967) listed the following criteria and requirements for a reproducible plaque assay:-

1. There must be no plaques in the absence of virus.
2. The characteristic virus must be regularly re-isolated from a plaque in far higher concentration than from areas away from a plaque.
3. Plaques must follow a linear relationship with dose, i.e., the plaque count must be proportional to virus concentration, at least over a range within statistical practicability.
4. Plaques should be spread among cultures of one batch according to a Poisson distribution.
5. Plaque initiation must be inhibited by low concentrations of heated specific antiserum that will not inhibit growth of another virus in the same cell system, and not be inhibited by normal serum of the same source species.
6. Repeated assays of a preserved virus stock in cultures of different

DISCUSSION

batches should give the same titer.

As has been shown by the results, all of the criteria above, except number 2, have been demonstrated for the plaque assay system for Ad 5.

In regard to criterion 6, it was shown (see Table III) that reproducible results can be obtained, provided the assay conditions are rigidly controlled, most likely the pH of the overlay.

Plaque counts in general, are heavily biased by overlapping as plaque number is increased. Howes (1969) described the methods for correcting overlap bias of plaque counts which yield more reliable estimates of the numbers of plaque-forming units actually present in the samples. Nevertheless, as pointed out by Howes, the need of the overlap bias correction will depend on the magnitude of these biases and the level of precision required. They are unnecessary where plaques are very small relative to culture area and where they are also regular and clearly defined. The plaques of adenovirus fall in the former category. Therefore, the overlap bias correction is unnecessary. To further reduce the overlap bias, the plaque numbers should be in the lower third to half of the possible counting range, which terminates as cultures approach semi-confluency (See Table II).

Investigations on the effects of serum on plaque formation show that the type, concentration and batch of serum affect the formation of plaques to a significant degree. Aldcorn (1966) reported that none of several fetal calf serum samples were inhibitory to adenovirus growth. However, it was found that horse serum allowed the production of more plaques which were also larger than those obtained with fetal calf

serum, at a similar concentration. On the other hand, a different batch of fetal calf serum was found to be non-inhibitory for plaque formation by Ad 5 as shown in Table VIII. However, by lowering the concentration of horse serum from 15% to 10%, there was a significant increase in the number of plaques. This suggests that inhibitors for Ad 5 are present in at least some samples of fetal calf serum and also in horse sera, possibly less in the horse serum. This result is in agreement with the findings of Philipson (1961) who showed that, with chick and calf sera, there was a corresponding reduction in plaque numbers with increasing serum concentrations. Anti-viral inhibitors may be present in the batches of sera that were inhibitory to plaque formation. Consequently, regardless of the type of serum all batches of serum used for plaque assay should be routinely pre-tested for virus inhibitory properties.

There are two aspects regarding the nutrient requirements for plaque formation by adenoviruses. On the one hand, some nutrients are required for adenovirus growth; and on the other hand, some nutrients are essential for maintenance of KB cell viability under an agar overlay.

It has been shown that of all the amino acids contained in Eagle's basal medium, only arginine is absolutely required for the synthesis of infectious Ad 2 virus (Rouse and Schlesinger, 1967). However, experiments with plaque formation by Ad 5 show that extra arginine added to Eagle's MEM did not provide the nutrients necessary for the survival of the KB cells under the agar overlay. This does not imply that there were insufficient nutrients for virus growth. Instead, proteose peptone was found to be absolutely necessary to maintain the KB

cells in a viable state during the long incubation period required for plaque formation by adenovirus. It was also found that the plaque promoting factors in proteose peptone were dialysable. The KB cell (strain-2) cultures were viable under an overlay containing dialyzed proteose peptone, but there were fewer plaques, compared with plaques obtained under an overlay containing undialyzed proteose peptone. This implies that the dialysable small molecular weight peptides or amino-acids were responsible for enhancing virus growth and the undialysable large molecular weight substances were responsible for maintaining the viability of the KB cell cultures under the conditions of the plaque assay of adenovirus. It should be noted that in the absence of agar, proteose peptone is not necessary either for the growth of adenovirus or for maintenance of cell viability. However, the exact nature of the plaque promoting factor in proteose peptone is still unknown.

It is a commonly observed phenomenon that autoclaved agar, which contains sulphated polysaccharides, inhibits plaque formation of EMC and ECHO viruses (Liebhaber and Takemoto, 1961). In contrast, experimental evidence from this report demonstrates that autoclaved agar does not inhibit plaque formation by adenovirus type 5 compared to either agarose or Noble agar+DEAE-dextran. This is indirect evidence that sulphated polysaccharides in agar do not inhibit Ad 5 plaque formation, which confirms Kjellen's (1963) finding. Wild type poliovirus is also not affected by autoclaved agar (Normura and Takemori, 1960). However it is shown that a reduction in the concentration of Noble agar from 1% to 0.6% greatly enhances the plaque numbers of Ad 5. Similarly, a reduction in the concentration of agarose prolonged the viable state of the KB cells

under the agarose overlay. However, the mechanism which results in plaque enhancement when concentrations of agar and agarose are lowered is not clear and may be due to one or more of the following: (1) a reduction in concentration of the solidifying agent concurrently reduces the viscosity which may be accompanied by an increase in the oxidative metabolism of the cells, thereby allowing the multiplication of some virus variants that are more sensitive to low oxygen tensions. This was demonstrated for NDV by Baron et al. (1961). Though adenoviruses are not known to be dependent on the oxidative metabolism of the cells, this is not excluded as a possibility. (2) The lowering of the agar concentration may also be accompanied by a reduction in the concentration of some other undefined virus inhibitors present in both agar and agarose.

Therefore, the lowest concentration of agar that forms a firm gel at the incubation temperature of 37°C. should be used in a plaque assay in order to obtain the highest possible eop.

Another factor which is generally found to inhibit plaque formation is the neutral red dye. The data presented show that neutral red (1/62,500) rapidly inactivates Ad 5 photodynamically, which conforms in general to the findings of Hiatt (1960). The neutral red also drastically reduces the capacity of the KB cells to support the growth of adenovirus in the dark.

The inactivating action of neutral red is, therefore, not restricted to the photodynamic mechanism alone, as is generally believed. Since the neutral red prevents the development of adenovirus in the dark, the spread of the virus, i.e., the reinfection of the surrounding cells, is also expected to be reduced considerably, if not halted altogether.

On the basis of the results, it is suggested that very little virus development takes place after neutral red is incorporated, and hence the later increase in number and size of plaques represents solely the response of the cells to pre-existing infection. Since dead KB cells lose the ability to retain the neutral red dye, the focus of infected KB cells is ultimately killed, and upon release of the dye, is seen as a plaque.

Experiments clearly demonstrate that the pH of the virus diluent, within the range of 6.6 to 7.5, does not affect the adsorption efficiency of Ad 5. However, it was found that the presence of DEAE-dextran in the adsorption fluid inhibits plaque formation, which indirectly indicates that the dextran inhibits virus adsorption. Since only one experiment was performed, the results need cautious interpretation. This is contrary to its effects on poxvirus (Kim and Sharp, 1969). The mechanism of action is presently unknown.

Results demonstrate that the presence of kanamycin in the growth medium enhances plaque formation in relation to plaque numbers as shown in Table IX. This is indirect evidence that the adsorption of Ad 5 is enhanced when the KB cell monolayers are treated with kanamycin prior to infection with virus. Initially, the enhancing action was thought to be due to the inhibitory effects of kanamycin on the growth of PPLO. However, when KB cell strain-2 (PPLO free) was used, there was still an enhancement in the virus adsorption as shown by the higher plaque numbers. The above suggests that, in addition to its effects on PPLO, kanamycin also affects the KB cells, possibly by altering the adsorption sites on the KB cell membrane, which is reflected in a higher efficiency of adsorp-

tion of the virus. However, the data is insufficient to allow a firm conclusion concerning this point. To explore the effect more directly, it would necessary to study adsorption of virus with kanamycin incorporated in the adsorption fluid.

As shown by the results in Table XVIII and XIX, maximum plaque numbers were obtained when the pH of the overlay was adjusted to 7.1 for KB strain-1 and 7.2 for strain-2. This difference in the optimum pH of the overlay for the two KB cell strains may or may not be significant. Since direct comparisons between the two strains were not made, a conclusion cannot be made.

At pH values below 7.2 (KB cell strain-2), there was a gradual decrease in plaque numbers and size. On the other hand, at the alkaline pH of 7.3 and 7.5, there were fewer plaques, but these plaques were much larger in size. This phenomenon may be due to one or more of the following:-

1) Genetic variants of adenovirus type may occur. Some of them grow best at the alkaline range (pH 7.5) whereas for others the optimum pH is 7.1-7.2.

2) The alkaline pH may have facilitated the release of fully infectious virions from the host cells to reinfect surrounding cells sooner than at neutral pH values, which results in larger plaques.

3) The alkaline pH may have shortened the life-span of the focus of infected cells thereby revealing larger plaques.

Since there is a significant difference in the plaque numbers obtained under overlays with different pH values, the pH of the agar

overlay is of utmost importance in the plaque assay of adenovirus. The reproducibility of the plaque assay is obviously dependent on careful control of the pH of the agar overlay.

The experiments reported here demonstrate that adenovirus serotypes 3, 5, 8 and 12 which are representative viruses of the four adenovirus HA subgroups (Norrby, 1968) are able to form plaques under the optimum plaque assay conditions developed for adenovirus 5.

The results show that the plaque diameter of each serotype falls into one of three categories, namely, small, intermediate and large. This criterion has been used as another method of classifying or grouping of adenoviruses (Green, 1967). However, it should be pointed out that this method of grouping adenoviruses is valid only if the same strain of KB cells is used for the plaque formation of all the viruses, because the plaque size and number of each adenovirus may not be the same in different KB cell strains, as is shown by the findings of this project. These differences in plaque size and number may be attributed to differences in the inherent sensitivities of the KB cells to adenovirus growth.

With the exception of the highly oncogenic adenoviruses, Green (1967) reported that the HA subgroups I, II, and III form small intermediate and large plaques respectively. His results show that the plaques of Ad 8 were intermediate in size. However, the findings reported here demonstrate that Ad 8 forms comparatively small plaques. Without direct comparison of the two strains of Ad 8, this discrepancy can-

not be resolved.

Green made a study of the plaquing efficiency of 30 human adenoviruses and the ratios of virions to PFU ranged from 11:1 for Ad 3 to 2,300:1 for Ad 25. In the plaque assay of adenoviruses, he allowed an adsorption time of 90 minutes, incorporated neutral red 10 days after infection and used a total overlay of 15 ml. However, as shown by the results of this project, it is plain that the time of addition of neutral red, pH of the overlay, adsorption period can be adjusted so as to increase the sensitivity still further.

The low efficiency of plating of Ad 25 (Green, 1967) may be due to non-optimal assay conditions. It could be improved if factors such as serum concentration, type of serum, nutrient content and time of adsorption are taken into account. As it stands, the results presented by Green on the ratio of virions to PFU may not represent the results of maximum eop, and therefore, are not true estimates of the maximum plaquing efficiency of human adenoviruses.

At this juncture, it should be noted that this project was aimed at developing a plaque assay system that is able to detect as many infectious virus particles as possible. The modifications made have increased the sensitivity of the plaque assay system by about three-fold compared to the initial system, as shown in Table XIII. Although no virus particle concentrations were measured, and it was not therefore possible

to estimate the absolute plaquing efficiency, these studies have paved the way for such an investigation.

SUMMARY

SUMMARY

A plaque assay procedure for adenovirus type 5, that allows the production of consistent and reproducible results, is described.

The composition of the agar overlay affects plaque formation by Ad 5. Horse serum was found to be superior to fetal calf serum in allowing the formation of both more plaques and larger plaques. The optimum serum concentration was found to be 10%.

A supplement to Eagle's MEM, proteose peptone, was found to be essential for plaque formation by adenovirus. It maintains the KB cells in a viable state during the long incubation period required for plaque formation. The plaque promoting factors in proteose peptone are dialysable, but the factors for maintenance of cell viability under agar are not.

Indirect evidence demonstrates that the sulphated polysaccharides present in autoclaved agar are non-inhibitory for adenovirus. However, agar concentrations higher than 0.6% were found to be inhibitory to plaque formation.

It was shown that neutral red inactivates adenovirus type 5 photodynamically and that it inhibits virus growth in the dark. This justifies the addition of neutral red after plaques have formed.

The pH of the virus diluent, within the range of 6.6 and 7.5 does not affect the adsorption efficiency of Ad 5. DEAE-dextran was found to inhibit virus adsorption. On the other hand, by the exposure of the KB cells to kanamycin prior to virus infection, adsorption of Ad 5 was enhanced.

The optimum pH of the agar overlay was found to be 7.1 for KB cell strain-1, and 7.2 for strain-2, and extreme pH values inhibit plaque formation. However, although fewer plaques are formed at pH 7.5, the plaques formed are larger than at pH 7.2.

It was observed that the plaques for Ad 5 had different characteristics in the two KB cell strains, e.g., with KB cell strain-1, the plaque counts of Ad 5 increased from 10 on day 15 to 73 on day 26, whereas there was no increase in plaque numbers after day 15 when KB cell strain-2 was used. The plaques were also larger in diameter on a given day with the latter strain.

Under the optimum conditions for type 5 virus, the plaque characteristics of adenovirus types 5, 3, 8 and 12 were compared. Plaques formed by Ad 5 were large compared to the intermediate plaques formed by Ad 3 and the small plaques formed by Ad 8 and Ad 12.

In conclusion, an accurate and highly sensitive plaque assay system for Ad 5 has been developed by the investigation of the factors that affect plaque assay and using the optimal features of these findings. The optimal conditions, therefore, allow an even closer estimate of the inherent infectivity of adenovirus.

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APPENDICES

APPENDIX A

Growth Medium for KB Cell Monolayer Cultures

Eagle's "working stock" (See Appendix D)	6.1 ml.
Earle's BSS (1X)	80.4 ml.
Calf Serum	10.0 ml.
Kanamycin (3.33×10^4 µg/ml.)	1.5 ml.
Sodium Bicarbonate (0.5M)	2.0 ml.
Total volume	100.0 ml.

APPENDIX B

The Optimum Nutrient Agar Overlay

Proteose peptone (88 mg./ml.)	5.7 ml.
Eagle's "working stock" (See Appendix D)	6.1 ml.
Horse serum	10.0 ml.
Kanamycin (3.33×10^4 µg./ml.)	0.3 ml.
Earle's BSS (10X)	10.0 ml.
Earle's BSS (1X)	5.0 ml.
Double glass distilled water (Sterile)	10.9 ml.
	48.0 ml.
Noble agar (1.2% in glass distilled water)	48.0 ml.
	96.0 ml.
Sodium bicarbonate (0.5M)	4.0 ml.
Total volume	100.0 ml.
Neutral red (0.4% in distilled water)	0.8 ml.

Note: The first seven components made up to 2x concentration were heated to 45°C. and mixed with Noble agar which was cooled to 45°C. Neutral red was incorporated only in the second overlay as described in Materials and Methods.

APPENDIX C

Nutrient Agar Overlay

Proteose peptone (88 mg./ml.)	5.7 ml.
Lactalbumin hydrolysate (44 mg./ml.)	5.7 ml.
Tryptose phosphate broth (3 mg./ml.)	5.0 ml.
Eagle's "working stock" (See Appendix D)	6.1 ml.
Horse serum	15.0 ml.
Penicillin (20,000 IU/ml.) and	
Streptomycin (10,000 µg./ml.)	0.5 ml.
Earle's BSS (10X)	10.0 ml.
	48.0 ml.
Noble agar (2% in glass distilled water)	48.0 ml.
	96.0 ml.
Sodium bicarbonate (0.5M)	4.0 ml.
	100.0 ml.
Neutral red (0.4% in distilled water)	0.8 ml.

APPENDIX D

Procedure for making up Eagle's MEM

1. Hank's basal solt solution with no glucose.
2. 10% glucose solution.
3. The relatively soluble and more stable amino acids are dissolved in 50 ml. of Hank's BSS by heating to 75°C. to make a 100X concentrate:

L-arginine-HCl	0.503 gm.
L-histidine-HCl	0.16 gm.
L-isoleucine	0.26 gm.
L-leucine	0.26 gm.
L-lysine-HCl	0.29 gm.
L-methionine	0.075 gm.
L-phenylalanine	0.16 gm.
L-threonine	0.24 gm.
L-tryptophan	0.05 gm.
L-valine	0.23 gm.

4. The less soluble amino acids are dissolved in 50 ml. of 0.1N HCl:

L-cystine	0.12 gm.
L-tyrosine	0.18 gm.

5. The following relatively soluble B vitamins are dissolved in 50 ml. Hank's BSS:

choline chloride	0.05 gm.
i-inositol	0.10 gm.
nicotinamide	0.05 gm.
calcium-pantothenate	0.05 gm.
pyridoxal	0.05 gm.
riboflavin	0.005 gm.
thiamine	0.05 gm.

6. The following less soluble B vitamins are dissolved in 50 ml. Hank's BBS and the pH adjusted to 7 with a few drops of 0.5 N NaOH.

folic acid	0.005 gm.
biotin	0.005 gm.

Sterilization and storage:

The above preparations are combined in the following proportions, sterilized with a millipore (HA grade) filter, labelled as the Eagle's working stock-A and stored at 4°C.

100 ml. of #2
50 ml. of #3
50 ml. of #4
5 ml. of #5
50 ml. of #6

7. 1.46 gm. glutamine is dissolved in 50 ml. Hank's BSS and kept frozen. Sterilize with HA millipore and labelled Eagle's working stock-B. For use, 51 ml. of stock A are combined with 10 ml. of stock B to make one litre of Eagle's MEM (Eagle, 1959). The "working stock"

was not used after storage at 4°C. for more than one week.