cur. However ability of non-adapted strain to produce plaques refutes this conclusion. In retrospect it appears that no actual change in virus had occurred during serial passage, but the observation that CPE could be regularly produced after as long as 12 days led to use of above procedure and successful production of plaques appearing with this virus at relatively late time of 12 days after inoculation. The sudden appearance of plaques seems unusual; apparently the final change of CPE, resulting in loss of neutral red staining, must occur very suddenly with this system, resulting in overnight appearance of plaques.

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Agar Diffusion Method for Detection and Bioassay of Antiviral Antibiotics. (25617)

ERNEST C. HERRMANN, JR., JANIS GABLIKS, CLAIRE ENGLE AND PRESTON L. PERLMAN
Biochemistry Dept., Schering Corp., Bloomfield, N. J.

The discovery of antibacterial and antifungal antibiotics clearly indicates that microbial cultures contain a wealth of diverse biochemicals useful in infectious disease. Unfortunately, there has been little progress in the exploitation of such microbial products for treatment of human and animal viral diseases. A major reason for this failure may lie in the time-consuming, cumbersome and insensitive technics that until recently were a necessary feature of virus research. Such technics did not permit large-scale screening of microbial cultures important to the discovery of clinically useful antibiotics. Furthermore, even if a naturally occurring antiviral agent were discovered, there were no satisfactory bioassay systems available to cope with the hundreds of assays essential to successful fermentation and isolation of the active principle. The following is a description of a filter paper disc-agar plate technic, similar to that used in bacteriology as applied to screening and assay of antiviral agents. The suggestion that such application was possible was made by De Somer and Prinzie(1) in their description of a similar method used for measurement of poliovirus antibody. More recently, Porterfield (2) applied this approach to interferon bioassay.

Materials and methods. Preparation of virus-infected agar plates. Trypsinized chick embryo cells were prepared as described by Dulbecco(3), suspended in Melnick's lactalbumin hydrolysate-calf serum medium(4) with Earle's bicarbonate balanced salt solution, to produce 3.5×10^6 cells per ml. The cell suspension was adjusted to pH 7 with CO_2, then poured into acid dichromate cleaned, sterile Pyrex baking dishes ($10 \times 6\frac{1}{2}$ inches), 85 ml per dish. The dishes were sealed air tight with Saran Wrap (Dow Chemical Corp.) and the medium readjusted with CO_2 to a pH 7.5 if required. This second gassing is often necessary because of the rapid loss of CO_2 from the relatively high bicarbonate containing medium during incubation period. About 5 to 10 chick embryos, depending on their age, were required to produce enough cells for a single dish. Eleven-day-old embryos seemed to produce the best cell yield. After 48 hr incubation at 36°C the chick embryo cells grew into a confluent sheet; the medium was discarded and the cell culture infected with a 5 ml suspension of one of the following viruses: West Nile virus (Egypt 101 strain from Dr. Joseph Melnick, Baylor Univ.), vaccinia (WR, mouse neurotropic), herpes simplex (HF378) and Newcastle disease (California No. 11914) viruses obtained from American Type Culture Collection. The virus was incubated with the cells for 1 hr, then 65 ml of overlay medium con-

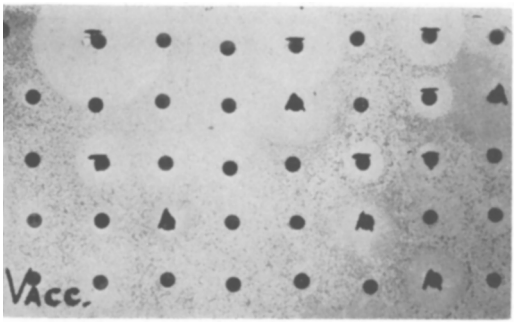

FIG. 1. Composite screening plate using vaccinia virus. Test discs intentionally used to show appearance of zones produced by a toxic substance (T) and zones resulting from an antiviral material (A).

taining 1.5% Noble's agar (Difco) was poured over the cell sheet. This medium contains the same constituents as Melnick's medium with the omission of phenol red and replacement of the 2% calf serum with Difco yeast extract (0.1% final concentration). The plates were again sealed with fresh Saran Wrap and further incubated 3 to 4 days, depending on the virus used, to permit formation of plaques. At the end of this period 36 ml of melted 1.5% agar containing 90 mg 100 ml of indonitro-tetrazolium chloride (Dajac) was poured over the primary agar base. Within 2 to 4 hr at 36°C the living cells reduced the tetrazolium salt to a purple-red formazan. Dead cells and hence virus-induced plaques were colorless. This staining technic, although expensive, has advantages over neutral red which also can be used. With neutral red the color is lost as soon as the cell dies while tetrazolium salt produces a permanent stain not affected by the later death of the cell. Further, light has no apparent deleterious effect on the tetrazolium system as has been observed with neutral red(5). *Preparation and application of filter paper discs.* A variety of number and letter designations, representing various test materials, were typewritten on strips of filter paper (Schleicher and Schuell, type 470), then punched out with a standard ¼-inch paper punch. The resulting ¼ inch discs were touched to the surfaces of either whole microbial liquid cultures or solutions of synthetic compounds. The discs were dried, sterilized with ethylene oxide, and placed on the hardened surface of the primary agar layer, remaining in place during the entire period of plaque development (3-4 days). The discs were later covered by the second, stain-containing agar overlay.

Results. Screening. Between 25 and 40 filter paper discs can be tested in a single baking dish (Fig. 1) making it possible to test large numbers of substances with relative ease. When a disc contained a substance inhibiting plaque formation, a distinct halo of deeply stained, living cells free of plaques (Fig. 2) was produced. The same photograph shows the disc containing the active principle slightly displaced so an inner, small zone can also be seen. This latter zone resulted from the presence of a cytotoxic agent, illustrating how toxicity of a test material can be easily distinguished from virus cytopathogenicity. These differences could not be readily determined if liquid medium tissue culture systems had been used. Care was taken to use enough virus to produce numerous plaques but not so much as to destroy the entire cell sheet. In this way the general condition of the cell sheet could be determined and toxic test materials detected. Plaque-free zones up to 40 mm in diameter were routinely produced by discs impregnated with unconcentrated, liquid bacterial cultures that contained inhibitors of plaque formation.

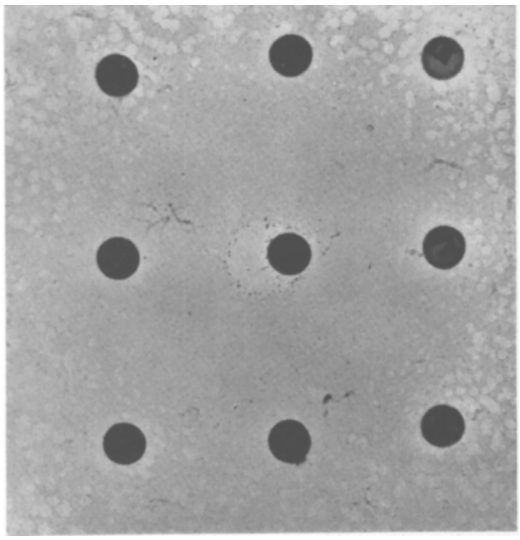

FIG. 2. Vaccinia virus plaque-free zone produced by a disc impregnated with a bacterial culture broth containing an antiviral substance.

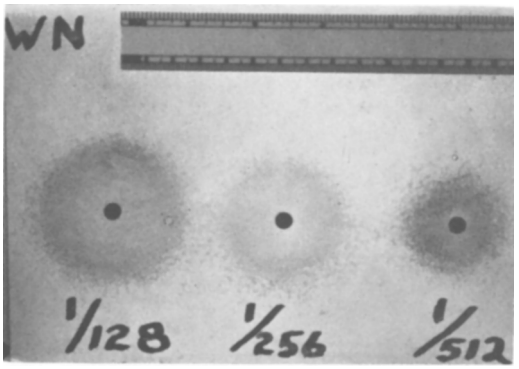

FIG. 3. Representative assay of an antiviral bacterial culture. Discs impregnated with indicated broth dilutions. In this example 10 times more West Nile virus was used than would be used in a routine screening test.

Bioassays were performed by impregnating paper discs with various 2-fold dilutions of an antiviral agent and placing the discs on the surface of a freshly prepared, virus infected, agar overlay culture. The size of the plaque-free zones produced were in proportion to quantity of active material present in the disc (Fig. 3). So far, it has not been possible to produce zones with regular, well-defined edges, even though a variety of virus concentrations have been used. Perhaps as much as a 10% error resulted from this inability to define accurately the zone sizes. Nevertheless, adequate bioassays were done. As the data show (Fig. 4), when zone diameter was plotted against dilution used, similar slopes resulted for a single antiviral agent no matter which virus was used. It was found best, however, to use discs containing enough active material to produce zones in the range of 20 to 50 mm for the most reproducible data. There was also some indication that concentrations of semipurified antiviral agents at less than 1 μg per disc produce zones in that range.

All the problems inherent in any agar diffusion method (6) are obviously present in this system. In addition, however, there are other problems unique to a tissue culture method. For example, it was not possible to use wide variations in pH in an effort to enhance diffusion of an antibiotic since the tissue culture cells were very sensitive to a non-physiological pH. Further, the cells were considerably more sensitive to non-specific toxic agents than bacteria would be in a similar test.

In the experiments reported here, the Pyrex baking dishes did not have perfectly even bottoms hence the depth of the agar varied, often resulting in asymmetric zone shapes. Despite these and other problems, the data produced readily answered various questions associated with fermentation and isolation of an antiviral microbial product.

It is, of course, usually only necessary to test one concentration of the agent in question once a standard has been established. At this point assays of any one material can be performed using a single disc and comparing the zone produced to that produced by a disc containing a standard quantity of the same substance. This permits bioassay of a very large number of samples with a minimum of effort.

Discussion. It now appears that the technics used so successfully in the search for and development of antibacterial antibiotics can be applied directly to the problems of virology. Not only can a very large number of materials be tested but the critical requirement for a bioassay can be fulfilled. Further, preliminary work already indicates that the

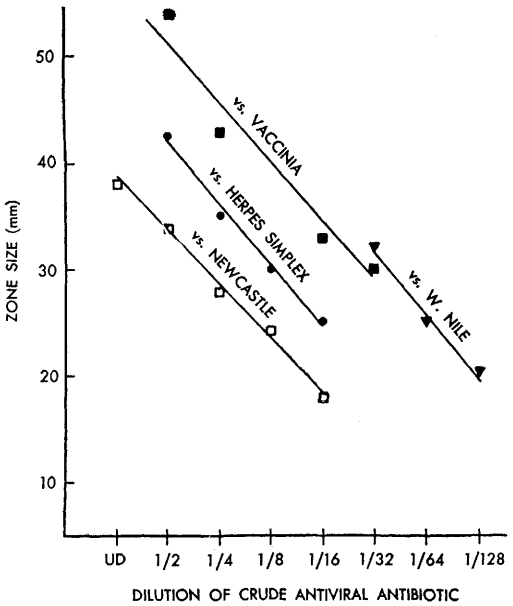

FIG. 4. Representative assay results of a single antiviral antibiotic tested against 4 different viruses.

bioautograph technic of developing paper chromatograms of antibacterial agents can also be applied to antiviral materials. It remains to be seen, however, whether antiviral substances discovered by this technic will eventually be useful in human and veterinary medicine. It is encouraging, in this regard, that at least one antiviral agent—isatin-thiosemicarbazone—which is highly active in vaccinia virus infected mice(7), also was extremely active in this tissue culture system. It can only be hoped that the success observed in discovering clinically useful antibacterial agents can be paralleled in virology.

Summary. A procedure has been developed whereby agar diffusion technics used in bacteriology can be applied to antiviral agents. Chick embryo cell monolayers were grown in Pyrex baking dishes, infected with virus and overlaid with agar. Filter paper discs impregnated with the test materials were deposited on the hardened agar surface. After proper incubation the virus produced plaques in the cell sheet, except in the area around a disc containing an antiviral agent. Since size of the plaque-free zone was proportioned to concentration of the antiviral agent present in the disc, bioassays were simply performed in exactly the same manner as done with antibacterial antibiotics.

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Activation of Histamine-Releasing Factor in Normal Rat Plasma by *E. coli* Endotoxin. (25618)

SHELDON E. GREISMAN
Depts. of Medicine and Physiology, University of Maryland School of Medicine, Baltimore

The mechanisms whereby Gram-negative bacterial endotoxins (somatic antigens) produce the various pathophysiologic alterations in the mammalian host(1) are poorly defined. The basic problem of whether endotoxins act directly at the cellular level, or through an intermediate step whereby composition of blood is altered, or both, is unresolved. The toxic activity of bacterial endotoxins, which are protein-lipid-polysaccharide complexes, appears to reside in the lipid-polysaccharide moiety(2). In a previous study(3), polysaccharides prepared from somatic antigens of *Salmonella typhi* and *Salmonella paratyphi* were included among a list of numerous high molecular weight polysaccharides capable of activating, *in vitro,* a factor in normal rat plasma with potent histamine-liberating activity. It seemed possible, therefore, that certain of the pathologic effects induced by bacterial endotoxin might be attributable to activation of such plasma histamine-liberating factors. The previous study, however, utilized large quantities of the bacterial polysaccharides (20 mg/ml rat plasma) and failed to specify whether these a) were combined with or dissociated from their lipid or protein components, b) exhibited the characteristic activities of endotoxin *in vivo,* or c) released some portion of histamine by direct tissue action. The present study was designed to determine whether smaller quantities of physiologically active, purified lipopolysaccharides from Gram-negative bacteria could activate plasma histamine-releasing factors and to exclude the possibility that such histamine release might be attributable to direct endotoxin-tissue interaction.

Materials and methods. The lower ileum was resected from normal 250 g guinea pigs