Gradient Plate Technique Applied to the Study of Antiviral Substances.* (31104)

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Viral inhibitors have been studied in virtually every system in which animal viruses can be cultivated. These systems are analogous to those used in antibacterial studies, with many of the same advantages and disadvantages. The gradient plate technique, first described for use in bacteriology by Szybalski(1) and later found to have great usefulness in investigation of antibiotic activity(2,3), is perhaps the only remaining antibacterial technique so far not applied to antiviral studies. The following is a description of the application.

Materials and methods. Monolayer cultures of human diploid fibroblast (WI-38, Flow Laboratories), rhesus monkey kidney (RMK, Flow Laboratories), and chick embryo fibroblast (CEF) cells were grown in 8.5-cm plastic petri plates (tissue culture type, Falcon). WI-38 cultures were grown in Eagle's basal medium(4) with 10% (v/v) fetal bovine serum and sodium bicarbonate (7.5 ml of 7.5% solution per liter of medium). RMK and CEF cells were grown in Melnick's lactalbumin hydrolysate-calf serum medium (5) with Earle's balanced salt solution (BSS) and 1 M tris-(hydroxymethyl)-aminomethane buffer, pH 7.6 (tris, Calbiochem; 1.6 ml/100 ml of medium)(6). Seeded petri plates were placed in Pyrex baking dishes (10 by 6½ inches), sealed airtight with Saran Wrap (Dow Chemical Corp.), and incubated at 36°C until confluent cell monolayers had formed. The cell cultures were then infected with 0.5 ml of one of the following viruses diluted in tryptose phosphate broth (Difco): from the American Type Culture Collection, herpes simplex (HF, egg passage), vaccinia (WR, egg passage), and poliomyelitis type 1/LS-a; from Dr. H. Wenner, University of Kansas, coxsackie A-9; from our own collection, coxsackie B-1 (obtained as a patient isolate from mouse brain pool and passaged 3 times in RMK cells); from the Communicable Disease Center, Atlanta, Georgia, ECHO type 14; and from Dr. E. M. Neumeyer, E. I. du Pont de Nemours and Co., Newark, Dela., influenza A/WSN (egg passage). After incubation at 25°C for 1 to 2 hours, unattached virus was removed by washing the monolayers twice with BSS. Cell monolayers were then overlaid with one or two layers of agar medium. With WI-38 and RMK, this consisted of twice-concentrated Eagle's basal medium, containing 20% (v/v) fetal bovine serum, sodium bicarbonate (3.0 ml of 7.5%/100 ml of medium), and 1 M tris buffer (pH 7.6, 2 ml/100 ml of medium), mixed with an equal volume of 2% Special Agar-Noble (Difco). CEF monolayers were overlaid with 1% agar medium as described by Simpson and Hirst(7) but without gelatin and phenol red.

A single layer of agar medium was used in preparing disc plates as previously described(8). Two layers of agar medium were used in preparing gradient plates.

The bottom agar overlay consisted of 9 ml added while the plates were slanted (about ⅛ inch) sufficiently so that the medium just covered the uppermost portion of the cell monolayer. When this agar overlay had solidified, the plate was placed in a perfectly level, horizontal position and a second 9-ml agar overlay was added; this contained the desired concentration of one of the following antiviral compounds: 5-iodo-2′-deoxyuridine (IUdR), guanidine·HCl, and 5-chloro-2′-deoxyuridine (CUdR) all obtained from International Chemical and Nuclear Corp.; 5-bromo-2′-deoxyuridine (BUdR) obtained from Calbiochem; 5-fluoro-2′-deoxyuridine (FUdR) from Hoffmann-La Roche; cytosine arabinoside (CA), isatin-β-thiosemicarbazone (IBT), and N-methyl-isatin-β-thiosemicarbazone (M-IBT) obtained from Nutritional Biochemicals; amantadine·HCl kindly supplied

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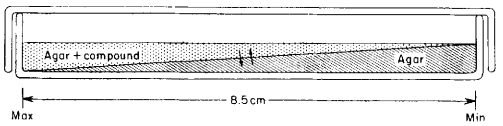

FIG. 1. Diagram of gradient plate. *Min* = low side of gradient (beginning of gradient) where, in effect, there was no antiviral compound. Arrows indicate a 2-fold dilution of antiviral compound (concentration was one-half the limit set by the initial concentration in the agar medium). *Max* = high side of gradient where concentration of antiviral compound was maximal.

by du Pont; and α-hydroxybenzylbenzimidazole (HBB) from Aldrich Chemical Co. Stock solutions of IUdR, amantadine·HCl, CA, and guanidine·HCl were made up in distilled water; CUdR, IBT, and M-IBT were dissolved in dimethylsulfoxide (Baker Chemical Co.); FUdR was dissolved in 95% ethyl alcohol; HBB was dissolved directly in the agar overlay medium. Stock solutions of all compounds were freshly prepared with the exception of IUdR and FUdR which were stored at −10°C. For use, 0.2 ml of stock solution was diluted in 8.8 ml of melted agar medium to produce the desired concentration. Preliminary trials showed that 0.2 ml of solvent alone in the second overlay had no observable effect on either cell monolayers or virus.

After the second (compound-containing) overlay had solidified (Fig. 1) the plates were again sealed in Pyrex baking dishes and incubated at 36°C for 3 days with herpes simplex or polio viruses and 4 days with all other viruses used. At the end of this incubation period, cell monolayers were vitally stained by addition to each plate of 6 ml melted (1%) Noble agar containing 0.11% 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT, Aldrich Chemical Co.)(8). Plaques were readily visible after overnight incubation at 36°C (Fig. 2).

The plaque inhibitory concentration (C_i) was expressed as the amount of antiviral compound, in micrograms per milliliter, calculated to be present at the point of complete plaque inhibition. The calculation is $D_1/D_2 \times C_{max} = C_i$, in which D_1 is the distance (cm) from the point of plaque inhibition to minimum of gradient, D_2 is the inside diameter of the petri plate (8.5 cm), and C_{max} is the initial concentration of compound in the second agar overlay. This calculation as well as the theory behind the gradient plate technique has been fully discussed by Szybalski(1).

Results and discussion. Generally, the antiviral activity observed with the gradient plate method agreed with results obtained by the disc plate technique (Table I). In agree-

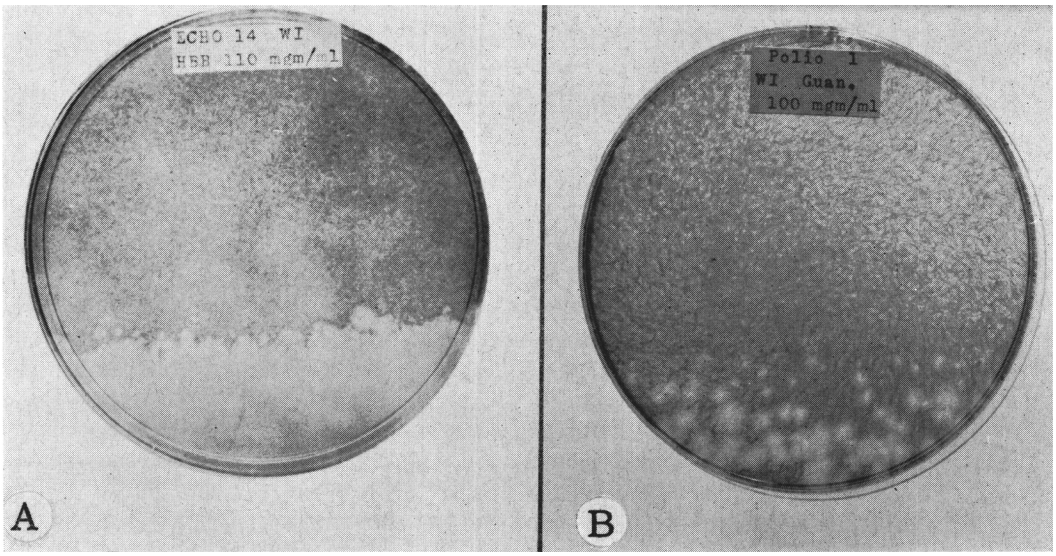

FIG. 2. Typical gradient plates. *A*, Inhibition by HBB (35 μg/ml) of echovirus plaque formation in WI-38 monolayer. *B*, Inhibition by guanidine·HCl (29 μg/ml) of poliovirus plaque formation in WI-38 monolayer.

TABLE I. Comparison of Gradient Plate Technique to Disc Plate Technique.

Compound	Max conc (μg/ml)	Virus	PFU* (× 10^2)	Cell system	Gradient plate technique inhibitory conc (μg/ml)†	Disc plate technique, plaque-free zone diam (mm)‡
IUdR	11.0	Herpes simplex	8	CEF	4.3 (5.2), 4.0 (5.4), 4.4 (5.1)	51
		Vaccinia	8	CEF	5.2 (4.5), 5.1 (4.6)	47
		Herpes simplex	100	CEF	5.7 (4.1), 6.1 (3.8)	50
		Vaccinia	100	CEF	—	46
BUdR	5.5	Herpes simplex	9	CEF	4.5 (1.3), 5.0 (.8)	48
		Vaccinia	9	CEF	1.9 (5.5), 1.9 (5.5)	53
CUdR	11.0	Herpes simplex	5	CEF	>11 (0)	trace
		Vaccinia	5	CEF	1.6 (7.3)	55
FUdR	11.0	Herpes simplex	150	CEF	>11 (0)	0
		Vaccinia	150	CEF	—	0
CA	11.0	Herpes simplex	3	CEF	5.8 (4.1), 5.2 (4.5)	46
		Vaccinia	3	CEF	2.1 (7.0), 1.8 (7.1)	54
IBT	6.6	"	5	CEF	1.3 (6.8), 1.2 (6.9)	58, 54, 62§
M-IBT	6.6	"	2	CEF	.9 (7.4)‖	43, 45, 42§
Amantadine	110	Influenza A/WSN	2	CEF	63 (3.6)¶	28
Guanidine	110	Polio 1	20	WI-38	29 (6.3)	37
		Coxsackie A-9	20	WI-38	75 (2.7)	0
		Echovirus 14	20	WI-38	>110 (0)	0
		Polio 1	3	RMK	25 (6.5)	29
		Coxsackie A-9	3	RMK	24 (6.6)	28
		" B-1	3	RMK	61 (3.8)	trace
		Echovirus 14	3	RMK	51 (4.5)	23
HBB	100	Polio 1	3	WI-38	50 (4.2)¶	18
		Coxsackie A-9	3	WI-38	22 (6.6)	37
		Echovirus 14	3	WI-38	35 (5.5)	35
		Polio 1	5	RMK	47 (4.5)	20
		Coxsackie A-9	5	RMK	26 (6.3)	52
		" B-1	5	RMK	27 (6.2)	42
		Echovirus 14	5	RMK	72 (2.4)	20

* Plaque forming units per 0.5 ml inoculum.

† Number in parentheses represents plaque-free zone, distance (cm) from point of inhibition to maximum of gradient (D_2-D_1).

‡ Disc treated with 100 μg of compound unless otherwise indicated.

§ Only 25 μg per disc; 8-mm toxic zone noted with IBT, 18-mm toxic zone with M-IBT.

‖ Toxic at 1.6 μg/ml.

¶ Inhibition incomplete; 95% of plaques inhibited compared to control.

ment with a previous report(6), FUdR was found to be inactive, perhaps as a result of the presence in chick embryo cells of an enzyme that degrades FUdR to the inactive fluorouracil(9). The data from gradient plates also indicated that vaccinia virus clearly was more susceptible to BUdR than was herpes simplex virus; in the gradient plate method the difference was at least 4.2 cm. In the disc plate method, there was only a 5-mm difference in plaque-free zones between these two viruses, a difference which easily can occur by error alone. In the disc plate method, poorly defined zone edges frequently made it impossible to detect as much as a 2-fold increase in drug concentration. The gradient plate method, however, exhibits a change in the plaque-free zone of at least 20 mm with each 2-fold increase in drug concentration. Therefore, poorly defined zone edges are not as serious a problem in this latter method.

The failure to obtain highly reproducible zone measurements in the disc plate method might be attributed to rapid diffusion of drug from the paper disc, resulting in a steep concentration gradient and a narrow area of optimal drug concentration surrounding the disc (10). In addition, other factors affect the

TABLE II. Reversal by Thymidine of IUdR Activity Against Herpes Simplex Virus in Gradient Plates.*

Max conc in gradient (μg/ml)		Plaque inhibitory conc of IUdR† (μg/ml)
IUdR	Thymidine	
22	.0	4.4 (6.8)
22	5.5	5.5 (6.4)
22	11.0	9.1 (5.0)
22	22.0	>22.0 (0.0)

* CEF monolayers were inoculated with 300 plaque forming units.
† Distance (cm) from point of inhibition to maximum of gradient given in parentheses.

diffusion of a compound from the impregnated disc, such as diffusion rate of the compound and tendency of the paper disc to retain compound(10). The gradient plate technique allows a gradual, proportional increase of drug concentration in the agar medium, extending over the entire cell monolayer. The slope of the gradient and the length of incubation do not seem to alter the results appreciably(1). However, it is important in the gradient plate method as well as in the disc plate method to use a virus dose that does not destroy the entire cell monolayer. This allows for examination of the living cells and ready detection of toxicity, due to test compound or some other factor, that might be confused with virus activity.

Different results might be expected when different host cells are used in the gradient plate technique; this has not yet been fully investigated. Certainly, the stability of the drug could also be a factor, as could the use of various media. Indeed, the incorporation of thymidine (distilled water stock solution stored at −10°C, Calbiochem) and IUdR in the same agar overlay completely eliminated IUdR activity against herpes simplex virus (Table II); hence, the presence of analogous antagonists in certain media must be considered. This method also has potential use in studying synergistic or additive effects of combinations of antiviral compounds.

Assays of guanidine against poliovirus and of IUdR against herpes simplex virus were performed in gradient plates by varying the initial concentration of the antiviral agent in the second agar overlay. These preliminary data (Fig. 3) suggest that the gradient plate method is capable of rather accurate assays, which has not always been the case with either tube dilution or disc plate methods. It should be noted that, although 3 gradient plates were used for each assay, this is not necessary in practice because a single gradient plate represents an infinite number of dilutions of the test compound. This is equally true of the disc plate method except that the effective inhibitory level of the compound is concentrated in a very narrow area.

One of the more significant uses for the gradient plate in bacteriology was the selection of antibiotic-resistant mutants(1). Similarly, the technique described here can be used in selecting strains of herpes simplex virus resistant to IUdR. Resistant plaques were readily observed in IUdR gradient plates but, as indicated previously(6), were not so readily seen in BUdR plates. Virus was isolated from the resistant plaques (made visible by overlaying the gradient plate with 3 ml of neutral red solubilized in distilled water [1:750]), passed twice in WI-38 liquid tissue cultures, and then used to infect CEF monolayers in gradient plates with increased initial concentrations of IUdR. As the data indicate (Table III), the isolated strains were highly resistant to IUdR as compared to the parent strain.

Summary. The gradient plate technique, originally used in bacteriology, was adapted to the study of virus inhibition by known antiviral compounds, including pyrimidine nucleosides, thiosemicarbazones, amantadine, guani-

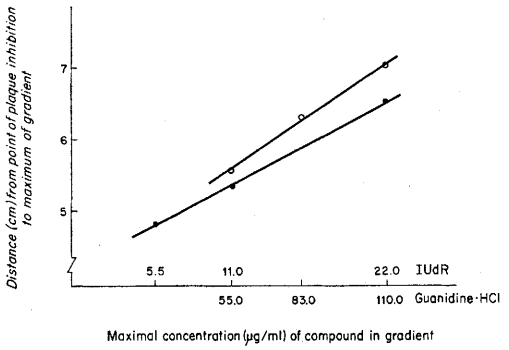

FIG. 3. Dose-response lines in gradient plate technique. Solid circles, IUdR assayed against herpes simplex (10^3 PFU/0.5 ml). Open circles, guanidine • HCl assayed against poliovirus (28 x 10^2 PFU/0.5 ml).

TABLE III. Effect of IUdR Against IUdR-Resistant Herpes Simplex in Gradient Plates.

Herpes simplex passaged strain*	Viral dose (PFU/0.5 ml)	Plaque inhibitory conc (μg/ml)
5C-R1	250	32
5C-R4A	700	>44
5C-R4A	70	>44
5C (parent strain)	800	4.4

* Virus originally isolated in IUdR plaque inhibition zone.

dine, and -hydroxybenzylbenzimidazole, all of which produced striking antiviral effects. Reversal of 5-iodo-2'-deoxyuridine (IUdR) activity by thymidine was readily quantified by using this method. The technique was applicable to the bioassay of IUdR and guanidine and to the ready detection and isolation of IUdR-resistant strains of herpes simplex virus.

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Effect of Testosterone on the Involution of Male Rats' Mammary Glands.* (31105)

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In previous studies, it was shown that the mammary glands of male rats could be stimulated to the growth of the duct system by 1 or 2 μg of estradiol benzoate (EB) for 20 days and the lobule-alveolar system by 2 μg EB and 6 mg progesterone (P) for 20 days. It was suggested that the male rat mammary gland was equipotential to the female gland in response to these ovarian hormones(1,2).

When the young are removed from lactating rats, it has been observed that the lobule-alveolar system quickly involutes back to a duct system with DNA values comparable to those of virgin ovariectomized rats(3). In studies on the prevention of the involutionary process by the ovarian hormones(4) and by oxytocin, lactogenic and hydrocortisone acetate(5) it was shown that EB + P was most effective.

The present study was designed to extend our observations upon the growth of the lobule-alveolar system with the ovarian hormones in male rats, then to determine whether testosterone would have the capacity to prevent involution of the glands so grown.

Materials and methods. Adult male rats of the Sprague-Dawley-Rolfsmeyer strain were castrated and maintained on Purina Lab Chow with tap water *ad libitum* in a constant environmental temperature of 78 ± 1°F. After a convalescent period of 15 days, the castrated rats were divided into 3 groups.

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