tain, we elected to perform a minimum number of experiments with morphine in order to utilize the available specimens for other purposes. However, the data presented here do indicate that this preparation may be useful for measurement of addiction liability, since nalorphine was able to antagonize the action of morphine.

Our studies with *E. virescens* were initiated because we needed a rapid bioassay for drugs which modify behavior and conditions did not permit us to undertake the more conventional bioassay methods. Our information is insufficient for correlation of emotional response of the fish with the observed results. Despite the shortcomings of this system which have been cited, two cardinal points are in its favor: (1) it can be used for precise and rapid bioassay of a variety of drugs, and (2) the fish respond to concentrations approximately the same as those which have effect in man. These properties have been shown to apply to neurotrophic polypeptides, lysergic acid diethylamide, methylphenidate, phenobarbital, morphine and nalorphine.

Summary. Studies with the electric transparent knife fish, *E. virescens,* have been extended to include observations on methylphenidate, pentylenetetrazole, phenobarbital, morphine, and nalorphine. Characteristic qualitative and quantitative responses were obtained with methylphenidate, phenobarbital, and morphine. Pentylenetetrazole produced no consistent effects. Nalorphine reversed the depressant effects of a preceding dose of morphine. The data suggested that the fish developed tolerance to morphine as well as physical dependence on this drug. The system studied appeared to be suitable as a bioassay technique. The observed drug effects were obtained with concentrations approximately the same as those which have effects in man.

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Effect of Environmental Changes upon Antiviral Action of Interferon.* (28757)

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Changes in the environment of cells have been reported to modify the antiviral action of interferon. Reduction of oxygen tension (1) and low pH(2) have been reported to increase the antiviral action of interferon. We have investigated the effect of changes in oxygen tension, pH, and temperature upon the action of interferon on vaccinia virus infection of chick cells. The effects of these changes upon the plating efficiency of vaccinia virus have also been studied.

Materials and methods. Interferon production. Chick interferon was prepared in chick fibroblast monolayers infected with large doses of Chikungunya virus as described by Ruiz-Gomez and Isaacs(3). Interferon-containing preparations were dialyzed against 0.1

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M HCl-KCl buffer, pH 2, overnight and then against Gey's balanced salt solution (BSS) to bring the pH to about 7.4.

Virus. A strain of vaccinia virus was employed as the assay virus. This strain of virus was similar to the Vl strain used by Lindenmann and Gifford(4) for development of plaques on chick fibroblasts without agar overlay.

Virus and interferon assays. Vaccinia virus and interferon were titrated according to the methods described(4,5) with slight modification to accommodate different sized bottles. Also, assays were carried out at 35° instead of 37° since the plating efficiency of vaccinia virus was thereby increased. Growth medium was decanted from culture bottles and interferon dilutions and virus dispensed in a final volume of 1.5 ml. Quadruplicate cultures were usually used for each dilution of interferon. The small amount of medium employed resulted in an average depth of slightly less than 1 mm. Bottles were incubated without disturbance for 44-45 hours at 35°; medium was decanted and monolayers stained with 0.1% crystal violet, washed with running tap water and inverted to dry. Monolayers were magnified 10X by use of an enlarger for plaque counting.

Medium. Growth medium for the establishment of chick monolayers consisted of Gey's BSS with 0.0025 M Tris, 5% calf serum, 0.25% lactalbumin hydrolysate, and 0.1% proteose peptone. Maintenance medium consisted of Gey's BBS with 0.11% sodium bicarbonate, 0.25% lactalbumin hydrolysate, 0.1% yeast extract, and 0.1% proteose peptone. For conditions requiring efficient pH control, Tris, 0.0025 M, and phosphate, 0.01 M, were added as supplementary buffers to the maintenance medium. These additions had no effect on the plating efficiency of vaccinia virus. To adjust this medium to different pH levels, the proportion of Na_2HPO_4 and NaH_2PO_4 was varied. In one experiment the pH was varied by altering the concentration of sodium bicarbonate.

Oxygen tension. To establish different oxygen tensions, cultures were placed in jars and mixtures of oxygen and nitrogen were allowed to flow through them for 5 minutes. The gas pressure inside the jar was slightly greater than atmospheric to insure against leakage. Jars were sealed and checked for retention of the gas pressure at the end of incubation time.

Cell cultures. Chick embryo fibroblasts were prepared by a slight modification of the technique previously described(4). Ten-day-old eviscerated chick embryos were trypsinized as described by Porterfield(6). Cells were suspended in growth medium and approximately 9×10^6 cells were dispensed in 3 ml volumes into soft glass, screw-capped 1 ounce bottles having a rectangular side of 18 cm^2. The cultures were incubated at 37° and employed 42 to 48 hours later. At that time there were about 3×10^6 cells per bottle as a monolayer.

Results. 1. Effect of temperature. Titrations of interferon and virus were prepared as usual but incubated at 35°, 37°, or 39° for 44 hours. Quadruplicate bottles were used at each temperature for each interferon level. Fig. 1 shows that more plaques developed at the lowest temperature employed. No significant difference in the percent inhibition of plaques by interferon could be found at these different temperatures (Table I), which is consistent with the findings of Ruiz-Gomez and Isaacs(7) for Chikungunya, Newcastle disease, and fowl plague viruses.

2. *Effect of pH.* Titrations of interferon and virus were prepared as usual except that the pH was varied by altering the proportion of NaH_2PO_4 and Na_2HPO_4 and maintaining the phosphate at 0.01 M. Tris, 0.0025 M was also included in the maintenance medium. A marked effect of pH on the plating efficiency of vaccinia virus was found with a narrow optimum range between 7.1 and 7.3. (Fig. 2). Some changes in pH occurred during incubation, especially with the lower pH levels used, and the pH levels plotted represent the average pH values obtained. No plaques were found when the pH was maintained at pH 6.8 or less, even though these pH levels were compatible with cell survival. When the percentage inhibition of plaques by interferon was measured, no significant difference in the action of interferon was found. These results were repeated several times. A repre-

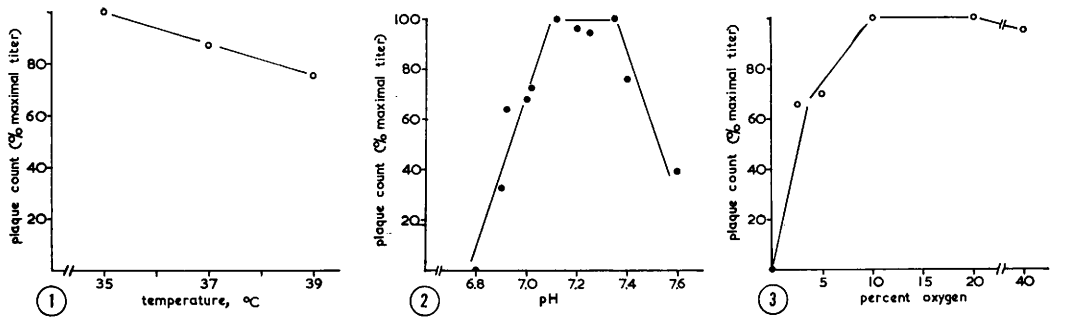

FIG. 1. Effect of temperature on plating efficiency of vaccinia virus on chick cells.
FIG. 2. Effect of pH on plating efficiency of vaccinia virus on chick cells.
FIG. 3. Effect of oxygen tension on plating efficiency of vaccinia virus on chick cells.

sentative experiment is shown in Table II. Similar results were found when the pH was varied by altering the concentration of bicarbonate to change the pH.

3. Effect of oxygen tension. Titrations of interferon and virus were prepared as usual except that the percentage of oxygen in the environment was altered. Medium, buffered with 0.11% sodium bicarbonate, 0.01 M phosphate and 0.0025 M Tris was employed since it was difficult to maintain the pH at low oxygen tensions. The effect of oxygen tension on the plating efficiency of vaccinia virus on chick cells is shown in Fig. 3. It was not possible completely to dissociate the effect of pH from the effect of oxygen tension on the plating efficiency of the virus since the pH was not well maintained when the percentage of oxygen was less than 2.5. Under anaerobic conditions the final pH was 6.7 and under 2.5% oxygen the final pH was 6.9. Under 5% or greater oxygen percentages, the pH at the end of the incubation time was found to be at least 7.0. There was no significant difference in interferon action when the percentage inhibition of plaques was measured (Table III).

Discussion. Vaccinia virus has several attributes which make it convenient for the assay of interferon in chick cells(5). In addition, it induced very little interferon in chick cells as compared with Chikungunya or Kumba viruses under comparable conditions (Gifford, Mussett and Heller, in preparation) and therefore the production of interferon during the assay would be less likely to be a complicating factor. Furthermore, for studies reported here, the lack of an agar overlay and the small amount of fluid permitted a more rapid diffusion of gases and nutrients and made the estimation of pH simpler.

In this study, it has been shown that changes in temperature, oxygen tension and

TABLE I. Effect of Temperature on Plating Efficiency of Vaccinia Virus on Chick Cells and on Interferon Action.

Temp of incubation	μl interferon preparation	No. of plaques	Avg No. of plaques ± S.D.*	% inhibition of plaques
35°	100	13, 30, 37, 12	23.0 ± 12.5	89
"	25	111, 99, 106, 80	99.0 ± 11.8	54
"	6.25	176, 157, 137, 158	157.0 ± 15.9	27
"	none	216, 218, 201, 230	216.5 ± 11.9	—
37°	100	33, 27, 24, 47	32.8 ± 10.0	82
"	25	105, 100, 100, 104	102.3 ± 2.6	46
"	6.25	110, 138, 126, 148	130.5 ± 16.7	30
"	none	188, 202, 159, 202	187.8 ± 20.2	—
39°	100	37, 29, 20, 18	26.0 ± 8.7	84
"	25	73, 56, 70, 76	68.8 ± 8.8	57
"	6.25	101, 94, 92, 130	104.3 ± 17.6	34
"	none	172, 150, 156, 158	159.0 ± 9.3	—

* S.D. = standard deviation.

pH altered the plating efficiency of vaccinia virus on chick cells. The action of interferon was not significantly affected by marked changes in these conditions when percentage inhibition of plaques was taken as the parameter of interferon action. The effect of

TABLE II. Effect of pH on Plating Efficiency of Vaccinia Virus on Chick Cells and on Interferon Action.

pH		μl interferon		Avg No. of	% inhibition
Initial	Final	preparation	No. of plaques	plaques ± S.D.*	of plaques
6.8	7.05	5.0	62, 58, 61, 52	58.3 ± 4.5	61
"	"	2.5	80, 86, 97, 94	89.5 ± 13.4	41
"	"	1.25	85, 119, 133, 127	116.0 ± 22.1	23
"	"	none	152, 168, 144, 135	149.8 ± 14.0	—
6.9	7.15	5.0	78, 63, 66, 43	62.5 ± 14.5	62
"	"	2.5	97, 114, 95, 98	101.0 ± 8.8	39
"	"	1.25	138, 120, 123, 142	130.8 ± 10.9	20
"	"	none	158, 158, 187, 159	165.5 ± 15.1	—
7.05	7.2	5.0	97, 72, 89, 67	81.3 ± 14.3	65
"	"	2.5	109, 138, 108, 111	116.5 ± 14.4	50
"	"	1.25	141, 155, 152, 156	151.0 ± 6.9	35
"	"	none	237, 225, 240, 234	234.0 ± 6.5	—
7.2	7.3	5.0	68, 71, 83, 108	82.5 ± 18.7	62
"	"	2.5	125, 143, 130, 108	126.5 ± 14.5	42
"	"	1.25	151, 181, 181, 187	175.0 ± 16.3	20
"	"	none	194, 215, 224, 242	218.8 ± 20.4	—
7.4	7.4	5.0	24, 32, 43, 34	33.3 ± 13.5	75
"	"	2.5	73, 58, 68, 67	66.5 ± 6.3	52
"	"	1.25	110, 82, 106, 86	96.0 ± 14.0	31
"	"	none	140, 131, 126, 128	131.5 ± 6.2	—
7.6	7.6	5.0	18, 17, 23, 25	21.5 ± 4.0	76
"	"	2.5	40, 42, 58, 38	44.5 ± 9.6	51
"	"	1.25	66, 71, 63, 58	64.5 ± 5.3	29
"	"	none	99, 67, 89, 107	30.5 ± 17.3	—

* S.D. = standard deviation.

TABLE III. Effect of Oxygen Tension on Plating Efficiency of Vaccinia Virus on Chick Cells and on Interferon Action.

Exp No.	% oxygen	μl interferon preparation	No. of plaques	Avg No. of plaques ± S.D.*	% inhibition of plaques
1	0	none	0, 0, 0, 0	0	—
	2.5	100	23, 20, 18, 19	20.0 ± 2.2	80
	"	50	46, 46, 36, 55	45.8 ± 7.5	54
	"	25	76, 68, 70, 50	66.0 ± 13.7	35
	"	none	120, 102, 81, 101	101.0 ± 15.9	—
	5.0	50	40, 45, 32, 42	39.8 ± 5.6	63
	"	25	60, 68, 67, —	65.0 ± 4.4	40
	"	none	96, 113, 114, 108	107.8 ± 8.3	—
	20.0	100	29, 30, 37, 23	29.8 ± 5.7	81
	"	50	56, 59, 67, 70	63.0 ± 5.5	59
	"	25	107, 106, 112, 103	107.0 ± 3.7	30
	"	none	153, 138, 163, 157	152.8 ± 10.7	—
2	20.0	100	79, 91, 90, —	86.7 ± 6.7	62
	"	50	118, 102, 139, 122	120.3 ± 15.2	48
	"	25	190, 173, 180, 162	176.3 ± 11.8	23
	"	none	244, 227, 231, 232, 212	229.2 ± 11.5	—
	40.0	100	71, 77, 79, 70	74.3 ± 4.4	66
	"	50	81, 95, 98, 79	88.3 ± 9.6	59
	"	25	162, 168, 148, 183	165.3 ± 14.5	24
	"	none	217, 210, 205, 235	216.8 ± 13.2	—

* S.D. = standard deviation.

temperature was consistent with the findings of Ruiz-Gomez and Isaacs(7) that for Chikungunya, fowl plague and Newcastle disease viruses there was no significant difference in the titer of interferon at 35° or 39°. The findings with respect to oxygen tension and pH are different from previously published interpretations(1,2). Since it is unlikely that interferon has a different mechanism of action for different viruses, it seems necessary to discuss the various reported observations and interpretations. Baron, Isaacs, and Porterfield(8) found that when tubes containing cells infected with a variety of viruses were partially filled with agar-containing overlay, viral plaques appeared over a limited depth below the surface, the size and number of plaques approaching zero as the depth increased. Various viruses differed in the depth to which they would produce plaques. They interpreted the effect as due to variation in oxygen requirement by different viruses. This interpretation seemed likely since plaques appeared earlier and at a greater depth when the oxygen tension was raised, and appeared later and to a lesser depth when the oxygen tension was lowered. Such an explanation, however, would not explain why both Chikungunya and Newcastle disease viruses developed at a greater depth under the bicarbonate containing overlay medium (0.013 M sodium bicarbonate) than in Tris medium (0.0025 M Tris) since it could not be anticipated that such differences would change oxygen diffusion. They could be explained by variations in pH since bicarbonate should be a better buffer under the conditions used. In addition, Isaacs, Porterfield, and Baron(1) reported a correspondence between the depth to which viruses would form plaques in agar filled tubes and their sensitivity to the antiviral action of interferon. It was concluded that interferon had a greater antiviral effect under reduced oxygen tension. Such agar-filled tubes would demonstrate, however, gradients other than oxygen partial pressure. Cells exposed to low oxygen pressures utilize glucose at a greater rate than cells exposed to higher pressures of oxygen with a concomitant decrease in pH. Such pH gradients can be readily seen in culture tubes filled with agar overlay containing phenol red. Other changes in the environment would occur as a consequence of the metabolic changes. Thus, one would anticipate gradients in glucose concentration and pH, among others, with changes in oxygen tension, or with increased depth under agar. Crude interferon preparations stimulate glycolysis(9) and therefore greater changes in pH might well occur in treated cultures. De Maeyer and De Somer(2) found an increase in the antiviral action of interferon when cells were infected in low bicarbonate medium which they attributed to decreased pH. In this study we could find no difference in the effect of interferon at different pH levels although changes in pH markedly altered the plating efficiency and presumably the growth of the virus. De Maeyer and De Somer(2) also reported that cells infected at low pH produced more interferon than at high pH. Since they used a relatively large challenge inoculum and waited for considerable cytopathology to read their interferon titers it is possible that interferon production during the assay may have been a complicating factor.

The rather narrow range of pH which permitted optimal plaque counts on chick cells implies that this factor should be carefully considered in assays of this virus. For example, as already mentioned, crude preparations of interferon have been shown to stimulate glycolysis with a concomitant decrease in pH. This decrease in pH could contribute to the inhibition of virus plaques and consequently yield a greater antiviral effect than the interferon preparation alone. The effect of pH on the replication of other viruses has been studied. However, it is not clear whether the reported effects were due to altered bicarbonate or hydrogen ion concentration since the alteration of either one would change the concentration of the other. Furthermore, cell metabolism in closed containers would yield bicarbonate. Gifford, Robertson, and Syverton(10) found a decreased yield of poliovirus in HeLa cells when the pH was maintained at 6.8 or less. In some experiments the only added bicarbonate was that present in the serum and

the medium was buffered with phosphate, phosphite and Tris. Vogt, Dulbecco, and Wenner(11) found that avirulent strains of poliovirus produced a smaller number of plaques than did virulent strains when plated under an agar overlay with reduced amounts of bicarbonate which they presumed was due to the lower pH. Hsiung and Melnick(12) also found this to be so but indicated that low bicarbonate concentration was the responsible factor. Both groups of workers (11,12) could not find any difference in the growth of the various strains of poliovirus in liquid medium with different bicarbonate concentrations. On the other hand, Sabin(13) and Lwoff(14) have obtained data in liquid medium which was comparable with that of Gifford *et al*(10). Mosley and Enders(15) have obtained evidence that low bicarbonate concentrations rather than low pH was responsible for inhibition of some poliovirus strains. In the present study the amount of bicarbonate added to the medium was rather high and the pH was adjusted by varying the proportion of Na_2HPO_4 and NaH_2PO_4. Similar effect on the plating efficiency of vaccinia virus was also obtained by altering the concentration of bicarbonate to change the pH. It is possible that the effects reported here, and related to pH, are due to alterations in bicarbonate concentration but more critical studies would have to be made to determine the relative importance of the two factors.

Summary. Changes in temperature, oxygen tension and pH were shown to have an effect upon the plating efficiency of vaccinia virus on chick cells. These changes had no significant effect on interferon action when the percentage inhibition of plaque formation was used as the parameter of interferon action.

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Dissociation and Cultivation of Chick Embryo Cells with an Actinomycete Protease. (28758)

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During studies on the growth and metabolism of chick embryonic pectoral muscle it was found that crystalline trypsin and ethylenediaminetetraacetic acid (EDTA) did not successfully separate the cells of this tissue. Consequently, a method was sought to dissociate rapidly chick embryonic muscle with physiological salt solutions of known chemical composition. Mintz(2) reported that 1 g/100 ml solutions of a crystalline enzyme obtained