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Poliovirus Replication and Cytopathogenicity in Monolayer Hamster Cell Cultures Fused with Beta Propiolactone-Inactivated Sendai Virus* (32668)

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Recently Enders and his associates (1) showed that poliovirus I replicates in primary chick embryo cells and in a line of hamster embryo cells exposed to a high concentration of Sendai virus largely inactivated by ultraviolet (UV) irradiation. It was suggested that poliovirus was incorporated into these naturally resistant cells during the process of fusion brought about by the Sendai virus while the natural barrier to infection presented by the cell surface was temporarily impaired. It was proposed that this technique might prove useful for the propagation of viral agents that hitherto have not been cultivated *in vitro*.

The potential value of the cell-fusion technique for such purposes would obviously be enhanced if it permitted the demonstration of cytopathic effects as well as replication of the agent. In the experiments of Enders and his associates a cytopathic effect could not be recognized with certainty. Although progressive degenerative changes were noted earlier in cells exposed to both polio and Sendai virus, those exposed only to the latter were also ultimately destroyed. Thus the specificity of the change in the poliovirus-exposed cultures remained in doubt. When these experiments were done it was known that the UV-inactivated Sendai virus concentrate contained traces of infectious virus. Later it was found by Dr. George Miller in unpublished experiments in our laboratory that the line of hamster cells employed exhibited cytopathic effects when exposed to unirradiated Sendai virus. Accordingly, the studies reported here were primarily undertaken to determine whether in the absence of all infectious Sendai virus specific cytopathic changes are induced by poliovirus in fused hamster cells. In addition, means were sought to simplify the original procedures which for use as routine were regarded as unnecessarily complicated. As will be described, complete inactivation of Sendai virus without loss of the fusion factor was achieved by addition of beta propiolactone (BPL) and simplification of the technique was effected by adoption of ordinary monolayer cultures instead of cell suspension. In monolayer cultures treated with Sendai virus inactivated in this way cytopathic effects induced by poliovirus were demonstrated.

Materials and Methods. Except as specifically noted hereafter materials and procedures were the same as described by Enders and associates (1). Two lots of poliovirus I (strain Brunhilde) were employed which exhibited titers of $10^{5.8}$ and $10^{6.5}$ ID_{50}/0.1 ml respectively, when assayed in cultures of the AHI line of grivet monkey kidney cells. Sendai virus was concentrated and resuspended ac-

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cording to Harris' procedure (2), except that the concentrate was centrifuged at 2000 rpm for 10 min just before inactivation with BPL. Hemagglutinin titer of the Sendai virus concentration ranged from 1:2460 to 1:10, 240. Inactivation by UV irradiation was done according to Harris' procedure (2). Hamster embryonic cells of the "I" line developed by G. Th. Diamandopoulos (3) were employed in the eighty-fifth to the ninety-fifth passage.

Inactivation of Sendai virus with BPL. A 10% solution of BPL[2] in 2× distilled H_2O was prepared immediately before use. Dilutions of the 10% solution were made in a saline biciarbonate solution (1.68 gm of $NaHCO_3$ and 0.5 ml of 0.4% phenol red solution were added to 100 ml of isotonic NaCl solution). Diluted BPL was added in the proportion of 1 part to 9 parts of concentrated Sendai virus. Final dilutions of BPL are recorded in the text. All materials employed were precooled by immersion in ice water prior to use. Dilutions of BPL were prepared as quickly as possible at 4°C. After addition of BPL to the concentrated Sendai virus, the preparation was shaken in a tightly closed container for 10 min at 4°C to insure complete mixing and then kept at 37°C for 2 hours. During this period it was shaken by hand at intervals of 10 min. The mixture was afterwards maintained overnight at 4°C to insure complete hydrolysis of BPL. The treated virus suspension was tested for residual infectivity using as inocula 0.1 aliquots of undiluted and diluted virus (1:100) introduced into the chorioallantoic cavity of 10-day chick embryos.

Storage of BPL-inactivated Sendai virus. In all experiments here described inactivated virus was stored at —65°C for short periods (2–3 weeks) before use. We have recently found that on more prolonged storage fusion capacity declines rapidly. It has now been determined that addition of bovine albumin in a final concentration of 0.5% prevents decay of the fusion factor for at least 5 weeks.

Inoculation and fusion of cells. Monolayer cultures were prepared by adding 100,000 to 300,000 "I" line hamster cells suspended in 1 ml Puck's medium to each of a series of 15 × 150 mm test tubes. After incubation at 36°C for 3 days, the growth medium was removed and replaced with 0.8 ml of Eagle's medium containing 2% fetal calf serum (FCS) at pH 7.0–7.2. To each culture 0.1 ml of poliovirus was added as routine and the tubes were placed for 1 hour at 36°C in a roller wheel turning at 0.3 rpm. In certain cases where large amounts of virus were required volumes of 1 ml were employed. The 0.1 ml inactivated Sendai virus concentrate was then added to each. Thereafter incubation at 36°C in the roller wheel was continued for 4 hours, when the fluid was removed and replaced with 1 ml of Eagle's medium containing 2% FCS. The air in each tube was subsequently replaced with a mixture of 5% CO_2 in air. The cultures were incubated in a stationary position at 36°C. In the first series of experiments (Table I) cultures were washed 6× with Hanks' solution without glucose after 16 hours. In later experiments (Tables

[2] Betaprone was purchased from Fellows Testagar Company, Detroit, Michigan.

TABLE I. Poliovirus Enhancing Effect and Fusion in Hamster Cells by Sendai Virus Treated with Beta Propiolactone.

		Poliovirus infectivity titer materials tested[b]	
Concentration of BPL (%)	Syncytia formation[a]	Wash (16 hours)[c]	Whole cult. (day 4)[d]
.03	+++	3.5	5.5
.06	+++	4.5	5.5
.13	+++	0.5	5.0
.25	+	3.0	3.5
.5	0	ND[e]	ND
1.0	0	ND	ND
UV-inactivated Sendai	++	2.5	5.0
No Sendai	0	0.8	2.8

[a] Estimated 90% fusion = +++; 25% fusion = +.

[b] Log ID_{50}/0.1 ml materials tested. Inoculum for all cultures was 0.1 ml stock poliovirus; titer, log ID_{50} = 5.8/0.1 ml.

[c] Cultures washed 6× at 16 hours after inoculation; titer of last washing.

[d] Whole culture frozen and thawed 3× on fourth day after inoculation; titer of fluid after removal of cellular debris.

[e] ND = not done.

TABLE II. Hamster Cell Monolayer Cultures Exposed to Poliovirus I with and without Addition of BPL-inactivated Sendai Virus: Poliovirus Infectivity Titers of Fluids at Various Intervals after Exposure.

		Poliovirus[a] and Sendai[b]			Poliovirus only		
Day	Material	Cult. 1	Cult 2	Cult 3	Cult 1	Cult 2	Cult 3
0	Wash[d]	0.5	1.5	1.0	1.0	1.0	0.5
0	Cells[e]	3.0[c]	—	—	2.5	—	—
1	Fluid[f]		3.5	4.0		2.5	3.0
2	Fluid		5.0	5.0		4.0	4.0
4	Fluid		5.5	6.0		4.0	4.0
8	Fluid		6.5	6.5		3.5	4.0
12	Fluid		5.5	5.5		3.0	3.5
16	Fluid		4.5	5.5		4.0	2.5
22	Fluid		4.0	4.5		2.0	nil[g]
26	Fluid		3.0	3.0		nil	nil
30	Fluid		3.5	3.5		nil	nil
35	Fluid		2.0	2.0		nil	nil
Cumulative titer			6.6	6.7		4.5	4.5

[a] Titer stock polio virus, log ID_{50} = 5.8/0.1 ml; 0.1 ml inoculum used.
[b] 0.1 ml of undiluted 0.13% BPL-inactivated Sendai virus used for fusion.
[c] Log ID_{50}/0.1 ml.
[d] Cultures washed 6×, 5 hours after inoculation.
[e] Cells frozen and thawed 3× immediately after washing 6× and resuspended 5 hours after poliovirus inoculation; titer of fluid after removal of cellular debris.
[f] Cultures washed 6× after each fluid change during 35 day period.
[g] No poliovirus detected.

II–IV) cultures were washed in the same manner after 5 hours.

Poliovirus cytopathic effect and replication. Cultures were daily observed for poliovirus cytopathic effect. Poliovirus infectivity titers of the fluid and of the whole culture were determined at various times. For the latter determination the culture was rapidly frozen and thawed 3×, employing an alcohol bath at about —-65°C and a water bath at 37°C. After centrifuging at 2000 rpm for 10 min the supernatant fluid was collected and tested.

Fixing and staining. Cultures were fixed in Bouin's fluid imbedded in collodion and stained with hematoxylin and eosin (4).

Experimental. Poliovirus growth enhancing effect and fusing properties of Sendai virus treated with serial dilutions of BPL. Serial twofold dilutions of BPL were prepared. An aliquot of each dilution was added to concentrated Sendai virus to give final concentrations of the reagent ranging from 0.03% to 1%. The capacity to induce fusion and to enhance replication of poliovirus of each preparation was determined according to the procedures outlined in Table I. The data presented in this table indicate that, as previously demonstrated in the case of UV-irradiated material, Sendai virus treated with BPL in concentrations ranging from 0.03% to 0.13% fully induced cell fusion. With higher concentrations fusion capacity was diminished or destroyed. It will also be noted that the poliovirus-enhancing capacity of Sendai virus exposed to the lower concentrations of BPL was not impaired whereas larger amounts proved inhibitory. It is also interesting to record here that the hemagglutinating capacity of the same concentrate was not diminished by treatment with BPL in concentrations ranging from 0.03 to 0.25% but was entirely destroyed by concentrations of 0.5 and 1.0%.

Lack of infectivity of Sendai virus concentrates treated with BPL. No evidence of replication of Sendai virus, as indicated by the development of hemagglutinin, was obtained after 2 egg passages of 3 concentrates treated with 0.06% BPL and of 3 concen-

TABLE III. Determination of Poliovirus Minimal Infective Dose in Fused Hamster Cells.

	Poliovirus and Sendai[b]			Fluids exposed to poliovirus only	
Log ID_{50} poliovirus inoculated[a]	Wash[d] (day 0)	Fluid[e] (day 2)	CPE	Wash (day 0)	Fluid (day 2)
7.5	2.0[c]	7.0	+	2.5	5.5
6.5	1.5	6.5	+	1.5	4.5
5.5	0.5	6.5	+	0.5	4.0
4.5	nil	nil	0	nil	nil
3.5	nil	nil	0	nil	nil

[a] Titer stock poliovirus, log $ID_{50} = 6.5/0.1$ ml.
[b] 0.1 ml of 1:5 dilution of Sendai virus inactivated with 0.06% BPL used as fusing agent.
[c] Log $ID_{50}/0.1$ ml.
[d] Cultures washed 6× at 5 hours after inoculation; titer of last washing.
[e] Titer of fluid collected on second day after inoculation.

TABLE IV. Determination of Poliovirus Minimal Infective Dose in Hamster Cells Using Infected Fluids from Fused and Nonfused Cultures as Inocula.

		Fluids exposed to poliovirus and Sendai[a]			Fluids exposed to poliovirus only	
	Log ID_{50} poliovirus inoculated	Wash[e] (day 0)	Fluid[f] (day 2)	CPE	Wash (day 0)	Fluid (day 2)
Poliovirus from fused cells[b]	6.5	2.0[d]	6.5	+	1.0	5.0
	5.5	0.5	6.5	+	0.5	3.5
	4.5	nil	6.5	+	nil	3.5
	3.5	nil	nil	0	nil	nil
Poliovirus from nonfused cells[c]	5.5	nil	6.5	+	0.5	3.0
	4.5	nil	0.5	0	nil	nil
	3.5	nil	nil	0	nil	nil

[a] 0.1 ml of 1:5 dilution of Sendai inactivated with 0.06% BPL used as fusing agent.
[b] Day 2 fluid from poliovirus infected, fused cultures: titer, log $ID_{50} = 6.5/0.1$ ml. (see Table III).
[c] Day 2 fluid from poliovirus infected, nonfused cultures: titer, log $ID_{50} = 5.5/0.1$ ml.
[d] Log $ID_{50}/0.1$ ml.
[e] Cultures washed 6× at 5 hours after inoculation; titer of last washing.
[f] Titer of fluid collected on second day after inoculation.

trates treated with 0.13% BPL. Allantoic fluids from embryonated eggs inoculated with comparable amounts of untreated Sendai virus concentrates demonstrated hemagglutinin titers of at least 1:160 at each egg passage. In addition to this lack of evidence for residual infectivity, it was found that hamster cell cultures exposed to undiluted and diluted BPL-treated Sendai virus showed no cytopathic effects attributable to this agent.

Viability of polykaryocytes formed by BPL-inactivated Sendai virus. Polykaryocytes resulting from the fusion of noninfected hamster cells with 0.13% BPL-inactivated Sendai virus and largely separated from mononuclear elements by differential sedimentation remained viable for at least 2 months as judged by morphological appearance. Moreover, such cells proved resistant to treatment with trypsin (0.05%) since in one experiment they were carried serially through 2 passages *in vitro*, employing Eagle's medium containing 10% FCS and trypsinization performed as routine for subcultivation of "I" cells. The total time

of cultivation of the polykaryocytes was 8 weeks. No indication was obtained that the number or size of the polykaryocytes increased during these passages. It was of interest to find during the course of this experiment that in contrast to the prolonged survival of polykaryocytes formed by BPL-inactivated virus, those induced by treatment with UV-inactivated Sendai survived for no longer than 2 weeks.

Persistence of poliovirus replication in cells fused with BPL-inactivated Sendai virus. In the experiments summarized in Table II, "I" cell monolayer cultures were fused with 0.1 ml of 0.13% BPL-treated Sendai virus after exposure to $10^{5.8}ID_{50}$ of poliovirus. Infectivity titrations of fluids from the cultures were done at intervals during a period of 35 days. Fluids from comparable inoculated unfused cultures included as controls were similarly titrated. At the beginning the amount of cell-associated poliovirus present was assayed by freezing and thawing one of each of the fused and unfused cultures 3×, 5 hours after the virus was added. Immediately before freezing the cultures were washed 6×. Subsequently at each change of medium the remaining cultures were washed 6× after removal of the old fluid for titration and before addition of the new.

The results in Table II show that the amount of poliovirus present in fluids from fused cultures removed at various intervals increased steadily to reach a maximum on the eighth day. The concentration of virus on the eighth day exceeded, by a factor of 3 logs, the cell associated virus present at the beginning of the experiment. Furthermore, it can be calculated that the total yield of virus over the entire period of observation represents at least a 10-fold increase in the total amount of virus originally introduced. In unfused cultures, in contrast, the maximum concentration was attained on the second day and exceeded that of the initial cell-associated virus by a factor of slightly over 1 log. Total virus recovered was approximately equivalent to the total virus originally inoculated. In both systems after the maximal fluid titer was reached, virus appeared in progressively smaller amounts for a considerable period. It was detectable at least through day 36 in fused cultures, and in unfused cultures through the

FIG. 1. "I" cells in monolayer, H & E stain; 2000×.

day 22. The data leave no doubt that viral replication occurred in the fused systems. A consideration of factors that may account for the apparently small increase and persistence of small amounts of virus in unfused cultures will be subsequently presented.

Demonstration of poliovirus cytopathic effect in hamster cells subsequently exposed to BPL-inactivated Sendai virus. In repeated experiments, optimal concentrations of Sendai virus inactivated with 0.06% BPL have been demonstrated to cause fusion of about 90% of hamster embryo cells growing in a monolayer such as illustrated in Fig. 1. Fusion has been apparent as early at 2–4 hours after addition of Sendai virus, with syncytial formation completed by 24 hours. Figure 2 shows the appearance of fused cells after 48 hours. Aggregates of nuclei with as many as fifty nuclei or more arranged as a rosette were numerous (Fig. 2 and 3). The cytoplasm about these aggregates often appeared to be continuous with that of others in the same area. When poliovirus in high multiplicities was added to the monolayer prior to fusion, characteristic cytopathic changes were subsequently noted (Fig. 4). The first of these consisted in the early disruption and fragmentation of the cytoplasm around the nuclear aggregate. This

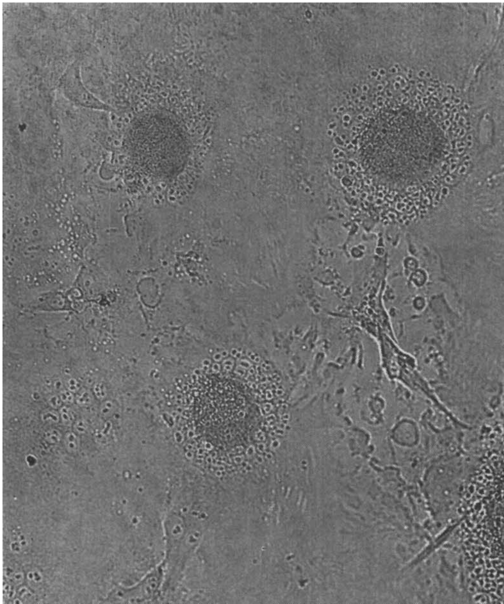

FIG. 2 "I" cells 48 hours after addition of Sendai virus, fresh preparation, showing syncytium with nuclear aggregates. 800×.

process advanced rapidly until only remnants of cytoplasm remained which often extended about the aggregate in elongated processes. Individual nuclei then became shrunken and pyknotic until all were thus affected. Eventually fragmentation of the nuclei and disintegration of the whole complex ensued. Such changes have been seen as early as 18–24 hours after fusion and were generally far advanced by 48 hours. They have not been noted later than 4 days after inoculation of the virus. When the cytoplasm appeared to be continuous between multiple nuclear aggregates, the entire complex became affected within 24 hours, with the exception of only a few associated monokaryocytes and small polykaryocytes (Fig. 4). Cytopathic effects of this character have been consistently observed in poliovirus-infected fused cultures. They have not been seen either in unfused, poliovirus-infected cultures or in uninfected, fused cultures, although in the latter very large polykaryocytes tend to retract and become detached from the glass within 2–3 days.

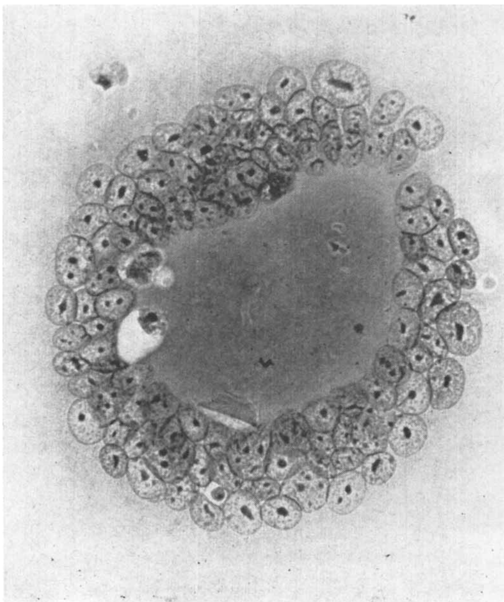

FIG. 3. "I" cells 48 hours after addition of Sendai virus, H & E stain. A nuclear aggregate. 2000×.

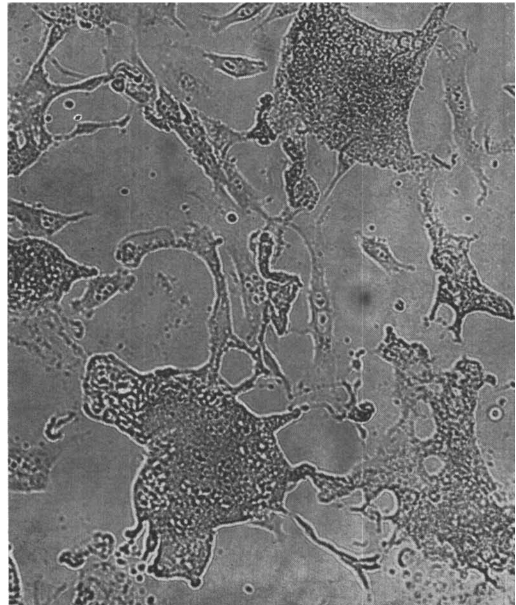

FIG. 4. "I" cells 48 hours after addition of poliovirus and Sendai virus, cytopathic effect of poliovirus, fresh preparation. 800×.

Minimal infective dose of poliovirus in fused cells. To determine the smallest quantity of poliovirus capable of replication in fused cells, dilutions of stock poliovirus were prepared and added to monolayer cultures. One set of cultures was fused with 0.1 ml of a 1:5 dilution of 0.06% BPL-treated Sendai virus

concentrate and another set served as unfused, inoculated controls (Table III). Cultures were washed 6×, 5 hours after inoculation of poliovirus and were then observed for 1 week for cytopathic effects. Fluids were collected on day 2 and day 7 for titration. In both fused and unfused cultures, poliovirus was found in fluids of day 2 in those cultures that were exposed to $10^{5.5}$ ID_{50} poliovirus or more. In fused as compared with unfused cultures the titer of poliovirus was at least 100× higher. Tests of 7-day fluids gave comparable results. Cytopathic changes were observed after 1–3 days only in fused cultures in which poliovirus replication was demonstrated.

Replication of poliovirus grown in fused and unfused cells. Replication of poliovirus derived from fused cell cultures as compared with that of virus from unfused cultures was determined in these systems, employing as inocula the 2-day fluids obtained in the experiment presented in Table III. The procedures and results are summarized in Table IV. Although the minimal infecting dose of virus from fused cultures was slightly lower, this difference cannot be regarded as significant. The findings demonstrate, however, as shown in the previous experiment (Table III) and by Enders and his associates (1) that a relatively large dose of virus is required to initiate infection in the fused cell system. In addition the results indicate that poliovirus can be passed serially in the fused cell system provided a sufficiently large inoculum is employed.

Discussion. Technically the results are of value since they provide more reliable and simple means of studying the enhancement of viral replication by cell fusion than those previously employed (1). Complete inactivation of the infectivity of Sendai virus by small amounts of BPL is easily accomplished without reduction in its fusion capacity. The reagent is thereafter rapidly hydrolyzed at 37°C, leaving products that are not viricidal and that in suitable concentrations appear to have little or no injurious effect on the "I" line of hamster cells, nor on human fibroblasts, chick embryo cells, or additional lines of hamster embryo cells, as we have determined in experiments not described here. Concentrated BPL-inactivated Sendai virus can therefore be employed as a cell fusing agent under circumstances in which neither the infectivity of the virus to be propagated nor the cellular substrate is noticeably affected. Accordingly, in experiments of this kind BPL-inactivated Sendai virus is to be preferred to UV-inactivated preparations which often contain residual infective virus and exhibit a varying degree of cell toxicity. The relative lack of cytotoxicity of BPL-inactivated Sendai virus would also seem to recommend it in other investigations, such as those on cell hybridization or in analyses of the chemical nature of the fusion factor itself.

The demonstration that cells growing in a monolayer undergo rapid and extensive fusion makes it unnecessary to use cell suspensions such as we employed in our original experiments, and in which repeated centrifuging is required. Furthermore, fusion in monolayer cultures appears to be usually more uniform and extensive.

Adoption of the monolayer culture and BPL-inactivated Sendai virus has enabled us to determine that poliovirus regularly induced cytopathic changes in fused cell complexes. In our earlier experiments, as already stated, degenerative changes in polykaryocytes exposed to this agent were observed, which at first were more pronounced than those seen in uninoculated cultures. However, because of the toxic and cytopathic effects of the UV-treated Sendai concentrate, we could not conclude that the more pronounced effects were induced by poliovirus. The recognition of specific cytopathic changes in BPL-fused hamster cells offers a convenient criterion for the replication of poliovirus and so increases the possible usefulness of the technique should it be found applicable to the cultivation of other cytopathic agents in resistant cell systems. From the biological point of view the close similarity between the cytopathic effects noted in fused resistant cells and in susceptible cells such as those of simian or human origin is not without interest. It suggests that the normal cellular factors which in the susceptible cell are affected by the virus in such a manner as to result in morphologically apparent injury are also present in the resistant cell.

The experiments here described contribute little toward the answers to two interrelated questions raised by our previous findings, i.e., what is the mechanism of enhanced poliovirus replication in fused systems of resistant cells? Are all the cells in the unfused systems we have studied completely resistant to this agent? It was originally postulated that in the presence of fusing cells poliovirus particles might be mechanically incorporated within the newly forming polynucleate complexes. The results presented in Table I afford further evidence that enhanced viral replication in the hamster cell system is dependent upon cell fusion, since the infectivity titers vary directly with the degree of fusion observed microscopically. These data, however, leave the manner in which the virus is introduced into the fusing complex still unclear. This might occur as we originally imagined, by physical incorporation of contiguous virions within the merging cytoplasm of individual cells, or as a result of focal impairment of the mononucleate cell membrane by the fusion factor or some other viral constituent, thus opening a way for the virus. A third mode of virus incorporation is suggested by the consistent finding in these as well as earlier experiments that a slight to moderate increase in poliovirus titers occurs in unfused systems. This increase may represent replication of the virus in a few susceptible cells or depend upon adsorption to resistant cells of viral aggregates in the original inoculum which subsequently are slowly dispersed. Which of these alternatives is correct must await further experimentation. However, the incorporation of such virus-associated cells, regardless of the nature of the association, into the syncytium might presumably serve to initiate the widespread infection observed.

The factors underlying the observed persistence of poliovirus over prolonged periods in both fused and unfused systems in slowly decreasing concentration, have also not yet been defined. Although the prolonged recovery of virus is *a priori* most readily attributable to its replication in a decreasing number of cells, preliminary results of experiments now in progress fail to support this hypothesis. We therefore intend to investigate the possibility that the slow dispersion of cell-associated aggregates may be responsible here as well as in the early apparent increase in titer found in unfused cell cultures.

Summary. Poliovirus can replicate and be passed serially in monolayer cultures of a line of hamster embryo cells subsequently fused with Sendai virus completely inactivated by low concentrations of beta propiolactone. Poliovirus fused into such a system produces a characteristic cytopathic effect.

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Intracranial Action of Oxytocin on Sodium Excretion by Conscious Dogs* (32669)

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Oxytocin is natriuretic in conscious dogs when the rate of urine flow is relatively low (1,2). Brooks and Pickford (1) found that small doses injected into the carotid circulation were natriuretic whereas doses 10–20 times as large were needed to produce com-

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