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Metabolism of Human Amnion Cell Cultures Infected with Poliomyelitis Virus. I. Glucose Metabolism During Virus Synthesis.¶ (23650)

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Human amnion cell cultures have been shown to be highly susceptible to infection with various polioviruses(1,2). Thus, a convenient system is at hand for studying metabolic changes in host cells during infection with the virus. In this work glucose uptake of infected and control cell cultures was measured, and with the aid of various metabolic inhibitors reactions important for virus reproduction were indicated.

Materials and methods. Preparation of amnion cell cultures. The technic deviates in a number of details from procedure described by Zitcer *et al.*(1), and modifications by Takemoto and Lerner(3), Weinstein *et al.* (4) and Lahelle(5). Human placentas† collected in sterile towels were brought to laboratory within a few hours after delivery. The amnion membrane was stripped off, washed several times with phosphate buffered saline (PBS) and blood clots were removed. The membrane was then cut into 3-4 pieces, put in Erlenmeyer flasks, and covered with pre-warmed Hanks' salt solution containing 0.25% trypsin (N.B. Co. 1/300), pH-7.6. After 1 hour, trypsin was poured off and replaced by fresh pre-warmed 0.1% trypsin solution (pH-7.6) and the amnion left overnight at room temperature. Flasks were always kept tightly stoppered. The next morning fresh trypsin solution was added and the flasks vigorously shaken by hand. This procedure was repeated 3-4 times, employing fresh (0.1%) trypsin solutions each time, till most cells were set free. The combined fluids were centrifuged at 800 rpm 8 minutes, and the sediment washed twice with sterile PBS. Total count of cells was determined in hemocytometer after staining with crystal violet solution(6), while percentage of non-viable cells was estimated from another sample stained with trypan blue(7). Suspensions showing more than 10% cells stained with trypan blue were discarded. Finally, cells were resuspended to contain 500,000/ml in a medium consisting of 4 parts of 0.5% lactalbumin hydrolysate in Hanks' solution and 1 part of calf serum. The pH of medium was adjusted to 7.4. As seed 0.5 ml of this suspension was used for tubes, and 13 ml and 50 ml for milk bottles and Roux flasks, respectively. All containers were stoppered with rubber and incubated at 37°C; tubes were kept in slightly slanted position. After 48 hours the medium was replaced by Hanks' solution containing 0.5% lactalbumin hydrolysate to which 2% calf serum and 0.5% chick embryo extract were added; 1 ml of this medium was dispensed into tubes. A confluent sheet of cells was obtained within 3 to 5 days. The maintenance medium for these cultures was composed as follows: 0.5% lactalbumin hydrolysate in Earle's salt solution and 2% of calf serum. All solutions and media contained 100 units of penicillin and 100 μg of streptomycin/ml. Mycostatin (30 units/ml) was added to membranes during

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overnight trypsinization and to all media. After infection of cell cultures with poliovirus, a medium was added which contained amino acids and vitamins in concentrations as used by Eagle for cultivation of HeLa cells(8). Hanks' solution and glucose 500 μg/ml. However, it contained no serum. *Monkey kidney cell cultures.*‡ Some experiments were performed with rhesus monkey kidney cells in addition to those using amnion cell cultures. For this purpose one Roux bottle containing a monolayer of trypsinized monkey kidney cells was used. The cells were harvested by the versene method(9), and grown in test tubes 3-4 days in medium 199, till monolayers were formed. *Virus.* The MEF_1 strain of poliovirus type II was used. Pools of the virus were prepared from infected amnion cell cultures grown in Roux bottles. The supernatant containing the virus was separated from cell debris by centrifugation, and its titer determined by 10-fold dilutions in test-tube-cultures by examination of the cytopathogenic effect. Two to 4 tubes were used dilution, and the $TCID_{50}$ was determined by Reed & Muench method(10). A titer of $10^{7.0}$/ml was usually obtained. The virus was preserved at –20°C. In a number of experiments inactivated virus was employed. For this purpose virus suspensions were heated in water bath for 30 minutes at 56°C. or for 5 minutes at 100°C. At both temperatures complete inactivation of virus was obtained. Neutralization of virus with immune monkey serum§ was also performed. Equal volumes of virus suspension and serum diluted 1:5 were mixed and kept at room temperature for 30 minutes. A control of normal monkey serum was also employed. No rise of virus titer was observed following infection of cultures with immune serum treated virus, whereas normal serum had no inhibiting effect on virus production. *Infection of cultures.* Pooled fluids of 10 test tube cultures or of one milk bottle were examined. Repeated counts gave values of 2 x 10^5 cells

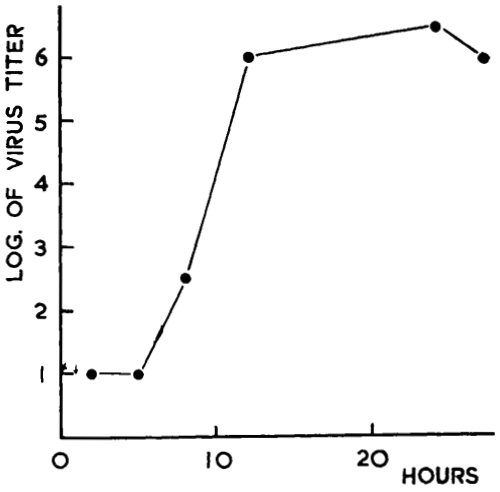

FIG. 1. Growth curve of MEF_1 strain of poliovirus type II in human amnion cell cultures. Cell cultures incubated with poliovirus for 1 hr at 37°C, then washed with PBS to remove unadsorbed virus and reincubated after addition of modified Eagle medium.

tube, and 2-3 x 10^6/milk bottle. Infection of cultures was carried out by adding sufficient virus to give a multiplicity of 5. The virus was left in contact with cell cultures for 1 hour at 37°C. The cultures were then washed 2-3 times with PBS to remove unadsorbed virus, the modified medium of Eagle was added and cultures reincubated at 37°C. Aliquots were taken immediately after addition of new medium and after various time intervals (1, 3, 5, 7, 9, and 24 hours). Uninfected control cultures were similarly treated. Appearance of cytopathogenic changes due to virus multiplication, was followed by microscopic examination. At the same time determinations for presence of glucose and phosphorus in the aliquots were also made. Glucose was examined by the Somogyi-Nelson photometric method(11) and phosphorus by the Fiske-Subbarrow method(12). *Inhibitors.* The influence of a number of inhibitors on glucose uptake by control and infected cell cultures was examined. Potassium cyanide (KCN), sodium azide (NaN_3), sodium fluoride (NaF), sodium monofluoroacetate (NaFAc), and monoiodoacetic acid (IAc) were used in concentration of M/100-M/1000. Solutions of inhibitors were prepared freshly in PBS. Inhibitors were added 0.1 or 0.2 ml to cell cultures immediately after addition of Eagle modified medium. Controls

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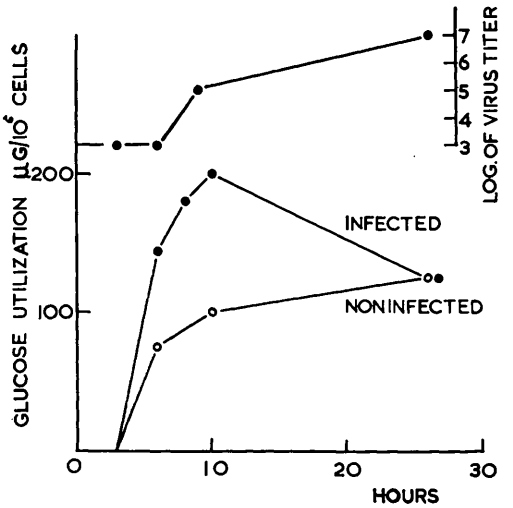

FIG. 2. Glucose uptake by non-infected and infected human amnion cell cultures during poliovirus production. Cell cultures incubated with poliovirus for 2 hr at 37°C, then washed with PBS to remove unadsorbed virus and reincubated after addition of modified Eagle medium.

were supplemented with same amounts of PBS which were found without effect on cell metabolism; aliquots for analysis were taken at intervals.

Results. Virus multiplication. A typical growth curve of the virus is given in Fig. 1. After a lag period of 3 hours the amount of virus in the culture fluid increased steadily until 12 hours after infection. In other amnion cultures release of the virus was delayed for 3-7 hours, but maximal titer of virus was reached about 12 hours thereafter.

Uptake of glucose. Aliquots that were used for determinations of virus titer were also analyzed for glucose content. Results are given in Table I and a typical curve in Fig. 2.

TABLE I. Uptake of Glucose by Infected and Control Human Amnion Cell Cultures.

Exp.		Hr postreincubation* 1	3	5	7	9	24
1	Infected	180†	160	240	320	400	480
	Control	100		120	260	320	400
2	Infected	0‡	200	240	380	200	
	Control	0	180	240	240	240	
3	Infected		145	180	200		125
	Control		75	145	100		125
4	Infected		142	105	105		137
	Control		0	67.5	167		228
5	Infected	0		7.5	45.0		77.5
	Control	0		25.0	27.5		77.5
6	Infected	0		0	12.5	22.5	22.5
	Control	0		0	0	12.5	35.0

* Cell cultures incubated with poliovirus for 1 hr at 37°C, then washed with PBS to remove unadsorbed virus and reincubated after addition of modified Eagle medium.

† Results of cumulative glucose disappearance are expressed in $\mu g/10^6$ cells originally present in culture.

‡ 0 = no glucose consumed.

TABLE II. Glucose Uptake by Human Amnion Cell Cultures, Non-infected and Infected with Active and with Neutralized Poliovirus.

Hr post-reincubation*	Cell cultures: Non-infected	Infected	Infected with neutralized virus
1	.0†	10.7†	.0†
3	.0	23.6	.0
5	.0		.0
7	.0	40.7	.0
9	.0	30.1	.0
21	15.7	.0	22.5
28	29.0	.0	22.5

* Cell cultures incubated with poliovirus for 1 hr at 37°C, then washed with PBS to remove unadsorbed virus and reincubated after addition of modified Eagle medium.

† No. represents uptake of glucose in %, cumulative from time 0, postreincubation.

Different amnion preparations showed considerable variations in glucose uptake. However, in all experiments the infected cell cultures consumed glucose more vigorously than non-infected cultures. It seems of significance to note that this increased glucose uptake precedes liberation of virus into the culture fluid, and it ceases during liberation of virus from injured cells. On the other hand, an undisturbed utilization of glucose is found in control cell cultures. In some experiments, virus release was accompanied by an increase of reducing substances in the culture fluids. No stimulation of glucose uptake was observed when cells were brought in contact with heated inactivated virus or when virus production was prevented by addition of specific antiserum (Table II).

Utilization of inorganic phosphate. As with glucose, the uptake of phosphorus was higher in infected cultures as compared to the controls. However, with liberation of free virus, a simultaneous release of inorganic

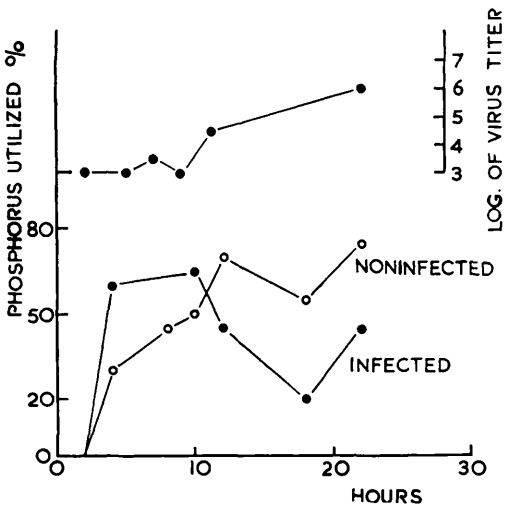

FIG. 3. Uptake of inorganic phosphorus by non-infected and infected human amnion cell cultures during virus production given as percentage of phosphorus in medium added after washing. Cell cultures incubated with poliovirus for 1 hr at 37°C, then washed with PBS to remove unadsorbed virus and reincubated after addition of modified Eagle medium.

phosphate was found in the culture fluids (Fig. 3).

Effect of inhibitors on glucose metabolism and virus multiplication in amnion cell cultures. Potassium cyanide (M/1000) neither affected the viability of cell cultures nor the synthesis of poliovirus within infected cell cultures; virus yield was equal in cyanide treated and untreated cultures. Both onset and extent of the characteristic cytopathogenic effect were the same in presence or absence of the inhibitor. However, addition of cyanide stimulated glucose utilization of infected cultures in comparison to the controls (Fig. 4). Addition of NaN_3 (M/1000) and NaFAc (1 mg/10^6 cells) did not affect, within observation period of 48 hours, the viability of amnion cell cultures. In the presence of inhibitors the same cytopathogenic changes appeared as in infected non-treated cultures. As in the case of cyanide, glucose utilization was increased in infected cultures by addition of azide or NaFAc. In contrast to the effects of cyanide, azide and fluoroacetate, NaF and monoiodoacetic acid (IAc) depressed glucose uptake in both control and infected cell cultures (Table III). Cultures so treated showed early cytological damage, but no cytopathogenic changes attributed to virus multiplication were observed. No multiplication of virus was found to occur in cell cultures inhibited by IAc.

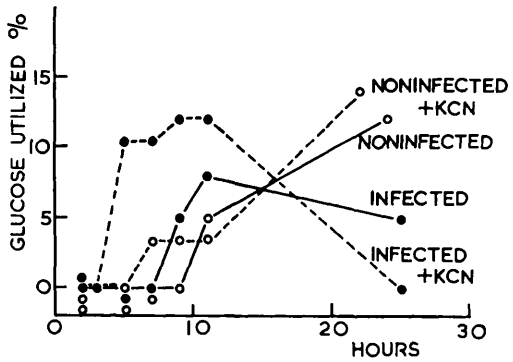

FIG. 4. Effect of cyanide (M/1000) on glucose uptake by non-infected and infected human amnion cell cultures given as percentage of glucose in medium added after washing. Cell cultures incubated with poliovirus for 1 hr at 37°C, then washed with PBS to remove unadsorbed virus and reincubated after addition of modified Eagle medium.

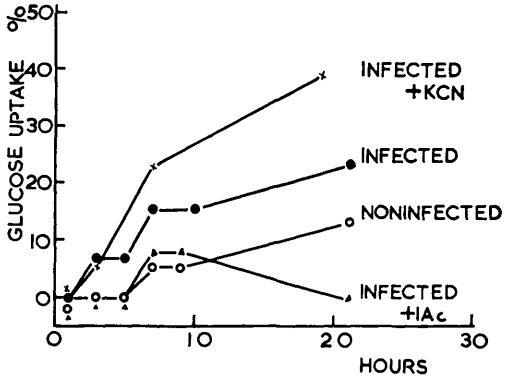

FIG. 5. Glucose uptake by non-infected and infected monkey kidney cell cultures given as percentage of glucose in medium added after washing. Effect of cyanide (M/1000) and iodoacetic acid (M/100) on glucose uptake by infected cells. Cell cultures incubated with poliovirus for 1 hr at 37°C, then washed with PBS to remove unadsorbed virus and reincubated after addition of modified Eagle medium.

Glucose metabolism in infected and control monkey kidney cell cultures. The same methods as used for human amnion cell cultures were employed. Kidney cell cultures infected by poliovirus showed stimulation of glucose uptake as compared to non-infected cell cultures. The data are given in Fig. 5. As with amnion cell cultures KCN (M/1000) stimulated and IAc (M/100) inhibited glucose uptake by infected cell cultures.

TABLE III. Inhibition of Glucose Uptake by M/1000 Iodoacetic Acid and NaF in Human Amnion Cultures, Non-infected and Infected by Poliovirus.

Hr post-reincubation*	Infected cultures	Non-infected cultures	Infected + NaF	Non-infected + NaF	Infected + IAc	Non-infected + IAc
3	35 †	0	0	0	0	0
5	"	0	14.7	0	14.7	0
7	"	35	"	14.7	"	0
9	44.8	"	"	"	"	14.7
19	64.7	64.7	35	35	"	"

* Cell cultures incubated with poliovirus for 1 hr at 37°C, then washed with PBS to remove unadsorbed virus and reincubated after addition of modified Eagle medium.

† Results of cumulative glucose disappearance are expressed in $\mu g/10^6$ cells originally present in culture.

Discussion. Experiments on growth of MEF_1 poliovirus in human amnion cell cultures indicated that a latent period of 3-7 hours, during which infected cells apparently propagated virus without releasing it into the medium, followed adsorption of virus to cells. Thereafter, virus titer of the supernatant culture fluid increased rapidly to a maximum approximately 7-12 hours after the end of the latent period. Similar results have been obtained with monkey kidney and HeLa cell cultures(13,14), while Dunnebacke(15,16), working with amnion cultures, obtained a slower growth rate.

Glucose is utilized more rapidly by infected cells as compared to non-infected cultures. This is particularly evident in early hours(1-3) after infection, when the titer of the virus in the culture fluid is still low, and no cytopathogenic effect is seen (Fig. 2). With the manifestation of the cytopathogenic effect and the rise of the virus titer an increase in the reducing capacity of the culture fluid is found. This may be interpreted either as a release into the medium of cell-bound glucose from infected cells, or as a release of reducing substances other than glucose. Since glucose is an easily metabolizable substrate, the former possibility is preferred. When heat or antiserum inactivated virus was applied to cell cultures instead of active virus, glucose uptake was identical to that of the control cultures. The increased uptake of glucose may be, therefore, related to the presence of active virus production in the cells. These findings are in disagreement with those of Franklin *et al.*(17) who found a diminished uptake of glucose in poliovirus-infected cultures. In their work, however, tissue cultures from human embryonic brain and cord were used, and glucose determinations were made after much longer intervals. Eagle and Habel(18) who determined the need of glucose and glutamine for virus synthesis by HeLa cells, postulated that the carbons of these substrates participate in the synthesis of viral nucleic acids by the host cells. The increased uptake of glucose by human amnion cell cultures upon infection with poliovirus is in agreement with this hypothesis (18).

Glucose uptake by control and infected cell cultures was stimulated by KCN, NaN_3 and NaFAc, however, infected cells utilized glucose more rapidly. This increase in glucose uptake should be attributed to increase in rate of glycolysis of the infected cells induced by blocking Krebs' cycle reactions. This interpretation is also favored by the enhanced uptake of inorganic phosphate as well as the augmented incorporation of P^{32} by HeLa cells infected with poliovirus type I(19). Since virus multiplication is not affected by inhibition of oxidative reactions, the importance of glycolysis for virus synthesis becomes even more evident.

Gifford and Syverton recently reported(20) that anaerobic conditions allow replication of poliovirus in HeLa and monkey kidney cells. Levy and Baron(21) showed increased lactic acid formation by monkey kidney cultures infected with Saukett strain of poliovirus type III. The stimulation of lactic acid formation took place under conditions of anaerobic and aerobic glycolysis. Thus, a similarity to our results obtained with human

amnion and monkey kidney cell cultures is evident. We feel, however, that uptake of a substrate (glucose, phosphate) by infected cells is more intimately connected with the process of virus multiplication than is the release into the medium of a metabolic end product (lactic acid).

Findings similar to these were recently reported for adenoviruses in HeLa cultures(*22*). Therefore the increased glucose uptake may be regarded to be involved with virus reproduction in general. Although at present it cannot be eliminated with certainty that the described changes are a reflection of non-specific symptoms of cell injury rather than viral infection, the *early* stimulation of some metabolic processes favors the assumption that a specific response is involved.

Summary. 1. A typical growth curve of poliovirus type II in human amnion cell cultures is reported. 2. During the early hours of virus multiplication and prior to onset of cytopathogenic effect human amnion cells showed a higher uptake of glucose than uninfected controls. This uptake was retarded with the active release of virus into the culture fluid. 3. Phosphorus was taken up at an increased rate by cells soon after infection, and was released back into the medium with the rise in virus titer. 4. Inhibitors of the oxidative pathways of glucose metabolism affected neither cell maintenance nor virus reproduction. It is assumed that glycolytic rather than oxidative processes are important for reproduction of poliovirus within human amnion and monkey kidney cell cultures.

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