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Biochemical Changes in HeLa Cells Associated with Infection by Type 2 Adenovirus. (23592)

HILTON B. LEVY, WALLACE P. ROWE, LEROY F. SNELLBAKER AND JANET W. HARTLEY
U. S. Department of Health, N.I.H., Nat. Inst. of Allergy and Infectious Diseases, Bethesda, Md.*

Infection of tissue culture cells with poliomyelitis virus has been shown to result in a rapid alteration of cellular metabolism, with temporary increased production of lactic acid and suppression of uptake of radioactive glycine(1). In their overall effects on HeLa cells, adenoviruses present a marked contrast to the poliovirus system previously studied; whereas poliovirus appears to suppress cellular metabolism with overall inhibition of acid production(2), adenoviruses stimulate acid production by infected HeLa cells(3,4) and do not interfere with the incorporation of vital stains for long periods after complete cytopathic effects(5). Consequently, experiments were conducted to determine some of the biochemical changes accompanying adenovirus infection of HeLa cells. A high multiplicity of virus to cells was employed in order to obtain as nearly synchronous infection as possible. Experiments were designed to survey a broad area of biochemical activity, with more detailed observations to follow upon any positive observations. This report will deal with the effect of the time course of the infection on the production of lactic acid by HeLa cells, and on the rate of their uptake of P^{32} and glycine-2-C^{14}, as well as with the distribution of these isotopes among several biochemical fractions after 5 hours of infection.

Materials and methods. HeLa cell cultures. HeLa cells† were grown in 2 oz. or 32 oz. prescription bottles, in a medium consisting of 10% human serum in Eagle's basal medium (BME)(6). In the majority of experiments, all cultures were prepared from a single pool of cells, the tissues were re-fed at 2 or 3 days, and the cultures used after an additional 2 days; only cultures with confluent cell sheets were used. Immediately before virus inoculation, the growth medium was removed and the cultures were rinsed with BME or 3:1 Hanks-Simms solution(7, 8). The 2 oz. bottle cultures were washed 3 times with 5 ml, and the 32 oz. cultures 2 or 3 times with 20 ml. Maintenance medium was 5% chicken serum in Eagle's basal medium, in a volume of 5 ml for the 2 oz. bottles and 40 or 50 ml for the 32 oz. bottles. All media contained penicillin, 250 u/ml, and streptomycin, 250 μg/ml. *Virus.* The Ad. 6 strain of adenovirus type 2, was used throughout. Virus pools were prepared in HeLa or KB cell(9) cultures: 4 pools were used, each of which had an infectivity titer for HeLa cells of $10^{8.5}$ $TCID_{50}$ per ml. The 2 oz. bottle cultures received 0.2 ml, and the large bottle cultures 1.6 ml of the undiluted virus suspension. This dose of virus produced complete cytopathogenic effects with beginning loss of cells into the culture medium within 18 to 24 hours after inoculation of the HeLa cell cultures. Control cultures were inoculated with an equal volume of medium from uninfected cultures of the same type as were used for production of the virus. Both virus and control inocula were clarified by centrifugation at 2,000 rpm for 10 minutes. *Radioactivity.* Radioactive inorganic phosphate was received from the Atomic Energy Commission, Oak Ridge, Tenn. Glycine-2-

* Lab. of Infect. Dis.

† Seed cultures were obtained from Microbiological Associates, Bethesda, Md.

C^{14} was purchased from Tracerlab, Inc., Boston, Mass. The amino acid generally had a specific activity of 200 μc in about 14 mg of compound. Aliquots of the material to be counted were deposited into weighed stainless steel planchets, dried, reweighed and counted. Where necessary, corrections were made for self absorption of the C^{14}. In some experiments both P^{32} and glycine-2-C^{14} were added to the same tissue culture. In such cases, the combined P^{32} counts and C^{14} counts of the sample were determined first and then the counts capable of penetrating an absorber of 24.2 mg/cm^2 were measured. This latter absorber permitted about 80% of the beta particles from P^{32} to come through with virtually none of the beta particles from C^{14} penetrating. By suitable calculation, the counts per minute for P^{32} and C^{14} were obtained. *Lactic acid* was determined by the method of Barker and Summerson(10). *Nucleic acids* were determined by the Schneider method(11) using diphenylamine as the chromogenic agent for DNA(12), and phloroglucinol(13) for RNA. *Phosphorus:* The method of Gomori(14) was used. *Two basic types of experiment* were performed. In the first type, the effect of infection of HeLa cells with type 2 adenovirus on the uptake of glycine-2-C^{14}‡, on the uptake of P^{32} and on the production of lactic acid was studied at selected times after infection. In the second type the incorporation of the isotopes into several biochemical fractions of infected and control cells was compared after 5 hours of infection. Four experiments of the first type were performed, and 3 of the second. The results of all experiments were essentially in agreement, with the three fractionation experiments furnishing additional confirmatory evidence for the experiments dealing with isotope uptake by whole cells. One experiment of each type will be described in detail. In order to indicate the extent of variation from one experiment to the next, data of another experiment, similar except for minor details, will also be presented.

‡ "The uptake of glycine-2-C^{14}" in the text, means uptake of the radioactivity of glycine-2-C^{14} and not necessarily the uptake of the intact compound.

Results. Effect of type 2 adenovirus on metabolic activities of HeLa cells during 96 hours of infection. The medium was poured off from one hundred twenty 2 oz. prescription bottles containing a fairly heavy continuous sheet of HeLa cells. The cells were washed 3 times with 2 ml of medium and then fed with 5 ml of maintenance medium. To half the flasks was added 0.2 ml of virus, representing about $10^{7.8}$ $TCID_{50}$. The ratio of virus to cells was about 70. To the other flasks, which served as controls, was added 0.2 ml of medium of the type in which the virus was suspended. This medium had served as nutrient for HeLa cells for a few days in order to have it comparable to the virus inoculum. The bottles were placed at 37°C and allowed to incubate. Groups of 8 infected bottles and 8 control bottles were preselected for removal after increasing periods of time. Thirty minutes before a given group of infected and control bottles were scheduled to be removed, they received 0.2 ml of a solution containing 0.1 μc of glycine-2-C^{14} and 0.1 μc of inorganic P^{32} in saline. When these bottles were taken for analysis, the growth medium was poured off, the cells were scraped into the medium with a rubber tipped policeman, like suspensions were pooled, the cells centrifuged out and washed 4 times with 40 ml each time of 0.85% NaCl in the cold. The medium was frozen and later used for lactic acid analysis. The packed cells were suspended in 0.5 ml of saline and transferred to stainless steel planchets, dried for 2 hours at 130°C, and weighed. The observed weight was corrected for the weight of NaCl arising from the saline to give the net weight of cells. The amount of radioactivity, as C^{14} and as P^{32} was determined as described under methods, and the data expressed as cpm/mg dry weight of tissue. The data for the uptake of the radioactivity of glycine are summarized in Fig. 1 and for the uptake of P^{32} in Fig. 2.

Since in all cases the radioactive solutions were in contact with the cells only for the 30 minute period just prior to their removal, what is shown in Fig. 1 and 2 is the effect of time of infection on the rate of uptake of the isotope, not on the cumulative uptake.

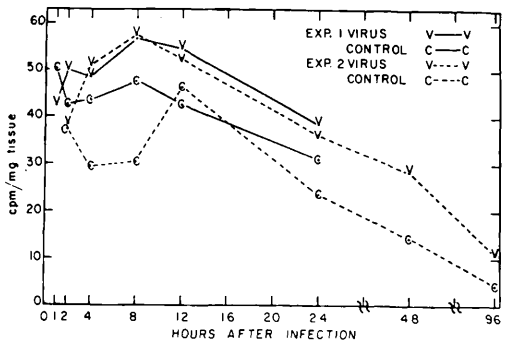

FIG. 1. Effect of type 2 adenovirus on uptake of radioactive glycine by HeLa cells.

It will be seen that early in the infection, and before the time when new adenovirus is thought to be produced(5,15), there was a definite increase in the uptake of P^{32} and C^{14}. This stimulative effect of the virus was still manifest after 48 hours and possibly longer.

The effect of virus infection on the glycolysis of the cells was determined in the following way. The amount of lactic acid present in the fluid immediately after addition of virus (zero time) was found by taking 8 infected and 8 control bottles at the time of infection and removing cells as described above. Lactic acid content was determined in these fluids. This was used as a blank figure to subtract from the amount of lactic acid found in the samples taken off after varying periods of infection. The net amount of lactic acid so calculated represents the amount of lactic acid accumulated in the fluids in the period of time subsequent to the initiation of the experiment. The data from the experiment described here as well

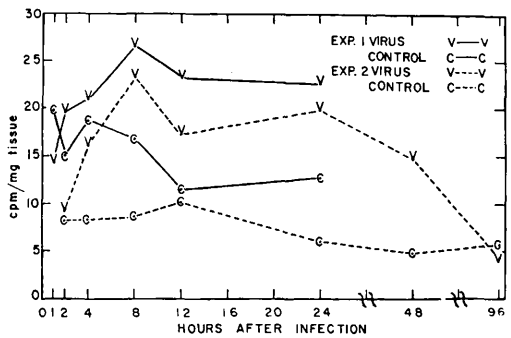

FIG. 2. Effect of type 2 adenovirus on uptake of radioactive inorganic phosphate by HeLa cells.

as those of another experiment are represented in Fig. 3, where the accumulated lactic acid per mg of cells is plotted against time after addition of the inoculum.

An increased lactic acid accumulation was always found in the infected cells as compared with controls. In the experiments shown in Fig. 3, this rise was apparent after 20-24 hours, but in other experiments it was seen as early as 8 hours after infection. It will be noted that in Exp. 3 there was a marked rise in lactic acid accumulation after 24 hours of incubation, while such rise was not seen in Exp. 1. Since the glucose of the original medium is probably used up by 24-36 hours, one possible interpretation of the difference between the two experiments is that the cells used in Exp. 3 had the capacity to produce lactic acid from some other substrate, while those of Exp. 1 either did not

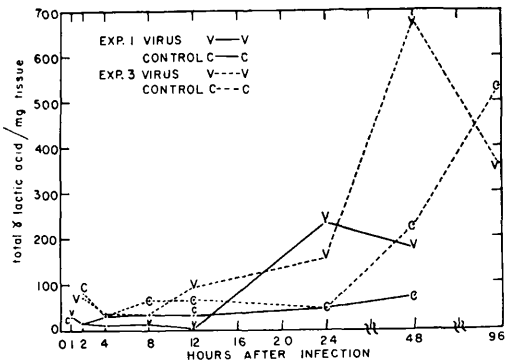

FIG. 3. Effect of type 2 adenovirus on lactic acid production by HeLa cells.

or destroyed the lactic acid so formed more rapidly. However, we have no evidence on this point.

Biochemical fractionation. The data above deal with the uptake of radioisotope by the whole cells; this uptake is of course a reflection of a weighted average of the uptakes by the different biochemical fractions. We studied the effect of infection on the uptake of the radioisotopes by several gross biochemical fractions.

The medium was poured off twenty 32 oz. Blake bottles containing solid sheets of HeLa cells. The cell sheet in each bottle was washed twice with 20 ml of Hanks-Simms solution, and 40 ml of Eagle's basal medium

containing 5% chick serum were then added. To half of the bottles were added 1.6 ml of virus and to the other half 1.6 ml of used growth medium similar in composition to that of virus inoculum. The virus/cell ratio was about 50. The bottles were placed at 37°C for 4½ hours, at which time half of the infected and half the control bottles received 2 μc of glycine-2-C^{14} and the other half received 8 μc of P^{32}. The 20 Blake bottles were thus divided as follows: 5—infected cells + P^{32}; 5—control cells + P^{32}; 5—infected cells + glycine-2-C^{14}; 5—control cells + glycine-2-C^{14}. At the end of an additional 30 minutes the fluid was poured off and the cells were scraped into 20 ml of 0.85% NaCl. Comparable cells were combined and centrifuged lightly in the cold. The cells were washed by centrifugation 3 additional times with 40 ml each time of cold 0.85% NaCl. The packed cells were made up in 0.85% NaCl to 10 ml, 1 ml was plated into weighed stainless steel planchets, while another 1 ml was taken off and stored in a freezer for the determination of total phosphorus. To the remaining 8 ml of cells was added 0.8 ml of 100% trichloracetic acid solution, and the flasks were allowed to stay overnight in the refrigerator. This and subsequent treatment of the cells is described qualitatively in the accompanying flow sheet, along with the analyses performed on the indicated fraction.

The data from 2 experiments are summarized in Tables I, II and III. In Table I are given the data relevant to phosphorus quantitation and to P^{32} uptake; in Table II those relevant to uptake of the radioactivity of glycine-2-C^{14}; in Table III, the individual and averaged values of RNA and DNA concentration.

In all 3 experiments, uptake of P^{32} into all fractions (with the occasional exception of lipid) was stimulated by the virus. On the other hand, the stimulatory effect of virus on the uptake of glycine-2-C^{14} by the cells was actually a reflection of the increased radioactivity in the acid soluble fraction, and the other 3 fractions were affected not at all or inhibited slightly. No reproducible effect of the virus on the concentration of P in any of the fractions was found, nor was any effect found on the concentration of RNA and DNA as determined by colorimetric methods for their specific sugars.

Discussion. The appearance of metabolic changes within 2 to 4 hours after inoculation is somewhat surprising in view of the reportedly slow rate of absorption of adenovirus and the 19 to 21 hour latent period before increase in intracellular virus(5,15).

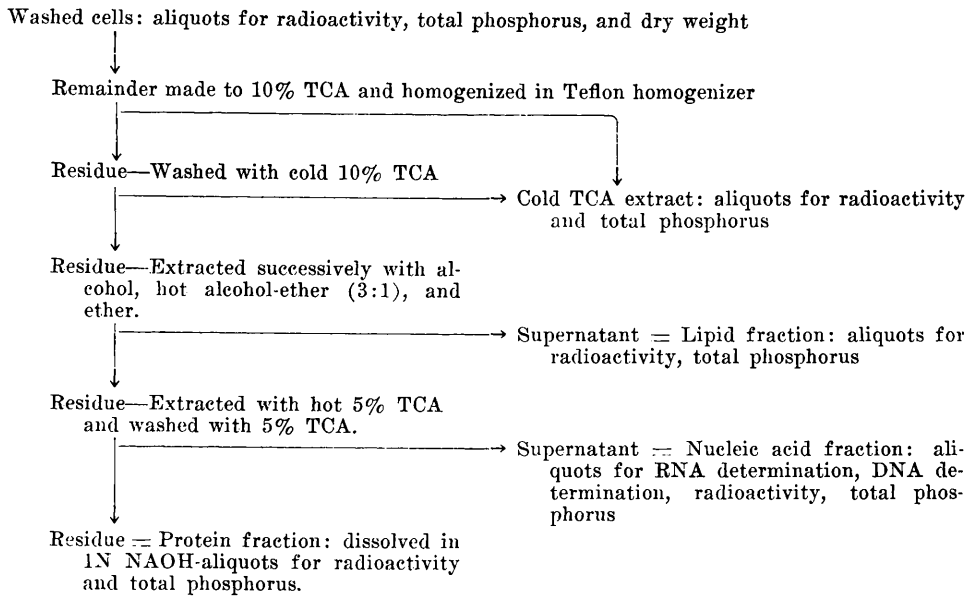

TABLE I. Effect of Type 2 Adenovirus on Phosphorus Uptake by Fractions from HeLa Cells after 5 Hours of Infection.

Fraction	Exp.	cpm/mg IV	cpm/mg V	Ratio Inf./Cont. IV	Ratio Inf./Cont. V	γP/mg IV	γP/mg V	cpm/mg P IV	cpm/mg P V
Whole cells	Infected	545	1114	1.18	1.57	15.9	17.0	34,270	65,500
	Control	461	708			14.4	17.6	32,010	40,200
Cold TCA	Infected	320	710	1.17	1.57	3.6	3.1	88,880	22,900
	Control	273	451			3.1	3.2	88,060	14,090
Lipid	Infected	5.2	12.7	1.00	1.30	2.7	3.6	1,962	3,520
	Control	5.2	9.7			2.9	3.5	1,793	2,770
Nucleic acid	Infected	86	74	1.26	1.68	5.2	6.9	16,538	10,700
	Control	68	44			5.9	5.3	11,525	8,300
Residue	Infected	25.1	38	1.78	1.90	6.6	.9	3,803	42,200
	Control	14.1	20			5.0	.7	2,802	28,500

However, since large doses of adenovirus comparable to those used in the present study can produce specific cytopathic effects within 2 to 4 hours after inoculation(16), it is apparent that adenoviruses can produce profound effects long before the appearance of infectious virus. That the observed metabolic changes were not simply the result of permeability changes is indicated by the markedly different patterns observed with phosphorus and glycine.

A difficulty in relating the observed results to the growth cycle of the virus is the problem of obtaining synchronous infection of most of the cells in the culture. Although a high multiplicity of virus to cells was used, results of studies by other workers indicate that synchronous infection probably was not obtained; thus, Harford *et al.*(17), using the electron microscope, found virus particles in only 5 to 20% of HeLa cells infected with adenovirus. Droz and Chany,§ using autoradiographic technics, observed increased isotope uptake in only a small proportion of HeLa cells infected with a large dose of adenovirus. If only a small proportion of cells are affected metabolically, the degree of metabolic change is all the more striking, since the data obtained here show the net effect on the culture as a whole. It should be emphasized that the biochemical fractionations were performed only at 5 hours; it is quite possible that a different pattern would have been observed at a later time when infectious virus is being liberated.

The observed metabolic effects possibly could be associated with the early cytopathic changes seen when large doses of adenovirus are used, and both might, in turn, be attribu-

§ Personal communication.

TABLE II. Effect of Type 2 Adenovirus on Uptake of Glycine-2-C^{14} by Fractions from HeLa Cells after 5 Hours of Infection.

Fraction		Exp. IV cpm/mg	Exp. IV Ratio Inf./Cont.	Exp. V cpm/mg	Exp. V Ratio Inf./Cont.
Whole cells	Inf.*	130.5	1.40	227	1.60
	C	93.0		142	
Cold TCA	Inf.	18.0	1.71	40	1.67
	C	10.5		24	
Lipid	Inf.	8.6	1.06	10	.9
	C	8.1		11	
Nucleic acid	Inf.	4.7	.80	3.6	.81
	C	5.9		4.4	
Residue	Inf.	54.0	1.05	82	.84
	C	51.2		97	

* Inf. = Infected; C = Control.

TABLE III. Effect of Type 2 Adenovirus Infection on Nucleic Acid Concentration of HeLa Cells; 3 Experiments. Figures expressed as % of dry wt.

	RNA, %	Avg, %	DNA, %	Avg, %
Infected	6.1, 4.7, 5.2	5.3	3.9, 3.7, 4.5	4.0
Control	5.5, 4.7, 5.9	5.3	3.5, 3.5, 4.8	3.9

table to the presence in the fluids of soluble antigens or other byproducts of virus multiplication. However, 3 preliminary pieces of evidence suggest that the early metabolic effects are the result of infection. First, stimulation of uptake of P^{32} was prevented by antiserum to type 2 adenovirus and not by normal rabbit serum. Second, a preparation of type 2 adenovirus whose infectivity had been destroyed by a combination of ultraviolet light irradiation and heating, but which still produced the early cytopathic changes, did not cause metabolic stimulation, whereas the untreated preparation produced the usual increase in turnover in the same test. Third, experiments with type 2 adenovirus in monkey kidney tissue culture, in which early cytopathic changes are not produced, gave metabolic results similar to those obtained in HeLa cells.

Some points of similarity and some points of difference might be pointed out in comparing the effects reported here with those found with type 3 poliovirus in monkey kidney tissue culture(1). In the latter system, an increased lactic acid production was manifest in the infected cultures within 1-2 hours after addition of the virus, which phenomenon was not seen with the adenovirus-HeLa cells until 8-24 hours. One might be inclined to associate this difference with the slower rate of absorption of adenovirus(5,18), but the effects of adenovirus on uptake of radiophosphate and radioglycine were seen within 2-3 hours.

Insofar as the uptake of radioglycine in the 2 virus-cell systems is concerned, some marked differences were noted. In the polio-kidney system, the uptake of radioglycine was strongly inhibited in all the fractions, and, consequently, in the whole cells. With the adenovirus-HeLa system, the uptake of radioglycine by the whole cells was stimulated, but this effect was restricted to the acid soluble subfraction, with the other fractions showing no effect or a slight inhibition. Experiments in these laboratories to determine the effects of poliovirus on the uptake of P^{32} by monkey kidney tissue culture cells have not given reproducible results, so no comparison can be made with such effects in the adenovirus-HeLa system. Preliminary experiments with poliovirus in HeLa cells suggest that its behavior there is similar to its behavior in kidney cells.¶

This group of experiments was designed to survey a broad area of metabolic activities to see if any effects of adenovirus could be found. Currently, investigations are under way to examine in more detail those effects that have been noted.

Summary. Infection of HeLa cells with a large inoculum of adenovirus type 2 produced a rapid and prolonged stimulation of cellular uptake of the radioactivity of inorganic P^{32} and glycine-2-C^{14}, with no change in concentration of P, RNA, or DNA. In fractions taken 5 hours after inoculation, the increased rate of P^{32} uptake was observed in each of the biochemical fractions tested, minimally in lipid. Glycine uptake was increased only in the acid-soluble fraction. In contrast to the early alteration of radioisotope uptake, increased production of lactic acid was not observed until 8 to 24 hours after infection.

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Incorporation of C^{14} of Acetate-1-C^{14} and Pyruvate-2-C^{14} into Brain Cholesterol in the Intact Rat. (23593)

P. J. McMILLAN, G. W. DOUGLAS AND R. A. MORTENSEN (Introduced by V. C. Bohr)
Department of Biochemistry, School of Medicine, College of Medical Evangelists, Loma Linda, Cal.

Srere *et al.*(1) found that slices prepared from newborn rat brain were able to convert acetate to cholesterol. With slices from adult animals, however, no formation of cholesterol from acetate could be detected. In the present work, the incorporation of labeled acetate and pyruvate into brain cholesterol was studied in the intact rat by injecting the labeled compounds into the cisterna magna. Under these conditions, appreciable incorporation of both acetate and pyruvate was observed in rats up to 1 year of age.

Methods. Male Sprague-Dawley rats were injected with sodium acetate-1-C^{14} (Tracerlab) and sodium acetate-1-C^{14} (Nuclear Instrument and Chemical Corp.) in isotonic saline by a technic previously described(2). Both compounds had a specific activity of 1 mc per millimole. Unless otherwise indicated, all injections were made under Nembutal anesthesia. A stock laboratory diet was provided *ad libitum* throughout the experiments. At stated intervals after injection, the animals were killed by decapitation. The brains were removed and saponified in alcoholic KOH for 4 hours. The saponifiable and unsaponifiable fractions were separated by extracting the mixture with petroleum ether, evaporating the extract to dryness, and then extracting the residue with hot ethanol, the procedure being similar to that described by Van Bruggen *et al.*(3). Each ethanol extract was made to a volume of 25 ml, and aliquots were used for the determination and isolation of cholesterol. The cholesterol was precipitated from the ethanol extract as the dibromide, regenerated by debromination with zinc dust, and recrystallized from cold methanol, as described by Schwenk and Werthessen(4). Suspensions of the purified product in a 4:1 methanol-water mixture were then deposited for counting on 5 sq. cm aluminum disks by centrifuging the suspensions in a special plating assembly(5). Ordinarily a deposit of about 5 to 10 mg was obtained. Some loss occurred in the plating process, owing to the small but appreciable solubility of cholesterol in the methanol-water mixture and to the tendency of some of the solid to cling to the surface of the supernatant fluid. The actual amount of cholesterol plated was determined in each case by weighing. All determinations of radioactivity were carried out in a windowless gas flow counter. Corrections for the self-absorption of cholesterol were made according to the data for wax(6). The cholesterol content of the ethanol extracts was found by the method of Sperry and Webb(7). Most of the results are expressed as the % incorporation of C^{14} into cholesterol, calculated as the total activity of the cholesterol found in the ethanol extract divided by 1% of the administered activity. Since some degradation of brain cholesterol is known to occur during saponification(8), the % incorporation figures thus obtained must be somewhat lower than the actual values.

Results. Comparison of intracisternal and intraperitoneal injections of acetate-1-C^{14}. After preliminary experiments had indicated that adult brain cholesterol could be labeled *in vivo* with acetate-1-C^{14}, an experiment was performed in which different routes of