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Accumulation of Organic Acids by HeLa Cells Infected with Type 4 Adenovirus.* (23113)

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That fluids from HeLa cell cultures infected with an adenovirus were more acidic than those from companion uninfected cultures was noted during early studies of the characteristics of these agents(1,2). Increased acidity of the media of infected cultures accompanied extensive cytopathogenic changes effected by the adenoviruses. Preliminary reports indicated that lowered pH of cultures infected with types 1-4 adenoviruses was a reflection of an increased quantity of lactic acid in the fluid, and the accumulation of acid was correlated with increased utilization of glucose by the infected cells(2).

This investigation was initiated to establish further the identity of acid or acids produced by uninfected and infected HeLa cells, and to determine whether an increased accumulation of acids other than lactic acid resulted from type 4 adenovirus infection. It is the purpose of this communication to report that lactic, pyruvic, acetic, and alpha ketoglutaric acids increase in fluids of HeLa cell cultures infected with this agent.

Methods. Tissue culture. HeLa cells were propagated in medium consisting of human serum, 40%, and Hanks' balanced salt solution (BSS), 60%, as described previously(3, 4). Cultures in tubes were initiated with 50,000 cells, and were used experimentally in approximately 24 hours. For infection, cultures were washed 3 times with 2 ml of Hanks' balanced salt solution per wash, and to each tube was added 0.8 ml of maintenance mixture composed of 67.5% Scherer's amino acid-vitamin mixture(5), 25% tryptose phosphate broth, and 7.5% chicken serum(4). To estimate pH of fluids, phenol red, 0.002%, was present in all media. *Viruses.* Type 4 aden-

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ovirus was propagated in HeLa cell cultures and its infectivity titer determined by methods previously described(3.4). To identify the acids and determine quantity of each present in the maintenance mixtures, cultures were infected with $10^{2.0}$ to $10^{3.5}$ 50% infectious doses (ID_{50}) of virus and the infected fluids or infected cells and fluids from cultures were pooled 4 to 6 days after infection. In each experiment fluids alone or cells and fluids (maintenance mixture) from uninfected cultures incubated for a similar period were also pooled. At the time that infected and uninfected cultures were harvested for determination of acids present, cell counts were not done since many cells in the infected cultures had fallen from the glass and an indeterminate number had lysed. Counts done on a large number of tube cultures after maintenance mixture had been added indicated that the variation in cell number between tubes prepared at the same time was less than 10%. *Initial identification and quantitative analyses of lactic acid* were done with culture fluids from uninfected and infected cultures using the method of Hullin and Noble(6). To identify further the carboxylic acids, chromatographic technics were employed using both cells and fluids from uninfected and infected HeLa cultures. To disrupt HeLa cells in suspension and obtain material for separation of acids, 1 ml of cell suspension was acidified to pH 2-3 with 10 N H_2SO_4 (usually a single drop of acid was required) and mixed with 2 g of celite by grinding with a pestle in evaporating dish. The mixture was then placed upon a 10 g celite column and the acids were eluted consecutively with a series of butanol-chloroform mixtures as described by Swim and Krampitz(7). Numerous experiments were done with prepared mixtures of known acids to determine the exact fraction in which each of the carboxylic acids would be eluted from the column under the conditions employed. Quantitative measurements for each acid fraction were made by titration with 0.01 N NaOH using phenol red as the indicator. During the titration procedure CO_2-free air was bubbled through the solution to obtain complete mixing. Lactic acid and keto-acids were also determined by colorimetric methods. Because the presence of phenol red in tissue culture fluids interfered with colorimetric determination of keto-acids, the data were valid only when samples for analysis were removed from the celite column. Contents of tubes considered to contain alpha keto-glutaric acid and possibly traces of succinic acid were pooled after separation from the celite column. The total volume was placed in an ice bath and an air jet was directed on the surface of the liquid until the chloroform layer had completely evaporated and the water phase was reduced to 1 or 2 ml. The sample was then diluted appropriately, the hydrazone prepared(8) and measured colorimetrically. By keeping the sample cool and retaining the compound as an acid and not the sodium salt, stability was maintained and reproducible results realized. When culture fluids were extracted with ether and the acids then separated on the column, results were in good agreement with values obtained when ether extraction was not employed. Therefore, extraction with ether was not used for most analyses. Compounds were further identified qualitatively by ascending paper chromatography using as solvent either a mixture of 85% secondary butyl alcohol, 5% formic acid and 10% water, or 75% methanol plus 25% saturated ammonium carbonate. With each chromatographic determination known acids were tested concomitantly with the homogenates from uninfected and infected cultures. These experiments were conducted at room temperature. Spots were developed by spraying the chromatograms with neutral brom cresol green indicator. *Glucose determination.* The quantity of glucose in maintenance mixture and in fluids from infected and uninfected HeLa cell cultures was measured by the method of Park and Johnson(9). Amount of glucose utilized by HeLa cells was computed by subtracting quantity of glucose present in the uninfected or infected culture fluid from that in maintenance mixture incubated at 36°C for the same period as the cultures.

Results. Comparison of fluids from uninfected and adenovirus infected HeLa cell cultures clearly indicates the greater acidity of maintenance fluid from the infected cultures.

TABLE I. Quantity of Lactic Acid Produced and Glucose Utilized by Uninfected and Type 4 Adenovirus Infected HeLa Cells.

Exp. No.	HeLa cell culture*	Lactic acid produced, μM	Glucose utilized, μM
1	Uninfected	1.4	
	Infected†	4.7	
2	Uninfected	4.7	
	Infected†	7.0	
3	Uninfected	.9	1.4
	Infected†	3.2	6.4
	Infected‡	2.5	4.8

* Incubated at 36°C for 6 days.
† Infected with $10^{3.0}$ ID_{50}.
‡ " " $10^{2.0}$ " .

The progressive decrease in pH of infected cultures to a pH of 6.5-6.8 has been correlated with viral multiplication and subsequent cytopathogenic changes.‡ Chemical analysis of fluids from control and infected cultures indicated that a marked accumulation of lactic acid occurred. Results from studies carried out with the type 4 adenovirus are presented in the tables. Data from 3 typical experiments presented in Table I illustrate the range of differences between quantity of lactic acid in fluids from infected and uninfected cultures. Studies were next done to determine whether glucose was utilized by viral infected cells in amounts commensurate with the quantity of lactic acid which accumulated; the results from one such study presented in Table I indicate that this was the case. It should be noted, however, that in control and infected cultures the amount of glucose metabolized was 3 to 4 times more than could be accounted for by the quantity of lactic acid produced. The results of these limited studies suggest that with HeLa cells, uninfected or infected, although lactic acid is a principal end-product of glucose metabolism, mechanisms other than anaerobic glycolysis are also involved.

The data presented in Table I were the results of determinations done on materials obtained by pooling 2 to 4 cultures. Analyses were next done with pools of cells and fluids made from 20 to 30 uninfected or infected cultures. For viral infection $10^{3.5}$ ID_{50} per culture of type 4 adenovirus was employed. Cultures were harvested 4 days after viral infection when practically all cells of the infected cultures had undergone characteristic cytopathic changes. To determine whether a variety of carboxylic acids accumulated as a result of viral infection, acids were separated and measured as described above. Glucose determinations were not done in these experiments. The results from 5 individual experiments are summarized in Table II. These data further point out the increased amount of lactic acid found in viral infected cultures. In addition, pyruvic and alpha ketoglutaric

TABLE II. Quantity of Organic Acids in Uninfected and Type 4 Adenovirus Infected HeLa Cell Cultures Determined by Chromatographic and Colorimetric Technics.

		Micromoles acid* determined by						
		Titration technic†				Colorimetric technic		
Exp. No.	HeLa cell culture	Acetic	Pyruvic	Lactic	α-keto-glutaric	Pyruvic	Lactic	α-keto-glutaric
1§	Uninfected	6.2	2.7	5.4	1.8	nt‡	nt	nt
	Infected**	9.6	3.1	7.4	2.2	nt	nt	nt
2‖	Uninfected	5.0	0	3.9	.7	nt	nt	nt
	Infected**	5.9	4.5	10.1	1.5	nt	nt	nt
3¶	Uninfected	6.2	1.0	3.1	.1	nt	3.3	nt
	Infected**	10.5	3.7	6.2	1.4	nt	6.1	nt
4¶	Uninfected	5.2	.2	6.1	.6	.9	6.4	.8
	Infected**	9.9	1.2	13.2	2.1	1.4	nt	2.3
5¶	Uninfected	1.5	0	2.1	0	0	2.9	0
	Infected**	4.6	2.6	5.4	1.5	2.2	5.9	1.6

* Acids separated on a celite column. † Titrated with .01 N NaOH. ‡ nt = not tested. § Cultures incubated at 36°C for 3 days. ‖ Cultures incubated at 36°C for 4 days. ¶ Cultures incubated at 36°C for 6 days. ** Infected with $10^{2.5}$-$10^{3.0}$ ID_{50} of Type 4 adenovirus.

‡ Unpublished data.

TABLE III. Identification by Paper Chromatography of Organic Acids in Uninfected and Type 4 Adenovirus Infected HeLa Cell Cultures. Comparison of R_f values and known acids.

Solvent	Acid determined	R_f values: Known (control)	R_f values: HeLa culture* Uninfected	R_f values: HeLa culture* Infected
Butyl alcohol - formic acid - water†	Acetic	.08	.08	.08
	Lactic	.8	—	.8
	α-ketoglutaric	.05	.05	.05
Methanol - saturated $(NH_4)_2CO_3$‡	Acetic	.19	—	.19
	Pyruvic	.58	—	.58
	Lactic	.72	—	.71

* Materials from Exp. 5, Table II.
† 85% secondary butyl alcohol, 5% formic acid and 10% water.
‡ 75% methanol and 25% saturated $(NH_4)_2CO_3$.

acids were consistently present in greater quantity in cultures infected with type 4 adenovirus; acetic acid also accumulated in greater amounts in the majority of infected cultures although the increase over that measured in uninfected cultures was not as marked as that of the other acids identified. The increased accumulation of lactic, pyruvic, and alpha ketoglutaric acids was also demonstrated by colorimetric methods.

The identity of these carboxylic acids was further confirmed by the use of paper chromatographic technics. An identification of compounds in culture homogenates from one experiment (Table II, Exp. 5) is summarized in Table III. The R_f values of the acids from uninfected and viral infected cultures corresponded very closely with R_f values of known solutions of lactic, pyruvic, acetic, and alpha ketoglutaric acids. On paper, a spot which corresponded to succinic acid could also be identified. This acid could not be measured in the eluate from the celite column because the small amount present was probably in the fraction with alpha ketoglutaric acid. There were no spots on the chromatograms which could not be identified. These data indicate that the acids obtained by fractionation of culture materials on the celite column were indeed the acids considered to be present and lend strong support to the findings described above.

Discussion. That adenovirus infection of HeLa cells results in an accumulation of organic acids in the culture media is demonstrated by the experimental data exemplified in this paper with type 4 viral infection. The acids identified as being present in uninfected and infected culture fluids were lactic, pyruvic, acetic, alpha ketoglutaric, and probably very small amounts of succinic. The increased accumulation of lactic acid and concomitant increased utilization of glucose by infected cells imply that adenovirus infection of HeLa cells results in a stimulation of glycolysis. The pathways of carbohydrate metabolism by HeLa cells have not yet been elucidated, so that the mechanism by which pyruvic, acetic, and alpha ketoglutaric acids accumulate and their relationship to the mode of viral action is not clear. The presence of the enzymes of the Krebs citric acid cycle in normal HeLa cells has recently been demonstrated(10), but their role in uninfected or adenovirus infected HeLa cells has not been studied.

The evidence presented does not permit the conclusion that the increased glycolysis and accumulation of carboxylic acids is an inherent part of viral synthesis. The phenomenon described by this investigation may rather reflect cell injury resulting from viral infection and could perhaps be initiated also by cell damage due to non-viral noxious agents. It is clear, however, that injury of cultured cells in general and HeLa cells in particular by a variety of other viruses, such as western equine encephalomyelitis(11), poliomyelitis (12-14), Coxsackie§, ECHO§ and Newcastle disease viruses,¶ does not cause increased accumulation of acids. Conversely, infection

¶ Unpublished data.

§ Steigman, A. J., Benyesh, M., Brown, R., and Melnick, J. L., personal communication.

with these agents results in a decreased production of acid. It is noteworthy that infection of HeLa cells by the latter viruses listed culminates in death and lysis of the host cells. In contrast, adenoviruses do not appear to lyse infected HeLa cells(2) and it has been suggested that these respiratory tract viruses do not actually kill the cells in which they multiply(2). It can be deduced, therefore, that the increased glycolysis and accumulation of carboxylic acids require a particular type of viral infection or cell injury. Whatever may be the mechanism of the increased accumulation of organic acids and utilization of glucose, the evidence is clear that the initiation of this phenomenon is an intrinsic characteristic of the adenoviruses and a constant effect upon HeLa cells which they infect.

Summary. Fluids from cultures infected with type 4 adenovirus were markedly more acid than fluids from non-infected control cultures. The lowered pH of infected cultures resulted from an accumulation of lactic, pyruvic, acetic and alpha ketoglutaric acids. The acids were separated by chromatography on a celite column, and measured by colorimetric and acid-base titration technics. Confirmation of the identity of the organic acids was obtained by paper chromatography. Concomitant with the increased accumulation of acid in fluids of infected cultures, an increased utilization of glucose by cells of the virus-infected cultures occurred.

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Guanine and Guanosine Deaminase Activity of Rat Mammary Gland Homogenates Through Pregnancy and Lactation.* (23114)

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Kirkham and Turner(1,2) reported changing levels of rat mammary gland DNA and PNA during pregnancy and lactation and in the mammary gland stimulated to growth with estrogen and progesterone. In rabbit mammary gland Yamamoto and Turner(3) showed increases in total DNA as glandular growth occurred. McShan *et al.*(4) have shown very striking increases in PNA content of pigeon crop gland following treatment with lactogenic hormone. Williams and Turner (5) reported apparent involvement of PNA in the action of lactogenic hormone on the mammary gland. From these studies PNA

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