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Effect of Poliomyelitis Virus on Mitochondria Phospholipids Of the HeLa Cell.* (28212)

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The uptake of radiophosphorus by HeLa cells exposed to the poliomyelitis virus was accelerated into the nucleic acid and total phospholipid fractions(1). Increased phospholipid synthesis has been observed to occur, associated with a number of other viral infections: skin of rabbits infected with Shope virus(2), chicken skin infected with fowlpox(3,4), mouse brain as a consequence of infection with Theiler's GD VII virus(5), and KB cells with adenovirus(6).

The total phospholipids of the HeLa cell after poliomyelitis virus exposure have been fractionated by chromatography in an attempt to investigate which of these fractions are involved during lipid phosphorylation (7). In the present investigation an attempt has been made to separate mitochondria of HeLa cells after poliomyelitis infection and fractionate the phospholipids by chromatography to ascertain if these lipids of this cellular fraction are involved in this process.

Materials and methods. HeLa cells were grown in growth medium in 200 ml culture flasks, in a stationary state at 36° according to the procedure of Syverton *et al.*(8). After 14 days, the growth medium was removed and cells rinsed 3 times with modified Hanks' solution(8) containing 0.1% glucose, 0.8% NaCl and 0.04% KCl. Fifteen minutes before virus inoculation, the growth medium was discarded and the HeLa cells were rinsed 3 times with 10 ml aliquots of modified Hanks' solution(8). The culture flasks to be inoculated with the virus received 8 ml of maintenance medium(8), and controls 9 ml. The maintenance medium consisted of 90 parts of maintenance solution(8) and 10 parts of chicken serum. Cultures inoculated with virus received, in addition to the maintenance medium, 1.0 ml of poliomyelitis virus† ($10^{7.42}$ $TCID_{50}$) ($TCID_{50}$ = the dilution of the virus just sufficient to kill 50% of the 5-day-old cultures in the test). Culture flasks were incubated for 4 hours. Thirty minutes before the cells were harvested 1.0 ml $NaH_2{}^{32}PO_4$ containing 80 μc was added. This time interval corresponds to the ascending part of the specific activity time curve, where synthesis of phospholipids occurs in the HeLa cell greater than breakdown(9).

At the time of harvesting the cells were scraped from the wall of 10 culture flasks with a rubber policeman and pooled and mixed in modified Hanks' solution(8), and an aliquot was removed for cell counting. The remainder of the cell suspension was centrifuged in the cold at 2000 × *g* for 3 minutes. The supernatant fluid was poured off and cells were homogenized in a Potter-

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† Type I (Mahoney strain).

TABLE I. Phospholipids Fractions of HeLa Cells Exposed to Poliomyelitis Virus.

Time of virus exposure, hr	Phosphatidyl inositol	Sphingomyelin	Lecithin	Phosphatidyl serine	Phosphatidyl ethanolamine
	Specific activity* of virus infected cells/control cells				
2	1.48	1.04	−.38	−.85	2.28
4	1.15	1.27	2.16	1.25	2.18
6	2.24	6.08	3.26	2.28	1.68
10	1.01	−.69	−.24	−.28	−.29

$NaH_2{}^{32}PO_4$ was added to medium 30 min before cells were harvested.

* Counts/min/μg P. Values given are avg of 2 to 3 determinations on 5 pooled cell cultures.

Elvehjem tissue grinder with Teflon pestle in ice-cold 0.25 M sucrose containing 0.00018 M $CaCl_3$. The homogenate fraction was separated into cellular fractions by the method of Hogeboom *et al.*(10) and Griffiths and Pace(11). The homogenate was centrifuged at 600 × *g* for 15 minutes to remove the nuclei. The mitochondria were separated from the supernatant by centrifugation at 12,500 × *g* for 25 minutes in an International Model HR-1 centrifuge (856 head). The mitochondria were checked for the contamination of microsomes by the absence of glucose-6 phosphatase(12). There was no nuclear contamination detectable by phase-contrast microscopy.‡ The lipids from the mitochondria were extracted 2 times with boiling ethanol and 3 times with ethanol-ethyl ether (2:1). The pooled lipid extracts were evaporated with Evapo-Mix (Rinco Instrument Co.), the water removed with Freeze Dryer (VirTis Co., Inc.) and the residue purified with chloroform(13). Phospholipids were chromatographed on glass paper impregnated with silicic acid(14). The solvent system was diisobutyl ketone:acetic acid:water:benzene (160:50:8:7, v/v). The chromatograms were dried, stained with Rhodamine 6G and the phospholipids identified with ultraviolet light. The individual phosphatides were hydrolyzed and extracted with 1.5 N methanolic HCl. The hydrolysate was evaporated to dryness, phosphorus(15) and radioactivity(16) determined. From these results and the radioactivity measurements, specific activities of the individual phospholipids were calculated. Additional HeLa cells were infected with poliomyelitis at 2, 4, 6 and 10 hours to investigate a time study of viral exposure to HeLa cell on lipid phosphorylation. Thirty minutes before cells were harvested $NaH_2{}^{32}PO_4$ was added, cells harvested, and total lipids extracted, chromatographed on silicic acid-impregnated glass paper, hydrolyzed and phosphorus(15) and radioactivity(16) determined.

Results and discussion. Table I shows the effect of time of virus exposure on uptake of the isotope into total phospholipid fraction of the HeLa cell. There is a progressive increase of lipid phosphorylation in all lipid fractions with extended viral infection up to 6 hours. The reason for, or the fate of the newly formed phospholipid molecule during viral infection is not known. Viral multiplication after cellular inoculation is known to reach a maximum after 3-6 hours. It is probably for this reason we failed to observe any increase in lipid phosporylation after 10 hours of viral infection. Table II shows the specific activities of phospholipid fraction of the mitochondria of HeLa cells exposed to poliomyelitis virus. This study was made after 4 hours of viral exposure, since it had previously been demonstrated at this time interval that all of the total cellular phospholipid fractions were increased after viral exposure(7). To evaluate the statistical significance of the results, the *t* test of significance(17) was applied to the ratio of the specific activities of phospholipid fractions of the virus infected cells/control cells. There is an increase of lipid phosphorylation in all lipid fractions except sphingomyelin of the mitochondria after 4 hours of viral exposure. The average increase over the control cells following viral infection in all phospholipid fractions except sphingomyelin was statistically significant ($P<0.01$).

‡ Dr. J. J. Taylor, Director of Electron Microscope Laboratory.

TABLE II. Specific Activities* of Mitochondria Phospholipids of HeLa Cells Exposed to Poliomyelitis Virus.

	Phosphatidyl inositol	Sphingo-myelin	Lecithin	Phosphatidyl serine	Phosphatidyl ethanolamine	Phosphatidic acid
Control	26.68	1.21	.94	1.34	.94	—
Virus	24.28	1.08	1.33	1.54	1.41	—
Control	20.80	1.27	.58	.93	1.45	14.15
Virus	27.00	.96	1.02	1.28	1.71	16.09
Control	23.62	1.06	.28	.64	.55	20.82
Virus	45.46	1.63	.65	.41	1.07	44.33
Control	10.93	.77	.27	.25	.76	10.97
Virus	12.53	.47	.32	.59	.76	11.22
Control	13.20	—	.84	.71	1.06	15.44
Virus	25.80	—	1.01	2.12	1.52	16.08
Control	11.40	—	.24	.27	.33	7.90
Virus	20.90	—	.76	.36	1.09	15.20
Control	12.90	.55	.36	.57	.89	8.22
Virus	23.60	1.17	.65	.67	1.16	17.13
Avg Ratio of Specific Activity of Virus Infected Cells/Control Cells.						
	$1.56 \pm .42$ $P < .01$	$1.19 \pm .57$	$1.84 \pm .70$ $P < .01$	$1.58 \pm .80$ $P < .01$	$1.70 \pm .86$ $P < .01$	$1.56 \pm .54$ $P < .01$

Cells were incubated 4 hr with and without poliomyelitis virus. ^{32}P was added to the medium 30 min before cells were harvested. Each control and virus experiment represents 10 pooled cell cultures.

* Counts/min/μg P. Figures preceded by $\pm$ sign indicate stand. dev. P is degree of probability for a chance occurrence of this increase.

This increase of lipid phosphorylation associated with viral synthesis may be reflection of increased oxidative phosphorylation of mitochondria. Mitochondria are composed of 21% phospholipids on a dry weight basis (18,19) and a number of enzymes involved in oxidative phosphorylation contain phospholipids(20,21). Recently Fleischer *et al.* (22) have demonstrated that mitochondria which have been nearly depleted of phospholipids lose nearly all their respiratory activity, and this is restored by addition of phospholipids. There is increased labeling of ^{32}P with extended viral infection of HeLa cells in the cellular nucleotides, nucleic acids and total phospholipids(23). Butler *et al.*(24) have demonstrated in *Bacillus megaterium* that there is an intimate relationship between phospholipids and protein biosynthesis. Levy (25) has demonstrated in HeLa cells after one hour of infection with poliovirus there is an increase in nucleolar RNA turnover with tritiated H^3-uridine and H^3-cytidine. He was able to detect after 2 hours of infection, specific fluorescent antibody in the cytoplasm of the HeLa cell which continued to increase for 5 hours after infection. Salzman *et al.*(26) have demonstrated that free nucleotides of the HeLa cell are the major source of viral RNA and there was no significant direct utilization of the cellular RNA for viral synthesis. Cellular synthesis of RNA from the free nucleotides and protein requires ATP and most of the ATP is produced in the mitochondria during oxidative phosphorylation. The period of time for viral RNA and protein synthesis in the cell is between 3 and 6 hours after infection(27). It is during this time interval (4 hours) that we observe lipid phosphorylation in the mitochondria associated with viral infection. This increase of lipid phosphorylation associated with viral synthesis apparently may be a reflection of increased oxidative phosphorylation of the mitochondria.

Summary. The uptake of radiophosphorus (^{32}P) into mitochondria phospholipids of the HeLa cell with and without poliomyelitis virus infection has been investigated. The uptake of radiophosphorus by HeLa cells exposed to the poliomyelitis virus was accelerated into all mitochondria phospholipids except the sphingomyelin fraction.

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Further Studies on the Nature and Source of the Conjugated Bile Pigments. (28213)

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Bilirubin is formed primarily from degradation of senescent erythrocytes in the reticuloendothelial system(1,2). The manner in which bilirubin is chemically altered and the sites at which it is conjugated for ultimate excretion in the bile have been the topic of much study and controversy. The work of Bollman and Mann(3) on the effect of hepatectomy in dogs showed that, despite absence of the liver, part of the serum bilirubin reacted directly in the Van den Bergh test. It was subsequently demonstrated that water-insoluble bilirubin, prior to its excretion in the bile, is conjugated with glucuronic acid(4) and sulfate(5) forming water-soluble excretory conjugates of bilirubin. The indirect Van den Bergh reaction occurs with free bilirubin whereas a direct reaction is obtained when diazo reagent is added to pigments 1 and 2 obtained by reverse-phase partition chromatography of bile or jaundiced serum. Pigment 1 has been described as bilirubin monoglucuronide whereas pigment 2 is bilirubin diglucuronide and perhaps bilirubin sulfate(6). Although confirmed in rats, the presence of bilirubin sulfate has not been confirmed in dogs or humans(7).

It has been reported(8) that the pigment obtained by reverse-phase partition column chromatography from the plasma of the hepatectomized dog (pigment 1) appeared to have the structure of bilirubin monoglucuronide and was of extrahepatic origin. Exactly where pigment 1 is formed extrahepatically and whether it is formed intrahepatically have not been determined. Nosslin(9) has recently supported the concept that pigment 1 is a complex of one mole of bilirubin diglucuronide (pigment 2) and one mole of unconjugated bilirubin as opposed to the concept of a bilirubin monoglucuronide structure. Pigment 2