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Effect of X-Radiation on Uptake of P^{32} into Individual Nucleotides of HeLa Cells.* (25993)

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Separation of the acid soluble phosphorus compounds of transplanted mouse mammary carcinoma and determination of their relative specific activities 2 hours after injection of radioactive phosphate have been described (1). It was demonstrated that 4500 r (in air) localized to the tumor brought about a lowering of relative specific activities of some polyphosphonucleotides. Because these tumors contained a variety of cells, it was thought that the results obtained might be due to changes in metabolism of cells other than those of the mammary carcinoma. To investigate the effect of non-malignant cells, the work was repeated employing Ehrlich ascites carcinoma grown in peritoneal cavities of susceptible mice. These cells can be isolated as a relatively homogenous line. The results of this study are being described elsewhere(2). As a further step in employment of a pure cell line, we have continued our studies using HeLa cells grown and irradiated *in vitro*. This latter investigation is the subject of this report.

Materials and methods. Cultures of HeLa cells(3) were propagated in Eagle's basal medium(4) supplemented with 10% human serum. Cells were harvested by rinsing with bicarbonate buffered glucose-KCl-NaCl solution followed by dispersal with 0.05% trypsin (Difco 1:250) as previously described(5). The trypsinized cells from about 100 bottles were pooled and centrifuged at 200 g for 10 minutes. Cells were resuspended in 25 ml of 10% human serum, 90% Hanks' balanced salt solution and enumerated. The suspension was divided and one-half containing at least 10^8 cells was subjected to irradiation. The bottle containing the cells was placed on its side on pressed wood backing material. The surface of the medium was 35 cm from the focal spot of the x-ray tube. The x-ray machine was operated at 220 KP, 15 MA with no filter (HVL 0.35 mm Cu). A total dose of 5000 r including backscatter was delivered at surface of medium. Cells were then incubated for 2 hours at 37°C, 4 ml of a solution of disodium hydrogen phosphate containing 200 μc of P^{32} having been added to each 30 ml of suspension. After centrifugation 200 g for 10 minutes, supernate was discarded. The cells were resuspended in 20 ml of Hanks' balanced salt solution and recentrifuged. The washed cells were homogenized with 4 volumes of ice cold 0.6 N perchloric acid in a glass Potter-Elvehjem homogenizer. After refrigeration for 30 minutes at 4°C, the homogenate was centrifuged in the cold at 1300 g for 30 minutes. Supernate was decanted off, and precipitate washed with 5 ml of 0.2 N perchloric acid. The combined extract and the washings were neutralized with cold 5 N potassium hydroxide and refrigerated overnight. After removal of potassium perchlorate by centrifugation, aliquots were taken for determination of radioactivity, total phosphorus(6), and nitrogen(7). The neutralized extracts were placed on twin columns of Dowex 1 (formate) X 8, 200-400

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mesh and eluted by gradient elution with formic acid and ammonium formate buffers as previously described(1). For preparations representing 2 x 10^9 cells, a column size of 33 cm x 2.4 cm was used. Volumes of eluants used were as follows: water, 1 bed vol.; 0.2 M formic acid, 2 vol.; 0.5 M formic acid, 1 vol.; 1 M formic acid, 1 vol.; 2 M formic acid, 5 vol.; 4 M formic acid, 2 vol.; 0.2 M ammonium formate in 4 M formic acid, 6 vol.; 0.4 M ammonium formate in 4 M formic acid, 2 vol.; 0.8 M ammonium formate in 4 M formic acid, 4 vol. Rate of flow was 5-6 ml in 25 minutes. An automatic scanning device was used to monitor radioactivity of the fractions eluted. Those showing radioactivity were carefully counted and their extinctions at 260 mμ determined. Values obtained were plotted against volume of eluate. Fig. 1 shows graphs obtained with 2 columns on which identical extracts were fractionated and demonstrates the reproducibility of the method. Fig. 2 shows the fractionation of extracts of (A) non-irradiated cells, (B) irradiated cells. Fractions forming each peak were pooled and a number of aliquots used for determination of specific activity of the phosphorus compound. Since inorganic phosphorus was eluted along with uridylic acid and inosinic acid, specific activity of the inorganic phosphorus from each column was determined by the method of Ernster and associates(8). In Table I activity of each peak is expressed as a percentage of specific activity of the inorganic phosphorus. The remainder of each pool was lyophilized, and residue paper - chromatographed. Where amount of material was sufficient, the identity of the phosphorus compound was corroborated by determining absorption ratio 250/260 and 280/260 mμ and by rechromatographing with known controls. The protein residue remaining from extraction with perchloric acid was further extracted with boiling chloroform-methanol mixture (2:1 by volume). Specific activity of the extracted lipid material was determined.

Resutls. In the earlier experiments 21 phosphorus compounds were separated as illustrated in Fig. 1. As the technic of the column chromatography was improved it became apparent that several of these peaks could be further resolved. In Fig. 2, 24 peaks are shown. Table I gives identities of these peaks as far as we have been able to determine them. Relative specific activity (r.s.a.) is specific activity in counts per minute per μg of phosphorus as a percentage of specific activity of the inorganic phosphorus.

The neutral phosphorus fraction which passed through the Dowex 1 column formed a smaller proportion of total acid soluble phosphorus than it did in extracts from ascites cells or from mouse mammary carcinoma (1,2).

The most prominent difference between irradiated and non-irradiated cells was shown by peak XVI. The phosphorus in this peak from irradiated cells formed 9.8% of total acid soluble phosphorus while from non-irradiated it was only 4.9%. The r.s.a. was markedly changed from 0.65 in non-irradiated to 3.8 in irradiated cells.

A difference was shown in the r.s.a. of adenosine triphosphate (ATP). It was only 3.7 in irradiated as compared to 22.7 in non-irradiated cells, and total amount of ATP also was lower in irradiated cells. Amount of phosphorus isolated in the previous peak XXI was higher in irradiated cells, which might suggest there was imperfect resolution of the 2 peaks. However, it should be noted that the r.s.a. of peak XXI was the same in both irradiated and non-irradiated cells.

The r.s.a. of the total phospholipid extracted from the residue showed no difference between irradiated and non-irradiated cells.

From Table I it would appear that there were differences in r.s.a. values of peaks VI, VIII, XI and XX from irradiated and non-irradiated cells. However, the significance of these differences is questionable because of the small amount of material available for analysis. It is worthy of note that peak XIII is much more prominent in HeLa cells than in either mammary carcinoma or Ehrlich ascites cells.

Discussion. The spectrum of phosphorus containing metabolites extractable by perchloric acid from HeLa cells propagated *in*

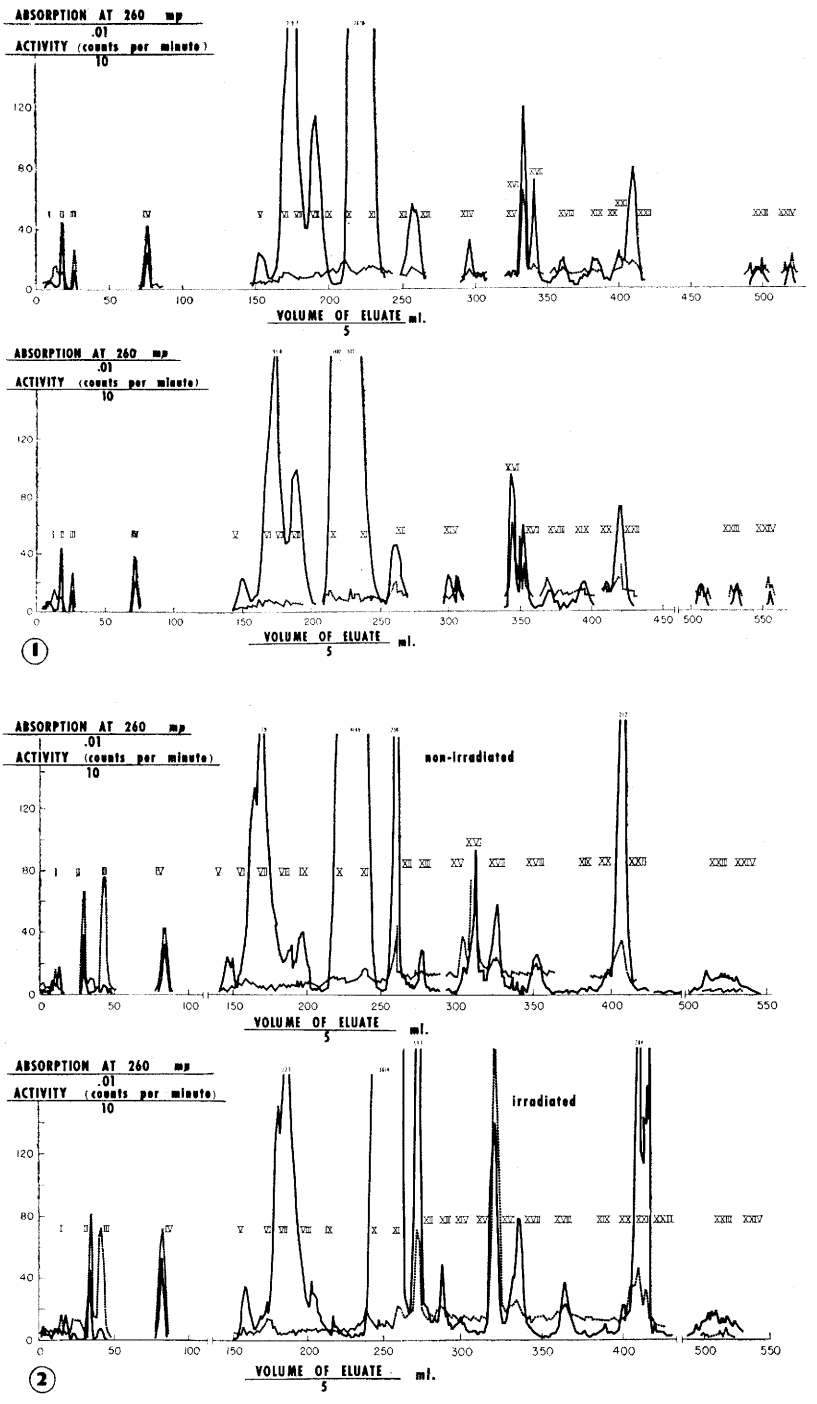

FIG. 1. Acid soluble nucleotides from HeLa cells. Upper and lower curves were obtained by elution of identical preparations from 2 columns. Dotted lines indicate absorption at 260 mμ. Solid lines indicate activity of P-32.

FIG. 2. Acid soluble nucleotides from irradiated and non-irradiated HeLa cells. Dotted lines indicate absorption at 260 mμ. Solid lines indicate activity of P-32.

TABLE I. Relative Specific Activities of Phosphorus Compounds Isolated from Irradiated and Non-Irradiated HeLa Cells.

Peak No.	Compound	Relative spec. activity A. Non-irrad.	Relative spec. activity B. Irrad.	Phosphorus as % of total acid soluble phosphorus (A)	Phosphorus as % of total acid soluble phosphorus (B)
I	?	1.4			
II	Cytidine monophosphate	1.4	1.3	2.1	3.1
III	Diphosphopyridine nucleotide	.4	3.4	1.2	1.6
IV	Adenosine monophosphate	8.0	1.9	.9	1.3
V	?	14.7	18.3	.6	.7
VI	Guanosine monophosphate*	69.7	19.0	.4	1.5
VII	Hexose " *	56.2	53.4	1.5	2.6
VIII	?	30.6	12.4	.3	1.1
IX	?	26.8	8.6	.8	.2
X	Inosine monophosphate*	77.5	92.7†	11.5	22.7
XI	Uridine "	76.4	87.0†	21.0	12.5
XII	Adenosine diphosphate	22.4	26.6	2.2	3.0
XIII	?	4.2	3.1	.6	.7
XIV	?				
XV	?				
XVI	Uridine diphosphoacetylglucosamine*	.65	3.8	4.9	9.8
XVII	" diphosphate glucose*	2.3	2.6	1.4	2.2
XVIII	Guanosine diphosphate*	1.8	3.5	.9	.86
XIX	Cytidine triphosphate*				
XX	Uridine diphosphateglycuronic acid	.6	18.6	.8	.6
XXI	?	5.1	5.1	.6	3.1
XXII	Adenosine triphosphate	22.7	5.7	8.6	1.2
XXIII	Guanosine " *	8.8	6.9		
XXIV	Uridine " *				

* Identity of the compounds has not been proven with certainty.

† These values do not represent true r.s.a. of inosine monophosphate and uridine monophosphate because they were not resolved from inorganic phosphate.

vitro for long periods is found to be quite similar to that of transplanted mouse mammary carcinoma(1) and Ehrlich ascites tumors(2). However, it seems that the proportion of total activity of the acid soluble phosphorus appearing in certain compounds is different. Thus, the unidentified peak XIII seems much more prominent in HeLa cells than in both mammary and Ehrlich cells grown *in vivo*. Relative specific activities of adenosine monophosphate, guanosine triphosphate and uridine triphosphate seem to be lower than in extracts of cells grown *in vivo*. The most prominent effect of irradiation is the reduction of adenosine triphosphate in both amount and activity, indicating a reduction in availability of this form of high energy phosphorus.

That this was likely due to reduction of oxidative phosphorylation is suggested by the increase of activity of the diphosphopyridine nucleotide. The significance of an accumulation of highly radioactive uridine diphosphoacetyle glucosamine in the irradiated cells is difficult to assess.

Summary. Separation of 24 acid soluble phosphorus compounds from irradiated and non-irradiated HeLa cells is described. Relative specific activities of 19 of these compounds are given. Irradiation of the cells produced an increase of relative specific activity and amount of uridine diphosphoacetylglucosamine, and a decrease in amount and specific activity of adenosine triphosphate.

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