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EFFECT OF DIGITONIN AND DIGITOXIN ON THE PHOSPHOLIPID METABOLISM OF MAMMALIAN TISSUE CULTURE CELLS*

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Abstract—The effects of digitonin and digitoxin on the phospholipid metabolism of mammalian tissue culture cells have been studied. Digitonin stimulates the incorporation of inorganic orthophosphate-^{32}P into the total phospholipid. The phospholipid was then separated on silicic acid–impregnated glass paper chromatography, and the specific activity of each phosphatide was calculated. It was found that digitonin caused a linear increase in the specific activity of phosphatidyl inositol and diphosphatidyl glycerol at the time periods studied. The increase in the specific activities of phosphatidyl choline and phosphatidyl ethanolamine was delayed but to the same extent (more than twofold) after 120-min incubation. The specific activities of phosphatidyl serine and sphingomyelin were low and not significantly affected.

The increase was similar in specific activity values of phospholipids of digitonin-treated HeLa cells as that of the controls in the nuclear, mitochondrial, and microsomal fractions.

Digitonin failed to stimulate the incorporation of palmitic-1-^{14}C acid into the phospholipids of HeLa cells. A nonsteroid surface-active agent (Triton X-100) did not stimulate the incorporation of $^{32}P_i$ into the phospholipids of HeLa cells, but other steroids, desoxycholate, and estradiol did stimulate such incorporation of $^{32}P_i$.

Digitoxin had little effect on the phospholipid labeling of HeLa cells. However, digitoxin stimulated the incorporation of $^{32}P_i$ into phosphatidyl inositol and sphingomyelin fraction of human heart culture cells. The specific interaction of various steroids with the target-cell membranes and its relation to phospholipid metabolism is discussed.

PHOSPHOLIPIDS are found in the membranes,[1] mitochondria, nuclei, and microsomes[2, 3] of the cell. Since phospholipids are major constituents of biological membranes and play a role in the structure and function of the cell, any modification in their properties will have important biological consequences. It has been shown that steroids such as estrogens,[4] vitamin D,[5] and cardiac glycosides[6–8] stimulate the incorporation of inorganic orthophosphate-^{32}P into phospholipids in rat uterus, intestinal mucosa, and heart ventricle respectively. Karnovsky *et al.*[9] have shown that digitonin and other surface-active agents cause polymorphonuclear leukocytes, monocytes, and alveolar macrophages to manifest the metabolic changes characteristic of phagocytosis. More

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recent studies indicate that digitonin mimics the action of polyene antibiotics,[10] which are known to penetrate lipid monolayers.[11] Since digitonin is a surface-active steroid derivative, it is interesting to see whether it affects the phospholipid metabolism at the cellular level.

The present report describes the effects of digitonin on mammalian tissue culture cells. Evidence indicates that digitonin stimulates the incorporation of $^{32}P_i$ into phospholipids of HeLa cells. The effect of digitoxin on the phospholipid metabolism of HeLa and heart tissue culture cells has also been studied for comparison.

METHODS

Cell growth. Tissue culture HeLa and human heart cells were originally obtained from Microbiological Associates, Bethesda, Md. The cells were grown as monolayers in 1000-ml culture bottles and were overlaid with 50 ml of growth medium which was made up of Hank's balanced salt solution containing 10 per cent human serum, 10 per cent glucose, 2 per cent yeast extract, and 1·4 per cent $NaHCO_3$ (pH 7·2). To the solution was added an amount of penicillin and streptomycin to yield a final concentration of 100 units and 100 μg/ml respectively. An antifungal agent (mycostatin 50 μg/ml or fungizone 2 μg/ml) was added routinely once a month but was absent when the cells were planted for experimental studies.

The cells were grown in a stationary state at 37°, and the growth medium was changed every other day. The inoculum of cells per bottle was from 0·5 to $1{\cdot}0 \times 10^{-6}$, and by the time of harvesting, approximately 5 to 10×10^{-6} cells were in each bottle.

Incubations with digitonin and digitoxin and inorganic orthophosphate-^{32}P. Cells aged 6 to 9 days were used in these studies. One day before harvesting the cells, the culture medium was replaced by 45 ml of fresh growth medium. Digitonin, digitoxin, or one of the other compounds tested was dissolved in 95% ethanol to provide various concentrations; 0·5 ml of the alcohol solution was added to the medium. Alcohol control cell cultures received the same volume of 95% ethanol as did the drug-treated cell cultures.

Inorganic orthophosphate ($NaH_2{}^{32}PO_4$, carrier-free), obtained from the Oak Ridge National Laboratory, Oak Ridge, Tenn., was diluted with GKN solution (Hank's solution containing 11% glucose, 0·8% NaCl and 0·04% KCl), and was sterilized before adding to the drug-treated, alcohol control, and blank control cell cultures. Five milliters $^{32}P_i$ containing 150 μc was added to the medium directly after the addition of the drugs. The final concentration of $^{32}P_i$ in the medium was 3 μc/ml. The cells were then incubated for 15–240 min.

Cell harvesting and homogenization. At the end of incubation periods, the growth medium was removed and the cells were rinsed with Hank's solution. The cells were harvested, pooled, and suspended in ice-cold 0·25 M sucrose solution containing 0·00018 M $CaCl_2$ and were pooled in a Potter–Elvehjem tissue grinder. After centrifugation, the sedimented cells were taken up with 10 volumes (2–3 ml) of sucrose–$CaCl_2$ solution and were homogenized with a Teflon pestle for 1 min.

Cell fractionation. The homogenate was brought to 9 ml with the sucrose–$CaCl_2$ solution. The suspension was centrifuged at 450 *g* for 10 min. The sediment was resuspended in sucrose–$CaCl_2$ solution and the homogenization and centrifugation were repeated twice. The final sediment was considered as nuclear fraction. The pooled supernatant fractions were centrifuged at 10,000 *g* for 15 min in a Servall

automatic superspeed refrigerated centrifuge, type RC-2, to sediment the mitochondria. The remaining supernatant was, in turn, centrifuged at 80,000 *g* for 90 min in a Spinco (Beckman) model L preparative ultracentrifuge to sediment the microsomal fraction. Biochemical and electron microscopic examinations were carried out during the process of cell fractionation. The mitochondrial fraction showed no glucose 6-phosphatase activity[12] and contained no microscopically ribosome-like granules (free or membranes attached). When examined microscopically, the microsomal fraction contained solely smooth and rough-surfaced endoplasmic reticulum and free ribosomal particles. Absence of nuclear contamination in the mitochondrial and microsomal fractions was also indicated by DNA analysis.

Extraction and chromatographic separation of phospholipids. The lipids of whole-cell homogenates and the separated nuclear, mitochondrial, and microsomal fractions were extracted once with 4 ml of 95% ethanol at 65° for 3 min and twice with boiling ethanol:ethyl ether, 2:1 (v/v). Additional extractions failed to give any detectable lipid phosphorus. The solvents were quickly removed in a rotary evaporator (Evapo-Mix, Rinco Instrument Co.) at 50° and 50 mm Hg in vacuum, and the residue was lyophilized in a freeze-dryer (VirTis Co., Inc.) overnight. The lipid extract was further purified by dissolving in chloroform:methanol, 1:1 (v/v) and the individual phosphatides were separated by chromatography on silicic acid-impregnated glass filter paper.[13] The solvent system was diisobutyl ketone:acetic acid:water:benzene (160:50:8:7). The chromatography was carried out at 20° for 6 hr. The chromatograms were dried and stained with rhodamine 6G. The phosphatides were identified under ultraviolet light, chemical spot tests and synthetic standards, and purified lipids as references.[13] Synthetic standards were L-α-lecithin (β,γ-dipalmitoyl), L-α-cephalin (β-γ-dipalmitoyl) (Calbiochem, Los Angeles), and purified phosphatidyl inositol, phosphatidyl serine, phosphatidic acid, and diphosphatidyl glycerol (Pierce Chemical Co., Rockford, Ill.). If the lipids did not separate or overlapping occurred, chromatograms were repeated with less lipid phosphorus.

Radioactive and quantitative determinations of phosphatides. The encircled individual phosphatide chromatograms were cut out and placed in a 1-in.-diameter steel planchet for radioactivity analysis. If the spots were larger than the planchets, they were cut in half and each half placed in a steel planchet and total radioactivity determined. The radioactivity of the spots was counted directly under FD-1′ thin-window flow counter with the SC-100 Multi/Magic sample changer (Tracerlab, Inc.).

After the determination of radioactivity, the chromatograms were placed in Pyrex test tubes (15 × 125 mm), and were eluted with 5 ml of 3 N methanolic HCl for 50 min in a water bath at 63°. The elution was repeated twice with 5 ml of 2 N methanolic HCl, and the combined eluates were evaporated to dryness under infrared light.

The quantitative determination of lipid phosphorus was performed by a modification of the colorimetric method of Shin[14] and Bartlett.[15] After the hydrolysate was evaporated to dryness, 1·0 ml of 18 N sulfuric acid was added. Digestion was carried out for 30 min on a medium gas flame. After the tube was cooled, two drops of 30% H_2O_2 were added and the tube was heated for 30 min. Excess peroxide was removed by adding 2 drops of 5% urea solution and heated again for 15 min. The tube was cooled and 2·0 ml distilled deionized water and 1 ml of 5% ammonium molybdate were added. After mixing, 0·25 ml of Fiske–SubbaRow reagent[14] was added and the solution was thoroughly mixed. The tube was placed in a water bath and was heated at 100° for

10 min. The tube was cooled and centrifuged to precipitate any insoluble residues. The optical density was determined at 800 mμ in a Cary model 15 recording spectrophotometer (Applied Physics Corp.). A linear curve for lipid phosphorus (0·2–16·0 μg) was observed.

Nucleic acids and protein nitrogen determination. The dried residue remaining after lipid extraction was treated with 5 ml of cold trichloroacetic acid (TCA) to extract the acid-soluble phosphorus fraction.[16] Total nucleic acids were then extracted from the remaining residue with 3 ml of 5% trichloroacetic acid for 30 min at 90°, according to the procedure of Logan *et al.*[17] The concentration of total nucleic acid was determined in a Beckman model DU spectrophotometer at 268·5 mμ.[17] DNA was determined with the use of the *p*-nitrophenylhydrazine method of Webb and Levy.[18] RNA was obtained from the difference between total nucleic acids and DNA.

Protein nitrogen of the residue after lipid and nucleic acid extraction was determined by means of the ultramicro-Kjeldahl method.[19]

The specific activity of phosphatides (counts/min/μg lipid phosphorus) was calculated from the phosphorus and radioactivity analysis.

Measurement of mitochondrial swelling. Mitochondria were obtained from the livers of male Sprague–Dawley rats according to the procedure of Lardy and Wellman.[20] The mitochondrial pellet so prepared was suspended in cold 0·25 M sucrose to a final volume equivalent to the weight in grams of the liver used (1 ml/g). The measurement of mitochondrial swelling was performed without delay in order to eliminate the aging effect.[21] To each of the matched 19 × 150 Kimax culture tubes, 6 ml of 0·25 M sucrose solution was added. Digitonin and digitoxin in 95% ethanol were added to separate tubes. The final concentrations of digitonin were 0·6 and 2·6 × 10^{-5} M. The final concentrations of digitoxin were 0·4 and 1·3 × 10^{-6} M. Alcohol and blank controls were performed simultaneously. Inorganic orthophosphate in a final concentration of

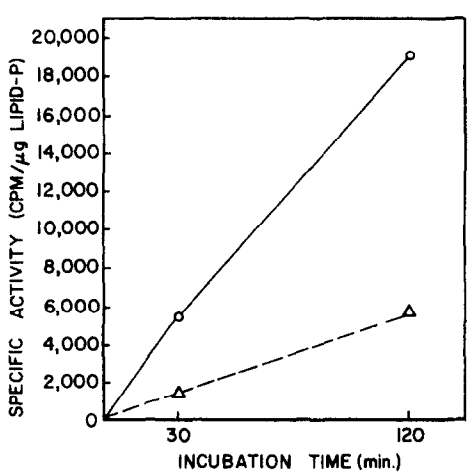

FIG. 1. Effect of digitonin on the incorporation of $^{32}P_i$ into the total phospholipids of HeLa cells. Six-day old HeLa cells, grown as monolayer in growth medium containing 10% of human serum were incubated with digitonin (3·3 × 10^{-5} M) and $^{32}P_i$ (3 μc/ml). Two bottles of cells were used for each designated incubation time interval; (△——△), alcohol control; (○——○), digitonin-treated. Other experimental details are given under Methods.

$4{\cdot}0 \times 10^{-3}$ M was used as a swelling agent for comparison.[22] To each of the tubes, 0·1 ml of the mitochondrial suspension (0·4 mg nitrogen-equivalents) was added and the solution was mixed. The optical density at 515 mμ ($O.D._{515}$) was recorded in a Coleman Jr. spectrophotometer every 2 to 3 min for periods up to 6·5 hr at room temperature. Decreases in $O.D._{515}$ were taken as a measure of swelling.

RESULTS

Effect of digitonin on the incorporation of $^{32}P_i$ into the total phospholipids of HeLa cells

Figure 1 illustrates the time course of $^{32}P_i$ incorporation into digitonin-treated and alcohol control HeLa cells. The stimulatory effect of digitonin was apparent throughout the two incubation periods. The specific activities of the total phospholipids from digitonin-treated HeLa cells were increased threefold over the controls. The pattern of the stimulation is very similar to that observed with estrogen on rat uterus lipid.[4]

The effect of various concentrations of digitonin on the incorporation of $^{32}P_i$ into the phospholipids of HeLa cells is seen from the data of Table 1. The greatest stimulation of the incorporation of the isotope occurred with 1×10^{-5} M concentration of

TABLE 1. EFFECT OF VARIOUS CONCENTRATIONS OF DIGITONIN ON THE INCORPORATION OF $^{32}P_i$ INTO TOTAL PHOSPHOLIPIDS OF HELA CELLS

Digitonin concentration (molarity)	Specific activity (counts/min/μg lipid-P)
Alcohol control	1015
5×10^{-7}	1220
1×10^{-6}	1840
1×10^{-5}	2090
2×10^{-5}	1900
5×10^{-5}	540
1×10^{-4}	200

HeLa cells were grown as monolayers in a growth medium containing 10% human serum for 6 days. Cells were incubated with digitonin at various concentrations and $^{32}P_i$ (3 μc/ml) for 2 hr. One bottle of cells was used for each experiment. Other experimental details are given under Methods.

digitonin. At very high concentrations lysis of the cells and inhibition of the uptake of the isotope results.

The individual phosphatides were separated by paper chromatography in order to ascertain which phosphatides accounted for the radioactivity. Figure 2 illustrates the time study of incorporation of $^{32}P_i$ into the individual phosphatides of control and digitonin-treated HeLa cells. From the patterns of specific activity curves, three groups of phosphatides could be distinguished in respect to the digitonin effect. A progressive increase in stimulation of $^{32}P_i$-labeling was observed in phosphatidyl inositol and diphosphatidyl glycerol. A delayed stimulation was observed in phosphatidyl choline and phosphatidyl ethanolamine which are the major phosphatides of HeLa cells. A third group composing sphingomyelin and phosphatidyl serine, which appeared to be very low in specific activity in the control groups, was not significantly stimulated by digitonin within the incubation period studied. The pattern of incorporation of $^{32}P_i$ into individual phosphatides of control and digiton stimulated HeLa cells

resembles that of phagocytic polymorphonuclear leukocytes[23] and the effect of vitamin D on the rat intestinal mucosa.[5] Ethanol at a concentration of 0·6% did not alter the specific activity of phospholipid in HeLa cells as compared to blank controls (Fig. 2). It has been shown that ethanol has no direct effect on phospholipid synthesis *in vivo*.[6, 24]

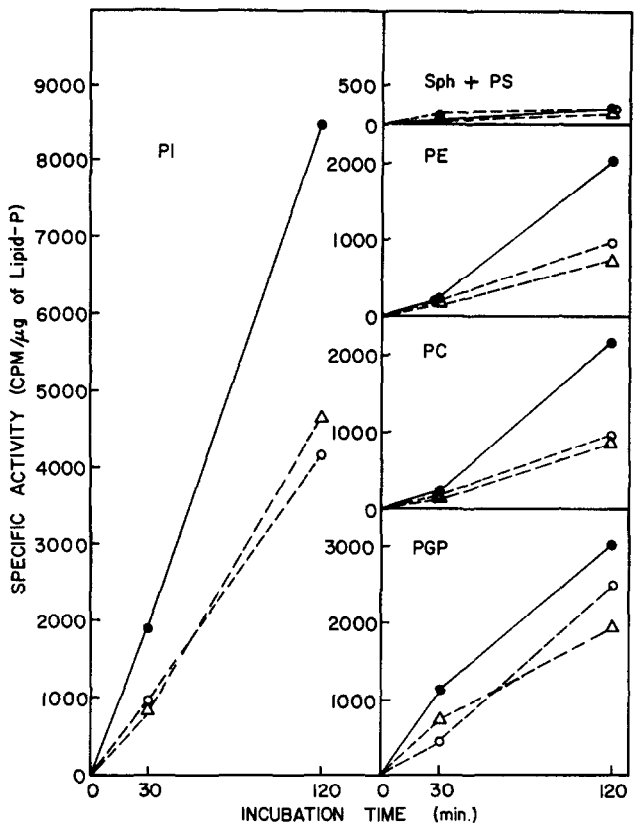

FIG. 2. Effect of digitonin on the incorporation of $^{32}P_i$ into various phosphatides of HeLa cells. Six-day old HeLa cells grown as monolayer in growth medium containing 10% of human serum were incubated with digitonin ($2{\cdot}4 \times 10^{-5}$ M) and $^{32}P_i$ (3 μc/ml). Three bottles of cells were used for each designated incubation time interval; (○——○), blank control; (△——△), alcohol control; (●——●), digitonin-treated. Abbreviations are: PI, phosphatidyl inositol; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PGP, diphosphatidyl glycerol; Sph, sphingomyelin; and PS, phosphatidyl serine. Other experimental details are given under Methods.

Effect of digitoxin on the incorporation of $^{32}P_i$ into the lipids of HeLa and human heart muscle cells

Digitoxin at low concentration ($0{\cdot}5 \times 10^{-6}$ M) was found to increase slightly the specific activity of phospholipids of HeLa cells during the first 30-min incubation period (Fig. 3). However, when the incubation extended to 120 min, the specific activity of phospholipids decreased in the digitoxin-treated cells. Nicholls *et al.*[8] have observed that ouabain, another cardiac glycoside, at concentration of 10^{-5} M, at first

enhances and later inhibits the incorporation of labeled $^{32}P_i$ into rabbit brain slices incubated in Krebs–Ringer medium containing glucose. Studies *in vivo* have shown that digitoxin stimulated the incorporation of $^{32}P_i$ into certain phospholipids of heart ventricle[6] and in heart and liver mitochondria.[7]

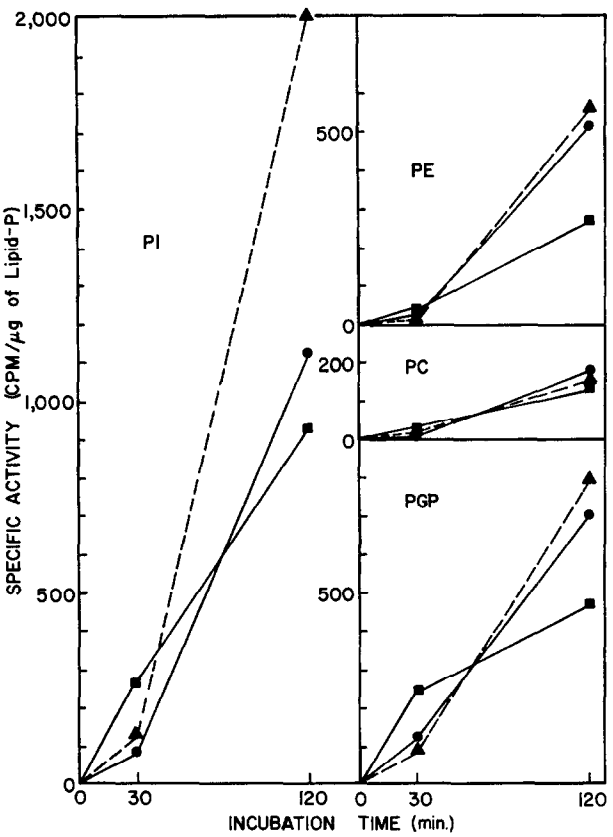

FIG. 3. Effect of digitoxin on the incorporation of $^{32}P_i$ into various phosphatides of HeLa cells. Seven-day old HeLa cells grown as monolayer in growth medium containing 10% of human serum were incubated with digitoxin and $^{32}P_i$ (3 μc/ml). Two bottles of cells were used for each designated incubation time interval; (▲——▲), alcohol control; (●——●), $1{\cdot}3 \times 10^{-5}$ M and (■——■), $0{\cdot}5 \times 10^{-6}$ M of digitoxin. Abbreviations as in Fig. 2. Other experimental details are given under Methods.

Since digitoxin has its major pharmacological action on the heart, the effect of digitoxin on human heart tissue culture cells was studied. Digitoxin stimulated the incorporation of the $^{32}P_i$ into phosphatidyl inositol and sphingomyelin fractions of human heart tissue culture cells, as shown by the increase of the specific activity values (Table 2). Other phosphatides were not significantly affected by digitoxin.

Chemical composition of control and drug-treated HeLa cells

The data in Table 3 indicate that no change of total RNA, protein, or phospholipid content (per μg DNA) of HeLa cells was observed when the cells were incubated either with digitonin or with digitoxin for 120 min. The composition of the phosphatide

fraction of total phospholipids from control and drug-treated HeLa cells, as obtained from the separations on silicic acid-impregnated glass paper chromatography, is given in Table 4. In digitonin-treated HeLa cells, a greater per cent of total lipid phosphorus occurs as phosphatidyl inositol and diphosphatidyl glycerol. No significant differences were observed between the lipids of digitoxin-treated and control HeLa cells with respect to the composition of the phosphatide fractions.

TABLE 2. EFFECT OF DIGITOXIN ON THE INCORPORATION OF $^{32}P_i$ INTO PHOSPHOLIPIDS OF HUMAN HEART CELLS IN CULTURE

Incubation time (min)	Material added	Specific activities of phospholipids*				
		PI	SPH	PC	PE	PA
		(counts/min/μg lipid-P)				
30	Alcohol	80·0	20·5	1·9	10·9	
	Digitoxin + alcohol	61·3	67·8	2·2	4·6	
120	Alcohol	52·8	44·4	5·9	6·2	
	Digitoxin + alcohol	87·2	131·2	8·4	7·8	64·0
240	Alcohol	176·9	84·3	16·4	33·6	663·4
	Digitoxin + alcohol	585·3	357·0	18·6	40·1	698·0

Six-day old human heart tissue cells grown as monolayer in growth medium containing 10% of human serum were incubated with digitoxin ($1·0 \times 10^{-6}$ M) and $^{32}P_i$ (3 μc/ml). Three bottles of cells were used for each designated incubation time interval. Other experimental details are given under Methods.

* Abbreviations are: PI, phosphatidyl inositol; SPH, sphingomyelin; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; and PA, phosphatitic acid.

TABLE 3. EFFECTS OF DIGITONIN AND DIGITOXIN ON THE CHEMICAL COMPOSITION OF HELA CELLS

Material added	RNA μg / DNA μg		Protein μg* / DNA μg		Phospholipid μg† / DNA μg	
	Incubation time (min)					
	30	120	30	120	30	120
None	2·03	2·16	13·48	13·33	3·93	3·80
Alcohol	2·19	2·16	13·01	13·78	3·71	3·64
Digitonin + alcohol ($2·4 \times 10^{-5}$ M)	2·18	2·23	12·67	13·41	3·60	3·71
Alcohol	2·20	2·18	11·11	12·10	3·99	4·09
Digitoxin + alcohol ($0·48 \times 10^{-6}$ M)	2·19	2·36	11·37	12·83	3·13	3·30
Digitoxin + alcohol ($1·3 \times 10^{-5}$ M)	1·79	1·98	10·61	11·66	4·22	3·75

HeLa cells were grown as monolayer in growth medium containing 10% of human serum for 6 days. Three bottles of cells were used for each designated incubation time interval. Other experimental details are given under Methods.

* μg Protein-N × 6·25.

† μg Lipid-P × 25.

Distribution of lipid-^{32}P in subcellular fractions of control and digitonin-treated HeLa cells

To ascertain whether or not specific components of the cells were affected by digitonin, the distribution of $^{32}P_i$ into the individual phosphatides of the subcellular fractions was studied. The results, as shown in Table 5, indicate that the increased incorporation of $^{32}P_i$ into phospholipids was approximately of the same extent in the three

TABLE 4. PER CENT COMPOSITION OF TOTAL PHOSPHOLIPID CONTRIBUTED BY EACH PHOSPHATIDE FROM DIGITONIN- OR DIGITOXIN-TREATED HELA CELLS

Material added	Incubation time (min)	Per cent of total lipid phosphorus				
		PI	SPH + PS	PC	PE	PGP
None	30	3·84	6·10	62·00	22·80	5·20
Alcohol	30	4·65	4·95	63·00	22·80	4·84
Digitonin + alcohol	30	5·46	5·65	59·70	23·20	5·90
None	120	3·64	5·70	58·80	27·00	4·85
Alcohol	120	4·47	4·74	60·70	25·50	4·61
Digitonin + alcohol	120	5·51	5·76	60·60	22·10	6·00
Alcohol	30	2·81	5·81	62·18	26·53	2·54
Digitonin + alcohol	30	2·97	4·69	60·77	28·91	2·66
Alcohol	240	3·35	5·03	61·52	27·61	2·49
Digitonin + alcohol	240	3·79	4·63	57·71	30·52	3·33
Alcohol	30	4·28	4·85	70·50	16·50	3·96
Digitoxin + alcohol (1)	30	4·40	4·10	71·50	16·50	3·48
Digitoxin + alcohol (2)	30	4·40	5·18	74·50	15·50	3·50
Alcohol	120	4·45	5·00	69·00	17·20	4·14
Digitoxin + alcohol (1)	120	4·37	4·05	72·60	15·60	3·42
Digitoxin + alcohol (2)	120	4·60	5·40	70·00	16·40	3·68

HeLa cells were grown as monolayer in growth medium containing 10% of human serum for 6 days. One bottle of cells was used for each designated incubation time interval. The final concentrations were: digitonin, $2{\cdot}4 \times 10^{-5}$ M; digitoxin, (1) $0{\cdot}5 \times 10^{-6}$ M, and (2) $1{\cdot}3 \times 10^{-5}$ M.

Abbreviations as in Table 2 plus: PS, phosphatidyl serine; and PGP, diphosphatidyl glycerol.

subcellular fractions isolated. The increases in $^{32}P_i$-incorporation were observed in phosphatidyl inositol, diphosphatidyl glycerol, phosphatidyl choline, and phosphatidyl ethanolamine fractions from nuclei, mitochondria, and microsomes after the incubation with digitonin.

The effect of digitonin and digitoxin on the integrity of isolated mitochondria

The isolated mitochondria from rat liver swelled readily in the presence of an adequate amount of inorganic phosphate in 0·25 M sucrose solution, as shown in Fig. 4. Neither digitonin nor digitoxin, at the concentrations studied, induced mitochondrial swelling within the 6·5-hr observation period. When phosphate was added to the digitonin-or digitoxin-treated mitochondria, swelling took place with a pattern resembling the standard curve of phosphate-induced mitochondrial swelling. This would suggest that digitonin and digitoxin, at the concentrations tested, do not affect the integrity of mitochondria or the swelling mechanism produced by inorganic

TABLE 5. EFFECT OF DIGITONIN ON THE INCORPORATION OF $^{32}P_i$ INTO SUBCELLULAR LIPID FRACTIONS OF HELA CELLS

Cellular fraction	Incubation time (min)	Material added	Specific activity (counts/min/μg lipid-P) PI	Ratio over control	PC	Ratio over control	PE	Ratio over control	PA	Ratio over control
Nuclei	30	Alcohol	351·1		25·9		19·4		85·3	
		Digitonin		1·67		0·83		1·55		2·11
		+ alcohol	587·3		21·6		30·0		179·6	
	240	Alcohol	848·1		172·0		140·8		366·7	
		Digitonin		2·56		4·48		3·08		1·55
		+ alcohol	2169·7		771·1		432·9		586·6	
Mitochondria	30	Alcohol	361·5		34·1		8·6		124·2	
		Digitonin		1·91		0·70		0·63		1·22
		+ alcohol	690·3		23·7		5·4		151·2	
	240	Alcohol	2656·2		245·5		177·1		496·2	
		Digitonin		1·72		2·86		2·06		1·69
		+ alcohol	4578·1		702·4		364·2		740·9	
Microsomes	30	Alcohol	314·3		15·8		18·8			
		Digitonin		1·89		0·64		0·54		
		+ alcohol	593·8		10·1		9·9			
	240	Alcohol	2452·0		322·4		243·5			
		Digitonin		2·48		1·36		2·68		
		+ alcohol	6088·2		438·6		651·5			

Nine-day old HeLa cells grown as monolayer in growth medium containing 10% of human serum were incubated with digitonin ($2·4 \times 10^{-5}$ M) and $^{32}P_i$ (3 μc/ml). Three bottles of cells were used for each designated time interval. Subcellular fractions were obtained by means of differential centrifugation. Other experimental details are given under Methods.

Abbreviations as in Table 2.

phosphate. Digitonin, however, tends to enhance the swelling effect of the latter compound, as shown in Fig. 4.

Effect of various agents on the incorporation of $^{32}P_i$ into phospholipids of HeLa cells

In order to ascertain whether or not any biological specificity was associated with the stimulatory response of HeLa cells to digitonin as a surface-active substance, a

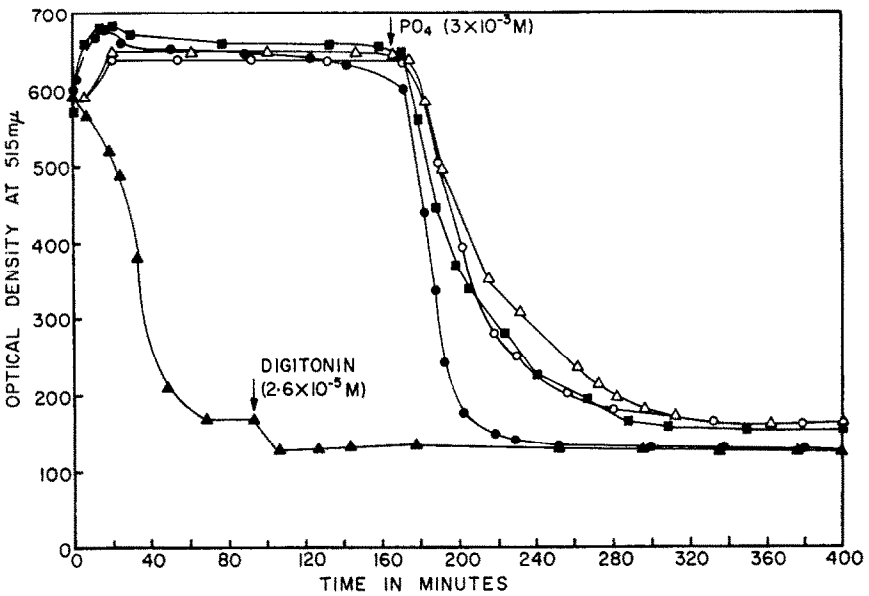

FIG. 4. Effect of digitonin and digitoxin on the integrity of isolated rat liver mitochondria in sucrose solution. Inorganic phosphate ($4{\cdot}0 \times 10^{-3}$ M) was used as a swelling agent for comparison. Each tube contained 6 ml of 0·25 M sucrose and 0·1 ml of freshly prepared mitochondria. Digitonin ($2{\cdot}6 \times 10^{-5}$ M) or digitoxin ($1{\cdot}3 \times 10^{-6}$ M), and 0·6% ethanol, was added and the change in optical density measured at various time intervals. Additional PO_4 and digitoxin were added as indicated by the arrows; (▲——▲), PO_4; (○——○), blank control; (△——△), alcohol control; (●——●), digitonin; (■——■), digitoxin. Other experimental details are given under Methods.

nonsteroid surface-active agent (Triton X-100), a surface-active steroid (desoxycholate), and a steroid hormone (estradiol) were tested. It can be seen from Table 6 that at low concentrations (1×10^{-5} M) both estradiol and desoxycholate stimulated the incorporation of $^{32}P_i$ into the phospholipids. When the concentrations were increased to 1×10^{-4} M, the stimulatory effect of the steroids disappeared. Triton X-100 suppressed the incorporation of $^{32}P_i$ into phospholipids of HeLa cells at both high (0·1%) and low (0·001%) concentrations.

Effect of digitonin on the incorporation of palmitic-1-^{14}C acid into phospholipids of HeLa cells

In order to learn whether digitonin stimulates the incorporation of precursors other than ^{32}P into phospholipids, experiments were performed with ^{14}C-labeled fatty acids, HeLa cells were grown as monolayers in growth medium containing 10% human serum for 6 days. Cells were incubated with digitonin (1×10^{-5} M in alcohol),

palmitic-1-^{14}C was added to each flask (0·06 μc/ml), and the cells were incubated for 2 hr. Control cells were incubated with alcohol and palmitic-1-^{14}C. Digitonin failed to stimulate the incorporation of palmitic-1-^{14}C into total phospholipids. The specific activities (counts/min/μg lipid-P) of the control and digitonin-treated cells were 1000 and 970 respectively. This observation would suggest that the effect of digitonin on

TABLE 6. EFFECT OF VARIOUS AGENTS ON THE INCORPORATION OF $^{32}P_i$ INTO PHOSPHOLIPIDS OF HELA CELLS

Compounds	Concentration	Specific activity (counts/min/μg lipid-P)	Concentration	Specific activity (counts/min/μg lipid-P)
Alcohol		3435		3114
Triton X-100 + alcohol	0·001%	1755	0·1%	440
Desoxycholate + alcohol	1×10^{-4} M	3121	1×10^{-5} M	3580
Estradiol + alcohol	1×10^{-4} M	3550	1×10^{-5} M	4060
Digitonin + alcohol	1×10^{-4} M	2100	1×10^{-5} M	6000

HeLa cells were grown as monolayers in a growth medium containing 10% human serum for 6 days. Cells were incubated with various compounds at various concentrations and $^{32}P_i$ (3 μc/ml) for 2 hr. One bottle of cells was used for each experiment. Other experimental details are given under Methods.

the incorporation of $^{32}P_i$ into phospholipids is specific in the biosynthesis of these lipids.

DISCUSSION

From the results of this investigation it is concluded that digitonin stimulates the incorporation of $^{32}P_i$ into phosphatides of HeLa cells. This effect may be related to the observation that digitonin causes phagocytic cells to manifest the metabolic changes characteristic of phagocytosis[9] which took place with simultaneous increase in the turnover of the cellular phospholipids.[23] The present study leads to the suggestion that the stimulatory effect of digitonin on the phospholipid metabolism might be a general phenomenon, related to the surface-active property of this compound. The increase in incorporation of $^{32}P_i$ could be related to the "reorientation" of the lipids in the biological membranes of the cell when digitonin is added. Bangham and Horne[25] and Dourmashkin *et al.*[26] have demonstrated by electron microscopy changes in membranes of erythrocytes and tissue cells after treatment with surface-active steroid glucosides. There are intimate relationships between phospholipid and protein molecules which serve either as structural units of the biological membranes or as coenzymes attached to the membranes.[27] Alteration of membranous phospholipids and/or cholesterol molecular structure by digitonin may affect the configurations of the juxtapositional enzyme protein; thus their active sites may be masked or revealed. Digitonin or other steroid surface-active agents have been shown to stimulate[28–30] or inhibit[28, 31, 32] enzymic activities. Enzymes responsible for phospholipid synthesis have been shown to be stimulated by surface-active agents desoxycholate[33] and Cutsum (diisobutyl phenoxypolyethoxyethanol).[34] Thus it is not unlikely that digitonin may

stimulate the incorporation of $^{32}P_i$ into phospholipid by activating the corresponding enzymes because of the reoriented membranous phospholipid molecules.

The amphipathic property of digitonin offers the possibility that the biological membrane may disintegrate under the influence of this substance.[35] Surface-active agents have been shown to produce "blebs" or blisters on cell membrane followed by lysis of the cell. This was observed, however, only at high concentrations.[36] It has recently been shown that certain cells treated with digitonin at a concentration up to 8×10^{-3} M showed no visible cytolysis.[37] Davison and Danielli[38] have demonstrated that digitonin at nonhemolytic concentration did not cause ion leakage of erythrocytes. The concentration of digitonin used in this study ($2{\cdot}4 \times 10^{-5}$ M) is of the same order as the concentrations of physiologically active steroids in other studies.[5, 8] It appears, thus, that the concentration of digitonin employed here should permit the integrity of the cell to be retained. It is of interest that digitonin, at a concentration less than $2{\cdot}6 \times 10^{-5}$ M, did not cause the volume change of isolated rat liver mitochondria in sucrose solution nor interfere in the swelling caused by inorganic phosphate.

By far the most interesting point is the stimulatory effect of digitonin on the labeling of phosphatides of HeLa cells, which resembles the effects of certain other steroids on the phospholipid metabolism of various tissues, e.g. estrogenic hormones on rat uterus,[4] vitamin D on intestinal mucosa of rats,[5] ouabain on rabbit brain and sea gull salt gland slices,[8] as well as the effect induced by phagocytosis on polymorphonuclear leukocytes[23] and acetyl choline on the secretion of pancreas[39] and the salt gland of the albatross.[40] Sastry and Hokin[41] recently reported that the increased incorporation of $^{32}P_i$ into phosphatidic acid and phosphatidyl inositol in polymorphonuclear leukocytes undergoing phagocytosis is not secondary to an elevated specific activity of adenosine triphosphate during phagocytosis. The similarities are listed as follows: (a) the primary effect observed was an increase in incorporation of $^{32}P_i$ into phospholipids; (b) the stimulation of $^{32}P_i$-incorporation involves most or all phosphatides; (c) the pattern of $^{32}P_i$-labeling of the individual phosphatides was very characteristic, the incorporation of the isotope into phosphatidyl inositol and diphosphatidyl glycerol was rapid within a short period of incubation, a lag period was observed in the labeling of phosphatidyl ethanolamine and phosphatidyl choline, and the uptake of the isotope into sphingomyelin was not significant; (d) the stimulation was observed in all subcellular fractions studied and to approximately the same extent for a given phosphatide; (e) stimulation of $^{32}P_i$-incorporation into nonlipid organic phosphates was not observed in the case of vitamin D_5 and ouabain[8] studies, and was delayed with estrogen[4] and digitonin studies. All these observations suggest the generalized effect of certain steroids on the metabolism of phospholipid in various cellular structures. From the data in Table 6 it is apparent that the nonsteroid surface-active agent Triton failed to stimulate the incorporation of $^{32}P_i$ into the phospholipids of HeLa cells. The other steroids, desoxycholate, and estradiol stimulated the incorporation of $^{32}P_i$ into the phospholipids. Deoxycholate, a surface-active agent, has been shown to stimulate the enzymes involved in phospholipid synthesis.[33] It would appear from these results that the stimulatory effect of digitonin on phospholipid biosynthesis may be related to its steroid structure and its surface-active property.

In contrast to digitonin, digitoxin did not significantly affect the specific activity of phospholipid of HeLa cells at either low or high concentration. However, digitoxin stimulated the incorporation of $^{32}P_i$ into phosphatidyl inositol and sphingomyelin

fraction of human heart culture cells. This may suggest the structural specificity of the various steroids in regard to their effect on the cell membranes. As is shown by Aizawa and Mueller,[4] natural estrogens (estrone, estradiol, and estriol) stimulate the incorporation of $^{32}P_i$ into phospholipids of rat uterus, adrenal corticosteroids, and progesterone; and the nonsteroid diethylstilbestrol, on the other hand, suppresses the incorporation. Recently, Bangham *et al.*[42] have further indicated the structural specificity of various biologically active steroids on the permeability change of artificial bilayer phospholipid membrane systems.

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