Stimulation by serum of ^{32}P incorporation into the phospholipids of Ehrlich ascites cells

Peter B. Schneider

Thorndike Laboratory and Department of Medicine, Harvard Medical School, and Beth Israel Hospital, Brookline, MA 02215

Abstract Ehrlich ascites cells adapted for suspension culture were incubated with $^{32}PO_4^{3-}$ in a Krebs-Ringer–HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonate) buffer with or without 5% calf serum. Specific activity was determined for cellular [γ-^{32}P]ATP, total phospholipids, and the individual phospholipids fractionated by two-dimensional thin-layer chromatography. Serum stimulated the incorporation of ^{32}P into the phosphatides with a 2–3-fold increase in specific activity of phosphatidylcholine and phosphatidylethanolamine. Phosphatidylinositol showed a moderate increase. Phosphatidic acid showed an increase that could be accounted for by a parallel small increase in the specific activity of precursor [γ-^{32}P]-ATP. The effect of serum could be discerned as early as 10 min after its addition. Complete inhibition of protein synthesis by cycloheximide or puromycin did not interfere with the serum stimulation of ^{32}P incorporation into lipids, indicating that the serum effect is not dependent on synthesis of new protein. Incubation with palmitic acid caused a marked increase of phosphatidylinositol specific activity, little change in specific activity of phosphatidic acid but a tripling of its total amount, and no changes in the other phosphatides. Oleic acid caused only a small increase in phosphatidylinositol specific activity and no significant changes in the other lipids.

Supplementary key word turnover

Phospholipids are ubiquitous cell constituents, important for both cell structure and activity as components of cell membranes. The cellular mechanisms involved in the control of phosphatide metabolism and turnover are thus of considerable interest, and investigations into the nature of these mechanisms should help elucidate the basic cell processes of growth and activity. Previous studies have focused primarily on increases of phosphatide turnover subsequent to increases of cell activity in such systems as the thyrotropin stimulation of thyroid (1–3), acetylcholine stimulation of pancreas (4), insulin stimulation of adipose tissue (5), acetylcholine stimulation of brain (6), and phagocytosis of starch granules by leukocytes (7). It has also been shown that phospholipid turnover is increased when cultured confluent 3T3 cells are stimulated to grow by the addition of serum (8). Another model system for the investigation of the effects of growth factors on cell processes is that of Ehrlich ascites cells in suspension culture where growth is dependent on the presence of serum (9). I have found that, in these cells, phosphatide turnover also is stimulated by serum. In this paper I report the patterns and some details of this phosphatide response.

METHODS

Cell cultivation

Ehrlich ascites cells adapted for growth in suspension culture (10) were the kind gift of Dr. E. Henshaw. These cells were grown at 37°C in spinner flasks (Bellco Glass, Inc., Vineland, NJ). The growth medium consisted of Eagle's Minimum Essential Medium (Grand Island Biological Co. Grand Island, NY) supplemented with 0.1% D-glucose, 75 U/ml of penicillin G, 50 μg/ml of streptomycin, and 5% dialyzed calf serum. The cells were grown at a density of $4–12 \times 10^5$/ml and were resuspended in fresh medium daily. Cells to be used for an experiment were grown as above but in the absence of serum for 24 hr prior to the experiment.

Cell incubations

Cells were harvested by centrifugation, resuspended in a minimum of saline, and added to 8 ml of incubation medium in 25-ml flasks in a shaking water bath. The medium was Krebs-Ringer–HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonate) buffer (pH 7.4 at 37°C) containing 10 mM D-glucose, 1.2 mM PO_4^{3-}, 10–20 μCi/ml ^{32}P, and the appropriate test substances. Final cell density was 10^7/ml. At the end of the incubation the contents of the flask were transferred to conical glass homogenizer tubes and centrifuged for 1 min at about 1500 *g*. The medium was carefully aspirated and the cells were

homogenized in 0.5 ml ice-cold 2% $HClO_4$. Aliquots of the acid extract were taken for [γ-^{32}P]ATP assays and the residue was extracted for lipid analyses. The specific activity of the terminal phosphate of ATP was selectively measured by an enzymatic method (11, 12) in which the transfer of the γ-P of ATP to [^{3}H]glycerol is measured.

When added to the incubations, palmitic and oleic acid were added as warmed sodium salts to the albumin or serum to be used in the incubation. These were then added to the incubation buffer. Palmitic acid (Fisher Scientific Co., Pittsburgh, PA, mp 61–62°C) and oleic acid (K&K Laboratories, Plainview, NJ 99+%) chromatographed as single spots on silica gel H plates developed with either chloroform–methanol–2 M ammonium hydroxide 60:45:4 or with petroleum ether–ethyl ether–acetic acid 60:40:1.

Phosphatide analysis

The acid ($HClO_4$) precipitate was treated with 1 ml of methanol and then twice with 2 ml of chloroform–methanol 1:1 (v/v) to extract lipids. One ml of chloroform and then 2 ml of 0.1 M $MgCl_2$ (in 0.01 M HCl) were added. The chloroform phase was washed twice with 2 ml of "artificial upper phase" (13) and used for lipid analysis. The specific activity of the crude phosphatides was determined by analyzing an aliquot for phosphorus (14) and for ^{32}P activity by liquid scintillation counting (12). Fractionation of phospholipids was accomplished as previously described (12) by two-dimensional thin-layer chromatography on Silica Gel H (Analabs, North Haven, CT) plates developed with chloroform–methanol–1 M ammonium hydroxide 50:50:5 (v/v/v), followed by chloroform–methanol–pyridine–conc. formic acid–water 100:10:32:12:1 (by volume). The phosphatide spots were located by radioautography, ninhydrin spray, and the Zinzadse spray for phosphorus (15), and were identified by comparison with appropriate standards (12). The spots were individually scraped from the plates for analysis. Spots corresponding to lysophospholipids and other minor phospholipids were not collected. Specific analysis of plasmalogens was not undertaken and these may have been partially lost by hydrolysis in the perchloric acid used for precipitation. However, a similar extraction procedure using trichloroacetic acid (16) was reported to show no difference in the fractionated lipids (including plasmalogens) compared to extraction with chloroform–methanol directly without use of the acid. The spots from the thin-layer plate were digested with 0.4 ml of 70% (w/w) $HClO_4$ and analyzed colorimetrically for phosphorus. The blue solution from colorimetry was bleached with alkaline ammonium peroxydisulfate, diluted with water, and used directly for ^{32}P determination in the aqueous phase by counting Cerenkov radiation (17). All ^{32}P determinations were normalized to a specific activity of 10,000 cpm/nmol of P substrate in the initial incubating medium. For convenience in working up a larger number of samples in some experiments (Table 3, Fig. 1) a partial fractionation was employed using one-dimensional thin-layer chromatography with chloroform–methanol–1 M ammonium hydroxide 60:40:4.5 (v/v/v) on Silica Gel H. This system is adequate for the analysis of phosphatidylethanolamine, phosphatidylinositol, and phosphatidylcholine. The first two lipids run discretely while phosphatidylcholine is variably overlapped by phosphatidylserine. However, since both the total amount and the total radioactivity of phosphatidylserine was only 10–20% that of the phosphatidylcholine, the error in the specific activity determinations of phosphatidylcholine is less than about 10%.

RESULTS

Preliminary experiments showed that Ehrlich ascites cells incubated in the absence of protein had a tendency to clump and that this could be obviated by the addition of 5 mg/ml of bovine serum albumin. However, the use of fatty acid-poor albumin resulted in slightly lower (about 5%) baseline ^{32}P incorporation into phosphatides than the use of Cohn fraction V. Moreover, the addition of both fatty acid-free albumin and serum in the incubations reduced the ^{32}P incorporation slightly below that seen with serum alone. On the other hand, the addition of fraction V to serum caused no change. Therefore, control incubations contained 5 mg/ml of bovine serum albumin (fraction V).

Table 1 shows the effect of serum, under several conditions, on the incorporation of ^{32}P into total and individual phospholipids as well as into the terminal P of ATP of Ehrlich ascites cells. Typical experiments are listed, but for further statistical analysis the results of pooled experiments are given in **Table 2.** In individual experiments the deviation from the mean of duplicate determinations was within 5% for the specific activities of ATP and total phosphatides and within 10% for all the individual lipids except for phosphatidylserine and sphingomyelin. This deviation ranged 10–30% for phosphatidylserine and 10–60% for spingomyelin, due primarily to variations in the low total radioactivity recovered for these lipids. The results listed in Table

TABLE 1. Effect of serum on incorporation of ^{32}P into the phosphatides of Ehrlich ascites cells

Exp.	Conditions	Calf Serum (5%)	[γ-^{32}P]ATP	Total ^{32}P-Labeled Lipids	Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidic Acid	Phosphatidylinositol	Phosphatidylserine	Sphingomyelin
			^{32}P sp act (cpm/nmole)							
1.	Standard (1 hr)	−	3310	170	27	150	2240	360	21	3.4
		+	3140	210	62	310	2040	440	22	3.7
2.	Supplemented with MEM amino acids & vitamins	−	2970	80	16	51	1060	130	6	1.5
		+	3290	120	61	125	1240	180	9	2.6
3.	Supplemented with 0.5 mM palmitate	−	2480	210	21	67	1690	430	8.7	1.0
		+	2790	250	66	100	1990	450	11	2.0
4.	Standard (4 hr)	−	2710	240	140	320	1820	440	130	15
		+	4000	530	600	670	1920	740	230	63

Ehrlich ascites cells were incubated at a density of 10^7/ml for 1 hr (4 hr in exp. 4) in the standard system consisting of 8 ml of Krebs-Ringer HEPES buffer (pH 7.4, 37°C) with 0.01 M glucose and 15 μCi/ml of ^{32}P. Conditions were varied from standard, as indicated, by the addition of the amino acids and vitamins of Eagle's Minimum Essential Medium (MEM) or palmitate. Calf serum was added where listed and the other flasks contained 5 mg/ml of bovine serum albumin (Cohn fraction V). All results are normalized to a specific activity of 10,000 cpm/nmol of the ^{32}P substrate. Each value is the mean of duplicate incubations and lipid fractionations.

2 include variations between experiments that were generally higher than those between duplicates within the experiment.

The specific activity of [γ-^{32}P]ATP was measured since, as a precursor of the phosphatide phosphorus, any increase in its specific activity would be reflected as an apparent stimulation of phosphatide turnover. The effect of serum on the [γ-^{32}P]ATP was relatively variable from experiment to experiment and in group 1, Table 2 the increase was not statistically significant. This point was tested in a larger series of observations on 12 pairs of incubations (1 hr) which revealed that serum produced an average increase of specific activity of 12% ± 3.1 (SEM), which is significant with $P < 0.005$ when analyzed by the paired t test. Similar observations on six pairs of incubations in which the medium was supplemented with the amino acids and vitamins of minimum essential medium showed an increase of 14% ± 1.7 (SEM) ($P < 0.001$) in the [γ-^{32}P]ATP specific activity due to serum. The variability of the serum effect was much less in replicate incubations within an experiment, suggesting that the quantitative differences in the effect of serum on ATP specific activity might have been due more to uncontrollable factors in the growth history of the cell batches than due to variations during the test incubations.

Serum causes a consistent stimulation of ^{32}P incorporation into total phosphatides. In the fractionated lipids the stimulation was most marked in phosphatidylcholine and phosphatidylethanolamine, which doubled or tripled their specific activity. Sphingomyelin and phosphatidylserine showed variable increases in specific activity in 1 hr incubations, the interpretation of which was hindered by the large experimental errors in these determinations.

TABLE 2. Effect of serum on incorporation of ^{32}P into the phosphatides of Ehrlich ascites cells[a]

Exp. Group	[γ-^{32}P]ATP	Total ^{32}P-Labeled Lipids	Phosphatidyl choline	Phosphatidylethanolamine	Phosphatidic Acid	Phosphatidylinositol	Phosphatidylserine	Sphingomyelin
	change of ^{32}P sp act due to serum (% of control ± SEM)							
1.	110 (6.2)	126 (1.9)[b]	244 (16.9)[b]	226 (25)[c]	90 (7.4)	120 (5)[d]	118 (14)	182 (41)
2.	116 (3.2)[c]	139 (7.9)[c]	407 (18)[b]	213 (17)[c]	110 (12)	154 (10)[c]	260 (79)	280 (73)
3.	114 (1.9)[b]	122 (9)	309 (10)[b]	136 (6)[c]	112 (9)	93 (12)	107 (20)	222 (43)[d]
4.	125 (9.6)[d]	194 (10)[b]	282 (45)[c]	262 (21)[b]	111 (8)	139 (13)[d]	216 (13)[b]	258 (53)[d]

Experimental conditions are indicated by corresponding experiment number in Table 1. For statistical analysis the results of several experiments were pooled. The effect of 5% serum is expressed as the percent of a control incubation without serum. Levels of significance were determined by the two-tailed paired t test (five pairs for group 1; six pairs for groups 2, 3, 4).

[a] Analysis of pooled experiments.
[b] $P < 0.001$.
[c] $P < 0.01$.
[d] $P < 0.05$.

However, in prolonged (4 hr) incubations there was a more significant increase in the specific activity of these two lipids. The changes in phosphatidic acid specific activity were small and can, for the most part, be explained by changes in specific activity of the precursor ATP. This is demonstrated by calculating the ratios of the phosphatidic acid to ATP specific activities; in each of the experiments of Table 1 this ratio in the presence of serum was not significantly elevated above that of the control. Serum increased phosphoinositide specific activity in standard incubations or when supplemented with amino acids and vitamins. These changes, of the order of 20–50% after 1hr, are more than can be attributed to changes in either ATP or phosphatidic acid, both of which are precursors of the phosphoinositide phosphorus. In incubations supplemented with palmitic acid, increases in phosphatidylinositol activity were not significant.

Although these experiments were designed for the determination of specific activity, the total amount of phosphatidylcholine recovered was reasonably reproducible, varying only within 10% between duplicate incubations and between sets incubated with and without serum. It thus appears that within those limits serum did not cause an increase in the amount of phosphatidylcholine. Similarly, serum had no effect on the total amount of phosphatidylethanolamine, phosphatidylinositol, phosphatidic acid, or sphingomyelin within about 15%, and had no effect on phosphatidylserine within about 30%.

To elucidate the effects of fatty acids per se on phosphatide turnover in Ehrlich ascites cells, incubations were carried out with palmitic and oleic acids. Palmitic acid maximally stimulated ^{32}P incorporation at a concentration of 0.5 mM (5% albumin) with no further change of effect up to about 3 mM. Oleic acid had little effect on total phosphatide ^{32}P up to a level of 0.5 mM (5% albumin), at which point it inhibited incorporation of the isotope. Accordingly, in the fractionation experiments (**Tables 3** and **4**), a level of 0.5 mM palmitic acid or 0.2 mM oleic acid was chosen. Palmitic acid had no effect on ^{32}P incorporation into [γ-^{32}P]ATP, phosphatidylcholine, or phosphatidylethanolamine. It appeared to decrease phosphatidylserine and increase sphingomyelin specific activities. Phosphatidylinositol showed a marked increase in ^{32}P. The response of the phosphatidic acid specific activity to palmitate was modest, but a striking finding was the tripling of the amount of phosphatidic acid, an increase far beyond analytical error. Because of this increase phosphatidic acid contributed significantly to the rise of ^{32}P of the unfractionated phosphatides. The effect of oleate was quite different with little or no effect on any component except for a moderate rise seen in the phosphoinositide specific activity, a moderate rise in the phosphoinositide and phosphatidylserine specific activity, but an inhibition of phosphatidic acid specific activity and total amount.

The time course of the incorporation of ^{32}P into the total phosphatides and the phosphatidylcholine of Ehrlich ascites cells and the response to incubation with serum is shown in **Fig. 1.** The stimulation of phosphatidylcholine turnover became evident as early as 10 min after the addition of serum, while this effect was masked in the unfractionated lipids until about 20 min. The response of phospha-

TABLE 3. Effect of fatty acids on incorporation of ^{32}P into the phosphatides of Ehrlich ascites cells

Exp.	Fatty Acid	[γ-^{32}P]ATP	Total ^{32}P-Labeled Lipids	Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidic Acid	Phosphatidylinositol	Phosphatidylserine	Sphingomyelin
				^{32}P sp act (cpm/nmol)					
1	–	2850	78	22	74	1390	59	15	0.4
	+ Palmitate 0.5 mM	2790	384	20	72	1730	320	9	1.4
2	–	2100	70	46	94	1520	40	24	2.4
	+ Oleate 0.2 mM	1890	69	52	105	1370	60	36	2.5
				recovered lipid ^{31}P (nmol/10^7 cells)					
1	–		200	49	21	2.8	12	13	15
	+ Palmitate 0.5 mM		214	54	23	9.4	15	14	14
2	–		210	53	22	1.2	11	8	14
	+ Oleate 0.2 mM		207	53	18	1.0	13	9	14

Ehrlich ascites cells were incubated at a density of 10^7/ml for 1 hr in 8 ml of Krebs-Ringer HEPES buffer (pH 7.4, 37°C) with 0.01 glucose and 15 μCi/ml of ^{32}P. All incubations contained 5 mg/ml of fatty acid-free bovine serum albumin and, where indicated, a fatty acid. All results are normalized to a specific activity of 10,000 cpm/nmol of the ^{32}P substrate. Each point is the mean of duplicate incubations and lipid fractionations.

TABLE 4. Effect of fatty acids on incorporation of ^{32}P into the phosphatides of Ehrlich ascites cells[a]

Exp. Group	[γ-^{32}P]ATP	Total ^{32}P-Labeled Lipids	Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidic Acid	Phosphatidylinositol	Phosphatidylserine	Sphingomyelin
		change of ^{32}P sp act due to fatty acid (% of control ± SEM)						
1.	98 (1)	249 (2.1)[b]	129 (23)	100 (4.5)	133 (12)	676 (93)[c]	58 (4)[c]	255 (63)
2.	90 (1.2)[c]	104 (3.2)	137 (16)	110 (3.3)	85 (4.6)[d]	194 (26)[d]	165 (7.9)[c]	115 (13)
		change of recovered lipid ^{31}P due to fatty acid (% of control ± SEM)						
1.		105 (1.3)[d]	92 (10)	97 (5.8)	299 (23)[c]	110 (11)	96 (10)	95 (2.8)
2.		103 (4)	103 (6.8)	89 (10)	71 (4.8)[c]	112 (5.5)	106 (28)	95 (5.1)

Experimental conditions are indicated by corresponding exp. number in Table 3. Statistical analysis as in Table 2 (4 pairs for each group).

[a] Analysis of pooled experiments.

[b] $P < 0.001$.

[c] $P < 0.01$.

[d] $P < 0.05$.

tidylethanolamine, which was also measured in this experiment, was parallel to that of the phosphatidylcholine, being first noted at about 10 min. The other lipids were not individually studied in this sequential manner but, in separate experiments with observations only at 15 min after addition of serum, no stimulation of turnover in those lipids was noted.

The relationship between the serum stimulation of protein synthesis and of phosphatide turnover was examined by incubations with inhibitors of protein synthesis **(Table 5).** Protein synthesis was completely inhibited by cycloheximide or puromycin. In contrast, except for a decrease baseline specific activity caused by 10^{-3}M puromycin, these inhibitors had no effect on the turnover of the measured phosphatides nor on the serum stimulation of that turnover.

DISCUSSION

The presence of serum in the incubation medium of Ehrlich ascites cells is necessary for their growth and multiplication and it is known that an early effect of serum addition is the stimulation of protein synthesis (9). We have found that another early effect of serum is the stimulation of ^{32}P incorporation into the phospholipids of the cells. Fractionation of the phosphatides showed that phosphatidylcholine and phosphatidylethanolamine were most markedly stimulated by serum; there was a lesser effect on phosphatidylinositol and no selective effect on phosphatidic acid. Phosphatidylserine and sphingomyelin specific activities were increased by serum in prolonged (4 hr) incubations although a more acute effect at 1 hr might have been clouded by the high variability of the results. A delayed response in these two lipids may reflect the role of phosphatidylcholine as a precursor for both phosphatidylserine and sphingomyelin as postulated by Diringer et al. (18, 19). In pulse-chase experiments with cultured mouse fibroblasts they found that changes in ^{32}P activity of phosphatidylcholine were reflected in phos-

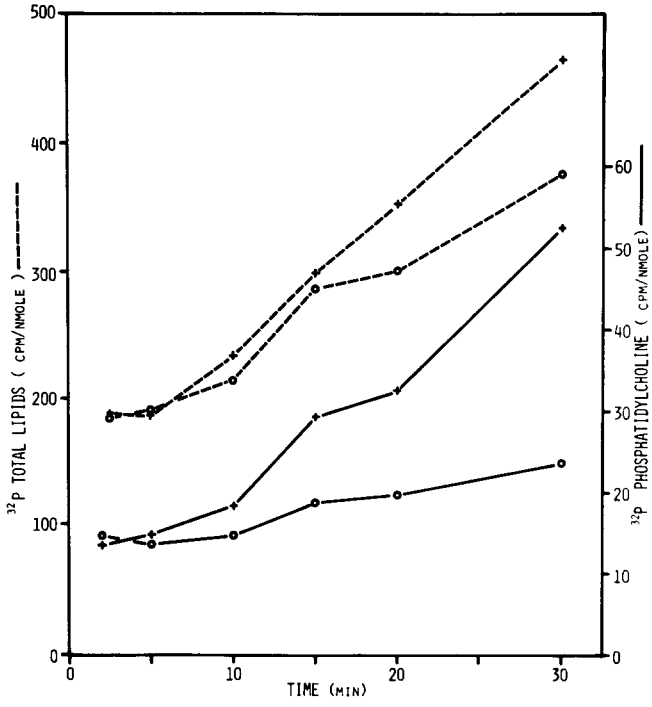

Fig. 1. The figure shows the time course of the incorporation of ^{32}P into total lipids (– – –) and phosphatidylcholine (——) of Ehrlich ascites cells incubated with (+) or without (0) 5% calf serum. Cells at a density of 10^7/ml were incubated in 2 ml Krebs-Ringer HEPES buffer (pH 7.4, 37°C) containing 0.01 M glucose and 10 μCi/ml of $^{32}PO^{3-}_4$. All incubations contained 5 mg/ml of bovine serum albumin (fraction V). Serum was added 10 min after the start of the incubation and timing for the displayed graphs was begun with that addition.

TABLE 5. Effect of inhibition of protein synthesis on the serum stimulation of phosphatide turnover in Ehrlich ascites cells

Inhibitor	Conc.	Calf Serum	Protein Synthesis	[γ-^{32}P]ATP	Total ^{32}P-Labeled Lipids	Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidylinositol
	M	*5%*	*% of control*			*cpm/nmol*		
None		0	100%	2040	62	16	56	130
		+	141	2840	109	47	100	230
Cycloheximide	3.5×10^{-5}	0	3	1990	62	23	51	134
		+	5	2690	110	47	99	234
	3.5×10^{-4}	0	0.6	2200	61	16	53	120
		+	0.7	2440	104	53	100	230
Puromycin	10^{-4}	0	18	2140	74	16	57	150
		+	14	2820	120	45	94	280
	10^{-3}	0	0	1800	47	9	41	97
		+	0.4	2680	103	29	67	225

Ehrlich ascites cells at a density of 10^7/ml were incubated in Krebs-Ringer HEPES buffer (pH 7.4, 37°C) containing 0.01 M glucose and the indicated inhibitor. At 30 min 10 μCi/ml of ^{32}P and, where listed, calf serum were added and the incubatin continued for another 1 hr. Specific activity of ^{32}P-containing compounds was determined and normalized to a specific activity of 10,000 cpm/nmol of the ^{32}P substrate. Protein synthesis was determined in parallel by measuring the incorporation of L-[^{14}C]lysine into total cell protein (16).

phatidylserine and sphingomyelin only after a delay of several hours, which was compatible with a precursor–product relationship of these lipids.

The pattern of response of the Ehrlich cell lipids to serum is in marked contrast to the response of hormonally stimulated tissues. In these latter systems, which include in vitro effects of acetylcholine on brain (20), acetylcholine on pancreas (4), acetylcholine on avian salt gland (21), insulin on adipose cells (5), and thyrotropin on thyroid (2, 3), the major lipids affected are phosphatidylinositol and phosphatidic acid with lesser or no response in the other phosphatides. Similarly, when phagocytosis is stimulated in leukocytes, phosphatidic acid and phosphatidylinositol turnover are primarily affected (7). This difference in response between Ehrlich ascites cells and the other tissues may reflect a basic functional difference: serum stimulates growth and multiplication of the Ehrlich cells while in the other systems, at least acutely, cellular activity is primarily stimulated. Thus, increases in the turnover of phosphatidylcholine and phosphatidylethanolamine may reflect stimulation of structural membranes involved in growth while increases in phosphatidylinositol and phosphatidic acid may reflect changes in those membranes concerned primarily with cell activity including such events as transmembrane transport or formation of zymogen granules or lysosomes.

Ehrlich ascites cells also respond differently than confluent 3T3 cells in which, as Cunningham (8) reported, the addition of serum caused equal increases in each of the phospholipids with no selective effects. Diringer and Koch (22), in pulse-chase experiments with cultured embryonic mouse cells, also observed that addition of serum increased the turnover of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol. In their experiments the responses of each of the three lipids were approximately comparable but were not prominent for several hours. It is not presently clear how much of the difference between our results and those of the above investigators is due to differences in the cell lines themselves (e.g. normal vs. carcinoma cells) or in the experimental design (e.g., monolayer vs. suspension culture).

Within our experimental limits we did not observe net synthesis of lipid. Obviously, as cells grow, a net synthesis must take place and it is possible that a small increase of phosphatides did occur within our assay limits of 10–15%. But it is also possible that the acute effect of serum is to cause membrane restructuring and lipid turnover preparatory to actual growth and need not entail net synthesis in this time interval.

The site of action of serum in stimulation of the ^{32}P incorporation into phosphatidylcholine and phosphatidylethanolamine appears to be beyond ATP in the biosynthetic sequence (ATP → phosphorylcholine → CDP-choline → phosphatidylcholine or, ATP → phosphorylethanolamine → CDP - ethanolamine → phosphatidylethanolamine). An effect on the transport of PO_4^{3-} across the cell membrane or an effect on ATP turnover can be excluded. If these processes were affected, all phosphatides should have

shown parallel increases in specific activity reflecting the changes in their precursors. Furthermore, the increases in the specific activity of phosphatidylcholine and phosphatidylethanolamine were greater than those observed in the [γ-^{32}P]ATP. On the other hand, changes in phosphatidic acid were quantitatively parallel to those in the [γ-^{32}P]ATP and thus could be explained merely by variations in ATP turnover. By similar reasoning, since increases in phosphatidylinositol specific activity due to serum were greater than those in the phosphatidic acid, the effect of serum is probably distal to phosphatidic acid in the sequence: phosphatidic acid → CDP-diglyceride → phosphatidylinositol. These arguments suppose the existence of relatively uniform precursor pools although multiple compartments cannot be explicitly excluded. It is theoretically possible, for instance, that the changes observed in phosphatidylinositol could reflect changes in a very small, high specific activity, pool of phosphatidic acid while direct observation of that pool is masked by a larger compartment of phosphatidic acid.

The mechanism of action of the serum is undetermined. We can, at least, rule out the possibility that serum produces its effect via synthesis of new enzymes or other proteins, since the effect is a very early one and is not influenced by inhibition of protein synthesis. As far as phosphoinositide goes, the serum effect may, at least in part, be due to its content of fatty acids. This is plausible in view of the marked effect of palmitic acid itself on ^{32}P incorporation into phosphatidylinositol and the finding that, in the presence of excess palmitic acid, serum has little additive effect. However, serum is much more active than albumin (fraction V), which contains the bulk of the fatty acids. This suggests that fatty acids are not the sole stimulating factor for phosphatidylinositol although differences in the fatty acid composition of serum and fraction V may play a role in our experiments.

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REFERENCES

1. Morton, M. E., and J. R. Schwartz. 1953. The stimulation in vitro of phospholipid synthesis in thyroid tissue by thyrotrophic hormone. *Science.* **117:** 103–104.
2. Freinkel, N. 1957. Pathways of thyroidal phosphorus metabolism. *Endocrinology.* **61:** 448–459.
3. Schneider, P. B. 1972. A site of action of thyrotropin. *J. Biol. Chem.* **247:** 7910–7914.
4. Hokin, L. E., and M. R. Hokin. 1955. Effects of acetylcholine on the turnover of phosphoryl units in individual phospholipids of pancreas slices. *Biochim. Biophys. Acta.* **18:** 102–110.
5. DeTorrontegui, G., and J. Berthet. 1966. The action of adrenalin and glucagon on the metabolism of phospholipids in rat liver. *Biochim. Biophys. Acta.* **116:** 477–481.
6. Hokin, L. E., and M. R. Hokin. 1955. Effects of acetylcholine on phosphate turnover in phospholipids of brain cortex in vitro. *Biochim. Biophys. Acta.* **16:** 229–237.
7. Karnofsky, M. L., and D. F. H. Wallach. 1961. The metabolic basis of phagocytosis. *J. Biol. Chem.* **236:** 1895–1901.
8. Cunningham, D. D. 1972. Changes in phospholipid turnover following growth of 3T3 mouse cells to confluency. *J. Biol. Chem.* **247:** 2464–2470.
9. Kaminskas, E. 1972. Serum mediated stimulation of protein synthesis in Ehrlich ascites tumor cells. *J. Biol. Chem.* **247:** 5470–5476.
10. Van Venrooij, W. J. W., E. C. Henshaw, and C. A. Hirsch. 1970. Nutritional effects on the polyribosomal distribution and rate of protein synthesis in Ehrlich ascites tumor cells in culture. *J. Biol. Chem.* **245:** 5947–5953.
11. Schneider, P. B. 1968. An enzymatic assay for ATP and determination of the specific activity of their terminal P. *Anal. Biochem.* **28:** 76–84.
12. Schneider, P. B. 1970. Stimulation of thyroidal phospholipid turnover by osmotic shock. *J. Biol. Chem.* **245:** 6281–6284.
13. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissue. *J. Biol. Chem.* **226:** 497–509.
14. Bartlett, G. R. 1959. Phosphorus analysis for column chromatography. *J. Biol. Chem.* **234:** 466–468.
15. Dittmer, J. C., and R. L. Lester. 1964. A simple specific spray for the detection of phospholipids on thin-layer chromatograms. *J. Lipid Res.* **5:** 126.
16. Wallach, D. F. H., J. Soderberg, and L. Bricker. 1960. The phospholipids of Ehrlich ascites carcinoma cells; composition and intracellular distribution. *Cancer Res.* **20:** 397–402.
17. Schneider, P. B. 1971. Determination of specific activity of ^{32}P-labeled compounds using Cerenkov counting. *J. Nucl. Med.* **12:** 14–16.
18. Diringer, H. 1973. Phospholipid metabolism in mammalian cells. Kinetic data suggests a biosynthesis of phosphatidylserine via phosphatidylcholine. *Hoppe-Seyler's Z. Physiol. Chem.* **354:** 577–582.
19. Diringer, H., W. D. Marggraf, M. A. Koch, and F. A. Anderer. 1972. Evidence for a new biosynthetic pathway of sphingomyelin in SV 40 transformed mouse cells. *Biochem. Biophys. Res. Comm.* **47:** 1345–1352.
20. Hokin, L. E., and M. R. Hokin. 1958. Acetylcholine and the exchange of inositol and phosphate in brain phosphoinositide. *J. Biol. Chem.* **233:** 818–821.
21. Hokin, L. E., and M. R. Hokin. 1960. Studies on the carrier function of phosphatidic acid in sodium transport. *J. Gen. Physiol.* **44:** 61–85.
22. Diringer, H., and M. A. Koch. 1974. Kinetic studies on the phospholipid metabolism of embryonic mouse cells under conditions of stimulated or restrained growth. *Hoppe-Seyler's Z. Physiol. Chem.* **355:** 93–97.