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# Characterization of Phosphatidylthreonine in Polyoma Virus Transformed Fibroblasts†

Diana Mark-Malchoff,‡ Guido V. Marinetti,* G. Donald Hare, and Arnold Meisler

ABSTRACT: A threonine phospholipid in polyoma virus-transformed hamster embryo fibroblasts has been characterized as phosphatidylthreonine. The identification has been made by chemical and enzymatic hydrolysis. Acid hydrolysis of the phospholipid produces free threonine. Mild alcoholysis produces a water-soluble derivative having the properties of glycerophosphorylthreonine. Hydrolysis with phospholipase C produces phosphorylthreonine which on prolonged acid hydrolysis yields threonine. Phosphatidylthreonine in the cell is more accessible to reaction with fluorodinitrobenzene than is phosphatidylserine. Phosphatidylthreonine also has been found as a major aminophospholipid in two other polyoma-transformed hamster cell lines and in the BHK-21/C13 line including the PVT-3 and TS-3 lines, the latter derived from BHK cells. Only a trace amount of phosphatidylthreonine occurs in normal liver, kidney and spleen of the adult mouse, in normal liver and kidney of the adult hamster, in whole mouse and hamster embryos, and in mouse 3T3 cells and $SV_{40}$-transformed 3T3 cells.

Threonine has been found in egg yolk lipids (Rhodes & Lea, 1957). Phosphatidylthreonine has been reported to occur in tuna muscle (Igaraski et al., 1958). The complete characterization of phosphatidylthreonine was not carried out in these previous studies.

In a recent paper we reported the marked elevation of a threonine phospholipid in polyoma transformed hamster embryo fibroblasts (Mark-Malchoff et al., 1977). This lipid was provisionally identified as phosphatidylthreonine. We now have characterized this lipid as phosphatidylthreonine by chemical and enzymatic hydrolysis.

## Experimental Procedure

Polyoma virus transformed hamster cells including the HFT-91[1] cells (HTC-3049-91TC, Hare, 1967) and PVT-3 cells (HTC-3049-3, Hare, 1964) and hamster embryo cells (HEF) were grown in tissue culture as described previously (Mark-Malchoff et al., 1977). TS-3 cells were obtained from Dr. Walter Eckhart of the Salk Institute and have been maintained at 32 °C. The BHK-21/C13 line of baby hamster kidney was obtained from the American Type Culture Association. Mouse 3T3 (Swiss) and SV-40 transformed 3T3 cells were originally obtained from Dr. Howard Green. These cell

† From the Departments of Biochemistry, Microbiology and Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642. *Received January 17, 1978.* This work was supported by Contract 1-CP-45611 from the Cancer Institute and Research Grant HLB-02063 from the National Institutes of Health.

‡ Present address: Department of Medicine, Endocrinology-Metabolic Unit, University of Rochester, Rochester, New York.

[1] Abbreviations used: HFT, polyoma virus transformed hamster embryo fibroblasts; HEF, normal hamster embryo fibroblasts; FDNB, 1-fluoro-2,4-dinitrobenzene; TNBS, 2,4,6-trinitrobenzenesulfonate; TNP, trinitrophenyl; DNP, dinitrophenyl; TLC, thin-layer chromatography; PS, phosphatidylserine; PT, phosphatidylthreonine; PE, phosphatidylethanolamine; ET, ethanolamine.

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TABLE I: Phosphatidylserine and Phosphatidylthreonine Content of Lipids of HEF and HFT Cells.[a]

| | nmol per $10^6$ cells | | |
|---|---|---|---|
| | PS | PT | % PT |
| HEF cells | 3.90 ± 0.61 | 0.11 ± 0.001 | 2.7 |
| HFT cells | 1.65 ± 0.34 | 1.39 ± 0.11 | 45.7 |

[a] Approximately $10^7$ HEF and HFT cells were washed with buffer and the lipids extracted with chloroform–methanol. The lipid extracts were washed by the Bligh–Dyer technique. The lipids were sonicated in 3 N HCl and hydrolyzed for 2 h at 100 °C. The HCl hydrolysate was extracted with ether to remove fatty acids, cholesterol, and acid stable lipids. The HCl phase was evaporated to dryness. The residue was dissolved in 0.07 M citrate buffer (pH 2.2) and aliquots were analyzed on the Spinco amino acid analyzer using type W-1 spherical sulfonated styrene resin. HEF cells are normal hamster embryo fibroblasts. HFT-91 cells are cells previously designated HTC-3049-91TC (Hare, 1967). [b] Values represent the mean ± standard deviation of two experiments.

lines are described elsewhere (Todaro & Green, 1963). The cells were washed with buffer and the lipids were extracted with chloroform–methanol as described previously (Bligh & Dyer, 1959). The lipids were subject to sodium methoxide degradation (Marinetti, 1962), acid hydrolysis in 3 N HCl, and enzymatic hydrolysis with purified phospholipase C. Phospholipase C (*B. cereus*) was a gift sample from Dr. L. L. VanDeenen, University of Utrecht, The Netherlands. Conversion of the amino-phospholipids to their dinitrophenyl derivatives and their analyses were carried out by treatment in chloroform–methanol–water containing $NaHCO_3$ and 2,4-dinitrofluorofluorobenzene (Baumgarten et al., 1974; Gordesky et al., 1975; Marinetti & Love, 1976). Thin-layer chromatography of the phospholipids and the dinitrophenyl derivatives was carried out on Merck-Darmstadt silica gel coated glass plates using the solvent chloroform–methanol–acetic acid–water (65:25:2:4, v/v). Analysis of ethanolamine, serine, threonine, *O*-phosphoethanolamine, *O*-phosphoserine, *O*-phosphothreonine, glycerophosphorylserine, and glycerophosphorylethanolamine was carried out on the Beckman Spinco amino acid analyzer. Serine, threonine, glycerophosphorylserine, and glycerophosphorylethanolamine were obtained from Sigma Chemical Co. Ethanolamine was obtained from Eastman Kodak and redistilled before use. *O*-Phosphoserine, *O*-phosphothreonine, and *O*-phosphoethanolamine were obtained from Calbiochem.

## Results

*Serine and Threonine Content of HEF and HFT Lipids.* Our previous observation on the marked elevation of threonine (representing PT) in the HCl hydrolysate of lipids from HFT91 cells was confirmed in the present study (Table I). It is noteworthy that the lipids of both HEF and HFT cells were washed by the Bligh–Dyer method and were found to contain no free serine or threonine. Only after HCl hydrolysis of the lipids were serine and threonine observed, representing PS and PT.

*Reaction of Lipids with FDNB and TNBS.* When HEF and HFT cells were reacted with FDNB and TNBS, and the lipids were extracted and separated by TLC, the patterns obtained for the DNP-lipid derivatives and for the ninhydrin-positive components are shown in Figure 1. HEF and HFT cells yield two DNP components (labeled bands 1 and 2) and two ninhydrin-positive bands which were identified as PE and PS. Band 2 of the HFT lipids moved with a higher $R_f$ value than band 2 of the HEF lipids. Bands 1 and 2 from HEF and HFT

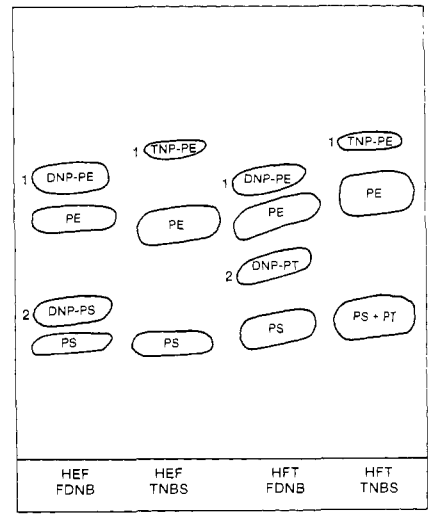


FIGURE 1: Reaction of HEF and HFT cells with FDNB and TNBS. Approximately $10^7$ HEF cells were treated with 2 mM FDNB or 2 mM TNBS in 20 mL of 120 mM $NaHCO_3$–40 mM NaCl buffer (pH 8.5) for 1 h at 23 °C. The cells were washed three times and extracted with chloroform–ethanol (1:1). The extracts were washed by the Bligh–Dyer method and the lipids subjected to TLC on silica gel coated plates using chloroform–methanol–acetic acid–water (65:25:2:4, v/v). The TLC plate was sprayed with ninhydrin to detect PE, PS, and PT. The yellow DNP derivatives of PE, PS, and PT were scraped off, eluted with methanol, and hydrolyzed in 3 N HCl (see Figure 2).

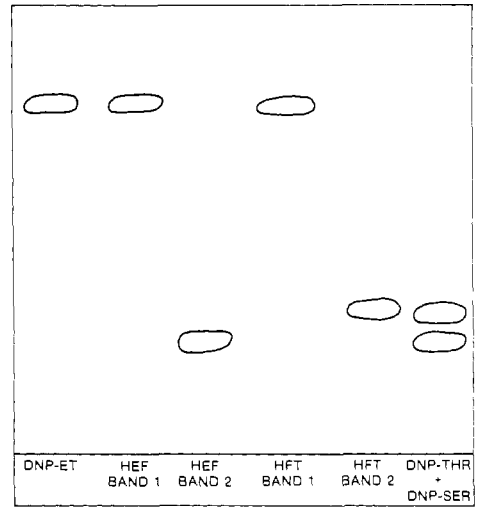


FIGURE 2: Acid hydrolysis of the dinitrophenylated phospholipids from HEF and HFT cells. The yellow bands 1 and 2 (DNP-PS, DNP-PT, and DNP-PE) shown in Figure 1 were scraped out of the TLC plate and eluted with methanol. The methanol was evaporated off and the lipids were sonicated in 3 N HCl and hydrolyzed for 2 h at 100 °C. The HCl phase was extracted with ethyl acetate and chromatographed on silica gel coated plates in the solvent given in Figure 1. Standard samples of DNP-Ser, DNP-Thr, and DNP-ET were run on the same plate.

cells which were reacted with FDNB were hydrolyzed with 3 N HCl and extracted with ethyl acetate. The yellow components in the ethyl acetate extracts were analyzed by TLC (Figure 2). Band 1 from both cells yielded DNP-ethanolamine which was derived from DNP-PE. Band 2 from HEF cells yielded DNP-Ser, whereas band 2 from HFT cells yielded DNP-Thr. These were identified by comparison with authentic standards. Band 2 from HEF cells represents DNP-PS, whereas band 2 from HFT cells represents DNP-PT.

In the previous experiment intact cells were reacted with FDNB. In a second experiment, lipids were extracted from HEF or HFT cells, and then were reacted with FDNB and

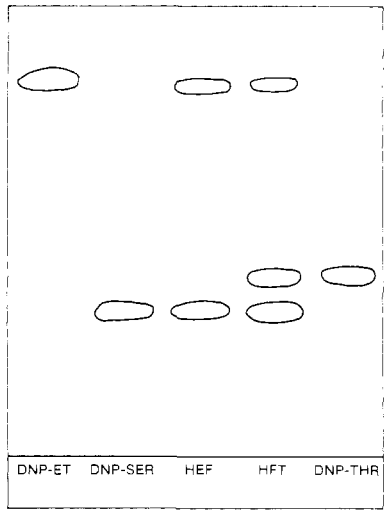


FIGURE 3: Identification of ethanolamine, serine, and threonine in the lipids of HEF and HFT cells by conversion to their DNP derivatives. HEF and HFT cells were extracted three times with $CHCl_3$:MeOH (1:1) and the extracts washed by the method of Bligh & Dyer. The washed lipid extracts were treated with 3 N HCl for 2 h at 100 °C. The acid hydrolysates were extracted with ether to remove fatty acids and cholesterol. The HCl phase was evaporated to dryness under nitrogen. The residues were dissolved in 5 mL of 120 mM $NaHCO_3$ and reacted with 2 mM FDNB for 2 h. The DNP derivatives were extracted with ethyl acetate and separated by TLC using solvent system $CHCl_3$:methanol:$H_2O$:HOAc (65:25:4:2). Standards of DNP-ET, DNP-Ser, and DNP-Thr were run simultaneously.

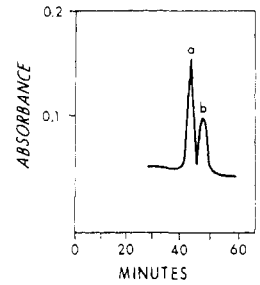


FIGURE 4: Amino acid analysis of the methoxide hydrolysis products of HFT lipids. The lipids of $10^7$ HFT cells were extracted by the Bligh & Dyer method and then hydrolyzed with 0.1 M $NaOCH_3$ in 5 mL of MeOH at 23 °C for 30 min. Glacial acetic acid (0.2 mL) was added; the total mixture was diluted with 2 volumes of water and extracted with ether to remove the methyl esters of fatty acids, sterols, and alkali-stable phospholipids. The aqueous phase was evaporated to dryness and the residue was dissolved in 2 mL of 0.07 M sodium citrate buffer (pH 2.2). Aliquots were run on the Spinco amino acid analyzer using type W-1 spherical sulfonate polystyrene resin which is routinely used for the separation of acidic and neutral amino acids. Peak "a" is a mixture of glycerophosphorylserine and glycerophosphorylthreonine. Peak "b" is glycerophosphorylethanolamine.

hydrolyzed with 3 N HCl. The aqueous HCl phase was extracted with ethyl acetate and analyzed by TLC. It can be seen in Figure 3 that lipids from HEF cells yield two DNP derivatives which migrated identically as DNP-ethanolamine and DNP-Ser. However, lipids from HFT cells contained an additional DNP-component which migrated identically as DNP-Thr. These results show that HFT lipids contain threonine and when cells are reacted with FDNB this lipid reacts to form DNP-PT but PS in the same cells does not react with FDNB. Therefore PS and PT have a different topological arrangement in the HFT cell. Neither PS nor PT reacts with TNBS (Figure 1) which is a relatively nonpenetrating probe. With both HEF and HFT cells only TNP-PE was observed. However, with both TNBS and FDNB not all the PE or PS reacts indicating some of these phospholipids are masked and not available to these probes, even when excess probe is used.

*Reaction of Lipids with Sodium Methoxide.* In order to characterize the threonine phospholipid, a sample of HFT lipids was hydrolyzed at 23 °C with 0.1 N $NaOCH_3$ in 5 mL of methanol for 60 min. This mild alkaline hydrolysis removes the fatty acids from the phospholipids as their methyl esters and converts the glycerolphospholipids to their water-soluble glycerophosphoryl-base derivatives. Thus PE yields glycerophosphorylethanolamine and PS yields glycerophosphorylserine. If the threonine phospholipid is PT, it will yield glycerophosphorylthreonine. However, if the threonine phospholipid is the threonine-*O*-acyl derivative of phosphatidylglycerol, it will yield free threonine and glycerophosphorylglycerol. If threonine occurs as the plasmalogen phosphatidylthreonine it will yield lysophosphatidylthreonine after treatment with $NaOCH_3$. If it occurs as the dialkanyl lipid it will be refractory to $NaOCH_3$ treatment.

The methoxide reacted lipid mixture in 5 mL of methanol–chloroform was treated with glacial acetic acid to neutralize excess $NaOCH_3$ and diluted with 2 volumes of water. The aqueous phase was evaporated to dryness and dissolved in 0.07 M sodium citrate buffer (pH 2.2) and run on the Spinco amino acid analyzer. Standards of serine, threonine, ethanolamine, *O*-phosphoserine, *O*-phosphothreonine, *O*-phosphoethanolamine, glycerophosphorylserine, and glycerophosphorylethanolamine were also run. A standard of glycerophosphorylthreonine is not available at this date. The methoxide hydrolysis products yielded two peaks on type W-1 resin (Figure 4). Component "a" ran identically as the standard glycerophosphorylserine and component "b" ran identically as glycerophosphorylethanolamine, Peak "a" is a mixture of glycerophosphorylserine and glycerophosphorylthreonine. These compounds have such a similar structure that they are not resolved on this column. It is noteworthy that no free serine or threonine was observed after mild alkaline hydrolysis. These data rule out any serine or threonine-*O*-acyl esters of phosphatidylglycerol. Moreover, TLC analysis showed that nearly all the PT, PS, and PE were degraded by $NaOCH_3$ treatment indicating very little or no plasmalogens or dialkanyl forms of these phospholipids.

*Reaction of Lipids with Phospholipase C.* Lipids from 0.7 × $10^7$ HFT cells were washed by the Bligh–Dyer method and treated with phospholipase C as described in the legend to Figure 5. The analysis of the phospholipase C water-soluble hydrolysis products of the HFT lipids showed 2 peaks (Figure 5A). The first peak (peak "a") ran identically as authentic *O*-phosphoserine and *O*-phosphothreonine. These two compounds are acidic and have structures which are so similar that they are not resolved on this ion-exchange column. The second peak (peak "b") ran identically as *O*-phosphoethanolamine. On prolonged HCl hydrolysis it yielded ethanolamine.

In order to determine whether the peak "a" (Figure 5A) contained *O*-phosphothreonine the sample was hydrolyzed with 6 N HCl for 48 h at 100 °C. The hydrolyzed sample showed a small residual amount of peaks "a" and "b" representing unhydrolyzed *O*-phosphothreonine and *O*-phosphoethanolamine, since these phosphate esters are more refractory to hydrolysis than is *O*-phosphoserine. The two new peaks, however, were free threonine (peak "c") and serine (peak "d") (Figure 5B). On this column, ethanolamine is retained and will not appear. Control time studies on the extent of hydrolysis of *O*-phosphoserine, *O*-phosphothreonine, and *O*-phosphoethanolamine in 6 N HCl at 100 °C showed that after 48 h the *O*-phosphoserine was completely hydrolyzed to free serine and

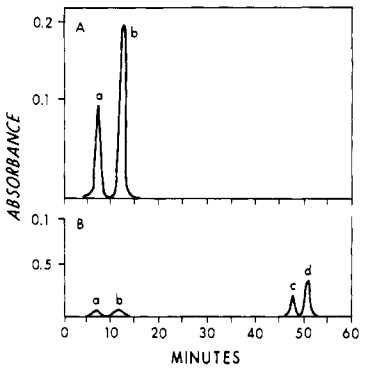


FIGURE 5: Amino acid analysis of phospholipase C hydrolysis products of HFT lipids. Lipids of HFT cells were extracted by the method of Bligh & Dyer and sonicated in 0.05 M Tris buffer (pH 7.4) made 10 mM in $CaCl_2$ and 0.25 mM in $MgCl_2$. The sonicated lipids were treated with 7 IU of pure phospholipase C for 2 h at 37 °C. The reaction mixture was then extracted with ether. The water phase was heated for 2 min at 100 °C and then centrifuged. The supernatant was evaporated under nitrogen to dryness, dissolved in 0.07 M citrate buffer (pH 2.2), and filtered through a 0.22-μm Millipore filter. The filtrate was divided into two equal samples. One sample was analyzed on the amino acid analyzer using W-1 resin. The other sample was hydrolyzed with 6 N HCl for 48 h at 100 °C, the HCl was evaporated to dryness, and the residue dissolved in citrate buffer and analyzed on the amino acid analyzer using W-1 resin. (A) Analysis of the water soluble hydrolysis products released by phospholipase C. (B) Analysis of the 48-h 6 N HCl hydrolysate of the products given in A. (a) *O*-phosphoserine + *O*-phosphothreonine; (b) *O*-phosphoethanolamine; (c) threonine; (d) serine.

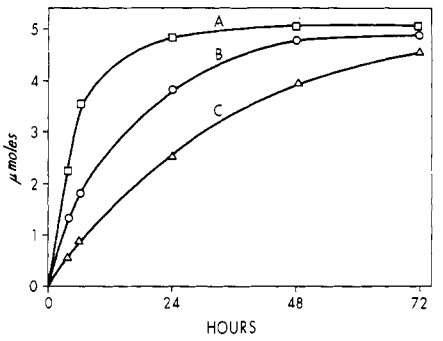


FIGURE 6: Acid hydrolysis of phosphate esters of serine, ethanolamine, and threonine. Five micromoles of *O*-phosphoserine, *O*-phosphoethanolamine, and *O*-phosphothreonine were dissolved in 1 mL of 6 N HCl in glass ampules, flushed with nitrogen, and sealed. Hydrolysis was carried out at 100 °C for 4–72 h. Aliquots were removed for analysis of inorganic phosphate. Curves A, B, and C represent the hydrolysis of *O*-phosphoserine, *O*-phosphoethanolamine, and *O*-phosphothreonine, respectively. The ordinate shows the appearance of inorganic phosphate.

phosphate whereas the *O*-phosphothreonine was only 85% hydrolyzed (Figure 6). The *O*-phosphoethanolamine was 95% hydrolyzed under these conditions. Therefore peak "a" (Figure 5A) is a mixture of *O*-phosphoserine and *O*-phosphothreonine.

The above experiments provide clear evidence that the threonine phospholipid is PT. This characterization is based on the following: (a) the washed lipid extract contains no free threonine or serine; these amino acids are liberated only after HCl hydrolysis of the lipids; (b) treatment of HFT cells with FDNB gives a DNP-lipid which migrates faster than DNP-PS and which on acid hydrolysis yields DNP-Thr; (c) treatment of HFT lipids with FDNB followed by HCl hydrolysis yields DNP-Ser and DNP-Thr, whereas similar treatment of HEF cells yields DNP-Ser; (d) treatment of HFT lipids with phospholipase C yields a water-soluble derivative which migrates identically as a mixture of *O*-phosphothreonine and *O*-phosphoserine on the Spinco amino acid analyzer. Hydrolysis of this mixture with HCl for 48 h yields both serine and threonine.

We have analyzed the PE, PS, and PT content of a variety of cells and find elevated PT levels in PVT-3 cells, in polyoma virus temperature sensitive transformed cells (TS-3 cells) and to a lesser extent in BHK-21 cells (Table II).

It is of further interest that the PT/PS ratio and hence the percent PT in TS-3 cells is sensitive to the temperature of incubation in parallel to changes in growth characteristics as described by Dulbecco & Eckhart (1970). This cell line was derived for the BHK21/C13 line by injection with the ts-3 temperature sensitive mutant of polyoma virus. The PT/PS ratio (the percent PT) of TS-3 cells grown at 36 °C decreased significantly when compared with the same cells maintained at 32 °C. We find mainly PE and PS in lipids of 3T3 cells and SV40-3T3 cells (Table III). Only a trace amount of PT was detected. We also have analyzed as controls, lipids from normal mouse and hamster embryo fibroblasts and lipids from liver, kidney, and spleen of adult hamsters and mice and find mainly PE and PS (Table III). Again, only a trace amount of phosphatidylthreonine was detected. It is noteworthy that the threonine phospholipid has been characterized as PT only in HFT cells. We presume that the threonine found in washed lipid extracts of other cells represents PT.

TABLE II: Phosphatidylserine and Phosphatidylthreonine Content of PVT-3, TS-3, and BHK-21 Cells.[a]

| | nmol/mg of cell pellet[b] | | PT/PS molar | |
|---|---|---|---|---|
| | PS | PT | ratio | % PT |
| PVT-3 cells | 3.36 | 1.55 | 0.46 | 31.6 |
| BHK-21 cells | 12.6 | 3.37 | 0.27 | 21.1 |
| TS-3 cells, 32 °C | 1.67 | 2.96 | 1.77 | 63.9 |
| TS-3 cells, 36 °C | 5.88 | 2.11 | 0.36 | 26.4 |

[a] PVT-3 cells are polyoma virus transformed hamster cells designated as HTC-3049-3 cells (Hare, 1964). BHK-21-C-13 cells are baby hamster kidney cells which have been selected for tissue culture and which may be abnormal. TS-3 cells are BHK-21 cells transformed by a temperature sensitive (ts-3) polyoma virus (Dulbecco & Eckhart, 1970). These transformed cells lose certain characteristics at 36–37 vs. 32 °C. [b] Cell pellet after lipid extraction. The lipids were extracted and hydrolyzed as given in the text.

TABLE III: Phosphatidylserine and Phosphatidylethanolamine Content of 3T3 and SV-3T3 Cells and Lipids from Tissues of Mouse and Hamster.[a]

| | nmol/μmol of total lipid $P_i$ | |
|---|---|---|
| | PS | PE |
| 3T3 cells[b] | 65 | 266 |
| SV-3T3 cells[b] | 62 | 218 |
| Hamster embryo | 50 | 283 |
| Mouse embryo | 68 | 283 |
| Hamster liver | 21 | 112 |
| Hamster kidney | 32 | 280 |
| Mouse liver | 12 | 182 |
| Mouse kidney | 44 | 415 |
| Mouse spleen | 92 | 407 |

[a] Tissue lipids were extracted by the Bligh-Dyer method and hydrolyzed with 3 N HCl for 2 h and analyzed on the Spinco amino acid analyzer for amino acids and ethanolamine. [b] 3T3 cells are normal mouse fibroblast cells. SV-3T3 cells are mouse fibroblast cells transformed by SV40 virus.

## Discussion

This paper provides data which characterizes the threonine phospholipid of polyoma virus transformed HFT cells as PT. This lipid is barely detectable in normal HFT cells but occurs at about the same level as PS in HFT cells. Another interesting observation is the difference in reactivity of PT and PS in HFT cells. When the isolated lipids of HFT cells are reacted with FDNB, both PS and PT react. However, when intact HFT cells are reacted with FDNB and the lipids are extracted and analyzed it is found that only PT reacts with FDNB. Therefore PT is available to react with FDNB in intact cells but PS is not. On the contrary, some PS of HEF cells does react with FDNB, demonstrating a different arrangement of PS in HEF cells as compared to HFT cells. Moreover, neither PS nor PT reacts with TNBS, a probe which does not readily penetrate the cell membrane. Therefore we conclude that both PS and PT are localized on the inner surface of the plasma membrane and/or on membranes inside the cell and that PT is more exposed than is PS. The mechanism which leads to an increase in PT in transformed HFT cells remains to be determined.

Of the different cell types we have analyzed, PT is a major amino-phospholipid in polyoma virus transformed cells (HFT, PVT-3, and TS-3 cells). Normal cells and tissues from hamster and mouse and other cell types such as mouse 3T3 and their SV40-transformed derivatives contain only a trace amount of PT. However, we have found an appreciable amount of PT in BHK-21 cells, a line which has been selected for continual growth in tissue culture. The cells which have survived may be considered to be transformed since it has been demonstrated that BHK21/C13 cells which grow in an oriented pattern in culture are capable of producing tumors on inoculation into adult hamsters, (Defendi et al., 1963).

At this time, this is insufficient evidence to conclude whether the regulation of the quantity of PT in the various polyoma-transformed hamster cells is related directly to the action of the viral genome or is an alteration in phospholipid metabolism common to "transformed" hamster cells and independent of the oncogenic stimulus. The presence of an increased PT level in the BHK21 cell strongly suggests that this altered phospholipid metabolism is not directly controlled by the virus. On the other hand, the temperature sensitivity of the PT/PS ratio in the TS-3 cells could reflect a modulation of phospholipid synthesis by either the ts function of the inducing virus or by a cell regulating function which is sensitive to environmental temperature and totally independent of the virus genome. Studies of other hamster tumor cells induced by different ts mutants of SV40 and polyoma virus as well as hamster cells with temperature sensitive growth controls will help to determine the mechanisms.

Virus transformed cells are reported to have altered lipid composition. A review of the lipids in normal and tumor cells grown in culture has recently been published by Howard & Howard (1975). These lipid changes involve cholesterol, phospholipids, and glycolipids. Our finding of an elevated PT content in polyoma virus transformed cells represents a novel observation since this phospholipid has not been recognized widely as a naturally occurring lipid. How and why this novel phospholipid is elevated in transformed hamster cells remain to be determined.

## Acknowledgments

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