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Research Article

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In 300 mOsm media major phospholipids other than the inositides were not labeled. Small amounts of label appeared in certain trace phospholipids, notably phosphatidic acid. In 150 mOsm media, labeling of inositides was moderately increased, that of trace phospholipids enormously so. The increased labeling was not solely due to thrombocytolysis since (*a*) platelet disruption by sonication or freeze-thawing abolished ^{32}P incorporation into phospholipids and (*b*) in timed studies, restoration of osmolarity to 300 mOsm by addition of hypertonic sorbitol blunted the enhancement effect of previous 150 mOsm exposure. Lowering K and compensatorily increasing Na concentration of 300 mOsm media also stimulated ^{32}P labeling of inositides and, to a lesser extent, the trace phospholipids. However, the pattern and degree of stimulation were not as strikingly […]

Quantification of Human Platelet Inositides and the Influence of Ionic Environment on Their Incorporation of Orthophosphate-^{32}P

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ABSTRACT Platelets are a rich source for the study of inositol lipids in man. The substitution of an EDTA-KCl solution for the water component of the Bligh and Dyer procedure permitted quantitative extraction of polyphosphoinositides. The latter, with monophosphoinositide, were found to comprise, on a molar basis, 6.7% of total platelet phospholipids. Study of the incorporation of orthophosphate-^{32}P into platelet phospholipids was further simplified by separating eight ^{32}P-labeled lipids, including the inositides, with a single chromatographic development on formaldehyde-treated paper. Particular attention was paid to the influence of ionic environment on the pattern and degree of labeling.

In 300 mOsm media major phospholipids other than the inositides were not labeled. Small amounts of label appeared in certain trace phospholipids, notably phosphatidic acid. In 150 mOsm media, labeling of inositides was moderately increased, that of trace phospholipids enormously so. The increased labeling was not solely due to thrombocytolysis since (*a*) platelet disruption by sonication or freeze-thawing abolished ^{32}P incorporation into phospholipids and (*b*) in timed studies, restoration of osmolarity to 300 mOsm by addition of hypertonic sorbitol blunted the enhancement effect of previous 150 mOsm exposure. Lowering K and compensatorily increasing Na concentration of 300 mOsm media also stimulated ^{32}P labeling of inositides and, to a lesser extent, the trace phospholipids. However, the pattern and degree of stimulation were not as strikingly altered as in the osmolarity studies.

These data show that drastic alterations of ionic environment can sharply influence the platelet's ability to incorporate orthophosphate-^{32}P into its phospholipids.

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INTRODUCTION

Interest in the phosphoinositides has centered around their distribution and metabolism in brain tissue. Folch and Woolley were the first to show the presence of bound inositol in brain cephalin (1) and later described the isolation of DPI [1] from this crude fraction (2). Subsequently in 1954, Dawson found that brain DPI rapidly incorporated $^{32}P_i$ in vitro (3).

Proof of the active turnover of the inositol lipids in tissues other than brain began in 1955 with the studies of Hokin and Hokin (4) on the stimulation by acetylcholine of $^{32}P_i$ incorporation into phosphoinositide of pancreas slices. Later, it became apparent that in various other tissues, liver (5, 6), erythrocytes (7, 8), leukocytes (9–11), and kidney (5, 12), the in vitro incorporation of $^{32}P_i$ was at a very high rate in the inositides.

A similar pattern has been found in studies with human platelets; only phosphoinositide and a lipid tentatively identified as PA were shown to be labeled in vitro by $^{32}P_i$ (9, 11, 13). By contrast, all major PL appeared to have been labeled in the platelets of patients who had

[1] *The following abbreviations are used in this paper:* MPI, monophosphoinositide; DPI, diphosphoinositide; TPI, triphosphoinositide; PPI, polyphosphoinositides, TPI plus DPI; LPA, lysophosphatidic acid; PG, phosphatidylglycerol; PA, phosphatidic acid; CDPDG, cytidine diphosphate diglyceride; DPG, diphosphatidylglycerol (cardiolipin); PC, phosphatidylcholine (lecithin); PE, phosphatidylethanolamine; PS, phosphatidylserine; PL, phospholipids; NL, neutral lipids; FA, fatty acid; G3P, glycerophosphate; P_i, orthophosphate.

been treated with ^{32}P for polycythemia vera (14) or chronic granulocytic leukemia (9). More recently it has been found that the addition of succinate stimulates incorporation of $^{32}P_i$ into phosphoinositide in vitro (15), thus establishing a link between oxidative phosphorylation and the phosphorylation of this lipid. Further, it has been shown lately that human platelet homogenates can convert PA to CDPDG (16) and the latter to MPI (17).

The present work was undertaken principally to determine whether human platelets have, in addition to the parent MPI, significant amounts of PPI, and if, in fact, the latter are responsible for most of the $^{32}P_i$ labeling of the inositide fraction in vitro. A second objective was to identify all platelet PL which can be labeled in vitro by $^{32}P_i$. A third goal was to examine the influence of medium osmolarity and Na and K concentration on ^{32}P labeling of the PL.

METHODS

Preparation of platelet suspensions. The platelet procurement differed in the following two ways from that previously described (18, 19). (*a*) Instead of acid-citrate-dextrose, a citrate–citric acid solution having a citrate concentration of 40 mmoles/liter and a pH of 6.8 was added in a proportion of 1 ml for each 8 ml of platelet-rich plasma before sedimenting the platelet pellet. (*b*) The platelet pellet was washed and resuspended in a buffer containing 5 mM K, 45 mM Na, 20 mM citrate, and 125 mM Tris at a pH of 7.0 and an osmolarity of 300 mOsm.

The platelet count (20) of the usual concentrate was 6×10^6 per mm^3; 1 mg platelet protein as determined by the biuret method (21) was equivalent to 5×10^8 platelets. No concentrate contained more than 100 leukocytes or 500 erythrocytes per mm^3. In some cases, platelets were disrupted by freeze-thawing or sonication as previously described (19).

Chemicals. Carrier-free $^{32}P_i$ ($NaH_2{}^{32}PO_4$) was purchased;[2] its radiochemical purity was verified by ascending paper chromatography in an isopropanol–trichloroacetic acid–13 N ammonia–water (75:5:0.2:25; v/w/v/v) system. All other chemicals and solvents were of analytical purity. Chloroform and methanol were redistilled before use.

Reference compounds. TPI was prepared from pig brain by the method of Dittmer and Dawson (22) and was shown to be identical with a sample of cow brain TPI which was a gift of Dr. R. M. C. Dawson. DPI was isolated from pig brain by the method of Folch (2). MPI was purchased[3] and purified by preparative chromatography on and elution from formaldehyde-treated paper (see below). PA was obtained by hydrolysis of egg lecithin with cabbage leaf extract (23) as a source of phospholipase D (E.C. 3.1.4.4); PG was furnished by the same procedure by adding an excess of glycerol to the incubation (23). Both PA and PG were purified by preparative thin-layer chromatography (TLC) on oxalic acid–impregnated Silica Gel G (24). Synthetic DPG was prepared by Dr. P. P. M. Bonsen; CDPDG was a gift of Dr. J. N. Hawthorne.

[2] Philips-Duphar, Putten, The Netherlands.

[3] Koch-Light Laboratories, Ltd., Poyle, Colnbrook, Buckinghamshire, England.

Incubation conditions. All incubations were at 37°C in an automatic, shaking water bath. Unless otherwise indicated, the time of incubation was 90 min; the gas phase was room air. 4 mg platelet protein was added to all incubations with the incorporation of $^{32}P_i$ into all identified platelet PL having been shown to increase linearly up to a protein concentration of 8 mg per incubation. The usual 300 mOsm incubation (2 ml) had the following composition: glucose (5 mmoles/liter); K (45 mEq/liter); Na (4.5 mEq/liter); Tris (125 mmoles/liter); citrate (2 mmoles/liter); PO_4 (2 mmoles/liter); 10^8 cpm ^{32}P as $NaH_2{}^{32}PO_4$ (SA 25×10^6 cpm $^{32}P/\mu$mole PO_4).

When necessary, medium osmolarity was varied by replacing part of the 0.05 M KCl–0.125 M Tris buffer with distilled water. In some studies 150 mOsm incubations were abruptly restored to isosmolarity by addition by 0.1 ml of 3 M sorbitol at various time intervals. Osmolarities were verified by the vapor pressure method at 37°C.

For experiments in which Na and K concentrations were varied, appropriate mixtures of 0.05 M KCl–0.125 M Tris and 0.05 M NaCl–0.125 M Tris buffer were used; the final Na and K concentrations were verified by flame photometry.

Control of bacterial contamination. PG is a major PL of many bacteria. To exclude significant bacterial contamination as a cause for our finding of ^{32}P-labeled PG, all buffers and added substrates were autoclaved before use. Random cultures at the start and end of incubations over time intervals of 15–180 min in no case showed more than 10 bacterial colonies per ml, a level of contamination which could not account for the observed ^{32}P labeling of PG.

Extraction of PL. The adopted method was a modification of the simple Bligh and Dyer procedure (25). To the 2 ml incubation mixture was added 0.5 ml of a solution of 0.1 M EDTA–Na_2 and 2.0 M KCl. This was followed immediately by 5 ml methanol and then 2 ml chloroform to keep a single phase, which was held at 20°C for 2 hr before being broken by the addition of 2 ml chloroform and 1 ml of the EDTA–KCl mixture. After centrifugation, the chloroform layer was removed, and the overlayer was washed three times with 3 ml of chloroform. The combined chloroform layers, holding a quantitative harvest of ^{32}P-labeled PL, were taken to dryness in a rotary evaporator in a nitrogen atmosphere, and the lipid residue was dissolved in 1 ml of a chloroform-methanol-water (75:25:2; v/v/v) mixture (26) in preparation for paper chromatography (see below).

To verify the completeness of this extraction, the final overlayer was centrifuged at 4000 *g* for 15 min to sediment the protein. The latter was subjected to the acid extraction procedure (22). In rare instances small amounts of labeled TPI (5–10% of the total) were recovered by this maneuver. The remaining aqueous-methanol layer was similarly extracted, but in no case yielded ^{32}P-labeled lipids.

Quantification of inositides. To quantify the TPI, DPI, and MPI concentrations in human platelets, freshly prepared platelet concentrates having at least 120 mg platelet protein were extracted and subjected to chromatography on formaldehyde-treated paper, using slight modifications of the method of Hörhammer, Wagner, and Richter (27) following suggestions of Letters (28) and Kai and Hawthorne (29).

The dried chromatograms were stained with Nile Blue (27, 28). The inositides were quantitatively eluted from individual paper strips by subjecting the latter to 18 hr of descending chromatography with the solvent mixture used to develop the original chromatogram (27–29). The eluates were analyzed for phosphorus by the method of Bartlett (30). A phosphorus determination was also done on the

total lipid extract and the eluate from an entire lipid track on the chromatograms.

Radioactivity assay. Formaldehyde-treated paper chromatograms were radioautographed by exposure to X-ray films[4] for 10–15 days. The spots on the film were traced on the paper chromatogram with carbon paper. The tracings were cut out and placed directly into a vial containing 16 ml of a standard scintillation mixture in preparation for counting. Results were corrected for decay to the day of the experiment and were expressed as counts per minute per PL.

Identification of labeled PL. To identify the compounds represented by the spots on the radioautograms, incubations were done on a preparative scale. After lipid extraction and separation, the formaldehyde-treated papers were scanned[5] and marked according to the limits of each well-defined peak before being cut into strips corresponding to the center of each peak. The radioactivity was then quantitatively eluted as described above.

Samples of each eluate were subjected to the following three types of chromatographic procedures: (*a*) formaldehyde-treated paper as described above, (*b*) thin-layer chromatography (TLC) on ammonium phosphate–impregnated Silica Gel H (19), and (*c*) TLC, on oxalic acid–impregnated Silica Gel G (24). Authentic internal and external reference compounds were also applied. The chromatograms were scanned or radioautographed. To localize the PL, the papers were stained with Nile Blue, the TLC with iodine.

The remainder of each eluate was taken to dryness and redissolved in 1 ml of 1% toluene in methanol in preparation for alkaline hydrolysis according to Dawson (31). The chromatographic (32) and high voltage electrophoretic[6] (33) mobilities of the water-soluble products of ^{32}P-labeled PL and authentic reference compounds were compared on parallel tracks. The chromatograms and electropherograms were scanned before staining with periodate-Schiff.

RESULTS

Quantification of inositide family. Fig. 1 shows that with the application of sufficient lipid extract, all three inositides were clearly visualized on the stained formaldehyde-treated paper chromatogram. Collectively the inositide family made up 9.67% of the total PL phosphorus, and on a molecular basis, 6.68% of the total platelet PL (Table I). Their molar ratios were roughly 4:2:1 (MPI/DPI/TPI). Using previous data relating thrombocytocrit to platelet numbers (34), it was calculated that on a wet weight basis, human platelets contain 1.06, 0.49, and 0.19 μmole of MPI, DPI, and TPI, respectively, per g wet weight. PA, PG, LPA, DPG, and CDPDG were not visualized by iodine staining of the platelet lipid tracks on the TLC systems (Fig. 2); hence, their concentrations were not quantified.

Isolation and identification of ^{32}P-labeled compounds. As shown by radioautography (Fig. 3), chromatography on formaldehyde-treated paper provided good separations among eight labeled compounds. Tentative identification of the radioactivity peaks localized by scanning and radioautography was established by comparison with the mobilities of authentic reference compounds (Fig. 2). The R_F values for the inositide family (Figs. 1–3) were similar to those which have been previously reported (35). P_i, G3P, and ATP had R_F values of 0.15 or less.

Three chromatography systems were used as cross references to identify tentatively CDPDG, LPA, PG, PA, and DPG, and to exclude labeling of other PL (Fig. 2). For example, the R_F values of LPA and PS were similar on formaldehyde-treated papers, but on the TLC systems it was possible to establish that LPA was, in fact, the PL that was labeled.

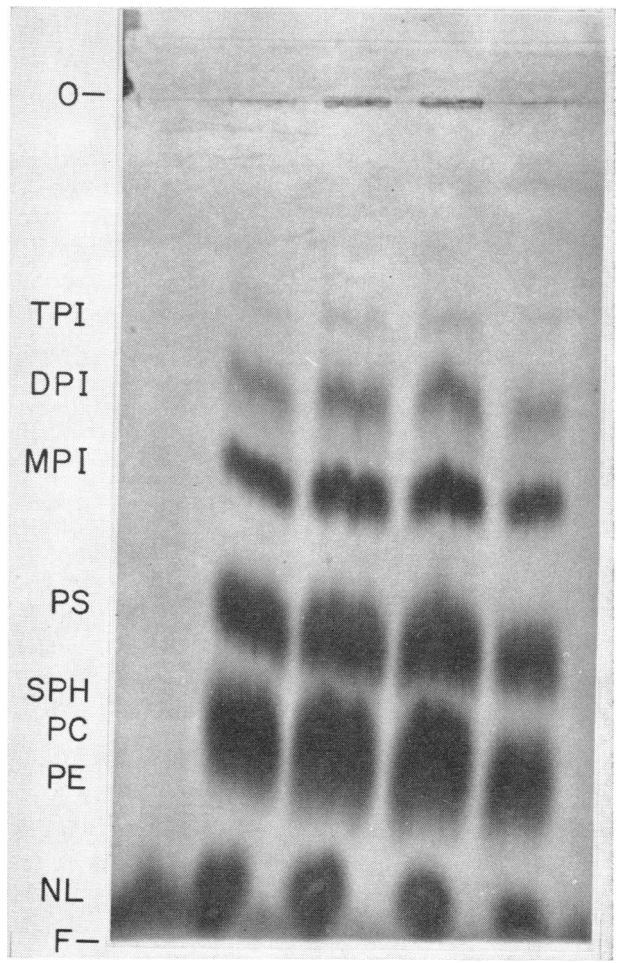

FIGURE 1 Photograph of human platelet lipids, separated by formaldehyde-treated paper chromatography and stained with Nile Blue. The outer tracks contained the lipid extract equivalent of 12 mg platelet protein; the inner, the equivalent of 16 mg. With the heavier amount of lipid extract, the TPI band was better visualized. O = origin; SPH = sphingomyelin; F = front.

[4] Ilford Red Seal 75 FW; Ilford Ltd., Ilford, Essex, England.

[5] Automatic TLC radioactivity scanner; Berthold, Wildbad, Germany.

[6] Pherograph type Mini 65; L. Hormuth, Heidelberg, Germany.

Final identification of six labeled PL (inositides, LPA, PG, PA) was proved by the alkaline hydrolysis studies. Identification of CDPDG and DPG remained only tentative.

Conditions affecting incorporation of $^{32}P_i$ into PL. Our laboratory has shown that the oxidation of glucose and selected Krebs cycle substrates by human platelets is considerably augmented in hypotonic as compared with isotonic incubation media (18). Those results, supported by data from other laboratories (see reference 18), suggested that in hypotonic media, permeability of the plasmalemmal or mitochondrial membranes towards certain low molecular weight compounds is enhanced, which permits greater access of energy substrates to oxidative systems. This raised the possibility that access of phosphate to its metabolic pathways could be similarly enhanced, thus affording a new method for examining turnover of the phosphate moiety of human platelet PL.

The uptake of $^{32}P_i$ into PL was shown to be essentially linear for 90 min in 300- and 150-mOsm incubations (Fig. 4). However, there was a striking difference in the pattern and degree of uptake at the tested extremes of osmolarity (Figs. 3–5). These results were virtually unaffected over a broad pH range, 6.8–7.4 (data not shown), the lower limits of which were not exceeded even in the hypotonic incubations with their diminished complement of buffer.

TABLE I
Inositide Content

	Human platelets		Rat*		
				Brain	Pancreas
	% total PL phosphorus	*% total PL*	*μmoles/g wet weight*		
MPI	3.96 ±0.41	4.09 ±0.48	1.06	2.22	3.07
DPI	3.62 ±0.89	1.87 ±0.46	0.49	0.25	0.03
TPI	2.09 ±0.52	0.72 ±0.18	0.19	0.40	0.06

Each analysis was done on a lipid extract derived from four 500-ml blood samples from four separate ABO-compatible donors. See Methods for a description of procedures for lipid extraction, separation, and elution from the paper chromatograms. Phosphorus analyses were done by the method of Bartlett (30).

Shown are the means ±SEM of three separate analyses. Results are given in three ways: per cent of the total PL phosphorus; per cent of total PL (molar basis); and μmoles/g wet weight. For comparison with the latter, values are shown for rat brain and pancreas, the two most inositide-rich tissues in a series of 11, as reported recently by Dittmer and Douglas (41)*.

At 300 mOsm, $^{32}P_i$ was incorporated into MPI, DPI, and TPI, and to a much lesser extent, the other PL (Figs. 3–5). Similar results were obtained at 275, 250,

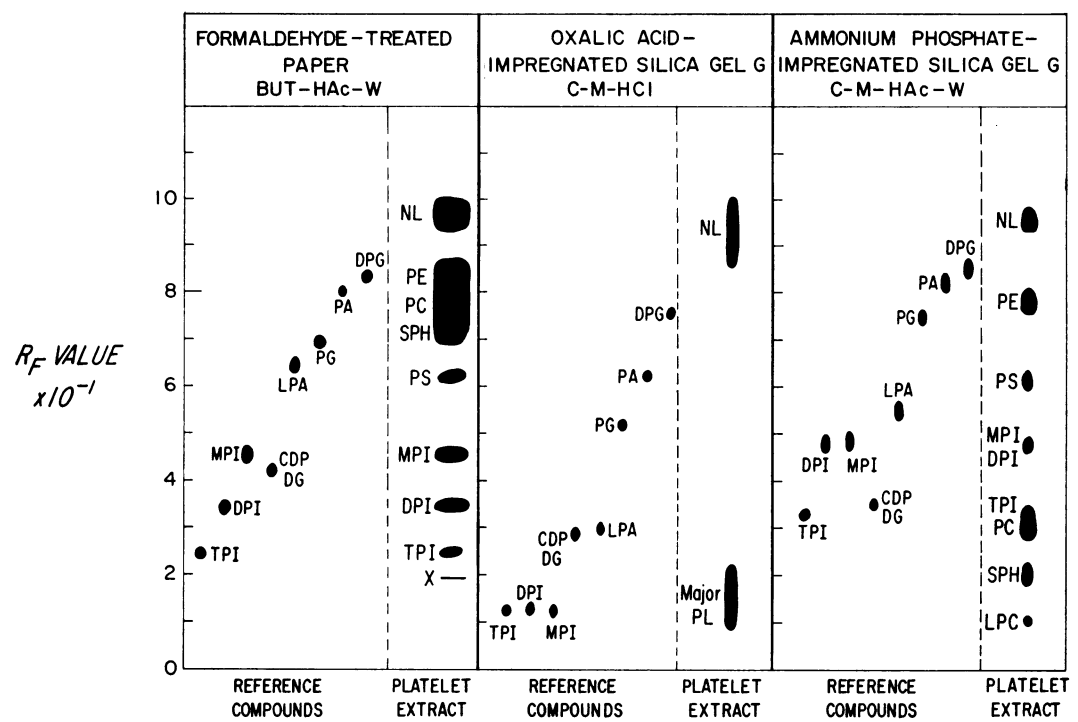

FIGURE 2 Diagrams of chromatography systems used as cross references to identify tentatively the ^{32}P-labeled PL. From left to right are shown: a formaldehyde-treated paper chromatogram and thin-layer chromatograms on oxalic acid–impregnated Silica Gel G and ammonium phosphate–impregnated Silica Gel H. PG, PA, and DPG were not visualized by iodine staining of the platelet lipid track. X refers to an unidentified ^{32}P-labeled compound (see Fig. 3).

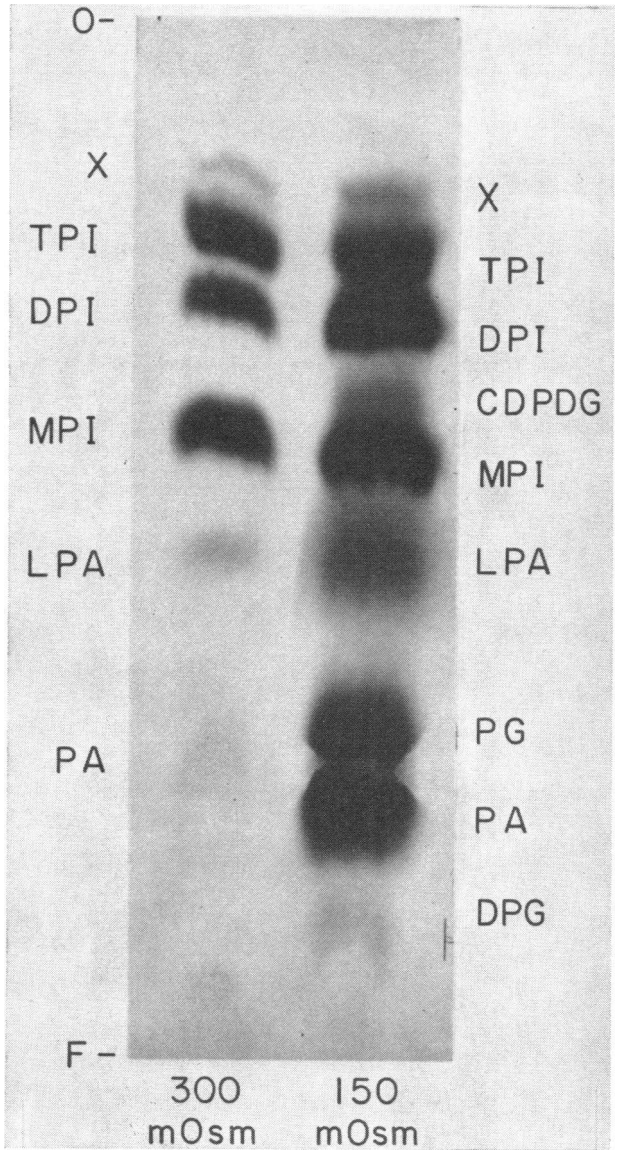

FIGURE 3 Radioautograms of ^{32}P-labeled human platelet PL separated by formaldehyde-treated paper chromatography. The pattern and degree of labeling were strikingly different at the tested extremes of osmolarity. X refers to an unidentified spot.

225, 300, and 150 mOsm. However, below 200 mOsm the labeling of the PL was enormously increased (25-fold in the case of PA).

It should be emphasized that the sharp upturn of the curve in Fig. 5 begins in a zone of medium osmolarity which defines the critical lytic volume of discrete cells such as platelets (36) and erythrocytes (37). As expected, the platelets were markedly swollen and distorted in 150 mOsm media. This suggested the possibility that other types of thrombocytolysis would produce findings similar to those found with 150 mOsm–treated platelets. However, severe mechanical disruption by freeze-thawing or sonication virtually abolished $^{32}P_i$ uptake into PL.

The effect of hypotonic exposure was further investigated in the experiments depicted in Fig. 6. In these timed studies with 150 mOsm incubations, correction of medium osmolarity and cell size by the addition of hypertonic sorbitol was found to blunt the enhanced ^{32}P labeling of all PL. The results with LPA, PG, and PA were similar; the earlier sorbitol was added, the greater the decrease in labeling compared with the control. By contrast, the labeling of MPI was unaffected if sorbitol was added after 5 min incubation with $^{32}P_i$ at 150 mOsm. The same effect was noted with DPI if sorbitol addition was delayed beyond 30 min. On the other hand, TPI appeared to lose label after restoration of osmolarity by sorbitol addition.

It is useful to point out that restoration of osmolarity of the incubation at zero time caused platelets to behave, with respect to ^{32}P labeling of PL, as if they had never been exposed to 150 mOsm media. Importantly, this "recovery" from 150 mOsm exposure occurred in media whose pH and salt content did not change as the result of sorbitol addition.

In further experiments, the effect of modifying medium Na and K concentrations was examined. It was found that lowering the K and compensatorily increasing the Na concentration stimulated the uptake of $^{32}P_i$ into the inositide family and, to a lesser extent, LPA, PG, and PA (Fig. 7). However, the pattern and degree of stimulation were not as strikingly altered as in the osmolarity studies.

DISCUSSION

We direct attention to two aspects of these studies with human platelets: the new method for quantitative extraction of inositides, and the effect of ionic environment on incorporation of $^{32}P_i$ into inositides and other PL.

Extraction and separation techniques. Our extraction method borrows from the observations of two laboratories: Dawson's (38) work, which showed the tightness of the Ca^{2+}-TPI-protein binding, suggested to us the use of an excess of EDTA as a successful competitor with TPI for Ca^{2+}, and the work of Garbus, DeLuca, Loomans, and Strong (5) suggested the use of an excess of KCl to drive the freed TPI and the other PL, including the trace compounds, to the chloroform layer of a two phase extraction system. Actually, Folch, Lees, and Sloane Stanley were the first to suggest that the ionic content of the overlayer could influence the capturing of lipid by the underlayer in a two phase system (39). This principle has also recently been applied by Michell, Hawthorne, Coleman, and Karnovsky (40), who used 2 M KCl to extract the PPI of rat brain. However, it is worth

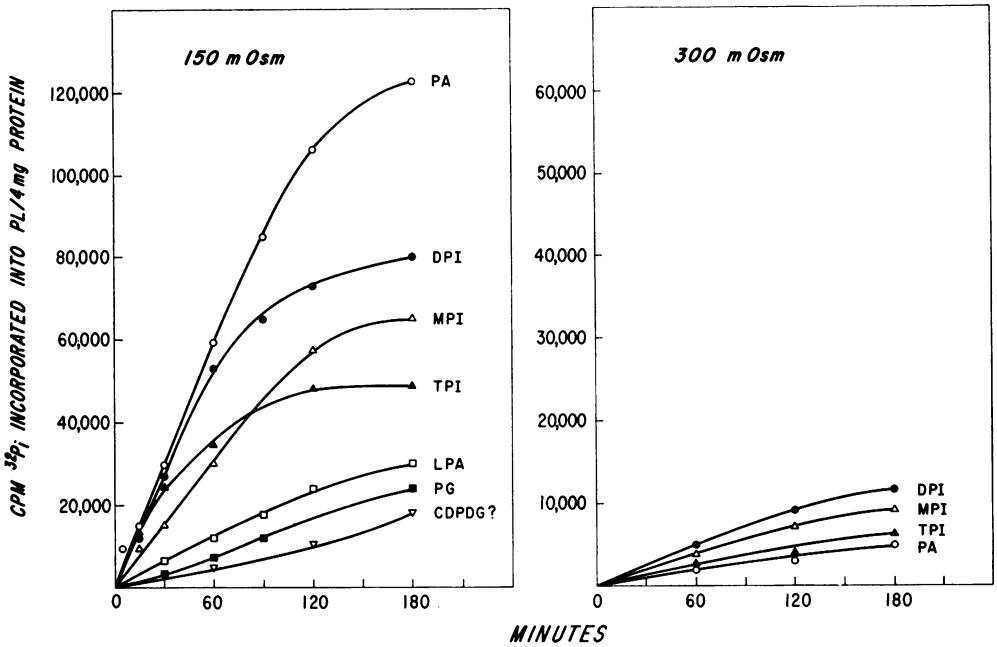

FIGURE 4 Time course studies on the incorporation of $^{32}P_i$ into human platelet PL in 150 and 300 mOsm media. The studies at the two osmolarities were done in parallel. For details, see Methods. Shown are the means of two experiments.

emphasizing again that with 2 M KCl alone we were not able to extract quantitatively the PPI of human platelets.

Our chromatographic procedure improves upon previous reports (27–29) only in that it offers the possibility for simple quantification of several ^{32}P-labeled PL other than inositides.

Inositide composition of human platelets. It is apparent that the inositides of human platelets taken collectively must be considered as a major PL class (Fig. 1; Table I); our previous studies, employing standard extraction methods, failed to recover at least one-third of the inositol-containing lipids (24).

On a wet weight basis, platelets are perhaps the richest readily harvestable source of the inositide family in man. In fact, if one can extrapolate from Dittmer and Douglas' ranking of the inositide content of 11 rat tissues (41), human platelets have nearly the same content of PPI as rat brain, a tissue known to be heavily endowed with these compounds (Table I).

It should be pointed out, however, that our extraction technique differs considerably from that used by Dittmer and Douglas, who employed the classical acid extraction procedure (22), and perhaps most importantly, used only fresh tissue (41). The latter may account for the rather heavy content of DPI in our platelets, since the platelet procurement method used (18, 19) requires nearly 3 hr, and even at 0–4° C this interval may permit a considerable breakdown of TPI. The studies of Dawson and Eichberg (42) and Sheltawy and Dawson (43) have shown that fresh frozen rat brain furnishes a higher yield of PPI than 15 min old tissue, an observation which they attributed to postmortem enhancement of hydrolytic activity.

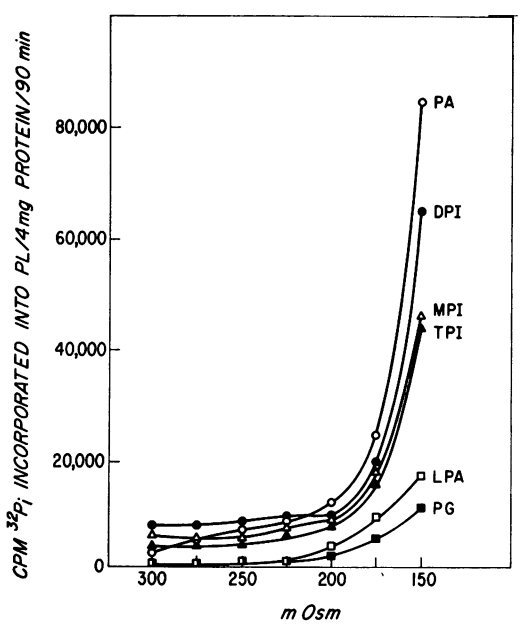

FIGURE 5 Effect of medium osmolarity on the incorporation of $^{32}P_i$ into human platelet PL. Between 200 and 150 mOsm there was a sharp upturn in the labeling of all PL. Shown are the means of two experiments.

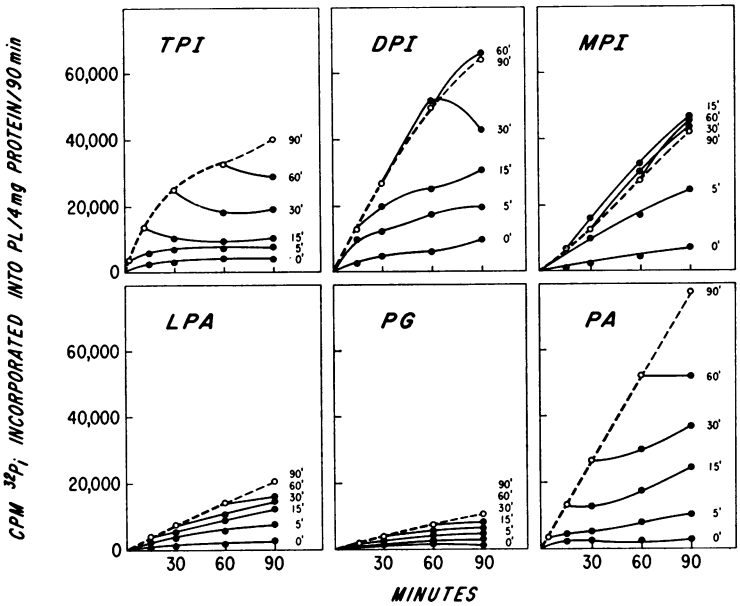

FIGURE 6 Time studies on the effect of restoring osmolarity to normal on $^{32}P_i$ incorporation into PL by platelets suspended in 150 mOsm media at the start of incubation. There were seven groups of four incubation flasks each. Among these were two sets of control 90-min time studies. To one of these sets, 0.1 ml of 0.075 M NaCl was added to each flask at the start of the incubation, and the reactions were stopped at 15, 30, 60, and 90 min. In a second control set, 0.1 ml of 0.15 M sorbitol was substituted for the NaCl addition. Each of the five remaining sets of four flasks received 0.1 ml of 3 M sorbitol at various time intervals: zero time, 5, 15, 30, and 60 min after the start of the incubation. For other details, see Methods. Both controls gave nearly identical curves, and only the sorbitol control (O---O) is shown. The remaining curves (●——●) are designated by the time at which 3 M sorbitol was added to restore osmolarity to 300 mOsm (0; 5; 15; 30; 60). Shown are the means of two experiments.

Incorporation of $^{32}P_i$ into inositides and other PL of human platelets. When $^{32}P_i$ is administered therapeutically, all of the major PL are labeled (9, 14),[†] presumably in the megakaryocytes. However, an extramegakaryocytic *de novo* pathway for synthesis of major PL in vitro has clearly been shown by Lewis and Majerus (44) and Cohen, Derksen, and van den Bosch (19) in studies using glycerol-^{14}C as a precursor. It is, therefore, difficult to explain why none of the major PL exclusive of the inositides can be labeled by $^{32}P_i$ in vitro (9, 10) (Figs. 3 and 4). A difference in the pattern of in vivo and in vitro labeling of PL by $^{32}P_i$ was first emphasized by Marinetti, Erbland, Albrecht, and Stotz in studies with liver (45).

It should be recalled that the phosphate moiety of PE, PC, and sphingomyelin is derived from phosphorylethanolamine and phosphorylcholine via their CDP derivatives (46, 47), and not from PA, which loses its phosphate in the classical *de novo* sequence (46). It is considered, therefore, that under the selected in vitro conditions, no labeling of phosphorylcholine or phosphorylethanolamine occurs, or that the labeled products are not used to form the corresponding CDP derivatives from which PC, sphingomyelin, and PE are synthesized.

On the other hand, the phosphate moieties of MPI, PG, and DPG derive from PA via CDPDG, hence remain with the parent molecular skeleton throughout their synthesis (48–50). Recently, in fact, the sequences leading to CDPDG (16) and MPI (17) have been shown in human platelets. Further branching of this pathway could account for the ^{32}P labeling of PG and DPG (Fig. 3).

That platelets contain kinases capable of phosphorylating the inositol ring of MPI was established by finding $^{32}P_i$ labeling of DPI and TPI. However, as previously mentioned, the relative labeling of these two compounds could have been obscured by enzymatic breakdown of TPI during the isolation or incubation of platelets as has been found recently in studies with swine

[†] Cohen, P., and J. W. W. Lisman. Unpublished data.

erythrocytes (8). In any case, the degree of labeling of DPI and TPI suggests their rapid turnover and implies the existence of a phosphomonoesterase-phosphokinase cycle for interconversions among the inositides.

The possible pathways for derivation of the ^{32}P label in PA (51, 52) and LPA (52, 53) have been well described and are beyond the scope of this discussion. It is useful to point out that labeled LPA possibly may have derived from nonenzymatic breakdown of PA.

It is also worth reemphasizing that we have not found measurable amounts of LPA, PG, or PA in platelets (Fig. 2); their labeling by $^{32}P_i$ remains the only evidence for their existence in these cells.

Effect of medium tonicity on $^{32}P_i$ incorporation into platelet PL. In common with temperature and pH optima, the notion that there could be optima related to medium tonicity may have limited usefulness. Yet, it is clear from these (Figs. 3–5) and previous studies (18) that medium osmolarity cannot be ignored while probing for metabolic activities in platelet suspensions. Although a biochemical explanation of the observed phenomena must await further study, it is useful for a start to consider some of the more obvious consequences of manipulating medium osmolarity.

That the effect of lowering medium osmolarity was probably not secondary to changes in cation concentration or pH was shown by the experiments in which sorbitol, without modifying either of these variables, was able to blunt the effect of hypoosmolarity at various time intervals after the start of incubation (Fig. 6). Further, severe hypotonic exposure cannot be equated with mechanical disruption since freeze-thawing or sonication abolished ^{32}P labeling of PL. The latter observation suggests that the general framework of cell architecture must be retained if there is to be optimal uptake of $^{32}P_i$ into PL. It is known, for example, that certain enzymes are released from mitochondria by mechanical disruption but remain attached with hypotonic exposure (54).

The possibility is considered that marked hypoosmolarity of the incubation medium results in an increased production or availability of "high energy" ^{32}P ($\sim^{32}P$) as a donor for PL phosphorus, and/or an increased turnover or net synthesis of PL. The latter could result from the demands for new membrane synthesis caused by stretching of the cell, analogous to the stress placed upon the leukocyte membrane during phagocytosis (11). However, the incorporation of oleic acid-1-^{14}C into PC, a major membrane constituent, is not similarly affected by hypotonic exposure (18). Thus, we believe it is more likely that medium hypoosmolarity affects the handling of phosphate by the cell, i.e., in some way facilitates penetration of the plasmalemma by free or activated phosphate.

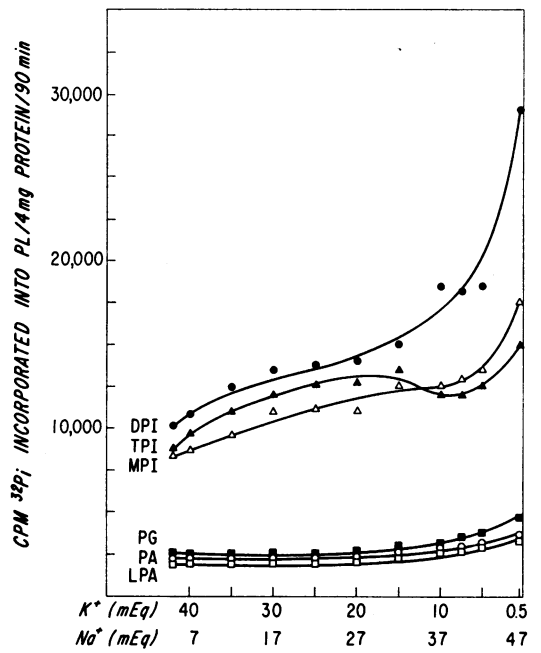

FIGURE 7 Effect of medium Na and K concentrations on the incorporation of $^{32}P_i$ into human platelet PL. These incubations were at 300 mOsm. For other details, see Methods. Shown are the means of two experiments.

The hydrated phosphate ion does not readily penetrate biological membranes; this may be accounted for by its large diameter (55). It is possible to envision greater phosphate penetration through the stretched membrane of hypotonically swollen cells. However, phosphate penetration of the plasmalemma is generally thought of as relying on its participation in numerous kinase systems. Thus, both mechanisms, organic phosphorylation(s) or diffusion of the free anion, could account for increased penetration of $^{32}P_i$ which then could lead to an increased production or specific activity of $\sim^{32}P$. The latter as $AT^{32}P$ would derive from glycolysis or oxidation of glucose or FA. It has been shown that glucose oxidation for which phosphorylation is a precondition can be considerably augmented in hypotonic media (18). Part of this effect is to compensate for a parallel failure of FA oxidation. However, even when FA oxidation is restored in hypoosmolar media by adding (−)-carnitine, glucose oxidation still remains above control values, which suggests that increased penetration of glucose is taking place (18). Thus, the increased availability of $\sim^{32}P$ for PL biosynthesis in hypoosmolar media possibly may be explained by increased glucose penetration or utilization since in the present experiments no (−)-carnitine was added.

Effect of Na and K concentrations on $^{32}P_i$ incorporation into platelet PL in isotonic media. As with other PL, the function of the inositides is not known. To support their participation in an active metabolic process, as opposed to a mere structural function, there are three observations. First, considering the lipophilic end of the molecule, there is evidence that the FA have a very high turnover rate (19). Second, on the hydrophilic end, there would appear to be a rather active phosphorylation-dephosphorylation cycle which is probably directly linked to energy metabolism (15). Third, in contrast with other major PL, inositides have a rather high degree of water solubility (38) which could enable them to shuttle rapidly between lipid and water compartments as carriers of water soluble substances requiring facilitated transport across membrane barriers.

These points bring to mind the requirements for Na/K pumping (a not inconsiderable burden for human platelets [56]) for which the inositide family could be linked to, or identical with, the involved ATPase system. Hokin and Hokin have suggested this possibility with respect to PA and inositides (57). However, this hypothesis has not been proved, and others have been proposed (58, 59). Lately, in fact, it has been shown that phosphorylation of the inositides of swine erythrocytes is at a considerably lower order of magnitude than hydrolysis of ATP by the Na plus K–stimulated ATPase and is unaffected by wide variations in the concentration of medium Na or K (8).

Our results show that high K and low Na concentrations in the medium decrease $^{32}P_i$ incorporation into the inositides and several other PL (Fig. 7). Similar results have been found in studies on hen brain slices (60).

Obviously, if one holds to the view that inositides have a universal function as specific Na and K carriers, the variations among the findings with hen brain, swine erythrocytes, and human platelets are presently irreconcilable. However, it should be considered that the medium Na/K ratio may, in addition to altering pumping requirements, influence production, utilization, or availability of $\sim^{32}P$. This latter view has been summarized by Sheltawy and Dawson (see reference 60), who suggest that raising the Na/K ratio of the medium in some way increases availability of AT^{32}P for a variety of phosphorylations including, but not exclusively involving, the inositides. As previously cited, this view could also apply to the hypoosmolarity experiments in which a global enhancement of ^{32}P labeling of PL was observed.

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