BBA 66458

COMPARATIVE STUDIES OF THE RESPONSES OF RAT LIVER MICROSOMAL GLUCOSE-6-PHOSPHATASE AND INORGANIC PYROPHOSPHATE–GLUCOSE PHOSPHOTRANSFERASE TO PHOSPHOLIPASE C TREATMENT AND PHOSPHOLIPID SUPPLEMENTATION*

ROY E. SNOKE AND ROBERT C. NORDLIE

The Guy and Bertha Ireland Laboratory, Department of Biochemistry, University of North Dakota School of Medicine, Grand Forks, N.D. 58201 (U.S.A.)

(Received May 24th, 1971)
(Revised September 13th, 1971)

SUMMARY

1. Comparative studies of the responses of Glc-6-*P* phosphohydrolase and PP_i–glucose phosphotransferase activities of rat liver microsomal glucose-6-phosphatase (EC 3.1.3.9) to *in vitro* phospholipid modifications have been carried out. Various conditions for assay of enzymic activities were employed, and results compared.

2. Based on activity assays performed at pH 6 in the absence of added detergent, both activities decreased progressively and in parallel fashion with increasing length of exposure of microsomes to the action of phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3). These activities of such treated preparations were increased by the addition of micellular dispersions of supplemental phospholipid (rat liver microsomal phospholipid preparations or, in one study, various classes of purified phospholipids). These observations provide yet another piece of evidence in support of catalysis of these two enzymic activities by a single microsomal enzyme.

3. The degree of restoration of activities towards original control levels through phospholipid supplementation following phospholipase C exposure of microsomes was much less when based on activity values obtained in the presence of the detergent deoxycholate than in its absence.

4. Enzymic activities of phospholipid-supplemented, phospholipase C-treated preparations were quite insensitive to stimulation by deoxycholate, while those of unexposed preparations were highly stimulated by this same detergent.

5. The pH–activity profile for PP_i–glucose phosphotransferase activity obtained after phospholipase C exposure and phospholipid supplementation resembled strongly that noted with detergent-activated, otherwise untreated microsomal pre-

Abbreviation: HEPES, N-2-hydroxyethylpiperizine-N′-ethanesulforic acid.

* The data presented are from a doctoral dissertation submitted by Roy E. Snoke in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Graduate School of the University of North Dakota, January 1970. A preliminary report has appeared[1].

parations, displaying a maximum at pH 5.6 in comparison with that at pH 4.5 exhibited by untreated microsomal preparations.

6. These observations are interpreted to indicate that phospholipid supplementation following phospholipase C exposure of rat liver microsomes does not lead to the restoration of microsomal glucose-6-phosphatase in its original form. Further, it is concluded that, in view of these findings and other observations in the literature, a reconsideration of the whole subject of the involvement of phospholipids in glucose-6-phosphatase action appears to be in order.

INTRODUCTION

During the past 20 years a number of interesting reports[2–7] implicating microsomal phospholipids in glucose-6-phosphatase (glucose-6-phosphate phosphohydrolase EC 3.1.3.9) function have appeared*. On the basis of work in our own laboratory[8] and that of STETTEN[9], beginning in 1963, this important biological catalyst is now known to possess a multiplicity of functions (see reviews in refs. 10–12), including potent PP_i–glucose phosphotransferase activity. As part of our continuing program of studies relating to the catalytic properties, physiological functions, and biological regulation of this multifunctional catalyst, we undertook, beginning in 1966, a systematic study of the comparative effects of various lipids and lipid modifications on hydrolytic and synthetic activities of the enzyme. Results of some of these studies, on the effects of *in vitro* phospholipid modifications, which appear particularly relevant with regard to the interpretation of a number of recent papers[3,5], are described here.

MATERIALS AND METHODS

Materials

Substrates, buffers, Glc-6-*P* dehydrogenase (D-glucose-6-phosphate: $NADP^+$ oxidoreductase, EC 1.1.1.49), and deoxycholate were obtained from sources indicated in earlier papers[8,13,14]. Lecithinase C (phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3)), Type I, prepared from *Clostridium welchii*, and 1,2-dipalmitin ("approx. 99% pure") were purchased from Sigma Chemical Co., St. Louis, Mo. This phospholipase C preparation, an $(NH_4)_2SO_4$ fraction prepared from a culture filtrate as described by MACFARLANE AND KNIGHT[15], previously has been employed in studies of enzymic phospholipid requirements by DUTTERA *et al.*[3] and MARTONOSI *et al.*[16]. Assays of the microsome-free supernatant following phospholipase C exposure for 15 min, as described in detail in MATERIALS AND METHODS below, by the ninhydrin method of LEE AND TAKAHASHI[17] failed to reveal any proteolysis, as also previously has been reported by MARTONOSI *et al.*[16] with a similar preparation of the enzyme from the same source and supplier. Chromatographically purified ("96 + % pure") bovine preparations of various phospholipids were obtained from

* While this work was in progress, a highly relevant report by DUTTERA *et al.*[3] appeared. An interesting, related paper by ZAKIM[5] appeared subsequent to the completion of the present studies, but prior to preparation of this manuscript for publication. Results from both of these laboratories are considered where appropriate throughout this paper.

Applied Science Laboratories, State College, Pa. Chloroform and methanol were "spectranalyzed" grade reagents purchased from Fisher Scientific Co., Fair Lawn, N.J., and Silica gel H was supplied by Brinkman Instruments, Inc., Westby, Long Island, N.Y. Distilled, deionized water was employed in the preparation of all aqueous solutions of reagents, and pH was adjusted as required with dilute NaOH and measured with a Beckman expanded scale meter.

Experimental animals

Young, adult male albino rats (150–250 g) were purchased from Sprague–Dawley, Inc., Madison, Wisc., and were maintained on tap water and Purina Lab Chow, *ad libitum*. It was decided that fed animals, rather than rats fasted for 48 h as employed by others[3], be utilized since the latter treatment has been shown by NORDLIE *et al.*[18] to produce unique, activity-discriminating, detergent modifiable responses of synthetic and hydrolytic activities of microsomal glucose-6-phosphatase. Studies carried out supplemental to those described in this paper indicated that Glc-6-*P* phosphohydrolase activity of microsomes from livers of fed animals were somewhat less responsive to phospholipid supplementation after phospholipase C exposure than were similarly treated preparations from animals which had been fasted for 48 h. For example, activity levels were observed to decrease to approx. 20% of original values following exposure of hepatic microsomes from either fed or fasted animals to phospholipase C for 15 min. The addition of 87 μg microsomal phospholipid P per mg microsomal protein to such exposed preparations from fasted animals restored activity completely (to 103% of original values). In contrast, identical supplementation restored activity of preparations from fed animals to only 64% or original values, an observation repeatedly confirmed in the studies described below and one which must be kept in mind when attempting to rationalize the various studies of this type[2–7] which have appeared in the literature.

Isolation of microsomal fraction

Livers were removed and homogenized, and microsomal fraction was isolated by conventional differential centrifugation technique[19], as in previous studies[14]. Microsomal preparations were suspended in 5 vol. of 0.25 M sucrose per g of original liver, and were stored frozen at $-15°$ (for less than 5 days) until used.

Extraction and isolation of microsomal phospholipids

Lipids were extracted from freshly isolated microsomal fraction by the method of FOLCH *et al.*[20]. Phospholipid was separated from the neutral lipid fraction by the silicic acid column procedure of BORGSTRÖM[21] as described by DUTTERA *et al.*[3]. The preparation, as a solution in chloroform, was stored under N_2 at $-15°$. The Folch washing procedure removes "essentially all non-lipid contaminants from total pure lipid extract"[20]. The silicic acid column procedure additionally provides for a 100% recovery of phospholipid of greater than 97.5% purity[21] from such lipid extracts.

Preparation of phospholipid micelles

Aliquots (approx. 80 mg of phospholipid) of the above solution of microsomal phospholipid in chloroform (or of commercial purified phospholipid in chloroform) were brought to dryness under a stream of dry N_2. Aliquots (10 ml) of 12.5 mM Tris–

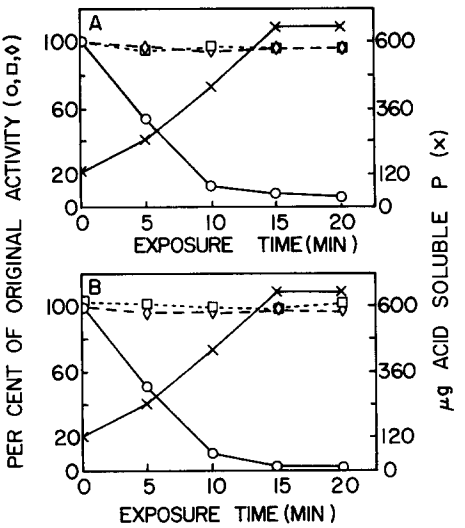

Fig. 1. Effects of phospholipase C treatment on microsomal Glc-6-*P* phosphohydrolase (A) and PP_i–glucose phosphotransferase (B) activity levels, and release of acid-soluble phosphorus. As described in MATERIAL AND METHODS, microsomal preparations were incubated with Tris buffer, phospholipase C and $CaCl_2$ for the indicated periods of time. Enzymic activity values determined after treatment with this complete system are indicated (○); results of control studies in which either $CaCl_2$ (□) or phospholipase C (◇) was omitted also are presented. Acid-soluble phosphorus levels present after various periods of incubation also are presented (×). Enzymic activity is expressed as per cent of activity obtained with microsomal preparation which had not been incubated with phospholipase C. Activity values in the latter case were 0.69 μmole Glc-6-*P* hydrolyzed, or 0.22 μmole Glc-6-*P* synthesized, per 5 min per mg microsomal protein.

HCl or *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (HEPES) buffer were then added and the samples dispersed by sonic disruption at 0°, under N_2, for 2.5 min with a Biosonic II probe sonicator (Bronwill Scientific Co., Rochester, N.Y.) operating at 75% of maximal intensity. Final pH values of the dispersions, which did not sediment when centrifuged at 25 000 × *g* for 10 min, were pH 7.5 (Tris–HCl buffer) and pH 7.0 (HEPES buffer). Such preparations were made shortly (less than 30 min) before use, and were kept in ice until added to microsomal preparations.

Phospholipase C treatment of microsomal preparations

Microsomes were incubated with 0.25 mM $CaCl_2$, 3.1 mM Tris–HCl (pH 7.4), and 0.05 mg phospholipase C per mg microsomal protein at 21° for the times noted in the legends to figures and tables. At the specified time, EDTA (pH 6.2), to a final concentration of 0.01 M, was added to chelate the Ca^{2+}, an essential cation for phospholipase action[2,22]. In certain cases(see for example Fig. 1) concomitant experimental controls were run in which either $CaCl_2$ or phospholipase C was omitted. After termination of the reaction, the contents of incubation mixtures were centrifuged at 27 000 rev./min for 50 min in the No. 30 rotor of the Spinco Model L preparative ultracentrifuge (3 000 000 *g*·min), after which the supernatant fractions were decanted and retained for assay of liberated acid-soluble phosphorus. The resulting microsomal pellet was resuspended in ice-cold 0.25 M sucrose, to a concentration of

4 to 5 mg microsomal protein per ml. Such preparations were maintained at 0° until assayed for enzymic activities.

Determination of liberated acid-soluble phosphorus

The action of phospholipase C, above, is to release phosphorylcholine (and some phosphorylethanolamine, *etc.*) from microsomal phospholipid. To determine the extent of such hydrolysis, 1-ml aliquots of the supernatant fraction resulting from the centrifugation described immediately above were mixed with 1.0 ml of 10% (w/v) trichloroacetic acid solution to precipitate soluble protein and centrifuged at 2500 rev./min for 10 min in a Model CL International Clinical centrifuge. Aliquots (0.5 ml) of this deproteinized solution were evaporated to dryness in a heating block over a flame, cooled, and assayed for P_i (see below).

Enzymic assays

The procedures basically were as described by NORDLIE AND ARION[14]. Incubations routinely were carried out, with shaking, for 5 min at 21°, except for the experiment depicted in Fig. 1 where the temperature was 30°. Assay mixtures, pH 6.0, contained in 1.5 ml, 40 mM sodium cacodylate buffer and either 6.7 mM sodium Glc-6-*P* (phosphohydrolase), or 6.7 mM sodium PP_i *plus* 160 mM D-glucose (PP_i–glucose phosphotransferase). A combination of 40 mM sodium cacodylate buffer and

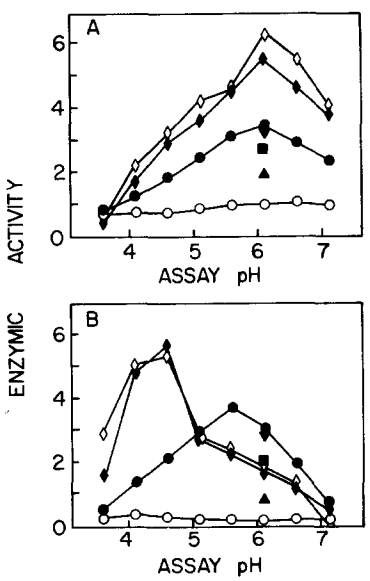

Fig. 2. Effects of phospholipase C treatment and phospholipid supplementation on levels of Glc-6-*P* phosphohydrolase (A) and PP_i–glucose phosphotransferase (B) activities assayed at various pH values. Microsomal preparations were incubated for 15 min with the complete phospholipase C system as described in the text (○, ●), or with the system *minus* phospholipase C (◇, ◆). Such preparations were then supplemented with microsomal phospholipid in HEPES buffer, pH 7 (◆, ●, ▲, ■, ▼), or with the suspending vehicle only (◇, ○). Supplemented phospholipid concentrations, expressed as mg phospholipid per mg microsomal protein, were as follows: ◆, ●, 1.80; ▼, 1.65; ■, 1.10; and ▲, 0.55 (1 mg phospholipid corresponds to 38 μg P). After 5 min preincubation of microsomal preparations with supplemental phospholipid at 21°, enzymic activities were assayed as described in the text. Specific enzymic activity is as defined in Table II.

40 mM sodium acetate buffer, at a variety of pH values, was employed in the studies depicted in Fig. 2. P_i liberated in the Glc-6-*P* phosphohydrolase reaction was assayed by the colorimetric method of FISKE AND SUBBAROW[23], while Glc-6-*P* produced in the phosphotransferase reaction was assayed spectrophotometrically with the aid of Glc-6-*P* dehydrogenase in the presence of excess $NADP^+$ (see ref. 14).

Other analytical techniques

Microsomal phospholipids, prepared as described above, were separated from one-another by chromatography on silicic acid by the method of PARKER AND PETERSON[24] as modified by MILLER AND CORNATZER[25]. P_i was determined on eluates from such chromatographic plates, and on acid-soluble phosphorus samples arising during phospholipase C incubations of microsomes (see above), by the method of FISKE AND SUBBAROW[23] after acid hydrolysis and adjustment of pH (ref. 25).

RESULTS

Effects of phospholipase C

Effects on enzymic activity and acid-soluble phosphorus levels. The effects on enzymic activity levels of exposure of rat liver microsomes to the action of phospholipase C for various periods of time are described in Fig. 1. Concomitant losses of both Glc-6-*P* phosphohydrolase (Reaction 1; see Fig. 1A) and PP_i–glucose phosphotransferase (Reaction 2; see Fig. 1B) progressive with increasing periods of phospholipase C treatment were observed. Little or no change in levels of enzymic activity was noted in companion, control studies in which either phospholipase C or $CaCl_2$, which is essential for phospholipase C activity[19], was omitted from incubation mixtures (see data indicated by ◇ and □ in Figs. 1A and 1B). The release of acid-soluble phosphorus (indicated by × in Figs. 1A and 1B) progressive with increasing duration of incubation of microsomes with phospholipase C *plus* $CaCl_2$ also is apparent from Fig. 1. This acid-soluble phosphorus represents principally phosphorylcholine[19] and some phosphorylethanolamine[26] released from microsomal phospholipid through the action of this phospholipase (see Eqn. 3).

$$\text{Glc-6-}P + H_2O \rightarrow \text{glucose} + P_i \quad (1)$$

$$PP_i + \text{glucose} \rightarrow \text{Glc-6-}P + P_i \quad (2)$$

$$\text{Phosphatidylcholine (or other phospholipid)} + H_2O \xrightarrow{Ca^{2+}} \alpha,\beta\text{-diglyceride} + \text{phosphorylcholine (or other corresponding phosphoryl derivative)} \quad (3)$$

Effects on microsomal phospholipid content. The effects of various periods of exposure of microsomal preparations to the action of phospholipase C on microsomal total phospholipid content and concentrations of various individual classes of phospholipids are described in Table I. Phospholipids were extracted, isolated from total microsomal lipid, separated into the various classes by thin-layer chromatographic procedures, and assayed as described in MATERIALS AND METHODS. The progressive decrease in total microsomal lipid phosphorus as the period of incubation with phospholipase C was increased is apparent from data in the second vertical column in this table. It is also obvious from the data in Table I that while the absolute con-

TABLE I

EFFECTS OF PHOSPHOLIPASE C TREATMENT ON THE PHOSPHOLIPID COMPOSITION OF RAT LIVER MICROSOMES

Microsomal preparations were incubated with phospholipase C, for the indicated periods of time, as described in the text. Lipids were extracted from resulting microsomal pellets by the chloroform–methanol procedure[20]; phospholipids were separated by thin-layer chromatography, and phosphorus assayed as described in the text.

Time of exposure to phospholipase C (min)	*μg total lipid P per mg microsomal protein*	*Phospholipid composition of microsomes**				
		Phosphatidyl-choline	*Phosphatidyl-ethanolamine*	*Phosphatidyl-inositol*	*Phosphatidyl-serine*	*Other*
0	14.3	58 (8.3)	23 (3.3)	13 (1.8)	2 (0.30)	4 (0.60)
5	10.3	54 (5.6)	26 (2.7)	11 (1.1)	3 (0.30)	6 (0.60)
10	3.1	38 (1.2)	28 (0.90)	25 (0.80)	3 (0.09)	6 (0.19)
15	2.8	17 (0.48)	37 (1.0)	37 (1.0)	3 (0.09)	6 (0.17)
20	1.7	11 (0.19)	39 (0.66)	39 (0.66)	6 (0.10)	6 (0.10)

* % of total lipid phosphorus. Values in parentheses present μg lipid phosphorus per mg microsomal protein.

centrations of all the individual classes of phospholipids, expressed in terms of μg lipid phosphorus per mg microsomal protein, decreased with increased length of incubation, phosphatidylcholine concentration diminished most rapidly. As a consequence of this specificity of hydrolysis, the relative proportion of total microsomal phospholipid represented by phosphatidylethanolamine and phosphatidylinositol (see "% of total lipid phosphorus" values in Table I) increased significantly as incubation progressed.

*Effects of phospholipid supplementation with phospholipase C-treated and unexposed microsomal preparations**

Supplementation with phospholipid of plant origin (asolectin, from soybeans) was for the most part employed by other workers[3,5] who previously have studied the effects of phospholipids on phosphohydrolase activity of this enzyme. In the present studies, rat liver microsomal phospholipid extracts routinely were employed, since it was felt, on the basis of possible specificity effects, that such preparations would be more appropriate for reactivation studies after phospholipid depletion of preparations of the enzyme from rat liver.

Fig. 2. describes alterations in Glc-6-*P* phosphohydrolase (Fig. 2A) and PP_i–glucose phosphotransferase (Fig. 2B) activities of microsomal glucose-6-phosphatase when exposed to phospholipase C and/or supplemented with micelles of total phospholipids extracted from rat liver microsomes. The addition of phospholipid (◆) to unexposed microsomes (◇) produced little or no significant change in the level of either activity. Exposure to phospholipase C for 15 min markedly decreased the

* Throughout this paper, the terms "unexposed preparation" or "unexposed microsomes" are used to refer as briefly as possible to microsomal preparations which were preincubated with 3.1 mM Tris–HCl buffer, pH 7.4, and 0.25 mM $CaCl_2$ in the absence of phospholipase C, and were otherwise treated as were phospholipase C-exposed microsomes as described in detail in the text. "Vehicle" refers to the suspending medium employed in the preparation of phospholipid micelles, which routinely was added to control samples in experiments in which phospholipid supplementation was studied.

enzymic activity (○); and as assayed at pH 6, a progressive increase in both activities was observed when increasing amounts of phospholipid (see ▲, ■, ▼ and ● in Figs. 2A and 2B) were added to the exposed preparations before assay. However, although both activities were significantly elevated, the extent of increase at pH 6 seemingly was in an activity-discriminating manner. Supplementation of exposed preparations with 1.65 mg or 1.80 mg phospholipid per mg microsomal protein increased the phosphotransferase activity to values greater than those for activity levels of unexposed preparations, while the phosphohydrolase activity was returned only partially towards unexposed activity levels.

Effects of varied assay pH on response patterns. It was felt that additional insight relating to the mechanism of action of phospholipid supplementation might be gained by measuring activity levels of the various preparations over a broad spectrum of assay pH values. These data also are presented in Fig. 2A and 2B. Phospholipid addition to unexposed preparations (1.8 mg phospholipid per mg microsomal protein) was without significant effect at any assay pH value. In addition, the same general patterns of response noted at pH 6 were observed throughout the entire pH range studied, in the case of phosphohydrolase activity (Fig. 2A). However, in marked contrast, the pattern of response obtained with the phosphotransferase activity was highly dependent on the pH value chosen for assay. The pH optimum for this synthetic activity of unexposed microsomes, pH 4.5 (see ◆ and ◇, Fig. 2B), shifted significantly towards neutrality upon phospholipid supplementation of phospholipase C-exposed microsomes, with a well defined maximum at pH 5.6 (see ●, Fig. 2B). Under such conditions, phosphotransferase activity assayed above pH 5 exceeded that of unexposed preparations (◇, ◆) while the activity assayed below pH 5 was much less than control activity values. Further, the entire pH–activity profile obtained under these conditions is highly reminiscent of that obtained either with partially purified enzyme[8] or after supplementation of microsomes with deoxycholate[18,27] or other detergents[27]. The possible significance of these observations will be considered further in the DISCUSSION, below.

Effects of individual classes of phospholipids. The responses of Glc-6-*P* phosphohydrolase and PP_i–glucose phosphotransferase activities to supplementation by a number of individual types of phospholipids are compared in Table II. These effects were studied both with unexposed and phospholipase C-treated microsomal preparations. Supplemented phospholipid concentration in each instance was 1.8 mg/mg microsomal protein. Equivalent concentration values, expressed as μg lipid P per mg microsomal protein, for each individual phospholipid, are given in Table II.

A number of valid conclusions appear justified on the basis of these data. With both unexposed and phospholipase C-treated preparations, similar patterns of response of both Glc-6-*P* phosphohydrolase and PP_i–glucose phosphotransferase activities to phospholipid supplementation were noted, providing yet another confirmatory piece of evidence in support of catalysis by a single enzyme. With unexposed microsomes, supplemental phosphatidylcholine, phosphatidylserine and total microsomal phospholipid were without significant effect on levels of either activity, while phosphatidylethanolamine and lysophosphatidylcholine produced significant increases in both. With phospholipase C-treated microsomes, in contrast, all supplementary phospholipids produced significant increases in both activities. The extents of response of both types of activities under the conditions described were greatest

TABLE II

COMPARATIVE RESPONSES OF Glc-6-*P* PHOSPHOHYDROLASE AND PP_i–GLUCOSE PHOSPHOTRANSFERASE ACTIVITIES OF PHOSPHOLIPASE C-EXPOSED AND UNEXPOSED MICROSOMES TO VARIOUS SUPPLEMENTAL PHOSPHOLIPIDS

Microsomes were incubated for 15 min with the complete phospholipase C system, or with the system *minus* phospholipase C ("unexposed microsomes"), EDTA added, and microsomal pellets recovered by centrifugation as described in the text. The various phospholipids or microsomal lipid (1.8 mg phospholipid per mg microsomal protein) dispersed in HEPES buffer, pH 7.0, were added, preparations were incubated for 5 min at 21°, and enzymic activities assayed as described in the text. The vehicle, HEPES buffer, pH 7.0, only, was added as indicated. Phospholipid content of microsomes was 14.3 and 2.8 μg lipid P/mg microsomal protein in unexposed and phospholipase C-treated preparations, respectively. Concentrations of individual, supplemented phospholipids (μg lipid P per mg microsomal protein) were as follows: phosphatidylcholine, 54.4; phosphatidylserine, 40.0; phosphatidylethanolamine, 73.6; lysophosphatidylcholine, 83.9; microsomal phospholipid, 68.4.

Addition	*Enzymic activity**			
	Unexposed microsomes		*Phospholipase C-treated microsomes*	
	Glc-6-P phosphohydrolase	*PP_i–glucose phosphotransferase*	*Glc-6-P phosphohydrolase*	*PP_i–glucose phosphotransferase*
Vehicle, only	6.7	2.9	1.0 (15)	0.18 (6)
Phosphatidylcholine	7.2	3.0	1.7 (23)	0.91 (31)
Phosphatidylserine	5.8	3.0	1.6 (28)	1.0 (33)
Phosphatidylethanolamine	9.5	8.5	2.9 (30)	2.6 (30)
Lysophosphatidylcholine	16.1	9.5	2.3 (17)	0.73 (8)
Microsomal phospholipid	6.5	2.9	3.4 (51)	3.7 (130)

* Enzymic activity = 10 × μmoles Glc-6-*P* hydrolyzed (phosphohydrolase) or synthesized (phosphotransferase) per 5 min per mg microsomal protein. Values in parentheses present 100 × activity with phospholipase C-treated preparation/activity observed with corresponding microsomal preparations unexposed to phospholipase C.

with total microsomal phospholipid, both a bit less pronounced with lysophosphatidylcholine, and only moderate with phosphatidylcholine or phosphatidylserine. Lysophosphatidylcholine, in the amount added, produced the smallest noted increase in phosphotransferase activity with phospholipase C-treated microsomes, in marked contrast with its effects with unexposed microsomes. The reasons for this difference are not presently apparent.

Responses of mannose-6-P phosphohydrolase and mannose-6-P–glucose phosphotransferase activities[27] *to phospholipid modifications.* Because of the interesting responses to the effects of phospholipase C treatment *plus* phospholipid supplementation noted with PP_i–glucose phosphotransferase activity (see, for example, Fig. 2B), two additional, also highly detergent-sensitive (T. L. HANSON AND R. C. NORDLIE unpublished observations) activities of microsomal glucose-6-phosphatase were chosen for the studies described in Table III. The responses of both activities to phospholipid supplementation of both phospholipase C-treated and unexposed microsomal preparations were studied. Little or no response to supplementation with phospholipids was noted with the latter preparation (compare the second with the first horizontal line of data in Table III), while, as with PP_i–glucose phosphotransferase, the supplementation of phospholipase C-treated microsomal preparations with approx. 2 mg microsomal phospholipid per mg microsomal protein produced elevations in activity levels to significantly greater than unexposed microsomal control values (compare the fourth with the first horizontal line of data in Table III). It thus appears that those activities of microsomal glucose-6-phosphatase most extensively

TABLE III

RESPONSES OF MANNOSE-6-*P* PHOSPHOHYDROLASE AND MANNOSE-6-*P*–GLUCOSE PHOSPHOTRANSFERASE ACTIVITIES OF MICROSOMAL GLUCOSE-6-PHOSPHATASE TO PHOSPHOLIPASE C-TREATMENT AND PHOSPHOLIPID SUPPLEMENTATION

Microsomes were incubated without ("unexposed") or with phospholipase C for 15 min, EDTA was added, and microsomal pellets were collected by centrifugation as described in the text. Phospholipid content of unexposed and phospholipase C-treated microsomes was 14.3 and 10.3 μg lipid P per mg microsomal protein. Microsomal phospholipid [2 mg phospholipid (76 μg phospholipid P) per mg microsomal protein] suspended in HEPES buffer, pH 7.0, or the buffer ("vehicle") only, was added and samples incubated at 21° for 5 min, followed by assay for enzymic activity. Assay incubations were for 5 min at 21°.

Preparation	*Enzymic activity**	
	Mannose-6-P phosphohydrolase	*Mannose-6-P–glucose phosphotransferase*
Unexposed microsomes *plus* vehicle	0.98 (100)	0.46 (100)
Unexposed microsomes *plus* microsomal phospholipid	1.0 (102)	0.41 (89)
Phospholipase C-treated microsomes *plus* vehicle	0.26 (27)	0.24 (52)
Phospholipase C-treated microsomes *plus* microsomal phospholipid	1.4 (143)	0.93 (202)

* Activities are expressed as in Table II. Values in parentheses present 100 × observed activity/activity observed with unexposed microsomes supplemented with vehicle, only.

activated by detergents — mannose-6-phosphatase and mannose-6-*P*–glucose phosphotransferase as well as PP_i–glucose phosphotransferase (see Fig. 2 and Table II) — are also considerably more responsive to activation by supplemental phospholipid after phospholipase C exposure of microsomes than is Glc-6-*P* phosphohydrolase (see Fig. 2 and Table II), which also is relatively less extensively activated by detergents[13,18,27–30].

Effects of deoxycholate. Deoxycholate[13,18,27–30] and a variety of other detergents[28,29], as well as high-pH treatment[31] and mechanical disruption[32], are known to produce activation of various activities of microsomal glucose-6-phosphatase. Such treatments particularly affect PP_i–glucose phosphotransferase activity, and produce significant shifts towards neutrality in pH–activity profiles, in a manner quite similar to that noted with phospholipid supplementation of phospholipase C-treated microsomes in the experiments depicted in Fig. 2B. For this reason, studies were carried out in which the responses of synthetic and hydrolytic activities of microsomal glucose-6-phosphatase to phospholipase C treatment and phospholipid supplementation, as assayed in the presence of supplemental deoxycholate (to 0.2%, w/v, in microsomal preparations; see ref. 27), were compared with responses to phospholipid modifications as assayed in the absence of this steroid detergent. The data obtained are presented in Table IV.

Rigorous consideration of the results of these experiments indicates to us that a new perspective is needed for the interpretation of the results of studies on the *in vitro* effects of phospholipases and supplemental phospholipids on activities of this enzyme. Activity data obtained in the absence of deoxycholate (vertical columns 1

TABLE IV

MODIFYING EFFECTS OF DEOXYCHOLATE ON THE APPARENT RESPONSES OF HYDROLYTIC AND SYNTHETIC ACTIVITIES OF MICROSOMAL GLUCOSE-6-PHOSPHATASE TO PHOSPHOLIPASE C TREATMENT AND PHOSPHOLIPID SUPPLEMENTATION

Microsomes were incubated for 15 min without and with phospholipase C, reactions terminated with EDTA, and microsomal pellets isolated by centrifugation, as described in the text. Phospholipid content of exposed and phospholipase C-treated microsomes was 14.3 and 2.8 μg lipid P per mg microsomal protein, respectively. Microsomal phospholipid [1.88 mg (72 μg P) per mg microsomal protein] dispersed in 12.5 mM HEPES buffer, pH 7.0, or the HEPES buffer only ("vehicle") was added as indicated and samples incubated at 21° for 5 min. Neutral sodium deoxycholate solution, to a final concentration of 0.2% (w/v) or an equivalent volume of distilled water was added as indicated. The preparations were then maintained at 0° for 30 min, and assayed for enzymic activities.

Preparation	*Enzymic activity**					
	Glc-6-P phosphohydrolase			*PP_i–glucose phosphotransferase*		
	Deoxycholate absent	*Deoxycholate present*	*100 × activity with deoxycholate/activity without deoxycholate (%)*	*Deoxycholate absent*	*Deoxycholate present*	*100 × activity with deoxycholate/activity without deoxycholate (%)*
	1	*2*	*3*	*4*	*5*	*6*
A. Unexposed microsomes *plus* vehicle	7.6	13.0	171	2.6 [0.34]	14.0 [1.1]	538
B. Unexposed microsomes *plus* microsomal phospholipid	7.0	14.0	200	2.6 [0.37]	17.4 [1.2]	669
C. Phospholipase C-treated microsomes *plus* vehicle	1.3 (17%)	2.0 (15%)	154	0.36 (14%) [0.28]	0.60 (4.3%) [0.30]	167
D. Phospholipase C-treated microsomes *plus* microsomal phospholipid	3.1 (41%) {238%}	3.6 (28%) {180%}	116	2.2 (83%) [0.71] {611%}	2.6 (20%) [0.72] {433%}	118

* Enzymic activity is expressed as in Table II. Values in brackets, [ ], indicate ratios of PP_i–glucose phosphotransferase/Glc-6-*P* phosphohydrolase. Values in parentheses indicate 100 × activity of designated preparation/activity observed in the absence of phospholipids with unexposed microsomes; *i.e.* % apparent recovery of activity initially present before phospholipase C treatment. Values in braces, { }, indicate 100 × activity with phospholipid supplementation/activity without phospholipid supplementation, for preparations in both cases exposed to phospholipase C, and are an index of the extent of activation of such phospholipase C-treated preparations by supplemental microsomal phospholipid.

and 4, Table IV), interpreted as in earlier studies[2–5], indicate (a) no response to supplemental phospholipids by preparations unexposed to phospholipase C (compare relevant data in horizontal line B with those in line A); (b) a significant lowering of activity levels after exposure of microsomes to phospholipase C (compare horizontal line C with line A); and (c) a partial (Glc-6-*P* phosphohydrolase) or nearly total (phosphotransferase) restoration to unexposed control activity levels following addition of supplemental microsomal phospholipids to phospholipase C-exposed preparations (compare horizontal line D values with those in lines A or B).

In contrast, the quantitative aspects of the latter responses are quite different when based on activity data obtained with deoxycholate-treated preparations (vertical columns 2 and 5, Table IV). Here, again, supplemental phospholipid exerts only small effects with unexposed microsomal preparations (see horizontal lines A and B), while nearly doubling Glc-6-*P* phosphohydrolase activity and increasing 5-fold phosphotransferase activity of phospholipase C-treated microsomes (compare data in horizontal line D with those in line C). However, most significantly, the absolute activity values obtained here with phospholipid-supplemented, phospholipase C-treated preparations (line D), still are only a fraction of those values obtained with original, unexposed microsomal preparations (line A) (*i.e.* 3.6 *versus* 13 original units for Glc-6-*P* phosphohydrolase; 2.6 *versus* 14.0 original units for phosphotransferase).

Additional insight relating to the effects of phospholipase C treatment and phospholipid supplementation also is provided by the data on the activating effects of deoxycholate on levels of enzymic activities under the various conditions (see vertical columns 3 and 6; Table IV). As previously noted with homogenates or microsomal preparations[13,18,27–30], the addition of deoxycholate, to 0.2%, w/v, activated both Glc-6-*P* phosphohydrolase and PP_i–glucose phosphotransferase activities of unexposed microsomes whether supplemented with phospholipid or not (see horizontal lines A and B). The detergent activated the phosphotransferase activity (column 6) to a considerably greater extent than the phosphohydrolase activity (column 3). The extent of activation by deoxycholate was, however, reduced after phospholipase C treatment of microsomes. Under these conditions the activations by detergent were approximately the same for both hydrolytic and synthetic activities (see horizontal line C). And when phospholipid was supplemented to such phospholipase C-exposed microsomes (horizontal line D, Table IV), only a very small amount of activation by detergent (16–18%), was observed.

These observations, along with the pH–activity profiles in Fig. 2, suggest to the authors that a combination of phospholipase C treatment *plus* phospholipid supplementation serves to convert existing molecules of glucose-6-phosphatase–phosphotransferase to the essentially fully activated form, and that therefore the superimposition of deoxycholate on such preparations has little further activating effect on remaining enzymic activity. This is in marked contrast to the effect of detergent-treatment on enzymic activities of glucose-6-phosphatase of unexposed microsomes.

Effects of products of phospholipase C action on enzymic activities

Soluble products. Various components of the phospholipase C incubation medium, as well as soluble products of phospholipase C action on microsomes[3]—phosphorylcholine, some phosphorylethanolamine, *etc.*—routinely were removed through centrifugation (see MATERIALS AND METHODS) prior to further supplemen-

Biochim. Biophys. Acta, 258 (1972) 188–205

TABLE V

EFFECTS OF ADDED DIGLYCERIDE ON HYDROLYTIC AND SYNTHETIC ACTIVITIES OF GLUCOSE-6-PHOSPHATASE IN THE ABSENCE AND PRESENCE OF DEOXYCHOLATE

Microsomal preparations, either exposed to phospholipase C for 5 min or unexposed, were supplemented with the indicated amounts of diglyceride and deoxycholate to 0.2%, w/v, (or distilled water) as indicated, and assayed for enzymic activity. Phospholipid content of unexposed and phospholipase C-exposed microsomes was 14.5 and 10.4 μg lipid P per mg microsomal protein, respectively. Specific details are presented in the text.

Diglyceride added (mg/mg microsomal protein)	*Enzymic activity**							
	Glc-6-P phosphohydrolase				*PP_i–glucose phosphotransferase*			
	Unexposed		*Phospholipase C exposed*		*Unexposed*		*Phospholipase C exposed*	
	Deoxycholate absent	*Deoxycholate present*	*Deoxycholate absent*	*Deoxycholate present*	*Deoxycholate absent*	*Deoxycholate present*	*Deoxycholate absent*	*Deoxycholate present*
0	4.16 (100%)	6.02 (100%)	2.86 (100%)	3.54 (100%)	1.14 (100%)	5.16 (100%)	0.77 (100%)	1.95 (100%)
0.11	4.02 (97%)	6.16 (102%)	2.86 (100%)	3.65 (103%)	1.02 (90%)	5.40 (105%)	0.77 (100%)	2.00 (102%)
0.21	4.26 (102%)	6.42 (107%)	2.84 (99%)	3.74 (106%)	1.02 (90%)	5.42 (105%)	0.77 (100%)	2.00 (102%)
0.42	4.07 (98%)	6.51 (108%)	2.86 (100%)	3.72 (105%)	1.09 (96%)	5.14 (100%)	0.72 (94%)	1.91 (98%)

* Enzymic activity is as defined in Table II. Values in parentheses indicate 100 × activity with indicated concentration of added diglyceride/activity without added diglyceride. Activity without diglyceride is taken as 100% in each situation.

tation and treatment of microsomes and assay for enzymic activities. DUTTERA *et al.*[3] previously have demonstrated that these materials were without significant effect on Glc-6-*P* phosphohydrolase activity. In experiments supplementary to the present study, no differences in levels of either Glc-6-*P* phosphohydrolase or PP_i–glucose phosphotransferase were noted, whether or not such soluble materials were removed by centrifugation following phospholipase C exposure of microsomes for 10 min as described in MATERIALS AND METHODS.

Diglyceride. In contrast with phosphorylcholine, which is watersoluble and hence may be easily washed out of phospholipase C-exposed microsomes as indicated above, diglyceride, the other product of phospholipase C action, remains with the membrane as droplets visible in the electron microscope[16]. The possibility thus existed that diglyceride in this form may not be very inhibitory since most of the enzyme may not be in contact with the diglyceride droplets, but that the detergent deoxycholate might bring the diglyceride into intimate contact with the enzyme and thus potentiate its inhibition of activities of glucose-6-phosphatase. If this were so, such effects could explain, in part, differences noted in the presence in contrast with absence of deoxycholate in the experiments described in Table IV, for phospholipase C-exposed microsomes would contain diglyceride while unexposed microsomal preparations would not. The following experiments accordingly were performed to explore the feasibility of such a possible rationalization of the differences in extent of recovery of enzymic activities noted in the presence in contrast with absence of deoxycholate, as described in Table IV.

Microsomal preparations, either exposed at 21° to the action of phospholipase C for 5 min as described in detail in the MATERIALS AND METHODS, or unexposed, were employed as enzyme source. Soluble products of phospholipase C action and other components of the incubation medium were removed by centrifugation as described above. Such microsomal preparations were then dispersed in 0.25 M sucrose and diluted to a concentration of 5 mg microsomal protein per ml. Various amounts of diglyceride (1,2-dipalmitin) which had been dispersed by sonication (5 min exposure in the cold, under N_2, in the Bronwill Biosonic II probe sonicator operating at 75% of maximal intensity) in a solution consisting of 9 parts of 12.5 mM HEPES buffer, pH 7, *plus* 1 part 95% ethanol were then added (see Table V) and the preparations allowed to stand 5 min at 21°. At this time deoxycholate solution, to a final concentration of 0.2%, w/v, (or an equal volume of distilled water) was added. The samples were maintained at 0° for 30 min and then assayed for enzymic activity.

The amounts of diglyceride added, 0, 0.11, 0.21, or 0.42 mg dipalmitin per mg microsomal protein, were calculated by analogy with the method employed by DUTTERA *et al.*[3] in similar studies. As indicated in Table I, incubation of microsomal preparations for 5 or 15 min under the conditions used here led, respectively, to a loss of 4.0 and 11.5 μg lipid P per mg microsomal protein. These values correspond, respectively, to 0.13 and 0.37 μmole diglyceride per mg microsomal protein. Assuming a 1:1 stoichiometric relationship between lipid P lost and diglyceride generated, and a molecular weight of 569 for 1,2-dipalmitin, it may be calculated that 0.075 and 0.21 mg 1,2-dipalmitin, respectively, correspond with those amounts of diglyceride formed per mg microsomal protein by phospholipase C action during 5 and 15 min of exposure to microsomes. Hence, it follows that those amounts of supplemental diglycerides added, as indicated in Table V, correspond with approx. 0×,

1.4×, 2.9×, and 5.8× that amount of diglyceride generated during a 5 min exposure of microsomes to phospholipase C, and to 0×, 0.5×, 1×, and 2× that amount of diglyceride produced per 15 min exposure of microsomes to phospholipase C.

As can be seen from the data in Table V, supplemental diglyceride exerted little or no effect on either synthetic or hydrolytic activity of glucose-6-phosphatase, either with unexposed or phospholipase C-treated microsomes. In no case, whether or not deoxycholate supplementation of microsomal preparations was involved, was a change in activity greater than 10% noted even with a relatively large excess of supplemental diglyceride. Potentiation by deoxycholate of inhibition of enzymic activities by diglyceride produced through phospholipase C action thus appears precluded as a possible basis for explaining the lesser apparent stimulation by the steroid detergent of enzymic activities of phospholipase C-exposed preparations as compared with unexposed microsomes, as described in Table IV.

Further, the results of the experiments described in Table V also contraindicate the possibility that deoxycholate may potentiate the inhibition of activities of glucose-6-phosphatase by small amounts of free fatty acid which may be formed from diglyceride through the action of acyl hydrolase endogenous to microsomes[33]. Inherent in the experimental design described above was the exposure of supplemental diglyceride to microsomal preparations for 5 min at 21° under the same conditions employed in the initial incubation of microsomes with phospholipase C (see above detailed procedural description). Any free fatty acid generated in 5 min through the action of microsomal acyl hydrolase on the excess supplemented diglyceride thus also was present, along with diglyceride, when supplementary deoxycholate was added and when enzymic activities were assayed.

DISCUSSION

The studies described in this paper on the effects of phospholipase C exposure and phospholipid supplementation of glucose-6-phosphatase differ conceptually from earlier investigations of DUTTERA *et al.*[3] and of ZAKIM[5] in that (a) they involve hepatic microsomes prepared from fed rats (rather than from beef[5] or from fasted rats[3], (b) phosphotransferase activity of the enzyme receives equal emphasis with Glc-6-*P* phosphohydrolase; (c) hydrolase and transferase activities involving mannose-6-*P* are included; (d) supplemental phospholipid from animal (rat liver microsomal extract) rather than plant (asolectin, from soybeans) sources was employed exclusively; (e) considerable emphasis is placed on responses of enzymic activities over a broad range of pH rather than at a single pH value; and (f) the modifying effects of detergent (deoxycholate) supplementation on patterns of response of enzymic activities to phospholipid modifications are considered.

Results of these studies represent an advancement beyond the findings of other investigators in that they (a) support the common identity of PP_i–glucose phosphotransferase and Glc-6-*P* phosphohydrolase; (b) provide novel, new insights regarding the nature of the effects of phospholipid modifications on the properties of glucose-6-phosphatase–phosphotransferase which indicate that the enzyme obtained following phospholipase C exposure and phospholipid supplementation is catalytically significantly different from the microsomal enzyme in its original form; and (c) raise serious questions (as have certain other recent studies of somewhat different

design[5,33,34]) regarding the obligatory requirement for phospholipids in glucose-6-phosphatase–phosphotransferase action. A detailed consideration of these points is made in the succeeding paragraphs where the ramifications of certain particularly relevant observations are elaborated upon.

In view of the various observations and considerations reported in this paper, and especially on the basis of observations described in Fig. 2 and in Table IV (as well as in Tables III and V), it is apparent that the interpretation in a mechanistic fashion of the results of the studies on the effects of phospholipid modifications on enzymic activities of this catalyst is extremely complex.

The various data presented above do indicate, as do those of earlier workers[3,5], that (a) exposure of microsomal preparations to phospholipase C does lead to a marked reduction in demonstrable enzymic activity, both hydrolytic and synthetic; and (b) supplementation of such phospholipase C-treated preparations with various micellular phospholipid preparations does effect an increase in activity. With respect to this latter point, it may be seen from the values in braces in horizontal line D of data in Table IV that the addition of microsomal phospholipid extracts to phospholipase C-treated preparations effects an increase in levels of both activities, whether or not deoxycholate is present. Increases in Glc-6-*P* phosphohydrolase activity from 1.3 to 3.1 units (an increase of 138%) and from 2.0 to 3.6 units (an increase of 80%) was noted in the absence and presence, respectively, of deoxycholate, while corresponding values for PP_i–glucose phosphotransferase were from 0.36 to 2.2 units (an increase of 511%) and from 0.60 to 2.6 units (a 333% increase).

However, it is also clear from a consideration of experimental observations, especially those described in Fig. 2 and Table IV (as well as in Table III) that (a) the activity of phospholipase C-treated preparations is catalytically different from that of unexposed microsomes, whether or not supplemental phospholipid has been added (see Fig. 2); and (b) that the extent of return towards activity values originally seen with unexposed microsomal preparations which is effected by phospholipid supplementation after phospholipase C exposure appears to be much less than that previously concluded to be the case on the basis of studies[3,5] in which activities were assayed without detergent. This latter conclusion is based on a comparison of activity levels with the various preparations observed with deoxycholate added (see columns 2 and 5, Table IV).

A possible rationalization of these differences noted in the presence and absence of deoxycholate on the basis of potentiation by the detergent of inhibition of glucose-6-phosphatase activities by products of phospholipase C action has been obviated experimentally, as considered in detail in the RESULTS.

It thus appears that phospholipase-treated, phospholipid—supplemented preparations may already be nearly maximally activated, while this is not true with unexposed preparations, either *plus* or *minus* supplemental phospholipid. As a consequence, the additional latent activity of the unexposed microsomes, but not that of the phospholipid-supplemented exposed preparations, may be brought out by deoxycholate.

While other interpretations of the data presented here are possible, it appears to the authors that the observations described may be explained most realistically as follows: Phospholipase C treatment effects a highly significant loss in measurable enzymic activity through an undefined action that does not involve inhibition by its

hydrolysis products (see Table V and accompanyign discussion). At the same time such phospholipase C action sensitizes the remaining enzyme molecules to activation by supplemental phospholipid. The addition of phospholipid to such preparations then activates the remaining potentially functional enzyme molecules in a detergent-like manner (see especially Fig. 2), to restore manifest activity values partially (but by no means completely) towards those originally observed.

On the basis of these observations, it appears that the whole question of the role of phospholipids in glucose-6-phosphatase–phosphotransferase is in need of a searching re-examination. This is particularly emphasized by the recent interesting observations of POLLAK *et al.*[34],* who have noted a marked (2–4-fold) increase in synthetic and hydrolytic activities of microsomal glucose-6-phosphatase concomitant with the depletion of microsomal lipid and an inhibition of these enzymic activities when phospholipid is added to such depleted preparations**.

ACKNOWLEDGEMENTS

This work was supported in part by grants from the Hill Family Foundation, the American Diabetes Association, Research Grant AM 07141 from the National Institutes of Health, U.S. Public Health Service, and Research Grant GB-14752 from the National Science Foundation. R.E.S. was supported in part by National Science Foundation Traineeship G2-231-1. The authors are indebted to Mr. R. Tello for excellent technical assistance, to Dr. J. E. Miller for valuable discussions relating to phospholipid analyses, and to Dr. J. K. Pollak for generously making available his interesting and highly relevant manuscript[34] prior to publication.

REFERENCES

1 R. E. SNOKE AND R. C. NORDLIE, *Abstr. 156th Natl. Meeting Am. Chem. Soc., Atlantic City, N.J., 1968*, Abstr. BIOL. 102.
2 H. BEAUFAY AND C. DE DUVE, *Bull. Soc. Chim. Biol.*, 36 (1954) 1551.
3 S. M. DUTTERA, W. L. BYRNE AND M. C. GANOZA, *J. Biol. Chem.*, 243 (1968) 2216.
4 G. FEUER AND L. GOLBERG, *Food Cosmet. Toxicol.*, 5 (1967) 673.
5 D. ZAKIM, *J. Biol. Chem.*, 245 (1970) 4953.
6 B. R. CATER, J. POULTER AND T. HALLINAN, *FEBS Lett.*, 10 (1970) 364.
7 M. C. GANOZA, Doctoral dissertation, Duke University, 1964, University Microfilms, Inc., Ann Arbor, Mich.
8 R. C. NORDLIE AND W. J. ARION, *J. Biol. Chem.*, 239 (1964) 1680.
9 M. R. STETTEN, *J. Biol. Chem.*, 239 (1964) 3576.
10 R. C. NORDLIE, *Ann. N.Y. Acad. Sci.*, 166 (1969) 699.
11 R. C. NORDLIE, in W. J. WHELAN, *Control of Glycogen Metabolism*, Oslo University Press, Oslo, 1968, p. 153.
12 R. C. NORDLIE, in P. D. BOYER, *The Enzymes*, Vol. 4, Academic Press, New York, 3rd ed., 1971, p. 543.
13 R. C. NORDLIE AND W. J. ARION, *J. Biol. Chem.*, 240 (1965) 2155.

* These observations recently have been confirmed in this laboratory (W. J. COLILLA AND R. C. NORDLIE, unpublished observations).

** On a somewhat different experimental basis, ZAKIM[5] has concluded that ". . . rather than being essential for activity, the natural phospholipid environment acts to constrain maximum potential enzyme activity". The present authors hold a somewhat similar view, recently having hypothesized[12] that ". . . conformational features, quite probably relating at least in part to the lipid–protein nature of the enzyme, may be intimately related to the selective control of synthetic and hydrolytic capabilities of the multifunctional enzyme". Very recently CATER AND HALLINAN[33] also have questioned the "necessity to posit phospholipid dependence for glucose-6-phosphatase".

14 R. C. NORDLIE AND W. J. ARION, *Methods Enzymol.*, 9 (1966) 619.
15 M. G. MACFARLANE AND B. C. J. G. KNIGHT, *Biochem. J.*, 35 (1941) 884.
16 A. MARTONOSI, J. DONLEY AND R. A. HALPIN, *J. Biol. Chem.*, 243 (1968) 61.
17 Y. P. LEE AND T. TAKAHASHI, *Anal. Biochem.*, 14 (1966) 71.
18 R. C. NORDLIE, W. J. ARION, T. L. HANSON, J. R. GILSDORF AND R. N. HORNE, *J. Biol. Chem.*, 243 (1968) 1140.
19 C. DE DUVE AND J. BERTHET, *Int. Rev. Cytol.*, 3 (1954) 225.
20 J. FOLCH, M. LEES AND G. H. SLOANE STANLEY, *J. Biol. Chem.*, 226 (1957) 497.
21 B. BORGSTRÖM, *Acta Physiol. Scand.*, 25 (1952) 101.
22 P. BEAUFAY AND C. DE DUVE, *Bull. Soc. Chim. Biol.*, 36 (1954) 1525.
23 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
24 R. PARKER AND N. F. PETERSON, *J. Lipid Res.*, 6 (1965) 455.
25 J. E. MILLER AND W. E. CORNATZER, *Lipids*, 4 (1969) 19.
26 J. DE GIER, G. H. DE HAAS AND L. L. M. VAN DEENEN, *Biochem. J.*, 81 (1961) 33P.
27 W. J. ARION AND R. C. NORDLIE, *J. Biol. Chem.*, 239 (1964) 2752.
28 R. E. SNOKE AND R. C. NORDLIE, *Biochim. Biophys. Acta*, 139 (1967) 190.
29 R. C. NORDLIE AND R. E. SNOKE, *Biochim. Biophys. Acta*, 148 (1967) 222.
30 R. C. NORDLIE, W. J. ARION AND E. A. GLENDE, JR., *J. Biol. Chem.*, 240 (1965) 3479.
31 M. R. STETTEN, S. MALAMED AND M. FEDERMAN, *Biochim. Biophys. Acta*, 193 (1969) 260.
32 R. C. NORDLIE, J. R. GILSDORF, R. N. HORNE AND R. J. PAUR, *Biochim. Biophys Acta*, 158 (1968) 157.
33 B. R. CATER AND T. HALLINAN, *FEBS Lett.*, 16 (1971) 137.
34 J. K. POLLAK, R. MALOR, M. MORTON AND K. A. WARD, in *Autonomy and Biogenesis of Mitochondria and Chloroplasts*, North-Holland Publ. Co., Amsterdam, in the press.