Regulation of Microsomal Enzymes by Phospholipids

I. THE EFFECT OF PHOSPHOLIPASES AND PHOSPHOLIPIDS ON GLUCOSE 6-PHOSPHATASE*

(Received for publication, May 15, 1970)

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SUMMARY

Incubation of bovine liver microsomes with partially purified phospholipase A from Naja naja venom inactivates the phosphohydrolase activities of glucose 6-phosphatase, but there is no quantitative correlation between hydrolysis of phospholipids and loss of enzyme activity. Furthermore, addition of EDTA to an incubating mixture of microsomes and phospholipase A completely stops hydrolysis of phospholipids, but does not halt the decline of glucose 6-phosphatase activity. These results indicate that hydrolysis of phospholipids by phospholipase A does not per se inactivate glucose 6-phosphatase. Instead, hydrolysis of phospholipids by phospholipase A produces an unstable form of the enzyme. The phospholipase A-treated form of glucose 6-phosphatase can be stabilized by asolectin and albumin, and at pH 5.75 by glucose-6-P or PPi.

In contrast to its effects on phosphohydrolase activities, conditions of phospholipase A treatment which lead to a 50 to 60% loss of these activities activate slightly the phosphotransferase activity. It was found, however, that the phospholipase A treatment led to a pH-dependent instability of phosphotransferase which could be separated from the instability of phosphohydrolase activities.

Phospholipids, in the form of asolectin, inhibit the phosphohydrolase activities of untreated microsomes whereas they activate after phospholipase A treatment. Albumin, which has no effect on the untreated enzyme, is a more effective activating agent of phospholipase A-treated glucose 6-phosphatase than asolectin. Asolectin activates, but albumin inhibits, the phosphotransferase activity of phospholipase A-treated microsomes. Since either albumin or asolectin can increase the phosphohydrolase activity of phospholipase A-treated microsomes to levels greater than those of untreated ones, their activating action cannot result from reversal of phospholipase A effects.

Phospholipase C treatment inactivates both the phosphohydrolase and phosphotransferase activities of glucose 6-phosphatase. This action does not seem to result from phospholipase C-induced instability of the enzyme. However, inactivation cannot be interpreted as indicating a phospholipid requirement for the enzyme since albumin, in addition to asolectin, activates phospholipase C-treated glucose 6-phosphatase, and the combined action of phospholipase C plus asolectin increases phosphotransferase activity to 50% greater than that of untreated microsomes.

It has been postulated that the microsomal enzymes glucose 6-phosphatase (EC 3.1.3.9) (1, 2), UDP-glucuronyl transferase (EC 2.4.1.17) (3), acyl-CoA-l-glycerol-3-P acyltransferase (4), and ATPase (5) require phospholipids for normal catalytic activity. The evidence for this requirement is that treatment of microsomes with phospholipases A and C leads to a decline in the activities of these enzymes (1-5), which is reported, are restored by phospholipids. These results, plus observations that phospholipids restore activity to aqueous acetone-extracted preparations of microsomal stearyl-CoA desaturase (6) and DPNH-cytochrome c reductase (7), suggest that phospholipids may be of general importance for the activity of microsomal enzymes. On the other hand, although the data cited are compatible with the conclusion that phospholipids affect the catalytic activities of some microsomal enzymes, it is not possible to state that there is an absolute requirement for phospholipids, as has been implied (1-5). That phospholipase-induced inactivations may have alternate interpretations is indicated, in fact, by data already available in the literature. For example, detergents, as well as phospholipids, restore to normal the activity of phospholipase C-treated ATPase (3), which suggests that the reactivation by phospholipids is nonspecific. Further, albumin prevents inactivation of glucose 6-phosphatase by phospholipase A without affecting the hydrolysis of phospholipids (2), and, although incubation of microsomes with phospholipase A at 20°C inactivates glucuronol transferase (3), digestion at 4°C does not (8). It may be that rather than inactivating through direct action hydrolysis of phospholipids by phospholipase A leads to unstable forms of some microsomal enzymes. With regard to this point, phospholipase A treatment in fact stabilizes, but does not inactivate directly, acyl-CoA-l-acyl glycerophosphorylcholine acyltransferase of rat liver microsomes (9). Other data which are not easily

* This investigation was supported in part by grants from the United States Public Health Service (HE 10027) and the National Science Foundation (GB 8248) to Dr. Thomas P. Singer, and from the Springer Fund of the University of California School of Medicine.
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reconciled with the conclusions drawn from studies with phos-
pholipases (2) are that detergent induced dissociation of micro-
somal lipids and proteins activates glucose 6-phosphatase (10–12).

Thus, although the postulated phospholipid requirement of mem-
brane-bound microsomal enzymes is teleologically satisfying,
several lines of evidence indicate that phospholipids could be im-
portant not only for maintaining the proper conformation of the
active sites of microsomal enzymes but also for their stability.
The exact effects of the phospholipid environment on catalytic
activity may vary in different enzymes, and each should be con-
sidered separately.

An understanding of the relationship between catalytic activity
and the phospholipid environment of each microsomal enzyme is
essential for elucidating mechanisms of action and physiological
regulation, the significance of membrane location, and the ways
in which activity can be altered pharmacologically. The meth-
ods for purifying microsomal enzymes also will depend ultimately
on the interrelations of phospholipids and activity. As a first
step in resolving some of these problems we have been examining
the effects of phospholipases on glucose 6-phosphatase activity.

Since this seems to be a multifunctional enzyme (10–14), in which
the activities are regulated in vivo in a selective, and sometimes
independent, manner (15–19), we have been interested not only in the
question of phospholipase inactivation, but also the possi-
abilities that phospholipases might have selective effects on the
activities of this enzyme and that phospholipids could be physi-
ologically important regulators of its activities.

EXPERIMENTAL PROCEDURE

Materials

Glucose-6-P (dissodium salt), NADP⁺, glucose-6-P dehydro-
genase (EC 1.1.1.49), bovine serum albumin (Cohn Fraction
IV), and phospholipase C from Clostridium welchii were pur-
chased from Sigma. Crude Naja naja venom was obtained from
the Ross Allen Reptile Institute.

Methods

Preparation of Microsomes—Fresh bovine liver was homoge-
nized in 5 volumes of 0.25 M sucrose for 30 sec in a Waring
Blender, and the homogenates were centrifuged at 10,000 × g for
10 min. The supernatant suspension was centrifuged at 50,000
rpm for 40 min in a Spinco 50.1 rotor, and the microsomal pellet
was resuspended in half of the original volume of 0.25 M sucrose.
The microsomes were washed twice, the final pellet was sus-
pended in 0.25 M sucrose at a protein concentration of 28 to 30
mg per ml, and stored at −20°.

Phospholipase Incubations—Phospholipase A from N. naja was
purified through the first Sephadex G-75 step by the method of
Cremona and Kearney (20). All incubations of microsomes with
phospholipase A were carried out at 23° in the presence of 80 mM
Tris, pH 8.00. The amounts of phospholipase A added per mg of
microsomal protein and the duration of incubation were differ-
ent in different experiments, and these conditions are noted in the
figures and text.

Crude phospholipase C from C. welchii was used without further
purification. Incubations with phospholipase C were carried
out at 23° in 80 mM Tris, pH 8.00, and 2 mM Ca²⁺. The amounts
of phospholipase C used and the duration of incubations are
noted in the figures and text.

Enzyme Assays—Unless otherwise noted, the following condi-
tions were used for all assays. For glucose 6-phosphatase ap-
proximately 1 mg of microsomal protein was incubated in 100
mM sodium acetate (pH 5.75) and 80 mM glucose-6-P for 5 min at
38°. The reaction was stopped by the addition of 10% (v/v)
trichloroacetic acid to a final concentration of 3.3%, and P₁
was assayed by the method of Sumner (21). For pyrophosphatase
approximately 1 mg of microsomal protein was incubated in 100
mM sodium acetate (pH 5.75) and 80 mM PP₁ for 5 min at 38°.
The reaction was stopped by the addition of 10% trichloroacetic
acid as above, and P₁ was measured by a modification of the
Lowry-Lopez method (22). For PP₁-glucose phosphotransfer-
ase approximately 1 mg of microsomal protein was incubated in 100
mM sodium acetate (pH 5.50), 400 mM glucose, and 80 mM
PP₁ for 5 min at 38°. The reaction was stopped by boiling the
 tubes for 3 min. The pH of each tube was adjusted to 7.5 and
after centrifugation, glucose-6-P was measured enzymatically in
the absence of Mg²⁺ (23). With the glucose-6-P dehydrogenase
used in this assay, 1 mole of NADPH was produced per mole of
glucose-6-P. The amounts of albumin and asolectin added to all
assay tubes are indicated in the tables and figures. All activities
are given as micromoles of substrate metabolized per min per mg
of microsomal protein.

Although most workers add EDTA to the media used for en-
zyme assay after phospholipase treatment, this was found not to
be necessary. At the pH values used for enzyme assays, there
was no residual phospholipase activity, and assays were linear
with time.

Preparation of Asolectin Micelles—Asolectin was dissolved in
CHCl₃-CH₃OH (2:1, v/v) and washed with 0.2 volume of H₂O.
The lower phase was removed, evaporated to dryness under N₂,
and then suspended in H₂O and sonically disrupted. The soni-
cally disrupted material was centrifuged at 40,000 rpm for 10 min
in a Spinco 40 rotor, and the supernatant solution was stored at
0°. An aliquot was digested in concentrated H₂SO₄ and ana-
lyzed for total phosphorus.

Proteins were determined by the biuret method (24) and fatty
acids by the method of Dole and Meinertz (25).

RESULTS

This work is part of studies initiated to establish methods for
the solubilization and purification of glucose 6-phosphatase.

Although most previous experiments have been limited to a study
of rat liver glucose 6-phosphatase, this tissue is not a suitable
source of enzyme for purification work. For this reason, we have
worked primarily with bovine liver which has the advantages of
supplying a large amount of microsomes and a higher specific
activity of glucose 6-phosphatase than rat liver. Preliminary
studies on rat liver microsomes indicate, however, that there
are no species differences between rat and bovine liver with re-
gard to the effects of phospholipids, albumin, and phospholipases
on the kinetic parameters of glucose 6-phosphatase.

Phosphohydrolase and Phosphotransferase Activities and Effects
of Albumin and Asolectin after Phospholipase A Treatment of Glu-
cose 6-Phosphatase—Glucose 6-phosphatase catalyzes the hy-
drosis of PP₁ (10–14) and phosphoramidate (26) in addition to
glucose-6-P, and can phosphorylate glucose with a variety of P₁
donors (10–14, 26). However, the effects of phospholipases on
all possible enzyme activities were not studied. Instead, only
the hydrolysis of glucose-6-P and PP₁, and the PP₁-glucose-phos-
photransferase reaction was investigated since the multiple ac-
tivities of glucose 6-phosphatase can be divided into two basic
types: a phosphohydrolase for which H₂O is the P₁ acceptor and a phosphotransferase for which glucose or another sugar (27) acts as P₁ acceptor.

The data in Fig. 1 indicate that, as with the rat liver enzyme (2) phospholipase A treatment leads to a decline in glucose 6-phosphatase activity. Pyrophosphatase activity also decreased to a similar extent. Further, the data show that, although albumin had no effect on the phosphohydrolase activities of untreated microsomes, its addition to the assay media increased the activities of the phospholipase A-treated enzyme to 60% greater than that assayed in the absence of albumin. The ratios of phosphohydrolase activities assayed in the presence and absence of albumin were similar after 5, 10, and 20 min of phospholipase A incubation, despite the variable loss of activity which was observed at these different times. In contrast to the effects of albumin, asolectin inhibited slightly the phosphohydrolase activities of untreated microsomes and had a considerably smaller enhancing effect, as compared to albumin, on the activities of the phospholipase A-treated form of glucose 6-phosphatase.

That phospholipase A treatment did not have similar effects on all glucose 6-phosphatase activities is evident from comparing the phosphohydrolase and phosphotransferase activities of phospholipase A-treated microsomes. For example, the kinetics of the phosphohydrolase assays was linear after phospholipase A treatment, but the phosphotransferase activity declined progressively (Fig. 2A). Also, although the phosphotransferase and phosphohydrolase activities of untreated microsomes were similar, the phosphotransferase activity was greater after phospholipase A treatment. Phospholipase A seemed to activate phosphotransferase (Fig. 2B). This effect was due in part to different pH optima for the untreated and phospholipase A-treated forms of the enzyme. Because of the nonlinear kinetics, however, it was not possible to determine quantitatively these differences between phosphohydrolase and phosphotransferase activities. Further, albumin and asolectin partially stabilized the phosphotransferase activity of the phospholipase A-treated microsomes (Fig. 2B), but it was not possible to distinguish activation from stabilization, nor to compare the effects of albumin and asolectin on phosphotransferase and phosphohydrolase activities. For these reasons, assay conditions were sought at which phospholipase A-treated PP₁-glucose phosphotransferase was stable. It was found that at 14° and pH 5.00, which was the apparent pH optimum for phospholipase A-treated and untreated enzyme, the

![Fig. 1. Effect of phospholipase A treatment on microsomal glucose 6-phosphatase (A) and pyrophosphatase (B) activities. Microsomes were incubated with 0.02 mg of partially purified phospholipase A per mg of microsomal protein under conditions described under "Methods." At the indicated times 1 mg of microsomal protein was removed for enzyme assays. Assay tubes contained 10 mg of albumin (□), 4 mg of asolectin micelles (△), or no additions (○).

![Fig. 2. A, effect of phospholipase A on kinetics of PP₁-glucose phosphotransferase reaction. Microsomes were incubated for 5 min with phospholipase A, as in Fig. 1. Phosphotransferase (□), pyrophosphatase (○), and glucose 6-phosphatase (●) were assayed on 1-mg aliquots of microsomal protein at pH 5.50, 38°. B, effects of albumin and asolectin on kinetics of phospholipase A-treated PP₁-glucose phosphotransferase reaction. Microsomes were incubated with phospholipase A and assayed as in A except that 10 mg of albumin (□) or 4 mg of asolectin (△) were added to the assay tubes. Activity of untreated microsomes is indicated by closed circles and phospholipase A-treated activity in the absence of albumin or asolectin addition by open circles.](http://www.jbc.org/content/4955)

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kinetics was linear (Fig. 3). When assayed with these conditions it was observed that conditions of phospholipase A incubation which consistently inactivated phosphohydrolase activity by 50% increased phosphotransferase activity to 15 to 20% greater than that of the untreated enzyme (Fig. 3; Table I). It also became apparent that, in contrast to their effects on the phosphohydrolase activities of phospholipase A-treated microsomes, asolectin, but not albumin, increased phosphotransferase activity (Fig. 3).

In view of the differential effect of phospholipase A on phosphohydrolase and phosphotransferase activities we tried to inactivate completely the former activity without altering the latter. However, after phosphohydrolase activity decreased to 40% of control, the phosphotransferase also began to decline. Both activities eventually disappeared completely.

Effect of Phospholipase A on Stablility of Glucose 6-Phosphatase— The design of the experiments used in the above section to test the effect of asolectin on the activities of phospholipase A-treated glucose 6-phosphatase was different from those used by other workers (2). In our studies aliquots of the microsomal phospholipase A incubation mixture were added directly to assay tubes which contained asolectin; the change in pH from 8.00 in the phospholipase incubation to 5.75 in the enzyme assay inhibited further phospholipase A action. In contrast, Duttera, Byrne, and Ganoza (2) stopped phospholipase A activity by adding EDTA and then previously incubated aliquots of the treated microsomes at 20°, pH 8.00, with and without phospholipids before assay. Under these conditions for asolectin treatment of phospholipase A-digested microsomes, Duttera et al. achieved 4-fold reactivations of glucose 6-phosphatase activity, a result significantly different from ours (Fig. 1). Since this discrepancy between our work and theirs is not only large quantitatively, but also could have an important bearing on the interpretation of the role of phospholipids in glucose 6-phosphatase activity, an experiment was done according to the conditions of Duttera et al., but with bovine instead of rat liver microsomes.

As the data in Fig. 4 indicate, phospholipase A-induced inactivation of glucose 6-phosphatase continued in the presence of 10 mM EDTA. On the other hand, addition of EDTA plus asolectin prevented the further decline of glucose 6-phosphatase activity. EDTA plus albumin had a similar effect. As a result, if it were assumed that EDTA prevented further inactivation of glucose 6-phosphatase by phospholipase A, comparison of the activities of phospholipase A-treated microsomes previously incubated with EDTA or EDTA plus asolectin would lead to the conclusion that asolectin increases significantly the activity of phospholipase A-treated enzyme. The data show, however, that the primary effect of asolectin is to stabilize rather than activate phospholipase A-treated glucose 6-phosphatase. The results suggest further that, if EDTA inhibits phospholipase A activity, then phospholipase A per se does not inactivate glucose 6-phosphatase but converts the native enzyme to an unstable form. In order to investigate this possibility, the effect of EDTA on hydrolysis of phospholipids by phospholipase A and the relation between the amount of hydrolysis and loss of enzyme activity were measured. As is evident from Fig. 5, there was no correlation between fatty

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**FIG. 3.** Kinetics of phospholipase A-treated PP₁-glucose phosphotransferase reaction at 14°. Microsomes were incubated with phospholipase A as in Fig. 2A. Portions, 2 mg, of microsomal protein were assayed for phosphotransferase activity at 14°, pH 5.00. Untreated, •; phospholipase A treated, ○; phospholipase A-treated plus 10 mg of albumin per assay tube, □; phospholipase A-treated plus 4 mg of asolectin per assay tube, △.

**Graph 3.** Comparison of effect of phospholipase A on activities of pyrophosphatase and PP₁-glucose phosphotransferase.

**Table I**

| Treatment               | Specific activity | Specific activity |
|-------------------------|------------------|------------------|
|                         | Pyrophosphatase  | PP₁-glucose      |
|                         |                  | phosphotransferase |

|          | Pyrophosphatase | PP₁-glucose phosphotransferase |
|----------|----------------|-------------------------------|
| None     | 0.121          | 0.087                         |
| Phospholipase A | 0.053          | 0.101                         |

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**FIG. 4.** Effect of EDTA (●) and EDTA plus albumin (□) or EDTA plus asolectin (△) on the glucose 6-phosphatase activity of phospholipase A-treated microsomes. Three tubes containing 30 mg of microsomal protein were incubated with 0.38 mg of phospholipase A. At the arrow, either EDTA (final concentration 10 mM in all tubes), EDTA plus 100 mg of albumin (pH 8.00), or EDTA plus 40 mg of asolectin was added to each tube. Mixtures were kept at 25°; 1-mg aliquots of microsomal protein were removed for assay of glucose 6-phosphatase at the indicated times.

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acid production and loss of glucose 6-phosphatase activity during incubation of microsomes with phospholipase A. Further, addition of EDTA to the phospholipase A incubation inhibited completely the hydrolysis of phospholipids, but again glucose 6-phosphatase activity continued to decline. Although the rate of glucose 6-phosphatase inactivation was diminished by EDTA, no such effect would have been seen if EDTA were added after 2 min of incubation of phospholipase A with microsomes. On the other hand, addition of EDTA prior to the addition of phospholipase A prevents both hydrolysis of phospholipids and inactivation of glucose 6-phosphatase. Thus, the results cannot be interpreted as resulting from contamination of phospholipase A with a proteolytic enzyme. These data indicate, therefore, that the major effect of phospholipase A on glucose 6-phosphatase is to produce an unstable enzyme.

Since phospholipase A-treated microsomes give linear rates of hydrolysis of glucose-6-P and PPI at pH 5.75, 35°, in the presence of 50 mM substrate, the phospholipase A treated form of glucose 6-phosphatase is either stabilized also by substrate or instability varies with pH. Although phospholipase A-treated glucose 6-phosphatase was slightly less stable at pH 5.75 than at 8.00, the phospholipase A-treated form of the enzyme was stabilized by substrate at pH 5.75. At higher pH values (Fig. 6), glucose-6-P did not prevent inactivation of the phospholipase A-treated enzyme.

Mechanism of Activation of Phospholipase A-treated Glucose 6-Phosphatase and Pyrophosphatase by Albumin and Asolectin—The data in the above section indicate that the major effect of phospholipase A on glucose 6-phosphatase is not to inactivate the enzyme but to produce an unstable form, which can be stabilized by asolectin or albumin, and, although complex, the action of phospholipase A may be visualized as resulting from the production of modified forms of native glucose 6-phosphatase with eventual production of an inactive form. In view of the fact that albumin activates the phosphohydrolase activities of the phospholipase A-treated form of glucose 6-phosphatase, it was important to determine whether this resulted from reactivation of a modified form of glucose 6-phosphatase, which would suggest that it acted by reversal of phospholipase A-induced effects. In order to study this aspect of the problem the effect of albumin on activity of phospholipase A-treated enzyme was measured before glucose 6-phosphatase activity declined. Microsomes were incubated for only 2 min with a small amount of phospholipase A (2 µg per mg of microsomal protein), conditions which did not inactivate glucose 6-phosphatase. On the other hand, this mild digestion with phospholipase A produced a form of glucose 6-phosphatase which was activated by albumin (Table II), and
comparison of the data in Table II with those in Fig. 1 shows that the per cent activation by albumin after phospholipase A treatment was the same on the fully active and partially inactivated forms of the phospholipase A-treated enzyme. These results indicate that a small amount of hydrolysis of phospholipids by phospholipase A is sufficient to produce maximum albumin activation and that the effects of albumin are not related to reversing phospholipase A induced damage to glucose 6-phosphatase. Although in comparison to albumin, asolectin had only a small activating effect on the phosphohydrolase activity of phospholipase A-treated glucose 6-phosphatase (Fig. 1), asolectin activation also cannot be considered to result from reversal of phospholipase A-induced defects in the enzyme. The evidence for this is that, after treatment of microsomes as in Table II, incubation with asolectin increased phosphohydrolase activity to 20% greater than that of the untreated enzyme.

**Effect of Washing on Albumin Activation of Phospholipase A-Treated Microsomes on Albumin Activation of Glucose 6-Phosphatase**—Albumin might activate phospholipase A-treated glucose 6-phosphatase by affecting directly the phospholipase A-treated form of the enzyme. Alternatively, phospholipase A itself might activate glucose 6-phosphatase, this effect being masked by hydrolysis products. Albumin could cause an apparent activation by removing these inhibitors. It has been reported, in fact, that fatty acids and lysolecithin inhibit intestinal glucose 6-phosphatase (28). If this were also the case for the bovine liver enzyme, it should be possible to incubate phospholipase A-treated glucose 6-phosphatase with albumin, remove the albumin by washing, and leave the enzyme in its “activated” state. As the data in Table III show, washing of phospholipase A-treated microsomes with sucrose alone did not alter the susceptibility of glucose 6-phosphatase to subsequent albumin activation. In contrast, after brief incubation of phospholipase A-treated microsomes with an amount of albumin needed to produce maximum activation of glucose 6-phosphatase and removal of albumin by washing, the enzyme no longer responded to albumin activation. This result suggests that the products of phospholipid hydrolysis inhibit phospholipase A-treated glucose 6-phosphatase but does not exclude the possibility that albumin acts directly on the enzyme. Thus, evidence for or against inhibition by hydrolysis products was also sought in another way.

If fatty acids or lysophosphatides (or both) are inhibitory, varying amounts of hydrolysis of microsomal phospholipids by phospholipase A should produce varying amounts of inhibition. As a result, the slope of the plot of per cent activation at any given albumin concentration against albumin concentration should be related to the extent of phospholipase A action. Microsomes were therefore incubated with low and high concentrations of phospholipase A, and glucose 6-phosphatase was assayed with varying amounts of albumin. As compared to microsomes incubated with a low concentration of phospholipase A, there was a lag in albumin activation of microsomes treated with the high concentration of phospholipase A (Fig. 7). The relative increase in per cent activation with increasing concentration of albumin, however, was the same for microsomes treated for 2 min with 3.9 µg of phospholipase A per mg of microsomal protein or for 5 min with 10 times this amount of phospholipase A.

**Effect of Phospholipase C on Activities of Glucose 6-Phosphatase**—Phospholipase C treatment progressively inactivated glucose 6-phosphatase of bovine liver microsomes (Fig. 8). However, in contrast to phospholipase A inactivation, the effect of phospholipase C seemed to be the direct result of the action of phospholipase C on the enzyme. The evidence for this conclusion is that at the pH used for phospholipase C digestion of microsomes the hydrolysis of glucose-6-P by the phospholipase C-treated glucose 6-phosphatase was linear with time, but glucose-6-P did not protect the enzyme from phospholipase C inactivation.
Asolectin restored the activity of phospholipase C-treated enzyme to control levels (Fig. 8), but this was not a specific restoration of the activity by added phospholipid in the sense of a reconstitution of holoenzyme since albumin also activated the phospholipase C-treated enzyme. In order to exclude the possibility that this albumin effect was due to contaminating phospholipids, the P,

content of a CHCl₃-CH₃OH (2:1, v/v) extract of an aqueous solution of albumin was determined and found to be 0.004 μg of CHCl₃-CH₃OH-extractable P,

per mg of albumin, an amount too small to account for activation on the basis of phospholipid contamination.

That phospholipase C treatment can have selective effects on the active site (sites) of glucose 6-phosphatase is indicated by the combined action of phospholipase C treatment and added phospholipids on PP₃-glucose phosphotransferase activity. In contrast to the differential effects of phospholipase A on the phosphohydrolase and phosphotransferase activities of the enzyme, phospholipase C treatment led to quantitatively similar inactivations of these activities (Table IV). However, after phospholipase C treatment, asolectin had a much greater activating effect on the phosphotransferase activity than the phosphohydrolase activities. In fact, the phosphotransferase activity increased nearly 3-fold, and in the presence of asolectin was 50% greater than that of untreated microsomes. The phosphohydrolase activities of similarly treated microsomes did not reach levels observed with the untreated enzyme.

Effects of Phospholipids on Glucose 6-Phosphatase—As indicated in Fig. 1, asolectin micelles inhibit the glucose 6-phosphatase and pyrophosphatase activities of untreated microsomes. An attempt was made to determine whether this was related to alteration of enzyme-substrate affinity, but plots of 1/v against 1/S were nonlinear. As a result, no estimates of the effect of phospholipids on Kₘ could be made. The nonlinearity of the Lineweaver-Burk plots seemed to be due in part to phospholipid inactivation of glucose 6-phosphatase which was prevented by high but not low concentrations of glucose 6-P (Fig. 9A). The mechanism of this inactivation is not clear, but it was found also to be pH-dependent (Fig. 9B).

| Treatment          | Addition to assay | Specific activity |
|--------------------|------------------|------------------|
|                    | Phospholipase C  | PP₃-glucose      |
|                    | Asolectin        | Phosphotransferase | Pyrophosphatase | Glucose 6-phosphate |
| None               | None             | 0.112            | 0.095           | 0.112              |
| Phospholipase C    | None             | 0.065            | 0.052           | 0.062              |
| Phospholipase C    | Asolectin        | 0.170            | 0.069           | 0.083              |

Although only the data for glucose 6-phosphatase are shown, asolectin had a similar action on the phosphotransferase activity of the enzyme, and the extent of inhibition and inactivation was identical for both activities. On the other hand, the phosphotransferase activity was not inhibited by asolectin at and above pH 5.00. At this pH, however, phosphotransferase activity is approximately one-third as much as at its pH optimum and of the phosphohydrolase activities. An asolectin effect on the phosphotransferase activity of untreated microsomes could be shown only under conditions which led to progressive inactivation of the phosphohydrolases.

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**Fig. 8.** Effect of phospholipase C on glucose 6-phosphatase. Microsomes were incubated with 0.14 mg of crude phospholipase C per mg of microsomal protein. Measurements of enzyme activity in the absence (○) and presence of albumin (□) or asolectin (△) were as in Fig. 1.

**Fig. 9.** A, effect of glucose-6-P concentration on asolectin inactivation of glucose 6-phosphatase. Enzyme assays were carried out in the presence of 3.8 mg of asolectin per tube at pH 5.50 with 80 mM (○) or 4 mM (□) glucose-6-P. B, effect of pH on asolectin inactivation of glucose 6-phosphatase. Assays were carried out in 80 mM glucose-6-P, pH 5.50 (○) or 5.00 (□).
Phospholipase A or C treatment changes the effects of asolectin on glucose 6-phosphatase. One difference between phospholipase-treated and untreated microsomes is that the phospholipase-treated enzyme is not progressively inactivated by asolectin in the presence of low concentrations of glucose-6-P. Another difference is that at high concentrations of glucose-6-P asolectin does not inhibit the glucose 6-phosphatase activity of phospholipase A- or C-treated microsomes. Thus, hydrolysis of phospholipids leads to major changes in the interactions between microsomal proteins and phospholipids.

After phospholipase A treatment plots of 1/v against 1/S for glucose 6-phosphatase activity were linear in the presence of asolectin; it was found that phospholipase A treatment decreased the $K_m$ of glucose 6-phosphatase for glucose-6-P, but incubation of these microsomes with asolectin restored the $K_m$ to its value in untreated microsomes (Fig. 10).

## DISCUSSION

**Relationship between Modification of Microsomal Phospholipids and Activities of Glucose 6-Phosphatase**—The present experiments indicate that treatment of microsomes with phospholipases A or C leads to the production of a variety of modified forms of glucose 6-phosphatase with differing kinetic properties and responses to albumin and asolectin. These results suggest that changes in the phospholipid environment can alter the conformation of glucose 6-phosphatase so as to increase or decrease maximum enzyme activity. Further, the fact that combined treatments with phospholipase A plus albumin or asolectin or phospholipase C plus asolectin increase the phosphotransferase and phosphohydrolase activities to greater levels than in the untreated enzyme indicates that rather than being essential for activity the native phospholipid environment acts to constrain maximum potential enzyme activity. This concept is consistent with the fact that detergents which clarify and disperse microsomal suspensions by modifying the normal protein-lipid interactions also increase the activities of glucose 6-phosphatase (10-12). Thus, with appropriate conditions, it can be shown that two different mechanisms for altering the phospholipids of microsomes increase the activities of glucose 6-phosphatase, reinforcing the conclusion that the interactions of phospholipids and glucose 6-phosphatase normally lower activity.

The differential effects of phospholipases, albumin, and pH on the phosphohydrolase and phosphotransferase activities indicate that the enzyme has different specific regions for interaction with glucose and H$_2$O which are either spatially distinct or result from different conformations of a single region, and, although the chemical basis for the effects of phospholipids on glucose 6-phosphatase is not clear, these data also are compatible with the hypothesis that the modulating action of phospholipids on glucose 6-phosphatase activities results from effects on the reactions of the postulated enzyme-Pi intermediate with glucose or H$_2$O. Kinetic evidence suggests the following mechanism for the multiple activities of glucose 6-phosphatase.

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\begin{align*}
\text{Glucose-6-P} & \rightarrow E-P + \text{glucose} \\
PP_i + E & \rightarrow E-P + P_i \\
E-P + H_2O & \rightarrow E + P_i \\
E-P + \text{glucose} & \rightarrow E + \text{glucose-6-P}
\end{align*}
\]

During incubation with phospholipase A at pH 8.00, the phosphohydrolase activities decline whereas the phosphotransferase increases slightly (Table I). Since Reactions 1a and 1b are common steps, and since glucose does not affect $K_{PP_i}$, the phospholipase A-induced decline of phosphohydrolase activity may result from a decrease in the rate of Reaction 2, which should be the rate-limiting step for phosphohydrolase activity in phospholipase A-treated microsomes. Albumin-induced augmentation of the phosphohydrolase activity of phospholipase A-treated microsomes, therefore, results also from an effect on Reaction 2. Moreover, since this albumin effect can be shown in phospholipase A-treated microsomes in which there has been no loss of phospholipase A activity, it is reasonable to conclude that Reaction 2 is the rate-limiting step in glucose 6-phosphatase of control microsomes. Since the phosphohydrolase and phosphotransferase activities of control microsomes are comparable, Reaction 3 is the rate-limiting step for the phosphotransferase reaction.

**Effect of Phospholipids on $K_m$ of Glucose 6-Phosphatase for Glucose-6-P**—Phospholipase A treatment lowers the $K_m$ of glucose 6-phosphatase for glucose-6-P, which is restored toward the level in untreated microsomes by incubation with asolectin (Fig. 10). Although the observed decline in $K_m$ is modest, it was measured in a preparation in which hydrolysis of approximately only 50% of microsomal phospholipids had occurred. More extensive removal of normal microsomal phospholipids might lead to a decrease in $K_m$, which is substantially lower than that due to phospholipase A treatment alone. Further, since the steady state concentration of glucose 6-P is approximately 0.5 mM in liver (30) and the $K_m$ of glucose 6-phosphatase for glucose-6-P is 2.4 mM, a decrease of the $K_m$ will have significant effects on the rate in vivo of hydrolysis of glucose-6-P. Thus, phospholipids are negative modifiers of glucose 6-phosphatase not only as a result...
of their effects on Reaction 2 but also because they increase the $K_m$ of the enzyme for glucose-6-P.

Implications for Physiological Regulation of Glucose 6-Phosphatase—Stadtman (31) has pointed out that regulation of glucose 6-phosphatase is "mandatory," and several lines of evidence indicate that glucose 6-phosphatase activity is regulated in a dynamic manner. For example, fasting produces a selective increase in the phosphorylase activity of the enzyme (15), and glucocorticoid-induced increases in glucose 6-phosphatase are only in part due to the synthesis of new enzyme (16). Further, alloxan diabetes increases the constraint on maximum enzyme activity, as indicated by the fact that the ratio of activity in the presence and absence of detergent increases in comparison to microsomes from normal livers (17). Fasting and alloxan diabetes (17-19) also increase the $K_m$ for glucose-6-P.

Nordlie has proposed several mechanisms by which regulation of glucose 6-phosphatase might be effected. Citrate (32) and HCO$_3^-$ (33) inhibit glucose 6-phosphatase, and long chain acyl-CoA compounds (34) increase selectively the phosphotransferase activities. However, the $K_m$ for citrate is 6 mm, which is 20-fold greater than the hepatic citrate concentration (25); and the effects of acyl-CoAs, which may result from their detergent properties, are probably negated by interaction with proteins other than glucose 6-phosphatase. Thus, the physiological significance of some of the proposed modulators of enzyme activity is questionable.

Our data on the effects of phospholipases, albumin, and ascorbic acid indicate that the type of control seen in fasted, glucocorticoid-treated, and alloxan diabetic animals could be related to alteration by microsomal phospholipids of the reaction between an enzyme-P$_i$ intermediate and H$_2$O or glucose, and to phospholipid effects on the affinity of the enzyme for substrates. Further, these data suggest a reason for the location of glucose 6-phosphatase in the microsomal membrane: an unconstrained glucose 6-phosphatase could short-circuit glycolysis and produce a lethal ATPase effect. By providing the enzyme with a phospholipid environment the activity is constrained in two different kinetic ways. Our experiments in vivo, those of other workers (10-12), and evidence in intact animals (15-19) suggest that appropriate signals could act to relieve in part the negative modifying effects of the phospholipid environment in order to maintain the blood sugar concentration within a narrow range.

Acknowledgments—The author wishes to thank Drs. Thomas P. Singer and Edna B. Kearney for many helpful discussions during the course of this work and for their critical review of the manuscript.

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J. Biol. Chem. 1970, 245:4953-4961.

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