Investigation of phosphofructokinase in normal and regenerating livers led to the discovery of an inactivating factor in the extracts of these livers. The inactivating factor was found to be a mixture of free fatty acids. The fatty acid compositions of the normal and regenerating livers are the same, but the concentrations of most of the fatty acids are at least 3 to 4 times higher in the latter.

Inactivation of phosphofructokinase by palmitate and oleate was investigated using purified rabbit muscle enzyme. Incubation of the enzyme with palmitate (250 μM) or oleate (50 μM) resulted in rapid inactivation of the enzyme with biphasic curves. The concentrations of oleate and palmitate required to produce 50% inactivation of the enzyme were 35 μM and 75 μM, respectively. Fructose-6-P (0.5 mM), MgATP, (1 mM), fructose-1,6-P₂ (1 mM), AMP (1 mM), and cyclic adenosine 3':5'-monophosphate (20 μM) protected the enzyme against inactivation when these metabolites were incubated with the enzyme before the addition of fatty acid. Bovine serum albumin (100 μM) and β-cyclodextrin (0.25 mM) also protected the enzyme against the inactivation. However, if the enzyme was inactivated by fatty acid, subsequent addition of the above metabolites or bovine serum albumin did not reactivate the enzyme.

Binding studies with [³H]oleate revealed at least three types of binding sites. The first site binds 2 to 4 mol of oleate/mol of enzyme. Oleate binding to this site did not seem to affect the enzyme activity. The second binding site binds 5 to 15 mol of oleate/mol of enzyme resulting in complete loss of the activity. This is followed by an increase in oleate binding to the third site of the enzyme. Sucrose density gradient centrifugation of oleate-inactivated enzyme indicated that the enzyme dissociated to the dimeric form. Similarly, centrifugation of [³H]oleate-treated enzyme revealed that all polymeric forms of phosphofructokinase bound approximately 6 to 8 mol of oleate/mol of enzyme. In the presence of fructose-6-P, oleate is bound to the polymers to a lesser degree and therefore protects against the fatty acid inactivation. Various polymers which are cross-linked with dimethylsuberimidate are also inhibited by oleate.

The hypothesis that glycolytic flux may be regulated by phosphofructokinase-fructose-1,6-bisphosphatase substrate cycling (1) has been supported by recent investigations of Lardy and co-workers (2). The factors influencing this cyclic process are still not known and are under extensive investigations. The possibility of phosphofructokinase-fructose-1,6-bisphosphatase interaction regulating the cycle has been investigated by Pogell et al. (3) and by Uyeda and Luby (4). Metabolites such as AMP also have been shown to reciprocally affect the phosphofructokinase and fructose-1,6-bisphosphatase activities (5, 6), and thus they are thought to regulate the futile cycle.

The observation that fatty acids increase glucose synthesis (7–10) suggested to Weber et al. (11) that free fatty acids may have a physiological role in effecting a transformation from glycolysis to gluconeogenesis. Further, Lea and Weber (12) have shown that fatty acids inhibit a number of glycolytic enzymes under in vitro conditions. More recently, Shaitir and Ruderman (13) have shown that linoleate inactivates a number of enzymes of glycolysis and lipogenesis in crude liver preparations.

In contrast to the effect on glycolytic enzymes, the gluconeogenic enzyme, fructose-1,6-bisphosphatase was found to be activated rather than inhibited by oleate (14). Thus, fatty acids appear to have a reciprocal effect on phosphofructokinase and fructose-1,6-bisphosphatase activities. During an investigation of phosphofructokinase in normal and regenerating rat liver, we observed the presence of an inactivating factor in these liver extracts. The present studies were undertaken to determine the nature of this inactivating factor, which has been shown to be a mixture of fatty acids, and to study in detail the possible mode of inhibition of purified phosphofructokinase by fatty acids. These studies include the binding of fatty acids and their effect on the enzyme activity as well as on the structure of protein, and the nature of fatty acids present in normal and regenerating liver.
EXPERIMENTAL PROCEDURE

Materials

Oleate was obtained from Sigma Chemical Co.; dimethylsulphoxide and dichloroacetic acid were purchased from Eastman Kodak Co., 10-51-HCl, HCl, NaCl from Nutritional Biochemicals Corp., and other chemicals were purchased from commercial sources.

Methods

Partial Hepatectomy—White male rats (Sprague-Dawley strain) weighing approximately 150 to 200 g were used in these experiments. Part of the liver was removed. The animals were returned to cages and given commercial diet (Purina products) and water ad libitum. At the end of 24 h, the animals were killed by cervical dislocation. The abdominal region was cut open quickly and the liver was perfused with 25 ml of ice-cold saline (0.9% NaCl solution). After perfusion the liver was excised and frozen at -70°C until further use.

Ether Extraction of Livers—Normal and regenerating rat livers (50 g each) were homogenized in 100 ml of ice-cold deionized water. The homogenates were extracted first with an equal volume of peroxide-free ether. A second extraction was performed using 100 ml of ether. The ether extracts were pooled and passed through a bed of anhydrous sodium sulfate. The ether was removed by evaporation under a stream of nitrogen. The extracts were kept in a vacuum desiccator, protected from light, and weighed every 4 h until a constant weight was obtained. The extracts were dissolved in chloroform just before use for silicic acid column chromatography.

Silicic Acid Column Chromatography—All operations were carried out at 4°C. A column of silicic acid (Unisal 100-200-mesh, 0.6 × 10 cm) was equilibrated with anhydrous chloroform. The extract (17 and 55 mg of lipid/50 g of liver for normal and regenerating liver, respectively) in 2 ml of chloroform was passed through the silicic acid column, and the column was washed with 50 ml of chloroform. The chloroform wash was collected and concentrated to dryness under a stream of nitrogen. The column was further washed with 50 ml of methanol, and this eluate was also concentrated to dryness under nitrogen.

Thin Layer Chromatography—Thin layer chromatography of chloroform eluate on silica gel plates was performed in thin layer chromatographic chambers, protected from light, and flushed with nitrogen before saturation with solvent system. The plates were developed in petroleum ether/ether/acetic acid (80/20/1) at room temperature. The lipid materials were detected by exposing to I2 vapor. The corresponding spots were scraped from the unexposed plates, extracted with ether, and tested for inactivating factor. The fraction containing the inactivating factor was rechromatographed on silica gel plates and eluted with ether/methanol/acetic acid (98/2/0.1). The extract (17 and 55 mg of lipid/50 g of liver for normal and regenerating liver, respectively) in 2 ml of chloroform was used. The extract was chromatographed on the thin layer with the solvent system. The free fatty acid fraction obtained from the thin layer chromatographic plates was the only fraction that showed inactivating phenomena with phosphofructokinase.

Fatty Acid Analysis By Gas-Liquid Chromatography—The free fatty acids obtained from the thin layer chromatogram were analyzed by gas chromatography. An internal standard, stearic acid (C18:0, 10.0 µg), was added to the eluate corresponding to the free fatty acids. The methyl ester of the fatty acids was prepared in a Teflon-lined screw cap culture tube as follows. The contents of the tubes were taken to dryness under nitrogen, and to each tube were added 1.5 ml of tetradecane, 2.0 ml of 14% BF3/methanol. The tubes were flushed with nitrogen and capped, and the samples were heated at 100°C for 30 min. After the samples were cooled, 20 ml of distilled water were added, and the samples were extracted three times with 3 ml of hexane. The combined hexane extracts were taken to dryness under nitrogen and redissolved in 50 µl of carbon disulfide. The fatty acid methyl esters were separated by gas chromatography. The operating temperature of the column was 185°C, and the injection temperature was 205°C. The columns were 6-foot glass columns with a 1/4-inch inner diameter, and packed with 10% SP-22-PS (Supelco Inc., Bellefonte, Penna.). Nitrogen was employed as the carrier gas at a flow rate of 25 ml/min at a pressure of 40 p.s.i. All analyses were performed in duplicate.

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Sucrose Density Gradient Centrifugation—The sedimentation properties of native and oleate-treated phosphofructokinase were determined by zonal centrifugation in a sucrose density gradient (20). The sucrose density gradients (5 to 20% sucrose), containing 0.5 M Tris-HCl, pH 8.0, 0.2 mM EDTA, and 10 mM dithiothreitol, were prepared using an automatic gradient maker. The native or oleate-treated enzyme (0.2 ml) was layered on top of the gradient (5 ml). Centrifugation was performed at 4°C in a Beckman model L2-65 ultracentrifuge with a SW50 rotor for approximately 15 hours at 31,000 rpm. The tubes then were removed from the bottom using an Auto Densi Flow (Beckman Instruments), and 0.15-ml fractions were collected. The phosphofructokinase activity of each fraction was determined, and the protein concentration was measured fluorometrically (excitation and emission wavelengths at 290 nm and 340 nm, respectively) according to the procedure of Uyeda.1

1 Unpublished procedure of K. Uyeda.
Cross-Linking of Phosphofructokinase—Phosphofructokinase was treated with the cross-linking reagent dimethylsuberimidate in a procedure similar to that employed by Lad and Hammes (21). Rabbit muscle phosphofructokinase (1 mg/ml) was dialyzed for 2 hours against 1 liter of 0.2 M triethanolamine hydrochloride (pH 8.1) containing 5 mM ATP, 5 mM MgCl₂, and 2 mM fructose-1,6-P₂. To the dialyzed enzyme (0.25 mg/ml) in the same buffer was added dimethylsuberimidate to a final concentration of 3 mg/ml. The reaction was allowed to proceed overnight at 4°C, and then was terminated by addition of ammonium chloride to a final concentration of 50 mM. Various polymers of this enzyme were separated by sucrose density gradient centrifugation (5 to 20%) in 0.1 M Tris-HCl (pH 7.0), 10 mM citrate, and 10 mM dithiothreitol. Other conditions of centrifugation were similar to those used by Lad and Hammes (21). Protein in the gradient fractions was monitored by the fluorometric method mentioned above.

RESULTS

Inactivation Factor in Ether Extract of Normal and Regenerating Rat Liver—Ether extracts of normal and regenerating rat livers representing two different physiological states were found to contain a factor(s) which inactivated the phosphofructokinase in vitro (Fig. 1). The regenerating liver contained at least 3 to 4 times more inactivating factor than that of normal liver extract based on the same weight of liver. This difference in the content of the factor between regenerating and normal livers is not due to the difference in efficiency of the extraction because when [⁹⁹⁷]oleate was added to the homogenates of both livers and extracted with ether, the ether extracts were found to contain the same amount of oleate.

Effect of Defatted Bovine Serum Albumin and Effecters on Ether Extract Inactivation of Phosphofructokinase—The inactivation of phosphofructokinase by the ether extract can be prevented by addition of defatted bovine serum albumin prior to the mixing of the extract with the enzyme. As can be seen from Table I, bovine serum albumin at a concentration of 100 μg almost completely protected the enzyme against the inactivation. The inactivation also can be prevented to varying degrees by the substrates, the products, or some of the effectors of phosphofructokinase. The maximal protection was obtained with fructose-6-P (1 mM), while ATP, fructose-1,6-P₂, cAMP, or AMP protected only 85 to 76% at the same concentration.

Chromatographic Separation of Components Present in Ether Extract—In order to determine the nature of the inactivating factor in the ether extracts from normal and regenerating liver, they were subjected to both silicic acid column chromatography and thin layer chromatography. The chloroform eluate from the silicic acid column was found to contain the inactivating material, while the methanol eluate contained no inactivating factor. The chloroform eluate of the column then was concentrated and further resolved on a silica gel thin layer plate using petroleum ether/ether/acetic acid (80/20/1) as the solvent system. The spots corresponding to Rₜ values of 0.3 to 0.5 contained the phosphofructokinase-inactivating material. This material was further subjected to thin layer chromatography on silica gel using a solvent system consisting of benzene/ether/methanol/acetic acid (90/21/3/2). The ether eluate of spots containing the inactivating factor corresponded to fatty acids, and these samples were analyzed for the type and the amount of fatty acids using gas-liquid chromatography.

The results from the analyses of the fatty acids in the above eluates from both the normal and the regenerating rat livers are presented in Table II. The fatty acids present at the highest concentrations in normal and regenerating livers were palmitic acid and oleic acid. Among the fatty acids shown, arachidonic acid was present in the lowest concentration in the normal liver. The regenerating liver contains 4- to 5-fold higher concentrations of most of the fatty acids and about 10-fold higher in arachidonic acid as compared to the normal liver. These results explain our initial observation that the ether extract of regenerating liver was 3 to 4 times more potent in inactivating phosphofructokinase.

Inactivation by Palmitate, Oleate, and Arachidonate—To elucidate the mechanism of fatty acid inhibition of phosphofructokinase, those fatty acids present in relatively high concentrations, namely, oleate and palmitate and also arachidonate, were chosen for detailed investigation. The rates of inactivation of phosphofructokinase by oleate and palmitate were first compared, and the results are shown in Fig. 2. When phosphofructokinase (7 μg/ml) was incubated with 50 μM each of oleate or palmitate, the rate of enzyme inactivation by oleate...
Interaction of Fatty Acids with Phosphofructokinase

TABLE II

Composition of free fatty acids in normal and regenerating rat livers

Fatty acids were extracted from normal and regenerating livers, resolved by silicic acid column chromatography and thin layer chromatography, and further analyzed by gas-liquid chromatography as described under "Methods." The values are representative of three different samples analyzed.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Normal</th>
<th>Regenerating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristic acid</td>
<td>C14</td>
<td>2.7</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>C16</td>
<td>19.9</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>C18</td>
<td>5.6</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>C18:1</td>
<td>16.9</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>C18:2</td>
<td>3.2</td>
</tr>
<tr>
<td>Linolenic acid</td>
<td>C18:3</td>
<td>2.3</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>C20:4</td>
<td>0.4</td>
</tr>
</tbody>
</table>

was about 20 times faster than that by palmitate. Moreover, the inactivation by oleate was complete, while that by palmitate was only 40% and no further inactivation of the enzyme was observed even after 30 min. The reaction was essentially complete at 2 min, as shown in Fig. 2 for three oleate concentrations. However, for all further inactivation experiments, a 15-min incubation time was used to ensure complete reaction.

Fig. 3, A and B, shows the comparison of inactivation of phosphofructokinase by different concentrations of oleate, arachidonate, and palmitate. Since it was possible that the effect of fatty acid was one of protein denaturation, we compared the inactivation of phosphofructokinase by fatty acid to the inactivation of the enzyme by a well known denaturant, sodium dodecyl sulfate. Oleate, arachidonate, and sodium dodecyl sulfate show (Fig. 3A) inactivation curves with initially little or no inactivation followed by progressively greater extent of inactivation with increasing concentration. On the other hand, palmitate appears to show (Fig. 3B) greater extent of initial inactivation, with progressively lesser extent of inactivation. Oleate and arachidonate are much more potent inactivators than palmitate, since 50% inactivation was achieved with 35 μM concentration of the former fatty acids while 75 μM palmitate was required for the same inactivation.

Protection against Oleate Inactivation—Protection against oleate inactivation by varying concentrations of substrates, product, and other effectors is shown in Fig. 4. cAMP offers the best protection among the effectors tested; 80% protection was obtained at about 15 μM. Fructose-6-P at 100 μM, which is close to the Kₐ value of this substrate (22), shows about 50% protection. While fructose-6-P (0.5 mM) or cAMP (0.05 mM) offered almost complete protection, MgATP (1 mM) and fructose-1,6-P₂ (1 mM) were able to protect phosphofructokinase only 60 to 70%. It is somewhat surprising that fructose-1,6-P₂ is not as effective as fructose-6-P in protecting the enzyme against the oleate inactivation, since it has been shown that fructose-1,6-P₂ binds very tightly to muscle phosphofructokinase (4) and also protects against cold inactivation of liver phosphofructokinase (23). Physiological concentrations of some of the effectors of phosphofructokinase were also tested for possible protection against oleate inactivation. A mixture of fructose-6-P (0.1 mM), glucose-6-P (0.5 mM), fructose-1,6-P₂ (1 mM), and ATP (1.0 mM) at physiological concentrations protected the enzyme completely, although separately they were effective only partially or not at all (Table III). β-Cyclodextrin, which has been shown to protect glucose-6-phosphate dehydrogenase from palmitoyl-CoA inactivation (24),
and bovine serum albumin also offered complete protection.

Attempts to Reactivate Oleate- and Palmitate-inactivated Phosphofructokinase Though some metabolites protected the enzyme from inactivation to varying degrees when present before the addition of fatty acid, their addition after the enzyme had been exposed to fatty acid failed to reactivate the enzyme. Oleate was added to the enzyme solution (7 μg/ml) at a concentration of 40 μM to produce about 75% inactivation. To this partially inactivated enzyme, fructose-6-P, fructose-1,6-P, ATP, and MgADP were added at the specified concentration with 10 mM dithiothreitol, and the mixture was incubated further at 35°C for 1 to 2 hours. Neither the metabolites nor 100 mM mercaptoethanol were able to reactivate the enzyme, as shown in Table IV. The presence of fructose-1,6-P at very low concentrations (5 μM) before inactivation, and then the subsequent addition of high concentrations of fructose-1,6-P, fructose-6-P, or ATP did not help in the reactivation of the enzyme. A mixture of glucose-6-P, fructose-6-P, ATP, and fructose-1,6-P at physiological concentrations also failed to reactivate the enzyme.

The above experiment was repeated with 10-fold more enzyme (70 μg), but the attempts to reactivate the inactivated enzyme at this higher protein concentration by various metabolites were also unsuccessful under these conditions. The addition of divalent metal ions like Mg²⁺ and Zn²⁺ alone, or in combination with other metabolites, also was not effective.

Incubation of the enzyme with fatty acid for 30 s, a much shorter time of exposure to fatty acids than above, and immediate addition of a mixture of metabolites only prevented further inactivation and still did not reactivate the inactivated enzyme. Changing the temperature of incubation to 0°C and adding metabolites within 1 min also did not help in reactivation.

The kinetics of inactivation of phosphofructokinase by palmitate appeared to be different from those of oleate and, therefore, it was thought possible to achieve reactivation of the enzyme in this case. However, many attempts similar to those for oleate-inactivated enzyme, were also unsuccessful in the palmitate-treated enzyme (Table IV).

Addition of bovine serum albumin or the fatty acid-binding cyclic oligosaccharide β-cyclodextrin also failed to reactivate the inactivated enzyme. Thus the binding of fatty acid to phosphofructokinase results in irreversible inactivation of the enzyme.

Molecular Species after Oleate or Sodium Dodecyl Sulfate Treatment—In order to determine whether the oleate inactivation results in dissociation of the enzyme, the enzyme (2.5 mg/ml) was treated with oleate (3.6 mM) and applied to a column of Bio-Gel A-0.5m. The elution profile is shown in Fig. 5. The marker enzymes included were citrate synthase (M₂ = 100,000) (25), lactate dehydrogenase (M₂ = 140,000) (26), and native phosphofructokinase (M₀ = 320,000) (27). In the oleate-treated sample the peak appeared before lactate dehydrogenase but after phosphofructokinase, indicating that the enzyme...
The unfolding of the protein. These results are consistent with the molecule but exposed upon inactivation of the enzyme due to binding. The last site may be initially buried in the enzyme site which binds 4 to 5 mol of fatty acid/subunit resulting in enzyme. This possibility was investigated by determining the dimer is formed by sodium dodecyl sulfate treatment under activity, an increased extent of binding of oleate can be seen, loss of activity, and a third nonspecific site with weaker loss of the enzyme activity. After the enzyme has lost its to 20 mol of oleate bound/mol of the enzyme cause complete loss of enzyme activity.

Results suggest at least three distinct types of binding sites for the fatty acid results in a linear type loss of the activity until 15 of enzyme, i.e. a specific site which binds 1 mol of fatty acid/subunit without affecting the activity, a second site which binds 4 to 5 mol of fatty acid/subunit resulting in loss of activity, and a third nonspecific site with weaker binding. The last site may be initially buried in the enzyme molecule but exposed upon inactivation of the enzyme due to unfolding of the protein. These results are consistent with the

Fig. 5. Bio-Gel column chromatography of oleate-treated phosphofructokinase. Enzyme solution (1.0 ml, 2.5 mg/ml) was treated with oleate (3.6 mM) or sodium dodecyl sulfate (SDS) (3.6 mM), and applied to a column of Bio-Gel A-0.5m (0.9 x 25 cm) which had been equilibrated with 0.05 M Tris-11PO4 buffer, pH 6.0, containing 0.2 mM EDTA and 5 mM diithiothreitol at 4°. Phosphofructokinase was eluted with the same buffer, and fractions of 1 ml were collected. The marker enzymes were native phosphofructokinase (PFK), lactate dehydrogenase (LDH), and citrate synthase (CS).

was dissociated to a protein with a molecular weight slightly larger than 140,000. This suggests that phosphofructokinase probably dissociated to a dimer with a molecular weight of 160,000, but not to the subunit (M, = 75,000). For comparison, sodium dodecyl sulfate-treated enzyme was also investigated. The elution profile indicates that a mixture of monomer and dimer is formed by sodium dodecyl sulfate treatment under similar conditions.

A sedimentation velocity run of oleate-treated phosphofructokinase (2.5 mg/ml of enzyme; 3.6 mM oleate) at 40,000 rpm for 90 min at 8°, gave a single symmetrical peak with a sedimentation coefficient of 6.6 S, which is slightly less than that of the enzyme dimer (7 S).

Binding Studies Using [H]Oleate—Since the inactivation curve for oleate (Fig. 3) was biphasic, a possibility exists that this fatty acid binds to two different binding sites on the enzyme. This possibility was investigated by determining the number of oleate molecules bound and the corresponding loss of enzyme activity. As the results shown in Fig. 6 indicate, the first 2 to 4 mol of oleate bound/mol of enzyme result in little loss of the enzyme activity. However, the succeeding binding of the fatty acid results in a linear type loss of the activity until 15 to 20 mol of oleate bound/mol of the enzyme cause complete loss of the enzyme activity. After the enzyme has lost its activity, an increased extent of binding of oleate can be seen, with further increase in added oleate concentration. These results suggest at least three distinct types of binding sites for oleate on the enzyme, i.e. a specific site which binds 1 mol of the fatty acid/subunit without affecting the activity, a second site which binds 4 to 5 mol of fatty acid/subunit resulting in loss of activity, and a third nonspecific site with weaker binding. The last site may be initially buried in the enzyme molecule but exposed upon inactivation of the enzyme due to unfolding of the protein. These results are consistent with the

biphasic titration curves of oleate inactivation as shown in Fig. 3.

We have attempted to plot these results by the procedure of Scatchard et al. (28) in order to determine the number of binding sites and the binding constants, but such plots were found to be inapplicable since oleate binding is essentially irreversible under these conditions.

Analysis of Partially and Fully Oleate-inactivated Phosphofructokinase Using Sucrose Density Gradient Centrifugation—In order to shed some light on the relationship between oleate binding and dissociation of phosphofructokinase, partially (50 and 75%) and fully (100%) inactivated samples were subjected to sucrose density gradient centrifugation as described under “Methods.” As can be seen from Fig. 7A, (in the control) the protein and the activity profiles suggest the presence of several aggregated forms of the enzyme. The peak at Fraction 12 is the tetrameric state of the enzyme with a M, = 320,000, and the other fractions between 2 and 8 are the polymeric forms of the enzyme. The peak fraction (Fraction 22) is the dimeric state of the enzyme which lacks enzyme activity. When the 50% inactivated enzyme was subjected to sucrose density gradient centrifugation, there was a reduction in the amount of protein associated with Fractions 3 to 15, as compared to the controls, and a significant increase in the protein at Fractions 21 to 25. The amount of protein associated with this peak increased further with the 75% inactivated enzyme. It is interesting to note that all polymeric forms, including those larger than tetramers, appear to be dissociated by oleate treatment. The completely inactivated enzyme showed only one broad protein peak in Fractions 18 to 22. The position of marker proteins such as pyruvate kinase (M, = 237,000) (29), aldolase (M, = 160,000) (30), and lactate dehydrogenase (M, = 140,000) (26) in the gradient indicated that the enzyme progressively dissociated with increasing inactivation by oleate, and the dissociated enzyme had a molecular weight approximately equal to that of aldolase, which corresponds to the dimeric state of phosphofructokinase. These results are in agreement with the data obtained using Bio-Gel A-0.5m column chromatography mentioned earlier. It is also interesting
that the progressive dissociation of higher polymeric forms to the dimer seems to involve stepwise dissociation via the tetramer, since the amount of the tetramer (Fraction 12) relative to those of higher polymeric forms remains fairly constant (Fig. 7, B and C). This disassociation of the polymeric form of phosphofructokinase may involve the exact reversal of the aggregation process of the enzyme described by Lad and Hammes (21).

In the partially inactivated enzyme, it was of interest to determine whether the active forms of the enzyme bind some oleate or none at all, since the binding studies indicated (Fig. 6) that 2 to 4 mol of oleate were bound/mol of enzyme without causing inactivation. Phosphofructokinase was treated with [14C]oleate and subjected to sucrose density gradient centrifugation. The amount of oleate bound to different polymeric forms of phosphofructokinase was determined from the radioactivity and the protein concentration of each fraction. As shown in Fig. 8, all forms of the enzyme, including polymeric forms of tetramer as well as dissociated forms, bind oleate, although in different amounts. Due to varying amounts of oleate bound, an accurate determination of mol of oleate bound/mol of enzyme is difficult. However, the amount of oleate bound per various polymeric states which have enzyme activity is relatively constant and approximately 6 to 10 mol of oleate bound/mol of enzyme (Fig. 8, A and B). In the dissociated forms of the enzyme which appear in the fractions above 20, the amount of oleate bound increases to 20 to 30 mol/mol of enzyme as shown in Fig. 8A. Further increase in the binding is demonstrated in these fractions above 18 in the 50% (Fig. 8B) and 100% inactivated phosphofructokinase (Fig. 8C).

Since fructose-6-P seems to protect the enzyme against oleate inactivation, it is of interest to determine whether any oleate is bound to the fructose-6-P-protected enzyme. The sucrose density gradient centrifugation of the enzyme which was fully protected by fructose-6-P from oleate inactivation (the concentration of oleate would have inactivated the enzyme about 50% in the absence of fructose-6-P) revealed (Fig. 8D) that 4 to 8 mol of oleate were bound/mol of enzyme. The average value appears to be somewhat less than that of unprotected enzyme (Fig. 8B). Thus, fructose-6-P may protect the enzyme from interacting with additional molecules of oleate at a site(s) which leads to the inhibition of the activity and dissociation of the enzyme.

**Allosteric Properties of Oleate-containing Phosphofructokinase**—In order to determine if the binding of oleate to the enzyme alters the allosteric properties of phosphofructokinase, the enzyme, reacted with oleate (at a concentration that would have produced 50% inactivation) in the presence of fructose-6-P, was examined for its sensitivity to ATP and citrate inhibition. The activity under optimal conditions (pH 8.0) was the same for the control and the oleate-treated enzyme. At pH 7.4 with 0.1 mM fructose-6-P in the reaction mixture, both untreated and oleate-treated enzymes showed identical ATP (Fig. 9A) and citrate (Fig. 9B) inhibition curves, 50% inhibition was obtained at approximately 1 mM ATP and 8 μM citrate. These results show that a limited number of oleate molecules bound to the enzyme do not alter the allosteric properties.

**Effect of Oleate on Cross-linked Phosphofructokinase**—The question arises whether the inactivation by oleate involves two steps: (a) the inhibition of the enzyme activity, followed by (b) inactivation due to the dissociation of the enzyme. In order to answer this question we have made cross-linked phosphofruc-
Interaction of Fatty Acids with Phosphofructokinase

A 25% INACTIVATED

B 50% INACTIVATED

C 100% INACTIVATED

D F6P PROTECTED

Fig. 8. Sucrose density gradient centrifugation of [3H]oleate-treated phosphofructokinase (PFK). Conditions of centrifugation were the same as those described under Fig. 7. Specific radioactivity of

The present study reveals that there are three types of fatty acid binding sites on the phosphofructokinase molecule, and that they may be classified according to the relative ease of binding as well as their effect on the enzyme activity and structure.

1. The first site is where the least number of fatty acids bind. The interaction of fatty acid at this site is probably hydrophobic interaction without significantly affecting the protein conformation, as indicated by no loss or change in the catalytic properties. The number of oleate molecules bound to these sites (2 to 4 mol/mol of enzyme) (Fig. 6) indicates that there is only one such hydrophobic site on each subunit of phosphofructokinase. This site is different from either the catalytic or the allosteric site, since the oleate binding causes no inhibition (Fig. 6) or change in allosteric properties of the enzyme (Fig. 9, A and B). It is interesting to note that even in the high polymeric forms of the enzyme this site is accessible to the fatty acids (Fig. 8, A, B, and D).

2. The second site is a site(s) in the enzyme where up to about 15 to 20 mol of oleate/mol of enzyme or 4 to 5 mol/subunit bind. These sites are essential for the enzyme activity. Oleate binding to these sites probably causes sufficient conformational changes to inactivate the enzyme by disrupting its tertiary structure, and possibly its quaternary structure as well. It is not clear whether or not this inactivation involves dissociation of the enzyme, but the observation that the cross-linked polymers of phosphofructokinase are also inhibited (or inactivated) suggests that the enzyme is inhibited without dissociation. Furthermore, the results shown in Fig. 7, B and C compared to those in Fig. 7D suggest no dissociation of the enzyme but rather inhibition of its catalytic activity. However, this interaction seems to render the enzyme susceptible to additional binding of fatty acid to other sites, which leads to dissociation of the enzyme. Fructose-6-P may protect these sites from reacting with fatty acid. These relatively high reactive sites for fatty acid on phosphofructokinase may be similar to those of other proteins such as bovine serum albumin, which has been shown to have eight sites for sodium dodecyl sulfate interaction (31). Further cooperative interaction results in additional binding at weaker sites as discussed below.

3. The binding of oleate to the third site(s) causes a drastic
present observation that relatively few molecules of fatty acid suggested by Srere (34) and Eger-Neufeldt et al. (35). The of the substrate cycle, it may act as a "physiological denatu-

enzyme and activating the latter. It is also interesting that fructose-1,6-bisphosphatase, i.e., by inactivating the former reciprocal effect of fatty acid on phosphofructokinase and gluconeogenesis. This may provide an explanation for the oleate in the activation of fructose-1,6-bisphosphatase during globulin. Thus, the possibility exists that phosphofructokinase activated fructose-1,6-bisphosphatase. A similar activation was

treated enzyme was added to initiate the reaction. B, effect of citrate on the activity of untreated (●—●) and treated (▲—▲) phosphofructokinase (PFK). The reaction mixture was the same as in A except ATP concentration was 0.9 mM. Enzyme solution (0.2 ml) was incubated with oleate or palmitate at 35° for 15 min. After this incubation period, metabolites or other agents were added and the incubation continued for an additional 1 to 2 hours at 35°. Aliquots were removed for phosphofructokinase assay.

can effectively inactivate and dissociate phosphofructokinase may be important in the turnover of this enzyme in vivo, especially since Doeken and Pette (36) have shown that phosphofructokinase turns over twice as rapidly as the other glycolytic enzymes in vivo. It is possible that the fatty acid inactivation of phosphofructokinase may result in increased susceptibility of the enzyme to protease digestion and thereby accelerate its degradation.

REFERENCES

Fig. 9. Effect of oleate treatment of phosphofructokinase in presence of fructose-6-P on the allosteric properties of the enzyme. Enzyme solution (0.2 ml, 0.5 mg/ml) was reacted with 0.5 mM fructose-6-P and 0.5 mM oleate at 35° for 15 min. A, effect of increasing ATP concentration on the activity of untreated (●—●) and treated (▲—▲) enzyme. The assay mixture contained 50 mM Tris-HCl, pH 7.4; 1.0 mM EDTA; 2.5 mM dithiothreitol; 10 mM ammonium chloride; 2 mM MgCl_2; 0.1 mM fructose-6-P; varying concentrations of ATP, and desalted enzyme mixture. An aliquot (20 μl) of untreated or oleate-

change in enzyme conformation and dissociates it to the dimeric form (Fig. 7, D and K). Further dissociation to the monomer does not occur. This is in contrast to the dissociation caused by sodium dodecyl sulfate (Fig. 5), which results in the formation of the monomer as well as dimer.

It is difficult to assess the physiological significance of fatty acid inactivation of phosphofructokinase. Although Lea and Weber (12) originally reported that fatty acids inhibit a number of glycolytic enzymes, our present studies strongly suggest that phosphofructokinase is irreversibly inactivated. Free fatty acids, even in the regenerating liver where their concentrations are 3 to 4 times higher than normal are unlikely to be negative effectors of phosphofructokinase since the effect is irreversible. The reversibility of inhibition is one of the requirements for an effector. Moreover, the inactivation is prevented by the presence of various positive effectors of the enzyme including fructose-6-P, cyclic AMP, etc. A mixture of glucose-6-P, fructose-6-P, ATP, and fructose-1,6-P_2 at physiological concentrations can also completely protect against inactivation (Table III).

More recently Carlson et al. (32) demonstrated that oleate and a cytosol protein of rat liver acting synergistically activated fructose-1,6-bisphosphatase. A similar activation was observed with protein such as phosphofructokinase and a-globulin. Thus, the possibility exists that phosphofructokinase or dissociated phosphofructokinase may serve as a carrier of oleate in the activation of fructose-1,6-bisphosphatase during gluconeogenesis. This may provide an explanation for the reciprocal effect of fatty acid on phosphofructokinase and fructose-1,6-bisphosphatase, i.e., by inactivating the former enzyme and activating the latter. It is also interesting that oleate fructose-1,6-bisphosphatase has been reported to inhibit phosphofructokinase activity (33).

In addition to the role fatty acids may play in the regulation of the substrate cycle, it may act as a "physiological denaturant," which accelerates degradation rates of enzymes, as suggested by Sere (34) and Eger-Neufeldt et al. (35). The present observation that relatively few molecules of fatty acid...
Interaction of Fatty Acids with Phosphofructokinase

Studies on the fatty acid inactivation of phosphofructokinase.
C S Ramadoss, K Uyeda and J M Johnston


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