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THE INTERACTION OF FATTY ACIDS WITH RABBIT LIVER AND MUSCLE GLYCEROL-3-PHOSPHATE DEHYDROGENASE

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Summary

Various fatty acids containing 10—22 carbons and including unsaturated derivatives were found to be inhibitors of rabbit liver and skeletal muscle *sn*-glycerol-3-phosphate dehydrogenase (*sn*-glycerol-3-phosphate:NAD^+ 2-oxidoreductase, EC 1.1.1.8). For the liver enzyme, the logarithm of the inhibition constant was linearly related to the number of carbon atoms in the saturated fatty acids whereas the muscle enzyme, which was generally more strongly inhibited, showed a nonlinear dependence. The liver and muscle enzymes also interacted differently with a series of unsaturated fatty acids for which a high degree of specificity was exhibited which was related to the position, configuration, and number of double bonds in the compound. A steady-state kinetic analysis shows that under some conditions, the kinetics of the NADH reduction of dihydroxyacetone phosphate by NADH in the presence of stearic acid do not follow simple Michaelis-Menten behavior but rather the velocity shows a sigmoidal dependence on fatty acid concentration and strong substrate inhibition. Stearic acid is a much poorer inhibitor of the NAD-dependent oxidation of glycerol-3-phosphate. At low substrate concentrations stearic acid is competitive with respect to NAD with an inhibition constant of 24 μM for stearic acid. In addition to the effect of fatty acids on the initial velocities of the enzyme-catalyzed reactions, preincubation of the enzyme with fatty acid leads to a slow, time-dependent irreversible inactivation of the enzyme which is prevented by the presence of NADH. The results are discussed in terms of the differences in the conformations of the hydrophobic binding sites on the two enzymes.

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Abbreviations used: glycerol-3-*P*, *sn*-glycerol-3-phosphate.

Glycerol-3-phosphate dehydrogenase (*sn*-glycerol-3-phosphate:NAD^+ 2-oxidoreductase, EC 1.1.1.8) has been found to exist in multiple molecular forms or isozymes in the tissues of various species [1—3]. The isozyme obtained from rabbit skeletal muscle has been relatively well-characterized yet the differences between it and the other rabbit isozymes have not been clearly defined. Lee and Choy [14] have shown that antibody prepared against the liver enzyme crossreacts with the enzyme from skeletal muscle but does not cross-react with the enzyme from heart tissue. On the other hand, studies of the amino acid compositions have shown [5] substantial differences between the liver and muscle enzymes although other workers have reported no differences in amino acid composition or isoelectricfocusing behavior between these forms of the enzyme [6]. The structural differences are thus unclear although distinct kinetic properties have been described for the rabbit liver and muscle isozymes [7,8].

Rabbit muscle glycerol-3-phosphate (glycerol-3-*P*) dehydrogenase is strongly inhibited by long chain alkyl compounds which interact at the coenzyme binding site [9] and its affinity for hydrophobic compounds has been exploited in the development of purification schemes [10]. In this report we present the results of a comparative study of the effect of a series of fatty acids on the kinetic properties of purified rabbit liver and skeletal muscle glycerol-3-*P* dehydrogenase. The fatty acids were used as molecular probes of the hydrophobic binding sites and the results are interpreted in terms of the topological features of the two enzymes.

Materials and Methods

Rabbit liver glycerol-3-*P* dehydrogenase was prepared from livers of New Zealand does [8]. The rabbit muscle enzyme and all biochemicals were obtained from Sigma Chemical Co. (St. Louis, Mo. U.S.A.). Stock solutions were prepared by dissolving the enzyme, which was stored as a suspension in ammonium sulfate, in buffer followed by filtration through a 0.9 × 15 cm Bio Gel P30 column (Bio-Rad Corp., Richmond, Ca). Both the liver and muscle isozymes were found to be homogeneous as judged by polyacrylamide gel electrophoresis in the presence [11] and absence [12] of SDS. Both enzymes had nearly identical specific activities (300 units/mg) and were indistinguishable on isoelectric focusing (pI = 6.5). Fatty acids were dissolved in ethanol. The concentration of ethanol in all assays was less than 10% (v/v), a concentration found not to be inhibitory.

Enzyme activity was measured in a buffered solution containing 50 mM triethanolamine-HCl/1 mM EDTA/1 mM mercaptoethanol (pH 7.5). This buffer was used in all solutions. The assay solution contained 0.1 mM NADH, 0.3 mM dihydroxyacetone phosphate and 0.1—0.5 units enzyme (which was added last). The rate of the reaction was measured by following the oxidation of NADH at 340 nm with a Beckman Model 25 spectrophotometer maintained at 30° C. All fluorescence measurements were made with a Farrand Model MK-1 fluorescence spectrophotometer in which the reactions were followed using an excitation wavelength of 340 nm with 5 nm slits and an emission wavelength of 460 nm with 10 nm slits. Reactions were carried out in 10 nm diameter

cuvettes containing 2 ml buffered solution in an eight-position turret cell holder maintained at 30° C.

Results

Saturated fatty acids were found to be effective inhibitors of both liver and muscle glycerol-3-*P* dehydrogenase. For a series of fatty acids ranging in chain length from 10—22 carbon atoms, the amount of fatty acid necessary to produce a 50% decrease in the original enzyme activity was found to be in the micromolar concentration range. In general, the inhibition increased with increasing chain length of the fatty acid although there are some exceptions to this trend, namely for lauric, stearic and arachidic acid inhibition of the muscle enzyme. As shown in Fig. 1, the logarithm of the inhibition constant is linearly related to the length of the fatty acid for the liver enzyme but for the muscle enzyme the plot exhibits a maximum and a minimum. Thus the two forms of the enzyme differ in specificity for saturated fatty acids.

The inhibition of the muscle and liver forms of the enzyme was also studied using a series of unsaturated fatty acids. Table I shows the effects of some monounsaturated fatty acids on the activity of the enzyme. As with the saturated fatty acids, quantitative differences are found in the behavior of the 2 isozymes. The degree of inhibition by the fatty acids is dependent upon the

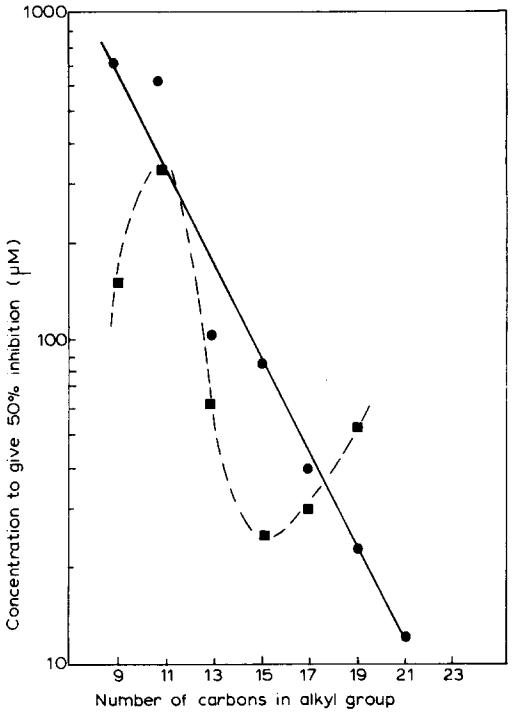

Fig. 1. Inhibition of glycerol-3-*P* dehydrogenase as a function of the chain length of fatty acids. The concentration of fatty acid required to give 50% inhibition of muscle (dashed line) or liver (solid line) enzyme is plotted versus the chain length of the alkyl portion of the fatty acid.

TABLE I

INHIBITION OF GLYCEROL-3-*P* DEHYDROGENASE BY MONOUNSATURATED FATTY ACIDS

Fatty acid number of carbon atoms	Name	Double bond	Concentration to give 50% inhibition * (μM)	
			Liver	Muscle
14	Myristoleic	cis-9	500	145
16	Palmitoleic	cis-9	160	48
18	Oleic	cis-9	38	25
18	Elaidic	trans-9	>>300	>500
18	Vaccenic	cis-11	58	20
20	Eicosenoic	cis-11	200	45
22	Erucic	cis-13	150	— **
24	Nervonic	cis-15	14	— **

* The concentration of fatty acid required to give 50% inhibition was determined by interpolation of a series of assays carried out at several concentrations of fatty acid.

** Not determined.

number of carbon atoms and the position and configuration of the double bond. In general the inhibition was more pronounced with the longer chain members of the series although several exceptions to this are evident. Elaidic, eicosenoic and erucic acids were found to be relatively poor inhibitors. An especially striking degree of specificity is seen in comparing the 18-carbon cis isomer (oleic) with the trans isomer (elaidic) which was found to require more than an order of magnitude higher concentration to achieve the same degree of inhibition. A change in the position of the double bond from C-9 in oleic acid to C-11 in vaccenic acid increases the concentration necessary for inhibition of the liver enzyme by about 50% but has a smaller effect on the muscle form of the enzyme.

Fatty acids in Table II are arranged to depict the effect of increasing the degree of unsaturation for the 18-carbon fatty acids. The inhibition constant remains essentially unchanged in this series for both the liver and muscle forms with oleic acid being a slightly more potent inhibitor than the other derivatives.

In addition to the immediate inhibition of the muscle and liver forms of the enzyme by fatty acids, a slow time-dependent inactivation was also found. Fig. 2 shows the activity remaining after the enzymes were preincubated with palmitic acid for various periods of time and then added to an assay solution. It was observed that both muscle and the liver forms of the enzyme were

TABLE II

INHIBITION OF GLYCEROL-3-*P* DEHYDROGENASE BY A SERIES OF 18-CARBON FATTY ACIDS

Fatty acid		Concentration to give 50% inhibition (μM)	
		Liver	Muscle
Stearic	(18 : 0)	40	31
Oleic	(18 : 1)	38	25
Linoleic	(18 : 2)	56	47
Linolenic	(18 : 3)	60	50

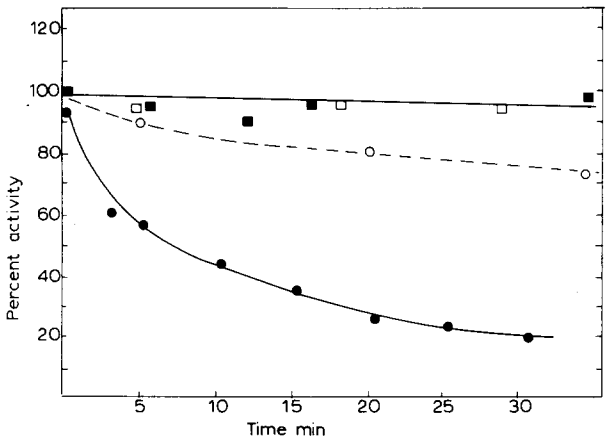

Fig. 2. Dependence of enzyme activity on preincubation time with palmitic acid. Rabbit muscle (○) or liver (●) enzyme (0.1 μM) was incubated at 30°C with 80 μM palmitic acid/50 mM triethanolamine/1 mM EDTA/1 mM mercaptoethanol (pH 7.5) for the indicated times and the diluted 20-fold for determination of enzymic activity. The preincubation was also conducted with the muscle (□) and liver (■) enzyme in the presence of 0.1 mM NADH.

inactivated in the presence of palmitic acid although the muscle enzyme appears to be more stable than the liver form. The presence of 0.1 mM NADH protected both forms of the enzyme from inactivation.

Initial velocity measurements were made as a function of substrate, coenzyme, and stearic acid concentrations using liver glycerol-3-*P* dehydrogenase. Fig. 3 shows the relationship between initial velocity and stearic acid

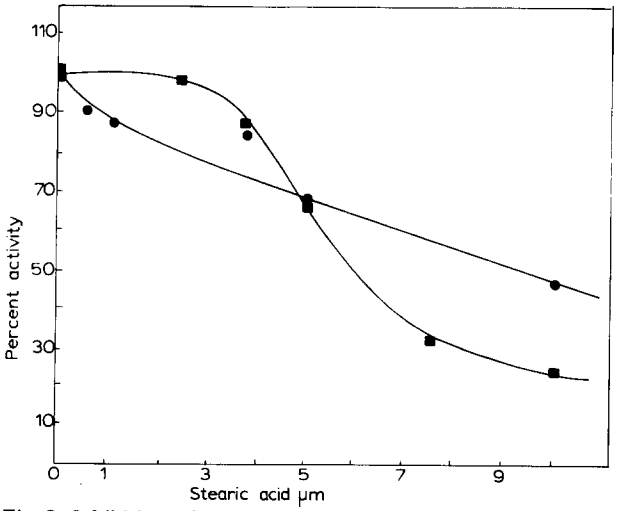

Fig. 3. Inhibition of the NADH catalyzed reduction of dihydroxyacetone phosphate reaction by stearic acid. Plot of percentage initial activity v. micromolar concentration of stearic acid. The concentrations of NADH were 5 μM (●) and 15 μM (■) and the concentration of dihydroxyacetone phosphate was 100 μM. The reaction mixture contained 2 ml of 50 mM triethanolamine buffer (pH 7.5)/2% (v/v) ethanol and the indicated amounts of dihydroxyacetone, NADH and stearic acid. The reaction was initiated by addition of an identical amount of enzyme to each reaction mixture and the decrease in fluorescence of NADH was followed with respect to time.

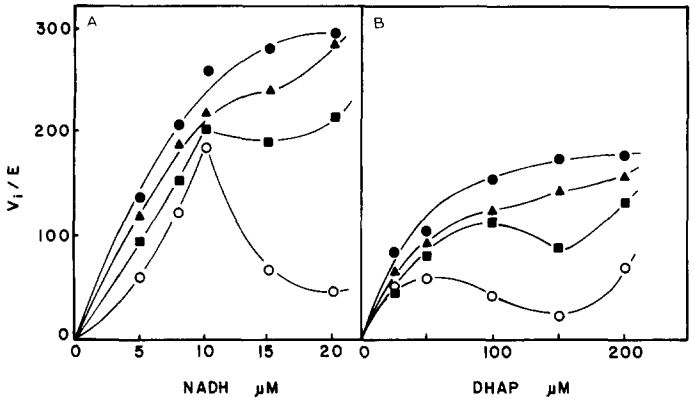

Fig. 4. A. Inhibition by stearic acid with NADH as the variable substrate. Plot of initial velocity per enzyme unit (V_i/E) v. micromolar concentration of NADH. The following concentrations of stearic acid were used: no stearic acid (●); 2.56 μM (▲); 5.14 μM (■); 10.2 μM (○). The concentration of dihydroxyacetone phosphate was constant at 100 μM. The reaction conditions were identical to those in Fig. 3. The units on the ordinate are μmol NADH consumed per min per enzyme unit. B. Inhibition by stearic acid with dihydroxyacetone phosphate as the variable substrate. Plot of initial velocity per enzyme unit (V_i/E) v. micromolar concentration of dihydroxyacetone phosphate (DHAP). The following concentrations of stearic acid were used: no stearic acid (●); 2.42 μM (▲); 4.84 μM (■); 9.68 μM (○). The concentration of NADH was constant at 3 μM. The reaction conditions were identical to those in Fig. 3. The units on the ordinate are μmol NADH consumed per min per enzyme unit.

concentration. For the sake of clarity, the curves at only two concentrations of NADH are included in Fig. 3 but these serve to illustrate the change from a hyperbolic to sigmoidal dependence of velocity on stearic acid concentration as the NADH concentration is increased from 5 to 15 μM.

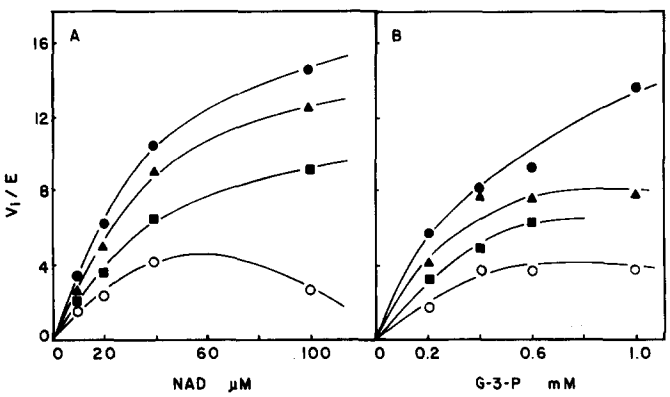

Fig. 5. A. Inhibition by stearic acid with NAD as the variable substrate. Plot of initial velocity per enzyme unit (V_i/E) v. μM concentration of NAD. The following concentrations of stearic acid were used: no stearic acid (●); 9.9 μM (▲); 29.1 μM (■); 47.6 μM (○). The concentration of *sn*-glycerol-3-phosphate was constant at 1 mM. The reaction mixture contained 2 ml 50 mM triethanolamine buffer (pH 7.5) and the indicated amount of *sn*-glycerol-3-phosphate, NAD and stearic acid. The ethanol concentration was 2%. The reaction was initiated by addition of a known amount of enzyme and the increase in fluorescence of NADH was followed with respect to time. B. Inhibition by stearic acid with *sn*-glycerol-3-phosphate as the variable substrate. Plot of initial velocity per enzyme unit (V_i/E) vs. μm concentration of *sn*-glycerol-3-phosphate (G-3-P). The following concentrations of stearic acid were used: no stearic acid (●); 9.9 μM (▲); 19.6 μM (■); 29.1 μM (○). The concentration of NAD was constant at 80 μM. Reaction conditions were identical to those in Fig. 5A.

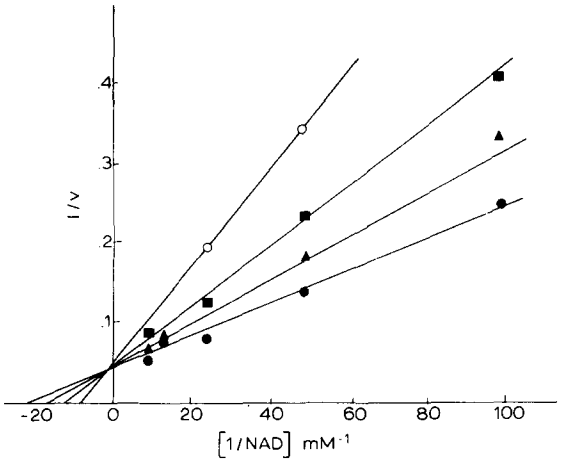

Fig. 6. Double reciprocal plot with stearic acid as inhibitor and NAD as the varied substrate. The following concentrations of stearic acid were used: no stearic acid (●); 9.9 μM (▲); 29.1 μM (■); 47.6 μM (○). The concentration of *sn*-glycerol-3-phosphate was constant at 1 mM. The reaction conditions were identical to those in Fig. 5.

Initial velocity measurements were also carried out as a function of substrate and coenzyme concentrations at various fixed concentrations of stearic acid. Fig. 4 illustrates the effect of varying NADH and DHAP on the velocity of the enzymic reaction. In the absence of stearic acid, the velocity is a hyperbolic function of NADH concentration, however, as the concentration of stearic acid is increased, the curves demonstrate increasingly strong substrate (NADH) inhibition. With DHAP, substrate inhibition is also seen at the highest concentrations of fatty acid.

The initial velocity as a function of substrate concentration was measured in the presence of stearic acid for the oxidation of glycerol-3-P by NAD as shown in Fig. 5. With either NAD or glycerol-3-P as the variable substrate, stearic acid was a negative effector for the reaction. At the lowest concentrations of NAD and stearic acid, the fatty acid appears to behave as a simple competitive inhibitor with respect to NAD (Fig. 6). The K_i value for stearic acid was calculated to be 24 μM.

Discussion

A comparative study was carried out on the inhibitory properties of a series of fatty acids toward the muscle and liver isozymic forms of glycerol-3-*P* dehydrogenase. In general, the muscle form of the enzyme was found to be inhibited more strongly than the liver form for nearly every fatty acid studied. For the liver enzyme the effectiveness of the inhibition increases directly with increasing chain length of the alkyl substituent in the series of saturated fatty acids from capric to behenic acid. This characteristic was not observed for the muscle enzyme. The differences between the two forms of the enzyme toward inhibition by fatty acids is exemplified in that a linear relationship between the logarithm of the inhibition constant and the number of carbons in the alkyl group of the inhibitor is obtained with liver glycerol-3-*P* dehydrogenase

whereas a nonlinear, S-shaped curve characterizes the muscle form of the enzyme. If it is assumed that the change of the logarithm of the inhibition constant (I_{50}) is linearly related to the free energy change by the following relationship,

$$\Delta\Delta G = -RT\,\Delta \ln I_{50} \quad (1)$$

where I_{50} represents the concentration of fatty acid required to give 50% inhibition of the enzyme activity, then the increment in free energy change, $\Delta\Delta G$, accompanying the interaction of a methylene group with the enzyme can be calculated. This value measures the energy change associated with the transfer of an aliphatic methylene group from its free state in solution to the state of absorption on the enzyme surface. A value of —0.20 kcal/mol is calculated per methylene group adsorbed onto the liver enzyme. This compares to the value of —0.38 kcal/mol obtained by Kim and Anderson [9] for the inhibition of the muscle enzyme by short chain fatty acids. These values are within the range suggested for interactions through dispersion forces [13] and are similar to the values obtained for hydrophobic interactions with yeast alcohol dehydrogenase [14,15] and with serum and eel cholineesterase [16]. The linear relationship between the logarithm of the inhibition constant and the alkyl chain length observed for the liver enzyme suggests that within this homologous series each additional methylene group produces the same increase in affinity. The structure of the surface on the liver enzyme therefore appears to be a large hydrophobic area or pocket where no steric restrictions are imposed on unbranched saturated fatty acids with a length of at least 22 carbons. In contrast to this, the muscle form of the enzyme does not bind aliphatic chains with the same affinity per methylene group over the entire range of fatty acid studied. This suggests the presence of a constriction or hydrophilic group on the enzyme at the binding site.

Unsaturated fatty acids generally are less effective than saturated fatty acids for inhibition of the enzyme. The inclusion of a double bond in the inhibitor may lessen the hydrophobic interactions between the effector and the enzyme. On the other hand, oleic acid is equally as effective an inhibitor as stearic acid toward both isozymes. Thus the conformation of the fatty acids is critical for interaction with the hydrophobic pocket and suggests that a bend may occur which accommodates the double bond in the pocket at approx. 9 carbons removed from the carboxyl group binding site of the fatty acid. Furthermore, for effective inhibition, the double bond must possess a cis configuration (oleic) rather than a trans configuration (elaidic). The introduction of additional double bonds into the fatty acids, as in the case of linoleic and linolenic acids, has little effect on the inhibition constant. The hydrophobic pocket on the enzymes may therefore conform to the stable conformation of these commonly occurring fatty acids. This idea is supported by the studies with the less commonly occurring fatty acids although distinctive specificities characterize the muscle and liver isozymes.

The concentration of fatty acids necessary to cause 50% inhibiton was in all cases less than the critical micelle concentration of the inhibitor. In general the critical micelle concentrations are slightly higher for unsaturated fatty acids than for the saturated compounds. In addition, the critical micelle concentra-

tion for elaidic acid is nearly identical to that of oleic acid [17] and thus the difference in inhibition by these two compounds is not due to simple differences in hydrophobicities but rather indicates a specificity for the cis derivative.

Glucokinase and phosphofructokinase have been reported [18,19] to exhibit a slow time-dependent inactivation when incubated with palmitic acid. This same phenomenon was found when glycerol-3-*P* dehydrogenase was incubated with this fatty acid. When the enzyme was preincubated with palmitic acid and then diluted into an assay solution, we observed a time-dependent inactivation which was not reversible by dilution, but was prevented by the presence of NADH. This result is consistent with the idea that hydrophobic compounds compete with coenzyme binding as found in this study and reported previously by Kim and Anderson [9] for short-chain fatty acids acting on muscle glycerol-3-*P* dehydrogenase.

The steady-state analysis of the liver enzyme kinetics demonstrates the effect of stearic acid on the catalytic activity of the enzyme. Kim and Anderson [9], in their study of the effect of fatty acids on the muscle enzyme, found that the fatty acids were competitive with respect to NAD. The same results were found here for stearic acid when the substrate concentrations were low. However, at higher concentrations of NAD and glycerol-3-*P* than those used by Kim and Anderson [9] and at high concentrations of stearic acid, significant deviations from simple competitive behavior were noted. In the presence of fatty acids, substrate inhibition was observed especially with NADH and dihydroxyacetone phosphate. Noteworthy is the fact that at a high concentration of stearic acid the curve describing the dependence of velocity on NADH is bell-shaped in contrast to the dependence of velocity on the concentration of the other substrates.

The unique shape of the substrate inhibition curves (Fig. 4) severely restricts the types of mechanisms which may apply to this inhibition. Such inhibition may result from binding of the substrate to the wrong enzyme form, from multiple substrate binding sites or from a change in kinetic mechanism at higher substrate concentrations. A mechanism explaining the dependence of velocity on the concentration of NADH would require at least a third-power dependence on the concentration of NADH. One way to achieve such a dependence is through the existence of three or more binding sites for NADH although binding studies show no evidence for this in the (dimeric) rabbit muscle enzyme [20,21]. Alternatively, kinetic models are capable of generating greater than a first-power dependence on substrate concentration with only a single substrate binding site. In particular, Ferdinand [22] has shown that an alternative pathway mechanism for a 2-substrate enzyme gives rise to apparent substate activation and inhibition. Such a mechanism is capable of explaining a variety of substrate saturation curves with up to two inflection points such as seen with NADH (Fig. 4). Since the substrate activation and inhibition only become marked in the presence of fatty acid, the effect of fatty acid within the context of this model would be to change the enzyme mechanism to an alternate pathway model in which the rate limiting step precedes the dehydrogenation step. Studies of rabbit muscle glycerol-3-*P* dehydrogenase have established [23,24] that this enzyme follows a sequential ordered mechanism under most

conditions. However, Bentley and Dickinson [24] have shown that at certain pH values and with the pseudo-substrate glyoxylate, the enzyme mechanism changes from a highly ordered to a more random addition of substrates. Therefore if fatty acids brought about a decrease in the rate of some of the substrate-binding steps, an alternate pathway mechanism could result along with the type of substrate inhibition that is observed. Additional kinetic and equilibrium binding studies would be necessary to establish the existence of such a mechanism.

Numerous genetic and physico-chemical studies have established the importance of isozymes in cellular differentiation and regulation. In the case of mammalian glycerol-3-*P* dehydrogenase, the existence of isozymic forms is without dispute although the exact number and properties of the isozymes is not clearly defined in all cases. The enzymes from rabbit and rat tissues have been the most thoroughly studied. Fondy and his coworkers have shown [3] the existence of multiple forms in the rat and Lee and Choy [4] have shown immunological differences between the rabbit heart and muscle isozymes. Conflicting reports have occurred regarding the chemical differences between the rabbit muscle and liver enzymes [5,6] although there appear to be discernible differences in kinetic properties [7,8]. The studies reported herein support the idea that the rabbit muscle and liver enzymes are functionally and structurally distinct. Furthermore, the strong inhibition of both isozymes and the high degree of specificity exhibited for certain fatty acids suggest that the commonly occurring fatty acids may play a role in the in vivo regulation of glycerol-3-*P* dehydrogenase as suggested for some glycolytic enzymes [25,18].

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