Studies on UDPG-Glycogen Transglucosylase. I. Preparation and Differentiation of Two Activities of UDPG-Glycogen Transglucosylase from Rat Skeletal Muscle*

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Two distinctly different activities of UDPG-glycogen transglucosylase were prepared from rat muscle in crude form. One activity did not require glucose-6-P, although stimulation could be detected at low concentrations of UDPG. This activity could be prepared by incubating a crude lyophilized enzyme preparation for 60 to 75 minutes at 30° in 0.05 M mercaptoethanol. The other activity, which was dependent on the presence of glucose-6-P, could be prepared by aging the muscle in the frozen state and by obtaining the enzyme associated with the 100,000 × *g* particulate fraction. Both activities were differentiated by kinetic measurements of the UDPG dependence in the presence of glucose-6-P and Mg^{++}. The apparent K_m under the varying conditions is given. Mg^{++} strongly stimulated the glucose-6-P independent activity, lowering the apparent K_m with no alteration in V. Glucose-6-P, Mg^{++}, or both together had the same action. The glucose-6-P dependent activity was not stimulated by Mg^{++} unless glucose-6-P was present. The stimulation due to glucose-6-P resulted from greatly increasing the V and, perhaps, from slightly decreasing the K_m.

Villar-Palasi and Larner (1960a,b, 1961) demonstrated that extracts prepared from rat hemidiaphragms incubated with insulin exhibited increased UDPG-glycogen transglucosylase[1] activity when measured in the absence of glucose-6-P. When measured in the presence of glucose-6-P, activities of extracts of control and insulin-treated diaphragms were both increased and did not differ. This increase in enzyme activity without glucose-6-P was also observed after diaphragms were incubated with insulin in the absence of glucose in the medium.

Steiner *et al.* (1961) reported a marked increase in transglucosylase activity in the livers of alloxan diabetic rats 2 to 4 hours after the injection of insulin. The differences noted in tissue extracts after pretreatment with insulin demonstrated an increased enzyme activity and a decreased sensitivity to glucose-6-P in the case of muscle. Differences in sensitivity to glucose-6-P did not appear to be explained by the total glucose-6-P content of the various tissue extracts or enzyme preparations (Villar-Palasi and Larner, 1961; Steiner *et al.*, 1961; Illingworth *et al.*, 1960; Rosell-Perez and Larner, 1962).

These facts suggested the possible existence of two different enzymic activities of UDPG glycogen transglucosylase, one acting independently of glucose-6-P and the other requiring this co-factor for activity.

The present paper describes the preparation in crude form of two different transglucosylase activities[2] from rat skeletal muscle. Kinetic studies are presented that clearly differentiate these two activities with regard to activation by both glucose-6-P and Mg^{++}. In the following paper (Rosell-Perez and Larner, 1962), studies with frog and toadfish muscle demonstrate that there is a marked species variation in the sensitivity of the enzyme to stimulation by glucose-6-P. This difference is also independent of the glucose-6-P content of the tissue.

MATERIALS AND METHODS

Analytical.—Glucose-6-P was determined with glucose-6-P dehydrogenase and TPN (as previously described) (Larner *et al.*, 1959). Transglucosylase activity was measured as radioactivity incorporated into glycogen from C^{14} glucose-labeled UDPG. The method, a modification of that of Schmid *et al.* (1959), has been described (Villar-Palasi and Larner, 1961). In

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[1] Abbreviations and short forms used are UDPG (uridinediphosphoglucose), TPN (triphosphopyridine nucleotide), Versene (ethylenediaminetetracetic acid), Tris (tris(hydroxylmethyl)aminomethane), Et-SH (mercaptoethanol), glucose-6-P (glucose-6-phosphate), PHMB (*p*-hydroxymercuribenzoate), transglucosylase (uridinediphosphoglucose glycogen transglucosylase), 3′,5′-AMP (cyclic adenylic acid), ATP (adenosine triphosphate).

[2] The use of the term "activity" rather than "form" is preferred at present because a distinction at the molecular level between the possibilities previously discussed (Villar-Palasi and Larner, 1961) is in progress (Friedman and Larner, 1962).

TABLE I
PURIFICATION OF RAT UDPG-GLYCOGEN TRANSGLUCOSYLASE[a]

Fraction	Volume (ml)	Activity (μmole/ml/hr)		Specific Activity (μmole/mg Prot/hr)		Recovery (%)		Ratio +G-6-P
		−G-6-P[b]	+G-6-P[c]	−G-6-P	+G-6-P	−G-6-P	+G-6-P	−G-6-P
1. Homogenate (1:10 w/v)	9,225	2.3	9.6	0.14	0.60	100	100	4.3
2. Supernatant fluid (Lourdes)	7,835	4.6	10.4	0.60	1.5	170[d]	92	2.5
3. $(NH_4)_2SO_4$ precipitate resuspended 1:10	760	18.2	51.6	0.64	1.81	65.8	45	2.8
4. Dialyzed fluid	750	17.8	38.2	0.72	1.5	63.5	32.5	2.1
5. Lyophilized powder	62.3 g							
6. Suspension 20 mg/ml		6.6	11.1	1.0	1.7	96.5[e]	41.6[e]	1.7
7. Supernatant fluid (International)		4.2	7.0	1.3	2.1	60	23.4	1.6
8. 100,000 × *g* particle fraction resuspended		4.4	7.6	8.6	15.5	63.3	24.9	1.8

[a] Purification steps as described in the text. [b] −G-6-P = assayed in the absence of glucose-6-P. [c] +G-6-P = assayed in the presence of glucose-6-P. [d] Increased activity in the absence of glucose-6-P may be explained by an enzymic transformation (Friedman and Larner, 1962). [e] Increased recovery of enzyme activity may be explained by activation by traces of $SO_4^=$ still remaining after dialysis (Rosell-Perez, 1962).

the present studies the reaction mixtures were as follows: buffer, 0.03 M Tris–0.003 M Versene; primer glycogen, 0.46%; 0.01 M glucose-6-P (when added); 0.008 M $MgCl_2$ (when added); UDPG at the indicated concentrations in the kinetic studies and 4.2×10^{-3} M in standard experiments. The total volume was 0.35 ml, and incubations were made at 30° for 5 to 10 minutes. Buffers were routinely neutralized with HCl to *p*H 7.8. Radioactivity was measured in an automatic gas flow counter and corrected for self-absorption to convert to μmole/mg of protein per hour.

Chemicals.—Glucose-6-P was supplied by Nutritional Biochemicals Corporation. Mercaptoethanol was obtained from Eastman Kodak Company; *p*-hydroxymercuribenzoate from Sigma Chemical Company; and Versene from Fisher Scientific Company as the disodium salt. Tris was obtained from J. Frederick Smith Chemical Company and was recrystallized one time from ethanol as described by Sutherland and Wosilait (1956).

EXPERIMENTAL

Enzyme Preparations and Purifications.—Transglucosylase was prepared from rat skeletal muscle, and the procedure can be summarized as follows:

1. Muscle homogenate: Muscle dissected from the hind legs and backs of Wistar strain rats was frozen in liquid nitrogen. Frozen muscle was homogenized in 10 volumes of 0.05 M Tris–0.0025 M Versene (*p*H 8.2) in the Waring Blendor.

2. Extract: The homogenate was centrifuged for 40 minutes at 8000 × *g* in the Lourdes refrigerated centrifuge. The supernatant fluid was recovered and had a *p*H of 7.8.

Precipitation with $(NH_4)_2SO_4$: Solid ammonium sulfate was added to the supernatant fluid to a concentration of 50%. While the salt was added, the solution was stirred mechanically for 1 hour (temperature: 0° to 3°). The precipitate was then allowed to settle overnight at the same temperature.

4. Dialysis: The precipitate was suspended in one-tenth of the original volume of buffer (0.05 M Tris–0.005 M Versene, *p*H 8.2) and was dialyzed for 3 hours against 0.002 M Versene brought to *p*H 8.0 with KOH.

5. Lyophilization: The dialyzed extract was lyophilized, and the fluffy material was ground to a fine powder and stored in the deep-freeze. It maintained its activity for a period of 3 months or longer. Direct enzymic analysis for glucose-6-P revealed only traces in the enzyme powder. Three analyses of two different preparations gave values that ranged from 4.8×10^{-6} M to 1.5×10^{-5} M when a 20 mg/ml suspension was analyzed.

The lyophilized powder (20 mg/ml) was suspended in 0.05 M Tris–0.005 M Versene buffer (*p*H 7.3–7.8), and was homogenized by hand in a glass homogenizer which was cooled in ice. It was then centrifuged at 3000 rpm in the International refrigerated centrifuge for 20 minutes. The activity remained in the supernatant fluid. If this supernatant fluid was recentrifuged in the Spinco centrifuge (Model L Preparative) at 100,000 × *g* for 2 hours, a very small, glassy pellet was obtained. When resuspended in 0.05 M Tris–0.005 M Versene–0.05 M mercaptoethanol buffer, the pellet was found to have the activity. The supernatant fluid had very little or no activity (Steps 6, 7, and 8, Table I). In Table I, two activities are recorded, one in the absence of glucose-6-P and the other in the presence of glucose-6-P. The ratio of activities with and without glucose-6-P was lowered during purification. The recoveries and purifications of the two activities were different.

Differential Inhibition by p-Hydroxymercuribenzoate.—SH groups of the enzymic protein ap-

peared to be involved in the activity because previous reports (Breckenridge and Crawford, 1960; Robbins *et al.*, 1959; Leloir and Goldemberg, 1960; Leloir *et al.*, 1959) showed a stimulation and stabilization of the enzyme in the presence of cysteine. Also, the recovery of activity in the Spinco centrifuge particulate fraction (100,000 × *g*) was increased in the presence of 0.05 M mercaptoethanol.

Further studies were done with an SH inhibitor. When the enzyme was treated with *p*-hydroxymercuribenzoate, there was an inhibition that was different when measured in the presence or absence of glucose-6-P (Fig. 1). After incubation (5 minutes at 30°) in the presence of 1×10^{-5} M *p*-hydroxymercuribenzoate, the activity in the absence of glucose-6-P disappeared; while in presence of this sugar phosphate, 30% remained. With 4.5×10^{-5} M *p*-hydroxymercuribenzoate, both activities were inhibited completely. Higher concentrations of *p*-hydroxymercuribenzoate (4×10^{-4} M) did not inhibit in the presence of 0.05 M mercaptoethanol.

Preparation of Glucose-6-P Independent[3] *Activity.*—Observations made after treatment of the suspension of lyophilized powder with mercaptoethanol showed that the enzyme activity was higher after a 5-minute incubation at 30° than at zero time. This led to experiments on the time course of enzyme activation in the presence of 0.05 M mercaptoethanol. Surprising results were obtained. Figure 2 shows the time course of enzyme activation in these experiments.

The activity of the enzyme incubated with mercaptoethanol measured without glucose-6-P increased with time, in contrast to the control incubated without mercaptoethanol. At the end of 1 hour of incubation in the presence of mercaptoethanol all the activity that was present was not stimulated by glucose-6-P. The activity stimulated by glucose-6-P had disappeared. The enzyme activity had been altered so that it did not respond to stimulation by glucose-6-P. Analysis of the glucose-6-P content of the enzyme before and after incubation with mercaptoethanol showed that there was no significant increase in this sugar phosphate accompanying the change in enzyme activity. Assuming a K_m for glucose-6-P of 6×10^{-4} M (Leloir *et al.*, 1959; redetermined in our laboratory), the initial content of glucose-6-P required to account for the activity in the absence of glucose-6-P would be 1.0×10^{-3} M, and after incubation with mercaptoethanol, it would be 3×10^{-3} M. The experimental values

[3] To distinguish the two types of enzyme activity we have selected the terms "independent" (I) and "dependent" (D). Although the independent enzyme is stimulated by glucose-6-P at low UDPG concentrations, it is active under these conditions without glucose-6-P. On the other hand, at high UDPG concentrations it is fully active without this sugar phosphate. These terms do not imply that glucose-6-P is the only anion that may be used to distinguish these two different enzyme activities.

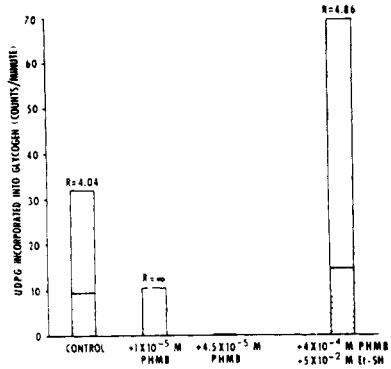

FIG. 1.—Inhibition of rat muscle transglucosylase by *p*-hydroxymercuribenzoate. After the inhibitor was added the enzyme preparations were incubated at 30° for 5 minutes and then assayed. R = ratio of activity +glucose-6-P/−glucose-6-P. Crossed-hatched bars represent activity in the absence of glucose-6-P.

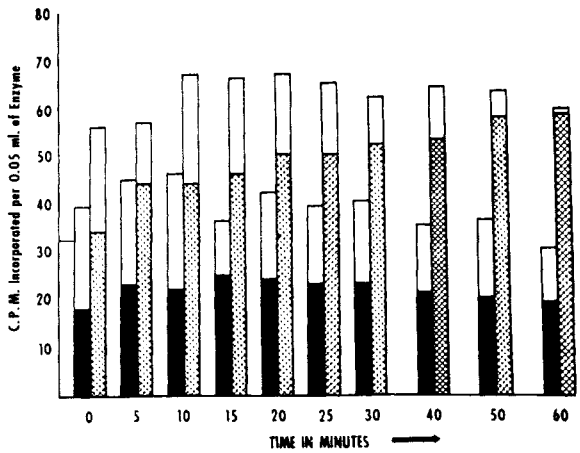

FIG. 2.—Time course of incubation of rat muscle transglucosylase [$(NH_4)_2SO_4$ powder] in the presence of 0.05 M mercaptoethanol. Total height of the bars represents activity in the presence of added glucose-6-P. Black bars represent activity in the absence of glucose-6-P and mercaptoethanol. Cross-hatched bars represent activity after mercaptoethanol incubation without glucose-6-P added. Enzyme assays as described in the text.

obtained were 1.1×10^{-5} M and 1.2×10^{-5} M, which are about 100- to 300-fold lower than those calculated. This indicated clearly that the activity present without added glucose-6-P was not accounted for by the glucose-6-P content of the enzyme and, further, that the increase in activity after incubation in the presence of mercaptoethanol was not due to an increase in glucose-6-P content.

Preparation of Glucose-6-P Dependent Activity.—NaCl (0.5 M) was added to the supernatant fluid (International, Step 7, Table I). No significant difference in the ratio of activity in the presence or absence of glucose-6-P was noted (control, 1.5; NaCl, 1.3). The particulate fraction (100,000 × *g*) was then collected as previously described.

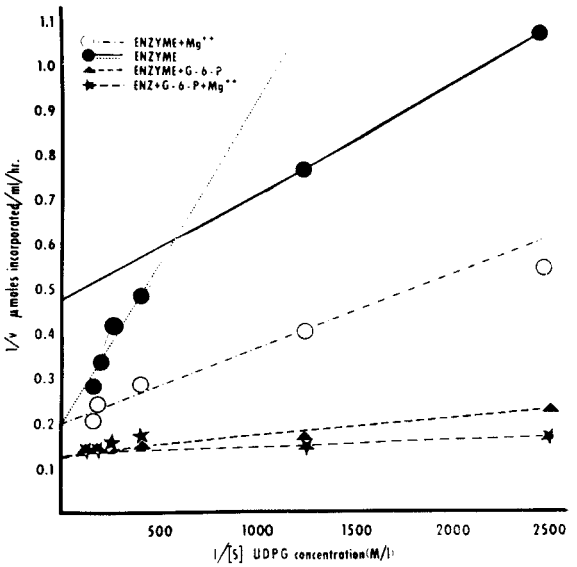

FIG. 3.—Reciprocal plot with 100,000 × *g* particle fraction obtained from ammonium sulfate crude preparation of rat muscle transglucosylase. The ratio of activity +glucose-6-P/−glucose-6-P was 3. Two slopes were drawn for the activity of the enzyme without added activator.

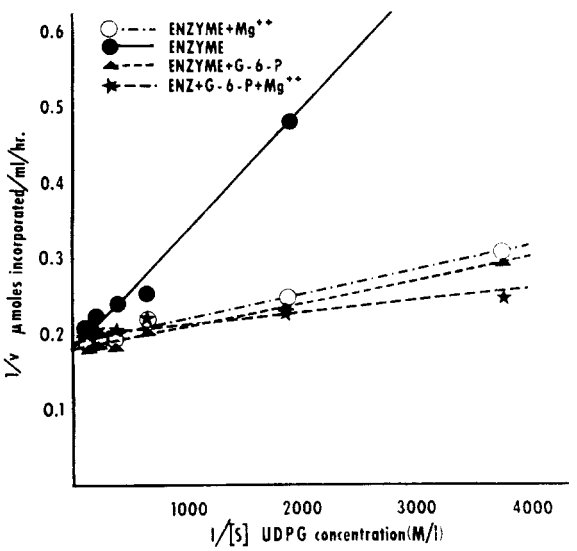

FIG. 4.—Reciprocal plot with UDPG-glycogen transglucosylase after an incubation period of 75 minutes of the ammonium sulfate powder extract in the presence of 0.05 M mercaptoethanol.

The pellet obtained from the tube containing 0.5 M NaCl was resuspended and assayed. The ratio of activity in the presence or absence of glucose-6-P had changed to 3.4, which indicates an enhanced sensitivity to stimulation by this sugar phosphate.[4]

This increased sensitivity to glucose-6-P stimulation became more apparent when aged enzyme preparations were studied. A similar effect was observed with frozen rat muscle stored in the deep-freeze. After 9 to 12 weeks of storage, the ratio of activity in the presence or absence of glucose-6-P of the 100,000 × *g* particulate fraction (suspended in Tris-Versene-mercaptoethanol buffer) rose to over 10 when assayed at the highest concentration of UDPG. In other words, after aging, the enzyme had little or no activity in the absence of glucose-6-P and was active only in the presence of this sugar phosphate.[5]

Kinetic Studies.—Kinetic studies were done to compare the two separated activities with similar enzyme preparations that had not been subjected to mercaptoethanol treatment or to aging.

A. UDPG DEPENDENCE OF THE MIXED ACTIVITIES.—Experiments were done with the suspended powder, as well as with the 100,000 × *g* particulate fraction described above. The apparent Michaelis constants were determined for UDPG, with and without glucose-6-P and with and without $MgCl_2$.

When enzyme preparations were used (suspended powder or 100,000 × *g* particulate fraction) with ratios of activity with and without glucose-6-P between 2 and 5, the reciprocal plot of activity in the absence of glucose-6-P and Mg^{++} gave curved instead of straight lines with two different slopes, as shown in Figure 3. The significance of this will be discussed later.

Linear reciprocal plots were obtained for the activities studied as a function of UDPG concentration in the presence of glucose-6-P and Mg^{++}.

B. UDPG DEPENDENCE OF THE TWO SEPARATE ACTIVITIES.

1. Kinetic studies of the glucose-6-P independent activity. Figure 4 shows the reciprocal plot of the enzymic activity after 75 minutes of incubation at 30° with 0.05 M mercaptoethanol.

Linear reciprocal plots were obtained under the conditions tested. The slope of the line obtained without any activator is similar to that obtained in the steepest slope of Figure 3. (Apparent K_m determined from the steepest slope = 3.6×10^{-3} M; K_m of this activity without glucose-6-P = 1.0×10^{-3} M.) Glucose-6-P, Mg^{++}, or both together decreased the K_m for UDPG, without a significant change in V. Activation by

[4] We do not imply that the activity of the enzyme had been transformed by this procedure. Analysis of this alteration has shown that the supernatant fluid separated from the particles contains a stimulatory anion which is inorganic phosphate. This, like $SO_4^{=}$ and sugar phosphates, stimulates the enzyme (Rosell-Perez, 1962).

[5] Incubation of this enzyme preparation in 0.05 M mercaptoethanol did not lead to the formation of a glucose-6-P independent activity as previously described with the lyophilized powder. The activation of mercaptoethanol was also not observed with the 100,000 × *g* particulate fraction obtained either from fresh muscle or from the ammonium sulfate powder (Table I, Step 8). This indicates that aging alone is not responsible for the loss of mercaptoethanol activation. These findings suggest that the activation with mercaptoethanol may be an indirect rather than a direct effect on the enzyme.

TABLE II
KINETIC CONSTANTS OF UDPG GLYCOGEN TRANSGLUCOSYLASE PREPARATIONS

		Enzyme Alone	Enzyme +G-6-P	Enz + Mg	Enz +G-6-P+Mg
G-6-P independent activity (mercaptoethanol-treated)	K_m (M UDPG)	1×10^{-3}	2×10^{-4}	1.7×10^{-4}	1×10^{-4}
	V (μmole/mg Prot/hr)	2.3	2.3	2.15	2.10
G-6-P dependent activity (Spinco particles from aged muscle)	K_m (M UDPG)	6×10^{-4} 3.6×10^{-3}[a]	4×10^{-4}	4×10^{-3}	2.5×10^{-4}
	V (μmole/mg Prot/hr)	0.27	1.8	0.38	1.8

[a] Value obtained with the first slope of the reciprocal plot in Figure 3.

glucose-6-P or Mg^{++} was noted at low concentrations of UDPG. Brown and Kornfeld (1960) reported studies in abstract form of a UDPG-glycogen transglucosylase from rabbit muscle, which appears to be similar to the glucose-6-P independent activity reported here.

2. Kinetic studies of the glucose-6-P dependent activity. Reciprocal plots of the UDPG dependence of the activity of the 100,000 × *g* particulate fraction prepared from aged muscle are shown in Figure 5.

Linear reciprocal plots were again obtained under the conditions tested. The activity of the enzyme without added glucose-6-P was extremely small even in the presence of Mg^{++}. Glucose-6-P produced a large activation. A large change in V in the presence of glucose-6-P was noted. There was very little or no stimulation in the presence of Mg^{++}. Both of these effects may be contrasted to the corresponding effects observed with the glucose-6-P independent enzyme preparation.

In Table II the kinetic constants obtained for both activities of UDPG - glycogen transglucosylase are summarized.

DISCUSSION

Two types of transglucosylase activity were prepared from rat muscle and were clearly differentiated. The preparation of the glucose-6-P independent activity was possible because of its greater stability and activation when the crude lyophilized enzyme was incubated with mercaptoethanol. The preparation of the glucose-6-P dependent activity in the 100,000 × *g* particulate fraction probably was possible because of its greater stability to storage and aging in the frozen state. In keeping with the greater stability in the presence of mercaptoethanol, the glucose-6-P independent activity appears to be more sensitive toward the SH reagent, *p*-hydroxymercuribenzoate. The various steps during the preparation of the enzyme gave different recoveries and purifications when the measurements were made in the presence or absence of glucose-6-P. If this sugar phosphate were the activator of a single enzyme, its activation might be expected to remain constant with purification.

Curved lines were observed in reciprocal plots when lyophilized powder or the 100,000 × *g* particulate fraction (Step 7 and Step 8, Table I) were used as an enzyme source. According to Reiner (1959), such kinetics may be interpreted to indicate two enzymes acting on the same substrate. When the lyophilized powder was incubated with mercaptoethanol, or when the 100,000 × *g* particulate fraction was prepared from frozen, aged muscle, the ratio of activity with and without glucose-6-P had either decreased to near 1, or increased to over 10. With these enzyme preparations, the reciprocal plots were no longer curved. Although other explanations for the curved lines may be possible, it would appear that an interpretation based on the presence of two enzymes acting on the same substrate is reasonable, since the kinetics were changed markedly

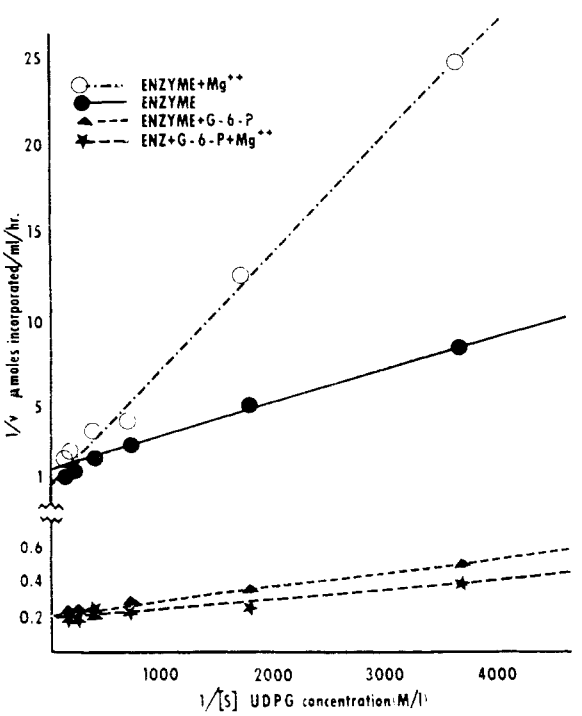

FIG. 5.—Reciprocal plot with enzyme prepared from rat muscle after aging and collecting the 100,000 × *g* particulate fraction as discussed in the text.

by treatments that did not alter the state of purity of the enzyme preparations.

The effect of glucose-6-P on the independent activity, *i.e.*, a lowering of the K_m without change in V, differs from that on the dependent activity, in which the major effect is to increase V greatly. A similar kinetic case of an altered V with no alteration in K_m was shown by Glaser and Brown (1957) for chitin synthetase activity when measured in the presence or absence of *N*-acetyl glucosamine.

The case of two enzymes acting on the same substrate and distinguishable on the basis of sensitivity to stimulation by an activator is not new. Muscle phosphorylase coexists in two forms, AMP dependent and AMP independent (phosphorylase *b* and *a*), which are interconvertible. Another case may be the phosphofructokinase of the liver fluke *Fasciola hepatica* reported by Mansour *et al.* (1961). This enzyme can be activated rapidly in soluble fractions of homogenates in the presence of 3′,5′-AMP, ATP, and Mg^{++}, with a decrease in the apparent K_m for fructose-6-P.

The experiments presented here on mercaptoethanol treatment of the crude lyophilized powder (Fig. 1), as well as experiments demonstrating enzyme activation in crude extracts before addition of ATP-Mg^{++}, suggest that a conversion reaction may be present which is opposite to that observed after ATP-Mg^{++} (Friedman and Larner, 1962).

In a recent paper Steiner (1961) presented evidence for a reversible inactivation and reactivation of liver transglucosylase. The lack of clear emphasis on simultaneous measurements of activity with and without glucose-6-P make it difficult to relate Steiner's work to that presented here. In muscle, conversion of I to D activities appears to depend on a phosphorylation reaction involving ATP (Friedman and Larner, 1962). On the other hand, the high Q_{10} of the enzyme inactivation noted by Steiner suggested a denaturation of the enzyme protein. This was associated with an altered binding to glycogen. These apparently divergent lines of evidence may be explained if a phosphorylation or dephosphorylation of the enzyme protein is associated with a conformational change that results in an altered enzyme activity and in an altered ability to bind glycogen.

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