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CHARACTERISTICS OF THE DEPHOSPHORYLATED FORM OF PHOSPHORYLASE PURIFIED FROM RAT LIVER AND MEASUREMENT OF ITS ACTIVITY IN CRUDE LIVER PREPARATIONS

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Summary

The phosphorylated form of liver glycogen phosphorylase (α-1,4-glucan : orthophosphate α-glucosyl-transferase, EC 2.4.1.1) (phosphorylase *a*) is active and easily measured while the dephosphorylated form (phosphorylase *b*), in contrast to the muscle enzyme, has been reported to be essentially inactive even in the presence of AMP. We have purified both forms of phosphorylase from rat liver and studied the characteristics of each. Phosphorylase *b* activity can be measured with our assay conditions. The phosphorylase *b* we obtained was stimulated by high concentrations of sulfate, and was a substrate for muscle phosphorylase kinase whereas phosphorylase *a* was inhibited by sulfate, and was a substrate for liver phosphorylase phosphatase. Substrate binding to phosphorylase *b* was poor (K_M glycogen = 2.5 mM, glucose-1-*P* = 250 mM) compared to phosphorylase *a* (K_M glycogen = 1.8 mM, K_M glucose-1-*P* = 0.7 mM). Liver phosphorylase *b* was active in the absence of AMP. However, AMP lowered the K_M for glucose-1-*P* to 80 mM for purified phosphorylase *b* and to 60 mM for the enzyme in crude extract (K_a = 0.5 mM). Using appropriate substrate, buffer and AMP concentrations, assay conditions have been developed which allow determination of phosphorylase *a* and 90% of the phosphorylase *b* activity in liver extracts. Interconversion of the two forms can be demonstrated in vivo (under acute stimulation) and in vitro with little change in total activity. A decrease in total phosphorylase activity has been observed after prolonged starvation and in diabetes.

Abbreviations used: MES, 2(*N*-morpholino)ethane sulfonic acid; PIPES, piperazine-*N*,*N*′-*bis*(2-ethane sulfonic acid); HEPES, *N*-2-hydroxyethylpiperazine-*N*′2-ethane sulfonic acid.

Introduction

Liver glycogen phosphorylase (α-1,4-glucan : orthophosphate α-glucosyltransferase, EC 2.4.1.1) is present both as a dephosphorylated and a phosphorylated enzyme and they are interconvertible by a kinase and phosphatase. Since first studied by Wosilait and Sutherland [1] it has generally been accepted that the dephosphorylated form is completely inactive and cannot be assayed even in the presence of AMP. A similar conclusion was reached by Appleman et al. [2] using purified enzymes from rabbit and pig liver. However, the phosphorylated form of phosphorylase is active and easily assayed in the absence of modifiers. AMP lowers the K_M for various substrates and only slightly increases the V of the purified enzyme from rabbit liver [3].

We have noted an approximately 30—40% stimulation of phosphorylase activity in liver extracts when AMP was added to the assay mixture. An assay system containing saturating concentrations of glucose-1-*P* and glycogen for the phosphorylated form of phosphorylase was used [4]. Others using slightly different assay systems [5,6] also have reported a similar amount of stimulation. In addition, we have found in our laboratory and have observed in other reported data [7], a considerable variation in the extent to which AMP stimulated phosphorylase activity in these extracts. In general, glucose administration to rats caused an increase in AMP-stimulatable activity and glucagon administration or anoxia caused a decrease in AMP-stimulatable activity. Furthermore, when phosphorylase phosphatase activity was measured in liver extract using endogenous substrate (Fig. 1) a residual amount of phosphorylase activity was always observed, an activity which had become more dependent on AMP than before the phosphatase reaction. Since the phosphorylated form of purified liver phosphorylase is only slightly stimulated by AMP [3], the reason for

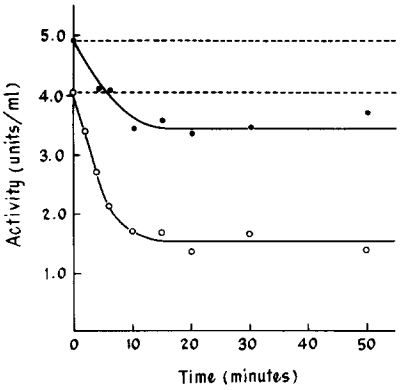

Fig. 1. Phosphorylase phosphatase activity on endogenous phosphorylase in the crude liver extract. A rat was given 25 μg/kg glucagon intravenously 15 s before sacrifice. A 1 : 3 homogenate in 50 mM glycylglycine buffer pH 7.4 was made and centrifuged at 8000 × *g* for 10 minutes. The extract was passed through a 50 ml bed volume Sephadex G-25 column and eluted with 50 mM glycylglycine (pH 7.4) [31]. The column-treated extract (now 1 : 6) was incubated at 20°C. At different time points, aliquots were diluted 1 : 5 with 50 mM MES (pH 6.1), 200 mM KF and assayed for phosphorylase activity using the standard assay. Open circles indicate phosphorylase activity in the absence of AMP; closed circles phosphorylase activity in the presence of AMP. Dotted lines are control incubations with 160 mM KF added at 0 time.

the large stimulation in liver extracts, the difference in AMP-stimulatable activity induced by hormonal or metabolic regulators, and the change in AMP dependency of phosphorylase activity upon incubation of these extracts (Fig. 1) could not be explained.

Since glucose-1-*P* concentration was low (16 mM) in the assay used by Appleman et al. [2] and high (67 mM) in our assay, the possibility was considered that the K_M of the dephosphorylated form of liver phosphorylase for glucose-1-*P* was high or that a high concentration of glucose-1-*P*, acting as a nonspecific anion, stimulated its activity similar to muscle phosphorylase *b* [8,9]. Therefore, we decided to study the kinetic properties of both forms of phosphorylase purified from rat liver with the goal of setting up an assay to measure the total amount as well as the phosphorylated form of phosphorylase in liver extracts.

Results from this study indicate that the two forms of the liver enzyme are similar in many respects to those of the muscle enzyme. Since the activity of both forms in liver can be measured under special conditions it is suggested that the nomenclature for the muscle enzymes "phosphorylase *a*" and "phosphorylase *b*", also be used for the liver enzymes. This terminology has been used in the present report.

An abstract of this work has been published previously [10].

Materials and Methods

Chemicals of the highest grade and auxillary enzymes were purchased from Sigma or Worthington Biochemical Company. Purified muscle phosphorylase kinase was obtained from Sigma as a lyophilized powder with a specific activity of 114 units per milligram of protein. A small amount of phosphorylase was found to be present as contaminant in this preparation and had to be removed. Glucagon hydrochloride was purchased as a lyophilized powder from Eli Lilly and Company and was dissolved in acid saline (0.001 M HCl) before use. Rabbit liver glycogen was routinely passed through a mixed-bed ion exchange column (Amberlite MB-3) before use. It was assayed against a glucose standard by the phenol sulfuric acid method [11] and its concentration was expressed as the molar equivalent of its glucose residues. One milligram of dry weight of glycogen per milliliter is equivalent to 5.95 mM glucose.

Glucose-1-*P*, ATP, AMP, UDPglucose, glucose-6-*P*, were prepared and standardized spectrophotometrically or enzymatically. Glucose-1-*P* and glycogen used for the assays were analyzed and found to be free from AMP (<0.001%).

Phosphorylase assays: The standard assay for liver phosphorylase was a modification of that of Danforth, Helmreich and Cori [4]. The assay mixture contained: 33 mM α(*N*-morpholino) ethane sulfonic acid (MES) buffer pH 6.1, 66.7 mM glucose-1-*P* containing tracer amounts of [^{14}C]glucose-1-*P*, pH 6.1, 40 mM glycogen, 133 mM KF. 2 mM AMP was included in the "plus" assay mixture and omitted from the "minus" assay mixture. After incubation at 30° C, the resultant labelled glycogen was measured by the method of Gilboe et al. [12].

Modified assay for liver phosphorylase a: The assay mixture contained 33

mM MES buffer pH 6.3, 15 mM glucose-1-*P* with tracer amounts of [^{14}C]glucose-1-*P*, pH 6.3, 20 mM glycogen, and 5 mM AMP.

Modified assay for liver phosphorylase b: The assay mixture contained 33 mM MES buffer pH 6.3, 273 mM glucose-1-*P* with tracer amounts of [^{14}C]glucose-1-*P*, pH 6.3, 80 mM glycogen and 5 mM AMP.

Enzyme activities also were determined in the reverse direction coupled with phosphoglucomutase and glucose-6-*P* dehydrogenase [3]. Since a high concentration of inorganic phosphate has been reported to inhibit the glucogen-6-*P* dehydrogenase activity [13], a two-step assay [9] was carried out when the inorganic phosphate in the assay was higher than 25 mM.

A unit of phosphorylase activity is expressed as one μmol of glucose-1-*P* incorporated into glycogen or glucose-1-*P* produced from glycogen per min at 30° C.

Protein determination was by the method of Lowry et al. [14], using bovine serum albumin as standard.

Results

Preparation and identification of phosphorylase a and phosphorylase b from rat liver

Enzymes were isolated according to the methods of Maddaiah and Madsen [3] and Appleman, Krebs and Fischer [2]. Results of the purification are summarized in Table I. As indicated by the ratio of activity using the standard assay with and without AMP, the purified phosphorylase *a* was only slightly stimulated by 2 mM AMP whereas phosphorylase *b* was strongly stimulated. The specific activity of the purified phosphorylase *a* was 50 μmol/mg/min, which is comparable to the reported value for rabbit liver phosphorylase *a* [3]. The specific activity of the phosphorylase *b* was 13 μmol/mg/min using the standard assay in the presence of AMP.

Incubation of the enzyme purified as phosphorylase *b* with purified rabbit muscle phosphorylase kinase, pH 8.6, in the presence of ATP · Mg^{2+} resulted in a three-fold increase in its activity (Fig. 2). The enzyme was converted from a form which is highly dependent on AMP to a form which is only slightly

TABLE I

PURIFICATION OF PHOSPHORYLASES *a* AND *b* FROM RAT LIVER*

Fraction	Phosphorylase *a*			Phosphorylase *b*		
	Total units** (μmol/min)	Units (—AMP) / Units (+AMP)	Specific activity** (μmol/min/mg)	Total units** (μmol/min)	Units (—AMP) / Units (+AMP)	Specific activity** (μmol/min/mg)
Extract	3580	0.91	0.30	2685	0.35	0.10
Glycogen pellet	665	0.85	5.93	443	0.43	1.1
Sephadex G-200	667	0.93	21.4	288	0.45	5.4
DEAE-cellulose	323	0.90	50.0	195	0.41	13.0

* Starting material: 120 g of rat liver.

** Activity measured with standard assay in the presence of AMP.

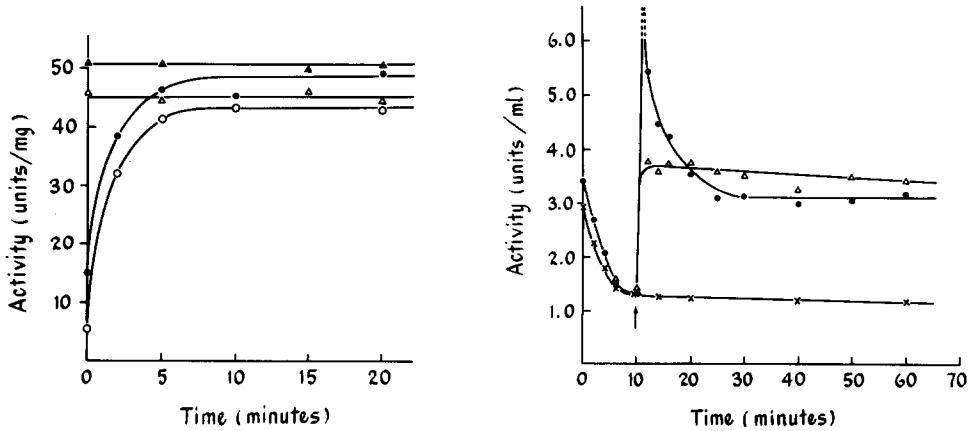

Fig. 2. Activation of purified liver phosphorylase *b* by rabbit muscle phosphorylase kinase. Incubation mixtures contained: 120 mM Tris · HCl (pH 8.6), 2 mM ATP, 20 mM magnesium acetate, 176 μg of purified muscle phosphorylase kinase, 100 μg of phosphorylase *a* (△,▲), or 175 μg of phosphorylase *b* (○,●), to give a final volume of 100 μl. Incubation was at 30°C. At different time points, aliquots were diluted and assayed immediately for phosphorylase activity using the standard assay condition. Symbols: activity measured without AMP (△,○), with AMP (▲,●).

Fig. 3. Phosphorylase phosphatase activity on purified liver phosphorylase *a* and phosphorylase *b*. Conditions of experiment were the same as that of Fig. 1. A 1 : 6 column-treated liver extract was incubated at 20°C. At 10 min of incubation, either buffer (X), or 2.7 units of purified phosphorylase *a* (●) or 1.3 units of purified phosphorylase *b* (△) was added (final volume = 0.58 ml) and incubation continued. At various time points aliquots were diluted with 50 mM MES, 200 mM KF (pH 6.1) and assayed for phosphorylase activity using the standard assay condition in the absence of AMP.

dependent on AMP, a characteristic of phosphorylase *a*. The activity of the enzyme purified as phosphorylase *a* was not changed by the kinase. Purified phosphorylase *a* was an excellent substrate for phosphorylase phosphatase in a liver extract whereas the enzyme purified as phosphorylase *b* was not acted upon by the phosphatase (Fig. 3). These data also indicated that purified phosphorylase *a* was not contaminated with phosphorylase *b* and vice versa, nor were phosphorylase kinase and phosphatase present in the purified preparations.

Appleman et al. [2] reported that phosphorylase *b* purified from pig liver was stimulated by a high concentration of Na_2SO_4 whereas phosphorylase *a* was inhibited. We also found this to be true with the two enzymes purified from rat liver using our standard assay conditions. However, we were able to detect considerable activity of phosphorylase *b* in the absence of Na_2SO_4 whereas Appleman et al. [2] reported no activity with the assay they used. The stimulation of phosphorylase *b* and inhibition of phosphorylase *a* activity by Na_2SO_4 was seen both in the absence and presence of AMP.

Based on the above evidence, it was concluded that the phosphorylase *b* we isolated from rat liver was indeed the dephosphorylated form of phosphorylase studied by other workers [2,15] and in our standard assay some phosphorylase *b* activity was being measured in the presence of AMP.

Properties of liver phosphorylases a and b

pH optimum. The pH profile of purified phosphorylase *b* was compared

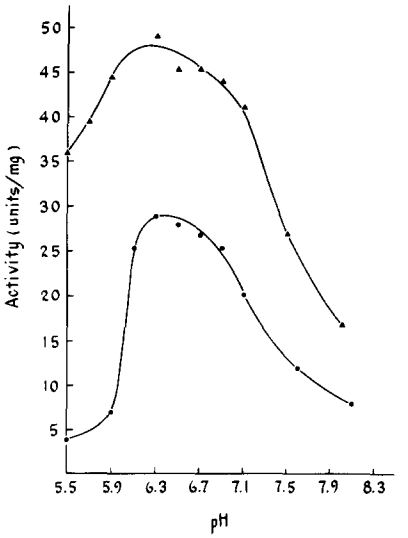

Fig. 4. pH profiles of purified phosphorylase *a* and phosphorylase *b*. For phosphorylase *a* (▲) the low substrate assay condition was used. For phosphorylase *b* (●), the high substrate assay condition was used. Buffers used were: MES, 2(*N*-morpholino)ethane sulfonic acid (pH 5.5—6.5); PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 6.5—7.0); HEPES, *N*-2-hydroxyethylpiperazine-*N*′2-ethane sulfonic acid (pH 7.0—8.0).

to that of phosphorylase *a* (Fig. 4). Both enzymes show a broad peak with maximal activity between pH 6.1 and 6.9.

Substrate binding. Kinetic parameters were determined using initial rates approximated by using low enzyme concentration and short assay time. The velocity of phosphorylase *b* activity was measured as a function of glucose-1-*P* at varying concentrations of glycogen (Fig. 5); and as a function of glycogen at varying concentrations of glucose-1-*P* (Fig. 6). The binding of glycogen to the enzyme was facilitated by the presence of glucose-1-*P*, and the binding constant was obtained at the point of convergence of the lines. Kinetic constants were calculated (Table II) from secondary plots obtained by replotting the slopes and intercepts of the above figures. Those for purified rat liver phosphorylase *a* also were determined and are included in the table for comparison. The data for phosphorylase *a* were obtained in imidazole rather than in MES buffer since a small degree of non-linearity was produced in the kinetics of phosphorylase *a* in the presence of MES. Nevertheless constants calculated were similar to those obtained in imidazole buffer. Under these conditions the turnover number of phosphorylase *b* approached that of phosphorylase *a*. However, the binding of glucose-1-*P* to phosphorylase *b* was poor and it would be difficult to saturate the enzyme with respect to this substrate.

Effects of salts and buffers. With enzymes purified from rat liver, fluoride as well as sulfate increased the K_M of phosphorylase *a* for glucose-1-*P* with a K_i value of about 50 mM (results not shown), but appeared to decrease the K_M of phosphorylase *b* for glucose-1-*P* (Table III). Among the buffers tested, α(*N*-morpholino) ethane sulfonate (MES) stimulated the activity of phosphorylase *b* more than maleate or β-glycerophosphate, due possibly to the presence of the

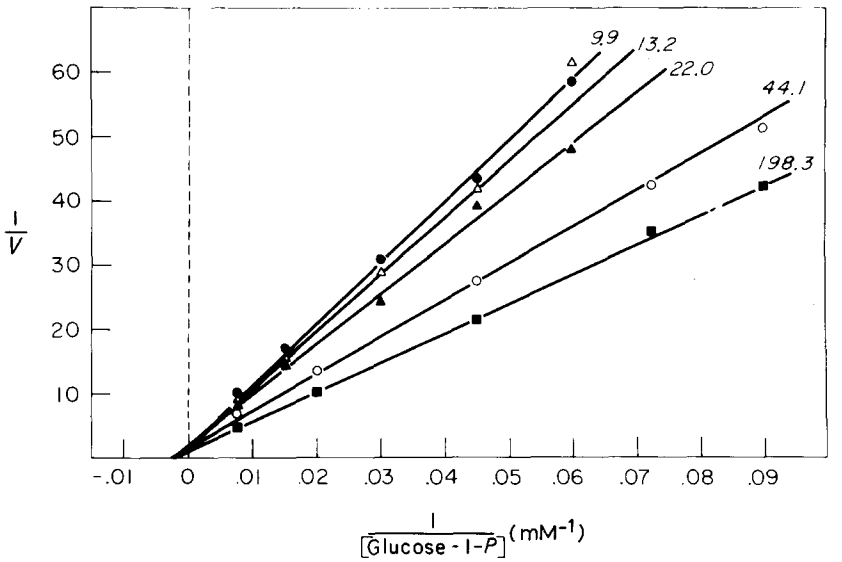

Fig. 5. Velocity of phosphorylase *b* activity as a function of glucose-1-*P* concentration at 5 levels of glycogen as follows: 9.9 mM (●), 13.2 mM (△), 22.0 mM (▲), 44.1 mM (○), 198.3 mM (■). Besides the various concentrations of substrates, reaction mixture also contained 34 mM MES (pH 6.1) and 3.3 μg purified phosphorylase *b* in a final volume of 180 μl. Velocity is expressed as units/ml reaction mixture. Incubation was for 2.5 min at 30° C.

sulfonate group of the buffer. The effect of MES was additive with KF at low substrate concentration but the stimulation was reduced at high substrate concentration. Phosphorylase *a* was not inhibited by 50 mM MES.

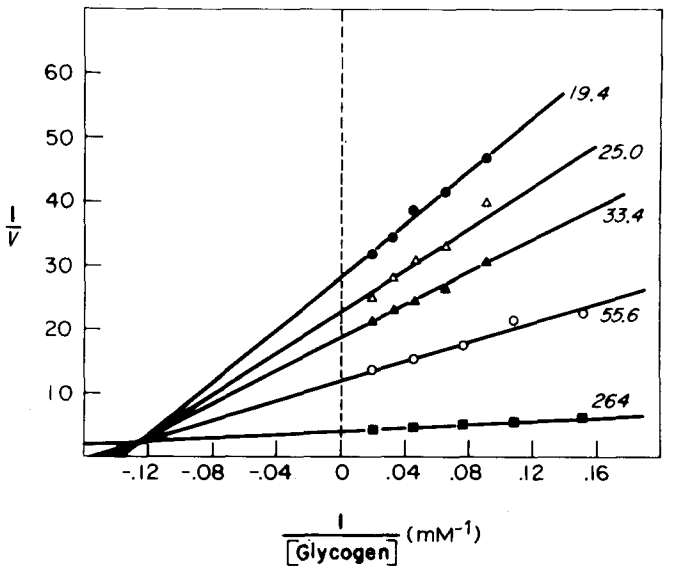

Fig. 6. Velocity of phosphorylase *b* activity as a function of glycogen concentration at 5 levels of glucose-1-*P* as follows: 19.4 mM (●), 25.0 mM (△), 33.4 mM (▲), 55.6 mM (○), 264 mM (■). Conditions same as for Fig. 5.

TABLE II

KINETIC PARAMETERS* OF LIVER PHOSPHORYLASE *a* AND *b*

	Phosphorylase *a***	Phosphorylase *b****
K_s^{G1P} (mM)	1.3	250
$K_s^{Glycogen}$ (mM)	3.4	9.5
K_M^{G1P} (mM)	0.7	250
$K_M^{Glycogen}$ (mM)	1.8	2.5
V (μmol/min/mg)	51	48

* The nomenclature of the Enzyme Commission has been used.
** Determined under the same conditions as [3].
*** Calculated from Figs. 4 and 5.

Effect of AMP. Under the standard assay condition, AMP had a small effect on the activity of phosphorylase *a* but greatly stimulated the activity of phosphorylase *b* (Table I). At a saturating glycogen concentration, 5 mM AMP did not change the V of phosphorylase *b* but brought about a positive cooperativity and increased the binding affinity for glucose-1-*P* (Fig. 7). The $S_{0.5}$ for glucose-1-*P* was calculated to be 80 mM from the Hill plot. In a liver extract, the $S_{0.5}$ of phosphorylase *b* for glucose-1-*P* was 60 mM in the presence of 5 mM AMP. At 270 mM glucose-1-*P*, AMP increased the velocity of the reaction as well as lowering the K_M for glycogen (Fig. 7). Half-maximal activation of phosphorylase *b* was found to occur at 0.5 mM AMP, both at 56 mM and 250 mM glucose-1-*P* (results not shown).

TABLE III

EFFECT OF SALTS AND BUFFERS ON PHOSPHORYLASE *b* ACTIVITY

Assays were done in the presence of 24 mM glycogen using a partially purified phosphorylase *b* preparation. Activity is expressed as units/mg protein.

Additions	2 mM AMP	Glucose-1-*P*	
		15 mM	270 mM
None (pH 6.3)	—	0.2	6.2
	+	0.4	9.5
Sulfate (200 mM) (pH 6.3)	—	0.7	8.0
	+	1.2	10.9
Fluoride (100 mM) (pH 6.3)	—	1.0	8.0
	+	1.5	10.9
MES (50 mM) (pH 6.3)	—	1.0	7.3
	+	1.4	10.2
MES (50 mM) (pH 6.3) with fluoride (100 mM)	—	1.5	8.0
	+	2.3	10.2
Maleate (50 mM) (pH 6.5)	—	0.2	6.1
	+	0.4	8.8
β-Glycerophosphate (50 mM) (pH 6.7)	—	0.4	7.3
	+	0.7	10.2

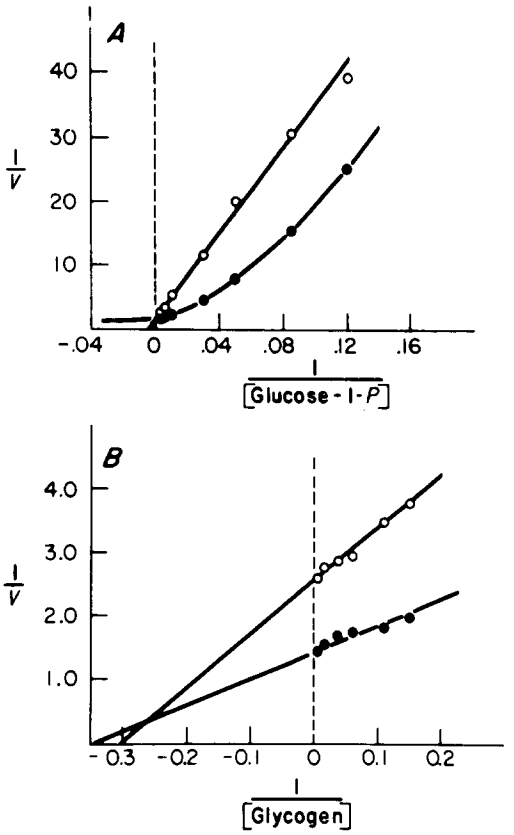

Fig. 7. Kinetics of AMP stimulation of phosphorylase *b*. (*A*) As a function of glucose-1-*P*. Glycogen concentration was 106 mM. Open circles indicate no addition; closed circles with addition of 5 mM AMP. (*B*) As a function of glycogen. Glucose-1-*P* concentration was 270 mM. Open circles indicate no addition; closed circles with addition of 5 mM AMP. Conditions same as for Fig. 5.

Reversibility. The reaction catalyzed by liver phosphorylase *b* was found to be readily reversible. The apparent K_M for inorganic phosphate was about 500 mM compared to the value of 2.1 mM for phosphorylase *a* [3]. AMP stimulated at each of the concentrations of substrate tested. Under the conditions of the assay, the enzyme favors glycogen synthesis as the V for this direction was three times that for glycogen degradation. Similar results have been reported for phosphorylase *a* [3].

Inhibitors. UDPglucose, ATP, ADP, glucose-6-*P*, caffeine, at concentrations between 1 and 3 mM and glucose at 9 mM were all potent inhibitors of purified phosphorylase *b*. Caffeine has previously been reported to inhibit muscle phosphorylase activity [16]. Because of the high K_M of phosphorylase *b* for glucose-1-*P*, it is difficult to obtain the exact K_i values of these inhibitors. Maddaiah and Madsen [3] reported that various nucleotides and sugar phosphates inhibited phosphorylase *a* purified from rabbit liver. This is confirmed in the present study using purified phosphorylase *a* from rat liver, and similar K_i values were obtained.

Modification of assay conditions for measurement of enzymes

Based on the information obtained, new assay conditions were established in order to measure the phosphorylated and dephosphorylated forms of liver phosphorylase in both crude and purified systems. The composition of these assay mixtures is listed in the Methods section. The standard assay we used initially has been modified so that the concentrations of substrates have been lowered for measurement of phosphorylase *a* and increased for measurement of phosphorylase *b*. AMP has been added to the high-substrate test mixture for the *b* form of the enzyme as it lowered the K_M for glucose-1-*P* and with a non-saturating concentration of glucose-1-*P*, it increased the reaction velocity of the enzyme. It was included in the low-substrate test mixture for the *a* form since it slightly stimulated activity. Six to ten percent of purified phosphorylase *b* activity was measured with the low-substrate assay. Potassium fluoride was omitted from the test mixtures. When added with the crude extract its concentration was kept below 50 mM in the assay.

Interconversions in vitro and in vivo

The modified assays were used for the measurement of interconversion reactions in a liver extract (Fig. 8). With the low-substrate assay, there was a drop in activity upon incubation to less than 15% of the original activity. An even lower level could be obtained if AMP was excluded from the assay mixture. This was in contrast to the 70% and 40% activity remaining detected earlier with the standard assay in the presence and absence of AMP, respectively (Fig. 1). With the high-substrate assay, there was only a small decrease (about 10%) in activity throughout the incubation when essentially all of the phosphorylase was converted to the *b* form. The addition of muscle phosphory-

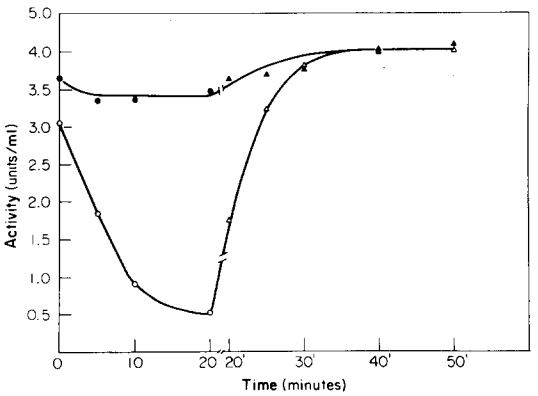

Fig. 8. Interconversion in vitro of liver phosphorylases in liver extract using modified assay conditions. Conditions of experiment were the same as that of Fig. 1. The column-treated extract was incubated at 20° C. At different time points aliquots were diluted 1 : 10 in 50 mM MES (pH 6.3), 50 mM KF and assayed for phosphorylase activity; with low-substrate (○), and high-substrate (●) assay conditions. After 20 min of incubation, to 0.7 ml of 1 : 6 extract was added 25 μmol KF, 600 μg muscle phosphorylase kinase, 6.4 μmol ATP, 20 μmol Mg^{2+}, 0.16 μmol cyclic AMP, 1.6 μmol theophylline to a final volume of 0.9 ml. Incubation was continued and at different time points, aliquots of incubation mixture were diluted 1 : 10 with 50 mM MES (pH 6.3) and 50 mM KF, and immediately assayed for phosphorylase activity: with low substrate (△); and high-substrate (▲) assay conditions. Results were corrected for dilution.

lase kinase with ATP · Mg^{2+} and potassium fluoride at this time caused an immediate conversion of the phosphorylase back to the *a* form, as indicated by the activity measured with the low-substrate assay. Total phosphorylase activity, as measured by the high-substrate assay was slightly increased (about 10%) by the action of muscle phosphorylase kinase. Similar results were observed with endogenous liver phosphorylase kinase; however conversion to the *a* form was not always complete.

When purified liver phosphorylase *b* was converted to phosphorylase *a* by muscle phosphorylase kinase, the specific activity as measured by the high-substrate assay was changed from 30 units/mg to 50 units/mg, the specific activity of the purified phosphorylase *a*, indicating that the high substrate assay was still inadequate to measure fully the purified phosphorylase *b* activity. This difference between the crude and purified systems could be explained by a lower requirement of phosphorylase *b* for glucose-1-*P* in the liver extract than in the purified form. Upon purification the enzyme may have been altered or the presence of other proteins and low concentrations of metabolites in the liver extract may modify the structure and properties of the purified enzyme. A protein-protein interaction has recently been demonstrated between purified muscle phosphorylase *b* and phosphorylase *b* kinase in the muscle extract [17].

The modified assays were used to demonstrate acute in vivo changes in phosphorylase activity in rat liver under different physiological conditions. Activity was compared before and after glucose and glucagon treatment in the same animal (Table IV). Using the low-substrate assay, which measures primarily phosphorylase *a*, there was a decrease following glucose and an increase following glucagon administration as expected. Using the high-substrate assay, the total activity of liver phosphorylase was not changed by the different

TABLE IV

MEASUREMENT OF PHOSPHORYLASE ACTIVITY IN LIVERS OF GLUCOSE- AND GLUCAGON-TREATED RATS WITH STANDARD AND MODIFIED ASSAY CONDITIONS

Fasted rats were anesthetized and the abdominal cavity opened. A lobe of liver was tied off (control) and immediately frozen. Glucose (250 mg/kg body weight) was injected via the posterior vena cava and allowed to circulate for 10 min. Then another lobe was tied off and quick-frozen. Glucagon (1 mg/kg body weight) was next injected into the same vein and one minute later another lobe was removed and frozen. A 1 : 13 homogenate was made in 100 mM KF/10 mM EDTA (pH 7.0). The homogenate was centrifuged at 8000 × *g* for 10 min and the extract obtained was diluted 1 : 3 with 50 mM MES buffer (pH 6.3)/50 mM mercaptoethanol and assayed for phosphorylase activity. Activity was expressed as units/g wet weight of liver. The values given represent the mean from 8 animals.

Assay condition	Control	Glucose-treated	Glucagon-treated
Standard (—AMP)	13.6	11.6	19.5
Standard (+AMP)	18.5	16.6	21.8
Ratio: $\frac{(-AMP)}{(+AMP)}$	0.73	0.70	0.89
Low-substrate	14.6	11.2	20.1
High-substrate	22.7	22.8	22.6
Ratio: $\frac{Low}{High}$	0.64	0.49	0.89

TABLE V

CHANGES IN TOTAL PHOSPHORYLASE AND PHOSPHORYLASE *a* ACTIVITY* IN LIVERS OF RATS UNDER DIFFERENT EXPERIMENTAL CONDITIONS

Preparation of tissues for analyses was the same as that described in Table IV.

Animals (no. of animals used in brackets)	Body weight (g)	Fresh		Frozen	
		Phosphorylase *a*	Total phosphorylase	Phosphorylase *a*	Total phosphorylase
Normal fed (6)	187 ± 10	15.3 ± 1.9	20.6 ± 1.9	12.2 ± 2.5***	20.2 ± 1.5
Normal fasted 24 h (6)	164 ± 5	12.4 ± 3.3	18.8 ± 2.6	7.6 ± 2.1***	15.4 ± 2.7***
Normal fasted 48 h (6)	149 ± 3	7.8 ± 1.7**	12.8 ± 1.5**	5.7 ± 1.0***	12.3 ± 1.6
Normal fasted 72 h (6)	134 ± 9	8.1 ± 3.4**	12.5 ± 0.8**	6.6 ± 2.0	11.1 ± 1.7
Normal fed (8)	<200		20.8 ± 1.7		
Normal fed (7)	>200		25.6 ± 3.0**		
Diabetic fed (10)	162 ± 21		14.5 ± 4.4**		
Diabetic fasted 16 h (4)	157 ± 17		14.9 ± 2.9**		
Adrenex fed (6)	237 ± 15		22.1 ± 2.3		
Adrenex fasted 48 h (8)	216 ± 16		13.8 ± 1.7**		

* Activities expressed as units/g wet weight ±S.D. of mean.
** $P < 0.01$ from normal fed.
*** $P < 0.05$ from fresh tissues.

treatments. The results using the original standard assay are shown for comparison.

These assays were also used to show changes in the total amount of phosphorylase activity in rat liver under different experimental conditions. Examples of such changes are shown in Table V. Liver samples which were quick-frozen with liquid nitrogen-cooled clamps and stored at −80°C generally showed a decrease in phosphorylase *a* with little change in total activity. There is a correlation of total phosphorylase activity with the size of the animals used. Prolonged fasting brought about a significant decrease in both phosphorylase *a* and total phosphorylase. Using endogenous phosphorylase kinase, Niemeyer et al. [18] also concluded from their studies that the low liver phosphorylase observed in animals fasted for 48 h was due to a decrease in total phosphorylase. A lower total phosphorylase activity was observed in diabetic animals, both fed and fasted. In adrenalectomized animals, the total phosphorylase was not different from normal controls. On fasting, the activity decreased to very low levels as was observed in control animals. Thus, insulin appears to be important in the maintenance of total phosphorylase in liver.

Discussion

The present studies indicate rat liver phosphorylase *b* is active and can be measured under special conditions, contrary to other reports in the literature. The negative results of previous investigations can largely be explained by the data we have obtained. Table VI summarizes various assay conditions reported in the literature and the specific activities of the purified liver enzymes obtained. In all these studies, failure to detect phosphorylase *b* activity can be explained by the low glucose-1-*P* concentrations used. The use of MES buffer and a high concentration of fluoride in our standard assay further stimulated enzyme activity. In our work, phosphorylase *b* measured under the conditions of Hedrick and Fischer [19], gave 25% of the activity obtained with our

TABLE VI

SPECIFIC ACTIVITIES OF LIVER PHOSPHORYLASE *a* AND *b* UNDER DIFFERENT ASSAY CONDITIONS

Investigators	Assay conditions	Phosphorylase *a*		Phosphorylase *b*		
		—AMP	+AMP	—AMP	+AMP[1]	+AMP[1] + SO_4^{2}
Appleman et al. [2] (Illingworth and Cori [32])	16 mM glucose-1-*P* 10 mg/ml glycogen 20 mM β-glycerol (pH 6.8) 15 mM cysteine · HCl	—	44	—	0	8.8
Wosilait and Sutherland [1]	36 mM glucose-1-*P* 4.0 mg/ml glycogen 100 mM NaF	—	—	2%	25%[3]	—
Sato and Weinhouse [6]	45 mM glucose-1-*P* 10 mg/ml glycogen 100 mM NaF 50 mM tris · maleate (pH 6.1)	—	—	10%	35%	100%[4]
Wolf et al. [15] (Hedrick and Fischer [19])	75 mM glucose-1-*P* 10 mg/ml glycogen 50 mM maleate (pH 6.5)	50	70	0	2	25
Tan and Nuttall:						
(A) Standard (Danforth et al. [4])	66.7 mM glucose-1-*P* (pH 6.1) 6.7 mg/ml glycogen 33.0 mM MES (pH 6.1) 133 mM KF	45	50	5	13	25
(B) Low-substrate assay. (this paper).	15 mM glucose-1-*P* (pH 6.3) 3.4 mg/ml glycogen 33.0 mM MES (pH 6.3) 5 mM AMP	—	45	—	0.3	—
(c) High-substrate assay. (this paper).	273 mM glucose-1-*P* (pH 6.3) 13.4 mg/ml glycogen 33.0 mM MES (pH 6.3) 5 mM AMP	—	52	—	30	—

[1] AMP concentration 1—2 mM.
[2] Na_2SO_4 concentration = 0.5—0.7 M.
[3] 100% represents specific activity of liver phosphorylase *a*.
[4] 100% represents activity measured in the presence of 1 mM AMP and 0.5 M Na_2SO_4.

high-substrate assay, whereas Wolf et al. [15] reported only 7%. The reason for this discrepancy is not clear.

Liver phosphorylase has been considered to be different from muscle phosphorylase in that an increase in molecular weight does not occur during conversion to the phosphorylated form [1] and AMP is incapable of stimulating activity of the dephosphorylated form in liver. However, Wang and Graves [20] have demonstrated that muscle phosphorylase *a* aggregates in the cold, and that tetramerization is an artifact and not necessary for activity. When muscle as well as liver phosphorylase become dephosphorylated, species are formed with reduced affinity for both glucose-1-*P* and inorganic phosphate. The presence of AMP restores the affinity of muscle phosphorylase *b* for substrates to the level of that of muscle phosphorylase *a*. AMP improved the binding of liver phosphorylase to glucose-1-*P* as shown in the present study, but a high concentration of substrate is still necessary for activity.

AMP appears to induce positive cooperativity in the binding of glucose-1-*P* to liver phosphorylase *b*. This is different from the effect of AMP on muscle [21] and liver [22] phosphorylase *a* where cooperative kinetics obtained in the presence of inhibitors are transformed into normal Michaelis-Menten kinetics. For muscle phosphorylase *b*, increasing AMP concentration decreases the K_M for the substrates; increasing the substrate concentration decreases the K_M for AMP [23]. Linear relationships were reported even though at low concentrations of AMP some departure from linearity was observed [24,25]. In the present study, no cooperativity was observed with liver phosphorylase *b* for glucose-1-*P* in 35 mM MES pH 6.1. In the presence of AMP, a conformational state was obtained which had higher affinity for the substrate and there was some cooperativity between the binding sites. This could be a distinctive feature for liver phosphorylase *b*, or perhaps the result of the special buffer used.

Engers and Madsen [8] observed that in the absence of AMP high concentrations of phosphate activated muscle phosphorylase *b*. The maximum activity was 30% of that obtained in the presence of AMP. This however was a non-specific effect as other anions high in the Hofmeister or lyotropic series activated muscle phosphorylase *b*. Anions low in the series inhibited its activity. Sealock and Graves [9] studied the effects of NaF and $NaClO_4$ (the anions of which are high and low in the series, respectively) on muscle phosphorylase *a* and *b*. They proposed a model in which the interaction of covalently bound phosphate or certain salts with a specific site on the surface of the enzyme results in conformational changes and therefore different catalytic and physical properties. The present results using liver phosphorylase *b* could also be explained by such a model.

When kidney phosphorylase becomes phosphorylated there also is no change in molecular weight [26]. Villar-Palasi et al. [27] and Medicus et al. [26] found that the dephosphorylated kidney enzyme has little or no activity in the absence of AMP but some activity could be obtained in the presence of AMP using assay conditions similar to our standard assay. Similar results have been reported for phosphorylases isolated from the spleen [28] and from Novikoff hepatoma [6]. It is not known whether more activity could be detected if the substrate concentrations were increased in the absence of AMP or if the activity of the dephosphorylated enzyme would approach that of the

phosphorylated enzyme if the glucose-1-*P* concentration was increased in the presence of AMP. If this is the case, the enzymes from the above organs are similar in many aspects to the rat liver enzymes reported in this paper.

It is apparent that with the assay conditions we used originally, both phosphorylase *a* and phosphorylase *b* activity were being measured simultaneously, but the conditions were not optimal for measurement of either. This, plus the different sensitivity of the two forms of the enzyme to AMP stimulation led to the difficulty we had in interpreting the in vivo data obtained with and without AMP following either hormone or glucose administration. The same problems and solutions also were illustrated in the data obtained when phosphorylase phosphatase activity was determined in vitro. Using the high and low substrate conditions, which are based on the substrate and modifier requirements for phosphorylase *a* and *b*, we now are able to determine physiological changes in the ratio of phosphorylase *a* and *b*, with little change in total activity (phosphorylase *a* + *b*).

True covalent modification of an enzyme such as the phosphorylation and dephosphorylation of phosphorylase is said to have occurred only if the total amount of enzyme remains unchanged. That the total amount of liver phosphorylase remained stable when changes in measurable phosphorylase occurred has been the assumption used by different workers. This generally is true after an acute stimulus with a short-term response. But the total phosphorylase activity may change under certain circumstances as shown in the present study. Therefore, in order to show that any change in phosphorylase activity is the result of an interconversion mechanism, total activity must be monitored in the same time period.

Maddaiah and Madsen [29] used the amount of phosphorylase activity after epinephrine treatment as an estimate of the total phosphorylase present in rat liver. Stalmans et al. [30] used the activity obtained after incubation of the crude extract with muscle phosphorylase kinase. The total activity measured with the high-substrate assay in the present study gave values similar to those reported by the above investigators but the technique is direct, rapid and convenient.

Addendum

After this paper was submitted for publication, Stalmans and Hers [33] reported assay conditions for total liver phosphorylase. Activity was determined in the presence of 1 mM AMP and 0.5 M sulfate pH 6.5 and a correction factor was applied to compensate for the sulfate inhibition of phosphorylase *a*.

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References

1 Wosilait, W.D. and Sutherland, E.W. (1956) J. Biol. Chem. 218, 469—481
2 Appleman, M.M., Krebs, E.G. and Fischer, E.H. (1966) Biochemistry 5, 2101—2107
3 Maddaiah, V.T. and Madsen, N.B. (1966) J. Biol. Chem. 241, 3873—3881
4 Danforth, W.H., Helmreich, E. and Cori, C.F. (1962) Proc. Natl. Acad. Sci. U.S. 48, 1191—1199
5 Bishop, J.S., Goldberg, N.D. and Larner, J. (1971) Am. J. Physiol. 220, 499—506
6 Sato, K. and Weinhouse, S. (1973) Arch. Biochem. Biophys. 159, 151—159
7 Bishop, J.S. and Larner, J. (1967) J. Biol. Chem. 242, 1355—1356
8 Engers, H.D. and Madsen, N.B. (1968) Biochem. Biophys. Res. Commun. 33, 49—54
9 Sealock, R.W. and Graves, D.J. (1967) Biochemistry 6, 201—206
10 Tan, A. and Nuttall, F.Q. (1974) Fed. Proc. 33, 1559
11 Dubois, M., Gilles, K.A., Hamilton, T.R., Rebers, P.A. and Smith, F. (1956) Anal. Chem. 28, 350—356
12 Gilboe, D.P., Larson, K.L. and Nuttall, F.Q. (1972) Anal. Biochem. 47, 20—27
13 Glaser, L. and Brown, D.H. (1955) J. Biol. Chem. 216, 67—79
14 Lowry, O.H., Rosebrough, A.L., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
15 Wolf, D.P., Fischer, E.H. and Krebs, E.G. (1970) Biochemistry 9, 1923—1929
16 Kihlman, B. and Overgaard-Hansen, K. (1955) Exp. Cell Res. 8, 252—255
17 Gergely, P., Vereb, G.Y. and Bot, G.Y. (1974) Acta Biochim. Biophys. Acad. Sci. Hung. 9, 223—226
18 Niemeyer, H., Radojkovic, J. and Perez, N. (1962) Arch. Biochem. Biophys. 97, 285—291
19 Hedrick, J.L. and Fischer, E.H. (1965) Biochemistry 4, 1337—1343
20 Wang, J.H. and Graves, D.J. (1964) Biochemistry 3, 1437—1445
21 Sagardia, F. (1964) Biochem. Biophys. Res. Commun. 17, 383—388
22 Stalmans, W., Laloux, M. and Hers, H.G. (1974) Eur. J. Biochem. 49, 415—427
23 Madsen, N.B. (1964) Biochem. Biophys. Res. Commun. 15, 390—395
24 Madsen, N.B. and Shechosky, S. (1967) J. Biol. Chem. 242, 3301—3307
25 Black, W.J. and Wang, J.H. (1968) J. Biol. Chem. 243, 5892—5898
26 Medicus, R. and Mendicino, J. (1973) Eur. J. Biochem. 40, 63—75
27 Villar-Palasi, C. and Gazquez-Martinez, I. (1968) Biochem. Biophys. Acta 159, 479—489
28 Kamogawa, A. and Fukui, T. (1971) Biochem. Biophys. Acta 242, 55—68
29 Maddaiah, V.T. and Madsen, N.B. (1966) Biochem. Biophys. Acta 121, 261—268
30 Stalmans, W., DeWulf, H., Hue, L. and Hers, H.G. (1974) Eur. J. Biochem. 41, 127—134
31 Stalmans, W., DeWulf, H., Lederer, B. and Hers, H.G. (1970) Eur. J. Biochem. 15, 9—12
32 Illingworth, B. and Cori, G.T. (1953) Biochem. Prep. 3, 1—5
33 Stalmans, W. and Hers, H.G. (1975) Eur. J. Biochem. 54, 341—350