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The Kinetics of Glycogen Phosphorylases from Brain and Muscle*

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SUMMARY

A kinetic study has been made of phosphorylase *a* from rabbit brain and of phosphorylase *b* from rabbit brain and muscle. In the case of brain phosphorylase *a* the kinetic data indicate strong cooperative interaction between glycogen and phosphate sites and between glycogen and adenylate sites. Under the experimental conditions there was found little evidence of interaction between phosphate and adenylate sites, although from a few experiments under different conditions and from the work of others, some interaction is possible. Although there are presumably at least two glycogen sites and two phosphate sites in the active form of the enzyme, there was no sign of interaction of one glycogen site with another or one phosphate site with another. The kinetic behavior of brain phosphorylase *a* is similar to that reported earlier for muscle phosphorylase *a* except for the failure to show the strong phosphate-adenylate interaction characteristic of the latter. The muscle enzyme also has much greater affinity for glycogen and somewhat lower affinity for adenylate and phosphate.

The kinetic behavior of phosphorylase *b* is more complicated than that of phosphorylase *a*. The data for muscle phosphorylase *b* can be adequately explained by a formulation involving two binding sites each for 5′-adenylate and inorganic phosphate, and one site for glycogen. Random order of addition is indicated with marked interaction between many of the sites. Of the 17 independent kinetic constants demanded by this formulation 10 have been provisionally evaluated. The kinetic pattern of brain phosphorylase *b* resembles that of the muscle enzyme but the apparent affinities for phosphate and adenylate are greater.

Raising the temperature to 38° profoundly decreases apparent affinity of all four enzymes for glycogen, with comparatively little effect on apparent affinity for phosphate.

Brain phosphorylase can exist in *a* and *b* forms as in the case of the muscle enzyme. Since, however, there is much less glycogen in brain, and since brain glycogen is probably not utilized except in emergency, it seemed desirable to see if there might not be differences in the kinetic properties of phosphorylases from the two sources. Direct comparison with muscle phosphorylase *a* was possible since it has already been studied in this laboratory with the same analytical techniques (1). However, this was not true for muscle phosphorylase *b* which has therefore been included in the present study in spite of the fact that several recent investigations have been made of its kinetics (2–4).

With the fluorometric assay system used it was possible to measure very low velocities. This in turn permitted exploration of a very wide range of substrate levels. Under these conditions certain kinetic features have become visible which would be difficult otherwise to perceive.

EXPERIMENTAL PROCEDURE

Enzyme Preparations

Muscle Phosphorylase b—This was prepared from 200 g of rabbit muscle essentially as described by Fischer and Krebs (5). Throughout most of the steps, reagents contained 1 mM EDTA and 10 mM β-mercaptoethanol. To remove amylase activity the enzyme was recrystallized six times and the final preparation of crystals was washed repeatedly at 0° with 1 mM 5′-AMP in 10 mM magnesium acetate.

Brain Phosphorylase a—Brain from seven rabbits (45 g) was homogenized at 0° in a Waring Blendor in 10 volumes of 0.015 M potassium phosphate buffer, pH 7, which contained 5 mM EDTA, 5 mM β-mercaptoethanol, and 0.2 mM 5′-AMP (Solution A). Each brain was homogenized separately within 2 min of the time of decapitation. AMP was included in the homogenizing fluid since it was found that this prevented partial conversion of the *a* to the *b* form. Mercaptoethanol was included since it protected the enzyme from partial inactivation during ammonium sulfate precipitation. During fractionation the temperature was maintained near 0°; precipitates were sedimented by centrifuging for 15 min at 15,000 × *g*.

To the supernatant fluid obtained by centrifuging the original homogenate was added solid ammonium sulfate to give a 1 M concentration. (At this and subsequent ammonium sulfate steps the pH was kept near 7 by adding NH_4OH.) The precipitate was discarded and the ammonium sulfate concentration was brought to 1.5 M. The precipitate (40% of the original activity) was dissolved and brought to a volume of 60 ml with Solution A. Ammonium sulfate was added to give a concentration of 1.2 M. The precipitate was discarded and the ammonium sulfate con-

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centration was raised to 2.5 M. The precipitate was dissolved in 4 ml of Solution A and was dialyzed overnight against a large volume of 0.1 M Tris-HCl buffer, pH 8.3, containing 15 mM K_2HPO_4, 5 mM EDTA, and 5 mM β-mercaptoethanol. The activity was 14 moles per kg of protein per hour when measured at 25° and pH 7.0 in the presence of 20 mM K_2HPO_4, 5 mM glycogen (as total glucosyl units), and 0.002 mM 5′-AMP, plus the other components of the assay system described below. This represented an 8-fold purification with 29% yield. The activity with 0.002 mM 5′-AMP was 73% of that with 0.5 mM 5′-AMP indicating the presence of 20 to 25% phosphorylase *b*. This contamination did not interfere with the kinetic measurements, since the 5′-AMP levels used were never high enough to permit a significant contribution by the phosphorylase *b* to the phosphorylase *a* activity.

Brain Phosphorylase b—Brain from five rabbits (32 g) was homogenized in 5 volumes of water containing 1 mM 5′-AMP and 5 mM β-mercaptoethanol. The supernatant fluid obtained by centrifuging for 10 min at 34,000 × *g* was incubated for 15 min at 38° which reduced phosphorylase *a* activity to less than 1% of the total. Saturated ammonium sulfate (pH 7) was added to give successive concentrations of 0.5, 0.8, 1.0, 1.3, and 1.7 M; the precipitates were removed at each step by centrifuging for 20 to 50 min at 34,000 × *g*. The 1.7 M precipitate contained 48% of the original activity with a 10-fold enrichment (13 moles per kg of protein per hour at 24° and pH 7.0 in the presence of 0.1 M imidazole-HCl, 10 mM K_2HPO_4, 7 mM glycogen, 0.3 mM 5′-AMP, plus the other components of the assay system below).

Approximately half of the sample (2 ml) was diluted to 22 ml with 0.05 M imidazole-HCl buffer, pH 6.8, containing 1 mM 5′-AMP and 10 mM β-mercaptoethanol (Buffer A). To this was added 25 mg of high molecular weight rabbit liver glycogen prepared according to Bueding and Orrell (6) and the mixture was centrifuged for 30 min at 34,000 × *g* (0°). The precipitate consisted of a tightly packed lower portion and an upper layer that was less well packed. This upper portion (0.9 ml) was removed and diluted with 1 ml of Buffer A. After standing for 30 min at 38° this was centrifuged as before but at 30°. To the precipitate was added 5 ml of buffer. This was allowed to stand overnight at 4°, heated for 30 min at 30°, and centrifuged as before at 30°. To the supernatant fluid (5 ml) was added an equal volume of saturated ammonium sulfate (pH 7). After centrifuging, the precipitate was dissolved in 60 μl of Buffer A. This particular fraction was only one of several active fractions obtained following the glycogen treatment. It was chosen for the kinetic studies because it had a very low ratio of amylase to phosphorylase activity. This fraction represented 8% of the activity of the portion treated with glycogen and had been purified 100-fold over-all (140 moles per kg of protein per hour).

Other Materials

Rabbit liver glycogen, used for all kinetic measurements, was obtained from Mann, and freed of nucleotides by dialysis at pH 4.8. All glycogen concentrations are calculated as total glucosyl units. Yeast glucose-6-P dehydrogenase of about 30% purity and crystalline muscle P-glucomutase were obtained from Boehringer and Sons.

Measurement of Phosphorylase Activity

All velocities were measured in the direction of glycogen breakdown as previously described (1). Unless otherwise stated the reagent was 1 ml of imidazole-HCl buffer, pH 6.9, containing 2 mM magnesium acetate, 0.5 mM EDTA, 0.025% bovine plasma albumin, 0.03 mM TPN^+, 10 μg per ml of crystalline P-glucomutase, and 1.6 μg per ml of glucose-6-P dehydrogenase (30% purity). The imidazole buffer was 0.1 M for muscle phosphorylase *b*, 0.05 M for the brain enzymes (see Table VI). (Most of the $(NH_4)_2SO_4$ was removed from the auxiliary enzymes by centrifuging stock suspensions and removing the supernatant fluid.) TPNH formation was followed directly in the Farrand fluorometer with sufficient sensitivity to measure as little as 10^{-7} M total product formation. The high sensitivity permitted valid measurements with very low substrate levels. As pointed out by Helmreich and Cori (2) high sensitivity is particularly important when glycogen levels are low, since velocities fall off as soon as appreciable chain shortening occurs. Because of the high sensitivity it was also possible to use very low enzyme levels. This was especially useful with the relatively crude brain preparations which were not entirely free of amylase contamination (see below). To make sure that difficulties on this score were avoided, each rate measurement was made with two different levels of enzyme.

In general, phosphorylase in a volume of 1 to 20 μl was added 2 min before the reaction was started by glycogen addition. Readings were started 2 min after glycogen addition and continued for 4 to 10 min. For rate measurements above or below room temperature, tubes were incubated in a water bath and read quickly at each time interval before appreciable temperature change could occur. Correction for the negative temperature coefficient of TPNH fluorescence (about 1.5% per degree) was made by means of TPNH standards prepared in the reagent and read at each temperature concerned.

Amylase Interference

Even many times recrystallized phosphorylase may contain traces of amylase, and crude preparations may be seriously contaminated with amylase activity. This enzyme as encountered in phosphorylase preparations has a very low Michaelis constant for glycogen; of the order of 10^{-7} M (based on total glucosyl residues). Consequently, a trace of amylase which might be overlooked when tested with a high level of glycogen can be very troublesome in a kinetic study with low glycogen levels. It is because of this problem that exposure of glycogen to phosphorylase was kept to a minimum by initiating the reaction with glycogen.

RESULTS

Brain Phosphorylase a

Velocity measurements were made with P_i and glycogen as variables at both 27° and 38° in the absence and in the presence of saturating levels of 5′-AMP. Reciprocal plots of velocity against both P_i and glycogen are linear within analytical limits (Figs. 1 and 2). According to limited studies (not shown), the same appears true for AMP, although in this case the apparent dissociation constants are so low that it has not been practicable to study an extreme range of velocities. Because of the linearity of the Lineweaver-Burk plots the data have been treated as though there were only single binding sites for each component. The possible forms of the enzyme are therefore *E*, *EG*, *EP*, and *EPG* in the absence of AMP, and *EA*, *EAG*, *EAP*, and *EAPG* in the presence of AMP (where *E*, *G*, *P*, and *A* represent enzyme,

glycogen, P_i, and AMP, respectively). Only the situations with zero AMP and saturating levels of AMP have been studied in any detail. Random addition and rapid equilibrium kinetics have been assumed as in a previous study of muscle phosphorylase *a* (1), and a recent study of liver phosphorylase (7). The calculations are made as described by Alberty (8), Frieden (9), and Dalziel (10) with the velocity equation

$$\frac{V}{v} = \frac{K_g K_p^G}{(g)\,(p)} + \frac{K_p^G}{(p)} + \frac{K_g^P}{(g)} + 1 \qquad (1)$$

where g and p represent glycogen and P_i, respectively, K_g = $(E)(g)/(EG)$ or $(EA)(g)/(EAG)$, $K_g^P = (EP)(g)/(EPG)$ etc. Reciprocal plots of velocity against substrate at several levels of

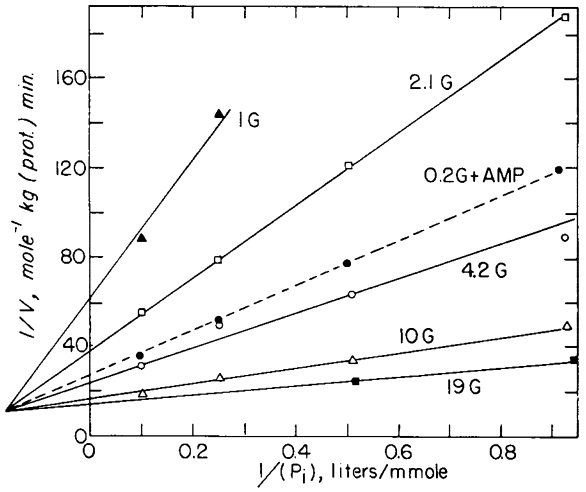

FIG. 1. Effect of P_i concentration on activity of brain phosphorylase *a* at five levels of glycogen (*G*) in the absence of AMP. Indicated concentrations are millimolar. The temperature was 27°. The *intersection* gives a value for $-1/(P_i) = -1/K_p$, where K_p, according to the formulation given in the text, represents the Michaelis constant for P_i when the enzyme is free of glycogen. For comparison one line is included showing the affect of AMP (0.01 mM) at a low level of glycogen.

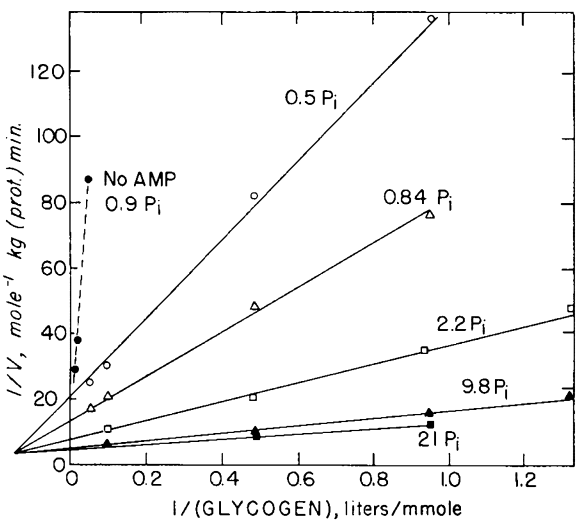

FIG. 2. Effect of glycogen concentration on activity of brain phosphorylase *a* at five levels of P_i in presence of 0.02 mM AMP. The temperature was 38°. The *intersection* gives a value for $-1/$(glycogen) $= -1/K_g$. For comparison one line is included with AMP omitted.

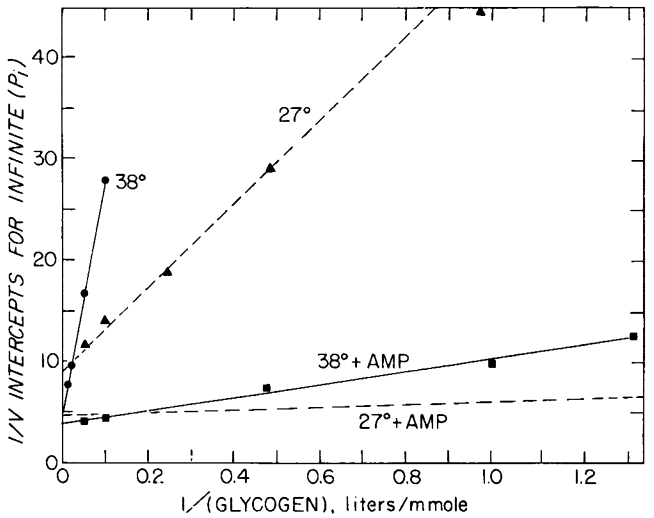

FIG. 3. Effect of glycogen and AMP concentration and of temperature on velocity with saturating levels of P_i. Intercepts from graphs such as those in Fig. 1 are plotted against the reciprocal of glycogen concentration. Velocity is expressed as moles per kg of protein per min. The *intersections* of these lines with the *abscissa* provide values for K_g^P, which according to the formulation assumed in the text represent Michaelis constants for glycogen when the enzyme is combined with P_i.

the second substrate intersect at $-1/(S) = -1/K_s$ (Figs. 1 and 2). This permits evaluation of K_p and K_g. The intercepts of $1/v$ for $1/(S) = 0$, at several levels of the second substrate, when replotted have slopes equal to K_g^P (Fig. 3), or K_p^G.

Alternatively, as pointed out by Dalziel (10), values for K_g^P, K_p^G, and $K_pK_g^P = K_gK_p^G$ (and therefore of K_p and K_g) can be obtained by plotting the slopes of Lineweaver-Burk plots against the reciprocal of the concentration of the second substrate (Fig. 4). In some cases evaluation is most accurate by the method illustrated in Figs. 1 to 3, in others that of Fig. 4 is more satisfactory. For example, the value for K_p^G at 27° in the absence of AMP from Fig. 4 is clearly not accurate, but can be suitably measured by intercept plots (Fig. 3). Similarly an estimate of K_g at 38° in the absence of AMP would be completely unsatisfactory by the method of intersections since the intersection is too close to the ordinate (not shown), but the method of Dalziel provides a valid figure.

By combining the two methods of evaluation, satisfactory estimates have been obtained, with two exceptions, for the four constants (one of which is of course not independent) at two temperatures in the presence and in the absence of AMP (Table I). The results indicate that the presence of one substrate markedly reduces the apparent binding constant for the other. This is particularly noticeable in the absence of AMP. AMP in turn profoundly increases the apparent affinity of the enzyme for glycogen (a 50-fold effect in the case of K_g). In contrast AMP decreases the affinity for P_i particularly when glycogen is present. The apparent dissociation constants for glycogen are strikingly increased by temperature, but the apparent P_i dissociation constants are little affected.

Although AMP has a marked effect on the Michaelis constants for glycogen, it has only a modest affect on the maximum velocity. Consequently the greatest percentage stimulation by AMP is observed at low glycogen levels (Table II). The stimulation by AMP is also more noticeable at elevated temperatures

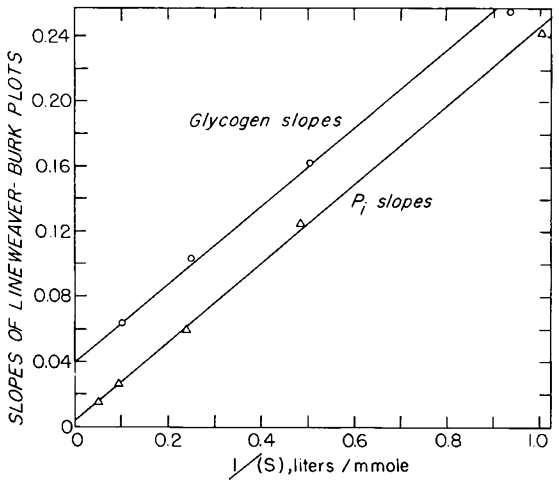

FIG. 4. Slopes of reciprocal plots of velocity and substrate concentration for brain phosphorylase *a*. The temperature was 27°, AMP was absent. The *P_i slopes* were obtained from Fig. 1. In this case $1/(S)$ represents the reciprocal of the glycogen concentrations shown in Fig. 1. The slopes are given as kilograms of protein × min per liter, *i.e.* the numerical values of the slopes of Fig. 1 have been multiplied by 0.001 to convert to rational units. If the intercepts with the ordinate are multiplied by the maximum velocities (intercepts of Fig. 3) values for K_g^P and K_p^G (in moles per liter) are obtained. The slopes of the lines in this figure multiplied by the maximum velocities provide estimates of $K_pK_g^P = K_gK_p^G$ (*cf.* Dalziel (10)). Note that the two curves shown are parallel as the theory demands.

TABLE I

Kinetic constants for phosphorylase a from brain

The symbols are defined in the text. For comparison, constants previously reported for muscle and liver phosphorylase *a* are also shown.

Enzyme	Temperature	AMP	K_p	K_p^G	K_g	K_g^P
		mM	*mM*			
Brain *a*	27°	0	6.2	0.60	44	4.3
Brain *a*	27	0.01	6.0	1.95	0.85	0.29
Brain *a*	38	0	5.5	0.6[a]	360[a]	45
Brain *a*	38	0.02	8.3	2.1	7.1	1.7
Muscle *a*[b]	25	0	>35	7	1.0	<0.2
Muscle *a*[b]	25	0.1	>20	1.1	0.26	<0.02
Muscle *a*[b]	38	0.1	14	2.2	1.7	0.3
Liver *a*[c]	30	1.0	11	0.82	13	0.93

[a] Rough estimates.
[b] Previously published data (1).
[c] From Maddaiah and Madsen (7).

because of the decreased affinity for glycogen. It will be seen in Table II that at body temperatures, with P_i and glycogen levels in the range encountered in brain, AMP can increase phosphorylase velocity 10- to 20-fold.

The apparent dissociation constants for AMP are very low, in the range of 10^{-7} M at 28° and 10^{-6} M at 38°. They are only moderately influenced by P_i and glycogen levels (Table II).

Comparison of kinetic constants with those previously reported for muscle phosphorylase *a* shows several major differences (Table I). (*a*) The brain enzyme has much larger apparent dissociation constants for glycogen. (*b*) The influence of AMP on these constants is more marked than for the muscle enzyme. As the result of these two factors K_g, in the absence of AMP, is 45-fold greater for brain *a* than for muscle *a*. (*c*) Brain enzyme has lower dissociation constants for P_i, except when the muscle enzyme is saturated with glycogen and AMP. (*d*) The influence of AMP on the P_i constants is *less* than for muscle *a*. In fact, with glycogen present, AMP has opposite effects on the two enzymes. (*e*) The apparent dissociation constants for AMP are smaller by a factor of 2 or 3 than for the muscle enzyme.

Maddaiah and Madsen have recently studied the kinetics of phosphorylase *a* from rabbit liver (7). In spite of several distinctive features the kinetic behavior is quite comparable to that of the brain and muscle enzymes (Table I). The apparent dissociation constants for P_i are similar to those for muscle. Also the liver enzyme resembles the muscle enzyme in that the reciprocal affects between P_i and glycogen are very marked, even in the presence of AMP. The dissociation constants for glycogen, however, are much larger than for either of the other enzymes. Maddaiah and Madsen also found that the effects of AMP are different. AMP lowers the apparent dissociation constants for both P_i and glycogen, but only by a factor of 2. Moreover the apparent dissociation constants for AMP are several 100-fold greater than for brain or muscle phosphorylase *a*. One further difference may be noted. We have found in a few preliminary experiments that liver phosphorylase does not exhibit the profound temperature phenomena seen with the muscle and brain enzymes.

Muscle Phosphorylase b

Kinetic measurements were made under conditions which required an extremely wide range of phosphorylase concentration (0.004 to 10 μg per ml). Consequently a series of rather dilute stock solutions of the enzyme was needed. It was found that activity decreased with time at 0° if the concentration was less than 500 μg per ml (Tris buffer, pH 8, with 0.02% bovine plasma albumin). The loss was not prevented by P_i, glycogen, mercaptoethanol, or increased albumin, but 0.1 mM 5′-AMP was completely protective for many hours, and was therefore included in making all subdilutions. This inactivation is presumably related to the cold inactivation phenomenon discovered by Graves, Sealock, and Wang (11) even though there are certain differences in the two instances. In the case of Graves *et al.* the loss in activity was observed at pH 6.0 but not at pH 6.8, the loss

TABLE II

Effect of 5′-AMP on brain phosphorylase a

The apparent dissociation constants are based on fragmentary experiments and should be regarded as somewhat provisional.

Temperature	P_i	Glycogen	Velocity		K'_{AMP}
			−AMP	+AMP[a]	
	mM		*% maximum*		*μM*
25°	0.5	0.5	0.6	11	0.23
18	1.1	2.0	6	24	0.08
18	10.0	5.8	23	56	0.09
28	1.1	2.0	4	29	0.08
28	10	5.8	30	100	0.09
38	1.1	2.0	0.8	14	0.7
38	10	5.8	8	84	0.9

[a] The concentration was 22 μM.

was prevented by glycogen as well as by AMP, and the rate of loss was not increased by dilution.

Velocity was measured as a function of AMP, glycogen, and P_i concentration. In each of three experiments one component was varied and the other two were held constant in four different combinations of high and low levels (Figs. 5 to 7). Absolute velocities covered a 20,000-fold range from one extreme to the other. Plots of reciprocal velocity against reciprocal AMP concentration were nonlinear at all levels of glycogen and P_i (Fig. 5). The same was true for P_i in the presence of a high level of AMP, although the curvature was less marked (Fig. 6). The nonlinearity is most convincing with a high level of AMP and low level of glycogen. Plots of reciprocal velocity against reciprocal glycogen concentration appeared to be linear within analytical limits, although there is a suggestion of deviation from linearity at the lowest glycogen levels (Fig. 7).

Clearly a very complicated kinetic situation exists. It is probable that the data could be made to fit a considerable number of different theoretical possibilities. Nevertheless it seemed worthwhile to explore one of these possibilities, hopefully one of the simplest. It seemed likely that the rapid equilibrium formulation that was applied to the *a* phosphorylases might be adapted to phosphorylase *b* as well. It was necessary to assume two binding sites for AMP, two for P_i, and one for glycogen. Although random order of addition was also assumed, interaction (positive or negative) between binding sites was permitted in the formulation. Each of the postulated two sites for AMP and P_i were taken to be equivalent, but capable of interaction. This would give a total of 18 forms of the enzyme symbolized by *E*, *EA*, *EAP*, through *EAAPPG* representing, respectively, free enzyme, and enzyme with one AMP site occupied, one AMP and one P_i site occupied, and up to all five sites occupied. It was

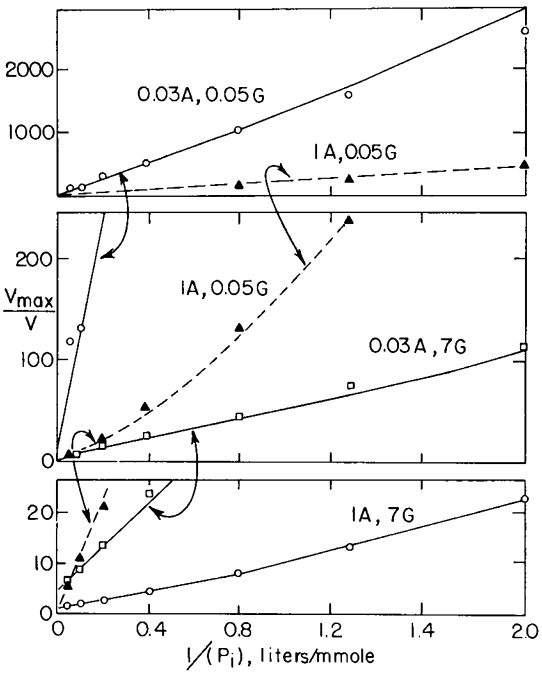

FIG. 6. Activity of muscle phosphorylase *b* as a function of P_i concentration. The *points* are observed velocities measured at 26°. The *lines* are calculated from Equation 2 and the constants of Table III. The indicated concentrations of 5′-AMP (*A*) and glycogen (*G*) are millimolar.

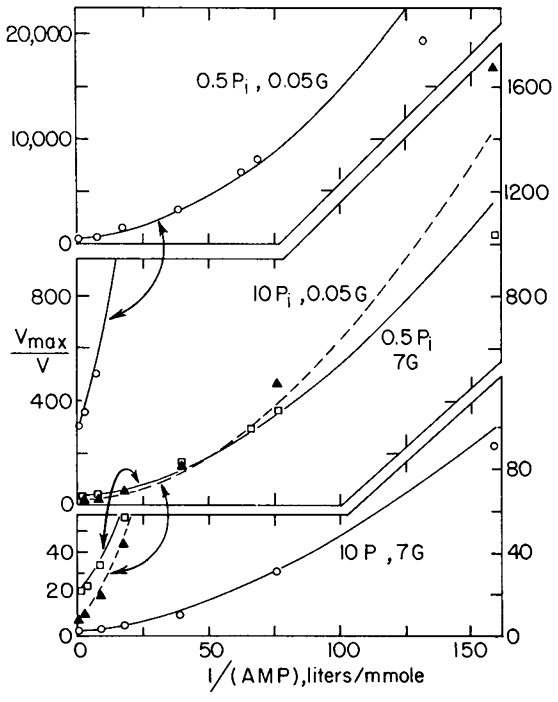

FIG. 5. Activity of muscle phosphorylase *b* as a function of 5′-AMP concentration. The velocities were measured at 23°. The indicated concentrations of P_i (*P*) and glycogen (*G*) are millimolar. (Glycogen concentration refers to total glucosyl units.) The *points* are observed, the *lines* are calculated from Equation 2. The constants of Table III were used in the calculation except that the K_g constants were all decreased by one-third. This was to compensate for the 3° difference in temperature (see "Temperature Effects").

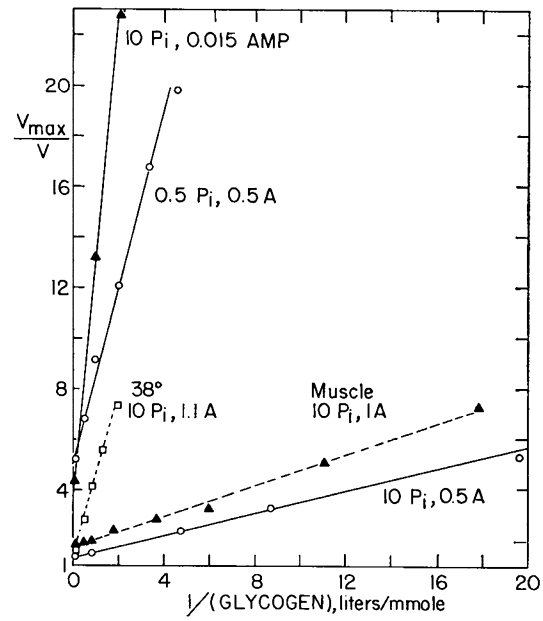

FIG. 7. Activity of muscle phosphorylase *b* as a function of glycogen concentration. The *points* are observed velocities at 23°. The *lines* are calculated from Equation 2 and the constants of Table III, except that the K_g constants were decreased by one-third to compensate for the 3° temperature difference. The indicated concentrations of 5′-AMP (*A*) and P_i are millimolar.

finally assumed that *EAAPPG* is the only active form of the enzyme. The resultant formulation is:

$$\frac{V}{v} = \frac{BCDFH}{aappg} + \frac{2CDFH}{appg} + \frac{DFH}{ppg} + \frac{2FH}{pg} + \frac{H}{g} + \frac{2JLFH}{aapg} + \frac{4LFH}{apg} + \frac{MNH}{aag} + \frac{2NH}{ag} + \frac{QRST}{aapp} + \frac{2RST}{app} + \frac{ST}{pp} + \frac{2T}{p} + \frac{2UWT}{aap} + \frac{4WT}{ap} + \frac{YZ}{aa} + \frac{2Z}{a} + 1 \quad (2)$$

In the equation v, V, a, p, and g represent, respectively, velocity, maximum velocity, and the concentrations of AMP, P_i, and glycogen. Each capital letter represents the dissociation constant for the component immediately below it, when the enzyme is lacking the components in the denominator to the *right* of the given component. For example, C represents the dissociation constant for AMP from *EAA*. Each term is numerically equal to the ratio between that enzyme combination which *lacks* all the components in the denominator and the complete (active) enzyme. For example, $BCDFH/aappg$ = (E)/(*EAAPPG*), and YZ/aa = (*EPPG*)/(*EAAPPG*).

According to the postulated formulation there would be 33 dissociation constants but only 17 of these are independent. Five of these were evaluated with reasonable confidence by extrapolation of the data to infinitely high concentrations for two components, and then evaluating the dissociation constant or constants for the third component. Because Lineweaver-Burk plots for glycogen were linear and because P_i markedly affects the apparent Michaelis constant for glycogen, it was possible to evaluate the dissociation constant for glycogen from *EAAG* with the use of data with high AMP and low P_i concentrations. For similar reasons the dissociation constants for P_i from *EAAPP* and *EAAP* could be estimated. The rest of the constants were obtained by trial and error to obtain the best fit to the observed data of Fig. 6. The reasonableness of the result may be judged by comparison of observed points in Figs. 5 to 7, and the theoretical lines of the figures which have been calculated from Equation 2 and the constants of Table III. The equation is more sensitive to some constants than to others and an attempt is made in the table to indicate this. The constants were calculated for 26°. Since as shown below, the Michaelis constants for glycogen are extremely sensitive to temperature, a somewhat arbitrary reduction of 33% in the glycogen constants was made for the experiments at 23° (see legend to Fig. 5).

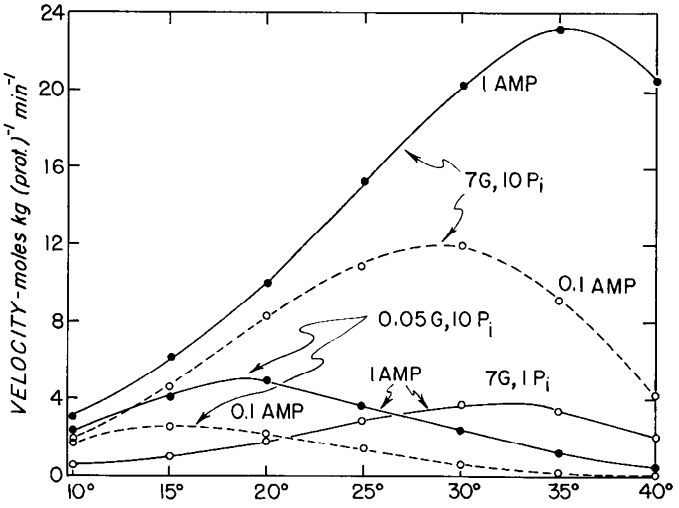

FIG. 8. Effect of temperature on activity of muscle phosphorylase *b*. The indicated concentrations of AMP, P_i, and glycogen (*G*) are millimolar.

TABLE III

Calculated dissociation constants for muscle phosphorylase b

The letters used to indicate the forms of the enzyme, *E*, *A*, *P*, and *G* represent, respectively, enzyme, AMP, P_i, and glycogen. The symbols *B* to *Z* are those used in Equation 2. The most secure values are boldfaced, the least secure are in parentheses. The constants apply to the enzyme at 26°.

Form of enzyme	Dissociation constant					
	K_a		K_p		K_g	
	Symbol	Value	Symbol	Value	Symbol	Value
		mM		mM		mM
EA	*B*	(0.04)				
EP				(0.09)		
EG						(1)
EAA	*C*	(0.01)				
EAP	*J*	**0.08**		(0.19)		
EAG	*Q*	(0.04)				(1)
EPG				(0.32)		**3.4**
EPP				(60)		
EAAP	*L*	**0.12**	*D*	**2.3**		
EAAG	*R*	(0.04)				**4.5**
EAPG	*U*	0.04		(0.32)		1.7
EAPP	*M*	(0.08)		(60)		
EPPG				**3.0**		(0.18)
EAAPG	*W*	0.04	*S*	**0.32**		**0.6**
EAAPP	*N*	(0.12)	*F*	60		
EAPPG	*Y*	**0.04**		3.0		(0.09)
EAAPPG	*Z*	**0.04**	*T*	**3.0**	*H*	0.03

If the formulation is valid the following conclusions might be drawn from Table III.

P_i Dissociation Constants—(*a*) The presence of a second P_i group on the enzyme increases the dissociability of the first P_i at least 10-fold, regardless of the presence or absence of AMP or glycogen (*e.g. T* against *S*, *F* against *D*). (*b*) Glycogen decreases the dissociation constants for P_i, especially if two P_i groups are present (*e.g. T* against *F*, *S* against *D*). (*c*) AMP has relatively little effect on P_i except that the presence of two AMP groups in the absence of glycogen appears to increase the dissociability of the first P_i group (*EAAP* against *EAP* or *EP* in the K_p column).

Glycogen Dissociation Constants—(*a*) AMP alone has little effect on glycogen dissociation. (*b*) A single P_i has little effect unless two AMP groups are already present (*EAAPG* against *EAAG*) but two P_i groups alone or in combination with AMP sharply decrease the dissociation constant. When two AMP groups are already present the addition of two P_i groups appear to decrease the constant 100-fold (*EAAPPG* against *EAAG*).

AMP Dissociation Constants—In contrast to glycogen and P_i, AMP dissociation is only moderately affected by other components. There is little interaction between the two presumptive AMP sites regardless of the presence of other components. In the absence of glycogen, P_i appears to increase

AMP dissociation constants by a factor of 2 or 3, and this effect is counteracted by the presence of glycogen.

Temperature Effects—As in the case of phosphorylase *a* temperature has an anomalous effect on phosphorylase *b* (Fig. 8). Whereas the temperature optimum is 36° with high levels of 5′-AMP, P_i, and glycogen, the optimum is only 16° if AMP and glycogen are sufficiently reduced. These are true effects on initial velocities and are not the consequence of stabilization of the enzyme by AMP or glycogen at higher temperatures. This was shown by abruptly changing the temperature in the middle of the assay. With low levels of AMP or glycogen, lowering the temperature immediately increased velocity to the extent predicted from Fig. 8; raising the temperature immediately decreased the velocity (*cf.* a graphic representation of this in the case of phosphorylase *a* (1)).

The temperature optimum is affected most by the glycogen level and least by that of P_i. Based on the velocities at the two glycogen levels, the apparent Michaelis constant for glycogen (with 1 mM AMP) is reduced nearly 50-fold (from 3.9 mM to 0.01 mM) by lowering the temperature from 40° to 10°. It is clear that the AMP level required for half-maximal activity is also greatly reduced at low temperature. Limited studies were made at 0° with a two-step assay system (not shown). At this temperature the velocity was almost as fast with 0.05 mM glycogen as with 7 mM (in the presence of 10 mM P_i, and either 1 mM or 0.02 mM AMP). On the other hand reducing P_i from 10 mM to 0.5 mM (7 mM glycogen and 1 mM AMP) reduced velocity by about the same degree at 0° as at 25° or 38°.

It is of interest to compare the kinetic constants for muscle phosphorylase *b* with previously published constants for muscle phosphorylase *a*. If it is assumed that the basic structure of phosphorylase *a* is comparable to one of the combined forms of phosphorylase *b*, then the best over-all correspondence is obtained if that form is *EAP* (Table IV). Thus, it is possible that the *b* form when combined with 1 molecule each of AMP and P_i, has a structure which resembles that of the free *a* form. However, a major difference between the two enzymes, which cannot be rationalized in this manner, concerns the AMP influence on Michaelis constants for P_i. There is a marked influence in the case of *a* and almost none for *b*.

Comparison with Other Studies

The nonlinearity of reciprocal plots of velocity against AMP concentration was noted by Helmreich and Cori (2) and by Morgan and Parmeggiani (3). These investigators also observed the favorable influence of AMP on the apparent affinity of phosphorylase *b* for glycogen and Helmreich and Cori demonstrated conversely the favorable effect of glycogen on apparent AMP binding. Although Madsen (4) reported a linear reciprocal plot of velocity and glucose-1-P concentration, it is significant that a nonlinear plot was obtained in the presence of inhibitory concentrations of ATP. As seen in Fig. 2, departure from linearity in the case of P_i is only detected at relatively low P_i levels.

There are several discrepancies between the present findings and those of Helmreich and Cori and of Morgan and Parmeggiani. Both of the latter groups reported that the Michaelis constants for P_i are lowered by increasing AMP concentration and conversely that increasing P_i concentration lowers apparent binding constants for AMP. This discrepancy can be largely explained by differences in Mg^{++} concentration during assay. (After deducting Mg^{++} bound by EDTA, free Mg^{++} levels were 1.5 mM in the present instance, and 8 and 20 mM, respectively, in the case of Helmreich and Cori and of Morgan and Parmeggiani.) As reported by Krebs and Fischer (12) and Madsen (13) a high level of Mg^{++} lowers the requirement for AMP. To see what consequence Mg^{++} might have on interactions between P_i and AMP, comparative measurements were made in reagents containing 1.5 and 9.5 mM free Mg^{++}. Velocities were measured both at pH 7.0 in the imidazole medium used for the present study, and at pH 7.5 in the Tris-buffered medium used by Helmreich and Cori. With 9.5 mM Mg^{++}, at both pH values, P_i and AMP had mutually favorable effects in regard to apparent binding affinity. In the pH 7.5 reagent with 1.5 mM free Mg^{++} the interaction was less, but still discernible. In the pH 7.0 reagent with 1.5 mM Mg^{++}, there was no significant interaction, in agreement with the more comprehensive study presented above.

Differences in assay conditions may also explain another discrepancy. The strong cooperative interactions of P_i and glycogen reported here were not observed by Helmreich and

TABLE IV

Comparison of calculated dissociation constants for phosphorylase a and b from muscle

Calculated constants for phosphorylase *b* from Table III have been selected to see if a match might be obtained with previously published (1) constants for phosphorylase *a*. The assumption was made that free phosphorylase *a* has properties comparable to the *b* form when the latter is combined with either $P_i(P)$, AMP(*A*), both (*AP*), or nothing (*O*).

Phosphorylase *a*			Phosphorylase *b*								
Form of enzyme	K_p	K_g	Form of enzyme	K_p when *X* is				K_g when *X* is			
				AP	*A*	*P*	*O*	*AP*	*A*	*P*	*O*
	mM			mM				mM			
EP	>35		*EXP*	60	0.32	60	0.09				
EAP	>20		*EXAP*	60	2.3	60	0.19				
EPG	7		*EXPG*	3.0	0.32	3.0	0.32				
EAPG	1.1		*EXAPG*	3.0	0.32	3.0	0.32				
EG		1.0	*EXG*					1.7	1	3.4	1
EAG		0.26	*EXAG*					0.6	4.5	1.7	1
EPG		<0.2	*EXPG*					0.09	1.7	0.18	3.4
EAPG		<0.03	*EXAPG*					0.03	0.6	0.09	1.7

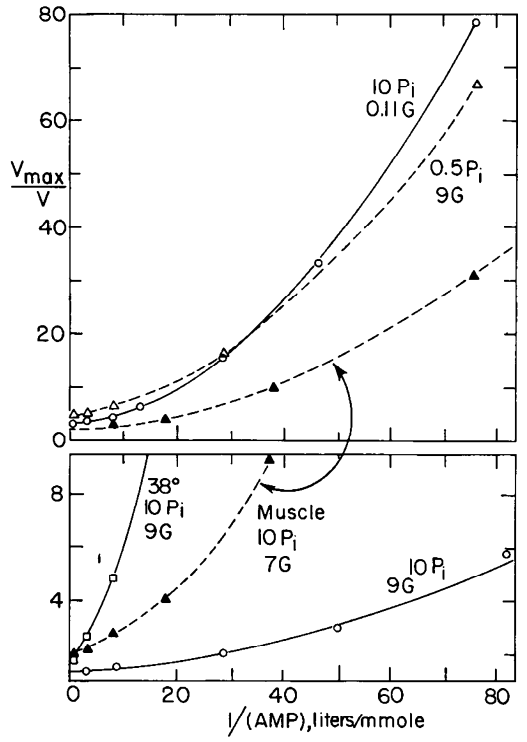

FIG. 9. Effect of 5'-AMP concentration on activity of brain phosphorylase activity at high and low levels of P_i and glycogen (*G*). The indicated concentrations are millimolar. The temperature was 24°. For comparison a curve for muscle phosphorylase *b* at high levels of P_i and glycogen is included, as well as a few points from a curve for brain phosphorylase *b* at 38° (also at high P_i and glycogen levels).

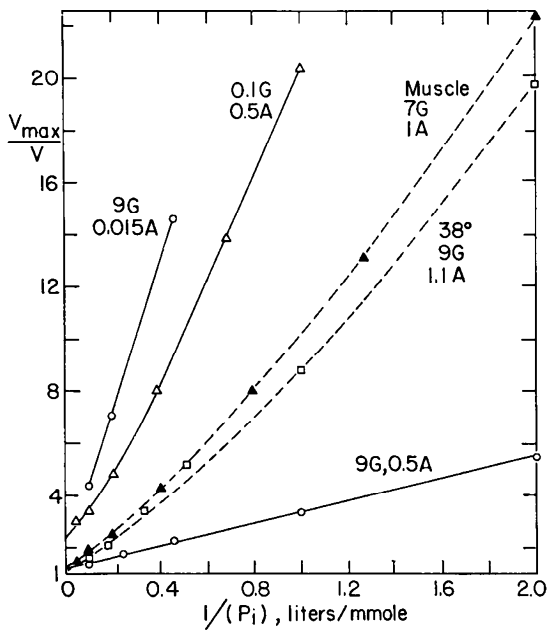

FIG. 10. Effect of P_i concentration on activity of brain phosphorylase *b* at high and low levels of glycogen (*G*) and 5'-AMP (*A*). The indicated concentrations are millimolar. The temperature was 23°. For comparison curves at high levels of AMP and glycogen are included for muscle phosphorylase *b* at 26° and brain phosphorylase *b* at 38°.

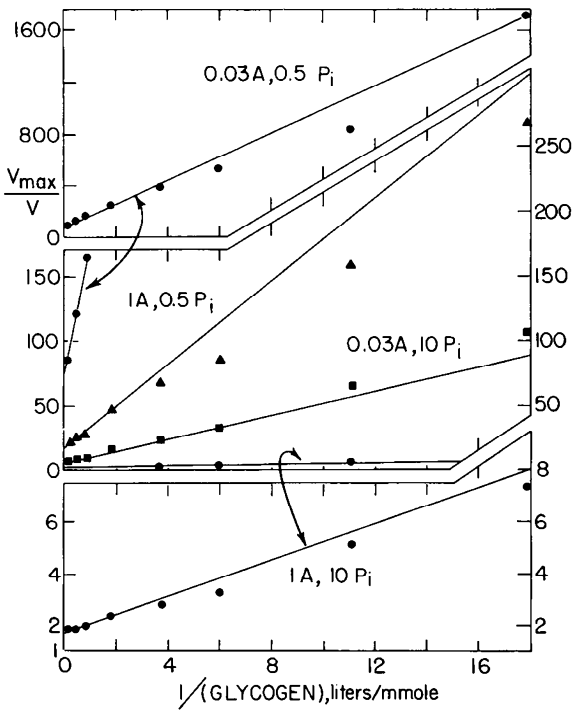

FIG. 11. Effect of glycogen concentration on activity of brain phosphorylase *b* at high and low levels of P_i and 5'-AMP (*A*). The indicated concentrations are millimolar. The temperature was 23°. For comparison curves at high levels of P_i and AMP are included for muscle phosphorylase *b* at 23° and brain phosphorylase *b* at 38°.

TABLE V

Comparison of apparent dissociation constants for 5'-adenylate from phosphorylase b of muscle and brain

K'_{AMP} represents the AMP concentration necessary to give activity equal to half of that activity (V') which would be maximal for the given conditions of temperature and substrate concentrations. B' and C' represent the apparent AMP dissociation constants necessary to satisfy the equation

$$\frac{V'}{v} = \frac{B'C'}{(\text{AMP})^2} + \frac{2C'}{(\text{AMP})} + 1$$

In the case of the brain enzyme these constants represent the best fit to the data (determined by trial and error). In the case of the muscle enzyme B' and C' were calculated by substitution of the constants of Table III in text Equation 2 and combining terms in $1/aa$ and those in $1/a$.

Phosphorylase	Temperature	P_i	Glycogen	K'_{AMP}	B'	C'
		mM		μM		
Muscle *b*	23°	10	7	100	43	42
	23	10	0.055	220	75	94
	23	0.5	7	100	44	42
	23	0.5	0.055	120	87	44
	38	10	7	380		
Brain *b*	23	10	9	26	60	6
	23	10	0.11	80	250	15
	23	0.5	9	50	120	15
	38	10	9	250	120	100

Cori (2), although Morgan and Parmeggiani did find that an increase in P_i lowered the Michaelis constant for glycogen (3).

Brain Phosphorylase b

This enzyme shows many qualitative similarities to muscle phosphorylase *b*. Reciprocal plots of velocity against AMP concentration are markedly curved (Fig. 9), and some of the reciprocal plots with P_i are also nonlinear (Fig. 10). Furthermore, reciprocal plots with glycogen (Fig. 11) are linear within analytical limits as was true for the muscle enzyme. In spite of these similarities there are at least two major quantitative differences between the enzymes, namely, Michaelis constants for P_i and apparent dissociation constants for AMP are substantially smaller for the brain enzyme.

Since the data are not as extensive as in the case of the muscle enzyme, no attempt has been made to evaluate individual dissociation constants. However, comparison has been made between the two enzymes on the basis of over-all (apparent) dissociation constants (Tables V and VI). The results suggest that of the two postulated AMP sites one binds AMP much more strongly in the brain enzyme than in the muscle enzyme. The analysis (Table V) indicates that the presence of 1 AMP molecule makes the 2nd AMP molecule bind more tightly. This seems to be true whether the enzyme is nearly saturated with glycogen and P_i or is largely depleted of one or the other substrate. It is of interest that it is this same site which appears to be most strongly affected by temperature. Thus there is a 16-fold increase in C' of brain (Table V) when the temperature is raised from 23° to 38°, whereas B' only increases 2-fold.

The Michaelis constants of the two *b* phosphorylases for glycogen (K'_g, Table VI) are, with one exception, quite similar both at 23° and at 38°. The exception indicates, in the case of the brain enzyme, a surprising dependence of the glycogen Michaelis constant on AMP concentration. When AMP concentrations were reduced to levels which are approximately equivalent for the two enzymes, K'_g increased 4-fold in the case of the muscle enzyme and 20-fold in the case of the brain enzyme.

Although the Michaelis constants for P_i (K'_p, Table VI) are smaller in the case of the brain enzyme, the percentage changes resulting from varying glycogen or temperature are roughly comparable for the two enzymes. However, the effect of AMP on K'_p is exceptional as it was on K'_g. Whereas there is little effect of AMP concentration in the case of the muscle enzyme, lowering AMP increases K'_p of brain phosphorylase roughly 6-fold.

Table VI

Comparison of apparent dissociation constants for glycogen and P_i from phosphorylase a and b of muscle and brain

K'_g and K'_p represent the concentrations of glycogen and P_i, respectively, necessary to give activity equal to half of that activity (V') which would be maximal for the given conditions. In the case of brain phosphorylase *b* at both temperatures, and muscle phosphorylase *b* at 38°, K'_g and K'_p are the directly observed values. In the case of the *a* phosphorylases these values were calculated by substitution of the constants of Table I in Equation 1 and rearranging. For example when glycogen is the variable

$$\frac{V'}{v} = \frac{V}{v(1 + K_p^G/(p))} = \frac{1}{(g)}\left(\frac{K_g K_p^G + (p)K_g^P}{(p) + K_p^G}\right) + 1 = \frac{K'_g}{(g)} + 1$$

In the case of muscle phosphorylase *b*, K'_g and K'_p were obtained by substitution of the constants of Table III in Equation 2 and calculating the P_i or glycogen levels to give $v = V'/2$ under the given conditions.

Enzyme	Glycogen constants					Phosphate constants				
	Temperature	AMP[a]	P_i	K'_g		Temperature	AMP[a]	Glycogen	K'_p	
				b	*a*				*b*	*a*
		mM					mM			
Muscle[b]	23° [c]	1.0	10	0.21	0.04[d]	23° [c]	1.0	7	6.5	1.1[d]
	23	1.0	0.5	0.87	0.19[d]	23	1.0	0.055	(40)	4.7[d]
	23	0.03	10	0.85	0.59	23	0.03	7	7.8	7.8
	23	0.03	0.5	1.18	0.95	23	0.03	0.055	(40)	29.0
	38	1.0	10	2.5	0.57[d]	35	1	7	(12)	2.7[d]
	38	0.1	10	4.5						
Brain[b]	23[e]	0.5	10	0.17	0.46[d]	26[e]	0.5	9	1.6	2.2[d]
	23	0.5	0.5	0.69	0.85[d]	26	0.5	0.11	4.5	4.8[d]
	23	0.015	10	3.0	6.1	26	0.015	9	(10)	3.0
	38	1.1	10	2.7	2.6[d]	38	1.1	9	5.6	2.7[d]

[a] These are the concentrations used for phosphorylase *b*; the levels for phosphorylase *a* are given in Footnote *d*.

[b] The brain enzymes were studied in 0.05 M imidazole-HCl buffer whereas the muscle enzymes were studied in 0.1 M imidazole-HCl buffer. To make sure that the difference in ionic strength did not seriously affect the results, measurements were made simultaneously with muscle phosphorylase *a* and *b* in both 0.1 M and 0.05 M imidazole-HCl reagents. Tests were made with 20 and 1 mM P_i, 7 and 0.3 mM glycogen, and two levels of AMP (0.3 and 1 mM for phosphorylase *b*, 0 and 0.1 mM for phosphorylase *a*). In all cases higher absolute activity was observed in the 0.05 M buffer, but none of the differences were such as to indicate that the buffer strength had more than minor effects on the kinetic constants.

[c] 25° in the case of phosphorylase *a*.

[d] The AMP levels were saturating in these experiments (0.02 to 0.1 mM); in the rest of the cases for phosphorylase *a*, AMP was absent.

[e] 27° in the case of phosphorylase *a*.

DISCUSSION

Comparison of Four Enzymes

It is now possible to compare the behavior of the *a* and *b* forms of both muscle and brain phosphorylase. In the case of the *a* forms, kinetic constants for P_i and glycogen have been calculated (Table I) for the infinite conditions in which the companion substrate is either completely absent or completely saturating. The data do not permit this presentation for brain phosphorylase *b* and in the case of muscle phosphorylase *b* the constants of Table III are not equivalent to the constants reported for phosphorylase *a*. Therefore comparison has been made on the basis of apparent kinetic constants under a variety of real rather than infinite conditions (Table VI).

The following conclusions seem warranted. (*a*) Under all observed conditions the kinetic constants for glycogen are smaller for muscle phosphorylase *a* than for brain phosphorylase *a*, whereas in the case of the *b* forms the constants are similar for brain and muscle enzymes. (*b*) The glycogen constants are decreased in all cases by an increase in P_i or AMP or by a decrease in temperature. (*c*) The *b* to *a* conversion lowers the glycogen constants for muscle enzyme but tends to increase these constants for brain enzyme. (*d*) In contrast to the glycogen constants, the P_i kinetic constants are larger for muscle phosphorylase *b* than for brain phosphorylase *b*. In the case of

the *a* forms, there is no consistent difference in regard to P_i constants between muscle and brain enzyme. (*e*) In all cases an increase in glycogen lowers the P_i constants, but AMP and temperature do not affect all four enzymes alike. In the case of muscle phosphorylase *a* and brain phosphorylase *b* the P_i constants are decreased by a decrease in temperature or an increase in AMP. With the muscle *b* enzyme there is a response to temperature but not to AMP; with the brain *a* enzyme, neither temperature nor AMP have much effect.

Multiplicity of Binding Sites—In case of the *b* forms of both muscle and brain phosphorylase the kinetic data suggest two interacting "homotropic" (14) binding sites for both P_i and AMP. Muscle phosphorylase *b*, at least, is recognized to be a dimer (15) capable of binding 2 moles of AMP (16). Therefore, it is reasonable to suppose that the two homotropic sites in each case are on separate monomers. The active form of muscle phosphorylase *a* is also reported to be a dimer (17), but there is no kinetic evidence for interacting homotropic sites for either AMP or P_i. These results suggest the following hypothesis: the addition of 1 P_i and 1 AMP molecule to phosphorylase *b* induces a change in configuration to one resembling that of phosphorylase *a*. (On the basis of bromthymol blue binding studies Ullman, Vagelos, and Monod (18) suggested that "the state stabilized by AMP in phosphorylase *b* already obtains in phosphorylase *a*.") The configuration induced by single AMP and P_i molecules has a relatively poor affinity for glycogen. When a 2nd molecule of P_i and of AMP are added to phosphorylase *b* each induces further changes in configuration which greatly improve glycogen binding. In the case of phosphorylase *a* there are also two binding sites for AMP and two for P_i but there is no interaction between the two AMP sites or between the two P_i sites. Nevertheless the addition of P_i or AMP (or both) improves the configuration in regard to binding of glycogen. Because of the complexity of the postulated conformational changes, increased temperature upsets the pattern possibly in a manner analogous to the "melting" of nucleic acid.

The general model for allosteric enzymes proposed by Monod *et al.* (14) could probably also be made to fit the kinetic situation. It would seem necessary however to introduce a third general form of the enzyme in addition to the postulated "*RR*" and "*TT*" forms. In fact these authors raised the possibility of intermediate forms. One difficulty, however, in fitting the data to the model of Monod *et al.* is that the addition of one P_i greatly decreases the apparent affinity for the second P_i, yet both the first and second P_i can increase the apparent affinity for glycogen. According to the model of Monod *et al.* if the addition of P_i to one site favors the addition of glycogen it should also favor the addition of P_i to its second site.

Physiological Implications—It is paradoxical that brain, with only 10% as much glycogen as muscle has phosphorylase with larger Michaelis constants for glycogen. It is tempting to attribute this to different roles for phosphorylase in the two tissues. Glycogen is definitely an emergency ration for brain, whereas muscle glycogen is regularly called upon during even moderate exercise. Consequently it may be desirable to have glycogen somewhat less accessible in brain than in muscle. The great sensitivity of the glycogen constants of muscle phosphorylase *a* to temperature may have physiological implications. Muscle may be forced to operate at temperatures well below 38°. In fact, shivering is part of the temperature control mechanism.

The distinctive features of liver phosphorylase reported by Maddaiah and Madsen might be explained teleologically by the fact that hepatic glycogen, unlike that of brain and muscle is not primarily intended for local consumption. Therefore, since 5′-AMP levels reflect local rather than systemic needs, control mechanisms based on 5′-AMP concentration are given less emphasis than in muscle or brain. The high Michaelis constant for glycogen found when P_i is low might represent a protection for the liver against complete depletion of glycogen unless an increase in P_i concentration signals a local emergency. It is known, for example, that it is difficult to reduce hepatic glycogen to levels as low as those of the brain.

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