The catalytic activity of phosphorylase *b* in the liver

With a note on the assay in the glycogenolytic direction

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1. The activity and the kinetic properties of purified hepatic phosphorylases *a* and *b* from rabbit and rat have been investigated in the glycogenolytic direction with a radiochemical assay. 2. In contrast with the *a* form, phosphorylase *b* has an absolute requirement for both AMP and a lyotropic salt. When the latter effectors are included, the *b/a*-form activity ratio remains low (0.03–0.15) at the hepatic concentration of P_i , because the *b* form has an exceedingly low affinity for this substrate. 3. Only phosphorylase *b* is significantly inhibited by glucose, glucose 6-phosphate and $MgATP^{2-}$. Assays in the presence of substrates, stimulators and inhibitors in the physiological concentration range indicate that glycogenolysis in the liver depends strictly on the conversion of phosphorylase *b* into *a*. Even at 1 mM-AMP the *b/a*-form activity ratio does not exceed 0.01. 4. Current spectrophotometric procedures for the glycogenolytic assay of phosphorylase in crude liver preparations are highly specific for the *a* form; the measurement of total phosphorylase (*a* + *b*) would require impractical modifications, and is better performed in the direction of glycogen synthesis.

The activity of glycogen phosphorylase is routinely determined in the non-physiological direction of glycogen synthesis. Specific assays for liver phosphorylase *a* and for total hepatic phosphorylase (*a* + *b* forms) are available (Stalmans & Hers, 1975; Lederer & Stalmans, 1976). They are based on an extensive knowledge of the catalytic properties of the two enzyme forms in the direction of glycogen synthesis (Appleman *et al.*, 1966; Maddaiah & Madsen, 1966; Stalmans *et al.*, 1974; Stalmans & Hers, 1975; Tan & Nuttall, 1975; Lederer & Stalmans, 1976). As to the physiological glycogenolytic direction, most of our information stems from the study of phosphorylase *a* by Maddaiah & Madsen (1966); the properties of the *b* form are virtually unknown (Tan & Nuttall, 1975; Stalmans, 1976; Guénard *et al.*, 1977). It is usually accepted that only the *a* form is able to catalyse the phosphorylase of glycogen in the liver (Hers, 1976; Stalmans, 1976; Hems & Whitton, 1980), but the participation of phosphorylase *b* has also been considered or postulated in specific conditions (Koster *et al.*, 1973; Hue *et al.*, 1975; de Barsey & Lederer, 1980; Sharma *et al.*, 1980). Notwithstanding this fragmentary knowledge, the glyco-

genolytic direction is occasionally used for the assay of 'total' phosphorylase activity as well as the *a* form in liver samples (Fernandes *et al.*, 1974; Thurston *et al.*, 1974; Walli *et al.*, 1974; Katz *et al.*, 1979).

In the present study we have compared the glycogenolytic activities of liver phosphorylases *a* and *b*, in the presence of various concentrations of substrates and of stimulators and inhibitors. The goals were (i) to estimate whether the *b* enzyme might sometimes become catalytically active *in vivo* and (ii) to decide on the adequacy of established assays for phosphorylases *a* and (*a* + *b*) in the glycogenolytic direction. Parts of this work have been published in a preliminary form (Stalmans, 1976; Gevers & Stalmans, 1980).

Materials and methods

Materials and analytical methods

Phosphorylase *b* and phosphorylase *b* kinase were purified from rabbit muscle as described by Fischer & Krebs (1958) and by Hayakawa *et al.* (1973) respectively. Particulate glycogen was purified from dog liver by the phenol method (Laskov & Margolias, 1963). It was peripherally labelled with [^{14}C]-glucosyl units as described by Gevers & Stalmans (1981). Purine derivatives were obtained from Sigma Chemical Co. DEAE-cellulose was type DE-52 from

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Whatman Biochemicals. All chemicals were the purest grade available from various suppliers. Glucose (Dahlqvist, 1961) and glucose 1-phosphate (Bergmeyer & Michal, 1974) were determined enzymically, and protein by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Enzyme assays

All enzyme activities were determined at 30°C. The properties of purified phosphorylases were examined in the glycogenolytic direction by the ^{14}C -assay method (Gevers & Stalmans, 1981), since the usual coupled spectrophotometric assay was unsuitable for several reasons detailed previously (Gevers & Stalmans, 1981). The assay mixture was a 'minimum physiological medium', containing 1% (w/v) ^{14}C -labelled particulate glycogen and 5 mM-potassium phosphate, pH 7.4. Other additions and modifications are indicated in the text. During purification, the activities of liver phosphorylases were measured in the reverse direction; one unit of phosphorylase *a* or *b* converts 1 μmol of substrate per min in the appropriate assay conditions (Stalmans & Hers, 1975). Phosphorylase kinase activity was determined from the rate at which purified muscle phosphorylase *b* was converted into the *a* form (Vandenhede *et al.*, 1979), in the presence of 10 mM-Hepes, pH 7.4.

The activity of glycogen hydrolases was estimated from the rate at which they degraded glycogen in the absence of P_i to products soluble in 66% (v/v) ethanol. The enzyme preparation (when necessary, freed from P_i by dialysis) was incubated for 30 min in the presence of 1% (w/v) ^{14}C -labelled particulate glycogen, 0.15 M-KCl, 0.3 mM-dithiothreitol and 20 mM-Hepes, pH 7.4, in a final volume of 0.2 ml. The reaction was arrested by the addition of trichloroacetic acid, and ethanol-soluble radioactivity was determined as in the ^{14}C -assay method of phosphorylase (Gevers & Stalmans, 1981). This procedure was considerably more sensitive for the detection of α -amylase than the assay based on the reducing power of enzyme products (Rick & Stegbauer, 1974).

Phosphorylases from liver

Phosphorylase *b*. Well-fed rabbits or Wistar rats were used for the isolation of an enzyme-glycogen complex from which phosphorylase *b* was further purified by DEAE-cellulose chromatography as described (Stalmans & Hers, 1975), with the following modifications. (i) The glycogen pellet was washed once by resedimentation. (ii) Instead of 1 mM-EDTA, a solution containing 50 mM-imidazole, pH 7.4, and 1 mM-dithiothreitol was used throughout the procedure, mainly to avoid alkaline denaturation of the enzyme on elution from the ion-exchanger with a gradient (0–0.6 M) of NaCl.

(iii) The fractions containing phosphorylase without phosphorylase kinase were pooled and concentrated by ultrafiltration; when required, the solution was filtered through Sephadex G-200 to separate phosphorylase *b* from a contaminating glycogen hydrolase. (iv) The final preparation was dialysed overnight against 50 mM-glycylglycine, pH 7.4, then for 2 h against the same buffer containing 66% (v/v) glycerol, and stored unfrozen at -20°C .

In the course of this work the enzyme has been purified three times each from rabbit and rat livers, with specific activities ranging from 4.4 to 9.1 units/mg of protein. Although in the past, several preparations of phosphorylases *b* and *a* have been stored frozen in glycylglycine buffer (Stalmans *et al.*, 1974; Stalmans & Hers, 1975; Lederer & Stalmans, 1976), we found that freezing in the presence of 50 mM-imidazole and 0.5 mM-dithiothreitol, pH 7.4, caused gross denaturation with complete loss of activity. When stored unfrozen at -20°C , the enzyme displayed a constant activity for at least 3 months.

Phosphorylase *a*. The purified *b* enzymes were converted into the *a* form with muscle phosphorylase kinase as previously described (Lederer & Stalmans, 1976); the completeness of the conversion was checked in parallel assays of *a*-form activity and total (*a* + *b*) activity. A single preparation of phosphorylase *a* has been isolated directly from rat liver as outlined by Stalmans *et al.* (1974), with modifications similar to those indicated above for the purification of the *b* form.

Results and discussion

Purification of liver phosphorylase *b*

The initial enzyme preparations were contaminated with phosphorylase kinase and with a glycogen hydrolase. The former enzyme interfered with the effect of MgATP^{2-} on phosphorylase *b*, and the latter caused high blank values in the ^{14}C -assay of phosphorylase. Phosphorylase kinase, when present in the glycogen fraction, was eluted from the DEAE-cellulose at the tail of phosphorylase *b*, and could be satisfactorily separated with a less steep gradient. Most of the glycogen hydrolase activity present in the liver homogenate was bound to the glycogen particles. Fig. 1 shows its behaviour during ion-exchange chromatography of the glycogen fraction from rabbit liver. The major part of the hydrolase remained associated with glycogen and was not retained on the exchanger. The minor adsorbed fraction was eluted just before phosphorylase *b*. When rat liver was used, the elution profile of the two enzymes from DEAE-cellulose overlapped, and a subsequent chromatography on Sephadex G-200 was required to separate phosphorylase *b* from the hydrolase of lower molecular

Fig. 1. Behaviour of phosphorylase *b* and of a 'glycogen hydrolase' during DEAE-cellulose chromatography of the glycogen fraction from rabbit liver

The glycogen fraction was isolated from 230g of rabbit liver, resuspended in 150 ml of imidazole/dithiothreitol/buffer, and the solution was passed through a column of DEAE-cellulose (bed volume, 250 ml), which was eluted with a linear NaCl gradient. O, Glycogen hydrolase (the first peak coincides with the unadsorbed glycogen); ●, phosphorylase *b*; ----, NaCl concentration in the effluent, as estimated by conductivity measurement.

weight (results not shown). The latter enzyme has the properties of α -amylase. It was stimulated by 0.15 M-NaCl. Analysis of the ^{14}C -labelled ethanol-soluble reaction products by chromatography on Sephadex G-15 revealed the absence of glucose, as expected from the action pattern of hepatic α -amylase (Brosemer & Rutter, 1961). The fact that rat liver contains at least five times more α -amylase than does rabbit liver (McGeachin & Potter, 1960; Rutter & Brosemer, 1961) may explain the separation problem encountered in the former case.

The effects of salt and substrates

The activities of phosphorylases *a* and *b* were initially determined in a 'minimum physiological medium' (1% glycogen and 5 mM- P_i , pH 7.4) plus AMP at 0.1 mM or 1 mM as indicated. In these conditions phosphorylase *b* was essentially inactive, unless a suitable salt was added. Fig. 2 illustrates that various potassium salts stimulate the *b* form according to the salting-out potency of the anion. These results are qualitatively similar to observations with muscle phosphorylase *b* (Engers & Madsen, 1968) and with the liver enzyme in the direction of glycogen synthesis (Stalmans & Hers, 1975); however, actual inhibition was only observed in the present study with the extremely chaotropic SCN^- . Sotiroidis *et al.* (1979) have reported an important stimulation of muscle phosphorylase *b* by some polycarboxylates, of which EDTA was

Fig. 2. Effect of anions on the activity of phosphorylase *b*. The activity of phosphorylase *b* from rabbit liver was determined in the presence of 1% particulate glycogen, 5 mM-potassium phosphate, 1 mM-AMP, and salts as indicated, at pH 7.4.

maximally effective; in our assay conditions, EDTA at up to 0.12 M behaved almost identically to the monocarboxylates acetate and formate (results not shown). In contrast with the importance of the type

of anion, a series of univalent chlorides (Li^+ , Na^+ , K^+ , Rb^+ , Cs^+ , NH_4^+) stimulated liver phosphorylase *b* to approximately the same extent at concentrations up to 0.15 M, where the recorded activities varied by a maximum of 12% (results not shown). In subsequent experiments, unless otherwise stated, 0.15 M-potassium acetate or fluoride was added to the assay medium. This approximates the intracellular cationic situation, and the anion serves as a highly effective substitute for the various intracellular contributors.

Although the addition of, e.g., 0.15 M-KF to the minimum physiological medium produced a several-fold increase in the activity of phosphorylase *b* (Fig. 2), the enzyme remained still poorly active when compared with phosphorylase *a*. As shown in Fig. 3, the basic reason is the very low affinity of the *b* form for the substrate P_i . In the presence of 0.15 M-KF (Fig. 3) or in the absence of added salt (results not shown) the saturation kinetics of rabbit liver phosphorylase *b* with P_i were clearly non-Michaelian.

Fig. 3. Effect of P_i on phosphorylase activity. The activity of phosphorylases *a* and *b* (a single preparation from rabbit liver) was determined in the presence of 1% particulate glycogen, 1 mM-AMP, 0.15 M-KF, 10 mM-Hepes and P_i , as indicated, at pH 7.4. ---- indicates the physiological P_i concentration in the liver (5 mM).

Substrate inhibition occurred above 0.25 M; the sigmoidal shape at low P_i concentrations was especially pronounced in the absence of added salt. Almost identical results were obtained with rat liver phosphorylase *b* (results not shown). The extremely lyotropic nature of P_i adds probably a positive salt effect to the saturation kinetics, as was previously concluded for the muscle enzyme (Engers & Madsen, 1968). We are unable to derive kinetic constants from these data. Tan & Nuttall (1975) reported a value of 500 mM- P_i for the apparent K_m of rat liver phosphorylase *b*.

Kinetic parameters for the saturation of phosphorylase *a* with P_i are listed in Table 1. In the presence of salts, the apparent K_m values were 6.6 mM and 2.4 mM for the rabbit enzyme in the absence and presence of AMP respectively. These values are in reasonable agreement with published data (1–3.5 mM) for the *a* enzymes from the liver of rabbit (Maddaiah & Madsen, 1966), dog (Kaufmann & Froesch, 1973; Van den Berghe *et al.*, 1973), mouse (Thurston *et al.*, 1974) and rat (Table 1). However, it is noteworthy that some potassium salts at 0.15 M (Cl^- and F^- , but not acetate) lower the affinity of phosphorylase *a* for P_i severalfold. The latter change is partially corrected by AMP, whereas none of these additions affects $V_{\max}$ greatly (Table 1). These observations imply that the affinity of liver phosphorylase *a* for P_i could be significantly lower *in vivo* than expected from the kinetic constants in the literature.

Throughout the present work we have used mildly isolated α -particulate glycogen, which is the natural substrate for liver phosphorylase. It has indeed been shown that the pattern of glycogen degradation by phosphorylase is markedly altered when the native structure of the polysaccharide is disrupted (Devos & Hers, 1979). The standard glycogen concentration in the present study (10 mg/ml) turned out to be above the apparent K_m value of either enzyme form isolated from both examined species; the constants of the *a* forms were 1–2 mg/ml; those of the *b* enzymes 4–7 mg/ml (Table 1). In comparable assay conditions Maddaiah & Madsen (1966) obtained a similar value (0.6 mg/ml) for rabbit liver phosphorylase *a* with alkali-treated glycogen. Thus the hypothesis of a marked influence of glycogen structure on the saturation kinetics of phosphorylase is not borne out by the experimental data.

The effect of inhibitors

When assayed in the minimum physiological medium plus a 0.15 M concentration of a lyotropic potassium salt and 1 mM-AMP, the *b/a*-form activity ratio was 0.03–0.15, according to the exact added salt and the particular enzyme preparation (see, e.g., Figs. 3–5). However, these values are susceptible to further modification if one takes into account the

Table 1. *Kinetic parameters for the saturation of liver phosphorylases with substrates, AMP and IMP*

Initial reaction rates of the two enzyme forms from rabbit liver were determined in the presence of 5 mM- P_i , 1% glycogen and AMP as shown (each of these ligands was varied in concentration in turn); salts and inhibitors were added as indicated. Except where specified (*), the values are means of at least two determinations; values in parentheses refer to a single determination with enzyme from rat liver. V_{max} is only indicated where a direct comparison is possible and meaningful. Hill coefficients (h) and AMP or IMP concentrations ($A_{0.5}$) at which $v = V_{max}/2$ were determined from Hill plots. 'Inhibitors' refers to a mixture containing 5 mM-glucose, 3 mM-ATP, 5 mM- Mg^{2+} , 0.2 mM-glucose 6-phosphate and 0.3 mM-UDP-glucose.

Variable	Additions			Kinetic parameters			
	Salt	AMP (mM)	Inhibitors	Phosphorylase <i>a</i>		Phosphorylase <i>b</i>	
				Apparent K_m (mM)	V_{max} (μ mol/min per mg of protein)		
P_i	—	—	—	6.6	4.6	See Fig. 3. and the text	
	—	1	—	2.4 (3.0)	5.8		
	0.15 M-Acetate	—	—	8.0	4.3		
	0.15 M-Acetate	1	—	2.3	4.9		
	0.15 M-KCl	—	—	29	5.6		
	0.15 M-KCl	1	—	6.9	5.9		
	0.15 M-KF	—	—	30	5.3		
	0.15 M-KF	1	—	19	5.5		
Glycogen				Apparent K_m (mg/ml)		Apparent K_m (mg/ml)	
	—	0.1	—	2.0			
	—	1	—	1.4 (1.5)			
	0.15 M-KF	0.1	—			4.2 (7.1)	
AMP	0.15 M-KF	1	—	1.0 (2.2)		5.1 (4.2)	
				K_a (μ M)		$A_{0.5}$ (mM)	h
	—	—	—	17			
	0.075 M- K_2SO_4	—	—			0.51	1.0
	0.15 M-KF	—	—			0.37*	1.0*
	0.15 M-KF	—	+			1.70*	1.79*
	0.15 M-Acetate	—	—	20†		0.36†	1.0†
IMP	0.15 M-Acetate	—	+	15†		1.35†	1.65†
	0.15 M-Acetate	—	—			1.65*	1.77*

† Data from Fig. 5.

divergent effect of inhibitors on the two enzyme forms. Potentially important inhibitors of liver phosphorylases include ATP and ADP, glucose, glucose 6-phosphate and UDP-glucose (Maddaiah & Madsen, 1966; Stalmans *et al.*, 1974; Tan & Nuttall, 1975). Other reported inhibitors (PP_i , fructose 1,6-bisphosphate, glycerol 1-phosphate) were not considered since the required concentration is several orders of magnitude above the hepatic level (Maddaiah & Madsen, 1966; Kaufmann & Froesch, 1973). As shown in Fig. 4, glucose at the basal concentration of 5 mM is the most important physiological inhibitor of phosphorylase *b*. Glucose 6-phosphate (0.2 mM) and 3 mM-ATP in the presence of 5 mM- Mg^{2+} were of secondary importance, whereas 0.3 mM-UDP-glucose did not inhibit. Phosphorylase *a* was not appreciably affected by the same concentrations of ligands (Fig. 4), though AMP was present at a 10-fold lower

concentration (0.1 mM). Rather puzzling is the observation that supraphysiological concentrations of UDP-glucose (1–3 mM) did inhibit phosphorylase *a*, but not at all the *b* form (Fig. 4). This paradoxical difference was reproducibly obtained with various enzyme preparations from rabbit and rat; it was observed in the presence of 1 mM-AMP as well as 0.1 mM; and it was identical when the reaction rate was monitored by enzymic determination of the product glucose 1-phosphate. Finally, phosphorylase *a* that was directly purified as such from rat liver (see the Materials and methods section) was also inhibited by UDP-glucose to a similar extent. We have no explanation for this exclusive inhibition of the *a* form, which, however, is quantitatively irrelevant at physiological concentrations of the ligand.

Kasvinsky *et al.* (1978) have demonstrated a negative nucleoside site, distinct from the AMP-

Fig. 4. Effect of glucose, glucose 6-phosphate, UDP-glucose and MgATP^{2-} on phosphorylase activity. The activity of phosphorylases *a* and *b* (a single preparation from rabbit liver) was determined in the presence of 1% particulate glycogen, 5 mM- P_i , 0.15 M-KF, AMP at either 1 mM (*b* form) or 0.1 mM (*a* form), and the indicated concentrations of the specified ligands, at pH 7.4. — indicates enzymic activities without added inhibitors. The filled symbols correspond to the basal hepatic concentrations of each ligand. The inhibitor concentration is indicated on a logarithmic scale.

Table 2. Effect of purine derivatives with or without glucose on the activity of phosphorylase *b*

The activity of phosphorylase *b* from rabbit liver (the same preparation as in Fig. 2) was determined in the presence of 1% particulate glycogen, 5 mM- P_i , 1 mM-AMP, 0.15 M-KF and additions as indicated, at pH 7.4.

Purine derivative (0.5 mM)	Activity (nmol/min per mg of protein)	
	Without glucose	+ 1 mM-Glucose
None	163	138
Adenosine	151	122
Inosine	143	119
Adenine	126	102
Hypoxanthine	120	99
Xanthine	121	98
Potassium urate	114	90
Allopurinol	109	78
Caffeine	40	10

bined, a strong inhibition of the enzyme in the direction of glycogen synthesis could be observed. Since no physiological ligand of the nucleoside site has been reported, we have screened some potential candidates for such an effect on phosphorylase *b* in the glycogenolytic direction (Table 2). At 0.5 mM, the degree of inhibition was greater for purines than for the corresponding nucleosides; the potency of urate approximated that of allopurinol. The inhibitory effect of 1 mM-glucose was rather strictly additive to that of the heterocyclic compounds. In the liver of primates urate (at approx. 0.25 mM) may contribute to the suppression of phosphorylase *b* activity. Rat liver produces allantoin and contains much less urate, which, however, accumulates to at least 0.15 mM during hypoxia (G. Van den Berghe, personal communication).

The effect of AMP and of IMP

An attempt was then made to define the activity of liver phosphorylases *a* and *b* in the presence of physiological concentrations of substrates, stimulators and inhibitors. Fig. 5 shows the effect of increasing AMP concentrations with or without a 'physiological' cocktail of inhibitors. In the absence of inhibitors the saturation of both enzyme forms with AMP followed strict hyperbolic kinetics, with

binding site, in phosphorylase *a* from liver and muscle. Glucose enhances the binding of purine derivatives (methylxanthines and allopurinol) to the former site. Thus when barely inhibitory concentrations of both caffeine and glucose were com-

Fig. 5. Effect of AMP with or without inhibitors on phosphorylase activity

The same enzyme preparation as in Fig. 4 was used. Enzyme activities were determined in the presence of 1% particulate glycogen, 5 mM- P_i , 0.15 M-potassium acetate and the indicated concentrations of AMP, without (○) or with (●) a physiological mixture of inhibitors, consisting of 5 mM-glucose, 0.2 mM-glucose 6-phosphate, 0.3 mM-UDP-glucose and 3 mM-ATP plus 5 mM- Mg^{2+} at pH 7.4. The kinetic constants derived from this experiment are listed in Table 1.

K_a values around 20 μ M for phosphorylase *a* and of 0.36–0.51 mM for phosphorylase *b* in the presence of various salts (Table 1). The mixture of inhibitors caused a modest inhibition of phosphorylase *a*, clearly non-competitive with AMP (Fig. 5 and Table 1). In contrast, the inhibitors altered markedly the saturation of the *b* form with AMP, by (i) increasing the degree of interaction between subunits; (ii) increasing $A_{0.5}$ and (iii) decreasing v at infinite AMP concentration (Fig. 5 and Table 1). As a result, the activity of phosphorylase *b* was completely suppressed at AMP concentrations up to 0.2 mM. Even at 1 mM-AMP the *b/a*-form activity ratio was only 0.007.

The K_a values for AMP mentioned above are in good agreement with published data obtained for the

reaction in the direction of glycogen synthesis; these range from 0.02 to 0.1 mM for phosphorylase *a* (Maddaiah & Madsen, 1966; Stalmans *et al.*, 1974) and from 0.5 to 1.25 mM for the *b* form (Stalmans & Hers, 1975; Tan & Nuttall, 1975; Lederer & Stalmans, 1976). The only previous data on phosphorylase *b* in the glycogenolytic direction (Guénard *et al.*, 1977) are at variance with the present observations. The latter authors reported (i) a K_a value for AMP (at 10 mM- P_i in the presence of 50 mM-KCl) of 75 μ M; (ii) an activity ratio (for in the absence/in the presence of AMP) of 0.4 in the same conditions; (iii) a K_m for P_i of 3 mM. These are the characteristics of phosphorylase *a*, which may have been a minor component of the enzyme preparation used by Guénard *et al.* (1977).

IMP was a weak activator of liver phosphorylase *b*, as compared with AMP; the saturation curve was markedly co-operative, and the $A_{0.5}$ value (1.65 mM) about 4-fold higher (Table 1). Also, v at saturating IMP concentration reached only 40% of that observed with AMP. These characteristics are closely similar to observations with muscle phosphorylase *b* (Black & Wang, 1968); again, they do not agree with the findings of Guénard *et al.* (1977), who reported a K_a value of 0.1 mM and a maximal effect approaching that of AMP.

Is phosphorylase *b* always inactive in the liver?

With the proviso that the existence of an unidentified potent stimulator of phosphorylase *b* cannot be excluded, our results indicate that the interconversion between phosphorylase *b* and *a* in the liver controls glycogenolysis in a strict 'on-off' fashion. However, in several situations a role has been proposed for phosphorylase *b* in hepatic glycogenolysis. There is the puzzling fact that patients with glycogen-storage disease due to hepatic phosphorylase kinase deficiency manifest usually a normal hyperglycaemic response to glucagon. de Barsey & Lederer (1980) have boosted the hypothesis of Koster *et al.* (1973) that phosphorylase *b* would be sufficiently stimulated by 5'-AMP, which might be generated by the catabolism of cyclic AMP. They point out that glucagon does rapidly increase the hepatic concentration of 5'-AMP in mice from 0.1 mM to 0.25 mM. However, even higher concentrations of AMP cannot be expected to endow phosphorylase *b* with sufficient activity (Fig. 5). We propose that this glycogen-storage disease may be due to a modified phosphorylase kinase, which acquires *in situ* a definite activity when phosphorylated by cyclic AMP-dependent protein kinase but may be unstable *in vitro*. Malthus *et al.* (1980) have recently reported that rats with a similar genetically determined deficiency are unable to mobilize any hepatic glycogen during starvation for 24 h. These animals should prove a useful model to

test hypotheses on the relationship between glucagon, phosphorylase and glycogenolysis.

There is also a series of observations with isolated liver tissue (slices, perfused organ, isolated hepatocytes) where glycogen synthesis, measured by the incorporation of labelled glucose into glycogen, occurred while the total glycogen content decreased (see Stalmans, 1976). Hue *et al.* (1975) made the important point that isolated hepatocytes often degrade glycogen even at very low concentrations of phosphorylase *a*. They suggested therefore that glycogenolysis in those conditions involved either phosphorylase *b*, or hydrolytic enzymes (α -amylase or α -glucosidase). Our approach, by elimination, implicates a hydrolytic pathway. In fact, it was established 20 years ago that in isolated liver tissue α -amylase was responsible for rapid glycogenolysis whenever cellular damage occurred (Rutter & Brosemer, 1961; Rutter *et al.*, 1961). Indirect evidence has recently also been adduced in favour of an extensive non-phosphorolytic degradation of glycogen in the liver of the neonatal rat, possibly through lysosomal α -glucosidase (DeVos & Hers, 1980).

Hypoxia induces glycogenolysis in the isolated liver; previous work indicated that the latter process could occur without conversion of phosphorylase *b* into *a* (Levine, 1965; Walli *et al.*, 1974). Though a recent re-investigation (Sharma *et al.*, 1980) clearly showed a rapid severalfold increase in the concentration of phosphorylase *a*, the authors left open the possibility that the high rise of AMP concentration would also enable phosphorylase *b* to catalyse glycogenolysis. This suggestion deserves closer attention because hypoxia induces complex changes in hepatic metabolites that may affect phosphorylase *b* (Hems & Brosnan, 1970; Weber *et al.*, 1977). Besides AMP, P_i also increases; there is a decrease in the concentration of inhibitory aden-

ine nucleotides (ATP + ADP); the total adenine content can drop by as much as $1 \mu\text{mol/g}$, and this loss is largely accounted for by an accumulation of IMP, which is hardly detectable in basal conditions; on the other hand, the inhibitor glucose 6-phosphate accumulates. We have therefore checked the activity of rabbit liver phosphorylases *a* and *b* in simulated conditions of pronounced hypoxia, on the basis of the data from Hems & Brosnan (1970) and from Weber *et al.* (1977). Besides 1% glycogen and 0.15 M-acetate, 10 mM- P_i , 5 mM-glucose, 0.8 mM-glucose 6-phosphate, 0.3 mM-UDP-glucose, 0.7 mM-ATP, 1.4 mM-ADP, 1 mM-AMP, 1 mM-IMP and 5 mM- Mg^{2+} were included. The *b/a*-form activity ratio was 0.01. We are bound to conclude that glycogenolysis in hypoxia depends on conversion of phosphorylase *b* into *a*. However, the extreme rates of glycogen breakdown during anaerobic perfusion of the liver [up to $10 \mu\text{mol/min per g}$ (Birch *et al.*, 1974)] may require the participation of hydrolytic glycogenolysis as well.

On the assay of liver phosphorylases

Phosphorylase activity in crude liver preparations is only occasionally determined in the glycogenolytic direction. Though Katz *et al.* (1979) have made some comparative assays, it remains obscure to what extent current glycogenolytic assays are adequate for the measurement of either phosphorylase *a* or total phosphorylase. It would be unwieldy to check each published assay procedure in detail, but a few conditions included in Table 3 may serve as a reference in the following discussion. Thurston *et al.* (1974) used 10 mM- P_i without AMP in an assay intended to be specific for phosphorylase *a*. The data in Table 3 indicate that the assay should be highly specific; this is confirmed by the recorded activities of hepatic phosphorylase, which cover a 22-fold range during various treatments *in vivo* (Thurston

Table 3. Activity of phosphorylase *a* and *b* in various assay conditions

The activity of the two enzyme forms (same preparation as in Fig. 3) was determined in the presence of 1% glycogen, 10 mM-Hepes, pH 7.4, and additions as indicated.

Additions			Activity ($\mu\text{mol/min per mg}$ of protein)		<i>b/a</i> form activity ratio
P_i (mM)	AMP (mM)	KF (0.3 M)	<i>b</i> form	<i>a</i> form	
5	0	—	~0	2.93	~0
5	0.1	—	0.003	4.10	<0.001
5	1	—	0.08	4.31	0.02
250	1	—	4.20	6.60	0.64
250	5	—	5.33	6.75	0.79
350	5	—	4.80	6.83	0.70
500	5	—	2.10	2.70	0.78
250	1	+	3.98	5.55	0.72
250	5	+	5.03	5.33	0.94

et al., 1974). In contrast, it would not appear feasible to measure phosphorylase *a* and total phosphorylase in an assay without and with 1 mM-AMP respectively, at 0.5 mM- P_i ; in these conditions, the two measured activities change in a strictly parallel way (Katz *et al.*, 1979), and the less than 2-fold increase in activity produced by AMP should be attributed to a stimulation of phosphorylase *a* (see Fig. 5). Assays of human liver homogenates in the presence of 10 mM- P_i appear also inadequate as a means to establish phosphorylase deficiency as the cause of glycogen-storage disease (Fernandes *et al.*, 1974).

As shown in Table 3, the activity of phosphorylase *b* starts to approximate that of the *a* form when P_i is increased to 0.25–0.35 M, and AMP to 5 mM. The supplementary addition of 0.3 M-KF causes some inhibition of phosphorylase *a*, and results in an almost equal activity of the two enzyme forms. However, it is not feasible to apply such conditions to the usual glycogenolytic assay (Maddaiah & Madsen, 1966), which monitors the production of glucose 1-phosphate by the reduction of NADP⁺, through the combined action of phosphoglucomutase and glucose 6-phosphate dehydrogenase; the latter enzyme is markedly inhibited by P_i at concentrations above 50 mM (Theorell, 1935; Glaser & Brown, 1955). In work with purified phosphorylases this problem can be circumvented by the use of a two-step procedure, where the produced glucose 1-phosphate is measured after suitable dilution (Engers & Madsen, 1968; Tan & Nuttall, 1975). However, the latter solution cannot be recommended for crude extracts, since some part of the glucose 1-phosphate is bound to be lost as glucose and as glycolytic intermediates beyond glucose 6-phosphate. The alternative radiochemical procedures that we have developed are not normally intended for use with crude enzymes either, as discussed previously (Gevers & Stalmans, 1981).

In conclusion, it is perfectly feasible to determine specifically phosphorylase *a* in crude liver preparations with current glycogenolytic assay methods. The measurement of total phosphorylase in liver homogenates is better performed in the direction of glycogen synthesis, where detailed procedures have been worked out for several species including man (Stalmans & Hers, 1975; Lederer & Stalmans, 1976).

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