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The inhibitory effect of phosphorylase *a* on the activation of glycogen synthase depends on the type of synthase phosphatase

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1. The activity of glycogen synthase phosphatase in rat liver stems from the co-operation of two proteins, a cytosolic S-component and a glycogen-bound G-component. It is shown that both components possess synthase phosphatase activity. The G-component was partially purified from the enzyme–glycogen complex. Dissociative treatments, which increase the activity of phosphorylase phosphatase manyfold, substantially decrease the synthase phosphatase activity of the purified G-component. 2. The specific inhibition of glycogen synthase phosphatase by phosphorylase *a*, originally observed in crude liver extracts, was investigated with purified liver synthase *b* and purified phosphorylase *a*. Synthase phosphatase is strongly inhibited, whether present in a dilute liver extract, in an isolated enzyme–glycogen complex, or as G-component purified therefrom. In contrast, the cytosolic S-component is insensitive to phosphorylase *a*. 3. The activation of glycogen synthase in crude extracts of skeletal muscle is not affected by phosphorylase *a* from muscle or liver. Consequently we have studied the dephosphorylation of purified muscle glycogen synthase, previously phosphorylated with any of three protein kinases. Phosphorylase *a* strongly inhibits the dephosphorylation by the hepatic G-component, but not by the hepatic S-component or by a muscle extract. 4. These observations show that the inhibitory effect of phosphorylase *a* on the activation of glycogen synthase depends on the type of synthase phosphatase.

Glycogenolysis and glycogen synthesis do not operate simultaneously at an appreciable rate in the liver of adult or foetal animals (Devos & Hers, 1974; Katz *et al.*, 1979; Postle & Bloxham, 1980). This energy-conserving situation results from the operation of several control mechanisms, one of which is the profound inhibition that phosphorylase *a* specifically exerts on glycogen synthase phosphatase (Stalmans *et al.*, 1971). The latter effect explains why the inactivation of phosphorylase is a prerequisite for the activation of glycogen synthase when the liver switches from glycogenolysis to glycogen deposition (Stalmans *et al.*, 1974*a*; Hue *et al.*, 1975). The mechanism operates when glycogen synthesis is induced by an increased glucose concentration (see Hers, 1981) or by insulin (see Stalmans & van de Werve, 1981), and is already in existence in the foetal liver (Gilbert & Bourbon, 1980).

The properties of this regulatory mechanism have also been explored *in vitro*. On incubation of crude gel-filtered liver extracts, the conversion of synthase *b* into *a* is preceded by a pronounced lag, which corresponds to the time required to convert phosphorylase almost completely into the *b* form. The inhibition appears to be specifically due to phosphorylase *a*, since the latency could be lengthened by the addition of the purified enzyme (but not by adding the *b* form), and was abolished by antibodies against liver phosphorylase (Stalmans *et al.*, 1971). The further study of the inhibitory effect of phosphorylase *a* has been hampered by difficulties encountered in the purification of liver synthase phosphatase. The inhibition is evident in isolated glycogen pellets (Stalmans, 1976; Laloux & Hers, 1979) or in a crude 'microsomal' fraction (Tan & Nuttall, 1978), but it could not be detected with a homogeneous multi-substrate phosphoprotein phosphatase of M_r 35000 (Killilea *et al.*, 1976).

Our previous work showed that synthase phosphatase activity stems from the co-operation of two protein components; the G-component is tightly associated with glycogen particles, whereas the S-component is present in the post-glycogen supernatant. When purified liver synthase *b* (type b_2 or b_3) is used as substrate, full synthase phosphatase

activity requires both components; the isolated S-component displayed only 10–15% of the synthase phosphatase activity present in a crude liver extract, and the isolated G-component was virtually unable to convert synthase *b* into *a*. Synthase phosphatase activity was completely restored when the two components were recombined. However, the mechanism of action of the G-component remained obscure (Doperé *et al.*, 1980). The present work describes a partial purification of the G-component, which is a synthase phosphatase that, in contrast with the S-component, is strongly inhibited by phosphorylase *a*. Further, we report that phosphorylase *a* does not affect the activation of synthase in extracts from skeletal muscle. It appears that sensitivity to phosphorylase *a* is linked to the type of synthase phosphatase.

Experimental

Materials and buffers

Dog liver glycogen synthase *b* was prepared as described by Doperé *et al.* (1980). Rabbit muscle glycogen synthase *a* was prepared essentially by method 2 of Nimmo *et al.* (1976). ^{32}P-labelled synthase *b* was prepared by incubating purified synthase *a* with the catalytic subunit of protein kinase, with a cyclic AMP- and Ca^{2+}-independent synthase kinase (F_A) or with phosphorylase *b* kinase, as described by Yang *et al.* (1980), or with casein kinases 1 or 2 (Itarte *et al.*, 1979; Huang *et al.*, 1982). The incorporation of [^{32}P]phosphate into synthase was measured as described by Vandenheede *et al.* (1980). The mean number of phosphate groups incorporated per subunit of synthase *b*, calculated as described by Embi *et al.* (1980), was as follows: cyclic AMP-dependent protein kinase, 1.7; F_A, 1.4; phosphorylase kinase, 0.4; casein kinase 1, 2.8; casein kinase 2, 0.9. All preparations of synthase *b* from liver and muscle were free of phosphorylase *a*.

Phosphorylase *a* was purified as such from dog liver (Stalmans & Gevers, 1981). Rabbit muscle phosphorylase *a* was prepared from the crystalline *b* enzyme as detailed by Yang *et al.* (1980), except that unlabelled ATP was used. Particulate glycogen was purified from dog liver by the phenol method (Laskov & Margoliash, 1963). Adrenaline was a commercial pharmacological preparation from Sterop (Brussels, Belgium). Phosphocellulose (type P1) was obtained from Whatman, and polyethylene glycol 6000 from BDH.

Buffer A contained 0.25 M-sucrose, 0.5 mM-dithiothreitol, 3.5 mg of particulate glycogen/ml and 0.1 M-glycylglycine, adjusted at room temperature to pH 7.4 (except when otherwise stated). Buffer B contained 0.25 M-sucrose, 0.5 mM-dithiothreitol and 50 mM-imidazole, adjusted at room temperature to pH 7.4.

Assays

Phosphorylase *a* was assayed as described by Stalmans & Hers (1975) and glycogen synthase (total and *a* form) as described by Doperé *et al.* (1980). One unit of these enzymes converts 1 μmol of substrate into product/min at 25°C. Phosphorylase phosphatase was measured as described by Doperé & Stalmans (1982). Protein was determined by the procedure of Lowry *et al.* (1951).

Synthase phosphatase was assayed by either the activation or the dephosphorylation of glycogen synthase. The former assay mixture (0.1 ml) contained liver synthase *b* (1 unit/ml), a suitably diluted preparation of synthase phosphatase, 50 mM-imidazole, pH 7.4, 0.25 M-sucrose, 0.5 mM-dithiothreitol, 5 mg of bovine serum albumin/ml, and either 5 mM-magnesium acetate plus 3 mM-AMP or 1 mM-magnesium acetate plus 5 mM-$(NH_4)_2SO_4$ as specified. Unless stated otherwise, the hepatic synthase *b* used as substrate corresponds to a pool of the forms termed b_2 and b_3 by Doperé *et al.* (1980). Dephosphorylation of ^{32}P-labelled glycogen synthase from muscle (0.4 mg/ml, i.e. about 2.5 units/ml) was measured in an otherwise identical assay mixture. During incubation at 25°C for up to 1 h, samples were withdrawn at regular intervals for the assay of synthase *a* (see above) or of acid-soluble radioactivity (Yang *et al.*, 1980).

Handling of animals and tissues

Extracts for activation of endogenous synthase. Male fed Wistar rats weighing about 250 g were injected subcutaneously with adrenaline (0.4 mg/100 g body wt.) 10 min before decapitation. The liver and/or thigh muscles were removed and chilled. The liver was homogenized in a Potter-Elvehjem tube in 2.5 vol. of ice-cold buffer A. The liver homogenate was centrifuged for 10 min at 8000 *g* in the cold, and 1 ml of the supernatant was filtered through a column of Sephadex G-25 (5 cm × 1.5 cm) equilibrated in buffer A without glycogen. The gel-filtered extract was then incubated at 25°C in the presence of either 1 mM-magnesium acetate and 5 mM-$(NH_4)_2SO_4$, or 5 mM-magnesium acetate and 3 mM-AMP.

The muscles were homogenized with an Ultra-Turrax blender (Janke and Kunkel) in 2.5 vol. of buffer A adjusted to pH 8. The muscle homogenate was centrifuged for 20 min at 8000 *g*. The supernatant, the pH of which was about 7.0, was gel-filtered and incubated exactly as described for the liver extract. The presence of glycogen in buffer A was essential to stabilize the total activity of glycogen synthase during the incubation of muscle

extracts, as previously reported by Gilboe & Nuttall (1972).

Preparations for assay of synthase phosphatase. Extracts to be assayed for synthase phosphatase activity with purified substrates were prepared from fed untreated rats. The livers and/or muscles were homogenized in 3 vol. of buffer B. The homogenate was centrifuged for 10 min at 8000 *g*, and the supernatant was incubated for 15 min at 25°C in the presence of 10 mM-caffeine to ensure the complete inactivation of phosphorylase. Where indicated, the preparations were then filtered on Sephadex G-25 equilibrated in buffer B.

The two components of synthase phosphatase were separated from the liver of overnight-starved glucagon-treated rats with the use of added glycogen as described by Doperé *et al.* (1980). Their concentration is indicated by comparison with the concentration of the homogenate from which they were derived. It was checked that the isolated components were free of phosphorylase *a*. Where indicated, the post-glycogen supernatant (containing the S-component) was filtered on Sephadex G-25 equilibrated in buffer B. The crude G-component was used at the stage of the isolated glycogen–protein complex. 'G-activity' is defined as the stimulatory effect that the G-component exerts on the activation of liver synthase *b* in the presence of S-component (Doperé *et al.*, 1980).

Partial purification of the G-component

A protein–glycogen complex was prepared from the livers of five rats as described by Doperé *et al.* (1980), and resuspended in a Dounce homogenizer in a solution containing 50 mM-Tris/HCl, pH 6.8, 0.5 mM-dithiothreitol and 20 mM-2-mercaptoethanol, to a maximum volume of 4 ml. This suspension was applied on a phosphocellulose column (70 ml; 2 cm × 22 cm) equilibrated in the same solution. The column was washed with the starting buffer until all the glycogen had passed through and the eluate was no longer opalescent. A gradient of NaCl (0–0.7 M) in the same medium was then applied. Most of the G-activity was co-eluted free of glycogen with the protein peak at 0.25 M-NaCl (Figs. 6*b* and 6*e*). The activity peak was pooled and allowed to precipitate for 30 min with 5% polyethylene glycol at 0°C. After centrifugation (10 min at 8000 *g*), the tiny pellet was resuspended in 1 ml of 50 mM-imidazole/HCl, pH 7.4, plus 0.5 mM-dithiothreitol and 20 mM-2-mercaptoethanol, and an equal volume of glycerol was added for storage without freezing at −20°C. Both glycerol and 2-mercaptoethanol were required for reasonable conservation of the G-activity (approx. 50% after 9 days). Several procedures have been tried to achieve a further purification, but were abandoned because of a negligible recovery.

Results

Latency in crude tissue extracts

When a gel-filtered extract from the liver of an adrenaline-treated fed rat was incubated in the presence of 1 mM-Mg^{2+} and 5 mM-sulphate, a pronounced lag preceded the conversion of glycogen synthase *b* into *a*. This lag corresponded to the time required for the near-complete inactivation of phosphorylase (Fig. 1). Similar patterns (not shown) were observed when 50 mM-imidazole buffer was used instead of glycylglycine, when Mg^{2+} was omitted, or when Mg^{2+} and sulphate were replaced by either 50 mM-KCl or 5 mM-P_i. In contrast, in the presence of 3 mM-AMP and 5 mM-Mg^{2+} no latency was apparent, and the inactivation of phosphorylase was blocked (Fig. 1). The patterns illustrated in Fig. 1 correspond basically to observations with similar preparations from mouse liver (Stalmans *et al.*, 1971).

A similar approach was used to explore the situation in skeletal muscle. However, owing to the significantly higher activity of phosphorylase phosphatase in muscle homogenates, the endogenous phosphorylase had already been largely or entirely inactivated in the freshly prepared gel filtrate (Fig. 2). Therefore purified phosphorylase *a* was

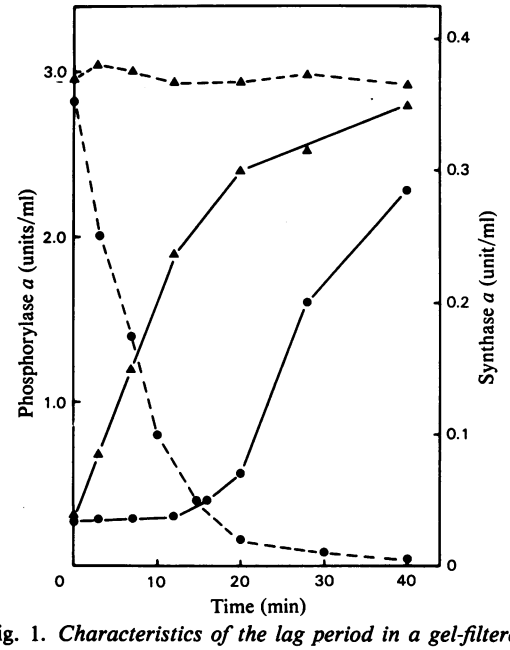

Fig. 1. *Characteristics of the lag period in a gel-filtered extract from rat liver*

The filtered extract was incubated in the presence of 1 mM-Mg^{2+} and 5 mM-sulphate (●) or 5 mM-Mg^{2+} and 3 mM-AMP (▲). ----, Phosphorylase *a*; ——, synthase *a*.

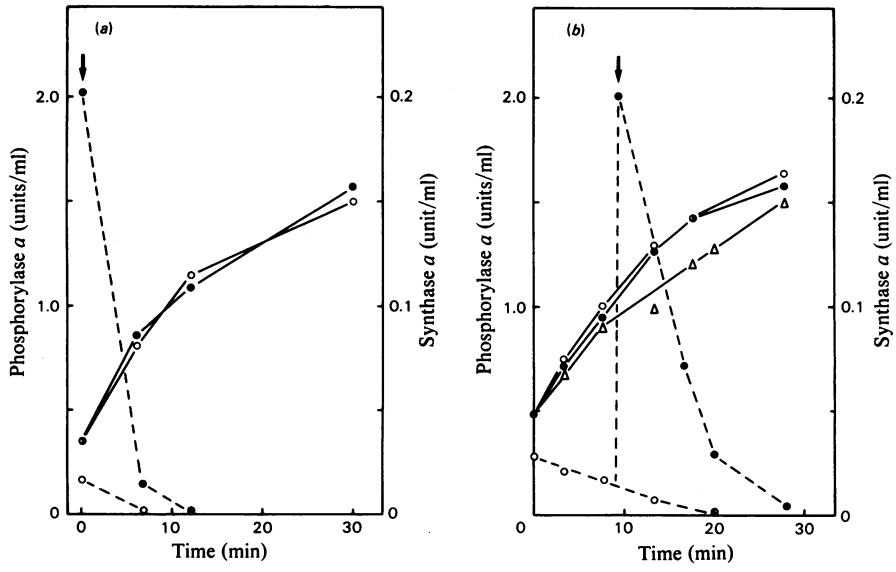

Fig. 2. *Lack of effect of phosphorylase a on the activation of glycogen synthase in a gel-filtered extract from rat skeletal muscle*

Filtered extracts were incubated without (open symbols) or with added phosphorylase *a* (filled symbols). The addition (arrow) was liver phosphorylase *a* (2 units/ml) in (*a*), and the same amount of muscle enzyme in (*b*). Incubation was in the presence of Mg^{2+} and sulphate (○, ●) or Mg^{2+} and AMP (△). ----, Phosphorylase *a*; ——, synthase *a*.

added either at the beginning of the incubation (Fig. 2*a*) or during the activation of glycogen synthase (Fig. 2*b*). In other experiments minor changes were introduced in the experimental design (omission of Mg^{2+} or of gel filtration; assay of synthase *a* in the presence of 0.25 mM-UDP-glucose instead of 5 mM). In no instance could we detect an effect of phosphorylase *a* on the rate of activation of glycogen synthase. Also noteworthy is the fact that AMP and Mg^{2+} caused a slight inhibition of synthase phosphatase in muscle extracts (Fig. 2*b*), in contrast with their specific anti-inhibitory effect in liver preparations (Fig. 1).

Activation and dephosphorylation of purified glycogen synthases by dilute extracts

For the purpose of comparison with the crude system, a dilute liver extract, free of phosphorylase *a*, was first used to activate the purified hepatic synthase *b* (Fig. 3). In the presence of Mg^{2+} and sulphate the activation of the synthase started immediately and proceeded at a constant rate; added phosphorylase *a* (2 units/ml) induced a pronounced lag, which lasted until the near-complete inactivation of phosphorylase. In the presence of AMP and Mg^{2+}, the conversion of synthase *b* into *a* started without lag and at an identical high rate whether or not phosphorylase *a* was added. Fig. 4 illustrates the effect of various concentrations of phosphorylase *a*. A family of parallel activation curves was generated which differed clearly in the duration of the lag phase. In contrast, the maximal degree of inhibition of synthase phosphatase was obtained with as little as 0·3 unit of phosphorylase *a*/ml.

Glycogen synthase *a* was purified from skeletal muscle and phosphorylated by protein kinase F_A (Vandenheede *et al.*, 1980). The subsequent dephosphorylation of ^{32}P-labelled synthase by a dilute liver extract in the presence of Mg^{2+} and sulphate was strongly inhibited by added phosphorylase *a* (1 unit/ml); the inhibition was cancelled in the presence of AMP and Mg^{2+}. When the experiment was repeated with a dilute gel-filtered extract from skeletal muscle, the dephosphorylation was wholly unaffected by the same amount of phosphorylase *a* (results not shown).

Studies with crude hepatic G- and S-components

The observations detailed below show that a crude (glycogen-bound) preparation of G-component from rat liver displays synthase phosphatase activity in various assay conditions. Some of these have allowed us to examine directly the effect of phosphorylase *a* on the separated S- and G-components.

(i) At a relative concentration of 2%, the crude G-component did not convert appreciably any

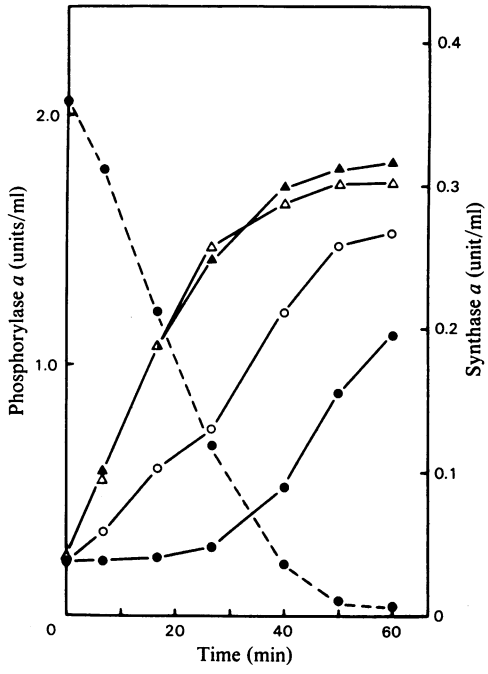

Fig. 3. *Effect of phosphorylase a on the activation of purified hepatic synthase by phosphatase present in a dilute liver extract*

Purified hepatic synthase *b* was incubated with a preincubated liver extract (final concn. 2%, w/v) in the presence of either Mg^{2+} and sulphate (○, ●) or Mg^{2+} and AMP (△, ▲), without (open symbols) or with (filled symbols) purified muscle phosphorylase *a* (2units/ml). ----, Phosphorylase *a*; ——, synthase *a*.

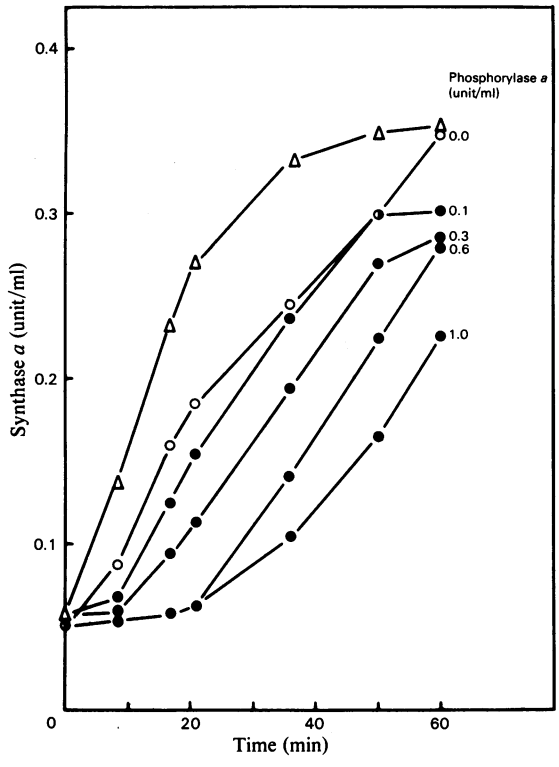

Fig. 4. *Concentration-dependence of the inhibition by phosphorylase a*

Experimental conditions and symbols are as in the legend to Fig. 3, except for the use of liver phosphorylase *a* at the indicated concentrations.

available type of liver synthase *b* into the *a* form (Doperé *et al.*, 1980). However, it increased the affinity of these preparations of synthase *b* for glucose 6-phosphate, in a time-dependent way (Table 1). In contrast, the S-component converted part of the synthase into the *a* form, but the remaining synthase *b* had an unchanged affinity for glucose 6-phosphate. Our results with the G-component are very similar to the observations of Ahmad & Huang (1981) on the partial dephosphorylation of muscle synthase by alkaline phosphatase, which resulted in important changes in the K_a for glucose 6-phosphate, without, however, significant production of synthase *a*.

(ii) Conversion of hepatic synthase *b* into *a* occurred in the presence of AMP and Mg^{2+}, or Mg^{2+} and sulphate, when the concentration of the crude G-component was increased 10-fold. In the presence of Mg^{2+} and sulphate, addition of phosphorylase *a* produced the typical transient latency (Fig. 5*a*). In contrast, the S-component, as present in a gel-filtered post-glycogen supernatant, was not inhibited at all by the same amount of phosphorylase *a* (Fig. 5*b*). These observations imply that the synthase phosphatase activity in concentrated G-preparations is not due to contamination by the S-component. When the two cell fractions were recombined (at a lower concentration), the rate of synthase activation was initially decreased to 15% by the addition of phosphorylase *a*, but it increased sharply after phosphorylase had been inactivated (Fig. 5*c*).

(iii) A time-dependent conversion of hepatic synthase *b* into *a* by low concentrations (2%) of crude G-component was observed when the incubation was done in the presence of 5mM-Mg^{2+} plus 2mM-glucose 6-phosphate (see also Fig. 6). Such high concentrations of the hexose phosphate tend to cancel the inhibitory effect of phosphorylase *a* (results not shown; Vandereycken *et al.*, 1975). The stimulatory effect of glucose 6-phosphate on the activation of glycogen synthase is well documented in the literature. It is considered to result from binding of the ligand to synthase *b* (Curnow & Larner, 1979).

(iv) The crude G-component, as well as the crude

Table 1. *Effect of the components of synthase phosphatase on the activation constant of hepatic synthases b by glucose 6-phosphate*

The preparations of liver synthase *b* were characterized with respect to K_m for UDP-glucose, $A_{0.5}$ for glucose 6-phosphate, and conversion into the *a* form by the S- and G-components (Doperé *et al.*, 1980). The synthases (1 unit/ml) were incubated in the presence of AMP and Mg^{2+} without or with crude S- and/or G-component at a relative concentration of 2%. The affinity for glucose 6-phosphate was then determined as described by Doperé *et al.* (1980). The concentration required for half-maximal stimulation by glucose 6-phosphate ($A_{0.5}$) and the Hill coefficient (*h*) were determined from Hill plots. Synthase *a* formed was measured in the regular assay and corresponded closely to the value obtained without glucose 6-phosphate in the kinetics assay. The limit for detection of an increase in synthase *a* was 10 munits/ml.

	Preincubation conditions		Kinetics with glucose 6-phosphate		
Type of synthase *b*	Addition	Time (min)	$A_{0.5}$ (mM)	*h*	Synthase *a* formed (munits/ml)
b_1	None	60	6.5	1.0	<10
	S	60	5.1	1.0	550
	G	60	2.9	1.0	15
	S+G	60	—	—	750
b_2	None	60	9.1	1.0	<10
	S	60	9.1	1.0	50
	G	60	4.9	1.0	<10
	S+G	60	—	—	340
b_3	None	60	12.6	1.7	<10
	S	60	15.8	1.6	35
	G	60	6.7	1.1	<10
	S+G	60	—	—	360
b_2	G	5	10.9	1.0	<10
	G	10	8.2	1.0	<10
	G	30	6.9	1.0	<10
	G	60	5.5	1.0	<10

S-component, were able to dephosphorylate substantially glycogen synthase from muscle which had previously been phosphorylated by any of five synthase kinases (see the Experimental section). The effect of phosphorylase *a* (2.5 units/ml) was examined on the dephosphorylation (in the presence of Mg^{2+} and sulphate) of synthase phosphorylated by cyclic AMP-dependent protein kinase, by phosphorylase kinase, or by kinase F_A. In each case the pattern was similar to that observed with liver synthase *b* as substrate (Fig. 5): dephosphorylation by the G-component was strongly inhibited, whereas dephosphorylation by the S-component was not measurably affected (results not shown).

Partial purification and properties of the G-component

The G-component has been partially purified from the enzyme–glycogen complex by chromatography on phosphocellulose and fractionation with polyethylene glycol (see the Experimental section). Fig. 6 shows the patterns obtained by assaying the phosphocellulose eluate for G-activity (i.e. activation of liver synthase *b* in the presence of added S-component), for direct manifestations of synthase phosphatase activity, and for phosphorylase phosphatase. The initial peak of all these activities coincided with the passage of glycogen. A second, rather broad, peak emerged together with most of the released protein; it was partially resolved into two peaks when labelled muscle synthase was used as substrate. However, phosphorylase phosphatase displayed a distinctly heterogeneous elution pattern.

Several harsh dissociative procedures (treatment with urea, with ethanol at room temperature, or with trypsin) lead to a manifold increase in the activity of phosphorylase phosphatase in crude liver preparations (for review, see Lee *et al.*, 1980). Killilea *et al.* (1976) reported a parallel increase of synthase phosphatase activity by ethanol treatment, but others found a substantial loss (Kikuchi *et al.*, 1977; Laloux *et al.*, 1978; Tan & Nuttall, 1978). It was therefore decided to examine the effect of these dissociative procedures on the purified G-component (Table 2). There was a complete loss of the G-activity, and a gross impairment of the capacity to dephosphorylate ^{32}P-labelled muscle synthase, in contrast with a variable (3–40-fold) increase in phosphorylase phosphatase activity.

Fig. 7 illustrates the pronounced inhibitory effect of phosphorylase *a* on the dephosphorylation of ^{32}P-labelled muscle synthase by the purified G-component. The lag depended on the continued presence of phosphorylase *a*, and it was cancelled in the presence of AMP and Mg^{2+}. Similar results (not shown) were obtained when the activation of liver synthase *b* by the purified G-component was examined.

Discussion

The biological significance of the latency that precedes the activation of glycogen synthase in gel-filtered liver extracts stems ultimately from the existence *in vivo* of a similar lag that corresponds to an extensive inactivation of phosphorylase (see the introduction). However, the importance and the nature of the phenomenon *in vitro* have been questioned on two grounds: (i) it would only be observed in very particular conditions; (ii) it would not be observed with purified enzymes. We first discuss these objections.

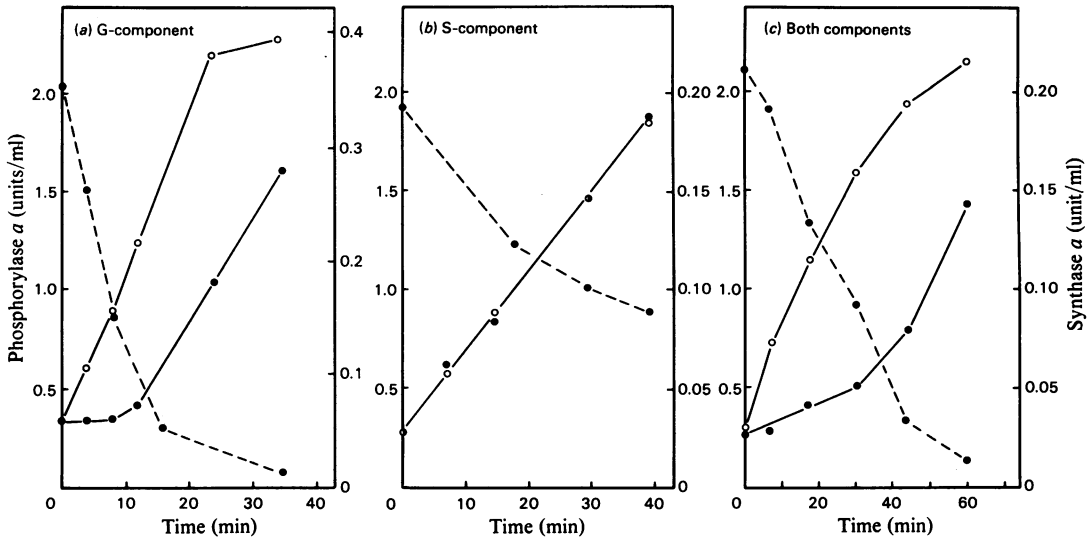

Fig. 5. *Effect of phosphorylase a on the activation of purified hepatic synthase by the two components of hepatic synthase phosphatase*

Synthase *b* was incubated with (*a*) a protein–glycogen complex (G-component) at a relative concentration of 20%; (*b*) a post-glycogen supernatant (S-component) at a concentration of 10%; (*c*) the combined fractions, each at a concentration of 5%. The added phosphorylase *a* (2 units/ml) was from muscle. Symbols are as in Fig. 3. The left-hand values in each panel refer to phosphorylase *a*, and the right-hand ones to synthase *a*.

Characteristics of the latency in liver extracts

It has been stated that the inhibition of synthase phosphatase by phosphorylase *a* depends on the presence of sulphate (Killilea *et al.*, 1976; Gilboe & Nuttall, 1982), and that it would not be observed when imidazole or Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer is used instead of glycylglycine (Curnow & Larner, 1979). One could argue then that a control mechanism 'should not be inferred from experiments in which a multiplicity of poorly understood factors influences the results' (Curnow & Larner, 1979). However, the former statements are inaccurate. The lag period is not evident in desalted preparations containing glycylglycine, imidazole or Hepes only (De Wulf *et al.*, 1970; Vandereycken *et al.*, 1975; Laloux & Hers, 1979). However, the lag is clearly observed when any of various anions is added, whether sulphate or phosphate at 5 mM (De Wulf *et al.*, 1970), sulphite, chloride or acetate (Vandereycken *et al.*, 1975), EDTA or ATP (Laloux & Hers, 1979). Univalent anions such as Cl^- may be ineffective at 5 mM (Patel & Ryman, 1972), but the addition of 50 mM- or 100 mM-KCl elicits a clear inhibitory effect of phosphorylase *a* on synthase phosphatase (Vandereycken *et al.*, 1975; the present paper). Also, there can be little doubt about the inhibition being manifest in imidazole buffer, whether 5 mM-sulphate is added (Laloux & Hers, 1979), or in addition 1 mM-Mg^{2+} (throughout the present work).

Unphysiologically high concentrations of AMP (3 mM) and Mg^{2+} (5 mM) abolish the latency (Fig. 1), as was previously observed with mouse liver extracts (Stalmans *et al.*, 1971). The effect of AMP may result from binding to phosphorylase *a*, which thereby becomes resistant to the action of phosphorylase phosphatase (Fig. 1), and apparently non-inhibitory to synthase phosphatase. The affinity of hepatic phosphorylase *a* for AMP is quite high, though it is decreased in the presence of other physiological effectors such as glucose (Stalmans *et al.*, 1974*b*). Presumably some of the phosphorylase *a* in the hepatocyte has AMP bound to it and should therefore not participate in the inhibition of synthase phosphatase. This consideration does not discount the physiological significance of the inhibition, which requires only a small fraction of the available phosphorylase *a* (Figs. 1 and 4). The presence of a small proportion of phosphorylase *a* in an AMP-associated form may, however, explain that, *in vivo*, unlike *in vitro*, the activation of glycogen synthase usually starts without a complete inactivation of phosphorylase.

Inhibition of purified hepatic synthase phosphatase

Killilea *et al.* (1976) have prepared from the liver,

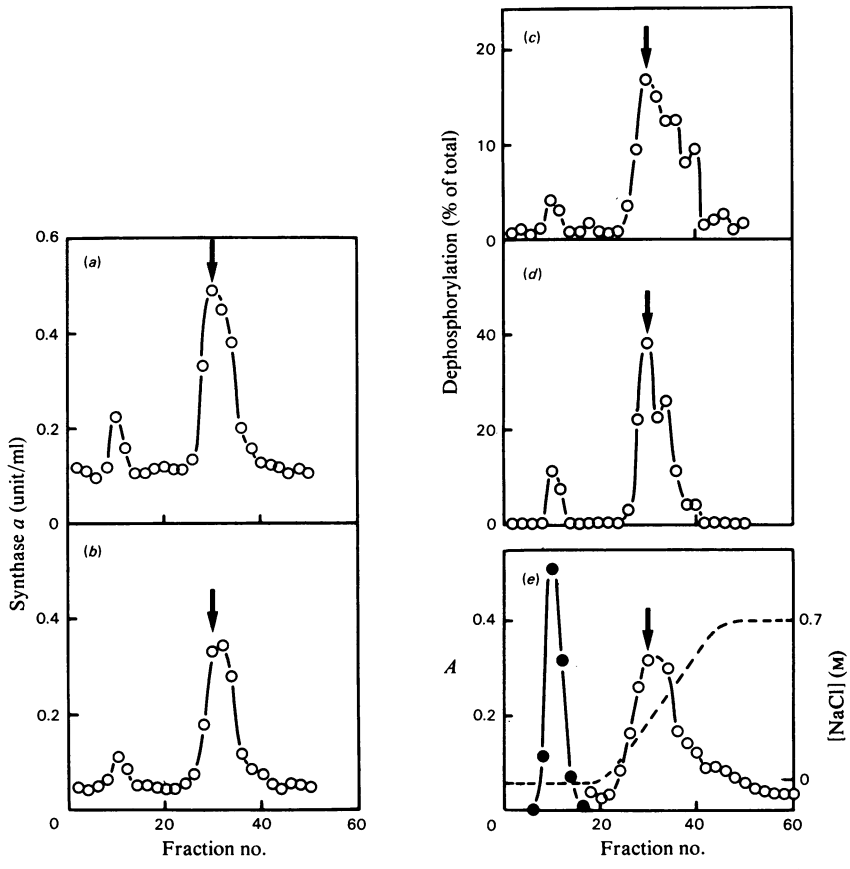

Fig. 6. *Chromatography of the crude G-component on phosphocellulose*

Chromatography of the protein–glycogen complex was performed as described in the Experimental section. Samples of the eluate (5 ml/fraction) were dialysed against a solution containing 50 mM-imidazole/HCl, pH 7.4, 0.5 mM-dithiothreitol and 20 mM-2-mercaptoethanol, for 2 × 45 min before the following assays: (*a*) synthase *a* formed from liver synthase *b* after 15 min of incubation in the presence of 5 mM-Mg^{2+} plus 2 mM-glucose 6-phosphate (the elevated 'baseline' values in this condition are due to some stimulation of synthase *b* by transferred glucose 6-phosphate); (*b*) G-activity: synthase *a* formed from the same substrate after 15 min of incubation in the presence of AMP and Mg^{2+} plus 2% crude S-component; (*c*) dephosphorylation of ^{32}P-labelled phosphorylase *a* after 5 min of incubation; (*d*) dephosphorylation of ^{32}P-labelled muscle synthase (phosphorylated by kinase F_A) after 10 min of incubation in the presence of AMP and Mg^{2+}. In each case, the general pattern was confirmed by incubation for a 3-fold longer time. Panel (*e*) illustrates the elution of glycogen (●, estimated from measurements of A_{600}) and of protein (○, determined at 280 nm), and the conductivity of the eluate (– – – –) indicating a NaCl gradient from 0 to 0.7 M. The arrows indicating fraction 30 have been added for the purpose of comparison.

by dissociative means, a phosphoprotein phosphatase of M_r 35000; this phosphatase acted on several substrates, including glycogen synthase *b* and phosphorylase *a*. However, the activation of glycogen synthase by this homogeneous phosphatase was only slightly, and competitively, inhibited by phosphorylase *a*. Furthermore, when the exact assay conditions used by Stalmans *et al.* (1971) were adopted, no trace of a latency period was observed. Killilea *et al.* (1976) suggested that some undefined factor in crude liver preparations might be responsible for the lag period that precedes the activation of glycogen synthase.

Our results do not support the latter hypothesis. When purified synthase *b* is used as substrate, purified phosphorylase *a* inhibits synthase phosphatase, as present in a crude extract, in an enzyme–glycogen complex, and as partially purified G-component. However, the S-component is not affected by phosphorylase *a*. Therefore the results of Killilea *et al.* (1976) could be satisfactorily explained by a cytosolic origin of their protein phosphatase.

Table 2. *Effect of various drastic treatments on the purified G-component*

A sample of the purified G-preparation was treated with trypsin as described by Doperé *et al.* (1980). Treatment with $(NH_4)_2SO_4$ plus ethanol was performed as described by Brandt *et al.* (1974). Incubation with 6M-urea was performed for 30min at 25°C. All the preparations were then dialysed for 4h as described in the legend to Fig. 6, before assay of the phosphatase activities as indicated in the legend to Figs. 6(*b*), 6(*c*) and 6(*d*), except that linear reaction rates were measured. The results are means of experiments with two different preparations of G-component.

	Activity (%) on		
Treatment of G-component	Liver synthase *b* + S-component	Muscle ^{32}P-labelled synthase *b*	^{32}P-labelled phosphorylase *a*
None	100	100	100
Trypsin	0	19	3900
$(NH_4)_2SO_4$ plus ethanol	0	14	1400
Urea	4	26	270

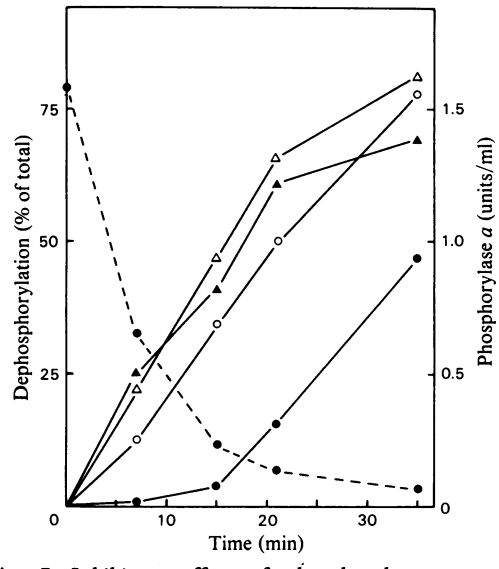

Fig. 7. *Inhibitory effect of phosphorylase a on the dephosphorylation of purified muscle synthase by purified G-component*

Purified muscle synthase *a* was phosphorylated by kinase F_A and then incubated with G-component purified by phosphocellulose chromatography and polyethylene glycol precipitation. The added phosphorylase *a* was from muscle. Symbols are as in Fig. 3, except that the continuous lines refer to dephosphorylation of glycogen synthase.

Alternatively, the native glycogen-associated synthase phosphatase might become insensitive to phosphorylase *a* as a result of the dissociative ethanol treatment applied by those workers. In our hands, however, the latter treatment resulted in a huge loss of synthase phosphatase activity, together with a 14-fold increase in phosphorylase phosphatase activity (Table 2); these opposite changes render it technically unfeasible to study the effect of phosphorylase *a* on the modified synthase phosphatase.

Comparison between liver and muscle

The control by phosphorylase *a* in the activation of glycogen synthase has mainly been documented for liver tissue (see the introduction), although its operation has also been demonstrated in leucocytes and in adipose tissue (Wang *et al.*, 1977; Saugmann & Esmann, 1977; Sobrino & Hers, 1980). As for skeletal muscle, the available evidence seems to exclude the possibility that the insulin-induced activation of glycogen synthase could be mediated by a decrease in phosphorylase *a* (Craig & Larner, 1964; Craig *et al.*, 1969; Adolfsson, 1973). To our knowledge there has been no previous report on the presence or absence of such a control mechanism in a crude muscle extract; in any case, we could not detect an effect of phosphorylase *a* in various experimental conditions. These negative results are in agreement with the finding by Villar-Palasi (1969) that the addition of phosphorylase *a* did not modify the affinity of a partially purified synthase phosphatase from muscle for its substrate.

The lack of control by phosphorylase *a* in muscle extracts could in principle result from a difference in the properties of any enzyme involved: phosphorylase *a*, synthase *b* or synthase phosphatase. That the source of phosphorylase *a* would account for the difference can be ruled out as a result of some direct comparisons (Figs. 2*a* and 2*b*; Figs. 3 and 4). Qualitatively the same results were also obtained whether activation of liver synthase *b* or dephosphorylation of ^{32}P-labelled muscle synthase was studied: the G-component was inhibited by phosphorylase *a*, but the S-component was not. The difference resides therefore in the properties of synthase phosphatase, which, in muscle extracts, is completely insensitive to phosphorylase *a*, and thus behaves like the S-component from liver. Taken together with the reported effect of insulin on skeletal muscle (see above), these results imply that the G-component, which confers on the liver the sensitivity to phosphorylase *a*, is absent or inoperative in muscle extracts and indeed in the intact muscle.

The loss of glycogen synthase phosphatase activity in the liver of the adrenalectomized starved rat has been traced to an almost complete loss of G-component (Doperé *et al.*, 1981). Work with foetal-rat liver has shown that the manyfold increase in synthase phosphatase activity during late gestation is caused by a glucocorticoid-induced synthesis of the G-component, and that the S-component is already largely or completely developed before glycogen synthesis occurs (Vanstapel *et al.*, 1980). Our present data illustrate again the regulatory significance of the G-component in the liver.

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