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REGULATION OF GLYCOGEN SYNTHETASE AND PHOSPHORYLASE PHOSPHATASE ACTIVITIES IN RAT ADIPOSE TISSUE

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Summary

Incubation of a rat adipose tissue homogenate causes a time and temperature dependent activation of glycogen synthetase (UDP glucose:glycogen 4-α-glucosyltransferase) and simultaneous inactivation of phosphorylase (1,4-α-D-glucan: orthophosphate α-glucosyltransferase, EC 2.4.1.1). Activation of glycogen synthetase at 15 and 23°C was preceded by a lag period. The duration of the lag period could not be correlated with significant changes in phosphorylase activity.

Addition of glucose and methylxanthines caused an increase in the rates of glycogen synthetase activation and phosphorylase inactivation. The effect on glycogen synthetase activation was mainly on the linear phase. Addition of AMP inhibited phosphorylase inactivation and accelerated glycogen synthetase activation. Addition of muscle phosphorylase *a* caused a prolongation of the lag period which lasted until phosphorylase *a* activity had decreased to the level originally present in the preparation.

It is concluded that in adipose tissue activation of glycogen synthetase is not dependent on prior inactivation of phosphorylase and that other factors should be looked for to explain the lag period preceding glycogen synthetase activation.

Introduction

The interconversion of glycogen synthetase (UDPglucose:glycogen 4-α-glucossyltransferase) and phosphorylase (1,4-α-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1) between a form, which is active under physiological conditions and an almost totally inactive form, has been shown to be due to phosphorylation and dephosphorylation catalyzed by a kinase and a phosphatase, respectively [1].

The factors regulating the activity of the kinase have been studied in many

tissues, including adipose tissue [1—3]. Hormonal activation of phosphorylase and concomitant inactivation of glycogen synthetase is mediated by changes in the intracellular concentration of 3′,5′-AMP through its action on protein kinase [1]. The phosphorylation of glycogen synthetase and of phosphorylase kinase by protein kinase provides one of the mechanisms for directing the flow of metabolites in the direction of glycogen synthesis or breakdown and avoiding waste of energy through the operation of futile cycles.

An additional safequard against the operation of futile cycles can be provided by the simultaneous operation of the phosphatases catalyzing the dephosphorylation of glycogen synthetase and phosphorylase. Such a mechanism, in which inactivation of phosphorylase, through the action of phosphorylase phosphatase, removes the inhibition of the activity of glycogen synthetase phosphatase has been proposed by Hers and his group for the liver enzyme system [4,5]. The finding by several authors, working on heart [6] or liver [7] phosphatase that an apparently homogeneous protein separated from these tissues could dephosphorylate glycogen synthetase as well as phosphorylase may provide an alternative explanation for the connection between activation of glycogen synthetase and inactivation of phosphorylase in these tissues.

Relatively little is known on the regulation of glycogen metabolism in adipose tissue [3,8,10,11]. In incubated rat adipose tissue insulin and glucose [3,12], as well as glucose alone [12], promote the conversion of glycogen synthetase to the *a* form. As in other tissues, the mechanism through which insulin effects activation of glycogen synthetase has not been fully elucidated [3]. Epinephrine stimulates the inactivation of glycogen synthetase and activation of phosphorylase through an effect on the kinase system and similar effects can be demonstrated by the addition of 3′,5′-AMP to adipose tissue homogenates [9].

The present investigation was undertaken to characterize the factors affecting the activity of the phosphatase(s) acting on glycogen synthetase and phosphorylase in rat adipose tissue, in order to achieve a better understanding of the interelations between activation of glycogen synthetase and inactivation of phosphorylase in this tissue.

Materials and Methods

Animals. Male albino rats of the Hebrew University strain, weighing 120—150 g, were used. The animals were kept on a standard laboratory diet (Amrod 931, Ambar, Hadera, Israel) containing 65% carbohydrate, 20% protein, 4% fat and 10% cellulose, minerals and inert material.

Materials. UDP-Glucose, glucose derivatives, glycogen, nucleotides, enzymes and buffers were purchased from Sigma Chemical Company. Diazyme (*Aspergillus niger* amyloglucosidase) was a product of Miles Chemical Co., Clifton, N.J. Diazyme solution was prepared according to the method of De Wulf et al., [13]. All other reagents were of analytical grade. UDP[U-^{14}C]glucose and [U-^{14}C]glucose-1-*P* were obtained from the Radiochemical Centre, Amersham, England. Shellfish glycogen was purified as described by Thomas et al. [14].

Preparation of enzyme solution. Epididymal adipose tissues of rats killed by cervical dislocation were homogenized with one volume (w/v) of 0.05 M Tris ·

HCl, pH 7.0. The homogenate was centrifuged at 8000 × *g* for 10 min at 4° C. The clear aqueous solution underneath the fat cake served as enzyme source. Protein concentration of this preparation was 8—10 mg/ml.

Glycogen synthetase activity. This was assayed as previously described [9]. In some experiments the radioactive glycogen was spotted on Whatman 31ET filter paper, as described by Thomas et al. [14]. Results with the two methods were identical.

Phosphorylase assay. The activity of phosphorylase was measured in the direction of glycogen synthesis by measurement of the incorporation of [U-^{14}C]-glucose-1-*P* into glycogen. The reaction mixture contained: 50 mM [U-^{14}C]glucose-1-*P* with specific activity of 0.01 μCi per μmol, 1% shellfish glycogen, 100 mM NaF, 1 mM 5′-AMP (when indicated) and 50 mM 2(*N*-morpholino)ethanesulfonic acid (MES) buffer pH 6.1 in a final volume of 0.060 ml. The reaction was started by addition of 0.010 ml of the enzyme preparation. Incubation was carried out for 10 min at 30° C, was terminated by spotting 0.050 ml of the reaction mixture on Whatman ET 31 filter paper, and carried through the procedure used for glycogen synthetase assay [14].

In vitro activation was carried out by preincubation of the enzyme solution for various periods up to 60 min. Preincubation was carried out at 23° C, unless otherwise stated.

Protein was assayed according to the method of Lowry et al. [15] following precipitation of the protein with 10% trichloroacetic acid. Bovine serum albumin was used as a standard.

One unit of glycogen synthetase or phosphorylase activity was defined as the amount of enzyme which catalyzes the incorporation of 1 μmol of substrate (UDPglucose or glucose-1-*P*, respectively) per min, into glycogen.

One unit of phosphatase activity was defined as the amount of enzyme which caused an increase in glycogen synthetase *a* activity or a decrease in phosphorylase *a* activity of one unit per min.

Results

In a freshly prepared adipose tissue homogenate glycogen synthetase activity is almost completely dependent on added glucose 6-phosphate (*b* form) whereas phosphorylase activity is mostly in the *a* form.

Incubation of the homogenate causes a time dependent activation of glycogen synthetase and a concomitant inactivation of phosphorylase. Both of these processes are reversible by addition of Mg · ATP (Fig. 1).

Dilution of the homogenate caused a marked decrease in the activity of glycogen synthetase phosphatase when dilution was with the buffer used for homogenization, but not when boiled homogenate was used for this purpose (Fig. 2). Phosphorylase phosphatase activity was not affected by dilution (not shown).

The effect of incubation temperature on the rate of glycogen synthetase activation and of phosphorylase inactivation, respectively, is shown in Table I. The rate of both processes is affected in an essentially similar manner by increasing the temperature of incubation. Nevertheless, some points should be noted: At 15° C glycogen synthetase activation starts after a lag period of about

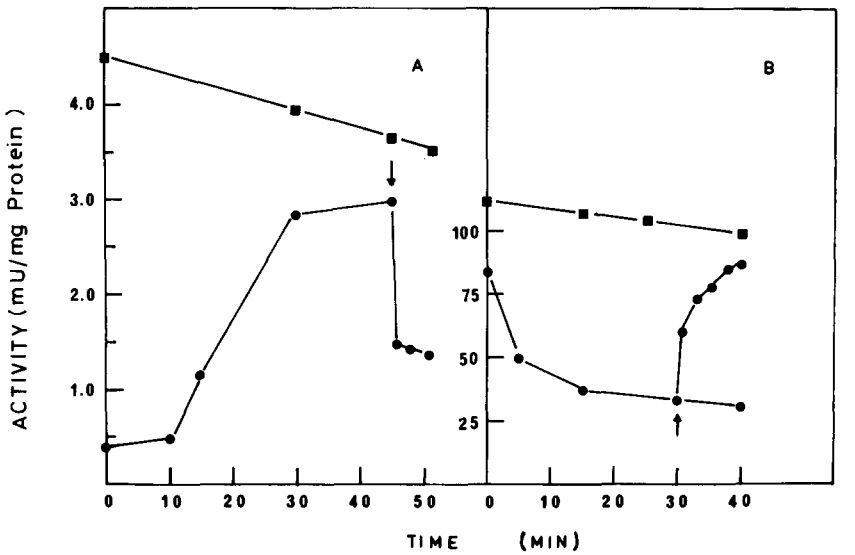

Fig. 1. Effect of preincubation on the activities of glycogen synthetase and phosphorylase. An adipose tissue homogenate, prepared as described in Materials and Methods was incubated at 23°C. At the time intervals indicated, samples were removed and immediately assayed for glycogen synthetase activity (A) with (■) or without (●) 5 mM glucose-6-*P* and for phosphorylase activity (B) with (■) or without (●) 1 mM AMP. At the time intervals indicated by an arrow ATP and Mg^{2+} were added to a concentration of 4 mM and 8 mM respectively.

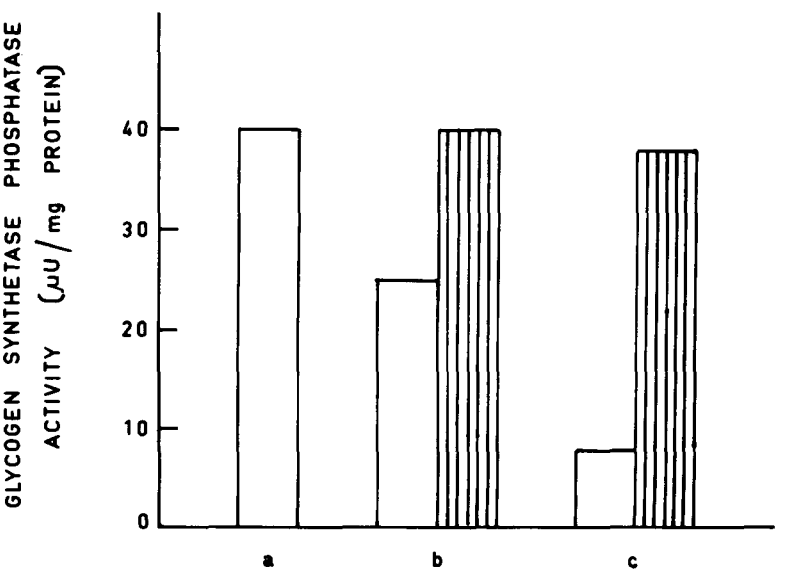

Fig. 2. Effect of homogenate concentration on glycogen synthetase phosphatase activity. Homogenates were prepared as described in Materials and Methods and incubated for 30 min at 23°C without further dilution (a) or after 1 : 1 (b) or 1 : 3 (c) dilution with homogenization buffer (empty bars) or the supernatant obtained after boiling and centrifugation of part of the homogenate (shaded bars). Phosphatase activity was calculated from the linear portion of the activation curve.

TABLE I

EFFECT OF INCUBATION TEMPERATURE ON ACTIVATION OF GLYCOGEN SYNTHETASE

Homogenates were incubated at the indicated temperatures. Samples were removed at the indicated time intervals and immediately assayed for glycogen synthetase *a* and phosphorylase *a* activities. Initial phosphorylase *a* activity was 96 munits/mg protein, total phosphorylase activity was 110 munits/mg protein. Synthetase *a* activity was 0.8 munits/mg protein at zero time. After 60 min of incubation at 15, 23, or 37°C, synthetase *a* activity was 2.8, 3.9 and 2.0 muints/mg protein, respectively.

Time (min)	Glycogen synthetase phosphatase activity μunits/mg protein			Residual phosphorylase activity * (%)		
	15°C	23°C	37°C	15°C	23°C	37°C
0—5	0	16	456	—	93	61
5—10	16	84	56	93	81	42
10—20	40	76	—64 **	82	66	35
20—35	36	32	—28	71	48	—
35—60	40	28	—8	64	41	31

* Residual phosphorylase activity refers to the percentage of the initial activity of phosphorylase *a* present at the end of the indicated time interval.

** (—) denotes a decrease in activity during the indicated time interval.

10 min, at 23°C the lag period is reduced to 5 min and at 37°C there was no apparent lag. Inactivation of phosphorylase proceeded at all temperatures without any lag. The beginning of glycogen synthetase activation following the lag period was not related to a significant decrease in phosphorylase *a* activity at any temperature. At 37°C the rate of synthetase activation was the most rapid but the maximal level of activity attained was lower than at 23°C, as a result of thermal inactivation of the enzyme. Under these conditions total enzyme activity, measured with 5 mM glucose 6-phosphate, decreased even more rapidly (not shown).

In view of the rapid deterioration of synthetase activity at 37°C (Table I) and even at 23°C (total activity, Fig. 1) the effects of various factors on the stability of both forms of the enzyme were investigated (Table II). Glycogen synthetase activity (measured with 5 mM glucose-6-*P*) in an homogenate pre-

TABLE II

EFFECTS OF METABOLITES ON STABILIZATION OF GLYCOGEN SYNTHETASE ACTIVITY

Adipose tissue was homogenized in 0.05 M Tris buffer containing 0.1 M NaF and 2 mM EDTA and incubated at room temperature or at 5°C for 24 h. Glycogen synthetase activity is expressed as percent of the initial activity.

Addition	Glycogen synthetase activity			
	a + *b*		*a*	
	2 h	24 h	2 h	24 h
none	52	16	67	32
5% albumin	61	19	60	23
1% glycogen	100	66	100	84
33% glycerol	70	60	72	58
25% sucrose	67	30	63	54
2 mM dithioerythritol	71	34	73	56
Dithioerythritol + glycogen	100	100	100	100

pared in 0.05 M Tris buffer containing 100 mM NaF and 2 mM EDTA, (to prevent changes in activity due to phosphatase or kinase activity) declined to about half within 2 h at 23°C. The decrease in enzyme activity was completely prevented by addition of glycogen (1%). Addition of albumin (5%), dithioerythritol (2 mM), glycerol (33%) or sucrose (25%) was less effective. After 24 h at 4°C synthetase activity without additions had declined to 16% of the initial activity; glycogen and glycerol were relatively efficient in preserving activity, whereas sucrose and dithioerythritol had only a slight effect and albumin was completely ineffective. The combination of glycogen and dithioerythritol could completely preserve enzyme activity for 24 h. The activity of synthetase *a* was somewhat less labile than that of the *b* form; The order of effectiveness of the various additions was similar to that found for the *b* form of the enzyme. The rapid decrease in total enzyme activity observed in preparations incubated at 37°C was not affected by addition of glucose 6-phosphate.

Glycogen which was very useful for stabilization of glycogen synthetase activity inhibited the synthetase phosphatase in a concentration dependent manner (Fig. 3). Phosphorylase phosphatase activity was only slightly affected by 4% glycogen, which almost completely inhibited synthetase activation.

Glycogen synthetase activation was maximal at pH 5.5—6.5 and was almost completely abolished at pH 5 or above pH 8. The optimum for phosphorylase phosphatase was at a somewhat lower pH. The lack of activation at pH 8.0 was not due to deterioration of glycogen synthetase *a*. Both the *a* and the *b* form were completely stable at this pH under the conditions of the experiment.

The effect of glucose on activation of glycogen synthetase and inactivation of phosphorylase is shown in Fig. 4. Addition of 10 mM glucose to the enzyme solution increased the rate of both activities. A smaller effect was also apparent at 5 mM, whereas increasing the glucose concentration up to 50 mM did not increase the effect beyond that obtained with 10 mM glucose. The effect of

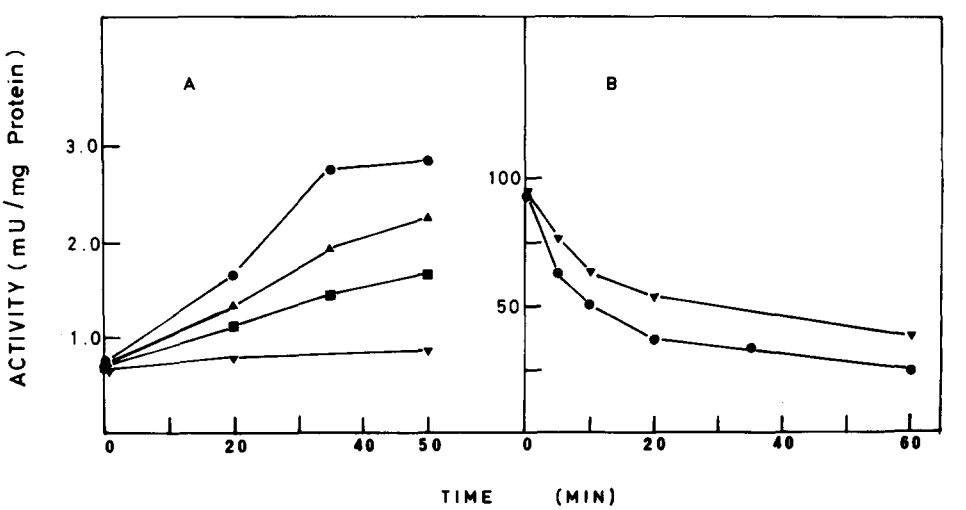

Fig. 3. Effect of glycogen on activation of glycogen synthetase (A) and inactivation of phosphorylase (B). Glycogen was added to the homogenate to a concentration of 0% (●) 0.1% (▲), 1% (■) and 4% (▲).

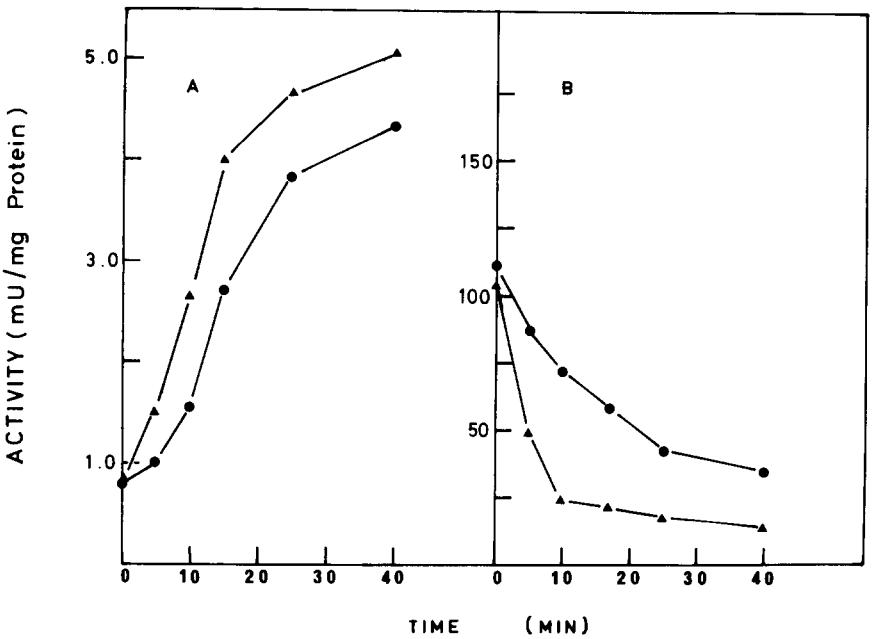

Fig. 4. Effect of glucose on activation of glycogen synthetase (A) and inactivation of phosphorylase (B). Homogenates were incubated (■) or without (●) 25 mM glucose. Other details as in Fig. 1.

glucose was to increase the rate of phosphatase activity during the linear phase, (10—20 min) rather than during the initial 10 min of activation, which included a lag period of several minutes (Fig. 2).

Addition of 25 mM galactose, mannose, fructose, xylose, 2 deoxyglucose, maltose, mannitol, dextran or alanine had no effect on the activation of glycogen synthetase or inactivation of phosphorylase.

The effect of caffeine (Fig. 5) was similar to that of glucose. The effect of caffeine on both activities was maximal at 1 mM and was not further increased by raising the concentration to 6 mM. The effect of theophylline was identical to that of caffeine (not shown).

In contrast to the effects of glucose and methylxanthines which increased the rate of activation of glycogen synthetase and inactivation of phosphorylase, respectively, AMP caused an increase in the rate of glycogen synthetase activation and a concomitant decrease in the rate of inactivation of phosphorylase. This effect was already apparent at 1.0 mM AMP and reached a maximum at 5 mM (Fig. 6).

Since the experiments in which the rate of inactivation of endogenous phosphorylase was modified by the action of metabolites, or through changes in the incubation temperature, did not reveal any connection between activation of glycogen synthetase and inactivation of phosphorylase, a further experiment was undertaken in which exogenous phosphorylase *a* was added to the homogenate. When phosphorylase *a* activity was raised to 3—4 times the endogenous level (Fig. 7) activation of glycogen synthetase was delayed, and began only when phosphorylase *a* activity in the homogenate had reached the original level.

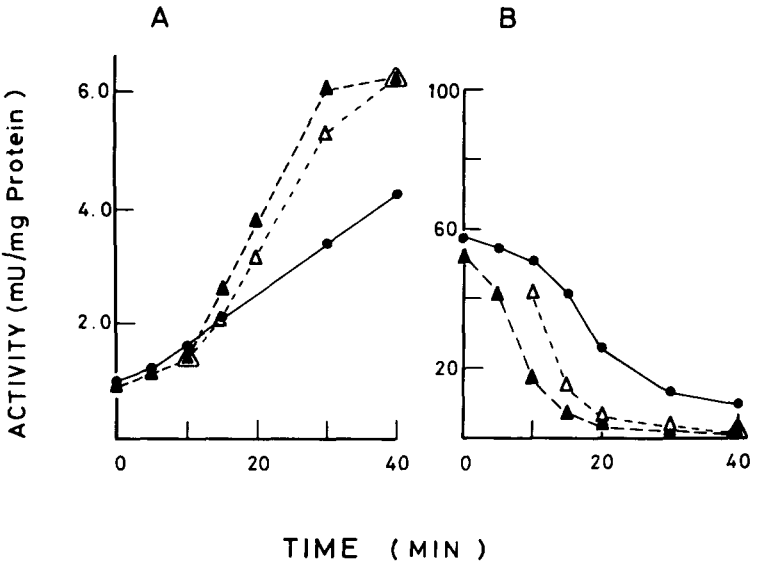

Fig. 5. Effect of caffeine on activation of glycogen synthetase and inactivation of phosphorylase. Homogenates were incubated with (▲△) or without (●) 1.25 mM caffeine. Caffeine was added to the homogenate at zero time (▲) or after 10 min of incubation (△). Details of the procedure as in Fig. 1.

This effect was specific for phosphorylase *a* and could only be demonstrated when phosphorylase *a* activity was raised to several times the endogenous activity.

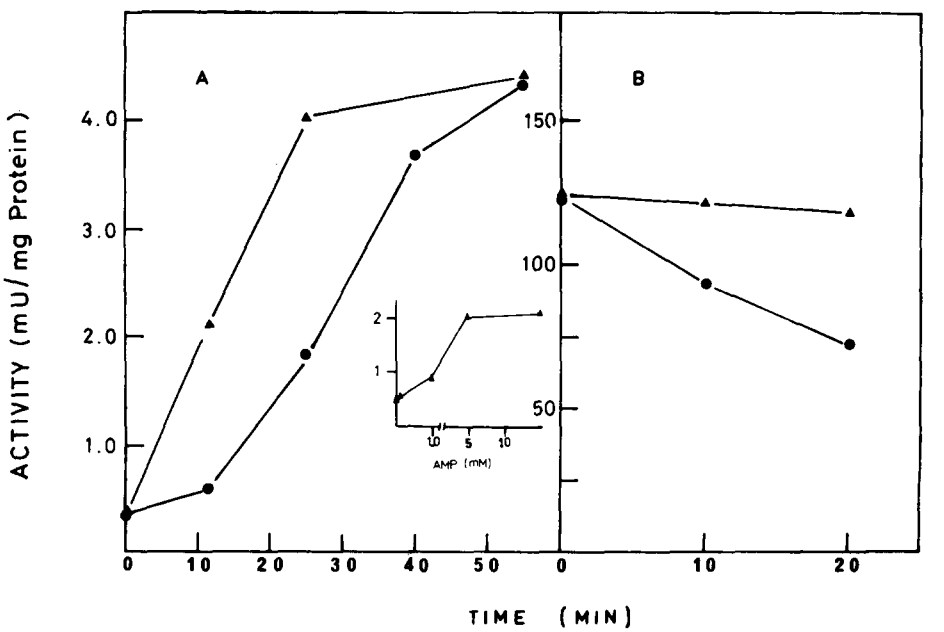

Fig. 6. Effect of AMP on activation of glycogen synthetase and inactivation of phosphorylase. Homogenates were incubated with (▲) or without (●) 5 mM AMP. Samples were removed at the times indicated, and assayed for glycogen synthetase (A) and phosphorylase (B) after removal of AMP with Dowex 1 X 400. The insert shows the effect of different concentrations of AMP on activation of glycogen synthetase.

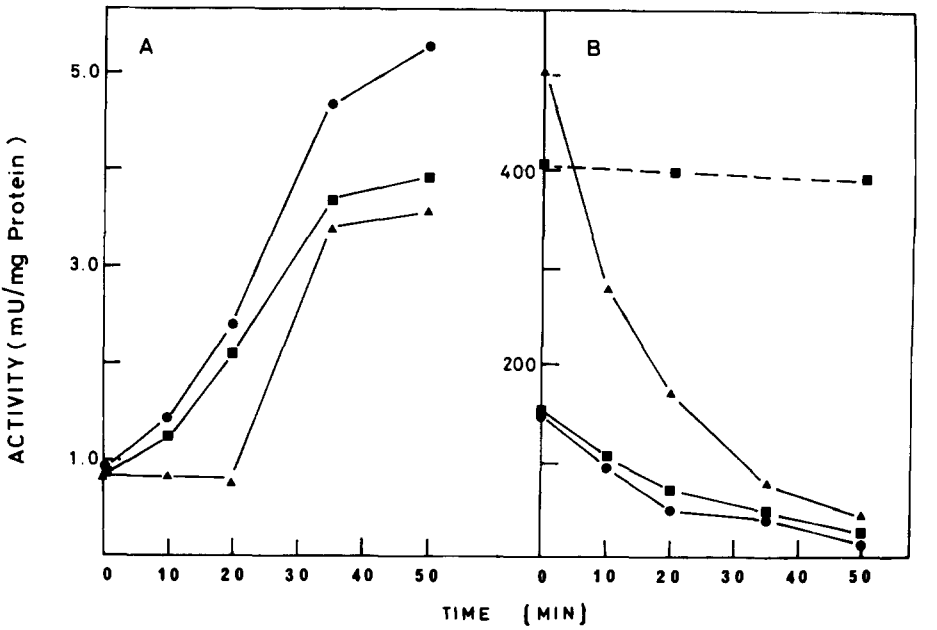

Fig. 7. Effect of added phosphorylase on activation of glycogen synthetase. Homogenates were incubated without any additions (●), with crystallized rabbit muscle phosphorylase *a* (▲) or *b* (■). Samples were removed at the times indicated and assayed for glycogen synthetase (A) or phosphorylase (B) activity. Phosphorylase was assayed with (- - - - -) or without (———) 1 mM AMP.

Discussion

The observation that in the homogenate both glycogen synthetase and phosphorylase were almost totally in the phosphorylated form does not necessarily reflect the in vivo situation. A similar relationship between the activities of the two enzymes has also been found in homogenates from livers of non-anesthetized mice, whereas a markedly lower activity of liver phosphorylase *a* was found when the animals were anesthetized and the liver was immediately frozen [5].

De Wulf et al. have suggested, on the basis of their experiments on mouse liver, that glycogen synthetase phosphatase is inhibited by phosphorylase *a*, so that an increase in the activity of synthetase phosphatase can occur only following a decrease in phosphorylase *a* activity [4,5].

In the present experiments no evidence was found to support the assumption that inactivation of phosphorylase *a* was a prerequisite for the initiation of in vitro activation of glycogen synthetase. Although inactivation of phosphorylase proceeded without a lag period, phosphorylase *a* activity was not significantly reduced at the time synthetase activation started; furthermore, the effect of substances, such as glucose and methylxanthines, which increased the rate of synthetase activation and phosphorylase inactivation was on the linear phase of glycogen synthetase activation, rather than on the duration of the lag period. This is in contrast to the results of De Wulf et al. who found that these substances caused a shortening of the lag period [4].

These observations have to be reconciled with the fact that addition of exogenous phosphorylase *a* was followed by a prolongation of the lag period preceding glycogen synthetase activation. Under these circumstances glycogen synthetase activation was inhibited but only so long as phosphorylase *a* activity exceeded the endogenous level. The phosphoprotein phosphatase found in heart [6] has a higher K_m for phosphorylase *a* than for synthetase *b*. If an analogous situation exists also in adipose tissue it is possible that with endogenous concentrations of phosphorylase *a* and synthetase *b* the phosphatase is simultaneously active on both substrates, whereas a large increase in phosphorylase *a* concentration may cause competitive inhibition of synthetase dephosphorylation.

Since the lag period preceding synthetase activation in adipose tissue cannot be ascribed to inhibition of glycogen synthetase phosphatase by phosphorylase *a*, it remains to be elucidated what other factors are responsible for this phenomenon. Further support for the assumption that phosphorylase *a* may not be the only factor involved in the lag period is provided by the observation that in a leukocyte preparation glycogen synthetase activation is also preceded by a lag period, although, almost all the phosphorylase activity in this preparation is in the *b* form (Nahas, N. and Gutman, A., personal communication).

It was recently proposed by Killilea et al. [16,17] that in vivo the liver phosphatase is in the form of an inactive enzyme-inhibitor complex. Release of lysosomal proteases during homogenization causes changes in the activity and molecular weight of the phosphatase-inhibitor complex. If such a process takes also place in other tissues it could be the factor responsible for the lag preceeding glycogen synthetase activation in adipose tissue and leukocytes.

It seems that a variety of factors, including the level of phosphorylase *a* activity, may contribute to the generation of the lag phenomenon in different tissues. In liver phosphorylase *a* activity appears to be the major (or only) factor responsible for the lag preceeding synthetase activation [5] wherease in extrahepatic tissues, such as adipose tissue and leukocytes, the relative importance of factors other than phosphorylase *a* activity may be greater.

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