Enzymes Regulating Glycogen Metabolism in Swine Subcutaneous Adipose Tissue. I. Phosphorylase and Phosphorylase Phosphatase†

Elizabeth Miller,‡ Bertil Fredholm,§ Richard E. Miller,# Daniel Steinberg, and Steven E. Mayer*

ABSTRACT: Glycogen phosphorylase from swine adipose tissue was purified nearly 700-fold using ethanol precipitation, DEAE-cellulose adsorption, AMP-agarose affinity chromatography, and agarose gel filtration. The purified enzyme migrated as one major and several minor components during polyacrylamide gel electrophoresis. Activity was associated with the major component and at least one of the minor components. The molecular weight of the disaggregated, reduced, and alkylated enzyme, estimated by polyacrylamide gel electrophoresis performed in the presence of sodium dodecyl sulfate, was 90,000. Stability of the purified enzyme was considerably increased in the presence of AMP. The isoelectric pH of the enzyme in crude homogenates was 6.3. The sedimentation coefficient of the purified enzyme (7.9 S) and that in crude homogenates (7.3 S) was determined by sucrose density gradient sedimentation. Optimal pH for activity was between pH 6.5 and 7.1. Apparent K_m values for glycogen and inorganic phosphate were 0.9 mg/ml and 6.6 mM, respectively. The K_a for AMP was 0.21 mM. Enzyme activity was increased by K_2SO_4, KF, KCl, and $MgCl_2$ and decreased by NaCl, Na_2SO_4, D-glucose, and ATP. Inhibition by glucose was noncompetitive with the activator AMP; inhibition by ATP was partially competitive with AMP. The purified enzyme was activated by incubation with skeletal muscle phosphorylase kinase. Enzyme in crude homogenates was activated by the addition of $MgCl_2$ and ATP; activation was not blocked by addition of protein kinase inhibitor, suggesting that phosphorylase kinase in homogenates of swine adipose tissue is present largely in an activated form. Deactivation of phosphorylase *a* by phosphorylase phosphatase was studied using enzyme purified approximately 200-fold from swine adipose tissue by ethanol precipitation, DEAE-cellulose chromatography, and gel filtration. The K_m of the adipose tissue phosphatase for skeletal muscle phosphorylase *a* was 6 μM. The purified swine adipose tissue phosphorylase, labeled with ^{32}P, was inactivated and dephosphorylated by the adipose tissue phosphatase. Dephosphorylation of both skeletal muscle and adipose tissue substrates was inhibited by AMP and glucose reversed this inhibition. Several lines of evidence suggest that AMP inhibition was due to an action on the substrate rather than on the enzyme. We have previously reported that the system for phosphorylase activation in rat fat cells differs in some important characteristics from that in skeletal muscle. However, both swine fat phosphorylase and phosphorylase phosphatase have major properties very similar to those described for the enzymes from skeletal muscle.

Adipose tissue contains at least three enzyme systems regulated by lipolytic hormones such as the catecholamines and glucagon: hormone-sensitive triglyceride lipase, glycogen phosphorylase, and glycogen synthase. It is well established that the hormone-sensitive lipase is activated by cyclic AMP-dependent protein kinase by phosphorylation of the lipase complex (Corbin et al., 1970; Huttunen et al., 1970a,b). The mechanisms by which the lipolytic hormones cause activation of phosphorylase and deactivation of glycogen synthase in adipose tissue have not yet been studied in similar detail. It seems likely that their regulation in adipose tissue is analogous to that so well studied in liver, skeletal muscle, and other tissues (Larner and Villar-Palasi, 1971; Fischer et al., 1971a,b). Certainly there is good correlation between epinephrine-stimulated increases in adipocyte concentrations of cyclic AMP and changes in the activity of all these enzyme systems (Khoo et al., 1973), yet there are indications that the adipose tissue systems may differ significantly in certain respects from those in other tissues. For example, while brief epinephrine treatment clearly activated both phosphorylase kinase and phosphorylase in muscle, in adipocytes it activated phosphorylase without an apparent increase in the phosphorylase kinase activation state (Khoo et al., 1972). These findings raise the question of whether and to what extent regulation of phosphorylase activity in adipose tissue is dependent upon deactivation by phosphorylase phosphatase. The presence of this enzyme has been demonstrated in rat adipocytes (Khoo et al., 1973). Again, the ability of insulin to prevent epinephrine-stimulated changes in phosphorylase activity in liver has been attributed to effects of the former on adenylate cyclase activity (Park et al., 1972), whereas in adipocytes studies of the correlation between insulin effects and cyclic

† From the Division of Metabolic Disease and the Division of Pharmacology, Department of Medicine, School of Medicine, University of California, San Diego, La Jolla, California 92037. *Received August 5, 1974.* This investigation was supported by Program Project Grant No. HL 12373 and Training Grant No. HL 05899 from the National Heart and Lung Institute.

‡ Present address: Department of Pharmacology, Indiana University School of Medicine, Indianapolis, Ind. 46202.

§ Present address: Department of Pharmacology, Karolinska Institute, S-10401, Stockholm 60, Sweden. Recipient of a U.S. Public Health Service International Fellowship (TWO 1876-01).

Supported by a Special Fellowship (HL 53159) from the National Institutes of Health and a grant from the Weight Watchers Foundation. Present Address: Department of Medicine, Veterans Administration Hospital, Case Western Reserve University, Cleveland, Ohio 44106.

AMP levels have yielded conflicting results (Jarett et al., 1972; Illiano and Cuatrecasas, 1972; Khoo et al., 1973). Progress in the elucidation of mechanisms regulating glycogen metabolism in adipose tissue would seem to call for better characterization of the enzymes involved.

There is an extensive, appropriate background for such investigations. Studies conducted in vivo indicate that swine will mobilize fatty acid when catecholamines are injected. Mersmann et al. (1974) have clearly demonstrated in vitro catecholamine stimulation of lipolysis in slices of swine subcutaneous back fat. White adipose tissue from rats contains a glycogenolytic system that is rapidly activated by catecholamines and by other lipolytic agents (Vaughan et al., 1959; Frerichs and Ball, 1962; Shafrir and Kerpel, 1964) with the activation of phosphorylase (Vaughan and Steinberg, 1965; Jungas, 1966). In muscle, calcium-dependent protein kinase (phosphorylase kinase) stimulates the conversion of phosphorylase *b* to *a* (cf. Krebs, 1973; Fischer et al., 1971a).

An enzyme capable of converting phosphorylase from its physiologically more active *a* form to the AMP-dependent *b* form was first described in skeletal muscle by Cori and Green (1943). Later this enzyme was shown to be a true phosphatase (Fischer et al., 1957; Krebs et al., 1959). It has been purified extensively from skeletal muscle (Hurd et al., 1966; Gratecos et al., 1974), but the mode of its regulation remains unknown. There is considerable controversy in the literature as to the control of this enzyme both in skeletal muscle (Chelala and Torres, 1970; Haschke et al., 1970) and in other tissues (Merlevede et al., 1969; Stalmans et al., 1970).

Similar systems have been described in a wide variety of tissues but no systematic studies have been carried out in adipose tissue, nor have the enzymes involved been purified from adipose tissue sources. The present report describes the purification of swine adipose tissue phosphorylase and phosphorylase phosphatase with characterization of some of their physical and catalytic properties, and demonstrates the phosphorylase kinase catalyzed conversion of purified phosphorylase *b* to phosphorylase *a*. The accompanying paper (Miller et al., 1975) describes the purification and characteristics of glycogen synthase from this same tissue.

Experimental Section

Materials. [γ-^{32}P]ATP was prepared by a modification (Soderling et al., 1970) of the method of Glynn and Chappel (1965) using $^{32}P_i$ obtained from Amersham-Searle.

Rabbit skeletal muscle was the source of the following proteins. Phosphorylase *b* was prepared by a modification (DeLange et al., 1968) of the method of Fischer and Krebs (1958); phosphorylase *b* kinase was prepared according to Krebs et al. (1964). Cyclic AMP dependent protein kinase was partially purified by the method of Walsh et al. (1968) through the first DEAE-cellulose column step. Protein kinase inhibitor was prepared by the method of Walsh et al. (1971) through the DEAE-cellulose step. Troponin B was a gift of Dr. Stephen Gross (cf. Gross and Mayer, 1973).

^{32}P-Labeled muscle phosphorylase *a* was prepared according to a slight modification of the method of Krebs et al. (1958): about 200 mg of phosphorylase *b* was incubated in 10 m*M* β-glycerol phosphate, 15 m*M* 2-mercaptoethanol, 8 m*M* magnesium acetate, 0.02 m*M* cyclic AMP, 1 μg/ml of cyclic AMP dependent protein kinase, 3 μg/ml of phosphorylase kinase, and 0.7 m*M* [^{32}P]ATP ($\sim 10^9$ cpm/μmol) at pH 6.8 in 2 ml for 1 hr. After termination of the reaction by addition of charcoal (Norit A) and removal of the charcoal by centrifugation, the phosphorylase was crystallized at 0° and the resuspended enzyme dialyzed 2 hr against 10 m*M* β-glycerol phosphate, 15 m*M* 2-mercaptoethanol, and 1 m*M* EDTA (pH 6.8) in a cellulose hollow fiber chamber (Bio-Rad, Los Angeles, Calif.). After a final crystallization the radioactivity not precipitable with trichloroacetic acid amounted to less than 1% of the total. Hormone-sensitive lipase, prepared as described by Huttunen et al. (1970b), was provided by Dr. John Khoo.

Shellfish glycogen was obtained from Sigma Chemical Co. and further purified by the procedure of Somogyi (1957) for use in the assay of phosphorylase. Nucleotides were obtained from P-L Biochemicals, Sigma Chemical Co., or from Boehringer-Mannheim. Other enzymes and substrates were from Sigma Chemical Co. or from Boehringer-Mannheim. DEAE-cellulose (Whatman DE-52) was obtained from Reeve-Angel. Sepharose 4B, Ficoll, Sephadex G-75, and Sephadex G-100 were from Pharmacia Fine Chemicals; agarose (Bio-Gel A, 0.5m) was obtained from Bio-Rad.

Methods. ANIMALS. Fed swine weighing 70–80 kg were anesthetized with pentobarbital and exsanguinated. The subcutaneous back fat was separated from muscle and skin, immediately frozen in liquid nitrogen, and stored at −80°.

ASSAYS. Unless otherwise indicated, phosphorylase activity was assayed by a modification of the method of Hardman et al. (1965) by measuring the conversion of glycogen to glucose 1-phosphate in the presence of inorganic phosphate. Standard assay mixtures (50 μl) contained 50 m*M* potassium phosphate (pH 6.7), 10 mg/ml of glycogen, 1 mg/ml of bovine serum albumin, with or without 2 m*M* AMP, and sufficient enzyme (in 5 μl) to produce 3–20 nmol of glucose 1-phosphate during the incubation at 30°. The assay was started by addition of enzyme and was linear for at least 40 min. The reaction was terminated by the addition of 5 μl of 0.7 *N* HCl. A solution (0.25 ml) containing 100 m*M* imidazole (pH 7.4), 2 m*M* magnesium acetate, 0.25 m*M* NADP, 5 μg/ml of phosphoglucomutase, 1 μg/ml of glucose-6-phosphate dehydrogenase, and 5 μ*M* glucose 1,6-diphosphate was added to each tube. After 15 min at 30°, the conversion of glucose 1-phosphate to 6-phosphogluconate was complete and 1 ml of a buffer containing 40 m*M* Na_2CO_3, 40 m*M* $NaHCO_3$, and 1 m*M* EDTA (pH 10) was added to each sample. Reduced pyridine nucleotide was quantified fluorometrically using a Farrand Mark I spectrofluorometer (excitation wavelength, 350 nm; emission wavelength, 450 nm; or a Turner filter fluorometer). One unit of activity is defined as that amount of enzyme catalyzing the formation of 1 μmol of glucose 1-phosphate/min. *Activity ratio* is defined as the quotient obtained by dividing the phosphorylase activity determined in the absence of AMP by that determined in the presence of AMP.

Phosphorylase activity was also measured in the direction of glycogen synthesis by a modification of the method of Danforth et al. (1962). The standard assay mixture (75 μl) contained 50 m*M* glucose 1-phosphate, 8 mg/ml of glycogen, 0.067 m*M* EDTA, 13.4 m*M* sodium β-glycerol phosphate (pH 6.7) with or without 3.33 m*M* AMP, and enough enzyme to produce 0.05–0.25 μmol of P_i during the incubation. The assay was linear for at least 60 min. Following incubation at 30°, 1.8 ml of 0.8% ferrous sulfate in 0.015 *N* H_2SO_4 was added to terminate the reaction. Color was developed by the addition of 0.2 ml of 6.6% ammonium molybdate·$7H_2O$ in 7.5 *N* H_2SO_4 and after 60 sec the absorb-

ance at 660 nm was determined using a Gilford Model 222 spectrophotometer. One unit of activity is defined as the amount of enzyme catalyzing the formation of 1 μmol of P_i/min.

Phosphorylase phosphatase activity was determined by the release of trichloroacetic acid-soluble ^{32}P from muscle ^{32}P-labeled phosphorylase *a* essentially according to Hurd et al. (1966). Unless otherwise indicated the assay mixture (40–80 μl) contained 50 m*M* Tris-HCl, 30 m*M* 2-mercaptoethanol, 1 m*M* EDTA, and 20 μ*M*[1] phosphorylase *a* at pH 7.8. The assay (30°) was linear with time for at least 15 min provided less than 20–25% of the substrate was consumed. Incubations of 5–10 min were generally used and were terminated by the addition of 9 vol of cold 10% trichloroacetic acid. Twenty minutes after addition of trichloroacetic acid, the samples were centrifuged and an aliquot of the clear supernatant solution was used for the estimation of radioactivity. One unit of phosphorylase phosphatase is defined as the amount of enzyme activity catalyzing the removal of 1 nmol of $^{32}P_i$ from ^{32}P-labeled phosphorylase *a* per minute at a substrate concentration of 20 μ*M* (Hurd et al., 1966).

Protamine phosphatase activity was determined by following the liberation of $^{32}P_i$ from [^{32}P]protamine. The assay mixture (100 μl) contained 20 m*M* Tris-Cl, 30 m*M* 2-mercaptoethanol, 0.5 m*M* EDTA, 50 m*M* imidazole-Cl (pH 7.5), 5 m*M* $MnCl_2$, and 0.07% bovine serum albumin. The reaction was terminated by the addition of 50 μl of 1% deoxycholate and 0.5 ml of 25% trichloroacetic acid. A 0.25-ml aliquot of the clear supernatant solution was counted in a Packard liquid scintillation spectrometer. In the absence of enzyme, less than 2% of the total protamine radioactivity was recovered in the supernatant fluid.

In the experiments on phosphorylation of phosphorylase (from skeletal muscle and adipose tissue) or of troponin B, the proteins were incubated with phosphorylase kinase, protein kinase, cyclic AMP, Mg^{2+}, [γ-^{32}P]ATP, and other additions under conditions described in detail in the Results section. At intervals aliquots of the reaction mixture were withdrawn and spotted on 2.3 cm diameter Whatman filter paper disks which were dropped into cold 10% trichloroacetic acid. The disks were washed seven times in 10% trichloroacetic acid and twice in ethanol and left to air dry and the radioactivity was measured in a Packard scintillation spectrometer.

PROTEIN DETERMINATIONS. Protein concentration was determined by the method of Lowry et al. (1951). Protein was precipitated with 10% trichloroacetic acid in the presence of 0.1% deoxycholate. Deoxycholate does not interfere with color development but greatly facilitates quantitative precipitation of small amounts of protein.[2] Bovine serum albumin was used as a standard.

ELECTROPHORESIS. Polyacrylamide disk gel electrophoresis was performed according to the method of Hedrick and Smith (1968) at pH 7.3. Bromophenol Blue was used as tracking dye. Phosphorylase activity was detected following electrophoresis by incubating slices of the gel in the standard assay mixture for 30 min at 30°.

Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS)[3] at pH 7.2 was performed according to the procedure of Weber and Osborn (1969). Bovine serum albumin, ovalbumin, and muscle phosphorylase *b* were used as standards.

SUCROSE DENSITY GRADIENT SEDIMENTATION. The method of Martin and Ames (1961) was used for sucrose density gradient sedimentation. Sucrose gradients (5–20%) were prepared in the presence of buffer containing 10 m*M* Tris-HCl (pH 7.6), 1 m*M* EDTA, 14.4 m*M* 2-mercaptoethanol, and 100 m*M* KCl. In studies using purified phosphorylase *b*, 20 m*M* AMP was added to the gradients to stabilize the enzyme. Yeast glucose-6-phosphate dehydrogenase (mol wt 101,600; $s_{20,w}$ = 6.1 S) was used as a reference standard (Yue et al., 1969).

ISOELECTRIC FOCUSING. Isoelectric focusing was performed as described in the instruction manual supplied by LKB in a 5–30% sucrose gradient with 2% Ampholine (pH 3–10 or 4–6 containing 15 m*M* 2-mercaptoethanol). Following focusing, fractions were collected and the pH of each fraction was determined using a Radiometer Model 600 pH meter. Prior to assay, fractions were neutralized with 1 *M* Tris-base or with 1 *M* HCl.

AMP-AGAROSE AFFINITY CHROMATOGRAPHY. N^6-(6-Aminohexyl)adenosine 5′-monophosphate was synthesized according to the method of Guilford et al. (1972) and covalently linked to 4% agarose (Sepharose 4B) as described by Cuatrecasas (1970). The AMP-agarose affinity column was synthesized and generously provided by Dr. Jack Dixon, Department of Chemistry, University of California, San Diego. The concentration of AMP was 2–3 μmol/ml of packed gel.

PURIFICATION OF ADIPOSE TISSUE PHOSPHORYLASE PHOSPHATASE. The methods were similar to those described by Hurd et al. (1966). The well-drained ethanol precipitate from swine fat was redissolved and treated with α-amylase (hog pancreas, Sigma), 20 μg/ml, for 16 hr at 4° and then diluted fourfold with standard buffer (20 m*M* Tris-HCl (pH 7.8), 5 m*M* EDTA, and 15 m*M* 2-mercaptoethanol). After centrifugation the supernatant fraction was applied to a DEAE-cellulose column (2.5 × 20 cm). After the column was washed with standard buffer the enzyme was eluted with a gradient of KCl (0–0.5 *M* in the buffer). The enzyme usually eluted at approximately 0.15 *M* KCl. The pooled fractions were either dialyzed against standard buffer and concentrated with Ficoll or dialyzed against water and concentrated by lyophilization. At this stage of purification the enzyme could be stored at −20° for several weeks without marked loss of activity. On the other hand, there was a rapid loss of activity at 4°. Finally, the enzyme was chromatographed on a 1.5 × 92 cm 10% agarose column (Bio-Gel A-0.5m, 200–400 mesh). The enzyme-containing fractions were pooled, dialyzed against water, and lyophilized. The lyophilized enzyme was resuspended in a small amount of buffer and insoluble material was removed by centrifugation. Overall purification was 100- to 200-fold with a yield of 5–20%.

The phosphorylase phosphatase reaction was linear with respect to concentration of purified enzyme (30–150 μg/ml) and with respect to incubation time for at least 10 min. Only at very high protein concentrations of the purified preparations were rates found to deviate from linearity. However, reaction rates in crude homogenates were not lin-

[1] The concentrations of phosphorylase given throughout the text are expressed in terms of the concentration of phosphorylase monomer (mol wt 93,000) and do not necessarily reflect the aggregation state of the enzyme.

[2] Dr. David Weinstein, personal communication.

[3] Abbreviation used is: SDS, sodium dodecyl sulfate.

ear even with small amounts of added protein (25 μg/ml). This suggested the presence of inhibitory material (see below). The optimal pH for the reaction was between 7.5 and 8.0 in both Tris-Cl and β-glycerol phosphate buffer. The enzyme preparation after ethanol precipitation sedimented in a sucrose gradient with a major peak of activity (4.05 S) and a minor peak (6.1 S). The DEAE fraction and the enzyme eluted from agarose showed only one component (4.1 S). This corresponds to a molecular weight of approximately 55,000 assuming a globular shape of the enzyme. Results from chromatography on 4% agarose, Sephadex G-75, and Sephadex G-100 were compatible with such a molecular weight. In three electrofocusing experiments the isoelectric point of the enzyme appeared to be between 4.8 and 5.0. The enzyme was quite temperature labile, as is the muscle enzyme (England et al., 1972; Nakai and Thomas, 1973). Thus, a 50% loss in activity occurred at 55° during a 15–20 min incubation at pH 7.5.

Potassium fluoride inhibited the activity of phosphorylase phosphatase (50% at 12 m*M*) competitively with the substrate phosphorylase. The divalent cations Mn^{2+}, Mg^{2+}, and Ca^{2+} (chloride salts) all inhibited the enzyme activity (50% at 2.1, 4.3, and 6.3 m*M*, respectively).

The inhibitor found in adipose tissue homogenate was heat stable (100° for 2 min), dialyzable, destroyed by 5′-nucleotidase, partially inactivated by adenylate deaminase, and bound by charcoal and an anion exchange resin. These data suggest that the inhibitor was a nucleotide, probably AMP.

Results

Purification of Phosphorylase. Phosphorylase was purified from crude homogenates of swine subcutaneous adipose tissue after powdering of the tissue in liquid N_2. Unless otherwise indicated, all procedures were performed at 4°. The standard buffer contained 20 m*M* Tris-HCl (pH 7.8), 5 m*M* EDTA, and 15 m*M* 2-mercaptoethanol.

STEP 1: CRUDE HOMOGENATE. Frozen swine adipose tissue was suspended in buffer in a ratio of 2 g of tissue per 3 ml of buffer. The suspension was homogenized in a Waring Blendor (180 sec), filtered through cheesecloth, and centrifuged (30,000*g* for 40 min). The floating fat was discarded and the infranatant fraction was filtered through glass wool. The pellet contained insignificant phosphorylase activity and was discarded.

STEP 2: 25% ETHANOL PRECIPITATION. The filtered infranatant fraction from step 1 was adjusted to 0.1 *M* KCl with the solid salt. After addition of oyster glycogen to achieve a final concentration of 0.5 mg/ml, absolute ethanol at −60° was added to the enzyme solution at 0° to achieve a final concentration of 25% ethanol (v/v). After 15 min at −5° in a salt–ice bath the mixture was centrifuged at 30,000*g* for 40 min. The supernatant fraction was discarded and the pellet was homogenized (glass–Teflon homogenizer) in a volume of buffer equal to one-fifth of the volume of the infranatant fraction from step 1. Insoluble material was removed by centrifugation (30,000*g* for 15 min) and the pellet was discarded.

STEP 3: DEAE-CELLULOSE ADSORPTION. Buffer-equilibrated DEAE-cellulose (Whatman DE-52, equal in packed bed volume to the volume of enzyme solution) was suspended in the enzyme preparation from step 2. After equilibration for 15 min with occasional mixing, the suspension was filtered on a sintered glass funnel and the moist DEAE-cellulose was washed in the funnel with two bed volumes of

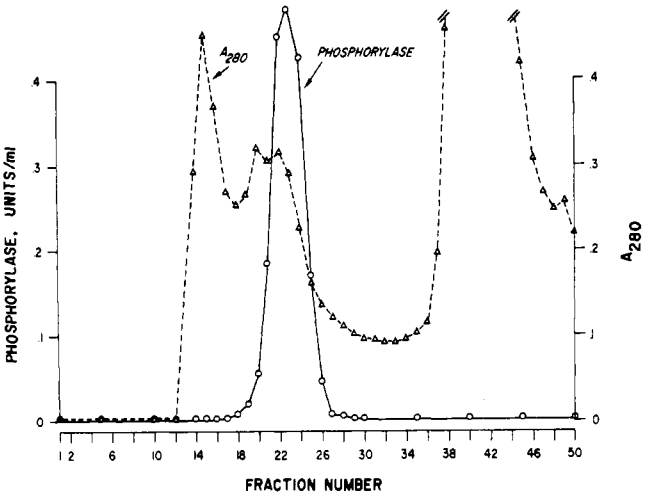

FIGURE 1: Elution profile of partially purified adipose tissue phosphorylase from a 10% agarose column (Bio-Gel A-0.5m, 200–400 mesh) (1.5 × 92 cm) equilibrated and eluted with standard buffer (step 6 of purification of Phosphorylase section): sample 5 ml containing 1.7 mg/ml of protein; fractions, 3 ml; flow rate, 3.2 ml/cm^2 per hr. Enzyme activity (○) was determined using the standard assay; absorbance at 280 nm (△).

standard buffer containing 70 m*M* KCl. Most of the phosphorylase activity appeared in the filtrate while nearly all of the glycogen synthase and phosphorylase phosphatase activity were retained by the DEAE-cellulose. The filtrates were pooled.

STEP 4: AMP-AGAROSE AFFINITY CHROMATOGRAPHY. The pooled filtrates from step 3 were applied (20–40 ml/hr) to the AMP-agarose column (2.0 × 5.5 cm) previously equilibrated with buffer. After sample application the column was washed with buffer until the 280-nm absorbance of the column effluent was equal to that of the buffer. Phosphorylase activity was eluted at about 21° with buffer containing 5 m*M* AMP. Optimal yields were obtained when the AMP-containing buffer was allowed to remain on the column for at least 30 min before starting the elution. The fractions containing most of the enzyme activity were pooled.

STEP 5: 25% ETHANOL PRECIPITATION. Step 2 was repeated on the pooled fractions from step 4 with the exception that KCl was omitted. The precipitate was suspended in a volume of buffer equal to 0.5% of the volume of the infranatant fraction from step 1.

STEP 6: 10% AGAROSE GEL FILTRATION. The enzyme preparation from step 5 was applied to a 10% agarose column (1.5 × 92 cm; Bio-Gel A-0.5m, 200–400 mesh) equilibrated with buffer. Enzyme was eluted with the same buffer and the fractions containing most of the phosphorylase activity were pooled (Figure 1). In some cases the pooled fractions were concentrated in dialysis tubing by surrounding the tubing with Ficoll.

Table I summarizes the results of a typical purification. Starting with the 30,000*g* infranatant fraction of step 1, the procedure resulted in a 689-fold enrichment of phosphorylase activity with a 38% overall yield. Enzyme prepared as described had little or no activity in the absence of AMP. During the first three steps of the purification procedure the activity ratio fell to zero from initial values as high as 0.5.

Stability of the Purified Phosphorylase. The purified enzyme stored at 4° in 20 m*M* Tris-HCl (pH 7.6), 14.4 m*M* 2-mercaptoethanol, lost nearly 40% of its original activity in 3 days and 90% in 10 days. In the presence of 2 m*M* AMP,

Table I: Purification of Swine Adipose Tissue Phosphorylase.

Fractionation Step	Vol (ml)	Total Act. (Units)[a]	Yield (%)	Total protein (mg)	Sp Act. (Units/mg)	Act. Ratio[b]
1. Crude extract	507	11.65	100	1619	0.007	0.27
2. 1st ethanol precipitation	98	11.09	95	295	0.038	0.06
3. DEAE-cellulose adsorption	238	8.59	74	179	0.048	0.013
4. AMP–Sepharose affinity chromatography	49	8.92	77	15.7	0.568	0
5. 2nd ethanol precipitation	2.8	5.82	50	4.76	1.22	0
6. Agarose gel filtration	11.5	4.43	38	0.92	4.82	0

[a] Standard assay conditions (Experimental Section) in the presence of 2 m*M* AMP. [b] Activity in the absence of added AMP divided by that in the presence of 2 m*M* AMP.

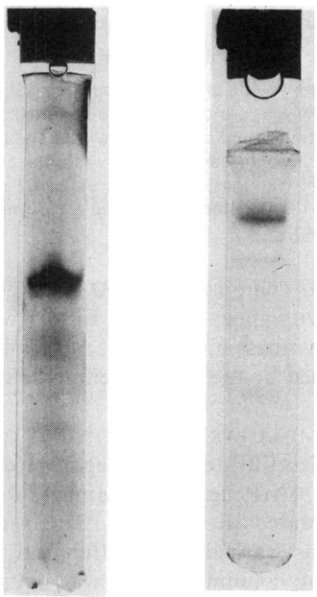

FIGURE 2: Polyacrylamide gel electrophoresis. (A) SDS gel (5% acrylamide; Experimental Section); 50 μg of purified adipose tissue phosphorylase from step 6 of Purification of Phosphorylase section. Electrophoresis was performed for a time twice that necessary for the tracking dye to reach the bottom of the gel. (B) Electrophoresis (7.5% acrylamide gel) performed at pH 7.3 in the absence of detergent until tracking dye reached the bottom of the gel; sample, 30 μg of phosphorylase from step 6 of Purification of Phosphorylase section.

however, the enzyme retained full activity after 10 days of storage at 4° and 50% after 45 days. Glycogen, bovine serum albumin, and glycerol also partially stabilized the enzyme activity at 4° but these compounds were less effective than AMP. Na_2SO_4, KCl, and EDTA did not stabilize the enzyme. Storage in the presence of 50 m*M* potassium phosphate increased the rate of loss of enzyme activity.

Incubation of the purified enzyme at room temperature for 18 hr resulted in no significant loss of activity. Incubation for 15 min at 40, 45, and 50° resulted in 0, 25, and 50% reductions in activity, respectively.

Freezing and thawing of the purified enzyme resulted in complete and irreversible inactivation. In contrast, rapid freezing and thawing of intact swine adipose tissue had no significant effect upon the amount of phosphorylase activity recovered in homogenates prepared from the tissue.

Homogeneity of Purified Phosphorylase. The enzyme from step 6 of the purification procedure was subjected to polyacrylamide gel electrophoresis at pH 7.2 in the presence of SDS or at pH 7.3 in the absence of detergent. Enzyme disaggregated with SDS, reduced with 2-mercaptoethanol, and alkylated with iodoacetate migrated as one major and one minor component during polyacrylamide gel electrophoresis performed in the presence of 0.1% SDS (Figure 2A). Assuming a proportionality between staining intensity and protein content, the major protein component was estimated to account for 80–90% of the protein detected on the gel. The molecular weight of the major protein component was approximately 90,000.

Enzyme from step 6 of the purification procedure migrated as one major protein component and several minor protein components during polyacrylamide gel electrophoresis performed at pH 7.3 in the absence of detergents (Figure 2B). Phosphorylase activity (determined on 1-mm slices of the acrylamide gel) was associated with the major component and with one of the more slowly migrating minor components in rough proportion to staining intensity. Conversion of phosphorylase from its AMP-dependent to its AMP-independent form (as described below) did not alter mobility of the enzyme during polyacrylamide gel electrophoresis performed at pH 7.3.

Sedimentation Coefficient and Isoelectric Point of Phosphorylase. The sedimentation coefficients ($s_{20,w}$) of the purified phosphorylase and of the enzyme activity in the first 25% ethanol fraction of crude homogenate were determined using sucrose density gradient centrifugation. The sedimentation coefficients for the purified enzyme and for the crude enzyme were similar, 7.9 and 7.3 S, respectively. When AMP was omitted from the gradients recovery of activity was markedly diminished; however, omission of AMP did not alter the observed sedimentation coefficient. The isoelectric point of phosphorylase activity in crude adipose tissue homogenates was pH 6.3 as estimated by isoelectric focusing.

Kinetic Parameters of Phosphorylase. The activity–pH profile of purified phosphorylase *b* was relatively broad with an optimum between pH 6.5 and 7.1. Substrate saturation kinetics were hyperbolic. The apparent K_m values for glycogen and potassium phosphate were 0.9 mg/ml and 6.6 m*M*, respectively. Saturation kinetics of the enzyme for the activator AMP was also hyperbolic. The K_a for AMP was 0.21 m*M*.

Effects of Inorganic Salts on Phosphorylase Activity. The effects of several inorganic salts on phosphorylase *b* activity were explored (Figure 3). NaCl and Na_2SO_4 produced inhibition of comparable degrees at all concentrations between 100 and 800 m*M*. Fifty percent inhibition occurred at 530 m*M* Na_2SO_4 and at 610 m*M* NaCl. $CaCl_2$ concentrations between 0.05 and 5 m*M* had no significant

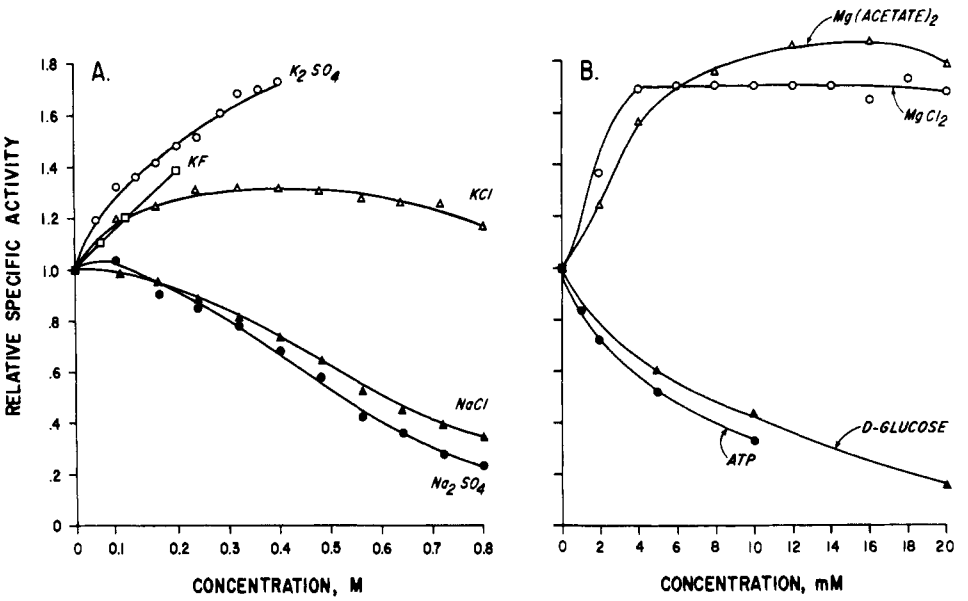

FIGURE 3: Effect of various substances on purified phosphorylase *b* activity. Standard assay system (Experimental Section) was used in the presence of various salts at concentrations indicated on the abscissa. (A) Each assay contained 1.25 milliunits of enzyme; K_2SO_4 (○); KF (□); KCl (△); NaCl (▲); Na_2SO_4 (●). (B) Each assay contained 1.0 milliunit of enzyme; $Mg(acetate)_2$ (△); $MgCl_2$ (○); D-glucose (▲); ATP (●).

Table II: Deactivation and Reactivation of Phosphorylase in Crude Homogenates of Swine Subcutaneous Adipose Tissue.

Addition	Act. Ratio	Total Act. (mU/mg)
Experiment 1[a]		
Prior to incubation		
None	0.23	31.0
After 30-min incubation		
None	0.08	30.9
Mg^{2+} (10 m*M*)	0.06	30.9
Mg^{2+} (10 m*M*) plus ATP (1 m*M*)	0.90	34.4
Experiment 2[a]		
Prior to incubation		
None	0.39	12.5
After 30-min incubation		
Protein kinase inhibitor (PKI) (1 μg/ml)	0.10	12.7
PKI (5 μg/ml) plus Mg^{2+} (10 m*M*)	0.17	9.5
PKI (5 μg/ml) plus Mg^{2+} (10 m*M*) plus ATP (1 m*M*)	0.96	12.5

[a] Crude adipose tissue homogenate (step 1 under Purification of Phosphorylase section) was incubated for 30 min at 25° in 10 m*M* Tris-HCl (pH 7.6), 0.1 m*M* EDTA, and 10 m*M* 2-mercaptoethanol and the indicated additions in a final volume of 1.0 ml. Aliquots were removed and assayed under standard assay conditions (Experimental Section).

effect upon enzyme activity (data not shown). K_2SO_4, KF, and KCl at concentrations of 0.2 *M* produced 50, 35, and 28% activation of phosphorylase *b*, respectively. At 0.4 *M* K_2SO_4 there was 70% activation of phosphorylase *b*. Maximal activation due to KCl was 32% at concentrations between 0.3 and 0.5 *M*. Above 0.5 *M*, KCl was a progressively less effective activator. Of the salts studied, $MgCl_2$ and magnesium acetate produced the greatest activation of phosphorylase *b*. At a concentration of 6 m*M* both Mg^{2+} salts produced 70% activation.

Interpretation of these salt effects is not straightforward. The data for Na^+ and K^+ salts taken together suggest that Na^+ ion may be an inhibitor of activity while SO_4^{2-} ion and to a lesser extent K^+ ion may be activators. It is noteworthy that 50 m*M* potassium phosphate is a constituent of the assay mixture, suggesting that K^+ may be an even more potent activator of the enzyme than indicated by these experiments. The effects of Cl^- ion are ambiguous and require additional study. The effects of Mg^{2+} salts upon adipose tissue phosphorylase *b* are similar to those observed for the skeletal muscle enzyme (Morgan and Parmeggiani, 1964).

Effects of Glucose 6-Phosphate, Glucose, and ATP on Phosphorylase Activity. At concentrations up to 3 m*M*, glucose 6-phosphate (in the absence of AMP) had no effect on adipose tissue phosphorylase *b* and phosphorylase *a* (prepared as described below). In the presence of 2 m*M* AMP, 5 m*M* glucose 6-phosphate inhibited adipose tissue phosphorylase *b* by only 16%.

Both glucose and ATP inhibited adipose tissue phosphorylase *b* (Figure 3). Under standard assay conditions, there was 50% inhibition of enzyme activity in the presence of 8 m*M* glucose or 5 m*M* ATP. When either glucose or ATP concentrations were held constant and AMP concentration was varied over subsaturating ranges of concentrations, families of AMP activity curves were generated. Double reciprocal plots of the data yielded families of intersecting lines. When glucose was the inhibitor, the lines intersected on the abscissa to the left of the ordinate. When ATP was the inhibitor, the lines intersected to the left of the ordinate and above the abscissa. These results indicate that glucose is noncompetitive and that ATP is partially competitive with the activator AMP.

Glucose was far less effective as an inhibitor of adipose phosphorylase *a*. Fifty percent inhibition of phosphorylase *a* occurred in the presence of 160 m*M* glucose. The mechanism by which glucose inhibits phosphorylase *a* was not studied.

Inactivation and Activation of Adipose Tissue Phosphorylase. The activity ratio of phosphorylase in crude homogenates of swine adipose tissue from fed animals ranged from 0.2 to 0.5. Incubation of the crude homogenate for 30 min in the absence or in the presence of Mg^{2+} resulted in a decrease in the activity ratio (Table II, experiment 1).

Upon addition of both $MgCl_2$ and ATP to crude homoge-

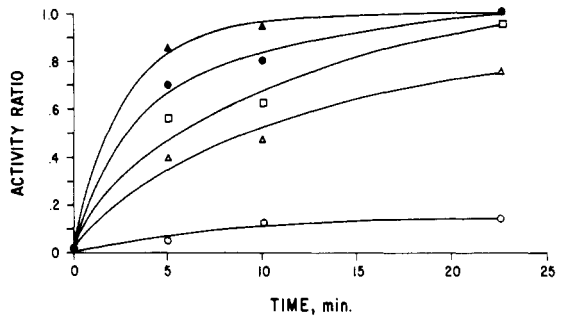

FIGURE 4: Conversion of purified phosphorylase *b* to phosphorylase *a* in the presence of various concentrations of muscle phosphorylase *b* kinase. Phosphorylase *b* (0.147 U/ml) was incubated at 30° for the indicated times in the absence (○) or in the presence of phosphorylase *b* kinase: 1.1 U/ml (△); 2.1 U/ml (□); 5.5 U/ml (●); or 11 U/ml (▲); 7.5 m*M* ATP, 15 m*M* $MgCl_2$, 1 mg/ml of glycogen, and 50 m*M* Tris-HCl (pH 8.2). Aliquots were assayed under standard assay conditions with the exception that 20 m*M* EDTA was present in the assay mixture. Total activity in the absence of phosphorylase *b* kinase decreased 39% during the incubation.

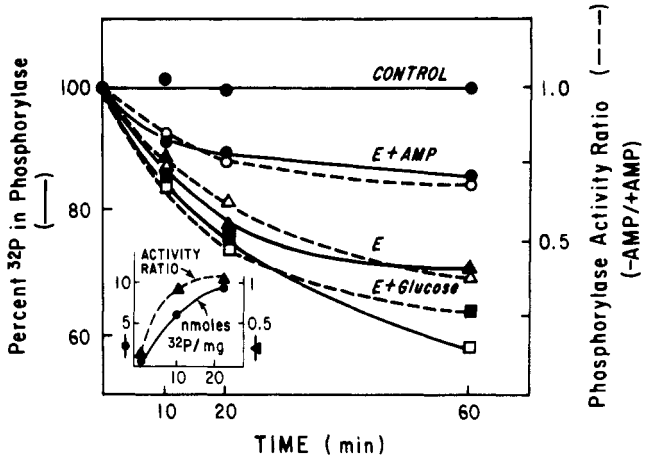

FIGURE 5: Dephosphorylation and inactivation of activated adipose tissue phosphorylase. ^{32}P-Labeled phosphorylase *a* was prepared by incubating 542 mU/ml of purified adipose tissue phosphorylase *b* in 0.7 ml of 10 m*M* β-glycerol phosphate, 15 m*M* 2-mercaptoethanol, 1 m*M* EDTA, 2 m*M* [γ-^{32}P]ATP (640 cpm/pmol), 50 m*M* cyclic AMP, 5 m*M* $MgCl_2$, 15 μg/ml of protein kinase, and 50 U/ml of phosphorylase kinase at pH 6.8. The time course of the phosphorylation and activation of the enzyme are shown in the insert. The sample was then dialyzed 18 hr against 1000 vol of 50 m*M* β-glycerol phosphate, 15 m*M* 2-mercaptoethanol, and 1 m*M* EDTA (pH 6.8). The phosphorylase activity ratio was 1.04, the activity was 581 mU/ml, and 10 nmol of ^{32}P was incorporated per mg of protein. The activated phosphorylase was then diluted 1:1 with phosphorylase phosphatase (1.05 mU/mg) in 50 m*M* Tris-Cl, 30 m*M* 2-mercaptoethanol, and 1 m*M* EDTA (pH 7.8) in the absence (E) or in the presence of 10 μ*M* AMP (E + AMP) or 10 m*M* glucose (E + glucose). The control contained no phosphorylase phosphatase. Aliquots of the reaction mixture were taken at the indicated times following the start of the incubation (30°) for the determination of phosphorylase activity and protein-bound ^{32}P radioactivity. Values are the mean of two determinations.

nates, previously incubated to reduce the activity ratio to 0.1 or below, there was a rapid increase in activity ratio to 0.9 or above. Neither exogenous cyclic AMP nor protein kinase was required for this activation. Furthermore, concentrations of protein kinase inhibitor, adequate to produce greater than 95% inhibition of endogenous cyclic AMP-dependent protein kinase, had no effect upon this activation (Table II, experiment 2), ruling out the possible participation of any endogenous cyclic AMP-independent protein kinase in this activation.

Table III: Increase in Activity Ratio and Total Activity of Purified Adipose Tissue Phosphorylase *b* Catalyzed by Muscle Phosphorylase *b* Kinase.[a]

Conditions	Time (min)	Phosphorylase *b* Kinase Added (U/ml)	Act. Ratio	Total Act. (U/ml)	Rel Act.
pH 8.2	0	0[b]	0.02	0.124	100
	5	0	0.05	0.093	100
	0	22.2	0.07	0.129	104
	5	22.2	0.88	0.170	183
	0	55.5	0.15	0.132	106
	5	55.5	0.95	0.170	183
pH 6.8, cyclic AMP, ATP–Mg, protein kinase	0	0[c]	0.08	0.542	100
	10	0	0.08	0.532	100
	0	50	0.08	0.550	102
	10	50	0.76	0.871	164

[a] Following complete conversion to the *a* form, the incubation mixture (pH 8.2, 30 mU of adipose tissue phosphorylase, 220 mU of phosphorylase kinase) was exhaustively dialyzed against 200 vol of buffer, 30 m*M* Tris-HCl at pH 7.8, or 30 m*M* β-glycerol phosphate at pH 6.8, 5 m*M* EDTA, and 15 m*M* 2-mercaptoethanol. [b] Purified phosphorylase *b* was incubated in the presence of nonactivated muscle phosphorylase *b* kinase, 50 m*M* Tris-HCl (pH 8.2), 7.5 m*M* ATP, 15 m*M* $MgCl_2$, and 1 mg/ml of glycogen at 30° for 0 or 5 min. Aliquots were removed and assayed under standard assay conditions (Experimental Section) with the exception that 20 m*M* EDTA was present. [c] Purified phosphorylase *b* was incubated in the presence of nonactivated phosphorylase *b* kinase, 30 m*M* β-glycerol phosphate (pH 6.8), 30 m*M* 2-mercaptoethanol, 1 m*M* EDTA, 2 m*M* ATP, 0.05 m*M* cyclic AMP, 5 m*M* $MgCl_2$, and 15 μg/ml protein kinase at 30° for 10 min. Aliquots were removed and assayed under standard assay conditions (Experimental Section) with the exception that 20 m*M* EDTA was present.

Conversion of Purified Phosphorylase b to Phosphorylase a by Skeletal Muscle Phosphorylase Kinase. Incubation of purified adipose tissue phosphorylase *b* with purified muscle phosphorylase kinase at pH 8.2 resulted in conversion to phosphorylase *a* by the criterion of decreasing dependence for activity on AMP. The conversion was kinase concentration and time dependent as shown in Figure 4. However, the ATP-Mg^{2+} dependent conversion of phosphorylase *b* to *a* also resulted in increased total (+AMP) activity (Table III). The conversion was qualitatively similar whether the phosphorylase kinase was activated by high pH (8.2) or by conversion of the kinase to its activated form at pH 6.8 by cyclic AMP and protein kinase (experiment 2, Table III). When [γ-^{32}P]ATP was used in the *b* to *a* conversion, 10 nmol of ^{32}P was incorporated into 1 mg of phosphorylase when the −AMP:+AMP ratio reached 1.0 (inset of Figure 5).

Deactivation and Dephosphorylation of Phosphorylase a by Adipose Tissue Phosphatase. [^{32}P]Phosphorylase *a* was prepared as described in the legend of Figure 5. After dialysis to remove adenine nucleotides the addition of partially purified swine adipose tissue phosphorylase phosphatase caused a progressive and parallel loss of protein-bound phosphate and conversion of phosphorylase *a* to *b* (Figure 5). This reaction was inhibited about 50% by 10 μ*M* AMP and was slightly augmented by glucose. The maximum release of $^{32}P_i$ was, however, only 40% with a reduction in the −AMP:+AMP activity ratio of the enzyme to 0.25. This suggests that proteins other than phosphorylase were labeled with ^{32}P (see below).

Effect of Nucleotides and Related Compounds on the Rate of Dephosphorylation of Phosphorylase a. Various

Table IV: Effects of ATP, Mg^{2+}, Cyclic AMP, and Protein Kinase (PK) on Phosphorylase Phosphatase Activity.[a]

Added Compound(s)	Act. (% of Control)	Range
ATP (3)	33	(22–43)
ATP + Mg^{2+} (2)	12	(5–18)
Mg^{2+} (2)	12	(11–13)
Cyclic AMP (2)	92	(82–102)
Protein kinase (PK) (2)	84	(78–89)
Cyclic AMP + PK (2)	86	(85–87)
Cyclic AMP + PK + Mg^{2+} (2)	10	(9–12)
Cyclic AMP + PK + ATP (2)	48	(45–52)
Cyclic AMP + PK + Mg^{2+} + ATP (3)	12	(4–19)

[a] Phosphorylase phosphatase (6 mU) and 0.15 nmol of [^{32}P]phosphorylase *a* were incubated for 5 min at 30° in a total volume of 75 μl of 50 m*M* Tris-Cl, 50 m*M* β-glycerol phosphate, 1 m*M* EDTA, and 30 m*M* 2-mercaptoethanol (pH 7.5) with the additions indicated. Values are mean and range of two–three (figures within parentheses) experiments carried out on different days. The concentrations of the added compound(s) were: 1 m*M* ATP, 6 m*M* $MgCl_2$, 0.01 m*M* cyclic AMP and 100 μg/ml of protein kinase.

nucleotides and related compounds were tested with regard to their ability to affect the rate of dephosphorylation by adipose tissue phosphorylase phosphatase of skeletal muscle [^{32}P]phosphorylase *a*. The inhibition caused by ATP, ADP, and AMP, as well as by the inhibitor from adipose tissue, was strictly competitive with phosphorylase *a* (results not shown). Calculated K_i values for AMP, ADP, and ATP were 6, 38, 1300 μ*M*, respectively. Part of the inhibitory activity of ATP and ADP was probably due to contamination by AMP or generation of AMP during incubation, because the degree of inhibition caused by ATP in crude preparations of adipose tissue was higher than in more purified preparations. Further, when [γ-^{32}P]ATP was added there was a good agreement between the degree of inhibition produced and the amount of [γ-^{32}P]ATP hydrolyzed.

A redissolved ethanol precipitate of crude swine adipose tissue homogenate was put on an AMP-Sepharose affinity column (2.0 × 5.5 cm). All of the added phosphatase (104%) appeared in the fraction eluted without AMP and no detectable activity was found to be subsequently eluted with AMP.

Creatine phosphate and cyclic AMP did not inhibit phosphorylase phosphatase at 1 m*M* concentration. Other nucleoside phosphates (5′-UMP, 3′-deoxy-AMP, and 5′-GMP) were considerably less effective than 5′-AMP. IMP inhibited the phosphatase 60% at 0.1 m*M*. This would explain the observation that adipose tissue extracts treated with adenosine deaminase still possessed considerable inhibitory activity. Adenosine, adenine, inosine, and hypoxanthine either had no effect or slightly activated phosphorylase phosphatase (15% or less). Theophylline caused a concentration-dependent activation (50% at 10 m*M*), as did glucose (50% at 10 m*M*) and glucose 6-phosphate (50% at 10 m*M*). Glucose 1-phosphate inhibited the rate of dephosphorylation 20% at 1 m*M*.

The inhibition by AMP was decreased by the presence of glucose (10 m*M*) and elevated temperatures, i.e. conditions under which the conversion of phosphorylase *a* to the dimeric form is favored (Bailey and Whelan, 1972).

Specificity of Phosphorylase Phosphatase. The action of the phosphatase on adipose tissue phosphorylase *a* was described above (Figure 5). England et al. (1972) have reported that phosphorylated muscle troponin is a substrate for muscle phosphorylase phosphatase. The 200-fold purified adipose tissue phosphorylase phosphatase preparation also catalyzed the rapid and complete dephosphorylation of phosphorylated troponin under conditions in which muscle phosphorylase was dephosphorylated to only a very minor extent, i.e. in the presence of adenine nucleotides (Figure 6). This again is compatible with the hypothesis that inhibition of the dephosphorylation of phosphorylase by nucleotides is the consequence of interaction with the substrate rather than with the enzyme itself. However, the possibility that phosphorylase phosphatase and troponin phosphatase activities reside in different proteins must also be considered.

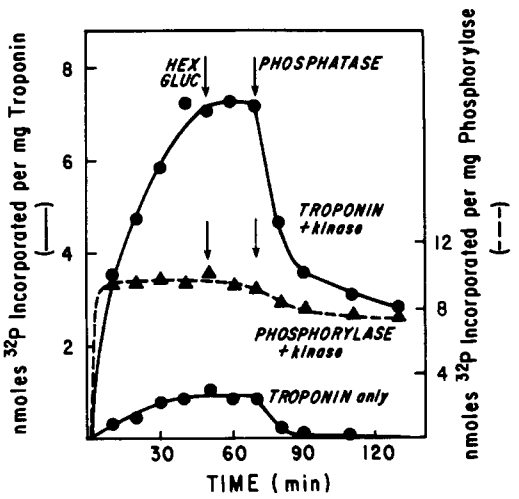

FIGURE 6: Troponin B and phosphorylase *b* from rabbit muscle were subjected to phosphorylation by muscle phosphorylase kinase followed by dephosphorylation by swine fat phosphorylase phosphatase. Troponin B (0.75 mg/ml) (●) or phosphorylase *b* (3.65 mg/ml) (▲) was incubated at 30° in a solution containing 30 m*M* Tris, 25 m*M* β-glycerol phosphate, 15 m*M* 2-mercaptoethanol, 2 m*M* magnesium acetate, 0.5 m*M* theophylline, 1 m*M* EDTA, 50 μg/ml of protein kinase, and 50 μ*M* cyclic AMP (pH 7.5). At zero time [γ-^{32}P]ATP (50 cpm/pmol, 1.2 m*M*) and 720 U/ml of phosphorylase kinase (kinase) were added. At 50 min hexokinase (Hex) and glucose (Gluc) were added (80 μg/ml and 5 m*M*, respectively). At 70 min purified swine fat phosphorylase phosphatase was added (0.4 U/ml). Aliquots of reaction mixture (50 μl) were spotted onto filter paper for ^{32}P-labeled protein determinations (see Experimental Section) at the times indicated by ● and ▲.

Effect of ATP, Mg, Cyclic AMP, and Protein Kinase on Phosphorylase Phosphatase Activity. There have been several reports in the literature indicating interconvertibility of molecular forms of phosphorylase phosphatase. Experiments with the adipose tissue enzyme are summarized in Table IV. ATP and Mg^{2+} separately inhibited the activity as did the combination. The addition of cyclic AMP, protein kinase, or both did not appear to affect the activity of the phosphatase nor the inhibition induced by ATP, by Mg^{2+}, or by both.

Discussion

Glycogenolysis and glycogen synthesis in adipose tissue have been shown to respond to epinephrine and to insulin in a manner similar to that observed in skeletal muscle, liver, and heart (Khoo et al., 1972, 1973). These processes have received little attention in comparison to the more obvious functions of adipocytes, the hydrolysis and resynthesis of triglycerides. Yet, during a cycle of fasting and refeeding glycogen virtually disappears and subsequently is resynthesized to far greater than normal concentrations (Steinberg et al., 1975). The mechanisms of activation and inactivation by covalent modification of phosphorylase and glycogen

synthase and other properties of these rate-limiting enzymes in adipose tissue are of considerable interest.

The present work provides information on the basis of which adipose tissue phosphorylase, and to some extent phosphorylase phosphatase, can be compared to these enzymes purified from other tissues from other species. Further direct comparative studies of the enzymes from swine muscle and liver have not yet been made. Such studies are indicated so that in addition to physical and enzymological comparisons the patterns of isozymes and immunological properties may also be examined.

Our final preparation of swine adipose tissue phosphorylase, purified almost 700-fold, showed a single major band on disk gel electrophoresis and that band contained almost all the phosphorylase activity. However, there were several minor protein bands migrating more rapidly and one more slowly. Thus, the final preparation was not homogeneous but was sufficiently pure to warrant study of its physical and kinetic properties.

The activity ratio of the purified enzyme was always zero although the activity ratio in crude homogenates ranged from 0.2 to 0.5. This is probably not due to selective purification of the *b* form but rather to the action of endogenous phosphorylase phosphatase during the early stages of purification. The activity ratio after the first ethanol precipitation had dropped from 0.27 to 0.06 but 95% of the *total* activity was recovered (Table I). In a 30-min incubation phosphorylase activity ratios in the crude homogenate fell from values of 0.2–0.4 down to 0.10 or lower, again with little or no change in total phosphorylase activity.

The sedimentation coefficient for the purified enzyme, 7.9 S, was similar to that reported for phosphorylase from muscle (Kent et al., 1958), spleen (Kamogawa and Fukui, 1971), and liver (Appleman et al., 1966). If the enzyme is a globular protein this would suggest a molecular weight of about 150,000. A molecular weight of 90,000 was estimated for the major component in the presence of SDS, suggesting that the native enzyme is made up of more than one subunit. In this regard the enzyme resembles that from muscle (Kent et al., 1958) and heart (Yunis et al., 1962), but not that from liver (Sutherland and Wosilait, 1956).

Adipose tissue phosphorylase *b* showed little or no activity in the absence of added AMP but was fully active in its presence like the muscle enzyme and unlike the nonactivated enzyme from liver, which does not exhibit activity even in the presence of AMP (Sutherland and Wosilait, 1956). The apparent K_a for AMP (0.21 m*M*) was similar to that reported for the muscle enzyme (0.09 m*M*) and for kidney phosphorylase (Villar-Palasi and Gazquez-Martinez, 1968). Evaluation of the possible physiologic importance of AMP as a regulator cannot be attempted without further study, especially in view of the marked effects that other tissue factors (e.g., glucose 1-phosphate, inorganic phosphate, and pH) may have on the apparent K_a (Fischer et al., 1971b). The same caveat applies to the other modifiers of activity studied: ATP, glucose, and glucose 6-phosphate. The inhibitory effects of ATP were competitive with AMP but the competition appeared to be of the mixed type rather than being strictly competitive as reported for the muscle enzyme (Fischer et al., 1971b). Glucose and AMP were strictly competitive but the high concentrations of glucose needed to obtain significant inhibition (50% at 8 m*M* under standard assay conditions) makes it most unlikely that glucose itself can be a functional intracellular regulator. Glucose 6-phosphate in the presence of a high concentration of AMP had no effect on swine adipose tissue phosphorylase *a* and only trivial effects on the *b* form. This is similar to what has been observed with rabbit skeletal muscle in the presence of a high concentration of AMP (Morgan and Parmeggiani, 1964). Swine skeletal muscle phosphorylase has also been reported to be little affected by glucose 6-phosphate (Hanabusa and Kohno, 1969).

The purified adipose tissue phosphorylase *b* was readily converted to the *a* form by incubation with purified rabbit skeletal muscle phosphorylase *b* kinase and ATP-Mg^{2+}. Activity ratios increased from less than 0.1 to greater than 0.9 indicating the completeness of the conversion. Total activity (i.e., the activity in the presence of AMP) also increased by 60 to 80%. This increase in total activity, implying a differential effect of AMP on the activated and nonactivated forms, is of a magnitude comparable to that reported to accompany activations of muscle phosphorylase (Brown and Cori, 1961; Krebs et al., 1958) but smaller than that accompanying activation of liver phosphorylase (Appleman et al., 1966) or of phosphorylase in homogenates of hormone-treated adipocytes (Khoo et al., 1973). Activation in crude homogenates of swine adipose tissue, utilizing endogenous phosphorylase kinase activity (only ATP and Mg^{2+} added), was accompanied by much smaller increases in total phosphorylase activity.

In crude homogenates, full activation of swine adipose tissue phosphorylase was effected with addition of ATP-Mg^{2+} alone. Addition of cyclic AMP and protein kinase was not necessary and the reaction was not inhibited by concentrations of protein kinase inhibitor adequate to inhibit protein kinase activity by 95%. Thus, it appears that, as in the case of rat adipose tissue homogenates (Khoo et al., 1972), there is enough endogenous active phosphorylase *b* kinase present to give maximal rates of phosphorylase activation. Whether or not phosphorylase kinase in swine adipose tissue is hormonally regulated remains to be determined.

It is clear from the foregoing that the properties of swine adipose tissue phosphorylase are generally similar to those of the skeletal muscle enzyme.

Although adipose tissue phosphorylase phosphatase was not purified to the same degree as the phosphorylase this enzyme also appears to have properties similar to those of the phosphatase isolated from skeletal muscle. The purification procedure was analogous to that used on muscle by Hurd et al. (1966). The isoelectric point (pH 5), the estimated molecular weight (55,000), and the K_m for muscle phosphorylase *a* (6 μM) are similar to the corresponding values reported for purified skeletal muscle phosphorylase phosphatase. As has recently been reported for the skeletal muscle enzyme (Nakai and Thomas, 1973), the adipose tissue phosphatase rapidly dephosphorylated phosphorylated troponin.

The inhibition of skeletal muscle phosphorylase phosphatase by AMP is thought to be mediated through conformational changes of the substrate (phosphorylase) rather than of the phosphatase (Bailey and Whelan, 1972; Nolan et al., 1964; Wang et al., 1965). At least in part these AMP and glucose effects appear to relate to the conversion of phosphorylase *a* from a dimeric to a tetrameric form, the latter being a poorer substrate for phosphorylase phosphatase. AMP and lowered temperature favor the tetrameric form while glucose and elevated temperature favor the dimeric form (Bailey and Whelan, 1972; Wang et al., 1965). Our own results with adipose tissue phosphorylase phosphatase

are consistent with these observations on the skeletal muscle enzyme. Further evidence for the hypothesis that AMP acts on the substrate was provided by the findings that: (1) troponin dephosphorylation was not affected by the presence of AMP and (2) adipose tissue phosphorylase but not phosphorylase phosphatase bound to the AMP–agarose affinity columns. The rate of dephosphorylation of purified adipose tissue phosphorylase was retarded by AMP and accelerated by glucose. This suggests that AMP and glucose induce conformational changes in adipose tissue phosphorylase as they do in the enzyme from muscle.

If it is assumed that the inhibitory activity of the crude adipose tissue extracts is due exclusively to AMP it can be calculated that the concentration of AMP in swine adipose tissue would be approximately 30 μmol/kg of fat. This concentration is similar to that found in rat epididymal fat pads (Saggerson and Greenbaum, 1970; Denton et al., 1966) and to that directly determined in swine adipose tissue (unpublished results). ATP and ADP concentrations in adipose tissue (unpublished observations) are probably insufficient to account for the inhibition of phosphorylase phosphatase observed in crude adipose tissue homogenates.

We have found no evidence that phosphorylase phosphatase exists in two interconvertible forms in adipose tissue. This conclusion is in agreement with the work on highly purified skeletal muscle phosphorylase phosphatase from Fischer's laboratory (Hurd et al., 1966; Gratecos et al., 1974). Active and inactive forms of the phosphatase have been reported from adrenal cortex (Merlevede and Riley, 1966), liver (Kalala et al., 1973), and skeletal muscle (Chelala and Torres, 1970). The degree of purification of these preparations differs from what we have achieved with adipose phosphorylase phosphatase and the much greater purification achieved by Gratecos et al. (1974) with muscle enzyme. This makes it difficult to reconcile the varying results at the present time.

The present results and the previous work of Khoo et al. (1973) make it clear that phosphorylase kinase from skeletal muscle is capable of transforming adipose tissue phosphorylase to a form not dependent upon AMP for activity. The control of phosphorylase kinase in fat appears to be different from that of the skeletal muscle enzyme. Thus, the ratio of phosphorylase kinase activity measured at pH 6.8 and 8.2 was considerably higher under basal conditions than it was in skeletal muscle or heart and incubation with epinephrine did not appear to change it (Khoo et al., 1973). Therefore, phosphorylase phosphatase activity may be an important means of regulating the fraction of adipose tissue phosphorylase that is in the *a* form. The ratio of phosphorylase activity measured in the absence to that in the presence of AMP is considerably higher (0.1–0.3) in adipose tissue than in muscle under basal conditions (Khoo et al., 1973), which is perhaps a reflection of high basal phosphorylase kinase and low phosphorylase phosphatase activities. The effects of epinephrine, insulin, and nutritional state on glycogen metabolism in adipose tissue may be mediated not only by cyclic AMP. Their effect also may be dependent upon the concentrations of AMP and other allosteric effectors that affect the conformational state of the substrate or on factors influencing directly the activity of fat cell phosphorylase phosphatase.

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