This study was initiated in order to elucidate further the role of protein phosphorylation in the regulation of glycogen synthase in vivo. Rat hearts were perfused with inorganic \[^{32}P\]phosphate and subsequently extracted under conditions which stabilized the glycogen synthase I/D ratio during the isolation of the enzyme. Throughout the standard Langendorff perfusion of up to 60 min, rat heart glycogen synthase existed 80% in the already phosphorylated D-form. We have previously shown (England, P. J., and Walsh, D. A. (1976) Anal. Biochem. 75, 429-435) that inorganic \[^{32}P\]phosphate in the perfusate equilibrates slowly with intracellular inorganic \[^{32}P\]phosphate and \[^{γ-32}P\]ATP, reaching only 5 to 10% equilibration in 60 min. In this present study it has been shown that perfusion with inorganic \[^{32}P\]phosphate led to a slow accumulation of \[^{32}P\]covalently bound to glycogen synthase. Epinephrine addition (2 \(μM\)) did not modify the extent of \[^{32}P\] covalently bound to glycogen synthase.

When purified rat heart glycogen synthase was phosphorylated with the catalytic subunit of cAMP-dependent protein kinase, sodium dodecyl sulfate gels of the resultant CNBr hydrolysates exhibited two \[^{32}P\]-phosphopeptides. These \[^{32}P\]-phosphopeptides co-migrated with those derived from either rat heart or rabbit skeletal muscle glycogen synthases that had been phosphorylated either by the rat heart cAMP-independent synthase kinase or the catalytic subunit of cAMP-dependent protein kinase. The cAMP-dependent and cAMP-independent enzymes, however, did preferentially phosphorylate the two sites of rat heart glycogen synthase at different rates. The same two \[^{32}P\]-phosphopeptides were obtained from rat heart glycogen synthase that had been phosphorylated in vivo.

Glycogen synthase (UDPglucose:glycogen \(α-4\)-glucosyltransferase, EC 2.4.1.11) is the rate-limiting enzyme for the synthesis of glycogen in mammalian tissues (1). Glycogen synthase exists in two interconvertible forms which can be distinguished by their dependence on glucose 6-phosphate for activity (3, 2). While the I-form has activity in the absence of glucose 6-phosphate, the D-form is dependent on glucose 6-phosphate and \[^{γ-32}P\]ATP, reaching only 5 to 10% equilibration in 60 min. In this present study it has been shown that perfusion with inorganic \[^{32}P\]phosphate led to a slow accumulation of \[^{32}P\]covalently bound to glycogen synthase. Epinephrine addition (2 \(μM\)) did not modify the extent of \[^{32}P\] covalently bound to glycogen synthase.

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**Experimental Procedures**

**Methods**

*Heart Perfusion*—Hearts from male Sprague-Dawley rats (240 to 260 g) were perfused essentially as described by England (15, 16). Briefly, hearts were removed under Nembutal-induced anesthesia and perfused by the Langendorff technique with Krebs-Henseleit bicarbonate-buffered media (17) containing 11 mM glucose but with the inorganic phosphate concentration reduced to 0.12 mM. The latter permits a higher intracellular specific activity of \[^{γ-32}P\]ATP to be achieved. The characteristics of phosphate uptake into the cardiac cell and the formation of \[^{γ-32}P\]ATP have been described previously (18). The hearts were perfused by drip-through with nonradioactive medium for an initial 2-min period and then perfused for 30 min with 17.5 ml of recirculating medium containing 0.1 to 0.4 mCi of inorganic \[^{γ-32}P\]ATP. Following the recycling perfusion with inorganic \[^{γ-32}P\]phosphate, the hearts were either perfused with control media for 10 to 20 s or stimulated with 2 \(μM\) epinephrine (drip-through perfusion) for an equivalent time period. Epinephrine administration had no effect on the specific activity of the intracellular \[^{γ-32}P\]ATP. Contractile force was measured at approximately 80% of the length tension curve with a force-displacement transducer (E and M Instrument Co.) attached by 00 silk thread to the apex of the heart. Flow of perfusion media was regulated by all-Teflon solenoids (Anzar Scientific Corp.) which also activated the physiograph for determinations of the interval of catecholamine stimulation. Perfusion temperature was 37°C and any heart that did not maintain a rate of greater than 230 beats/min was rejected. At the termination of the perfusion, hearts were freeze-clamped by the method of Wollenberger et al. (19) and powdered in a percussion mortar precooled in dry ice. Hearts were stored at \(-80°C\).

*Extraction*—The frozen, powdered tissue from each heart was rapidly homogenized at 4°C in a Potter-Elvehjem homogenizer containing 10 ml of an extraction buffer consisting of 30 mM Tris (pH 7.5), 30 mM KCl, 5 mM EDTA, 100 mM NaF, 45 mM 2-mercaptoethanol.
The modification of the method of Thomas et al. (20). The extract from units of pure a-amylase prior to immunoprecipitation of the glycogen to 1 g wet weight of tissue in 50 ml of extraction buffer containing 1 mg/ml of bovine serum albumin. The reaction was initiated by the addition of 0.02 ml of diluted extract to 0.10 ml of a solution containing 60 mM Tris (pH 7.7), 40 mM NaF, 12 mg/ml of glycogen, 5 mM EDTA, 5 mM [3H]UDP-Glc, and a 10 mM concentration of either Na3SO4 or glucose-6-P. 3H incorporation into glycogen was determined by pipetting 0.06 ml aliquots onto quenchers (1 x 1 cm) of filter paper (Whatman ET31); the papers were immersed in 0.6% ethanol at 0°C, then for 15 min washed three times (30 min each) with 6% ethanol and once (5 min) with acetone (20). The dried squares were counted in toluene scintillation fluid. The rate of 3H incorporation into glycogen was linear between 2 and 32 min. One unit of enzyme activity (per cent incorporation) refers to activity measured in the presence of 10 mM Na3SO4 or glucose-6-P. 14C incorporation into glycogen/min. The synthase activity ratio (per cent incorporation) refers to activity measured in the absence of 5'-AMP divided by synthase activity measured in the presence of 5'-AMP. The addition of up to 20 mg of charcoal/milliliter of tissue extract to remove 5'-AMP had no effect on the per cent of phosphorylase b measured.

Glycogen Synthase Immunoprecipitation—Antiserum was prepared in the goat against rabbit skeletal muscle glycogen synthase purified to homogeneity by the method of Soderling et al. (5). Immunoglobulins were precipitated from antiserum and normal serum by the addition of ammonium sulfate to 40% saturation. The solution was centrifuged at 10,000 × g for 10 min. The protein pellets were solubilized (to half the volume) with 0.2% Nonidet P-40 and 50 mM KCl, 5 mM EDTA, and 100 mM NaF and dialyzed against the same buffer for 24 h. No cross-reactivity of the antiserum could be detected with either phosphorylase a or phosphorylase b kinase.

Standard Isolation of Glycogen Synthase by Immunoprecipitation—For this isolation 0.4 ml aliquots of heart extracts were incubated with 3.0 ml of partially purified antiserum for 15 min at 30°C. After centrifugation for 15 min at 15,000 × g, 2.5 ml of partially purified antiserum was added to the supernatant. The solution was incubated for 25 min at 30°C, at which time 30 µg of rabbit skeletal muscle glycogen synthase were added as carrier protein. The solution was incubated for an additional 5 min at 30°C, and then for 90 min at 4°C. The immunoprecipitate was collected by centrifugation at 10,000 × g for 15 min at 4°C. The immunoprecipitate was collected by centrifugation at 30,000 × g for 15 min. The washed immunoprecipitates were solubilized by incubation for 30 min at 65°C in 200 µl of 10 mM sodium phosphate (pH 7.0), 1% SDS, 25% 2-mercaptoethanol, 5% glycerol, and 0.02% bromophenol blue and were electrophoresed on 6% gels (10 cm) by the procedure of Weber and Osborne (22). The gels were fixed in 7.5% acetic acid and then sliced in 2-mm sections. The slices were placed on filter paper squares, dried to 100°C, and counted in toluene scintillation fluid.

Purification of Rat Heart Glycogen Synthase—Rat heart glycogen synthase was partially purified from 10 g of tissue obtained from 12 animals. The minced tissue was homogenized in a Waring Blender with 60:50 ml of 50 mM Tris (pH 7.5), 100 mM NaCl, 2 mM EDTA, and 45 mM 2-mercaptoethanol (Buffer A). The homogenate was clarified by centrifugation for 20 min at 40,000 × g. The supernatant then was centrifuged for 90 min at 78,000 × g. Glycogen synthase was precipitated from the 78,000 × g supernatant by ammonium sulfate (20 g/100 ml). Following centrifugation for 15 min at 10,000 × g, the pellet was solubilized in 100 ml of 60 mM Tris (pH 7.5), 2 mM EDTA, 45 mM 2-mercaptoethanol, 5% sucrose (Buffer B). The solution was incubated for 1 h at 4°C with 180 ml packed volume of DE52 resin that had been equilibrated previously with Buffer B. The resin was poured in a column (25.5 × 45 cm) and the protein subsequently was eluted with a 500-ml linear gradient of 0 to 0.4 M NaCl in Buffer B. Glycogen synthase eluted at a conductivity of between 5.0 and 7.0 mmoles and was recovered from the pooled fractions by the addition of ammonium sulfate (20 g/100 ml). Following centrifugation for 15 min at 10,000 × g, the pellet was solubilized in 2 ml of 50 mM Tris (pH 7.5), 2 mM EDTA, 45 mM 2-mercaptoethanol, and 10% sucrose (Buffer C) and then applied to a Sepharose 4B column (25.5 × 90 cm) that had been previously equilibrated with Buffer C. The enzyme from the pooled fractions containing glycogen synthase was precipitated by the addition of ammonium sulfate and resolubilized by the same procedure as used following DEAE chromatography. After dialysis for 24 h against 1 liter of Buffer C the protein was stored frozen at −20°C. The partially purified rat heart glycogen synthase had an activity ratio of 0.96 and a specific enzyme activity of 2 units/mg. This preparation exhibited two major bands upon SDS electrophoresis, one of which was identified as the subunit of glycogen synthase and co-migrated with the skeletal muscle enzyme.

Purification of Glycogen Synthase Kinase(s)—Rat heart cAMP-independent glycogen synthase kinase(s) were partially purified by the method of the procedure of Schienert and Reitman (23). Twelve rat hearts were extracted by homogenization in a blender with 60 ml of Buffer A. The homogenate was centrifuged at 10,000 × g for 20 min at 40,000 × g. The supernatant was incubated for 40 min at 4°C with 4 ml of phosphocellulose resin that had been extensively washed with 1 n HCl, 1 n NaOH, distilled water, and then Buffer A. The resin was poured into a column and washed with 30 ml of 50 mM Tris (pH 7.5), 0.3 M NaCl, 2 mM EDTA, and 5% glycerol (Buffer D) to remove the catalytic subunit of cAMP-dependent protein kinase (23). The glycogen synthase kinases were step-eluted with Buffer D containing 0.7 M NaCl. The solution of cAMP-independent glycogen synthase kinases was concentrated to 1.5 ml with an Amicon filtration system, using a PM-10 filter, and stored at 4°C. The inhibitor protein of cAMP-dependent protein kinase had no effect on glycogen synthase kinase activity.

Phosphorylation of Glycogen Synthase—Phosphorylation of cAMP-dependent protein kinase was performed by the addition of 0.045 ml of glycogen synthase to 0.015 ml of a reaction mixture containing 166 mM NaF, 6 mM [γ-32P]ATP (1000 cpm/pmol), 20 mM MgCl2, and 8 µg of pure catalytic subunit of cAMP-dependent protein kinase. Rabbit skeletal muscle glycogen synthase (1.2 mg/ml) was in 125 mM Tris and 125 mM glycerol phosphate (pH 6.8). Rat heart glycogen synthase (0.9 mg/ml) was in Buffer C to which was added MgCl2 to a final concentration of 20 mM. cAMP-independent glycogen synthase was purified from the supernatant of 35 mg of rabbit skeletal muscle glycogen synthase by CAMP-independent synthase kinase(s) were partially purified by the method of Schienert and Reitman (23). Twelve rat hearts were extracted by homogenization in a blender with 60 ml of Buffer A. The homogenate was centrifuged at 10,000 × g for 20 min at 40,000 × g. The supernatant was incubated for 40 min at 4°C with 4 ml of phosphocellulose resin that had been extensively washed with 1 n HCl, 1 n NaOH, distilled water, and then Buffer A. The resin was poured into a column and washed with 30 ml of 50 mM Tris (pH 7.5), 0.3 M NaCl, 2 mM EDTA, and 5% glycerol (Buffer D) to remove the catalytic subunit of cAMP-dependent protein kinase (23). The glycogen synthase kinases were step-eluted with Buffer D containing 0.7 M NaCl. The solution of cAMP-independent glycogen synthase kinases was concentrated to 1.5 ml with an Amicon filtration system, using a PM-10 filter, and stored at 4°C. The inhibitor protein of cAMP-dependent protein kinase had no effect on glycogen synthase kinase activity.

Preparation of Cyanogen Bromide Peptides—Phosphoproteins from [32P]glycogen synthase labeled either in vivo or in vitro were obtained by CNBr hydrolysis. To ensure that all the CNBr phosphoproteins were derived from glycogen synthase and not from any contaminating phosphoproteins, samples of the phosphorylated glycogen synthase isolated by the procedures described above were routinely characterized by electrophoresis on 6% SDS gels (10 cm) as per Weber and Osborne (22). In all experiments gels of pure rabbit skeletal muscle glycogen synthase exhibited a single, sharp peak of radioactivity co-migrating with the enzyme. However, gels of partially purified rat heart glycogen synthase that had been phosphorylated in vivo with the enzyme.
vitro or of an immunoprecipitate prepared from rat heart perfused with inorganic $^3$P-phosphate occasionally exhibited small amounts of contaminating $^3$P-phosphoproteins. Therefore, the glycogen synthase in partially purified rat heart glycogen synthase phosphorylated in vitro and in immunoprecipitates of a rat heart perfused with inorganic $^3$P-phosphate was routinely further purified by preparative electrophoresis prior to CNBr digestion. In order to perform preparative electrophoresis the proteins were solubilized in 200 ml of 10 mM sodium phosphate (pH 7.0), 1% SDS, 2.5% 2-mercaptoethanol, 5% glycerol, and 0.09% bromphenol blue and electrophoresed on three gels. The region in the gels corresponding to the glycogen synthase was dissected ($R_g = 0.25$ to 0.40); each section was placed on top of a 6% SDS gel plug ($1 \times 0.6$ cm) and electrophoresed 30 h at 8 mA/gel, and the eluted protein was collected in dialysis tubing. Rat heart glycogen synthase was precipitated at 4°C by the addition of 100% trichloroacetic acid to a final concentration of 20%. Following centrifugation for 30 min at 10,000 $\times g$ the pellet was washed at 4°C with 5 ml of 10% trichloroacetic acid and two times with 7.5 ml of ether. The pellets were solubilized in 0.5 ml of 70% formic acid.

Cyanogen bromide digestion was performed by the method of Soderling et al. (10) and the phosphopeptide products were identified by SDS electrophoresis on 10% gels (10 cm) by the method of Weber and Osborne (22).

**cAMP Determinations**—Samples of frozen tissues (50 mg) were homogenized in 0.5 ml of 0.1 N HCl, and 0.15 ml of water was added. The samples were placed in a boiling water bath for 2 min and then centrifuged for 10 min at 27,000 $\times g$. A 0.5-ml aliquot of the supernatant was neutralized by the additions of 0.05 ml of 0.6 N NaOH and 0.01 ml of 2 M sodium acetate. The sample was clarified by centrifugation, and the supernatant was assayed for cAMP by the method of Brostrom and Kon (25).

**Materials**

The following chemicals were from Sigma Chemical Co.: cyanogen bromide, UDP-glucose, insoluble $\alpha$-amylase (235 units/g), $\alpha$-amylase type II A crystallized four times (870 unit/mg), glucose 6 phosphate, and ATP. UDP-$[U-^3$H]glucose and inorganic $^3$P-phosphate were purchased from ICN Pharmaceuticals, Inc. Prior to use, $\alpha$-amylase (1 mg/ml) was preincubated 30 min at 30°C in 1 mM glucose-6-phosphate, 150 mM KCl, 10 mM sodium phosphate (pH 7.0), 1% SDS, 2.5% 2-mercaptoethanol, and 0.09% bromphenol blue and electrophoresed on three gels. The region in the gels corresponding to the enzyme was dissected ($R_g = 0.25$ to 0.40); each section was placed on top of a 6% SDS gel plug ($1 \times 0.6$ cm) and electrophoresed 30 h at 8 mA/gel, and the eluted protein was collected in dialysis tubing. Rat heart glycogen synthase was precipitated at 4°C by the addition of 100% trichloroacetic acid to a final concentration of 20%. Following centrifugation for 30 min at 10,000 $\times g$ the pellet was washed at 4°C with 5 ml of 10% trichloroacetic acid and two times with 7.5 ml of ether. The pellets were solubilized in 0.5 ml of 70% formic acid.

**RESULTS**

[cAMP]Glycogen synthase, labeled during perfusion of rat heart, was isolated by immunoprecipitation for either control or epinephrine-stimulated tissue. In agreement with the results of others (11, 13, 14) glycogen synthase in extracts from unstimulated perfused heart exists mainly in the already phosphorylated D-form. (As indicated subsequently (vide Fig. 4) the phosphorylation (and, in consequence, activation) state of the enzyme, as measured, is not a result of covalent modification occurring during the extraction procedure.) Immunoprecipitation of glycogen synthase and activity assays were performed with rat heart extracts clarified by centrifugation for 15 min at 15,000 $\times g$. Under these conditions 85% of the total activity was soluble and the percentage of the enzyme in the I-form was the same as for the enzyme measured in the total homogenate (Fig. 1). At higher centrifugation speeds an increasing percentage of total activity was sedimented and the D-form was selectively removed from the supernatant.

**Effect of Epinephrine on cAMP, Phosphorylase, and Glycogen Synthase**—As reported by others for perfused rat heart (11) epinephrine stimulated cAMP accumulation, increased phosphorylase activity, but had negligible effect on glycogen synthase activity (Fig. 2). The control level of cAMP was 0.02 nmol/g (wet weight) of tissue. This level increased rapidly reaching a maximum of 1.11 nmol/g at 20 s after epinephrine administration. The percentage of phosphorylase $\alpha$ increased from a basal level of 12.4% to a maximum of 79.1% at 20 s. In the absence of epinephrine glycogen synthase was observed to be predominantly in the inactive form. The basal level of 79.7% D-form did not vary more than ±3.6% during 30 s of 2 $\mu$M epinephrine exposure, even though this concentration was shown to produce a maximal change in phosphorylase kinase activity. The basal level of glycogen synthase in the D-form was not altered when unmodified Krebs-Henseleit buffer (i.e. 1.17 mM KH$_2$PO$_4$ instead of 0.12 mM) was used, when glucose was deleted from the perfusate, nor when the perfusate temperature was varied from 35 to 38°C (data not shown).

**Glycogen Synthase Immunoprecipitation**—In the extract, rat heart glycogen synthase is bound to glycogen. The presence of this complex prevented quantitative and reproducible immunoprecipitation of the synthase; however, prior treatment of the extract with $\alpha$-amylase for 30 min at 0°C circumvented this difficulty. The characteristics of immunotitration and immunoprecipitation of glycogen synthase from $\alpha$-amylase-treated rat heart extract are presented in Fig. 3. Rat heart glycogen synthase activity was inhibited by the addition of rabbit anti-skeletal muscle glycogen synthase (Fig. 3A), but quantitative precipitation of the enzyme was obtained only with the subsequent addition of carrier skeletal muscle glycogen synthase (Fig. 3, B and C). In the experiment presented in Fig. 3A, rat heart extract was treated with insoluble polyacrylamide-bound $\alpha$-amylase to permit subsequent removal of

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**Footnote:**

1. T. E. McCullough and D. A. Walsh, unpublished observation.
In the experiments presented in Fig. 3, B and C, the heart extract was treated with normal sera (followed by centrifugation) prior to the addition of anti-glycogen synthase. Without this treatment the antibody-antigen-glycogen synthase complex also contained co-precipitating low molecular weight compounds. This observation is illustrated in Fig. 4A. The immunoprecipitated $^{32}$P-labeled protein was examined by SDS-gel electrophoresis. For immunoprecipitates isolated without prior treatment with normal sera, in addition to the band of radioactivity that co-migrated with glycogen synthase (88,000 daltons), two highly labeled low molecular weight compounds of less than 40,000 were detected. The same two low molecular weight compounds were present in samples treated with normal sera alone (Fig. 4A) or in a sample that had been incubated under identical conditions without addition of sera (data not shown); under neither of these latter two conditions was a band of $^{32}$P-labeled glycogen synthase present. Presumably the low molecular weight phosphocompounds are rapidly denaturing (or precipitating, or both) components not removed during the initial centrifugation but labile under the incubation conditions required for the immunoprecipitation. These compounds could be precipitated by centrifugation of the original homogenate at 40,000 x g for 15 min, but this procedure also removed a large amount of the glycogen synthase (vide Fig. 1). The addition of normal sera, although not essential for precipitation of the low molecular weight phosphocompounds, did enhance their removal. Routinely for the isolation of $^{32}$P-labeled glycogen synthase from rat heart the extracts were initially incubated with normal sera, the precipitate was removed by centrifugation, and glycogen synthase subsequently was precipitated by the addition of antisera. Under these conditions only one band of $^{32}$P-protein was detected in the immunoprecipitate isolated from $^{32}$P-perfused rat heart; this band co-migrated with both skeletal muscle glycogen synthase (identified by Coomassie protein stain, Fig. 4) and with rat cardiac glycogen synthase (detected by Coomassie stain and by $^{32}$P from in vitro labeling; data not presented).

The peak of [$^{32}$P]glycogen synthase was absent from the SDS gel of the immunoprecipitate when antisera was pre-treated with pure skeletal muscle glycogen synthase, thus adding additional confirmation that the $^{32}$P label identified by the SDS gel is covalently associated with the cardiac glycogen synthase and not coincidentally co-migrating. In all experiments reported in this manuscript the extent of $^{32}$P incorporation into glycogen synthase was determined from the SDS-gel electrophoresis profiles.

As indicated in Fig. 3 quantitive immunoprecipitation of rat heart glycogen synthase required the subsequent addition of skeletal muscle glycogen synthase as carrier. The possibility that either the added pure skeletal muscle enzyme or the endogenous glycogen synthase of the heart extract was being phosphorylated during the extractions and incubations required for immunoprecipitation was examined (Fig. 4B). A I-g rat heart contains 4.15 $\mu$mol of ATP (28). Therefore, with the assumption that there was no hydrolysis of endogenous ATP, exogenous high specific activity [$\gamma$-$^{32}$P]ATP was added to a homogenate of a nonradioactive heart to give a final specific activity of the total ATP pool of 30 cpm/$\mu$mol, a value equivalent to the specific activity of the endogenous [$\gamma$-$^{32}$P]-ATP obtained on perfusion of the heart with inorganic [$^{32}$P]-phosphate. Glycogen synthase was isolated from this supplemented extract by the standard immunoprecipitation technique. No $^{32}$P-protein was observed in the resultant immunoprecipitate (Fig. 4B). In a second control experiment 3 $\mu$g of $^{32}$P-labeled pure skeletal muscle glycogen synthase was added to 0.4 ml of a nonradioactive heart extract; 103% of the

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The α-amylase and enable the subsequent assay of glycogen synthase to be performed; in all other experiments soluble α-amylase was used. In a comparison experiment, approximately 27-fold more antiserum was required to inactivate 1 unit of rat heart glycogen synthase in an extract than to inactivate 1 unit of pure rabbit skeletal muscle enzyme (data not shown).
radioactivity was recovered in the resultant immunoprecipitate in a single sharp peak (Fig. 4C), establishing that the immunoprecipitation method quantitively isolated 32P-labeled glycogen synthase from rat heart extract and in addition demonstrating the absence of post-homogenization phosphatase or protease activity.

The conditions as defined by the experiments depicted in Figs. 3 and 4 formed the basis for the experimental procedure utilized in these studies for the determination by immunoprecipitation of 32P-labeled glycogen synthase from perfused heart; the full protocol is presented under "Experimental Procedures" (see "Standard Isolation of Glycogen Synthase by Immunoprecipitation").

The Specific Activities of 32P/Phosphate, γ-32P|ATP, and [32P]Glycogen Synthase During Perfusion—We have previously shown that equilibration of inorganic [32P]phosphate into the myocardium occurs slowly and that the transport of inorganic [32P]phosphate across the myocardial sarcolemma is the rate-limiting step in the formation of intracellular [γ-32P]ATP (18). The characteristics of the cardiac perfusion system used in this study are presented in Fig. 5. During perfusion the specific activity of the perfusate inorganic phosphate declined substantially and by 50 min it was 25% of that of the initial value. This occurred without a change in the extracellular inorganic phosphate concentration (0.12 mM), even though the perfusate inorganic phosphate concentration was 1/5th of the physiological value. Uptake of inorganic [32P]phosphate occurs as an exchange of intracellular and extracellular phosphate with no net efflux from the myocardium and no net loss of total cellular phosphate. Intracellular inorganic phosphate and the γ-phosphate of ATP are in rapid equilibrium (18). During 60 min of perfusion the specific activity of [γ-32P]ATP continued to increase (Fig. 5), but because of the extensive total exchangeable phosphate pool of the myocardial cell it only reached a specific activity that was 8.5% of that of the extracellular phosphate even after a 50-min perfusion. Most of the experiments presented in this manuscript were performed with hearts equilibrated with inorganic [32P]phosphate for 30 min, the specific activity of the [γ-32P]ATP attaining approximately 1/5th of that of the extracellular phosphate in the perfusate.

The interpretation of data for the incorporation of 32P into

![Fig. 4. SDS-gel electrophoresis of immunoprecipitates of glycogen synthase.](http://www.jbc.org/)

![Fig. 5. Relative changes in specific activities of perfusate inorganic [32P]phosphate (— — —) and intracellular [γ-32P]ATP (— — —).](http://www.jbc.org/)
glycogen synthase during cardiac perfusion is complex. As indicated by the experiment presented in Fig. 6, following a brief lag (< 10 min) uptake of $^{32}$P phosphate into glycogen synthase occurred throughout the 60-min perfusion. During this period the I/D ratio of the synthase remains constant; this $^{32}$P incorporation presumably represents an exchange rather than a net phosphorylation of the protein. Throughout this period the increase in the $^{32}$P content of glycogen synthase paralleled the increase in the specific activity of the intracellular [$\gamma$-$^32$P]ATP. To further elucidate this process hearts were perfused with 0.12 mM inorganic $^{32}$P phosphate for 30 min at which time KH$_2$PO$_4$ and Na$_2$HPO$_4$ were added to the perfusate so to reduce the specific activity of the perfusate inorganic $^{32}$P phosphate to a value equal to the specific activity of the intracellular [$\gamma$-$^32$P]ATP. Under these conditions the specific activity of the [$\gamma$-$^32$P]ATP remained constant during an additional 30-min perfusion (Fig. 6B). During this subsequent 30-min period, the radioactivity in glycogen synthase continued to increase at an incorporation rate of 0.3 mol of phosphate incorporated/90,000 daltons of enzyme/30 min (Fig. 6A).

The possibility that this slower increase in $^{32}$P-incorporated into glycogen synthase represented phosphorylation of newly synthesized enzyme was investigated. Perfusion for 30 min with 20 $\mu$m cycloheximide did not cause any reduction in the specific enzyme activity of glycogen synthase in the resultant heart homogenate (not shown). This level of cycloheximide effectively blocks protein synthesis in the perfused rat heart (30).

**Effect of Epinephrine on Phosphorylation of Glycogen Synthase**—As indicated previously (Fig. 2) epinephrine administration had no effect on the per cent of glycogen synthase in the D-form. In addition, the administration of 2 $\mu$m epinephrine for 10 or 20 s to a rat heart perfused with inorganic $^{32}$P phosphate caused no significant change in the amount of phosphate incorporated (Fig. 7). Under identical conditions both activation and phosphorylation of phosphorylase kinase and troponin I (10) are readily demonstrated.

**Comparison between Heart Glycogen Synthase Phosphorylated in Vivo, Heart Glycogen Synthase Phosphorylated in Vitro, and Skeletal Muscle Glycogen Synthase Phosphorylated in Vitro**—We have compared the in vivo sites of phosphorylation of rat heart glycogen synthase with the sites phosphorylated in vitro by the cAMP-dependent protein kinase and by the cAMP-independent glycogen synthase kinase(s). Additionally, these data have been compared to the already determined sites in skeletal muscle glycogen synthase.

Soderling et al. (10, 31) have determined by limited trypsin digests and by CNBr digests that there are two major phosphorylation sites in skeletal muscle glycogen synthase. Both sites can be phosphorylated by the cAMP-dependent protein kinase and by the cAMP-independent glycogen synthase kinase. However, the cAMP-dependent protein kinase preferentially phosphorylates a site which can be removed from glycogen synthase by mild trypsinization and is, therefore, termed the “trypsin-sensitive” site. The cAMP-independent glycogen synthase kinase preferentially phosphorylates a site which is not degraded by mild trypsin treatment and, therefore, is termed the “trypsin-insensitive” site. The two sites can also be distinguished by cyanogen bromide digestion of phosphorylated glycogen synthase and the subsequent separation of the phosphopeptides by SDS-gel electrophoresis. The larger CNBr phosphopeptide contains the “trypsin-sensitive” site and the lower molecular weight phosphopeptide contains the “trypsin-insensitive” site.

In agreement with Soderling et al. (10), SDS gels of CNBr digests of pure rabbit skeletal muscle glycogen synthase that had been phosphorylated by the pure catalytic subunit of cAMP-dependent protein kinase exhibited two peaks of radioactivity; of the radioactivity in the two peaks 67% was in the higher molecular weight phosphopeptide (Fig. 8A). Treatment of intact glycogen synthase with trypsin (1:125 w/w) for 30 min at 30°C prior to precipitation at 4°C with 10% trichloroacetic acid resulted in the elimination of the heavier CNBr phosphopeptide from the ensuing SDS gel (data not presented). An essentially identical pattern was obtained for partially purified rat heart glycogen synthase. The SDS-gel electrophoretic profile (Fig. 8B) of cyanogen bromide phosphopeptides exhibited two major peaks of radioactivity with mobilities identical to those obtained for the skeletal muscle enzyme; 66% of the radioactivity in the two major phospho-
peptides was in the larger species. Aliquots of the cyanogen bromide 32P peptides, purified rat heart and rabbit skeletal muscle glycogen synthases were phosphorylated by the catalytic subunit of cAMP-dependent protein kinase. Following preparative electrophoreses of the heart enzyme each sample was precipitated with trichloroacetic acid, washed with trichloroacetic acid and ether, solubilized with 70% formic acid, hydrolyzed with cyanogen bromide, dried under nitrogen, and electrophoresed on 10% SDS gels. The gels were sliced in 2-mm sections and counted for 32P. Details of the conditions for preparation and phosphorylation of enzymes, and for isolation and hydrolysis of 32P products are presented under "Experimental Procedures." In A, 30 μl of a CNBr hydrolysate of rabbit skeletal muscle glycogen synthase were applied to a gel. In B, 10 μl of a CNBr hydrolysate of rat heart glycogen synthase were applied to a gel. In C, 10 μl of rabbit skeletal muscle and 40 μl of rat heart glycogen synthase CNBr hydrolysates were applied to a gel.

An identical comparison has been performed of sites in rat heart and rabbit skeletal muscle glycogen synthase co-migrated with those from the rabbit skeletal muscle enzyme.

Peptides was in the larger species. Aliquots of the cyanogen bromide hydrolysates of both rat heart and rabbit skeletal muscle glycogen synthase were combined and electrophoresed (Fig. 8C). The phosphopeptides derived from rat heart glycogen synthase co-migrated with those from the rabbit skeletal muscle enzyme.

An identical comparison has been performed of sites in rat heart and rabbit skeletal muscle glycogen synthase phosphorylated in vitro by the cAMP-independent synthase kinase (Fig. 9). The phosphorylation reactions were performed in the presence of the inhibitor protein of cAMP-dependent protein kinase, present at a 300-fold excess of the amount necessary for 90% inhibition of all the kinase activity had it been cAMP-dependent. For skeletal muscle glycogen synthase phosphorylated for 6 min at 30°C, the SDS gel of the resultant cyanogen bromide hydrolysate exhibited one predominant peak of radioactive, which contained 78% of the radioactivity, which contained 78% of the radioactivity (Fig. 9A). Following phosphorylation for 30 min at 30°C, the radioactivity was observed to be in two major phosphopeptides (Fig. 9B); 65% of the radioactivity in these two phosphopeptides was in the lower molecular weight species. The two phosphopeptides phosphorylated by the cAMP-independent synthase kinase co-migrated with those phosphorylated by the cAMP-dependent enzyme (cf. Fig. 8A) but, in accord with the data of Soderling et al. (10), the cAMP-dependent enzyme preferentially phosphorylated the site in the larger peptide, whereas the cAMP-independent kinase preferentially phosphorylated a site in the smaller species. A similar, although less definitive result was observed for the cardiac glycogen synthase. Following phosphorylation of the cardiac glycogen synthase with the cAMP-independent kinase for 6 min, phosphate was incorporated predominantly into two phosphopeptides (Fig. 9C). The two major phosphopeptides co-migrated with those from skeletal muscle glycogen synthase as catalyzed by the cyclic nucleotide-independent kinase (cf. Fig. 9B) and with those from cardiac glycogen synthase as catalyzed by the cAMP-dependent protein kinase (cf. Fig. 9B). Seventy per cent of the radioactivity in the two phosphopeptides was in the lower molecular weight species (Fig. 9C). Following phosphorylation of cardiac glycogen synthase for a longer time period (30 min) with the cyclic nucleotide-independent kinase the radioactivity in the heavier phosphopeptide increased to over 50% of the total radioactivity in the two phosphopeptides (Fig. 9D). For the cardiac glycogen synthase particularly in comparison to the skeletal muscle enzyme, both the cAMP-dependent and the cAMP-independent protein kinase catalyzed the incorporation of low levels of phosphate into peptides other than those containing the two major sites.

The sites of glycogen synthase phosphorylated in the intact heart have been examined by an identical procedure. To ensure that the phosphopeptides identified were derived from glycogen synthase the 32P-labeled glycogen synthase was isolated from the perfused heart extract by immunoprecipitation, the intact protein electrophoresed on SDS gel, the band containing the 32P-labeled glycogen synthase was dissected out, and CNBr digestion was performed on the protein eluted from the gel (details of the methods used are given under "Experimental Procedures"). The SDS gels of the resultant CNBr phosphopeptides exhibited two major peaks of radioactivity of Rm 0.60 and 0.90 (Fig. 10A); 57% of the radioactivity was in the higher molecular weight species. These phospho-
Phosphorylation of Glycogen Synthase

Fig. 10. SDS-gel electrophoresis of cyanogen bromide 32P-
peptides. Rat heart [32P]glycogen synthase from 4 ml of an extract of a rat heart that had been perfused with 5 mCi of inorganic [32P]-
phosphate for 30 min was isolated by immunoprecipitation followed by preparative electrophoresis. Pure rabbit skeletal muscle glycogen synthase was phosphorylated by the catalytic subunit of cAMP-
dependent protein kinase. Cyanogen bromide phosphopeptides were prepared and electrophoresed. Full details of these methods are presented under "Experimental Procedures." In A, 50 µl of a CNBr hydrolysate of rat heart glycogen synthase phosphorylated in vivo were applied to a 10% SDS gel. In B, 25 µl of a CNBr hydrolysate of rabbit skeletal muscle glycogen synthase were applied to a 10% SDS gel. In C, 50 µl of rat heart and 25 µl of rabbit skeletal muscle glycogen synthase CNBr hydrolysates were applied to a gel.

peptides co-migrated with those obtained by in vitro phosphorylation of the glycogen synthase (Fig. 10, B and C).

DISCUSSION

In vitro studies utilizing glycogen synthase from liver, skeletal muscle, adipose, brain, spleen, and heart (32), and kidney (33) have demonstrated that the enzyme is phosphorylated and inactivated by CAMP-dependent protein kinase. In agreement with the consensus of observations by other investigators (11, 13, 14, 34, 35) our results indicate that rat heart glycogen synthase in the basal (unstimulated) tissue exists primarily in the D-form, indicating that it would be phosphorylated even in the absence of elevated cAMP levels. Studies with isolated diaphragm have demonstrated that agents known to elevate intracellular cAMP levels caused a rapid inactivation of glycogen synthase (36); however, conflicting results have been reported from studies on the intact heart. In the perfused rat heart epinephrine, which elevates CAMP, has been reported to increase, decrease, or have no effect (11, 13, 14) on the per cent of glycogen synthase in the I-form. In the in situ rat heart the per cent of glycogen synthase in the I-form was observed to increase (34) or decrease slightly (35) upon epinephrine administration. Under the experimental conditions used in this investigation epinephrine has no significant effect on glycogen synthase activity nor on the incorporation of phosphate into the enzyme.

Recently, glycogen synthase kinase(s) other than CAMP-
dependent protein kinase and its catalytic subunit have been described (6-8). These kinases have in common the ability to bind to phosphocellulose. However, they were eluted at differing ionic strengths of buffer. The skeletal muscle CAMP-
dependent independent synthase kinase(s) studied by Schlender and Reimann (23) and Itarte et al. (37) was fully eluted by 0.5 M NaCl or KCl. Only trace amounts of CAMP-independent synthase kinase activity could be detected with further elution by 1 M NaCl. In contrast, the CAMP-independent synthase kinase studied by Nimmo et al. (6), Brown et al. (38), and Soderling et al. (10) elutes from phosphocellulose at 0.5 to 1.0 M NaCl. For our study, we collected all the cardiac cyclic nucleotide independent glycogen synthase kinases that eluted from phosphocellulose between 0.3 and 1.0 M NaCl. The mechanisms whereby the activities of these kinases are regulated are as yet unknown.

Both the cAMP-dependent protein kinase and the CAMP-
dependent glycogen synthase kinase catalyze the conversion of skeletal muscle glycogen synthase from the I to the D activity form; each catalyzes the phosphorylation of the enzyme but the site preferentially phosphorylated is different for CAMP-independent and CAMP-dependent kinases. A minimum of four covalently different forms of glycogen synthase exist. These have been termed glycogen synthase a (dephospho form), glycogen synthase b1 and b2 (the monophospho forms phosphorylated in the CAMP-dependent site (trypsin-sensitive, high molecular weight CNBr digest fragment) or CAMP-independent (trypsin-insensitive, low molecular weight CNBr digest fragment) site, respectively), and glycogen synthase b1,b2 (diphospho form). The I/D ratio of each of the forms have been variously reported to be: a, 0.8 to 1.0; b1, 0.1 to 0.3; b2, 0.1 to 0.3; and b1,b2, 0.01 to 0.1 (2, 6, 10, 37). In addition, Soderling et al. (10) have reported that there is an additional site of phosphorylation on skeletal muscle glycogen synthase catalyzed by the CAMP-dependent kinase. It is apparent, therefore, that an understanding of the regulation of glycogen synthase requires not only the determination of the I/D ratio but also an elucidation of the site phosphorylated.

The data presented in this manuscript indicate that CNBr phosphopeptides derived from rat heart glycogen synthase phosphorylated in vitro are closely similar (or identical) to those of the skeletal muscle enzyme and that the apparent preferential sites of phosphorylation by the CAMP-dependent and CAMP-independent protein kinases are the same for the cardiac and skeletal muscle glycogen synthases (Figs. 8 and 9).

On perfusion of rat hearts with inorganic [32P]phosphate, there was an increase in radioactivity in glycogen synthase (Fig. 6). This increase in 32P in glycogen synthase was probably not due to phosphorylation of newly synthesized enzyme since there was no change in the specific enzyme activity of glycogen synthase in perfusions in the presence of cycloheximide at a concentration sufficient to block protein synthesis. The increase in 32P in glycogen synthase may have been due to either net phosphorylation of the protein or turnover of phosphate. Since predominantly only those sites known to regulate enzyme activity were observed to become phosphorylated (Fig. 10) and there was no measurable effect on enzyme activity (per cent I-form or total activity), this would argue against a change in the net phosphate (i.e. 32P + 32P) content; nevertheless, it should be recognized that the per cent I measurement is insensitive to change in phosphate content for enzyme already containing greater than 1 mol of phosphate/subunit. Estimates of the stoichiometry of the [32P]phosphate incorporated into glycogen synthase due to net turnover are complicated by five factors: (a) the specific activity of intracellular [γ-32P]ATP is changing during the perfusion, (b) the specific activity of phosphate incorporated into protein may be greater than that of the average specific activity of intracellular [γ-
2]ATP as it has been reported in skeletal muscle for the phosphorylation of phosphorylase (39), (c) cardiac glycogen synthase has not been obtained in a highly homogeneous form to permit a determination of the specific enzymatic activity, (d) although skeletal muscle glycogen synthase has been...
obtained in a highly purified form its specific activity has been
variously reported between 8 to 32 units/mg of protein, and
(e) the immunoprecipitation of cardiac enzyme requires the
addition of carrier protein, thus negating the possibility of
obtaining a specific radioactivity based on a protein determi-
nation of the immunoprecipitate. Within these limitations,
and based on the assumption of a specific enzymatic activity
for the cardiac glycogen synthase of 20 units/mg of protein, it
would be calculated that perfusion of the heart for 60 min
(time of Fig. 6) resulted in the incorporation (as turn-
over) of between 0.6 to 1.6 mol of phosphate/90,000-dalton
subunit of the enzyme. A more accurate calculation of the
moles of ^38 incorporated requires determination of the spe-
cific activity of the phosphate in the glycogen synthase; such
is technically restricted by available sample size.

The data for both skeletal muscle (Figs. 8A and 9 A and B)
and cardiac (Figs. 8B and 9, C and D) glycogen synthase
indicate that there is an apparent overlap of the site specific-
ities for both the cAMP-dependent and cyclic nucleotide-
dependent independent protein kinase in vitro. Nevertheless, there is a
selectivity for the sites, with the b, (high molecular weight
CNBr peptide) and the bZ (low molecular weight CNBr pep-
tide) being preferentially phosphorylated by the cAMP-de-
dependent and cyclic nucleotide independent kinases, respec-
tively. It is possible that under the more restrictive conditions
that may occur in the intact cell a higher degree of specificity
for each enzyme may be exhibited. If this were so, then the
phosphate turnover that occurs at both sites in vitro (Fig. 10A)
would be a result of a balance of each of the protein kinases
and the respective phosphatases. For the cAMP-regulated
system such an interpretation would infer that the cAMP-
dependent protein kinase would be exhibiting activity even in
the absence of hormonally elevated levels of cAMP; alterna-
tively, the phosphorylation of the b site may be catalyzed in
vivo by a cAMP independent enzyme. Under identical condi-
tions other substrates for the cAMP-dependent protein kinase,
phosphorylase kinase (40), a 27,000-dalton protein of sarco-
lemma (41), and troponin I (15, 16) remain essentially in the
dephosphorylated forms. Presumably this reflects differing
activities, specificities, and/or substrate-directed regulation of
the kinase and respective phosphatase(s).

Insulin elevates the percentage of glycogen synthase in the
I-form in perfused rat heart (42, 43) as well as other tissues. It
will be of great interest to determine using the methods
established in this report whether or not insulin adminis-
tration leads to a preferential loss of radioactivity from one site
only.

REFERENCES

1. Soderling, T. R., and Park, C. R. (1974) Adv. Cyclic Nucleotide
Res. 4, 283-333
2. Roach, P. J., and Larner, J. (1977) Mol. Cell. Biochem. 15, 179-
200
3. Larner, J., Villar-Palasi, C., and Brown, N. E. (1969) Biochim.
Biophys. Acta 178, 470-479
4. Schröder, K. K., Wei, S. H., and Villar-Palasi, C. (1969) Biochim.
Biophys. Acta 191, 272-278
5. Soderling, T. R., Hickenbottom, J. P., Reimann, E. M., Hunkeler,
F. L., Walsh, D. A., and Krebs, E. G. (1970) J. Biol. Chem. 245,
Phosphorylation of glycogen synthase in the perfused rat heart.
T E McCullough and D A Walsh

J. Biol. Chem. 1979, 254:7336-7344.