The effects of insulin and epinephrine on the phosphorylation of glycogen synthase were investigated using rat hemidiaphragms incubated with $^{32}$P phosphate. Antibodies against rabbit skeletal muscle glycogen synthase were investigated using rat hemidiaphragms incubated with $^{32}$P phosphate. After cleavage with CNBr two major $^{32}$P-labeled fragments were used for the rapid purification of the $^{32}$P-labeled enzyme under conditions that prevented changes in its state of phosphorylation. The purified material migrated as a single radioactive species ($M_r = 90,000$) when subjected to electrophoresis in sodium dodecyl sulfate.

Insulin decreased the $[^{32}P]$phosphate content of glycogen synthase. This effect occurred rapidly (within 15 min) and was observed with physiological concentrations of insulin (25 micromoles/ml). The amount of $[^{32}P]$phosphate removed from glycogen synthase by either different concentrations of insulin or times of incubation with the hormone was well correlated to the extent to which the enzyme was activated. Epinephrine (10 μM) inactivated glycogen synthase and increased its content of $[^{32}P]$phosphate by about 50%.

Cleavage of the immunoprecipitated enzyme with cyanogen bromide yielded two major $[^{32}P]$-labeled fragments of apparent molecular weights equal to approximately 28,000 and 15,000. The larger fragment (Fragment II) displayed electrophoretic heterogeneity similar to that observed with the corresponding CNBr fragment (CB-2) from purified rabbit skeletal muscle glycogen synthase phosphorylated by different protein kinases. Epinephrine increased $[^{32}P]$phosphate content of both fragments; however, the increase in the radioactivity of the smaller fragment (Fragment I) was more pronounced. Insulin decreased the amount of $[^{32}P]$phosphate present in Fragments I and II by about 40%. The results presented provide direct evidence that both insulin and epinephrine control glycogen synthase activity by regulating the phosphate present at multiple sites on the enzyme.

Over two decades ago the observation was made that insulin acted on rat diaphragm to increase glycogen synthase activity when measured in the absence of glucose-6-P but not in its presence (1). This finding led to the proposal that the enzyme existed in active (I) and inactive (D) forms. Epinephrine was later shown to decrease the percentage of glycogen synthase I activity (2). This provided a scheme for hormonal control of glycogen synthesis via modulation of glycogen synthase activity by insulin and epinephrine. The discovery of cAMP-dependent protein kinase in skeletal muscle which phosphorylated and inactivated glycogen synthase provided a mechanism by which the stable effects of epinephrine could be explained (3). By activating adenylate cyclase and increasing cAMP, epinephrine would activate cAMP-dependent protein kinase resulting in phosphorylation and inactivation of glycogen synthase. Insulin action has been more difficult to fit into the scheme as no effect of insulin can be demonstrated on the concentration of cAMP in skeletal muscle (4, 5).

A major criticism of previous studies of the hormonal regulation of glycogen synthase is that changes in the phosphorylation of the enzyme have not been directly measured but inferred from changes in kinetic properties of the enzyme. Glycogen synthase has multiple sites per subunit that can be phosphorylated in vitro. As many as 7 sites are phosphorylated in vivo, and the sequences of amino acids surrounding these sites have been identified (7). In addition to cAMP-dependent protein kinase, at least four other protein kinases have now been shown to phosphorylate glycogen synthase in vitro (8–10). These kinases exhibit different specificities for the different sites on glycogen synthase and their actions may generate a wide variety of phosphorylated forms of the enzyme (8). Furthermore, there is no guarantee that all of the protein kinases or phosphorylation sites important in the regulation of glycogen synthase have been discovered. Therefore, it is not possible to deduce the actual phosphorylation state of the enzyme from measurements of enzymatic activities.

It is important to determine which sites on glycogen synthase are phosphorylated or dephosphorylated as a result of hormone action. Together with the information that exists on the site specificities of the different protein kinases and phosphatases, it might be possible to identify which enzymes mediate the actions of insulin and epinephrine. Several approaches could be taken to identify changes in the phosphorylation states of individual sites on glycogen synthase promoted by hormones. We have used immunological procedures for the rapid purification of $[^{32}P]$glycogen synthase from hormonally treated rat hemidiaphragms which had been incubated with $[^{32}P]$phosphate. After cleavage with CNBr two
**Hormonal Control of Site-specific Phosphorylation**

**1P-labeled fragments were obtained as have been described in studies of purified rabbit skeletal muscle glycogen synthase (7, 10-12). More [1P]phosphate was associated with both fragments after treatment of muscles with epinephrine, although the hormone caused a greater percentage increase in the radioactivity of the smaller fragment. Insulin caused a 40 to 50% decrease in the content of [1P]phosphate in both fragments. The implications of multisite control of glycogen synthase by insulin and epinephrine are discussed in relation to proposed mechanisms of action of the hormones.**

**EXPERIMENTAL PROCEDURES**

**Incubation of Hemidiaphragms—Hemidiaphragms from 80- to 100-g male rats (Sprague-Dawley) were trimmed of extraneous tissue leaving a thin border of ribs as described by Goldberg et al. (13). Muscles (typically from 6 animals) were incubated at 23°C in 250 ml of medium A which was composed of 0.1 mM sodium phosphate and essentially the same concentrations of amino acids, vitamins, and other components as Dulbecco's modified Eagle's medium (14). All incubation media were gassed with a mixture of 95% O2/5% CO2 and were supplemented with penicillin G (20 µg/ml) and streptomycin (10 µg/ml). After 1 h hemidiaphragms were transferred to a single vessel containing homogenization buffer (A) (20 mg/ml of hemidiaphragm) and neutralized potassium perchlorate (0.2-0.4 M) (15). The tissue was incubated at 36°C for 5 h and then transferred to multiple incubation vessels (3 hemidiaphragms each) containing 6 ml of medium B. This medium was composed of 119 mM NaCl, 4.6 mM KCl, 2.4 mM CaCl2, 1.2 mM MgSO4, 48.5 mM NaHCO3, 2.0 mM potassium phosphate, 0.1 mM potassium phosphate, and 1.0 mM sodium phosphate and 1.0 mM potassium phosphate and 1.0 mM sodium phosphate (same specific activity as medium A). Tissue was incubated for 1 h in medium B before being frozen in liquid nitrogen. Unless otherwise specified, insulin (25 µU/ml) was added for the last 30 min and epinephrine (10 µM) for the last 10 min before the incubation was terminated.**

**Preparation of Extracts—Frozen muscles were manually ground using a porcelain mortar and pestle chilled with liquid nitrogen. Typically, powdered tissue from 3 hemidiaphragms was homogenized in homogenization buffer (1 ml) composed of 100 mM KF, 10 mM EDTA, 2 mM EGTA, 10 mM potassium phosphate, 100 mM benzamidine, 0.1% Triton X 100, and 50 mM Tris/HCl (pH 7.0 at 0°C). After centrifugation of homogenates at 20,000 g for 30 min, the supernatants were removed for assays of enzymatic activities and immunoprecipitations of [1P]labeled glycogen synthase.**

**Measurement of Glycogen Synthase and Phosphorylase Activities—**

Samples of the extracts were diluted 5-fold with homogenization buffer. Glycogen synthase activities were measured essentially as described by Thomas et al. (15) and phosphorylase activities as described by Gilboe et al. (16). For glycogen synthase activity, diluted samples (30 µl) were added to 60-µl aliquots of reaction mixtures composed of 20 mM EDTA, 25 mM KF, 10 mg/ml of rabbit liver glycogen, 100 mM Tris/HCl (pH 7.8 at 30°C) plus or minus 15 µM glucose-6-P. Phosphorylase reaction mixtures were composed of 200 mM KF, 10 mg/ml of glycogen, and 100 mM glucose-1-P (pH 6.1 at 30°C) plus or minus 3 mM AMP. Reactions were run at 30°C for 30 min. The amount of 14C-glucose ([U-1C]glucose (600 µCi/mmol) or [U-1C]glucose-1-P (16/2 µCi/mmol) participated in the incorporation of 14C into glycogen.**

**Measurement of Concentrations of AMP and Specific Activities of**

*[^1] The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; SDS, sodium dodecyl sulfate; CNBr, cyanogen bromide; Mapp, apparent molecular weight determined by polyacrylamide gel electrophoresis in the presence of SDS (see Ref. 12); anti-GS Ab, purified IgG fraction from guinea pigs immunized with glycogen synthase.
Insulin and epinephrine. Interestingly, slightly lower concentrations of antibodies were effective in removing glycogen synthase from the muscle extracts (Fig. 1B) than were needed to remove the purified enzymes from solution (Fig. 1A). At present we do not have a definitive explanation for this observation. In other experiments we have studied the binding of purified $^{32}$P-labeled glycogen synthase by determining the radioactivity retained by the bacteria following several washes to remove unbound enzyme. All of the $^{32}$P-labeled enzyme could be bound by the antibody and the binding could be inhibited by unlabeled glycogen synthase. Taken together, these results indicate that the heteroclonal antibodies obtained by immunizing guinea pigs with unphosphorylated glycogen synthase recognize both phosphorylated and nonphosphorylated enzyme.

Immuno precipitation of $^{32}$P/Gly cogen Synthase from Rat Hemidiaphragms—Extracts (300 $\mu l$) of hemidiaphragms incubated with $^{32}$P phosphate were incubated at 23 $^\circ$C with 150 $\mu g$ of either anti-GS antibodies or control IgG. As shown in Fig. 1B, the concentration of 0.5 mg/ml of anti-GS antibody is sufficient to bind 80 to 90% of the enzyme present. Although desirable, it was not possible to use a large excess of antibodies because the increase in nonspecific binding observed with increasing concentrations of immunoglobulins led to high levels of $^{32}$P-labeled contaminants in the immunoprecipitate. After 20 min the samples were washed three times in ice and each was added to a solution containing 0.1 mM ATP, 6 mM MgCl$_2$, 25% glycerol, 50 mM Tris/HCl (pH 7.5), and 1 $\mu$g/ml of C subunit of cAMP-dependent protein kinase. The phosphorylation reaction was terminated after 30 min by adding KF and EDTA to final concentrations of 100 and 10 mM, respectively. Under these conditions, glycogen synthase was phosphorylated to a level of 1.4 phosphates/subunit as determined from $^{32}$P incorporation in a sample incubated under identical conditions except in the presence of [y-$^{32}$P]ATP (1600 cpm/pmol). Another sample of enzyme was incubated as described above except in the absence of ATP and C subunit and is designated nonphosphorylated glycogen synthase. Increasing concentrations of control IgG (C, V) or anti-glycogen synthase antibodies (0, V) were incubated at 23 $^\circ$C in 30 $\mu$l of homogenization buffer containing 25% glycerol and 0.4 $\mu$g of either phosphorylated glycogen synthase (V, V) or nonphosphorylated enzyme (0, O). After 20 min 100 $\mu$l of a 40% suspension of inactivated bacteria were added to each sample and incubated at 0 $^\circ$C. The samples were centrifuged after 30 min to pellet the bacteria, and glycogen synthase activities (in the presence of 10 mM glucose-6-P) were measured in the supernatant. These activities and those observed in supernatants of samples incubated without immunoglobulins were used to estimate the percentage of glycogen synthase activity removed by each antibody concentration. B, hemidiaphragms were incubated in medium A (without $^{32}$P) and then transferred to medium B and incubated with 0 or with 260 milliunits/ml of insulin (A, D) or 10 $\mu$g epinephrine (O, D) before extracts were prepared as described under "Experimental Procedures." The activity ratios and total activities of glycogen synthase in the different extracts were as follows: control, 0.12, 149 milliunits/ml; epinephrine, 0.06, 153 milliunits/ml; and insulin, 0.26, 155 milliunits/ml. One unit of enzyme activity is that amount catalyzing the utilization of 1 umol of substrate in 1 min at 30 $^\circ$C. Samples (30 $\mu$l) of extract were added to solutions (20 $\mu$l) of increasing concentrations of control IgG (C, D) or anti-glycogen synthase antibodies (0, A, B) and incubated at 23 $^\circ$C for 20 min. Inactivated bacteria (100 $\mu$l of a 20% suspension) were added to each and incubated at 0 $^\circ$C. After 30 min the samples were centrifuged and glycogen synthase activities (in the presence of 10 mM glucose-6-P) were measured in the supernatants. The results presented represent the percentages of glycogen synthase activities removed by the antibodies.

An estimation of the amount of glycogen synthase activity immunoprecipitated under the standard conditions can be obtained from the results presented in Table 1. In these experiments extracts of control hemidiaphragms and muscles incubated with insulin and epinephrine were incubated with 0.5 mg/ml of control IgG or anti-GS antibodies before the bacterial suspension was added. After incubation and centrifugation to pellet the bacteria, glycogen synthase activity was measured in the supernatant. Essentially the same percentage of activity was removed from all three extracts by the antibodies. Furthermore, the activity ratios in the supernatant were inactivation by limiting the bacteria used to the smallest amount sufficient to bind all of the IgG present. Different preparations of S. aureus vary significantly with respect to binding capacity for IgG. The amount of the particular lot (Pansorbin, Lot 130155) used in the standard procedure was not sufficient to bind all of the control pig antibodies. This is the reason that the amounts of activity removed in the presence of the antibodies in the experiments presented in Fig. 1B are less than those removed at equivalent concentrations of antibody in the experiment shown in Fig. 1B, where excess S. aureus was used.

### Table 1

<table>
<thead>
<tr>
<th>IgG ($\mu$g/ml)</th>
<th>Activity Removal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>80</td>
</tr>
<tr>
<td>50</td>
<td>90</td>
</tr>
</tbody>
</table>

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1 Non specific contamination by $^{32}$P labeled proteins is proportional to the amount of bacteria used. We attempted to reduce this contamination by limiting the bacteria used to the smallest amount sufficient to bind all of the IgG present. Different preparations of S. aureus vary significantly with respect to binding capacity for IgG. The amount of the particular lot (Pansorbin, Lot 130155) used in the standard procedure was not sufficient to bind all of the control pig antibodies. This is the reason that the amounts of activity removed in the presence of the antibodies in the experiments presented in Fig. 1B are less than those removed at equivalent concentrations of antibody in the experiment shown in Fig. 1B, where excess S. aureus was used.
TABLE I

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<table>
<thead>
<tr>
<th>Fraction</th>
<th>Hormonal treatment</th>
<th>Control IgG</th>
<th>Anti-GS Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activity ratio</td>
<td>Total activity</td>
<td>Activity ratio</td>
</tr>
<tr>
<td></td>
<td>milliunits/ml</td>
<td></td>
<td>milliunits/ml</td>
</tr>
<tr>
<td>Supernatant</td>
<td>Control</td>
<td>0.27 ± 0.09</td>
<td>246 ± 17</td>
</tr>
<tr>
<td></td>
<td>Epinephrine, 10 μM</td>
<td>0.19 ± 0.07</td>
<td>183 ± 2</td>
</tr>
<tr>
<td></td>
<td>Insulin, 25 milliunits/ml</td>
<td>0.40 ± 0.09</td>
<td>196 ± 12</td>
</tr>
<tr>
<td>Pellet</td>
<td>Control</td>
<td>0</td>
<td>0.27 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>Epinephrine, 10 μM</td>
<td>0</td>
<td>0.14 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>Insulin, 25 milliunits/ml</td>
<td>0</td>
<td>0.44 ± 0.08</td>
</tr>
</tbody>
</table>

TABLE II

Effects of insulin and epinephrine on glycogen synthase

<table>
<thead>
<tr>
<th>Glycogen synthase</th>
<th>32P-labeled immunoprecipitate</th>
<th>Fragment II</th>
<th>Fragment I</th>
<th>Intact</th>
<th>Fragment II/ Fragment I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity ratio</td>
<td>Total activity</td>
<td>percentage of control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.24 ± 0.02</td>
<td>0.027 ± 0.002</td>
<td>100</td>
<td>100</td>
<td>6.9 ± 0.7</td>
</tr>
<tr>
<td>Epinephrine, 10 μM</td>
<td>0.16 ± 0.02</td>
<td>0.028 ± 0.002</td>
<td>150 ± 15</td>
<td>247 ± 11</td>
<td>3.6 ± 0.2</td>
</tr>
<tr>
<td>Insulin, 25 milliunits/ml</td>
<td>0.35 ± 0.01</td>
<td>0.025 ± 0.004</td>
<td>54 ± 4</td>
<td>61 ± 10</td>
<td>6.4 ± 1.0</td>
</tr>
<tr>
<td>Insulin, 25 milliunits/ml plus epinephrine, 10 μM</td>
<td>0.22 ± 0.03</td>
<td>0.025 ± 0.004</td>
<td>148 ± 11</td>
<td>198 ± 39</td>
<td>5.1 ± 0.5</td>
</tr>
</tbody>
</table>

unchanged from those in the whole extract. Interestingly, a readily detectable amount of glycogen synthase activity was observed in the washed bacterial pellets from samples incubated with anti-glycogen synthase antibodies. A constant percentage of activity was recovered in the different pellets and, again, the activity ratios were the same as in the whole extracts. These results provide additional evidence that the antibodies were not selectively removing specific forms of enzymes from solution and also indicate that the conditions of immunoprecipitation were sufficient to prevent posthomogenizational changes in the phosphorylation state of the enzyme.

Cyanogen Bromide Cleavages and Electrophoretic Resolution of 32P-labeled Proteins—To elute the 32P-labeled glycogen synthase from the antibody-protein A complexes, bacterial pellets were suspended in 70% formic acid (200 μl) and incubated at 23 °C for 15 min. After centrifugation at 12,000 × g for 5 min, the supernatants were collected and the pellets extracted once more with formic acid. Supernatants from the two extractions were pooled. Each sample of formic acid extract was divided into two equal volumes. To one was added 3-6 mg of CNBr. Both samples were sealed and incubated for 18 h at 25 °C. The samples were lyophilized and then reconstituted in 0.2 ml of H2O and evaporated to dryness in a centrifugal Bio-Dryer (VirTis). To each tube was added 30 μl of electrophoresis sample buffer composed of 10% glycerol, 120 mM β-mercaptoethanol, 0.5% SDS, and 12.5 mM Tris/PO4 (pH 6.8). After thorough mixing, the samples were incubated at 100 °C for 1 min and then allowed to cool to room temperature. The original extracts contained similar but unequal amounts of glycogen synthase. To correct for these differences sample buffer was added in appropriate volumes to normalize samples to equal amounts of total glycogen synthase activity which is presumably proportional to the amount of enzyme present. Since hormones did not change total synthase activity (Table II), the same results would be expected if samples had been corrected using measurements of extract protein. Polyacrylamide gel electrophoresis was performed using a modification of the procedures of Laemmli (23). Samples (20 μl) were added to slab gels (1.5 mm thick) formed with a linear gradient of acrylamide from 5 to 20% in the resolving gel. Molecular weights were estimated from the mobilities of the following standards: β-galactosidase (130,000), phosphorylase b (94,000), rabbit skeletal muscle glycogen synthase (85,000), bovine serum albumin (68,000), pyruvate kinase (57,000), glutamate dehydrogenase (55,000), ovalbumin (45,000), aldolase (40,000), lactate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), soybean trypsin inhibitor (21,000), β-lactoglobulin (18,400), myoglobin (17,200), and hemoglobin (15,500). For references on autoradiograms, purified rabbit skeletal muscle glycogen synthase and CNBr fragments that had been phosphorylated with [γ-32P]ATP and catalytic subunit of cAMP-dependent protein kinase were included. After electrophoresis was complete, gels were placed in a solution of Coomassie blue to stain protein (24) or were placed in 2 liters of 1% glycerol and 10% acetic acid for 90 min and then dried onto filter paper (Whatman 3MM).

Autoradiography and Analysis of Autoradiograms—Dried gels were placed in x-ray cassettes containing films (Kodak XAR-5) which were exposed at −85 °C for different periods of time depending on the amount of radioactivity present. After developing, the autoradiograms were cut into strips corresponding to individual lanes on the gel. Differences in optical density of strips from samples that had been incubated with immune and nonimmune IgG were determined at 540...
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Results presented were derived from these weights which are directly proportional to the areas beneath the respective peaks.

Other Materials—[U-14C]Glucose-1-P and [U-14C]glucose were obtained from ICN Radioisotope Division. UDP-[U-14C]glucose was prepared essentially as described by Tan (25). [γ-32P]ATP was from Amersham Corp. and [32P]orthophosphate was from New England Nuclear. Cyanogen bromide was obtained from Pierce Chemical Co. Purified catalytic subunit of cAMP-dependent protein kinase from beef heart was kindly supplied by Dr. David Braun (Bruteen University). Proteins used as molecular weight standards and other enzymes were obtained from Boehringer Mannheim. Highly purified insulin was a gift from Lilly. Glucose-1-P, UDP-glucose, rabbit liver glycogen, ATP, benzamidine, and other chemicals were from Sigma.

Electrophoretic Mobility of 32P-labeled Glycogen Synthase from Rat Hemidiaphragms—Antibodies against highly purified rabbit skeletal muscle glycogen synthase were used to immunoprecipitate glycogen synthase from extracts of rat hemidiaphragms that had been incubated with [32P]phosphate. Samples of the immunoprecipitate were incubated in 70% formic acid in the absence and presence of cyanogen bromide before being subjected to electrophoresis in the presence of SDS. The profile of a scan of the optical density of an autoradiogram corresponding to a sample of uncleaved immunoprecipitate is shown in Fig. 2A (panel i). Nearly all of the radioactivity was present in a single band, attesting to the specificity of the antibodies. The immunoprecipitate had slightly lower mobility than purified rabbit skeletal muscle glycogen synthase labeled with [32P]phosphate by incubation with [γ-32P]ATP and the catalytic subunit of cAMP-dependent protein kinase. Likewise treatment of the immunoprecipitate with CNBr resulted in the formation of two fragments of lower mobility than those obtained with the [32P]-labeled rabbit muscle enzyme (Fig. 2A, panel ii). The peak of lower mobility (Fragment II) of the immunoprecipitate is distinctly broader than that corresponding to the large fragment (CB-2) of the rabbit enzyme. As will be discussed later, multiple species of slightly different mobilities comprise Peak II. The mobilities of the immunoprecipitate and CNBr fragments are compared with those of other proteins of known molecular weights in Fig. 2B. The intact immunoprecipitate has an apparent molecular weight of 90,000; the two CNBr fragments have apparent molecular weights of 27,900 and 15,500.

Incubation of Hemidiaphragms with [32P]Phosphate—Since prepared. The 32P-labeled glycogen synthase was immunoprecipitated and treated with CNBr before electrophoresis as described under “Experimental Procedures.” A, profiles of scans of optical density from autoradiograms of uncleaved samples (i) or samples which were treated with CNBr (ii). The dotted lines were obtained using purified rabbit skeletal muscle glycogen synthase that had been phosphorylated with [γ-32P]ATP and C subunit of cAMP-dependent protein kinase. B, estimation of the molecular weights of the immunoprecipitated glycogen synthase and cyanogen bromide fragments. The standard curve was constructed using proteins of known molecular weights (●, see under “Experimental Procedures” for a description of standards). Rabbit skeletal muscle glycogen synthase (●) and its cyanogen bromide fragments II (□, 23,000) and I (▲, 13,500) have lower mobilities than the immunoprecipitated enzyme (V, 90,000) and its CNBr fragments II (□, 29,000) and I (▲, 15,500) from rat diaphragms (the numbers in parentheses denote apparent molecular weights). The molecular weight of purified rabbit skeletal muscle glycogen synthase I has been taken to be 85,000 Da (20).

![Graph of Electrophoretic Mobility of 32P-labeled Glycogen Synthase from Rat Hemidiaphragms](http://example.com/graph.png)
Hormonal Control of Site-specific Phosphorylation

Uptake and incorporation of $^{32}$P-phosphate into muscle phosphorylases occur slowly, relatively long incubations were required to allow incorporation of sufficient radioactivity into glycogen synthase. Under appropriate conditions, preparations of rat diaphragm may remain viable for several days in organ culture (26). In the experiments presented in Fig. 3A, rat hemidiaphragms were incubated in 250 ml of medium A (minus $^{32}$P) for 1 h. The muscles were then placed (0 h in the figures) in an incubation vessel containing medium A plus $^{32}$P-phosphate (2 ml/hemidiaphragms, 0.2 mCi/ml) and incubated for increasing periods of time before being transferred to tubes containing medium B plus $^{32}$P-phosphate (0.2 mCi/ml). Immediately after transfer, medium A (2 ml/hemidiaphragm) was withdrawn to maintain a constant tissue/medium ratio. After 1 h in medium B the tissue was frozen in liquid nitrogen; the times indicated represent total time of exposure to $^{32}$P-phosphate. Frozen tissue was powdered and processed to allow measurement of (A) glycogen synthase and phosphorylase activities, (B) concentrations of ATP and the specific activities of $\gamma$-$^{32}$PATP, and (C) relative amounts of $^{32}$P incorporated into glycogen synthase and phosphorylase fragments of the enzyme. The values presented in C represent the weights of peaks from photocopies of gel scans analyzed by the procedures described under “Experimental Procedures.” All results presented are the average values from two separate experiments.

**Hormonal Effects on the $^{32}$P Content of Glycogen Synthase in Hemidiaphragms Incubated with $^{32}$P-Phosphate**—The effects of insulin and epinephrine on the activity ratios of glycogen synthase were determined in hemidiaphragms incubated with $^{32}$P-phosphate for 6 h (Table II). Incubation with insulin for the last 30 min increased the ratio from 0.24 to 0.38, and epinephrine decreased the ratio to 0.16. The activity ratio in extracts from hemidiaphragms incubated with insulin plus epinephrine was quite similar to control. The results presented in Fig. 4 are consistent with the hypothesis that epinephrine increases phosphorylation of glycogen synthase and that insulin decreases the phosphate content of the enzyme.
zyme. In this representative experiment, $^{32}$P-labeled glycogen synthase was immunoprecipitated from extracts of hormonally treated tissues before being dissolved and subjected to electrophoresis in SDS. Autoradiograms were prepared and the tracks corresponding to control samples and samples from different hormonal treatments were scanned for optical density at 540 nm. Incubation of hemidiaphragms with insulin resulted in a decrease in the size of the peak of $^{32}$P-labeled glycogen synthase (Fig. 4). The size of the peak from samples of epinephrine-treated tissue was larger than the control peak, and the peak from samples of tissue incubated with insulin plus epinephrine was between control and epinephrine in size. These results were expressed as a percentage of the size of the control peak and averaged with results from three other experiments (Table II). These values indicate that epinephrine increased the $^{32}$P content of glycogen synthase by 50%, whereas insulin action resulted in a 44% decrease in the amount of $[^{32}$P]phosphate bound to the enzyme. Under the conditions used in this experiment, we have observed no effect of insulin or epinephrine on the specific activity of $[^{32}$P]ATP.$^2$

The effects of increasing concentrations of insulin are shown in Fig. 5. The decreases in $^{32}$P content of the enzyme correlated well with the increases in activity ratio. Concentrations of insulin as low as 25 milliunits/ml were effective. The effect of the hormone (25 milliunits/ml) was maximal after 15 min and could be observed after as little as 5 min of incubation (Fig. 6). Thus the dephosphorylation of glycogen synthase produced by insulin was rapid and occurred at physiological concentrations of the hormone.

To determine if insulin and epinephrine preferentially affected the amount of $[^{32}$P]phosphate present in sites found in different CNBr fragments of the enzyme, optical density scans were made of autoradiograms from gels of samples that had been cleaved with CNBr (Fig. 7). Incubation of the muscles with insulin resulted in a decrease in the total amount of radioactivity present in the fragments. In insulin samples, three peaks were most clearly resolved in the area corresponding to Fragment II in Fig. 1. Examination of the other scans reveals a consistent shoulder close to the position of the phosphorylated CB-2 of rabbit muscle glycogen synthase, and clearly more than one band may be seen in the pictures of the autoradiograms below the scans. For quantitation the different electrophoretic species have been considered collectively.

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**Fig. 5. Dephosphorylation of glycogen synthase by increasing concentrations of insulin.** Hemidiaphragms were incubated with $[^{32}$P]phosphate as described under "Experimental Procedures" before increasing concentrations of insulin were added. After 30 min the incubations were terminated and extracts were prepared. Glycogen synthase activities were measured and the $^{32}$P-labeled enzymes were immunoprecipitated from samples of the extracts. After electrophoresis, autoradiograms of the gels were scanned and changes in the amounts of radioactivity due to the different concentrations of insulin were determined. The results presented are the mean values ± S.E. from 5 experiments and represent the percentage changes in activity ratio (O) or $[^{32}$P]contents (Δ) of glycogen synthase produced by insulin. mU, milliunits.

**Fig. 6. Effect of increasing times of incubation on the dephosphorylation of glycogen synthase by insulin.** Hemidiaphragms were incubated in vitro in the presence of $[^{32}$P]phosphate for a total of 6 h. Insulin (25 milliunits/ml) was added at the appropriate time prior to termination of the incubation to give the period of exposure indicated. The results are expressed as described in the legend to Fig. 5 and represent the mean values ± S.E. from 3 experiments.

**Fig. 7. Electrophoretic analysis of the cyanogen bromide fragments of $^{32}$P-labeled glycogen synthase following incubation of skeletal muscle with insulin and epinephrine.** Samples of the immunoprecipitates used in the experiment shown in Fig. 4 were cleaved with cyanogen bromide before being subjected to electrophoresis. Shown below each optical density scan are reproductions of the autoradiograms from the gels of the CNBr cleavage products. The arrows show the location of the two CNBr fragments of glycogen synthase phosphorylated with the catalytic subunit of cAMP-dependent protein kinase.
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as Fragment II (Table II). Insulin decreased the \[^{32}P\]phosphate in Fragment I by 39%. The hormone also promoted a 46% reduction in the radioactivity associated with Fragment II. In samples from control tissue, the ratio of radioactivity present in Fragment II to that in Fragment I was about 7:1. Insulin did not significantly affect this ratio, indicating that the hormone did not differentially affect the phosphate content of the two fragments. Epinephrine increased the amount of radioactivity present in both fragments; however, it had a greater effect on the radioactivity present in Fragment I which resulted in a 50% decrease in the ratio of the \[^{32}P\] content of Fragment II to that of Fragment I. When epinephrine was added to tissue first incubated with insulin, the amount of radioactivity was increased in both Fragments I and II. In these experiments insulin had little, if any, effect on opposing epinephrine action or either the phosphorylation or the activity ratio of glycogen synthase.

DISCUSSION

The subunit of rabbit skeletal muscle glycogen synthase is subject to phosphorylation in vitro at a minimum of seven different sites through the action of at least five distinct protein kinases (for example, see Refs. (7–12). All seven sites characterized in vitro are contained in only two CNBr fragments of the enzyme (9). One site (site 2) is located in an NH\(_2\)-terminal fragment (CB-1) and the other six sites (sites 1a, 1b, 3a, 3b, 3c, 5b) in a COOH-terminal fragment (CB-2). It is known that the mobility of CB-2 on analysis by polyacrylamide gel electrophoresis in the presence of SDS is very sensitive to its phosphorylation pattern, and depending on the particular site occupancy, may display significant electrophoretic heterogeneity (7, 12).

The activity of glycogen synthase is a complex function of the degree and site distribution of its covalent phosphorylation. Though it is almost certain that hormones which cause stable changes in glycogen synthase activity do so by modifying its phosphorylation state, the site specificity of such hormonal control has yet to be fully defined. The present report describes experiments designed to study directly hormonal control of phosphorylation of glycogen synthase in rat diaphragm.

Our experimental approach was to incubate rat hemidiaphragms with \[^{32}P\]phosphate to allow labeling of glycogen synthase prior to treatment with insulin and/or epinephrine. \[^{32}P\]-labeled glycogen synthase was then rapidly purified by the use of specific antibodies. Most other studies of rat or rabbit skeletal muscle glycogen synthase phosphorylation in intact tissue have utilized conventional purification of the enzyme (28–35); one report of rat heart glycogen synthase does describe the effective use of \[^{32}P\]-labeling and immunoprecipitation although no effect of epinephrine on the \[^{32}P\] content of the enzyme was detected (36). Our method has several advantages when compared to conventional purification of glycogen synthase from whole animals. Treatment of isolated tissue avoids some of the complex interactions which are possible when injecting hormones into whole animals and which may obscure interpretation of the results. Rapid immunopurification is likely to be more reproducible, and certainly simpler, than large scale enzyme preparation, and some information can be obtained on the rate of phosphate turnover in the enzyme. Since the enzyme is labeled with \[^{32}P\]phosphate, detection of phosphorylation does not depend on prior chemical knowledge of the site so that novel phosphorylation sites could in principle be detected. Nonetheless, maximum interpretation of the data requires reference to what is known of the in vitro phosphorylation of the enzyme. The main disadvantage of our approach was that a steady state labeling of glycogen synthase with \[^{32}P\]phosphate was not achieved, even though the specific activity of the terminal phosphate of ATP had reached a plateau level. Thus, the absolute amount of phosphate per enzyme subunit cannot be determined. However, as discussed later, the observed changes in \[^{32}P\]phosphate associated with glycogen synthase were consistent with chemical measurements of the phosphate content of purified enzymes made in other studies.

Although rat skeletal muscle glycogen synthase has been less well characterized than the rabbit enzyme, the two enzymes are similar to several respects. Identical values of 85,000 Da have been reported for the molecular weights of both enzymes (20, 28). We have used conventional techniques to obtain a purified preparation of rat skeletal muscle glycogen synthase. This enzyme has the same mobility as the rabbit enzyme when subjected to electrophoresis on acrylamide gels in the presence of SDS. Phosphorylation of the rat enzyme with [\(^y\)^-\[^{32}P\]]ATP and the catalytic subunit of cAMP-dependent protein kinase, followed by treatment with CNBr, resulted in formation of two \[^{32}P\]-labeled fragments of the same electrophoretic mobility as those from the rabbit enzyme analogously phosphorylated. Furthermore, as demonstrated in this report, antibodies against rabbit skeletal muscle glycogen synthase cross-react with the rat enzyme. Although the assumption of total chemical identity between the two enzymes may be unwarranted, the demonstrated structural homologies justify the working hypothesis that phosphorylation of the rat enzyme closely resembles that of rabbit muscle glycogen synthase.

The \[^{32}P\]-labeled glycogen synthase immunoprecipitated from rat hemidiaphragms had \(M_{app} = 90,000\) (Fig. 2). Similar values have been obtained with mouse (37) and rabbit diaphragms. An initial concern was that limited proteolysis of a larger protein had generated the enzyme of \(M_{app} = 85,000\) during purification from either rabbit (20) or rat muscle. However, it is now known that the lower mobility form (\(M_{app} = 90,000\)) can be produced from the higher mobility form (\(M_{app} = 85,000\)) by phosphorylation of certain sites. Based on mobility differences of different phosphorylated forms of glycogen synthase, one might speculate that there was a relatively high degree of phosphorylation in sites 3 and possibly 5 in the immunoprecipitated rat enzyme (12). Similar considerations can explain the observation that the \[^{32}P\] labeled enzyme isolated from rat diaphragms gave rise to multiple phosphorylated species in the mobility range of CB-2 from purified rabbit enzyme since phosphorylation also critically affects the mobility of this fragment (7, 12). The resolution of three phosphorylated species of CB-2 (Fig. 7) indicates that the glycogen synthase subunit exists in muscle at a minimum of three distinct phosphorylation states, even after hormonal treatment.

A question remains concerning Fragment I of the rat diaphragm enzyme since it has a slightly lower electrophoretic mobility (\(M_{app} = 15,500\)) compared with CB-1 from purified rabbit glycogen synthase (\(M_{app} = 13,100\)) (Fig. 2B). One hypothesis is that these two small fragments are in no way...
analogous. If this was correct, then site 2 was neither labeled with \(^{32}P\)phosphate nor responded to epinephrine in the incubated diaphragm, and a hitherto unknown site(s) exists that was phosphorylated in response to epinephrine. This seems improbable, particularly since Parker et al. (31) provided compelling evidence for stimulation of site 2 phosphorylation by epinephrine in vivo. We, therefore, propose that the smaller rat diaphragm CNBr fragment is identifiable with CB-1, although the reason for the slight mobility difference is unknown. Some explanations could be the existence of a novel phosphorylation site in this region, slight NH$_2$-terminal proteolysis, or other unrecognized chemical modifications. Therefore, an important conclusion from this study is that, following incubation of rat hemidiaphragms, \(^{32}P\)phosphate was confined to the regions of the glycogen synthase molecule corresponding to CB-1 and CB-2, consistent with phosphorylation sites thus far identified in vitro.

In our experiments, epinephrine caused a 50% increase in the amount of \(^{32}P\)phosphate present in glycogen synthase (Table II). Epinephrine has been reported to increase chemically determined phosphate in the enzyme by 58% in perfused rat hindlimbs (28), and 41% (29), 64% (30), and 77% (31) in whole rabbits. Insulin caused, in the present experiments, about a 50% decrease in \(^{32}P\) content of glycogen synthase. Previous values, again for chemically determined phosphate, were decreases of 35% (32) and 15% (35) in whole rabbits and 21% in perfused rat hindlimbs (33). The similarity of our values to published results argues strongly that changes in the \(^{32}P\) content of glycogen synthase, as determined in the present study, provide a reliable index of changes in phosphorylation state.

The results presented in the present report provide direct information on the acute effects of insulin on the distribution of phosphate in glycogen synthase. An important conclusion is that insulin decreased the content of phosphate in both CB-1 (containing site 2) and CB-2 (containing sites 1, 3a, 3b, 3c, and 5). After insulin treatment, the electrophoretic heterogeneity of CB-2 became more clearly visible (Fig. 7) as though the relative proportions of different forms had been changed. These results are suggestive of dephosphorylation in sites 3 since the mobility of this fragment is most affected by changes in the phosphate content of these sites (7, 12). This interpretation is consistent with the recent finding that injection of 50% rabbits with insulin decreased phosphate in sites 3 (35). Indeed, Parker et al. (36) concluded that insulin treatment on glycogen synthase was confined to these sites. However, we observed an effect of insulin on decreasing \(^{32}P\)phosphate in CB-1 (Table II) indicating that insulin action in rat diaphragm involves dephosphorylation of at least one other site (presumably site 2).

In earlier work, Sheorain et al. (34) had shown that alloxan-induced diabetes in rabbits resulted in over a 2-fold increase in the phosphate content of what they defined as the "trypsin-insensitive domain" of the enzyme. Treatment of the diabetic animals for 4 days with insulin reversed the effects of diabetes, and administration of insulin to control animals for 4 days decreased the trypsin-insensitive phosphate by about 40%. However, these treatments with insulin must be considered long term and the effects observed may bear little relationship to the acute actions of the hormone. Indeed, it was reported that short term administration of insulin did not affect the phosphate content of glycogen synthase even though the hormone increased the activity ratio (34). This finding is clearly in conflict with previous results (32, 33, 35) and with those of the present report.

Treatment of rat hemidiaphragms with epinephrine led to an increase in the \(^{32}P\) contents of both CB-1 and CB-2, although the greatest increase was CB-1. In recent studies, Parker et al. (31) chemically analyzed the phosphate content of sites on glycogen synthase after rabbits were injected with epinephrine. These investigators concluded that phosphate was introduced into all sites examined, and the greatest increase was in site 2 (2.6-fold), a value very similar to that obtained in our study (2.5-fold) for the increase in the \(^{32}P\) content of CB-1, which contains site 2. From the results of Parker et al. (31), the collective increase in phosphate in sites 3, 5, and 1 was 64%, again similar to our value of 50%. In the work of Sheorain et al. (34), in which trypsin sensitivity alone was employed to dissect phosphorylation sites, a 2-fold increase in the trypsin-sensitive phosphate was observed following epinephrine treatment but little effect was seen on trypsin-insensitive phosphate. A question may be raised, however, as to whether sites 3 would register quantitatively in the trypsin-sensitive or trypsin-insensitive regions (35), so that direct comparison of our results with those of Sheorain et al. (34) is difficult.

An important generalization that can be made from the present study is that both insulin and epinephrine control the phosphorylation of multiple (at least two) sites on the enzyme. For epinephrine action, the same conclusion was drawn from studies of mouse diaphragms (37) and rabbit muscle (31). Phosphorylation in both CB-1 and CB-2 is consistent with the specificity of cyclic AMP-dependent protein kinase (9-11), which is believed to be involved in mediating epinephrine action. However, Parker et al. (31) also demonstrated a significant increase in the phosphorylation of sites 3, which cannot be phosphorylated by cyclic AMP-dependent protein kinase in vitro. Because we did not resolve sites 3 from sites 1 and 5, our data do not yet address this point and we cannot say whether factors besides cyclic AMP-dependent protein kinase must be invoked to explain epinephrine-mediated phosphorylation of rat diaphragm glycogen synthase.

The observed multisite control of glycogen synthase phosphorylation by insulin has several interesting implications that must be accounted for in any proposed mechanism by insulin action. Should insulin action be mediated directly by a single protein kinase, then the kinase would have to act on phosphorylation sites in both CB-1 and CB-2. Evidence has been presented that insulin may increase glycogen synthase phosphorylation activity in various tissues and several potential mechanisms have been discussed (38-41). Our results do not rule out an involvement of protein phosphatases in the hormonal regulation of glycogen synthase phosphorylation. In fact, the rapidity of the insulin effect (Fig. 6) relative to the incorporation of \(^{32}P\)phosphate into glycogen synthase (Fig. 3C) strongly suggests that the hormone increased phosphatase activity. The present results do dictate that, if phosphatase action alone is to explain insulin-mediated control of glycogen synthase phosphorylation, then the phosphatase(s) must be capable of dephosphorylating sites in both CB-1 and CB-2. It is, of course, quite conceivable that insulin action could involve control of both phosphatases and protein kinases. Continued work on the phosphorylation of glycogen synthase in intact muscle, with improved resolution of the phosphorylation sites, should provide more and more restrictions of the potential mechanisms of control by both insulin and epinephrine.

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J C Lawrence, Jr, J F Hiken, A A DePaoli-Roach and P J Roach


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