We have investigated the effects of insulin on the phosphorylation of glycogen phosphorylase in skeletal muscle. Rat epitrochlearis muscles were incubated in vitro with $^{32}$P, to label cellular phosphoproteins, before being treated with hormones. Phosphorylase, phosphorylase kinase, and glycogen synthase were immunoprecipitated under conditions that prevented changes in their phosphorylation states. Based on measurements of the activity ratio ($-\text{AMP}+/\text{AMP}$) and the $^{32}$P content of phosphorylase, 4–8% of the phosphorylase in untreated muscles appeared to be phosphorylated. Epinephrine promoted increases of approximately 4-fold in the $^{32}$P content and activity ratio. Neither these effects nor the epinephrine-stimulated increases in phosphorylation of glycogen synthase and phosphorylase kinase were attenuated by insulin. However, insulin at physiological concentrations rapidly decreased the $^{32}$P content of phosphorylase in muscles incubated without epinephrine. Results from peptide mapping experiments indicate that phosphorylase was phosphorylated at a single site in both control and hormone-treated muscles. The maximum effect of insulin on phosphorylase represented a decrease in $^{32}$P of approximately 50%. By comparison, the $^{32}$P content of glycogen synthase and the $\beta$ subunit of phosphorylase kinase were decreased by only 20 and 16%, respectively; the $^{32}$P content of the kinase $\alpha$ subunit was not affected by insulin. The results provide direct evidence that insulin decreases the amount of phosphate in phosphorylase and phosphorylase kinase. These findings have important implications with respect to both the regulation of glycogen metabolism in skeletal muscle and the mechanism of insulin action.

The level of glycogen in cells is determined to a large extent by the relative activities of glycogen synthase and phosphorylase, two enzymes controlled by phosphorylation (for review see Refs. 1–4). The nonphosphorylated ($b$) form of phosphorylase is inactive in the absence of AMP but is fully active in its presence. Phosphorylase $b$ is converted to the $a$ form by phosphorylase kinase, which phosphorylates a single serine located near the $\text{NH}_2$-terminal of phosphorylase. Phosphorylase $a$ is fully active in the absence of AMP, so that the $b$ to $a$ conversion may be followed by monitoring the increase in the $-\text{AMP}+/\text{AMP}$ activity ratio. Glycogen synthase is also phosphorylated in a single site by phosphorylase kinase (5, 6). However, synthase may be phosphorylated in vitro in at least nine additional sites and can be utilized as a substrate by 10 or more protein kinases, including CAMP-dependent protein kinase (4).

Epinephrine activates phosphorylase in skeletal muscle (7–9), thereby stimulating glycogenolysis. This hormonal effect is explained by the now classic phosphorylation cascade in which an increase in CAMP stimulates CAMP-dependent protein kinase which phosphorylates and activates phosphorylase kinase, resulting in the conversion of phosphorylase $b$ to $a$ (1, 2). Epinephrine also stimulates the phosphorylation of glycogen synthase, but in this case the enzyme is inactivated (3, 4, 10). Inhibiting glycogen synthesis when glycogenolysis is stimulated presumably limits the rate of "futile cycling" between glycogen and glucose-1-P. Insulin promotes the dephosphorylation and activation of synthase (11–16). To prevent substrate cycling, insulin might be expected to stimulate the inactivation of phosphorylase; but in previous studies the hormone had had little, if any, effect on the phosphorylase activity ratio (10, 14, 15, 17, 18).

Insulin stimulates the dephosphorylation of several intracellular proteins, in addition to glycogen synthase (19, 20). The mechanism is not yet known, but it seems clear that it must involve protein kinase inhibition or protein phosphatase stimulation. Finding that insulin decreased the phosphorylation of sites 3(a+b+c) in synthase in rabbit skeletal muscle (14), led to the suggestion that insulin inhibited GSK-3, a CAMP-independent protein kinase which selectively phosphorylates these sites (21). However, insulin action must involve more than inhibition of GSK-3 because the hormone decreases the phosphorylation of at least one additional site which is not phosphorylated by this kinase (15, 16). The effects of insulin on glycogen synthase would be consistent with increased protein phosphatase activity.

The phosphatases which dephosphorylate phosphoserine and phosphothreonine in proteins may be divided into two types, based on their ability to dephosphorylate the $\alpha$ and $\beta$ subunits of phosphorylase kinase (22). When the two subunits have been phosphorylated by CAMP-dependent protein kinase, Type I and II phosphatases preferentially dephosphorylate the $\beta$ and $\alpha$ subunits, respectively. A recent hypothesis is that insulin activates Type I phosphatase (23–26). Because the major portion of phosphorylase phosphatase activity in skeletal muscle is thought to be due to this phosphatase (24, 27), the hypothesis predicts that insulin would stimulate the conversion of phosphorylase $a$ to $b$. This prediction conflicts with the widely held view that insulin does not cause dephosphorylation of phosphorylase (10, 14, 15, 17, 18, 24).

It has been suggested that differences in phosphorylase $a$ activity in crude extracts might be difficult to detect, partic-
ularly at the low activity ratios that are found in nonstimulated skeletal muscle. In previous investigations with insulin, the phosphorylation state of phosphorylase had not been directly assessed but inferred from measurements of enzyme activity. In the present experiments we have used \( ^{32}P \)-labeling procedures to investigate the effects of insulin on the phosphorylation of phosphorylase, phosphorylase kinase, and glycogen synthase.

**EXPERIMENTAL PROCEDURES**

*Incubation of Epitrochlearis Muscles with \( ^{32}P \).*—Incubations of muscles were performed essentially as described previously (16). For this reason the following description is abbreviated. Male rats (Sprague-Dawley, 60–75 g) were anesthetized with ether before their epitrochlearis muscles were removed and mounted at resting length on stainless steel holders (5–6 muscles/holder). Before adding \( ^{32}P \), two 15-min incubations in medium (50 ml) were performed to wash out endogenous hormones and to reduce the phosphate content of the tissue (16). In all incubations, the medium was directly gassed by bubbling with 95% \( O_2 \), 5% \( CO_2 \) to ensure that the muscles were adequately oxygenated. The medium was identical to Dubecco's modified Eagle's medium except that the phosphate concentration was reduced to 0.1 mM. For \( ^{32}P \) labeling, muscles were transferred to a single 50-ml centrifuge tube (4–5 holders/tube) containing medium (5 ml per holder) supplemented with \( ^{32}P \), \( (0.8 \text{ mCi/ml}) \). Performing incubations with \( ^{32}P \), in the same tube before treatment with hormone, reduced the variability in \( ^{32}P \) incorporation among the groups, presumably by ensuring that the muscles were exposed to the same specific activity of \( ^{32}P \). After 4 h at 37 °C each holder, together with 5 ml of the medium, was transferred to an individual tube and incubated for 30 min without added hormones or with insulin and/or epinephrine. Muscles were then quick-frozen in liquid nitrogen, ground into powder, and stored at -80 °C until further processing.

**Immunoprecipitations**—Samples of the muscle powder were homogenized as previously described in homogenization buffer (approximately 10 ml/g powder) composed of 100 mMKF, 10 mM EDTA, 2 mM EGTA, 2 mM potassium phosphate, 1 mM benzamidine, and 50 mM Tris/HCl, pH 7.8. The homogenates were centrifuged at 10,000 \( \times g \) for 30 min, and the supernatants were then removed for immunoprecipitations. When \( ^{32}P \)-labeled muscles were used, it was not feasible to weigh the powdered tissue, and the protein concentrations differed somewhat among the samples. To compensate, the protein content of each sample was measured and adjusted to an equal concentration by using homogenization buffer.

Guinea pig antibodies against rabbit skeletal muscle phosphorylase \( b \), phosphorylase kinase, and glycogen synthase have been previously described (16, 28, 29). In the case of phosphorylase (28) and phosphorylase kinase (29), affinity purified Ab were used; for glycogen synthase, unfractionated antiserum that had been incubated at 56 °C for 30 min was used (16). Immunoprecipitations were performed essentially as described previously (16). However, glycogen synthase co-precipitated with phosphorylase and interfered with quantitation of \( ^{32}P \)-labeled phosphorylase (Fig. 1).

Phosphorylase and glycogen synthase are associated with glycogen particles (1–4). Incubating the extracts with human salivary \( \alpha \)-amy- lase before addition of the phosphorylase Ab reduced the amount of contaminating glycogen synthase suggesting that precipitation of particles (with both synthase and phosphorylase bound) accounted for the recovery of synthase with the phosphorylase Ab. However, some glycogen synthase still precipitated with these Ab even after amyloglucosidase treatment. It is unlikely that the phosphorylase Ab are binding glycogen synthase directly. The Ab were generated by immunizing guinea pigs with pure phosphorylase \( b \) (4 \( \times \) crystallized) and were affinity purified by using a column prepared from this enzyme (28). Furthermore, binding of the Ab to glycogen synthase electrophoretically transferred to nitrocellulose was not observed (Fig. 2), and we are unable to immunoprecipitate purified synthase by using the phosphorylase Ab.

Because \( ^{32}P \) glycogen synthase interfered with measurements of \( ^{32}P \) phosphorylase, the synthase was removed from the extract before addition of the phosphorylase Ab. In this case a sample (100 ml) of

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1 The abbreviations used are: EGTA, \( \text{[ethylenebis(oxyeth-}
\text{ylenenitrilo)]tetracetic acid} \); Ab, antibodies; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

2 J. Zhang and J. C. Lawrence, Jr., unpublished observations.

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**FIG. 1.** Coprecipitation of glycogen synthase (GS) and phosphorylase (Phos) with phosphorylase Ab. Muscles were incubated with \( ^{32}P \), for 4.5 h, then frozen. Immunoprecipitation from one sample (5 \( \mu l \)) of extract was performed using 5 \( \mu l \) of affinity purified phosphorylase b Ab (lane f). Another sample (130 \( \mu l \)) was incubated with glycogen synthase antisera (3 \( \mu l \)) at 23 °C for 30 min. Pansorbin (50 \( \mu l \) of a 10% suspension) was added, and the sample was centrifuged. The supernatant was removed, and 5 \( \mu l \) were incubated with the phosphorylase Ab before immune complexes were recovered using Pansorbin (lane e). The immunoprecipitated samples were then subjected to SDS-PAGE. A picture of the autoradiogram is presented. TD, tracking dye.

**FIG. 2.** Analysis of antibody specificity by immunoblotting. Samples of purified rabbit muscle glycogen synthase (100 ng, lanes 1, 6, and 11), rabbit muscle phosphorylase b (50 ng, lanes 2, 7, and 12) and a (50 ng, lanes 3, 8, and 13), and rat muscle phosphorylase b (100 ng, lanes 4, 9, and 14) and a (100 ng, lanes 5, 10, and 15) were subjected to SDS-PAGE and transferred to nitrocellulose sheets. The sheets were either stained with amido black (lanes 1–5) or incubated in buffer with either glycogen synthase antisera (1 \( \mu l/ml \), lanes 6–10) or phosphorylase Ab (1 \( \mu g/ml \), lanes 11–15) before Ab binding was detected using \( ^{32}P \)-labeled protein A as previously described (29). A picture of the autoradiograms and the sheet stained with amido black in the region surrounding glycogen synthase and phosphorylase is presented.

Extract was incubated with glycogen synthase antisera (3 \( \mu l \)) for 10 min at 23 °C. Inactivated Staphylococcus aureus (Pansorbin, 50 \( \mu l \) of a 10% suspension) was then added, and after incubation for 15 min at 0 °C, the samples were centrifuged. Under these conditions, the \( ^{32}P \)-labeled phosphorylase remained in the supernatant (Fig. 1). Consequently, in all subsequent experiments involving immunoprep-
tation of 32P-labeled phosphorylase from extracts of epitrochlearis muscles. 32P-labeled glyceron synthase was first immunoprecipitated. Depending on the protein, different volumes of extract were needed to obtain sufficient amounts of the 32P-labeled proteins. Generally, the equivalents of 5 μl of the extracts were used for phosphorylase, and 40 μl were used to obtain phosphorylase kinase. In all cases a sufficient amount of antibody was added to bind all of the respective proteins. For phosphorylase, 5 μg of Ab was added/5 μl of extract. As shown in Fig. 3, the amount of Ab is sufficient to remove all of the phosphorylase activity from solution. To identify 32P-labeled species shown in Fig. 3, this amount of Ab is sufficient to remove all of the phosphorylase activity. Generally, muscles, 32P-labeled glycogen synthase was first immunoprecipitated. As expected of a competitive interaction (Fig. 3), the antibody (Ab) binds to both phosphorylase Q and phosphorylase a as shown in Fig. 2. Results presented in Figs. 2 and 4 confirm this interpretation. The Ab could be used to immunoprecipitate purified 132P-phosphorylase a (Fig. 4). In this case immunoprecipitation was inhibited by increasing concentrations of unlabeled phosphorylase a or b, as expected of a competitive interaction (Fig. 4).

Measurements of Phosphorylase Activities—When enzyme activities were measured, muscles were incubated without 32P, and the muscle powders were homogenized in 100 mM KF and 10 mM EDTA, pH 7.0. Phosphorylase activity was measured in the direction of glycogen synthesis from glucose 1-P as described by Gilboe et al. (30). Samples (30 μl) were added to 60 μl of reaction mixtures (200 mM KF, 10 mg/ml glycerone, and 100 mM [U-32P]glucose-1-P (100,000 cpm), pH 6.1 at 30 °C, plus or minus AMP) and incubated for 20 min at 30 °C. Phosphorylase activity is expressed as total activity (measured in the presence of 5 mM AMP) or as an activity ratio (activity minus AMP/activity plus AMP). Activity is based on the amount of extract protein, determined by the method of Bradford (31).

Electrophoretic Analyses of Immunoprecipitated Proteins—PAGE in the presence of SDS was performed by the method of Laemmli (32). Samples were applied to slab gels formed with 7.5% acrylamide. After electrophoresis, proteins were stained with Coomassie Blue. In some cases, the amount of 32P in the immunoprecipitated protein was measured by slicing the stained band from the gels and measuring the radioactivity in a scintillation counter. In most experiments, relative band intensities were determined by optical density scanning and measurements of the resulting peak areas.

Phosphoamino Acid Analyses—32P-Labeled proteins were eluted from the gel, precipitated by using 10% trichloroacetic acid, and hydrolyzed in 5.7 N HCl for 2 h at 110 °C. Phosphoserine was resolved from phosphothreonine and phosphotyrosine by one-dimensional thin layer electrophoresis at pH 1.9 on cellulose thin layer sheets (E. Merck) essentially as described by Cooper et al. (33).

Reverse-phase High Performance Liquid Chromatography of the 32P-Phosphopeptide Resulting from Digestion of 32P-Phosphorylase with Chymotrypsin—Samples of immunoprecipitated phosphorylase were subjected to SDS-PAGE, and the gel was stained with Coomassie Blue. After destaining for just enough time (approximately 30 min) to locate the phosphorylase, the enzyme was sliced from the gel. Gel slices were placed in water for 15 min, then frozen in liquid nitrogen and ground into powder with a porcelain mortar and pestle. The powders were suspended in 100 μl of 1% SDS and 10 mM Tris/HCl, pH 7.8, and incubated with continuous mixing for 15 h at 37 °C. Supernatants were obtained after centrifugation at 13,000 × g for 10 min. The pellets were extracted two more times. Each time 100 μl of the buffer containing SDS was added and the samples incubated with mixing at 30 °C for 4 h before centrifugation and removal of the supernatants. Approximately 90% of the 32P present in the original gel slices was extracted under these conditions. Phosphorylase was precipitated from the supernatant by adding trichloroacetic acid to 10%. After centrifugation at 13,000 × g for 10 min, the pellets were rinsed twice with 200 μl of ether to remove the acid. Residual ether was removed under vacuum. To each sample, which contained approximately 5 μg of phosphorylase, was added 50 μl of 0.5 mg/ml chymotrypsin (Worthington), 1 mg/ml soybean trypsin inhibitor, and 0.1 mM ammonium bicarbonate, pH 8.0. The samples were incubated at 30 °C for 18 h before trichloroacetic acid was added to stop the reaction. At this point, less than 5% of the 32P remained insoluble in the acid. After centrifugation at 13,000 × g for 10 min, the supernatants were applied to a C-18 column (Vydac TP104, 4.6 × 250 mm) which had been equilibrated with buffer A (0.1% trifluoroacetic acid). During chromatography the flow rate was maintained at 1 ml/min, and 1-ml fractions were collected. The 32P-labeled fragment was eluted with an acetonitrile gradient generated by increasing the percentage of buffer B (0.1% trifluoroacetic acid, 99.9% acetonitrile) as follows: 0% for 15 min, 9–100% in 60 min, and 100% for 20 min.  

Fig. 3. Immunoprecipitation of phosphorylase from extracts of rat epitrochlearis muscles. Samples of epitrochlearis muscles, 0.1 g of tissue/ml in 100 mM KF, 10 mM EDTA, 2 mM EGTA, and 50 mM Tris/HCl, pH 7.8. After centrifugation at 10,000 × g for 30 min the supernatants were removed and samples (5 μl) were incubated for 1 h at 30 °C with increasing concentrations of either nonimmune IgG or phosphorylase Ab. Inactivated S. aureus (60 μl of a 10% suspension) suspended in 100 mM KF and 10 mM EGTA was added to bind the immune complexes, and the samples were incubated at 0 °C for 30 min. The samples were then centrifuged at 10,000 × g and phosphorylase activity (in the presence of AMP) was measured in the supernatants. The results presented are expressed as the percentage of activity removed from the extract by the antibodies.
The amount of 32P in each fraction was determined by scintillation counting.

Enzyme Purification—Glycogen synthase was purified in a dephosphorylated form from rabbit skeletal muscle as described by Takeda et al. (34). Phosphorylase b and phosphorylase kinase were purified from rabbit skeletal muscle as described by Fischer and Krebs (35) and Cohen (36), respectively. Rat skeletal muscle phosphorylase was purified and crystallized three times as described by Sevilla and Fischer (37).

\[ \gamma^{32}P \text{ATP Specific Activity} \] —Muscle powders (approximately 10 mg) were homogenized in 150 μl of 0.5 N perchloric acid (38). After centrifugation at 10,000 × g for 30 min, the supernatants were neutralized with potassium bicarbonate. The potassium perchlorate precipitate was removed by centrifugation. The specific activity of \( \gamma^{32}P \text{ATP} \) was determined by the method of England and Walsh (39).

Statistical Analyses and Expression of Changes Due to Hormones—Differences in 32P contents of phosphorylase or other immunoprecipitated proteins produced by insulin or epinephrine are expressed relative to the amount of 32P in proteins from muscles incubated without hormones. The percentage change in 32P content or activity due to hormonal treatments was determined from the following expression: 100% × (treatment value – control value)/control value. When a negative value was obtained, the negative sign was retained to signify the direction of the change. For example, −50% denotes a decrease of 50%. For experiments in which the absolute amounts of 32P were not measured, the significance of the difference between a control and treatment group was determined by the sign test (40), a nonparametric method of analysis. Student’s t test was used to evaluate the statistical significance of differences in phosphorylase activities.

Other Materials—Isotopically labeled compounds were obtained from Du Pont-New England Nuclear. Rabbit liver glycogen was obtained from Sigma and was purified before use by ion exchange (Amberlite MB-3) chromatography. Porcine insulin (27 units/mg) was a gift from Lilly.

RESULTS

The epitrochlearis is a small, very thin, muscle found in the forelimb. Under appropriate conditions, these muscles remain viable and retain hormone responsiveness when incubated in vitro (41). In addition, their ATP can be labeled to high specific activity by incubation in medium containing 32P, facilitating investigation of the hormonal control of protein phosphorylation (16). In the experiment presented in Fig. 5, muscles were incubated with 32P, and subsequently treated with insulin or epinephrine before extracts were prepared and subjected to SDS-PAGE. Epinephrine caused a severalfold increase in a 32P-labeled species (indicated by the arrow), which had the same electrophoretic mobility as rabbit muscle phosphorylase, a protein known to be phosphorylated in response to the hormone (7–9). Insulin decreased the amount of this 32P-labeled species. Therefore, experiments were performed to investigate the phosphorylation of phosphorylase, and its possible control by insulin.

Immunoprecipitation of Phosphorylase, Phosphorylase Kinase, and Glycogen Synthase from Extracts of Rat Epitrochlearis Muscles—Immunoprecipitations were performed from extracts of 32P-labeled muscles by using antibodies against phosphorylase, phosphorylase kinase, and glycogen synthase. Skeletal muscle phosphorylase exists as dimers or tetramers of identical subunits, denoted \( \alpha, \beta, \gamma, \) and \( \delta \), which have apparent molecular weights equal to approximately 145,000, 130,000, 45,000, and 18,000, respectively (43, 44). The \( \alpha \) and \( \beta \) subunits of the immunoprecipitated kinase were readily detected by protein staining (Fig. 6, lane 3), and the electrophoretic mobilities of these subunits were essentially the same as the corresponding subunits of the rabbit enzyme (lane 1). Two isozymes of phosphorylase kinase have been described, which differ in the apparent \( M_r \) of the \( \alpha \) subunit (45). The \( \alpha' \) subunit (apparent \( M_r \) equal to 140,000) is found in slow oxidative and fast oxidative glycolytic muscle fibers, which have almost none of the \( \alpha \) subunit containing isoforms (29, 46, 47). On the other hand, a fast glycolytic fiber may have both isozymes (29). Approximately 65% of the fibers in the epitrochlearis are of the fast glycolytic type, with almost equal numbers of fast oxidative glycolytic and slow oxidative types making up the remainder (48). The kinase from epitrochlearis muscles has approximately equal proportions of \( \alpha \) and \( \alpha' \) subunits (Fig. 6, lane 3), and the subunits were labeled to essentially the same extent with 32P (lane 5 and 6). Furthermore, when hormonal effects were detected, the 32P contents of the \( \alpha \) and \( \alpha' \) subunits appeared to change in parallel. Therefore, their 32P contents were not measured individually, and the results presented reflect the radioactivity found in both subunits. Approximately eight times more 32P was observed in the (\( \alpha + \alpha' \)) subunits than in the \( \beta \) subunits (Fig. 7A).

The dephosphorylated glycogen synthase subunit has an apparent \( M_r \) of 85,000 (34). At least three 32P-labeled species of immunoprecipitated glycogen synthase were detected (Fig. 6, lanes 14 and 15) that had electrophoretic mobilities slightly lower than that of the purified (nonphosphorylated) synthase standard (lane 12). Phosphorylation of synthase in the site 3 region retards the electrophoretic mobility of the protein, and multiple phosphorylated forms may be resolved by SDS-PAGE (49). Most likely because of the electrophoretic heterogeneity, Coomassie Blue-stained bands of the immunoprecipitated synthase were difficult to detect. Note that slightly different amounts of protein were applied to the gel (see legend to Fig. 5).
If phosphate turnover in a protein is sufficiently rapid, and if it is assumed that phosphate is incorporated only from the ~P position of ATP and that the ATP pool is uniformly labeled (8), then the ratio of specific activity of ~P-labeled protein to that of [~P]ATP should provide a good estimate of the stoichiometry of phosphorylation. Thus, phosphorylase appears to contain only 0.04 phosphates/subunit. After a 12-h incubation, the (a + a') and b subunits of phosphorylase kinase contain at least 2.1 and 0.25 phosphates/subunit, respectively (Fig. 7B). This sum is close to the value (2.2) that can be calculated from the results of Mayer and Krebs (8) for the phosphate content of the kinase in rabbit muscle.

Ideally, when using ~P-labeling procedures to investigate changes in protein phosphorylation, tissues would be incubated for a sufficient time to achieve steady state labeling of [~P]ATP and phosphoproteins. This was not feasible because the muscles become unresponsive to insulin after incubation for a time long enough to reach steady state labeling (16). We chose to incubate muscles for 4 h with ~P before hormonal treatments. At this time, sufficiently high specific activities of phosphorylase and phosphorylase kinase were achieved, and the muscles remained responsive to insulin. Furthermore, the specific activity of [~P]ATP is not changed by incubating the muscles for an additional 30 min with insulin or epinephrine (16).

Insulin-stimulated Dephosphorylation of Phosphorylase—Incubating muscles with insulin markedly decreased the ~P content of phosphorylase (Figs. 8 and 9). This effect of insulin was maximal after 15 min (Fig. 8A and B) and was associated with a decrease in the phosphorylase activity ratio (Fig. 8C). However, the effect of insulin on decreasing the activity ratio was relatively small in comparison to the decrease in phosphorylation (Fig. 8C, Table I). It should also be noted that the activity ratio (0.15) in control muscles was higher than the estimated fraction (0.04) of phosphorylated phosphorylase. Possible reasons for this discrepancy will be discussed later.

Insulin did not decrease the amount of phosphorylase immunoprecipitated. This may be seen in Fig. 8B, which is a picture of a Coomassie Blue-stained gel and corresponding autoradiogram in the region of the gel surrounding phosphorylase. Thus, it seems clear that the hormonal effect involves a decrease in the phosphate content of phosphorylase, rather than a decrease in the amount of the protein. Also consistent with this interpretation are results indicating that insulin was without effect on the total amount of phosphorylase activity (Table I).

The concentration of insulin used in the preceding experiments was intended to saturate insulin receptors. Experiments were also performed in which muscles were incubated with lower concentrations of the hormone (Fig. 9). An effect of insulin on decreasing the amount of ~P in phosphorylase was observed with 25 micromoles/ml, which is within the physiological range of insulin concentrations (i.e. 10–100 micromoles/ml). The maximum effect represented a 50% decrease in the amount of [~P]phosphorylase.

Effects of Insulin and Epinephrine on the Phosphorylation

It should be stressed that the numbers of phosphates/subunit were not measured directly and should not be taken as absolute values. Stoichiometries based on ~P contents are likely to be underestimated, since steady state labeling was not achieved. Furthermore, there are clearly different pools of ATP in the epitrochlearis muscle, which is composed of different muscle fiber types. The amounts of phosphorylase and phosphorylase kinase also differ greatly among fibers. Differences in the labeling patterns of both the proteins and [~P]ATP among different fiber types is possible. Such differences could introduce significant error into the estimation.
Insulin-stimulated Dephosphorylation of Phosphorylase

FIG. 7. Time course of the incorporation of 32P into phosphorylase, phosphorylase kinase subunits, and the γ-phosphate of ATP. Six muscles were frozen in liquid nitrogen after each of the following times of incubation in medium containing 32P (0.8 mCi/ml): 2, 4, 8, and 12 h. Samples of powdered muscle (approximately 10 mg) were homogenized in 0.5 M perchloric acid for [γ-32P]ATP-specific activity measurements. Other samples (approximately 30 mg) were homogenized in buffer at pH 7.8, and extracts were prepared. Phosphorylase and phosphorylase kinase were immunoprecipitated and subjected to SDS-PAGE together with increasing amounts (0.1–1 μg) of purified phosphorylase or phosphorylase kinase standards. After electrophoresis, the gels were stained with Coomassie Blue and optical density scanning was performed. The amounts of the proteins immunoprecipitated were determined by their staining intensities relative to those of the respective standards. The pmol of phosphorylase and the phosphorylase kinase (α + α′) and β subunits were calculated based on M, values of 97,000, 145,000, and 130,000, respectively. The amounts of 32P in the proteins were determined by scintillation counting after slicing the appropriate bands from the gel. A, the results presented are the specific activities (cpm/pmol) of [γ-32P]ATP (○), phosphorylase (●), and the (α + α′) (△) and β (■) subunits of phosphorylase kinase. B, the specific activities of the labeled proteins were divided by the specific activity of [γ-32P]ATP. The results presented are mean values ± S.E. from three experiments performed on different days. Error bars not shown fall within the height of the symbols.

FIG. 8. Effect of increasing times of incubation with insulin on the 32P content of phosphorylase. A and B, epitrochlearis muscles were incubated in medium containing 32P, for a total of 4.5 h, then frozen in liquid nitrogen. Group of six muscles each were exposed to insulin (250 milliunits/ml) for the final 5, 15, or 30 min of incubation. Phosphorylase was immunoprecipitated and subjected to SDS-PAGE. The gels were stained with Coomassie Blue and autoradiograms were prepared. A, relative changes in 32P content of phosphorylase were determined and expressed as % decreases. Values shown are means ± S.E. of (n) experiments performed on different days. B, a picture of a stained gel and autoradiogram of the region surrounding phosphorylase from a representative experiment is presented. C, muscles were incubated as described above, except that 32P was not added to the medium. Phosphorylase activity in both the absence and presence of 2 mM AMP was measured. Results represent the decrease in the -AMP/+AMP activity ratio and are means ± S.E. from (n) experiments performed on different days.

of Phosphorylase, Phosphorylase Kinase, and Glycogen Synthase—In 11 experiments in which muscles were incubated with 250 milliunits/ml insulin for 30 min, 32P in phosphorylase was reduced by approximately 40%, which was twice as large as the percentage decrease in 32P content of glycogen synthase in the same muscles (Table II). In six experiments in which the amounts of 32P were measured, incubating muscles with 2.5 milliunits/ml insulin for 30 min decreased 32P-labeled phosphorylase (cpm/mg extract protein) from 252 ± 53 to 165 ± 51 (p < 0.001, paired t test).

Insulin did not affect the amount of 32P in the α + α′ subunits of phosphorylase kinase but decreased the 32P content of the β subunits by 16% (Table II). Although the percent decrease in β subunit phosphorylation was comparable to the hormonal effect on synthase phosphorylation (Table II), it was relatively small. As another index of change in phosphorylation of the kinase subunits, the effect of insulin on the ratio of the 32P-labeled subunits was determined. Insulin decreased the ratio of β/(α + α′) from 0.036 ± 0.005 to 0.025
Insulin-stimulated Dephosphorylation of Phosphorylase

Effects of insulin and epinephrine on phosphorylase activity

Epitrochlearis muscles were incubated in low phosphate medium without $^{32}$P, for 4 h at 37°C before insulin (250 milliunits/ml), epinephrine (10 µM), or insulin plus epinephrine were added. The incubations were continued for 30 min before the muscles were frozen in liquid nitrogen. Phosphorylase activity was measured in extracts of the muscles. The activity ratio represents the activity measured in the absence of AMP divided by the activity observed in the presence of AMP. Total activity is that activity measured in the presence of 2 mM AMP and is expressed relative to extract protein. Values presented are means ± S.E. from $n$ experiments performed on different days.

| Hormones added | $n$ | Activity ratio (−AMP/+AMP) | Total activity (µmol/min/mg) |
|----------------|----|---------------------------|----------------------------|
| None           | 11 | 0.15 ± 0.01               | 1.30 ± 0.13                |
| Insulin, 250 milliunits/ml | 11 | 0.11 ± 0.01*              | 1.37 ± 0.11                |
| Epinephrine, 10 µM | 3  | 0.42 ± 0.08*              | 1.48 ± 0.31                |
| Insulin, 250 milliunits/ml plus epinephrine, 10 µM | 3  | 0.44 ± 0.08               | 1.13 ± 0.21                |

* $p < 0.01$ versus control, paired comparison.

Epinephrine was much more pronounced. In this case, the $^{32}$P content increased by more than 4-fold. Insulin did not counteract the effect epinephrine had on phosphorylase, which is consistent with the finding that insulin did not oppose the increase in phosphorylase activity ratio produced by epinephrine (Table I).

Phosphoamino Acid Analyses and Peptide Mapping of [32P] Phosphorylase—Immunoprecipitated phosphorylase was found to contain [32P]phosphoserine when phosphoamino acid analyses were performed after acid hydrolysis (Fig. 10). Little, if any, phosphothreonine or phosphotyrosine were present. Although the actual differences in amounts of phosphoamino acids are difficult to determine in this type of analysis, in three separate experiments, less [32P]phosphoserine was recovered in the phosphorylase sample from insulin-treated muscles. The acid hydrolysis did release some 32P, but this is expected since phosphoamino acids are slowly hydrolyzed under these conditions (33).

When rabbit muscle phosphorylase $a$ is digested with chymotrypsin, the phosphorylated serine is recovered in a 14-amino acid fragment (50). The fragment from rat muscle phosphorylase $a$ contains an aspartic instead of glutamic acid residue but is otherwise identical to the rabbit fragment (37). As might be expected, this difference has essentially no effect on the elution of the two fragments from a reverse-phase column (Fig. 11A). When phosphorylase immunoprecipitated from rat epitrochlearis muscles was digested with chymotrypsin, essentially all of the 32P eluted at the same acetonitrile concentration as the phosphorylated fragment from purified phosphorylase $a$. As in Fig. 11B, insulin decreased and epinephrine increased the amount of the 32P-labeled fragment.

DISCUSSION

The present findings indicate that insulin, at physiological concentrations, rapidly stimulates the dephosphorylation of phosphorylase in epitrochlearis muscles. The effect of insulin on decreasing the 32P content of phosphorylase was twice as large as its well-established effect on the dephosphorylation of glycogen synthase. Phosphorylase is phosphorylated at a single site by phosphorylase kinase (1). Yet, the possibility has not been excluded that the enzyme is phosphorylated in epinephrine-treated muscles in six of seven experiments, although the effect of epinephrine was not judged to be significant by the sign test.

The 32P contents of the $\beta$ and ($\alpha + \alpha'$) subunits were higher in

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TABLE II
Effects of insulin and epinephrine on the $^{32}$P contents of phosphorylase, phosphorylase kinase, and glycogen synthase

| Hormones                          | $n$ | Phosphorylase | Phosphorylase kinase | Glycogen synthase |
|-----------------------------------|-----|---------------|----------------------|------------------|
| Insulin, 250 milliunits/ml       | 11  | $-39.5 \pm 5.3^a$ | $-4.8 \pm 5.2$      | $-20.8 \pm 5.7^a$ |
| Epinephrine, 10 $\mu$M           | 7   | $442 \pm 121^b$   | $52.9 \pm 18.8$     | $62.4 \pm 8.5^b$  |

$^a$ $p < 0.01$, versus control.
$^b$ $p < 0.05$, versus control.

Fig. 10. Phosphoamino acid analysis of phosphorylase immunoprecipitated from $^{32}$P-labeled epitrochlearis muscles. Muscles were incubated with $^{32}$P, for 4 h. Incubations were then continued for 30 min in the absence of hormones (lane 1), or in the presence of 250 milliunits/ml insulin (lane 2), 10 $\mu$M epinephrine (lane 3), or the combination of insulin plus epinephrine (lane 4). Phosphorylase was immunoprecipitated and subjected to SDS-PAGE. A picture of the gel after staining with Coomassie Blue (A) and an autoradiogram of the region surrounding phosphorylase is presented (A). The $^{32}$P-labeled phosphorylase was eluted from the gel and hydrolyzed with HCl. Samples were then subjected to high voltage electrophoresis at pH 1.9, to optimize separation of phosphoserine from phosphothreonine and phosphotyrosine. The thin layer sheet was stained with ninhydrin to enable detection of the phosphoamino acid standards that had been added before electrophoresis. A picture of an autoradiogram is presented (C). The spots identified as Pser were coincident with the ninhydrin-stained phosphoserine standard in all four samples. The positions of the phosphothreonine (P-Thr) and phosphotyrosine (P-Tyr) standards and origins are indicated by arrows.

more than one site in vivo. Stimulation of the dephosphorylation of a site not involved in the a to b conversion was considered as an explanation of why the effect of insulin on decreasing $^{32}$P-labeled phosphorylase was larger than the effect on decreasing the phosphorylase activity ratio. However, this does not appear to be the case. The b to a conversion involves phosphorylation of a serine located 14 residues from the NH$_2$-terminal of phosphorylase (42, 50). $^{32}$PPhosphoserine was the only labeled phosphoamino acid detected in immunoprecipitated phosphorylase (Fig. 10), and all of the $^{32}$P was found in a chymotryptic fragment which eluted from a reverse-phase column in the same position as the peptide from $^{32}$Pphosphorylase a (Fig. 11).

While the percentage decrease in phosphorylase phosphorylation stimulated by insulin was relatively large, most of the enzyme was already dephosphorylated before the hormone was added. Nevertheless, in considering the significance of the effect of insulin, it is important to remember that there is over 10 times more phosphorylase than glycogen synthase in skeletal muscle. Consequently, most of the phosphorylase has to be inactive in order for net glycogen synthesis to occur. It might also be noted that the accumulation of glycogen in response to insulin occurs over many min to a few h. In contrast, when a large percentage of phosphorylase is activated, as with epinephrine or contractile activity, there can be an explosive burst of glycogenolysis resulting in almost complete degradation of glycogen within a few min (51).

Cycling between glycogen and glucose-1-P occurs even in resting muscle (52). When considered over a time course of h, even a small change in phosphorylase activity can determine whether there is net accumulation or degradation of glycogen. In a steady state cycle, a 50% reduction in the rate of degradation would lead to a 2-fold increase in the amount of glycogen. Because phosphorylase $b$ is almost completely inactive at the substrate and effector concentrations found in resting muscle (1), the small amount of phosphorylated phosphorylase probably accounts for much, if not all, of the basal glycogenolysis. Therefore, a decrease in the phosphorylated form is likely to be physiologically significant, even if the change involves only a small portion of the total phosphorylase.

A question remains of why phosphorylase inactivation in response to insulin was not previously detected (10, 14, 15, 17, 18). Possible explanations involve the methods for assessing the phosphorylation state of phosphorylase and the skeletal muscle preparation used. Measuring $^{32}$P content provides a different index of phosphorylation than measuring the activity ratio, as was done in the previous studies (10, 14, 15, 17, 18). It might be noted that a statistically significant decrease in activity ratio in response to insulin in perfused rat hindlimb was recently reported (53), and a decrease was detected in the present experiments (Table I). However, the effect of insulin on phosphorylase activity was much smaller than the decrease in phosphate (see Fig. 8, for example), and was consequently harder to detect.
Insulin-stimulated Dephosphorylation of Phosphorylase

The conditions used in the assay of phosphorylase activity ratio differ among the previous studies. The present measurements were performed in the direction of glycogen synthesis by using a relatively high (67 mM) concentration of glucose-1-P. At the low levels of phosphorylase a found in nonstimulated skeletal muscle, assaying activity at high substrate may provide a more sensitive index of changes in the phosphorylation of the enzyme. Fischer et al. (54) noted that when phosphorylase b was phosphorylated in vitro with phosphorylase kinase, and activity was measured at high concentrations of glucose-1-P, phosphorylase a activity ran well ahead of phosphate incorporation, reaching a maximum at 0.5 instead of 1.0 phosphates/subunit. It was suggested that this might be due to the formation of hybrids in which only one of the subunits in each phosphorylase dimer was phosphorylated. The a-b hybrids have properties intermediate to the a and b forms of the enzyme (54, 55). In particular, the hybrids are active at high glucose-1-P concentrations, even in the absence of AMP. In the absence of cooperativity in phosphorylation, a-b hybrids would be expected to predominate at the low phosphorylation states found in resting muscle. Such hybrids might explain, at least in part, why the activity ratio of 0.15 was considerably higher than the estimated phosphorylation state of 0.04 phosphates/subunit.4

While the substrate concentration may be important in detecting the effect of insulin on activity ratio, it cannot be the only explanation for the absence of the insulin response in some muscles. Incubating rat diaphragm muscles with insulin did not detectably decrease the activity ratio, even when assays were performed under the same conditions as the present experiments (15). Muscles may differ greatly in contractile and metabolic properties, due to differences in their fiber composition (56). Most of the fibers in the rat epitrochlearis muscle are of the fast glycolytic type (48). Insulin-stimulated rates of glucose transport (57) and glycogen synthesis (58, 59) are much more pronounced in muscles like the diaphragm, that contain a higher proportion of oxidative fiber types. On the other hand, the fast glycolytic fibers contain not only higher concentrations of glycogen (56) but also several times more phosphorylase kinase and phosphorylase (29, 56), so that they are able to mobilize the glycogen much faster. It seems possible that the control of phosphorylase phosphorylation by insulin might be more pronounced in glycolytic fibers than in oxidative fibers.

There is other evidence that in epitrochlearis muscles insulin has a more pronounced effect on inhibiting glycogenolysis than on stimulating glycogen synthesis. Challiss et al. (52) found that the hormone increased the rate of glycogen synthesis by 3-fold, while decreasing the rate of glycogenolysis by 7-fold. The present finding that insulin stimulated the dephosphorylation of phosphorylase to a greater extent than the dephosphorylation of glycogen synthase (Table II) provides a potential explanation of the more pronounced effect on inhibiting glycogenolysis.

Type I protein phosphatase is found in glycogen particles and is thought to be responsible for dephosphorylating enzymes of glycogen metabolism (27). One form of the phosphatase exists as a complex (60) of a catalytic subunit and a regulatory subunit, termed inhibitor-2 (61). The phosphatase is activated when the regulatory subunit is phosphorylated by GSK-3 (62). Phosphorylation of inhibitor-2 by casein kinase II markedly increases the ability of GSK-3 to phosphorylate and activate the phosphatase (63). DePaoli-Roach (63) first suggested that stimulation of casein kinase II was a potential mechanism by which insulin might activate Type I phosphatase, and there is evidence that insulin increases casein kinase II activity in some cells (25). In fat cells the phosphorylation of inhibitor-2 was found to be increased in response to insulin (24), indicating that the Type I phosphatase is a target of insulin action. Insulin has also been shown to stimulate the dephosphorylation of inhibitor-1, which would be expected to increase the activity of this phosphatase (64, 65). Very recently, Olivier et al. (26) reported that insulin activated Type I phosphatase in mouse 3T3 fibroblasts.

Finding decreased 32P in the β subunit of phosphorylase

FIG. 11. Reverse-phase HPLC of chymotrypsin fragments of [32P]phosphorylase. A, samples (100 μg) of phosphorylase b from rat and rabbit skeletal muscle were incubated with 0.5 μg of phosphorylase kinase for 1 h at 30 °C in 50 μl of a solution containing 8 mM MgCl₂, 30 mM β-mercaptoethanol, 100 μM [γ-32P]ATP (900 cpm/pmol), and 100 mM Tris/HCl, pH 8.2 at 30 °C. After 1 h trichloroacetic acid was added to 10%. The samples were placed on ice for 10 min, then centrifuged at 13,000 × g for 10 min. The pellets were rinsed twice with 500 μl of 10% trichloroacetic acid, and twice with 500 μl of ether. Residual ether was removed under vacuum, and to each sample was added 500 μl of 0.5 mg/ml chymotrypsin, 1 mg/ml soybean trypsin inhibitor, and 0.1 M ammonium bicarbonate, pH 8.0. After incubating at 30 °C for 18 h, trichloroacetic acid was added to 20%, and samples of the acid-soluble material were applied to a reverse-phase column. The [32P]phosphopeptides were eluted with a gradient of increasing acetonitrile. In B, rat epitrochlearis muscles were incubated in medium containing 32P for a total of 4.5 h, then frozen in liquid nitrogen. Groups of five muscles each were incubated without hormones (●), with 250 milliunits/ml insulin (○) for the last 30 min, or with 10 μM epinephrine (△) for the last 15 min. [32P]Phosphorylase was immunoprecipitated from extracts of the muscles and subjected to SDS-PAGE. The enzyme was eluted from slices of the gel, precipitated with trichloroacetic acid, and incubated with chymotrypsin as described under “Experimental Procedures.” The samples applied to the C-18 column contained the following amounts of [32P]: control, 277 cpm; insulin, 155 cpm; and epinephrine, 1570 cpm.
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kinase, but no change in the \( \alpha \) subunits is also suggestive of increased Type I phosphatase activity (Table II). However, in considering these results, it is important to note that the stimulation of \( \beta \) subunit dephosphorylation may have involved a site other than the one defining phosphatase specificity. Also, the high level of \( \alpha \) subunits might have obscured a small net change in phosphatase.

Nevertheless, stimulation of the phosphatase might explain why insulin had a greater effect on phosphorylase than on glycogen synthase or phosphorylase kinase. While the phosphatase would be expected to decrease phosphate in all three enzymes, an additional decrease in the phosphorylation state of phosphorylase would be expected due to the decrease in kinase activity resulting from dephosphorylation of the phosphorylase kinase \( \beta \) subunit.

As expected, epinephrine increased the phosphorylation of glycogen synthase, phosphorylase, and phosphorylase kinase (Table II). Insulin did not oppose the increase in phosphorylation stimulated by epinephrine. The reason for the lack of an effect of insulin in the presence of epinephrine is not clear due to the decrease in kinase activity resulting from dephosphorylation of the phosphorylase subunit.

In summary, the present experiments provide direct evidence that insulin stimulates the dephosphorylation of phosphorylase and the \( \beta \) subunit of phosphorylase kinase. Our results and those of Challis et al. (52) indicate that inhibition of glycogenolysis by insulin is important in controlling glycogen metabolism in epithelial cells incubated in vitro. Additional research is not needed to determine whether inhibition of glycogenolysis is involved in the stimulation of glycogen accumulation by insulin in vivo.

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