Further Studies on the Site Phosphorylated in the Phosphorylase *b* to *a* Reaction*

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Received December 18, 1963

Digestion of ^{32}P-labeled rabbit muscle phosphorylase *a* with various proteolytic enzymes has shown that the rate of release of phosphopeptides from the enzyme is greater than the overall rate of proteolysis, suggesting that the phosphorylation in the phosphorylase *b* to *a* conversion occurs on a particularly exposed or less highly organized region of the enzyme molecule. The phosphopeptides released by tryptic attack are not required for the enzymatic activity of the residual protein (phosphorylase *b'*). A phosphoserine-containing tetradecapeptide was isolated from a chymotryptic digest of phosphorylase *a* and its amino acid sequence was determined. This peptide and a phospho-octapeptide, also isolated from the chymotryptic digest, were shown to be substrates for purified phosphorylase phosphatase. The rate of enzymatic dephosphorylation of the tetradecapeptide was 2–3% of the rate observed for the phosphorylase *a* substrate; the octapeptide and hexa- and pentapeptides previously isolated from a tryptic hydrolysate of phosphorylase *a* were attacked at successively lower rates. Phosphorylation of the dephosphotetradecapeptide at a low rate in the presence of phosphorylase *b* kinase and [^{32}P]ATP was demonstrated.

In the previous publication (Fischer *et al.*, 1959), the site phosphorylated during the conversion of rabbit muscle phosphorylase *b* to *a* was shown to have the following sequence: Lys-Glu(NH_2)-Ileu-Ser(P)-Val-Arg. An identical phosphorylated hexapeptide was isolated from *human muscle* phosphorylase *a* (Hughes *et al.*, 1962a), which has an amino acid composition very similar to that of the rabbit enzyme (Appleman *et al.*, 1963). On the other hand, tryptic attack of rabbit liver phosphorylase yielded a phosphorylated hexapeptide with a different amino acid composition, namely, Arg_2, Glu(NH_2), Val_2, and Ser(P) (Appleman *et al.*, 1962). Limitation of material prevented complete determination of the sequence of this peptide, but preliminary data appeared to indicate that it might result from the conventional substitution of an arginyl for the lysyl residue and a valyl for the isoleucyl residue.

The phosphohexapeptide released during tryptic attack was shown to be dephosphorylated (though very slowly) by phosphorylase phosphatase, but the resulting dephosphopeptide could not be rephosphorylated by phosphorylase *b* kinase and ATP (Graves *et al.*, 1960).

It appeared of interest to pursue these studies in order to determine, for instance, if the affinity of phosphorylase phosphatase for the phosphopeptides would be increased if larger fragments were used as substrate. Also, could larger fragments of the dephosphopeptide be rephosphorylated by phosphorylase kinase and ATP—which would indicate that the activity of this enzyme is not dependent on the presence of certain missing groups or a particular conformation of the phosphorylase molecule.

Another point that deserved clarification concerned the residual protein remaining after tryptic attack (phosphorylase *b'*) shown (Cori and Cori, 1945; Keller, 1955) to be still enzymatically active when tested in the presence of AMP. The possibility remained that the phosphopeptides released by trypsin were still bound to the protein by noncovalent linkages, and conferred catalytic activity upon the phosphorylase *b'* moiety in a manner similar to that described (Richards, 1958) for ribonuclease and the S-peptide.

The present paper, which extends the sequence of the site phosphorylated during the phosphorylase *b* to *a* reaction to that of a tetradecapeptide, has attempted to answer some of the above questions.

MATERIALS

Crystalline ^{32}P-labeled Phosphorylase a.—This was prepared from three-times-crystallized phosphorylase *b* essentially as previously described (Fischer *et al.*, 1959) and freed of nucleotides by filtering the enzyme in solution through a 1:1 charcoal-cellulose mixture (Fischer and Krebs, 1958). Sodium borohydride reduction of phosphorylase *a* was performed as described by Kent (1959).

Phosphorylase Activity.—Phosphorylase activity was measured in 0.04 M sodium glycerophosphate–0.03 M cysteine buffer, *p*H 6.8, in the presence or absence of adenosine-5'-phosphate (AMP, 10^{-3} M in the reaction mixture) according to the methods of Illingworth and Cori (1953).

Phosphorylase b kinase was prepared as described by Krebs and co-workers;[1] the sedimenting fraction obtained in the step involving centrifugation at 100,000 $\times g$ was used throughout. *Phosphorylase a phosphatase* was prepared by a method to be described.[2] The preparation used was that obtained from the DEAE-cellulose chromatography step.

β,γ-Labeled [^{32}P]ATP, prepared by the method of Kielley and Kielley (1951), was used without further purification in the phosphorylase *b* to *a* conversion reaction. γ- Labeled [^{32}P]ATP, a gift from Mr. Robert DeLange, was prepared essentially according to the procedure of Tanaka *et al.* (1959) and purified by ion-exchange chromatography by a modification of the method of Hurlbert *et al.* (1954).

α-Chymotrypsin (thrice crystallized), *trypsin* (twice crystallized), *crystalline papain*, and *crystalline soybean*

* This research was generously supported by research grants from the National Institutes of Health, U. S. Public Health Service (AM-859) and the National Science Foundation (GB-239).

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[1] D. S. Love, K. A. Trayser, W. L. Meyer, E. H. Fischer, and E. G. Krebs, in preparation.

[2] S. S. Hurd, W. B. Novoa, E. G. Krebs, and E. H. Fischer, in preparation.

trypsin inhibitor were obtained from the Worthington Biochemical Corp. *Bacterial proteinase* (Nagarse) was obtained from the Biddle-Sawyer Corp. Crystalline, diisopropylfluorophosphate-treated *carboxypeptidase A* was a gift from Dr. Hans Neurath.

Methods

Chymotryptic Digestion of $NaBH_4$-reduced, ^{32}P-labeled Phosphorylase a.—^{32}P-labeled, reduced[3] phosphorylase *a*, AMP-free (5.9 g), was dissolved in 475 ml of distilled water and the *p*H of the solution was adjusted to *p*H 7.8 with dilute NaOH. To this was added 15 ml of a solution containing α-chymotrypsin (20 mg/ml) and soybean trypsin inhibitor (1 mg/ml), also at *p*H 7.8, which had been prepared and allowed to stand at room temperature 30 minutes prior to use. The trypsin inhibitor was added as a precaution against possible trypsin contamination of the chymotrypsin preparation because of the extreme susceptibility of the phosphorylated site of phosphorylase *a* to tryptic attack (Fischer *et al.*, 1959, and this paper). The digestion was performed at 25° with continuous stirring, and the *p*H was maintained between 7.5 and 7.8 (Radiometer *p*H-stat) by the continuous addition of 0.2 N NaOH from a buret. An additional 5 ml of chymotrypsin-trypsin inhibitor solution was added after 3.5 hours. The reaction appeared to be virtually complete after about 8 hours and was stopped after 8.5 hours by freezing in Dry Ice–ethanol and lyophilization.

Chromatographic Separation of Peptides.—The phospho- and pyridoxylphosphate (PLP) peptides[4] were partially purified by chromatography of the digest on a column (2.7 × 140 cm) of Dowex 50-X2, 200–400 mesh. The column was eluted at room temperature at 110 ml/hr with the aid of a Sigma pump. The column effluent was collected in 20-ml fractions with a Technicon fraction collector.

The buffers used for elution were 0.2 M pyridine-acetate, *p*H 3.1, and 2.0 M pyridine-acetate, *p*H 5.0, described by Margoliash and Smith (1962). The lyophilized chymotryptic digest was dissolved in the *p*H 3.1 buffer used for equilibration of the column. After removal by centrifugation of a small amount of insoluble material containing 7% of the total radioactivity, an equivalent of 5.6 g of phosphorylase with a radioactivity of 18.5×10^7 cpm (93%) was applied to the column. The column was first eluted with 2 liters of the *p*H 3.1 buffer, after which a linear gradient was established between the *p*H 3.1 and *p*H 5.0 buffers with 5 liters of the former in the mixing chamber and 5 liters of the latter in the reservoir. At the end of this gradient, elution was continued with an additional 0.7 liter of 4.0 M pyridine-acetate buffer, *p*H 5.1.

Elution of the peptides was followed by application of the ninhydrin method (Moore and Stein, 1954). The phosphopeptides were located by their radioactivity with the use of a Nuclear Chicago automatic gas-flow counter. The PylP-peptides were located visually by their fluorescence under ultraviolet light and spectrophotometrically at 325 mμ (Kent, 1959). The fractions were exposed to ultraviolet light only briefly, and the fractions containing PylP-peptides were kept in the dark or dim light during subsequent purification steps to minimize possible photodecomposition of these substances (Fischer *et al.*, 1963). The appropriate effluent fractions were pooled and concentrated to dryness on a rotary evaporator at 30–35° under reduced pressure.

Further Purification of Peptides.—The phospho- and PylP-peptide fractions from the ion-exchange column were further purified by gel filtration on a column (2.7 × 150 cm) of Sephadex G-25. The column was eluted at room temperature at a rate of 20–25 ml/hr, and the effluent was collected in fractions of 4–6 ml as described above. The peptides were eluted from the column with 0.05 M pyridine-acetate buffer at *p*H 3.3 (20.1 ml pyridine and 300 ml glacial acetic acid diluted to 5 liters with water) or with 1.0 M pyridine-acetate buffer at *p*H 8.0 (402.5 ml pyridine and 2.5 ml glacial acetic acid diluted to 5 liters with water). The peptides were located in the effluent fractions, and the appropriate fractions were pooled and concentrated, as described above. Since the same column was used repeatedly, it was washed thoroughly with the eluting buffer after each run.

The peptide fractions thus obtained were examined for purity by two-dimensional paper electrophoresis-chromatography, as described below. When necessary, the peptides were further purified by preparative paper electrophoresis and/or chromatography.

Two-Dimensional Paper Electrophoresis-Chromatography (Fingerprinting).—Paper electrophoresis was performed on Whatman 3MM paper according to the method of Ingram (1956). Electrophoresis of peptides was routinely run for 50–60 minutes at a potential of 43 v/cm in pyridine-acetate buffers at *p*H 6.5 or 3.6. Chromatograms were routinely run for 16–18 hours in 1-butanol–acetic acid–water (200:30:75) (solvent 1) or 1-butanol–pyridine–acetic acid–water (30:20:6:24) (solvent 2), in a descending system. Fingerprinting of amino acids was performed in a pyridineformate buffer, *p*H 2.2 (electrophoresis), and in solvent 1 (chromatography).

Peptides and amino acids were detected on paper with the ninhydrin reagent of Levy and Chung (1953). ^{32}P-phosphopeptides were also located by radioautography and PylP-peptides by their light blue fluorescence under ultraviolet light.

Amino Acid Analysis.—For amino acid analysis, peptides were hydrolyzed in thrice-distilled constant-boiling HCl (5.7 N) at 108–110° under reduced pressure (14 mm Hg) for 21–23 hours in sealed Pyrex glass tubes. Hydrolysates were first examined by fingerprinting, as described above, then analyzed quantitatively on a Technicon amino acid analyzer.

Peptide Structure.—The Edman degradation technique employed was that described by Acher *et al.* (1956) and by Light and Smith (1960). The phenylisothiocyanate (Matheson, Coleman and Bell) and pyridine (reagent grade) were redistilled just before use. Benzene (Eastman Chemical Co.) was washed with sulfuric acid. Ether (Squibb) was treated with $FeSO_4$ to remove peroxides; trimethylamine, 25% (practical grade), was obtained from Eastman Chemical Co. Phenylthiohydantoins were identified by paper chromatography on Whatman No. 1 paper in Solvents A, C (Sjöquist, 1953) and F (Edman and Sjöquist, 1956), and identification was made indirectly by analysis of the peptide remaining after each degradation step.

End-group analyses were also performed by the method of Sanger, as described by Fraenkel-Conrat *et al.* (1955).

Total Protein Determination.—Total protein was determined by the ninhydrin method following hydroly-

[3] $NaBH_4$-reduced phosphorylase *a* was used in this preparation so that the chymotryptic pyridoxylphosphate-peptide could be isolated for separate investigations.

[4] Abbreviations used are PLP, pyridoxal-5'-phosphate; PylP-peptide, pyridoxylphosphate peptide; BAL, 2,3-dimercaptopropanol; PTH, phenylthiohydantoin.

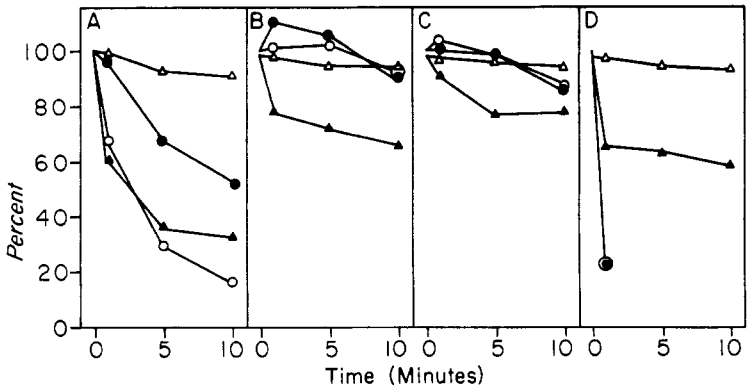

FIG. 1.—Relative rate of release of radioactivity and protein material from ^{32}P-labeled phosphorylase *a* with various proteolytic enzymes. AMP-free ^{32}P-labeled phosphorylase *a* (64.2 mg, 1417 cpm/mg in a total of 8.5 ml) was treated with 750 μg of trypsin (A), chymotrypsin (B), papain (C), and bacterial proteinase (D), respectively, corresponding to ratios of protease-phosphorylase of approximately 1:85. All reactions were run in a Radiometer *p*H-stat at *p*H 8.0; the tryptic attack was carried out in the presence of 0.01 M Ca^{2+} ions. At various times an aliquot sample of 0.3 ml was precipitated with 2.7 ml of 6% trichloroacetic acid and centrifuged; the supernatant solution was tested for radioactivity and ninhydrin-positive material after base hydrolysis. The results are expressed as per cent of total radioactivity (-▲-) and ninhydrin-positive material (-△-) remaining in the precipitate versus time of hydrolysis. Also indicated in the graphs are enzymatic activity measured with (-●-) and without (-○-) AMP, at a 1:500 dilution as indicated under Methods.

sis in 2.5 N NaOH for 2.5 hours according to the procedure of Hirs *et al.* (1956).

RESULTS

Release of Phosphopeptides from Phosphorylase a with Various Proteolytic Enzymes.—It was shown previously (Fischer *et al.*, 1959) that the release of a phosphorylated hexapeptide by trypsin was extremely rapid, occurring while more than 90% of the protein still precipitated in trichloroacetic acid. Since the hexapeptide itself was basic and since, because of the known specificity of trypsin, it had to be adjacent to yet another basic amino acid, the possibility was mentioned that its rapid release could result from an unusually high proportion of positive charges in this particular location. Alternatively, the phosphorylated site of phosphorylase *a* could be in a particularly exposed region of the protein molecule. In order to decide between these alternatives, ^{32}P-labeled phosphorylase *a* was subjected to the action of different proteolytic enzymes. At various times, samples were removed and tested for enzymatic activity, trichloroacetic acid–soluble material, and radioactivity. As can be seen in Figure 1, the rate of release of radioactivity was always greater than the overall rate of proteolysis.

Enzymatic Activity and Properties of Phosphorylase b'.—It is well known that when subtilisin splits a single peptide bond in ribonuclease (Richards, 1958), full enzymatic activity is retained as long as the S-peptide produced remains bound to the bulk of the protein (ribonuclease-A). Separation of S-peptide from ribonuclease-S results in total loss of ribonuclease activity, which is quantitatively recovered by recombination of the two components in equimolar proportions. It appeared of interest, therefore, to determine whether an analogous situation exists in the case of phosphorylase *b'* and the phosphopeptide. Preliminary experiments were carried out to determine the most appropriate procedure to separate the phosphopeptide from the bulk of the labile protein without irreversible inactivation of the latter. To this effect, ^{32}P-labeled phosphorylase *a* was attacked by trypsin; then, after stopping the reaction by addition of an excess of soybean trypsin inhibitor, the reaction mixture was subjected to (a) gel filtration on a column of Sephadex G-25, (b) dialysis against several changes of ice-cold 0.04 M glycerophosphate–0.03 M cysteine buffer, *p*H 6.8, and (c) precipitation with 2 volumes of saturated ammonium sulfate, *p*H 7.0, centrifugation, and dialysis of the precipitate as described above. In these three instances, although losses in specific activity (measured in the presence of AMP) were as high as 70%, increases in the ratio of activity units/cpm of approximately 3-, 6-, and 9-fold, respectively, were observed. It was noted, however, that when the incubation reaction mixture after tryptic attack was brought to *p*H 6.5, a heavy precipitate developed that contained most of the protein present. This procedure was therefore selected for a large-scale preparation of phosphorylase *b'*. To this effect, 352 mg of ^{32}P-labeled phosphorylase *a* (2000 units/mg, 760 cpm/mg) was attacked by 1.17 mg of trypsin (trypsin-phosphorylase, 1:300, w/w) at *p*H 7.8, 30° in a total of 48 ml. After 10 minutes the reaction was stopped by addition of an 8.6-fold excess (10 mg) of soybean trypsin inhibitor. Activity measured in the absence of AMP had fallen to 50 units/mg, indicating approximately 97% conversion to phosphorylase *b'*. Trichloroacetic acid precipitation of an aliquot sample followed by centrifugation showed almost quantitative release of counts in the supernatant solution; both acid and base hydrolysis of this solution and ninhydrin analysis of the hydrolysate showed the presence of less than 2% protein material. This remarkable selectivity of the tryptic attack was confirmed by a fingerprint of the supernatant solution (after elimination of the trichloroacetic acid by ether extraction) which revealed the presence of only two faint, nonradioactive, ninhydrin-positive spots, in addition to the three expected radioactive phosphopeptides A, B, and C, the latter being present in much greater amounts (see Fischer *et al.*, 1959).

The remainder of the protein in the incubation reaction mixture was precipitated by lowering the *p*H to 6.5–6.7 with 0.1 N acetic acid. The suspension was centrifuged, the pellet was suspended in water, dissolved by addition of 0.1 N NaOH to *p*H 7.8, and precipitated by lowering the *p*H to 6.5. This second

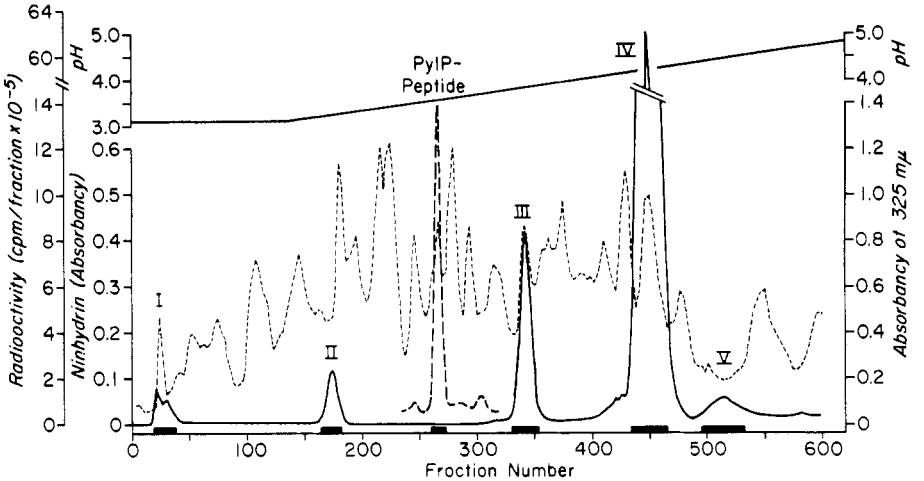

FIG. 2.—Dowex 50-column elution profile of phosphopeptides and pyridoxylphosphate (PylP)-peptide from the chymotryptic digest of ^{32}P-labeled $NaBH_4$-reduced phosphorylase *a*. The effluent fractions were tested for radioactivity (phosphopeptides), ———; absorbancy at 325 mμ (PylP-peptide), — — —; and ninhydrin-positive material, ·····. The shaded areas on the abscissa represent the fractions pooled.

pellet, when taken up in water and adjusted to *p*H 7.8, gave a clear solution containing 216 mg of protein (using an absorbancy index for phosphorylase of 1.19/mg/cm at 278 mμ, *p*H 6.8), corresponding to a recovery of 79%. Activities and counts of both these fractions are listed in Table I. As can be seen,

TABLE I
FORMATION OF PHOSPHORYLASE *b'* FROM ^{32}P-LABELED PHOSPHORYLASE *a*[a]

^{32}P-labeled Phosphorylase *a*	Specific Activity (+AMP) (units/mg)	Ratio Activity −AMP/+AMP	Specific radio activity (cpm/mg)	Units/cpm
Before tryptic attack	2000	0.74	760	2.6
After tryptic attack	1435	0.03	760	1.9
After two precipitations at *p*H 6.5	790	0	14	56

[a] Preparation as described in text.

despite a 60% loss in specific activity, there was a 20-fold increase in the activity units/cpm ratio, clearly indicating that the phosphopeptide released by trypsin is not required for the enzymatic activity displayed by phosphorylase *b'*. The 330 mμ absorbancy, indicative of the presence of pyridoxal-5'-phosphate, was barely visible with phosphorylase *b'*, perhaps as a result of partial resolution of the enzyme. In confirmation of an earlier report (Krebs, 1954) the binding constant of phosphorylase *b'* with AMP was essentially unaffected by the presence of protamine, contrary to what has been observed with phosphorylase *b*. Values of K_{AMP} of 7.4×10^{-5} and 5.0×10^{-5} were found for phosphorylase *b'* when measured in the presence and absence of protamine (125 μg/ml), as compared to 5.0×10^{-5} and 3.0×10^{-6} for phosphorylase *b*. Despite the similar binding constants of phosphorylase *b* and *b'* for AMP, the latter form of the enzyme could not be crystallized in the presence of this nucleotide.

Isolation of Phospho- and Pyridoxylphosphate-Peptides.—The elution diagram obtained from the ion-exchange column fractionation of the chymotryptic digest of $NaBH_4$-reduced ^{32}P-labeled phosphorylase *a* is shown in Figure 2. The radioactive (phosphopeptide) fractions are numbered I–V in order of their emergence from the column. The pooled fractions accounted for 1.5, 1.8, 7.0, 71.0, and 2.5%, respectively, of the radioactive sample applied to the column, for a total recovery in this step of 84%. No additional radioactive bands could be detected on the column. Fraction I was discarded since it was present in very small amount and obviously consisted of more than one radioactive component.

Essentially all the PLP derivative recovered from the column emerged in one peak, as previously reported (Kent, 1959), and in agreement with a fingerprint of the chymotryptic digest, which showed the presence of one major PylP-peptide, and only trace amounts of a second fluorescent spot. A radioautogram of the same fingerprint showed the presence of several phosphopeptides, as found by ion-exchange chromatography. The recovery of PylP-peptide, measured spectrophotometrically at 325 mμ in 0.1 N sodium acetate, *p*H 5.5, was 19.0 μmoles. This value was calculated from the molar absorbancy index of ε-*N*-pyridoxylphosphatelysine, which at *p*H 5.5 is 1.0×10^4 (Forrey, 1963). The amount recovered was 42% of the theoretical amount applied to the column (45 μmoles), based on a molecular weight for phosphorylase of 500,000 and the presence of 4 moles of PLP per mole of protein (Baranowski *et al.*, 1957; Kent *et al.*, 1958). Interference by the protein prevented a reliable direct estimation of the PLP derivatives present in the intact reduced protein and the chymotryptic digest.

Each of the peptide fractions was further purified by gel filtration on a column of Sephadex G-25 in 0.05 M pyridine-acetate buffer at *p*H 3.3, as described under Methods. Further purification of fractions III and V was achieved by passing them through the Sephadex column again, this time using 1.0 M pyridine-acetate, *p*H 8.0, as the eluting buffer. Fractionation of the peptides by gel filtration at both acid and alkaline *p*H was suggested by the work of Porath (1960). The Sephadex column elution diagrams for the PylP-peptide and the major phosphopeptide fractions, III and IV, are shown in Figure 3. Fraction IV was apparently partially resolved into two radioactive components, fractions IV-A and IV-B. A rerun of fractions IV-B, which contained some IV-A, through the Sephadex column at *p*H 3.3 resulted in

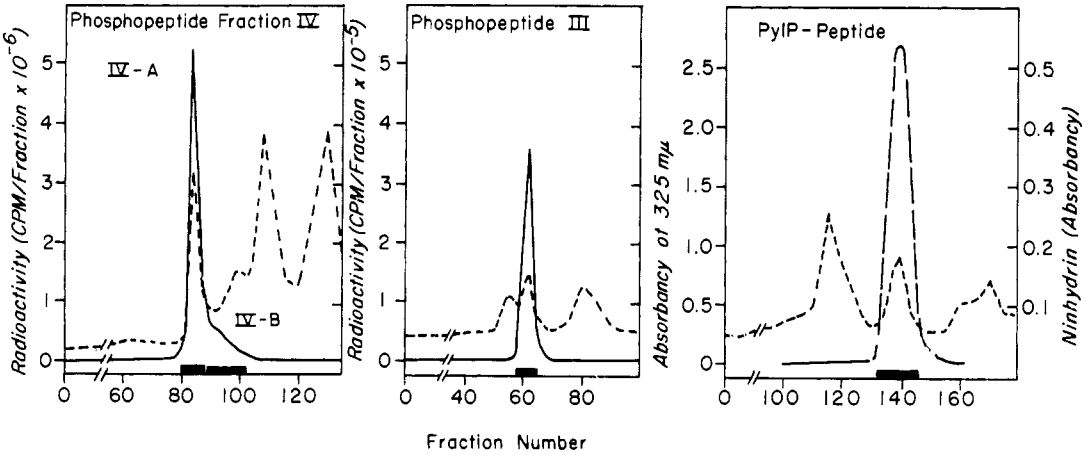

FIG. 3.—Sephadex G-25 column elution profile of phosphopeptides and the pyridoxylphosphate-peptide from the Dowex 50 column. The columns were eluted with pyridine-acetate buffers at *p*H 3.3 or 8.0, as described in the text. Radioactivity, absorbancy at 325 mμ, and ninhydrin-positive material are represented as in Figure 2. The ninhydrin color scale on the ordinate at the far right of the figure applies to all parts of the figure. The scale for radioactivity or absorbancy at 325 mμ is given at the left of each elution profile. The shaded areas on the abscissa indicate the fractions pooled.

nearly complete resolution of the two radioactive components. Although these results indicate that fractions IV-A and IV-B are different substances, they could not be distinguished from each other by various methods as described below.

Fractions II and V were both separated into two or more radioactive components by gel filtration at *p*H 3.3. Only the major component was retained in each case.

For the gel filtration steps, the calculated recoveries for fractions II, III, IV-A, IV-B, and V, based on radioactivity, are 64.7, 77.0, 83.7, 14.4, and 34.4%, corresponding to overall recoveries of 1.1, 4.9, 54.2, 9.3, and 0.5%, respectively. The recovery of the PylP-peptide in this step was 90% (17.2 μmoles), which represents an overall recovery of 38% of theoretical. The recoveries of the phosphopeptides are summarized in Table II.

TABLE II
RECOVERY OF PHOSPHOPEPTIDES FROM ^{32}P-PHOSPHORYLASE *a*

Preparation Step	Per Cent Recovery (as Per Cent of Total Radioactivity)[a]	
	Individual Step	Overall
Chymotryptic digestion	93	93
Ion-exchange chromatography[b]	84	77
Gel filtration[b]	91	70

[a] The recoveries in the paper-electrophoresis-chromatography step used for the final purification of the minor phosphopeptides are not included. [b] Recovery values are the sums of the values for the individual peptide fractions.

Each of the peptide fractions obtained from the gel filtration step was examined for purity by fingerprinting. Electrophoresis was performed at *p*H 6.5 and chromatography in solvent 1, as described above. Under these conditions, fractions IV-A and IV-B behaved as pure substances; only one component could be detected with ninhydrin reagent, which coincided exactly with the radioactive spot. It was noted that fraction II also behaved as a pure component; this peptide, however, did not give a color reaction with ninhydrin on paper or in the Sephadex column effluent.

Fractions III and IV and the PylP-peptide fraction were found to contain small amounts of contaminants and were further purified by electrophoresis on paper at *p*H 6.8. Only enough peptide III for amino acid analysis was purified; for studies with phosphorylase phosphatase the peptide fraction obtained from the gel filtration step was used. The small amount of fraction V isolated appeared to be homogeneous upon paper chromatography in solvent 1; however, it was resolved into several radioactive components in solvent 2. Estimated spectrophotometrically, approximately 16.0 μmoles or 93% of the PylP-peptide was recovered in this step (36% of the amount theoretically obtainable from phosphorylase). However, as estimated from amino acid analysis of the highly purified peptide, approximately 23 μmoles of the peptide was recovered in this step (51% of theoretical). The value obtained by amino acid analysis is probably more reliable because pyridoxylphosphate peptides have been repeatedly shown to undergo changes in their spectral properties (Fischer *et al.*, 1963).

Amino Acid Composition of Peptides.—The amino acid compositions of phosphopeptides III, IV-A, and IV-B are given in Table III. It can be seen that the composition of acid hydrolysates of IV-A and IV-B are the same, each with 14 amino acid residues. Peptide III is an octapeptide with a composition qualitatively very similar to that of the tetradecapeptides.

The amino acid compositions of the small amounts of peptide II and fraction V available were estimated visually on paper following two-dimensional separation by electrophoresis at *p*H 2.2 and chromatography in solvent 1. The approximate composition of peptide II is: serine (1), glutamic acid (1), glycine (1), valine (1–2), isoleucine (1), leucine (1), arginine (1–2). Except for the absence of lysine in this peptide, its composition is very similar if not identical with that of peptide III. The composition of fraction V could not be distinguished from that of peptides IV-A and IV-B, except that it contained small amounts of alanine, tyrosine, and phenylalanine. As already noted, a number of radioactive peptides were present in this fraction.

Problems of stability of the PylP-peptide from

TABLE III

AMINO ACID COMPOSITIONS OF PHOSPHOPEPTIDES IV-A, IV-B, AND III[a]

The analyses were performed on 0.2–0.4 μmole of peptide. The results are not corrected for destruction during acid hydrolysis.

Amino Acid	IV-A		IV-B		III	
	Calculated Molar Ratios	Estimated Residues	Calculated Molar Ratios	Estimated Residues	Calculated Molar Ratios	Estimated Residues
Aspartic acid	1.12	1	1.42	1		
Serine	1.54	2	1.51	2	0.79	1
Glutamic acid	3.00	3	3.01	3	1.06	1
Glycine	0.94	1	0.93	1	0.93	1
Valine	1.01	1	1.16	1	1.00	1
Isoleucine	1.01	1	1.03	1	0.96	1
Leucine	1.02	1	1.10	1	0.98	1
Lysine	1.85	2	1.63	2	1.00	1
Arginine	1.92	2	1.88	2	1.01	1

[a] Peptide IV-A also contained the equivalent of 0.2 residue each of proline and alanine; IV-B contained 0.3 residue of alanine, and III had 0.2 residue of aspartic acid. Other amino acids were absent or present in smaller amounts.

TABLE IV

SUMMARY OF SEQUENCE INFORMATION FOR PHOSPHOPEPTIDE IV-A

Reaction	Peptide Attacked		Product or Results	Electrophoretic Migration[a] (cm)
Sanger	IV-A		DNP-Ser	
Tryptic hydrolysis	IV-A	T-1	Ser-(Asp,Glu(NH_2),Glu)-Lys	−4.8
		T-2	Ser-(Asp,Glu(NH_2),Glu)-Lys-Arg	0.0
		T-3	Arg-Lys	+13.9
		T-4	Lys-Glu(NH_2)-Ileu-Ser(P)-Val-Arg	+2.0
		T-5	Glu(NH_2)-Ileu-Ser(P)-Val-Arg	−1.9
		T-6	Gly-Leu	0.0
Papain hydrolysis	T-1	P-1	Ser-(Asp,Glu[NH_2])	−6.2
		P-2	Ser-(Asp,Gly[NH_2]),Glu	−8.9
		P-3	Glu-Lys	−0.5
Edman	IV-A		Ser-Asp---	
Carboxypeptidase A	IV-A		---Gly-Leu	
	Sequence:		Ser-Asp-Glu(NH_2)-Glu-Lys-Arg-Lys-Glu(NH_2)-Ileu-Ser(P)-Val-Arg-Gly-Leu	+1.0

[a] Distance migrated on paper during electrophoresis at *p*H 6.5 for 50 minutes at 43 volts/cm after correction for electroendosmosis. The plus and minus signs indicate migration toward the cathode and the anode, respectively.

chymotryptic hydrolysates of reduced phosphorylase have left the results of previous studies on this peptide in doubt. In the present study, amino acid analysis of this peptide confirmed the structure as originally reported (Fischer and Krebs, 1959), namely, the substituted dipeptide ε-*N*-pyridoxylphosphatelysylphenylalanine.[5] Studies on the PLP-binding site of phosphorylase will be the subject of a future report.

Amino Acid Sequence of Phosphopeptide IV-A.— The amino acid sequence of peptide IV-A was elucidated by enzymatic and chemical-degradation techniques. Serine was identified as the amino-terminal amino acid by the Sanger method, as described. The peptide (0.5 μmole) was hydrolyzed with trypsin (0.3 mg) at *p*H 8.0 in a total volume of 0.3 ml. After a 2-hour incubation period at 37°, an aliquot was removed and examined by fingerprinting (*p*H 6.5, solvent 1). The remaining hydrolysate was stored frozen. Peptides were located on the paper with ninhydrin reagent, and phosphoserine-containing peptides were identified by radioautography. The peptides, designated T-1 through T-7, were isolated from the remaining hydrolysate and from a duplicate hydrolysate by preparative electrophoresis and chromatography on paper.

Quantitative amino acid analyses of aliquots (approximately 0.1 μmole) of peptides T-2 and T-4 showed molar ratios of aspartic acid (0.94), serine (0.59), glutamic acid (2.02), lysine (1.08), arginine (0.95) for T-2; and serine phosphate (0.89), glutamic acid (1.12), valine (0.99), isoleucine (1.02), lysine (0.85), arginine (1.01) for T-4.

The amino acid compositions of the remaining tryptic peptides were estimated visually from fingerprints. The compositions of peptides T-1 through T-6 are given in Table IV. The composition of T-7, as estimated from fingerprints, was identical with that of T-5. However, the former peptide, present in small amounts, did not give a color reaction with ninhydrin reagent and was more negatively charged at *p*H 6.5 than was the latter peptide.

Only peptides T-4, T-5, and T-7 contained phosphoserine. On the basis of the compositions and electrophoretic properties of these peptides, they were assigned the amino acid sequences previously determined for phosphopeptides C, B, and A, respectively, which were isolated from a tryptic hydrolysate of phosphorylase *a* (Fischer *et al.*, 1959). It was previ-

[5] The sequence, ε-*N*-PylP-Lys-Lys-Phe, recently reported (Hughes *et al.*, 1962b) appears to be incorrect. The error undoubtedly results from the partial decomposition of the pyridoxylphosphatelysyl residue, which is particularly noticeable after irradiation with ultraviolet light and acid treatment.

ously concluded on the basis of its failure to react with ninhydrin or dinitrofluorobenzene and its greater negative charge relative to peptide B, that peptide A arose from peptide B by the cyclization of the amino-terminal glutaminyl residue in B to form a pyrrolidonecarboxylic acid residue, with the resultant loss of the α-amino group. The results of the present studies are consonant with this conclusion.

The specificity of trypsin places the single lysyl residue of peptide T-1 at the carboxyl-terminus, and peptide T-2, by the same reasoning, must have the COOH-terminal sequence, Lys-Arg. Since T-2 has no net charge at *p*H 6.5, the aspartyl or one of the glutamyl residues must occur in the amidated form. Evidence for a glutaminyl residue positioned as shown in Table IV is described in subsequent paragraphs.

In an attempt to complete the amino acid sequence of T-1, it was incubated with papain at *p*H 4.25, since at this *p*H papain is known to split at glutamyl as well as glutaminyl residues (Smith and Kimmel, 1960; Nolan and Smith, 1962). The peptide (0.1 μmole) was incubated with papain (0.2 mg) and BAL (0.01 M) in 0.02 M sodium acetate buffer, *p*H 4.25 (0.1 ml total volume). The incubation was performed at 37° in a small tapered glass tube under nitrogen, which was used to prevent oxidation of BAL. After 3 hours the reaction mixture was subjected to two-dimensional paper electrophoresis-chromatography (*p*H 6.5, solvent 1). Three peptides (P-1, P-2, and P-3), free lysine, and a trace of free glutamic acid were detected on paper with the ninhydrin reagent. The amino acid composition of acid hydrolysates of the peptides, given in Table IV, were estimated from fingerprints.

The acid hydrolysate of P-3 contained equimolar quantities of glutamic acid and lysine. Since lysine is COOH-terminal in T-1, the sequence of the dipeptide can be written as Glu-Lys. A glutamyl rather than a glutaminyl residue is assigned on the basis of its slight anodic migration upon paper electrophoresis at *p*H 6.5. The overlap in the composition of peptides T-1, P-2, and P-3 establishes a glutamyl residue as the carboxyl-terminal residue in P-3, which is in agreement with the relative charges on these peptides.

To complete the sequence of the amino-terminal portion of fraction IV-A, 0.34 μmole of the peptide was subjected to the Edman degradation technique. The PTH-amino acid cleaved at each step was identified directly by chromatography on paper as described under Methods and indirectly by amino acid analysis of an aliquot (0.1 μmole) of the remaining peptide on the amino acid analyzer. The composition of the intact peptide is given in Table III. The molar ratios of the amino acids obtained after each of two degradation steps are:

Step 1: aspartic acid (1.11), *serine (0.89)*, glutamic acid (3.14), glycine (1.00), valine (0.95), isoleucine (1.09), leucine (1.19), lysine (1.54), arginine (1.88).

Step 2: *aspartic acid (0.54)*, serine (0.81), glutamic acid (3.00), glycine (0.98), valine (1.08), isoleucine (1.02), leucine (1.06), lysine (1.64), arginine (1.96).

Identification of the PTH-amino acids on paper showed that the second residue was an aspartyl rather than an asparaginyl residue. This information establishes the sequences of peptide P-1 as Ser-Asp-Glu-(NH_2).

As predicted on the basis of trypsin specificity and the sequence information from peptides T-2 and T-4, arginine was identified as the amino-terminal residue in T-3 by the Sanger method.

The specificity of chymotrypsin would place the single leucyl residue of peptide IV-A at the carboxyl terminus, and thus the sequence of T-6 may be written as Gly-Leu. Confirmation of this sequence was obtained by hydrolysis of peptide IV-A with carboxypeptidase A. A 0.3-ml reaction mixture containing 0.34 μmole of the peptide and 0.2 mg of carboxypeptidase A at *p*H 8.0 was incubated at 39°. After 1 hour an aliquot (0.05 μmole) was removed and examined by fingerprinting. After incubating an additional 2.5 hours, the reaction was stopped by the addition of 0.5 ml of 0.2 N sodium citrate at *p*H 2.2. Leucine and a minute amount of glycine were the only amino acids detected in the 1-hour hydrolysate. Quantitative analysis of the 3.5-hour hydrolysate showed the molar ratio of leucine to glycine to be 1.00 to 0.06. Other amino acids were absent or present in amounts too small to be measured.

From the above sequence information, together with the overlaps provided by the tryptic and papain peptides, as summarized in Table IV, the amino acid sequence of peptide IV-A can be written as shown in the table.

Although peptide IV-B appeared to be distinguishable from IV-A on the basis of its gel-filtration properties, the amino acid compositions of these peptides are identical (Table III). To compare the two peptides further, an aliquot of peptide IV-B was subjected to tryptic hydrolysis and the hydrolysate was examined by fingerprinting under the conditions described for IV-A. No difference between the two peptides could be detected by a comparison of the fingerprints.

Phosphorylase Phosphatase Attack on Phosphopeptides.—Previous studies (Graves *et al.*, 1960) have shown that phosphorylase phosphatase catalyzed the hydrolysis of phosphate from the hexapeptide and pentapeptide (corresponding to phosphopeptides T-4 and T-5, Table IV) isolated from a tryptic hydrolysate of phosphorylase *a*. Rates of hydrolysis of approximately 5 and 4%, respectively, of the rates of hydrolysis of phosphorylase *a* were reported. The enzyme demonstrated an almost absolute requirement for the carboxyl-terminal arginyl residue in these peptides for its activity; little or no hydrolysis occurred after removal of the arginyl residue. Moreover, phosphoserine itself and various phosphoserine-containing peptides were not attacked by the enzyme.

The isolation of phosphopeptides containing 8 (peptide III) and 14 amino acid residues (peptide IV-A) in the present study provided material for further studies on the nature of the enzyme-substrate interaction.

The rates of dephosphorylation of peptides IV-A, III, and T-5 by phosphorylase phosphatase were compared in the following way. Reaction mixtures containing 0.45 μmole of peptide (7225 cpm/μmole) in 0.8 ml of 0.03 M Tris–0.03 M cysteine buffer at *p*H 7.3 and 0.2 ml (0.44 mg) of enzyme were incubated at 30°. The phosphatase preparation used was the DEAE-cellulose column fraction described under Materials. The rate of hydrolysis of *p*-nitrophenylphosphate by this enzyme preparation under the above conditions was negligible. At intervals, aliquots of the reaction mixture with a radioactivity of 650 cpm were removed and pipetted into 0.3 ml of 1 N acetic acid on the surface of columns (0.6 × 3 cm) of Dowex 50-X2 to stop the reaction. The columns, which had been equilibrated with 0.2 N pyridine-acetate buffer at *p*H 3.1, were eluted with 4 ml of this buffer in small portions. The effluents, containing inorganic ^{32}P liberated from the peptide, were concentrated and their radioactivity determined. The re-

maining, unhydrolyzed portions of phosphopeptides IV-A, III, and T-5 were retained on the columns. Examination of the peptide T-5 preparation showed that during isolation it had been converted to the extent of about 50% to the corresponding peptide T-7, in which the amino-terminal glutaminyl residue had undergone cyclization. The latter peptide was not retained on the columns in the above experiment; the values obtained for inorganic ^{32}P in the column effluents were therefore corrected for the presence of this peptide. First-order plots of the results and the first-order rate constants (k_1) are given in Figure 4. From the curves, the reactions appear to follow first-order kinetics.

The Effect of AMP and Caffeine on Phosphorylase Phosphatase Activity.—Sutherland (1951) has reported that phosphorylase-inactivating enzyme (phosphorylase phosphatase) in liver, muscle, and other tissues was inhibited by AMP. With the purified muscle phosphatase, free of adenylic deaminase activity, Hurd and co-workers[2] obtained complete inhibition with 10^{-5} M AMP and measurable inhibition at concentrations as low as 10^{-6} M. Since it is known that the presence of AMP renders phosphorylase *a* more resistant to proteolytic attack (Graves *et al.*, 1960), it appeared likely that the inhibition of the phosphatase reaction resulted from alterations of the substrate (phosphorylase *a*) rather than of the enzyme (phosphatase). To test this possibility, phosphorylase phosphatase activity was measured in the presence and absence of AMP (Table V) using phosphopeptide IV-A as substrate. Activity assays were performed as described above. As seen in the table, no inhibition was observed at concentrations of AMP as high as 10^{-3} M. Only at 10^{-2} M AMP was a slight (23%) inhibition observed.

In view of the report (Sutherland, 1951) that caffeine at 2×10^{-4} M enhances the activity of phosphorylase phosphatase, it was also of interest to test the effect of this methylated purine on the rate of dephosphorylation of a phosphopeptide substrate. This was done with peptide IV-A, and it was observed that the rate of dephosphorylation was the same in the presence of 2×10^{-4} M caffeine as in its absence.

Phosphorylation of Dephosphopeptide IV-A by Phosphorylase b Kinase.—Earlier attempts to phosphorylate the dephosphorylated form of phosphopeptide C (hexapeptide) with phosphorylase *b* kinase and ATP, were unsuccessful (Graves, *et al.*, 1960). In the present study it was found that phosphorylase kinase catalyzed the phosphorylation of dephosphopeptide IV-A (hexadecapeptide), although at a very slow rate.

Dephosphopeptide IV-A was prepared as follows: 0.9 μmole of peptide IV-A was incubated with 0.9 mg of phosphorylase phosphatase at 30° for 3 hours under the conditions described. At the end of the reaction, most of the cysteine was oxidized by bubbling oxygen through the solution after adjusting the *p*H to 8 with 0.1 N NH_4OH, and the resulting cystine was removed by centrifugation. The product was isolated from the supernatant solution by electrophoresis on paper at *p*H 6.5 for 90 minutes at a potential of 43 v/cm. Only the product, a small amount of unhydrolyzed phosphopeptide, and cysteine were detected with ninhydrin. Under the above conditions the phosphopeptide migrated 4.6 cm and the dephosphopeptide 7.2 cm toward the cathode after correction for electroendosmosis. Identification of the dephosphopeptide was confirmed by a comparison of the amino acid compositions of the two peptides by fingerprinting after acid hydrolysis. The above isolation procedure was adopted when preliminary attempts to elute the dephosphopeptide from Dowex 1 or Dowex 50 columns were unsuccessful.

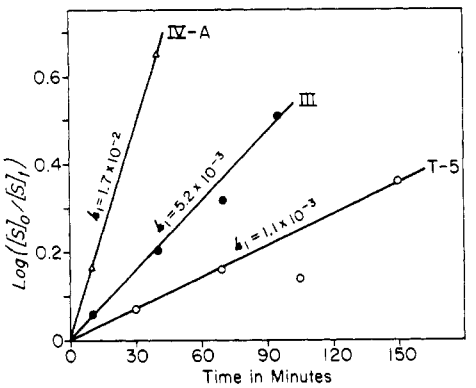

FIG. 4.—Rate of dephosphorylation of the phosphorylated tetradeca-, octa-, and pentapeptides (IV-A, III, and T-5, respectively) from phosphorylase *a* in the presence of phosphorylase phosphatase. The reaction mixtures contained 0.45 μmole of peptide and 0.44 mg of enzyme in a total volume of 1.0 ml at *p*H 7.3, as described in the text. The first-order rate constant, k_1, is given for each reaction.

The phosphorylation reaction mixture contained the following: dephosphopeptide IV-A (approximately 0.02 μmole), 0.17 μmole of γ-labeled [^{32}P]ATP (2.52×10^6 cpm/μmole), 0.05 ml of 0.02 M Tris-HCl buffer at *p*H 8.8, 0.05 ml of phosphorylase *b* kinase (0.018 mg) in 0.001 M cysteine, 0.05 ml of 0.01 M magnesium acetate, and water to a final volume of 0.2 ml. The reaction mixture was incubated at 30° for 60 minutes and then subjected to electrophoresis on paper at *p*H 6.5 for 2 hours at 43 v/cm. One major and three or four minor radioactive components were detected by radioautography; none of these was present in controls run without peptide or without enzyme. The identification of this substance as phosphopeptide IV-A was confirmed by isotopic dilution techniques. The peptide was eluted from the paper, mixed with 0.05 μmole of authentic nonlabeled phosphopeptide IV-A, and again subjected to two-dimensional electrophoresis-chromatography on paper. This time electrophoresis was carried out at *p*H 3.6 for 2 hours at 43 v/cm and chromatography was performed in solvent 2 for 22 hours. The presence of a single, ninhydrin-positive, radioactive substance was detected with ninhydrin reagent and radioautography. Under the above conditions the phosphorylated peptide migrated 20.6 cm toward the cathode during electrophoresis and 6.9 cm during chromatography. As estimated from the radioactivity recovered from the paper, 13% (2.7×10^{-3} μmole of the dephosphopeptide was converted to the phosphorylated form in 1 hour. Assayed under the reaction conditions described for the dephosphopeptide substrate, but with only 1/20 the amount of kinase, an equivalent amount of phosphorylase *b* was converted to phosphorylase *a* at the

TABLE V

THE EFFECT OF AMP ON THE HYDROLYSIS OF PHOSPHOPEPTIDE IV-A BY PHOSPHORYLASE PHOSPHATASE[a]

Final AMP Concentration (M)	Per cent Hydrolysis	Per cent Inhibition
None	44	0
10^{-4}	46	0
10^{-3}	44	0
10^{-2}	34	23

[a] Enzyme activity was measured as described in the text. The reaction time was 30 minutes.

TABLE VI
SUMMARY OF PHOSPHOPEPTIDES FROM PHOSPHORYLASE *a*

Peptide	Structure
IV-A	Ser-Asp-Glu(NH_2)-Glu-Lys-Arg-Lys-Glu(NH_2)-Ileu-Ser(P)-Val-Arg-Gly-Leu
III[a]	Lys-Glu(NH_2)-Ileu-Ser(P)-Val-Arg-Gly-Leu
II[a]	Glu(NH_2)-Ileu-Ser(P)-Val-Arg-Gly-Leu
T-4	Lys-Glu(NH_2)-Ileu-Ser(P)-Val-Arg
T-5	Glu(NH_2)-Ileu-Ser(P)-Val-Arg

[a] Amino acid sequence inferred by analogy with peptides IV-A, T-4, and T-5.

rate of 5.2%/min (under the conditions of the assay, performed as described by Krebs and co-workers,[1] the reaction with phosphorylase *b* follows zero-order kinetics). These results show that the dephosphopeptide is phosphorylated at a rate no greater than 1% that at which phosphorylase *b* is phosphorylated. However, the presence in the peptide-kinase reaction mixture of other ^{32}P-labeled substances not present in a control run without peptide suggests that the tetradecapeptide may have been partially degraded in the reaction mixture, perhaps by proteolytic enzymes still contaminating the purified kinase preparation. A more detailed study of the phosphorylation reaction awaits isolation of larger quantities of dephosphopeptide.

DISCUSSION

Previous studies have shown that the conversion of phosphorylase *b* to *a* by phosphorylase kinase and ATP results in the incorporation of 2 moles of phosphate per mole of phosphorylase *b* (4 phosphate groups incorporated per molecule of phosphorylase *a*) with the concomitant dimerization of the protein (Krebs *et al.*, 1958). It was concluded from studies on phosphoserine-containing peptides isolated from a tryptic hydrolysate of phosphorylase *a* that a unique seryl residue, presumably present in each of four very similar if not identical subunits, was phosphorylated in the conversion reaction (Fischer *et al.*, 1959). Isolation in high yield of the closely related phosphopeptides from the chymotryptic digest of phosphorylase *a* described here, which extends the amino acid sequence to 14 residues, supports this conclusion.

Susceptibility of phosphorylase *a* to proteolytic enzymes appears to vary considerably from one preparation to another, even though precautions are taken to subject the various phosphorylase samples to identical treatment prior to proteolytic attack (complete removal of AMP on charcoal in the presence of cysteine or other SH— compounds and removal of the mercaptopeptides by dialysis). This variation could perhaps depend upon the past history of the particular enzyme sample, the time after which crystalline phosphorylase *b* had been converted to phosphorylase *a*, the age of the phosphorylase *a* crystals, etc. Nonetheless, a study of the action pattern of various proteolytic enzymes on ^{32}P-labeled phosphorylase *a* has shown that in each case the rate of release of phosphopeptides was greater than the overall rate of proteolysis (Fig. 1). This and the ready availability of the phosphate ester bond to phosphorylase phosphatase strongly suggest that the phosphorylated site is in a particularly exposed position on the enzyme surface or, perhaps, in a less highly organized region of the molecule.

Selectivity was most remarkable in the case of trypsin, which produced phosphorylase *b'* (a form of the enzyme requiring AMP for activity) rapidly and in very high yields. No such AMP-dependent active material was produced by either chymotrypsin, papain, or bacterial proteinase; in the last case, loss of enzymatic activity (measured with or without AMP) was almost immediate.

No further information has been obtained on the relationship between phosphorylation and dimerization of phosphorylase *b*. Although the exposed nature of the phosphorylated sites suggests that they may be in a position to participate directly in the binding of the two protein moieties (perhaps as a consequence of the partial neutralization of a preponderance of positive charges in the immediate vicinity of the phosphoseryl residue), there is no evidence that this is so. On the contrary, study of the role of AMP in the activation and structure of phosphorylase that will be reported elsewhere rather suggests that phosphorylation of the protein might bring about conformational changes which expose the necessary binding sites.

The phosphopeptides isolated from phosphorylase *a* are summarized in Table VI. Although the amino acid sequences of phosphopeptides II and III were not determined, their compositions and the known sequences of peptides IV-A, T-4, and T-5 are consonant with the sequence assigned. It is evident that these peptides arose in small amounts, as a result of a trypticlike split in the chymotryptic digest, even though soybean trypsin inhibitor had been added. Since peptide II did not react with the ninhydrin reagent, the amino-terminal glutaminyl residue must have undergone cyclization, as previously reported in the case of T-5.

In a previous publication (Graves *et al.*, 1960) it was reported that the dephosphorylation of the phosphopenta- and phosphohexapeptides by phosphorylase phosphatase proceeded at a much slower rate than that at which phosphorylase *a* was attacked (4 and 5% of the latter, respectively). The values reported here (0.2, 0.5, 0.8, and 2.6% for the penta-, hexa-, octa-, and tetradecapeptide, respectively) are still lower, perhaps because a more extensive purification of phosphorylase phosphatase had been achieved meanwhile. Interesting, however, is the parallelism observed between size of the peptide substrate and the rate of reaction. The octapeptide (III) differs from the hexapeptide (T-4) in having a Gly-Leu sequence at the carboxyl terminus. The higher reaction rate of III may not be attributable to the Gly-Leu sequence per se, but rather to the fact that the free carboxyl group of the arginyl residue in the hexapeptide is blocked in the octapeptide. As already mentioned (Graves *et al.*, 1960), the phosphatase demonstrates a nearly absolute requirement for this arginyl residue. It would be of interest to know if the increase in rate reflects a particular affinity of the phosphatase for specific groups on the phosphopeptides which, according to Koshland's (1958, 1959a,b) "induced-fit" theory, would induce conformational changes in the enzyme resulting in the proper alignment of catalytic groups, or if an increase in size of the peptide chain alone would be sufficient, irrespective of the sequence of the amino acid side chains.

The demonstration that the presence of AMP in concentrations as high as 10^{-3} M had no effect on the

rate of reaction of phosphorylase phosphatase with the tetradecapeptide, whereas reaction with phosphorylase *a* as substrate was completely inhibited by 10^{-5} M AMP, clearly indicates that the nucleotide exerts its inhibitory effect by reacting with the protein substrate rather than with the enzyme itself. This result was not unexpected since it was already known that AMP, in addition to inhibiting the phosphorylase *a* to *b* conversion, brings about alterations in the phosphorylase molecule, resulting in considerably greater resistance to tryptic hydrolysis (Fischer *et al.*, 1959) and urea denaturation (Appleman, 1962).

The effect of caffeine on phosphorylase phosphatase was measured at only one concentration of the purine (2×10^{-4} M), shown by Sutherland (1951) to stimulate the dephosphorylation of active phosphorylase. Nonetheless, the finding that dephosphorylation of the tetradecapeptide was not enhanced under these conditions indicated that, here again, the effect of this agent is on the protein substrate rather than on the enzyme. Kihlman and Overgaard-Hansen (1955) have reported that muscle phosphorylase is almost completely inhibited by 10^{-2} M caffeine, and that the inhibition is reversed by AMP. However, at the concentration present in the phosphatase reaction mixtures, little or no inhibition of phosphorylase activity could be observed (W. B. Novoa, unpublished results). This suggests that caffeine may exert its activating effect on the phosphorylase phosphatase activity toward phosphorylase *a* by competing with endogenous AMP for binding sites on the phosphorylase molecule, and thereby reversing the AMP inhibition.

Phosphorylation of the dephosphotetradecapeptide by phosphorylase kinase and ATP clearly indicates that this reaction does not display an absolute requirement for a particular conformation of the protein substrate, but is dependent on the chemical composition of the peptide. Unfortunately, scarcity of the dephosphopeptide has not permitted further investigation of this reaction from a kinetic or mechanistic point of view. It would be of great interest to determine if certain of the conditions or factors affecting the phosphorylation of phosphorylase *b* (requirement for an "activated" form of kinase, difference between activity at *p*H 6.8 and 8.6, etc.[1]) are also operative when the dephosphopeptide is used as substrate. It may well be that, as shown here to be the case with phosphorylase phosphatase, some of these factors are only involved when the phosphorylase *b* protein is used as substrate.

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