The Purification and Properties of Rat Muscle Glycogen Phosphorylase*

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ABSTRACT: The purification and crystallization of rat muscle glycogen phosphorylase (α-1,4-glucan:orthophosphate glucosyl transferase, EC 2.4.1.1) are described and its properties compared to those of rabbit muscle phosphorylase. The purified enzyme has a specific activity of 85 ± 5 units per mg, corresponding to a turnover number of 15,700 moles of substrate consumed/mole of enzyme dimer per min when measured at 30° in the direction of glycogen synthesis. Molecular weights of 185,000 and 350,000 were calculated for the *b* (dimer) and *a* (tetramer) forms of the enzyme, both by sedimentation velocity and diffusion and by equilibrium measurements. As found for rabbit muscle phosphorylase (Seery, V. L., Fischer, E. H., and Teller, D. C. (1967), *Biochemistry 6*, 3315) the dimeric form of the enzyme appears to be slightly associated and the tetrameric form slightly dissociated. Amino acid analyses carried out on three separate preparations of rat phosphorylase gave essentially identical results. Comparison with amino acid analyses performed under similar conditions on rabbit phosphorylase indicated a considerable degree of similarity between the two muscle enzymes. Whereas no amino- or carboxyl-terminal group had been detected in any phosphorylase so far investigated, 1 equiv of isoleucine/enzyme monomer was released by carboxypeptidase A attack, without loss of enzymatic activity. Subsequent digestion with carboxypeptidase B released a single lysyl residue, indicating that rat phosphorylase possesses a lysylisoleucine sequence at its carboxyl end.

A phosphorylated tetradecapeptide was isolated from the site involved in the phosphorylase *b* into *a* conversion. Its amino acid sequence was found to be identical with that of a phosphotetradecapeptide isolated from rabbit muscle phosphorylase (Nolan, C., Novoa, W. B., Krebs, E. G., and Fischer, E. H. (1964), *Biochemistry 3*, 542), except for the conservative substitution of an aspartyl for a glutamyl residue found in the rabbit phosphopeptide. The above physical and chemical properties together with the finding that the interconversions of the *a* and *b* form of rat muscle phosphorylase are catalyzed by rabbit muscle phosphorylase kinase (EC 2.7.1.38) and phosphatase (EC 3.1.3.17) indicate extreme similarity between the two systems.

Of all the phosphorylases so far investigated, that from rabbit skeletal muscle has been the most thoroughly studied, so much so that this enzyme has come to be used as a standard reference in characterizing phosphorylases from other sources. Indeed, much is known about its chemical and physical characteristics and its complex mechanism of control has been extensively investigated. On the other hand, the rabbit does not lend itself too well to metabolic studies involving the intact animal; perhaps for this reason, and except for early studies by Velick and Wicks (1951) on the turnover of the enzyme, few such studies have been undertaken. And yet, several interesting problems concerning the biosynthesis of this protein suggest themselves, particularly as they may be related to the metabolism of pyridoxal 5′-phosphate. It is known for instance that more than 50% of the total vitamin B_6 present in skeletal muscle is bound to glycogen phosphorylase in the form of pyridoxal 5′-phosphate (see Krebs and Fischer, 1962), and yet, the exact role of this cofactor in conferring enzymatic activity to the protein is still obscure: both a direct catalytic role and one in which pyridoxal 5′-phosphate functions in stabilizing the active conformation of the enzyme have been postulated. Illingworth *et al.* (1960) and Eisenstein (1962) have shown that the level of muscle phosphorylase falls when rats are kept on a vitamin B_6 deficient diet. It appeared of interest to investigate the mechanism by which the biosynthesis and intracellular degradation of the enzyme is controlled, and whether or not the cofactor is involved in certain phases of this regulation.

With this purpose in mind, the isolation and characterization of rat muscle phosphorylase was undertaken. This manuscript reports the purification and crystallization of the enzyme; its physical and chemical properties including molecular weight determination, amino acid composition, pyridoxal 5′-phosphate content, end-group analysis, and sequence around the seryl residue involved in the phosphorylase *b* into *a* conversion have been determined and compared with those of rabbit

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muscle phosphorylase. This manuscript is the sixth of a series on the comparative studies on glycogen phosphorylase; for the previous work, see Appleman *et al.* (1963).

Materials and Methods

Male Sprague–Dawley rats were obtained from Lab Associates, Inc., Kirkland, Wash., and Simonsen Laboratory, Gilroy, Calif. Whatman DE-52 cellulose ion exchanger and Whatman No. 3MM chromatography paper were obtained from H. Reeve Angel, Inc., Clifton, N. J. Maleic anhydride and β-mercaptoethanol were obtained from Eastman Organic Chemicals; bovine serum albumin and pyridoxal 5′-phosphate from Sigma Chemical Co.; AMP and ATP from Pabst Research Biochemicals; sodium β-glycerophosphate and L-cysteine hydrochloride from Nutritional Biochemicals Co. Glucose 1-phosphate (Nutritional Biochemical Co.) was further purified by precipitation of contaminating P_i with $BaCl_2$, followed by removal of excess Ba^{2+} ions with K_2SO_4. The material obtained was recrystallized as the potassium salt from alcohol–water mixtures. Shell fish glycogen was obtained from Krishell Laboratories, Inc., Portland, Ore., and further purified by the Somogyi (1957) procedure. Dowex resins were obtained from Bio-Rad Laboratories and further treated as described by Schroeder (1967). Pyridine, *N*-ethylmorpholine, α-picoline, and phenyl isothiocyanate were redistilled. Precoated silica gel thin-layer plates (F 254) were obtained from Brinkmann Instruments, Inc.; dimethylaminonaphthalenesulfonyl (dansyl) chloride from Sigma Chemical Co.; L-(1-tosylamido-2-phenyl)-ethyl chloromethyl ketone treated trypsin from Gallard Schlesinger Chemical Manufacturing Co.; chymotrypsin, soybean trypsin inhibitor, and diisopropylphosphorofluoridate-treated carboxypeptidases A and B from Worthington Biochemicals; and Sephadex G-25 and G-50 from Pharmacia Fine Chemicals, Inc., Piscataway, N. J.

Enzyme Assay. Phosphorylase activity was routinely determined in the direction of glycogen synthesis by the procedure of Hedrick and Fischer (1965), in which one unit of activity is defined as the amount of enzyme causing the release of 1 μmole of P_i from glucose 1-phosphate per min at 30°, pH 6.5. Specific activities of approximately 85 units/mg were obtained for phosphorylase *b* in the presence of AMP and less than 0.5 unit/mg in its absence. For phosphorylase *a*, values of 85 and 56 units per mg were obtained with and without AMP, respectively.

Protein was determined by the procedure of Lowry *et al.* (1951), the microbiuret method of Itzhaki and Gill (1964), or by absorbancy at 280 mμ using an absorbancy index of $A_{280}^{1\%}$ of 12.5, as determined on the crystalline enzyme. In the latter instance, protein concentration was determined by the procedure of Walsh and Brown (1962) and refractometrically by use of the ultracentrifuge.

Amino acid analyses were performed by the procedure outlined by Moore and Stein (1963) using norleucine as an internal standard (Walsh and Brown, 1962). Three-times-recrystallized phosphorylase was dissolved in and extensively dialyzed against 5×10^{-2} M NaCl, then against several changes of twice-distilled water before analysis. Hydrolyses were carried out *in vacuo* (after repeated flushing with nitrogen) on triplicate samples (2.5 mg) obtained from different preparations of the enzyme at 108 ± 1° for periods of 16, 24, 48, 72, and 96 hr, according to the procedure of Moore and Stein (1963). After removal of HCl in a vacuum desiccator, the samples were analyzed on a Spinco Model 120 automatic recording amino acid analyzer. Supporting analyses (cysteine or cystine, tryptophan, and pyridoxal 5′-phosphate) were also performed on triplicate samples obtained from different preparations of the enzyme. Half-cystine was determined as cysteic acid following performic acid oxidation as described by Moore (1963), while free sulfhydryl groups were determined by the procedure of Ellman (1959) on native and sodium dodecyl sulfate denatured enzyme in which aliquot samples of the protein (3–5 mg, dialyzed as described for amino acid analysis) were titrated with 5,5′-dithiobis(2-nitrobenzoic acid). The final concentrations of reactants were $1–3 \times 10^{-5}$ M phosphorylase,[1] 6.2×10^{-4} M 5,5′-dithiobis(2-nitrobenzoic acid), 7×10^{-2} M sodium phosphate (pH 8.0), and 0.9% sodium dodecyl sulfate when added. Absorbancy readings were taken at 2-min intervals until the values remained constant.

Tryptophan was determined by both the spectrophotometric method of Bencze and Schmid (1957) and the chemical procedure of Spies and Chambers (1949).

Pyridoxal 5′-phosphate was determined by the phenylhydrazine method of Wada and Snell (1961). Pyridoxal 5′-phosphate was released from phosphorylase (1–5 mg) by precipitating the protein with 3×10^{-1} M perchloric acid (final concentration). The mixture was left standing at room temperature for 30 min to ensure complete release of the cofactor from the protein; the precipitated enzyme was then removed by centrifugation and the supernatant was analyzed.

Disc gel electrophoreses were run as described by Ornstein and Davis (1963); activity stains were carried out according to Davis *et al.* (1967) following a modification of the procedure of Allen and Hynick (1963).

Sedimentation and Molecular Weight Studies. Triplicate equilibrium sedimentation runs were carried out using multiple cells with 2-mm solution columns according to the procedure of Yphantis (1964). The runs were performed at 20° at a speed of 11,000 rpm for phosphorylase *a* and 13,000 rpm for phosphorylase *b* in the Spinco Model E analytical ultracentrifuge with interference optics, until equilibrium was attained. The protein concentration was 0.5 mg/ml in a 2×10^{-2} M sodium glycerophosphate–3×10^{-2} M β-mercaptoethanol–5×10^{-2} M NaCl buffer (pH 6.8). A modified Nikon microcomparator was used to measure the photographic plates of the equilibrium pattern. Data were processed on the 7094 computer with a program prepared by Teller *et al.* (1969). A partial specific volume of

[1] Unless otherwise stated, the molarity of phosphorylase will be expressed as that of the monomer, *i.e.*, 92,500 daltons/l.

0.736 cc/g obtained from the amino acid composition of the enzyme was used in the calculations.

Sedimentation velocity experiments were carried out at protein concentrations of 2, 3, 4, 5, 7, and 9 mg per ml, and the sedimentation coefficients calculated by rectilinear extrapolation to zero concentration using the least-squares procedure. The diffusion coefficient was determined using the synthetic boundary method by running samples at low speed in the ultracentrifuge; six protein concentrations were also used to allow for extrapolation to infinite dilution.

Interconversions of Phosphorylases b and a. Rat muscle phosphorylase *b* was converted into the *a* form and crystallized according to the method of Krebs *et al.* (1964) for the rabbit enzyme using purified rabbit phosphorylase kinase. When γ-labeled [^{32}P]ATP was used in the conversion and ^{32}P incorporation was measured, aliquots were removed and precipitated with trichloroacetic acid. The precipitate was dissolved in 0.1 N NaOH, reprecipitated in and washed once more with trichloroacetic acid, and finally redissolved in 90% formic acid. The protein concentration of the formic acid solution was determined spectrophotometrically, and the radioactivity was measured by counting an aliquot in a dioxane scintillant with the Packard Tri-Carb scintillation counter. Phosphorylase *a* was reconverted into the *b* form by the action of purified rabbit muscle phosphorylase phosphatase using the procedure of Hurd (1967).

End-Group Analyses. Amino-terminal determinations were performed according to the carboxylation procedure of Stark and Smyth (1963). Lyophilized phosphorylase (0.5–0.75 μmole)[1] was dissolved in 5 ml of 8 M urea containing 1.2 M *N*-ethylmorpholine acetate (pH 8.0) and carbamylation was carried out with KCNO for 12 hr at 50°. A control sample of phosphorylase was subjected to the same treatment except that no urea or cyanate was added.

Carboxyl-terminal analyses were performed both by hydrazinolysis according to the procedure of Braun and Schroeder (1967) and carboxypeptidase digestion. Anhydrous hydrazine was prepared by vacuum distillation of 95% hydrazine (City Chemical Co., New York) over NaOH pellets. Phosphorylase was dialyzed extensively first against 0.1 M KCl, then distilled water. Aliquots (25 mg, 0.27 μmole) were transferred to thick-walled hydrolysis tubes, lyophilized, and further dried for 24 hr at 65° over P_2O_5 in a vacuum oven. Dry Amberlite CG-50 (50 mg) was added to each sample followed by 1–2 ml of anhydrous hydrazine. The tubes were flushed with nitrogen, sealed under vacuum, and heated for various lengths of time at 80°. The free amino acids produced during the reaction were separated from the hydrazides by chromatography on Dowex 50-X2 and the fractions were analyzed on the Spinco amino acid analyzer. For carboxypeptidase digestion, phosphorylase *b* (0.2–1 μmole) was dialyzed against several changes of 0.2 M *N*-ethylmorpholine acetate (pH 8.0) and then treated with varying proportions of carboxypeptidase A, B, or both at room temperature. At various time intervals, aliquots containing 0.2 μmole of phosphorylase were removed and precipitated with 25% trichloroacetic acid to stop the reaction. After the protein was removed by centrifugation, the supernatant was extracted three times with an equal volume of ether to remove excess trichloroacetic acid, dried in a vacuum desiccator, and analyzed for free amino acids on the amino acid analyzer. Controls containing carboxypeptidase alone were treated in a similar way. When sodium dodecyl sulfate denatured phosphorylase was used, the same procedure was followed except that 0.5% sodium dodecyl sulfate was included in the dialysis step and during the digestion.

Separation and Identification of Peptide. For peptide mapping, 0.1–0.5 μmole of material was applied to Whatman No. 3MM paper (56 × 47 cm) and chromatographed in butanol–acetic acid–water (4:1:5, v/v) using phenol red as a marker. The paper was dried, then subjected to electrophoresis at pH 3.6 (pyridine–acetic acid–water, 1:10:289, v/v) for 1 hr at 2500 V. The peptide maps were developed by dipping in a 0.25% ninhydrin solution in acetone. When ^{32}P-labeled peptides were examined, autoradiograms were prepared with Kodak Medical X-Ray Film.

For ion-exchange chromatography, columns of Dowex 50W-X2 or Dowex 1-X2 (0.6 × 60 cm) were prepared as described by Schroeder (1967), and developed with volatile buffers. Peptides were determined in the column effluent by subjecting aliquots to alkaline hydrolysis followed by reaction with ninhydrin as described by Hirs *et al.* (1956).

Amino acid sequences in peptides were determined using Edman's phenyl isothiocyanate reaction for sequential degradation plus dansylation for amino-terminal group determination (Gray, 1967). Dansylamino acids were separated by either thin-layer chromatography on precoated silica gel plates in a solvent system consisting of chloroform–ethanol–acetic acid (38:4:3, v/v) or by paper electrophoresis at pH 3.6 at 2500 V for 1–3 hr.

Results

Purification and Crystallization of Rat Striated Muscle Phosphorylase. Male Sprague–Dawley rats were stunned by a blow on the head and decapitated. The animals were rapidly skinned and eviscerated; the muscle removed from the bones, placed in ice, ground in a meat grinder using a coarse plate, and homogenized for 1 min in 2.5 volumes of 10^{-3} M sodium glycerophosphate (pH 7.5). The homogenate was left standing at room temperature for 15 min, filtered through two layers of cheesecloth, then through glass wool, and the filtrate was cooled in ice. The pH, usually about 6.5, was lowered to 5.4 by slow addition of 1 N acetic acid. The resulting turbid suspension was centrifuged at 1400*g* for 40 min in an International Model PR-2 centrifuge. The supernatant was filtered through large, coarse, fluted filter paper (S & S, No. 520-5-1/2), and the pH was raised to 6.8 by addition of solid $KHCO_3$.

The above solution was brought to 0.41 saturation by addition of 800 ml of ammonium sulfate (saturated at 0°) per l. of the supernatant. This suspension was left standing overnight during which time the phosphorylase

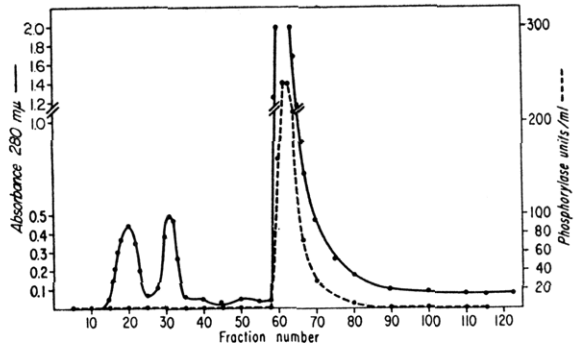

FIGURE 1: Elution pattern of rat skeletal muscle phosphorylase *b* from Whatman DE-52 cellulose column chromatography. The dialyzed ammonium sulfate fraction containing 350 mg of phosphorylase *b* in a total of 1.2 g of protein was applied to a 2.5 × 100 cm column and eluted as described in the text; 10-ml fractions were collected.

sedimented. The clear supernatant was removed by suction, and the precipitate was centrifuged at 3500*g* for 40 min in a Lourdes or Sorvall RC-2 centrifuge. The pellets were collected, redissolved in a minimum volume of 5×10^{-3} M sodium glycerophosphate (pH 7.0), and either dialyzed overnight against the above buffer or passed through a Sephadex G-25 column in order to remove the remaining ammonium sulfate.

The protein fraction (approximately 15 mg/ml) was spun once more at 3500*g* for 40 min to remove some denatured protein; the supernatant solution was applied to a 2.5 × 50 cm DEAE-cellulose column that had been equilibrated with 2×10^{-3} M sodium glycerophosphate–2×10^{-3} M β-mercaptoethanol–10^{-3} M EDTA (pH 6.8). A linear gradient was set up by slowly mixing 1 l. each of the initial and final buffer (containing 5×10^{-2} M sodium glycerophosphate–5×10^{-2} M mercaptoethanol–10^{-3} M EDTA, pH 6.8); Figure 1 shows a typical elution pattern.

The fractions containing phosphorylase activity were combined and concentrated to a protein concentration of approximately 30 mg/ml in an Amicon ultrafiltration cell (Amicon Co., Cambridge, Mass.) using a UM-1 Diaflo membrane. Magnesium acetate and AMP were added to the protein solution to yield final concentrations of 5×10^{-2} and 5×10^{-3} M, respectively. The enzyme crystallized upon standing on ice for several hours. Dissolution of the crystals and recrystallization were carried out as described for the rabbit muscle enzyme (Fischer *et al.*, 1958).

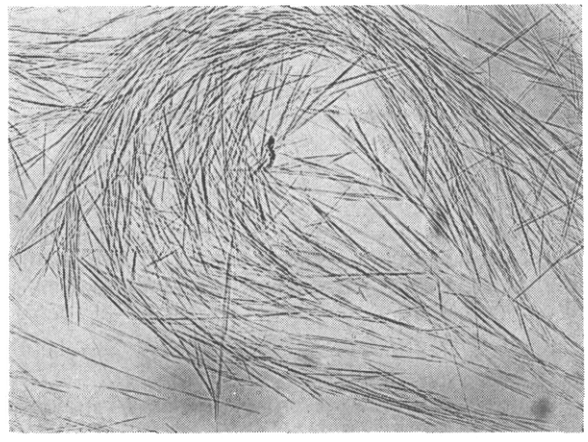

FIGURE 2: Crystalline phosphorylase *b* from rat skeletal muscle; ×600.

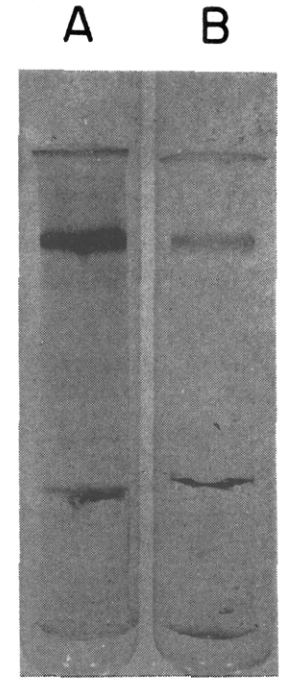

FIGURE 3: Acrylamide gel electrophoresis of purified phosphorylase *b* from rat muscle. Gel (6%) and protein (100 μg) were applied per gel. A, protein stain; B, activity stain.

A typical purification is summarized in Table I and the crystals are shown in Figure 2; acrylamide gel patterns of the crystalline phosphorylase are presented in Figure 3.

Amino Acid Analyses. Amino acid analyses of rat muscle phosphorylase were carried out on three separate preparations of the crystallized enzyme at five times of hydrolysis. For the sake of simplification, only the averages for each series are given in Table II. As usual, simple averages over the five times of hydrolysis were taken for most amino acids. For serine, threonine, and ammonia, extrapolation to zero time of hydrolysis was carried out, whereas for valine and isoleucine, maximum value obtained after prolonged hydrolysis was used. When the values obtained were compared to those previously reported for rabbit muscle phosphorylase, slight differences were observed. Since the analysis of the latter enzyme was carried out several years ago using a one-column chromatographic system (Technicon autoanalyzer), it was felt desirable to repeat the analysis under precisely the same conditions as described above for the rat enzyme. For the sake of simplification, only the averages for the rabbit protein are presented in Table II. In Table III, a comparison is made of the integral number of residues for the rat enzyme with that of other phosphorylases, calculated on the basis of a monomer molecular weight of 92,500 for all enzymes.

Sulfhydryl Groups. The number of sulfhydryl groups

TABLE I: Purification of Rat Skeletal Muscle Glycogen Phosphorylase.[a]

Fraction	Vol (ml)	Total Act. Units	Protein (g)	Sp Act.	% Yield
Crude extract	4500	1.47×10^4	47.8	3.1	100
Neutralized acid supernatant	4300	1.23×10^4	25.0	4.9	83
0.41 ammonium sulfate pellet	64	0.81×10^4	2.7	30.6	55
Pooled and concentrated fraction from DEAE-cellulose chromatography	25	0.64×10^4	0.8	81.0	45
First crystals				80.5	
Second crystals				82.0	

[a] The starting material was 2100 g of skeletal muscle from male Sprague–Dawley rats.

TABLE II: Amino Acid Composition of Glycogen Phosphorylase.

Amino Acid	Rat Phosphorylase (moles/92,500 g) Av of Series 1	Av of Series 2	Av of Series 3	Total Av	Rabbit Total Av
Lysine	43.1	43.5	43.6	43.41 ± 0.29	43.44 ± 0
Histidine	19.2	19.9	19.4	19.49 ± 0.36	19.29 ± 0.19
NH_3[a]	74.6	82.5	78.8	78.62 ± 2.67	71.35 ± 2.43
Arginine	59.8	60.3	60.4	60.17 ± 0.27	58.98 ± 0.94
Aspartic acid	96.0	93.8	95.2	95.03 ± 0.60	90.83 ± 1.30
Threonine[a]	31.9	31.5	31.1	31.49 ± 0.39	31.65 ± 0.18
Serine[a]	29.3	27.6	27.5	28.15 ± 0.6	27.34 ± 0.75
Glutamic acid	91.3	90.1	91.2	90.88 ± 0.74	94.38 ± 1.12
Proline	37.3	34.9	35.9	36.02 ± 0.90	39.32 ± 0
Glycine	45.3	43.9	44.7	44.65 ± 0.45	45.88 ± 0.18
Alanine	59.4	57.4	58.9	58.58 ± 0.82	60.30 ± 0
Valine[a]	53.6	51.6	53.5	52.91 ± 0.85	56.74 ± 0.56
Methionine	22.5	22.6	21.6	22.23 ± 0.47	20.22 ± 0.37
Isoleucine[a]	48.1	46.5	49.8	48.04 ± 1.8	44.94 ± 0.38
Leucine	74.3	71.6	74.7	73.53 ± 1.2	75.28 ± 0.37
Tyrosine	34.4	33.9	34.4	34.24 ± 0.21	33.90 ± 0.18
Phenylalanine	35.9	34.4	35.2	35.15 ± 0.49	35.58 ± 0.37
Half-cystine[b]	7.97	7.94	7.33	7.72 ± 0.25	7.86 ± 0.5
Tryptophan[b]	13.0	14.1	12.5	13.20 ± 0.57	11.95 ± 0.43
Pyridoxal 5′-phosphate	0.94	0.93	0.96	0.94 ± 0.02	0.95 ± 0.03

[a] Determined as described in text. [b] Determined as described under Methods.

present in the protein was determined by the Ellman procedure as described in the Methods section. Figure 4 shows the time course of a typical titration of the enzyme in the presence and in the absence of sodium dodecyl sulfate. For the native enzyme 3.2 ± 0.2 sulfhydryls were titrated per 92,500 g, while 7.2 ± 0.3 groups were found for the sodium dodecyl sulfate denatured protein. Performic acid oxidation of rat phosphorylase yielded 7.6 ± 0.4 cysteic acid residues per 92,500 g. From these data a value of eight half-cystines per enzyme monomer appears most reasonable, with no indication for the presence of disulfide bonds.

Pyridoxal 5′-Phosphate. Rat muscle phosphorylase was shown to contain 1.0 ± 0.1 mole of pyridoxal 5′-phosphate per 92,500. The *b* form of the enzyme could be resolved under the exact conditions described by Shaltiel *et al.* (1966) for the resolution of rabbit muscle phosphorylase *b;* in both instances, loss of enzymatic activity paralleled loss of pyridoxal phosphate. Resolution was first order, with a half-life of 6.3 min similar to that reported for the rabbit enzyme. The apoenzyme had a residual activity ranging from 0 to 5% of that of the native enzyme depending upon the duration of the resolution and also on such factors as trace contamina-

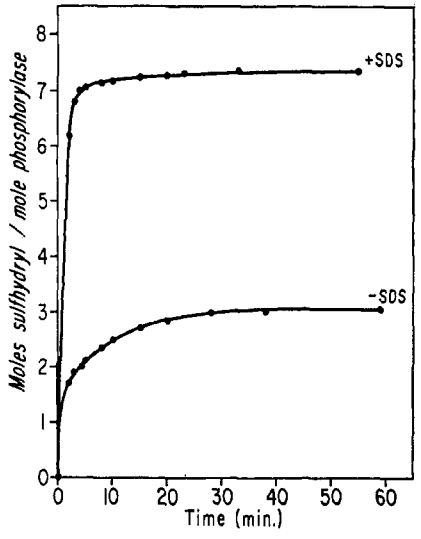

FIGURE 4: 5,5′-Dithiobis(2-nitrobenzoic acid) titration of rat muscle phosphorylase *b* in the presence or absence of sodium dodecyl sulfate. Conditions are described under Methods.

tion by AMP which was shown to block resolution. Residual activity corresponded to residual PLP in the protein. Usually phosphorylase activity could be fully restored by incubating the apoprotein for 10 min at 37° with pyridoxal phosphate at pH 7.0 in a buffer containing either β-mercaptoethanol or L-cysteine (Hedrick *et al.*, 1966). The apoprotein could be titrated with pyridoxal phosphate, showing exact proportionality between the amount of pyridoxal phosphate added and the appearance of enzymatic activity (Figure 5).

Molecular Weight. High-speed sedimentation equilibrium experiments were carried out on rat phosphorylase *b*. Figure 6 illustrates a representative distribution of molecular weights as functions of protein concentration. Weight-average molecular weight (M_w = 195,534) and number-average molecular weight (M_n = 193,606) (average of three cells) were obtained, similar to the values described for rabbit muscle phosphorylase *b* (Seery *et al.*, 1967). This close agreement between these values provides further evidence for the homogeneity of the preparation.

By contrast, high-speed sedimentation equilibrium runs on phosphorylase *a* yielded significant differences between number- and weight-average molecular weights (346,000 and 367,000, respectively), indicating molecular heterogeneity of the preparation. A similar conclusion was drawn from diffusion experiments described below. Since there was absolutely no indication of phosphorylase *b* (or any other) contamination in the phosphorylase *a* preparation, heterogeneity was attributed to dissociation of tetrameric phosphorylase *a* to a dimeric species. Earlier ultracentrifugation studies carried out on rabbit muscle phosphorylase *a* led to the same interpretation (Seery *et al.*, 1967). The dissociation of phosphorylase *a* to an active dimeric species under the influences of salts, glycogen, or glucose was previously described by Wang and Graves (1963, 1964) and Wang *et al.* (1965).

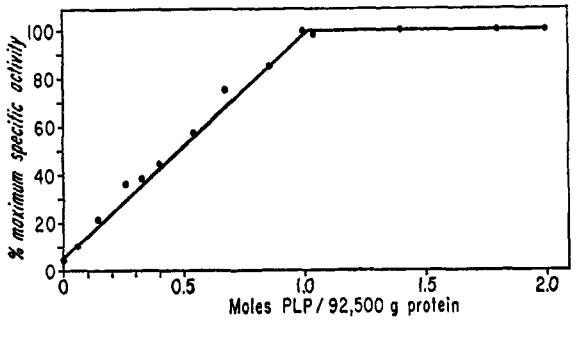

FIGURE 5: Pyridoxal 5′-phosphate titration of rat muscle apophosphorylase *b*. The details are described in the text.

TABLE III: Comparative Amino Acid Composition of Skeletal Muscle Glycogen Phosphorylases from Rat, Rabbit, Human, and Frog.

Amino Acid	Residues per 92,500 g of Protein[c]			
	Rat	Rabbit	Human[a]	Frog[b]
Lysine	43	43	47	42
Histidine	19	19	23	20
Ammonia	79	71	68	66
Arginine	60	59	63	53
Aspartic acid	95	91	94	90
Threonine	31	32	32	34
Serine	28	27	24	34
Glutamic acid	91	94	88	84
Proline	36	39	33	43
Glycine	45	46	46	47
Alanine	59	60	62	55
Half-cystine	8	8	8	11
Valine	53	57	56	57
Methionine	22	20	21	20
Isoleucine	48	45	46	43
Leucine	74	75	75	70
Tyrosine	34	34	33	34
Phenylalanine	35	36	39	35
Tryptophan	13	12	11	12
Total amino acid residues	794	797	800	785

[a] Data from Appleman *et al.* (1963). [b] Recalculated from the data of Metzger *et al.* (1968). [c] Calculated for a monomer molecular weight of 92,500 for all enzymes. All values were rounded off to the nearest integer.

Single symmetrical peaks were obtained for both phosphorylases *b* and *a* in sedimentation velocity experiments (Figure 7). Both forms of the enzyme exhibited some dependence of sedimentation upon concentration; extrapolation to zero concentration by a least-squares equation of the form $s_{20,w} = s^0_{20,w}(1 - KC)$, where K expresses this concentration dependency (slope), gave values of 9.07 ± 0.08 S for phosphorylase

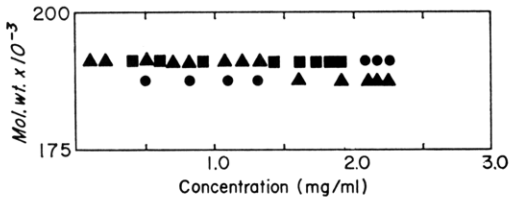

FIGURE 6: High-speed equilibrium run of rat muscle phosphorylase *b;* representative distribution of molecular weight moments as obtained from the computer program of Teller *et al.* (1969). ■ represents $M_{n,x}$; ▲ represents $M_{w,x}$; and ● represents $2M_{n,x} - M_{w,x}$. Phosphorylase *b* (0.5 mg/ml) was centrifuged in a 0.02 M sodium β-glycerophosphate, 0.03 M β-mercaptoethanol, and 0.05 M NaCl buffer, pH 6.8, 20°; 14,291 rpm at sedimentation equilibrium.

b and 13.94 ± 0.01 S for phosphorylase *a*. The negative slopes for the concentration dependence of the sedimentation coefficient, more noticeable in the case of phosphorylase *a* than of phosphorylase *b* ($K = 4.2 \times 10^{-3}$ *vs.* 2.3×10^{-3} ml per mg for phosphorylase *b*), are less pronounced than those obtained for most globular proteins (Creeth and Knight, 1965). This indicates that both forms of the enzyme are involved in an association equilibrium. Combining sedimentation coefficients with diffusion coefficients of $D_{20,w} = 4.44 \pm 0.04$ and $3.88 \pm 0.04\ 10^{-7}$ cm^2 per sec^{-1} for phosphorylase *b* and *a*, respectively, and a partial specific volume of 0.736 ml/g gave molecular weights of 191,000 and 337,000 g per mole for these two forms of the enzyme in good agreement with the values of 185,000 and 370,000 recently obtained for rabbit muscle phosphorylase *b* and *a* (Seery *et al.*, 1967). As found for this enzyme, the dimeric *b* form of rat phosphorylase appears to be slightly associated and the tetrameric *a* form, slightly dissociated.

Both sedimentation equilibrium and velocity carried out on rat phosphorylase dissolved in 6 M guanidine hydrochloride indicated molecular heterogeneity. In Figure 8, reciprocal weight-average, number-average, and *z*-average molecular weights as a function of phosphorylase *b* concentration in 6 M guanidine hydrochloride are illustrated. Treatment of the data in this

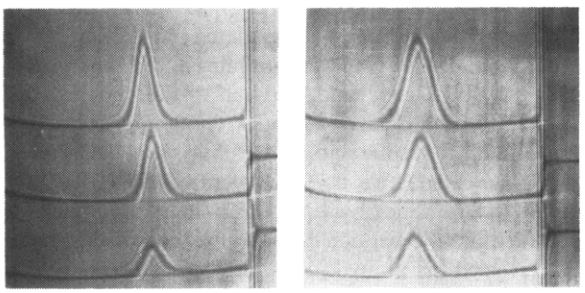

FIGURE 7: Sedimentation velocity patterns of rat muscle glycogen phosphorylase in 0.05 M sodium glycerophosphate, 0.05 M β-mercaptoethanol, and 0.1 M NaCl (pH 7.0). Both phosphorylase *b* and *a* were run at 8, 5, and 3 mg per ml, respectively. Sedimentation at 52,640, 20°, for 24 min (from right to left).

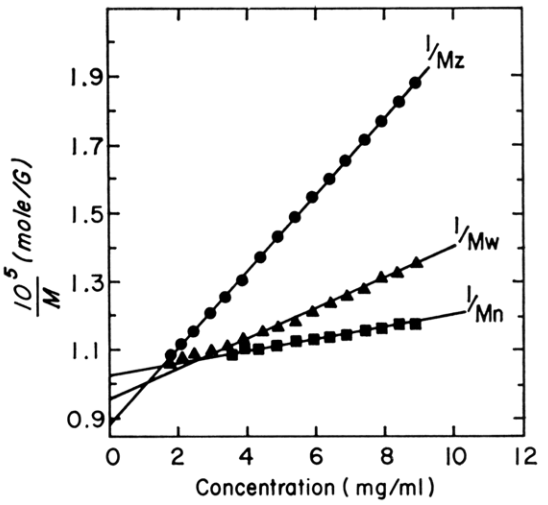

FIGURE 8: Reciprocal number-average, weight-average, and z-average molecular weights as a function of concentration of rat phosphorylase *b* in 6 M guanidine hydrochloride, 0.005 M Tris, and 0.01 M β-mercaptoethanol. ■ represents $M^{-1}{}_{n,x}$; ▲ represents $M^{-1}{}_{w,x}$; and ● represents $M^{-1}{}_{z,x}$.

fashion assumes homogeneity of the protein and eliminates thermodynamic nonideality by extrapolation to infinite dilution. The plots yielded straight lines with slopes proportional to the virial coefficients and intercepts determined by the reciprocal molecular weight of the material. At infinite dilution, values of $M_n = 93{,}721 \pm 949$ g/mole, $M_w = 101{,}112 \pm 166$ g/mole, and $M_z = 113{,}379 \pm 340$ g/mole were obtained when a partial specific volume of 0.736 ml/g was used in the calculations. Once more, these data are quite similar to those obtained with the rabbit muscle enzyme in guanidine hydrochloride. They are consistent with, but do not prove, the assumption that the 92,500 molecular weight species (enzyme monomer) represents the smallest subunit for this enzyme.

Interconversions of Phosphorylase b and a. Phosphorylase *b* from the rat could be converted into the active *a* form by purified rabbit muscle phosphorylase kinase. Using γ-labeled [^{32}P]ATP in the conversion reaction, 0.75 mole of phosphate was incorporated per 92,500 g of protein; Figure 8 shows the appearance of phosphorylase *a* activity (measured in the absence of AMP) with uptake of phosphate. Curiously, a parallel experiment run under precisely the same conditions on rabbit phosphorylase *b* showed that both activation and phosphate incorporation proceeded at only half the rate observed with the rat enzyme (Figure 9). It is not known whether this difference is significant or not or if it merely reflects the state or "past history" of the enzyme preparations involved. Purified rat phosphorylase *a* could be reconverted into the inactive *b* form by the action of purified rabbit muscle phosphorylase phosphatase, emphasizing once more the lack of species specificity of the phosphorylase kinase and phosphatase from this animal.

Rat muscle phosphorylase *b* and *a* are active in the range of pH 5.7–8.3 with optimum activity between pH 6.3 and 6.5. Simple kinetic studies yielded K_m (AMP) of 2×10^{-4} M and K_m (G-1-P) of 7.5×10^{-3} M for phos-

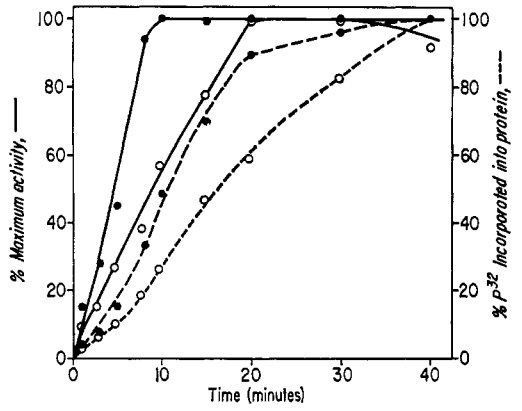

FIGURE 9: Conversion of rat muscle (●) and rabbit muscle (○) phosphorylase *b* into *a* catalyzed by rabbit muscle phosphorylase *b* kinase. Conditions as described in the text. Solid line represents phosphorylase *a* activity (measured in the absence of AMP); broken line represents uptake of ^{32}P.

phorylase *b*, essentially identical with the values obtained for the rabbit enzyme under the same conditions.

Carboxyl-Terminal Studies. No amino or carboxyl end group could be detected in rabbit muscle phosphorylase under a variety of conditions and approaches, a finding that further complicated the understanding of the subunit structure of this enzyme (Appleman *et al.*, 1966). It was therefore of added interest to reexamine this problem with the rat enzyme. The carboxyl-terminal group of rat muscle phosphorylase *b* was investigated both by chemical and enzymatic procedures. Carboxypeptidase A (1:200 molar ratio) released 0.93 ± 0.05 equiv of isoleucine in 10 min at 25° when added to both native and sodium dodecyl sulfate denatured rat phosphorylase. No further release of amino acid occurred by prolonging the incubation. When, however, carboxypeptidase B was added to the preceding reaction mixture in the presence of sodium dodecyl sulfate, an additional 0.9 ± 0.10 equiv of lysine was liberated. No release of lysine was observed either when the native enzyme was used (no sodium dodecyl sulfate) or in the absence of carboxypeptidase A.

The presence of an isoleucyl residue at the carboxyl end of the molecule was confirmed by hydrazinolysis, which released 0.6 equiv of this amino acid in 40 hr and 0.65 after 95 hr of treatment. No other free amino acid could be detected. Likewise, no free amino acid was generated when *rabbit* muscle phosphorylase was subjected to the same treatment. It is concluded that the enzyme contains a lysylisoleucine sequence at its carboxyl end.

No amino-terminal group could be detected by the cyanate procedure of Stark and Smyth (1963), carried out as described under Methods.

Sequence of Phosphoserine-Containing Peptide. The site phosphorylated during conversion of rat muscle phosphorylase *b* into *a* was isolated and characterized as follows. Phosphorylase *a*, prepared from phosphorylase *b* using γ-labeled [^{32}P]ATP (450 mg, 5.5 × 10^6 cpm/μmole corresponding to *ca.* 44,000 cpm/mg), was dialyzed against 0.1 M NH_4CO_3 (pH 8.0) and digested for 19 hr at 35°, pH 8.0, with chymotrypsin added in a 1:100 weight ratio. Soybean trypsin inhibitor was added

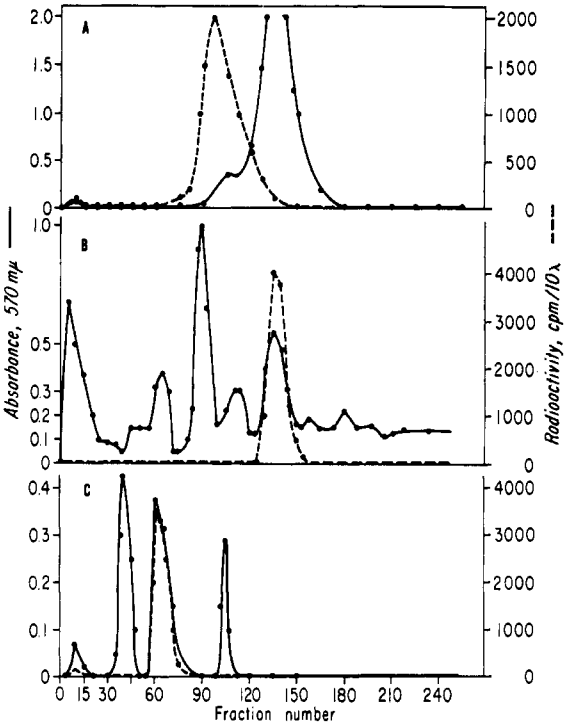

FIGURE 10: The chromatographic separation of the phosphorylated tetradecapeptide from rat muscle phosphorylase *a*. A = chymotryptic digest of rat muscle phosphorylase *a* chromatographed on a Sephadex G-50 column (2.5 × 90 cm) equilibrated and developed with 1 M acetic acid. Elution was carried out at room temperature, at a flow rate of 0.4 ml/min; the effluent was collected in 2-ml fractions and assayed for radioactivity and ninhydrin-staining material. B = Dowex 50-X2 chromatography of fraction A-1. The column was eluted with 20 ml of pH 3.1 pyridine–acetate buffer before the gradient consisting of 100 ml of pH 3.1 and 200 ml of pH 5.0 buffers was begun. Fractions (1 ml) were collected at a rate of 0.25 ml/min. C = Dowex 1-X2 chromatography of fraction B-1 using the α-picoline–*N*-ethylmorpholine–pyridine acetate buffer gradient from pH 8.4 to 5.0 of Schroeder (1967).

to chymotrypsin in a 1:25 molar ratio to inhibit possible trypsin contamination. At various times, samples were withdrawn and analyzed for ^{32}P released. After 5.5 hr (63% counts released), a second addition of chymotrypsin was made and after 19 hr (97% counts released) the digestion was terminated by addition of 1 M acetic acid. The sample was lyophilized and taken up in 10 ml of 30% acetic acid, and half of the material was applied to each of two Sephadex G-50 columns equilibrated with 1 M acetic acid. The profile is shown in Figure 10A. The radioactive fractions were combined and lyophilized; the residue was taken up in pH 3.1 pyridine–acetate buffer. A peptide map of a portion of this material revealed ten ninhydrin-staining spots, one of which was radioactive.

The remainder of the phosphopeptide fraction was chromatographed on Dowex 50W-X2 as described under Figure 10B; the radioactive fractions were again combined and lyophilized. A peptide map showed the presence of two major and five minor ninhydrin-staining spots; radioactivity coincided with one of the major spots. This fraction was further purified by chromatography on Dowex 1-X2 (Figure 10C). The phosphopep-

TABLE IV: Summary of Data for the Phosphopeptide Sequence of Rat Muscle Phosphorylase.

Reaction		Results
Composition (μmoles)		Lys (0.109), Arg (0.110), Asp (0.109), Ser (0.084), Glu (0.105), Gly (0.048), Val (0.051), Ile (0.054), Leu (0.057)
Tryptic hydrolysis	T-1	Ser,Asp_2,Glu,Lys
	T-2	Ser,Asp_2,Glu,Lys,Arg
	T-3	Arg,Lys
	T-4	Lys,Glu,Ile,Ser(P),Val,Arg
	T-5	Glu,Ile,Ser(P),Val,Arg
	T-6	Gly,Leu
NH_2 terminal (Dansyl)		Ser
Dansyl–Edman		Ser-Asp-Gln-Asp-Lys-Arg (- - -)
COOH terminal (carboxypeptidase A–Dansyl)		(- - -) Gly-Leu
Dansyl–Edman of T-4		Lys-Gln-Ile-Ser(P)-Val-Arg
Sequence		Ser-Asp-Gln-Asp-Lys-Arg-Lys-Gln-Ile-Ser(P)-Val-Arg-Gly-Leu
Sequence of rabbit muscle phosphorylase phosphopeptide[a]		Ser-Asp-Gln-*Glu*-Lys-Arg-Lys-Gln-Ile-Ser(P)-Val-Arg-Gly-Leu

[a] Nolan *et al.* (1964).

ide was pure at this point, as shown by peptide mapping and amino acid analysis.

The amino acid composition for this peptide and the approach used for solving its structure and amino acid sequence are summarized in Table IV. The sequence presented is identical with that obtained earlier (Nolan *et al.*, 1964) for the phosphorylated site of rabbit muscle phosphorylase, except for the conservative substitution of an aspartyl for a glutamyl residue as italicized in Table IV.

Discussion

From the purification scheme and the physicochemical properties described herein for rat muscle phosphorylase, it is clear that this enzyme is very similar to that obtained from rabbit skeletal muscle. Isolation followed the general method of Fischer *et al.* (1958) for the rabbit enzyme, except that the heat treatment at alkaline pH introduced to destroy contaminating phosphorylase phosphatase and kinase was replaced by a DE-52 column chromatography step. As observed for the rabbit enzyme (Krebs and Fischer, 1955), the phosphorylase content per gram of rat muscle increases with the size of the animal (from approximately 60 mg of phosphorylase/100 g of muscle for a 100-g rat to 150 mg of phosphorylase/100 g of tissue for a 350-g animal). Specific activity of the crystalline product (85 ± 5 μmole of GIP converted/min per mg) is the same as that for the rabbit enzyme, corresponding to a turnover number of 15,700 per mole of enzyme dimer.

A value of $A_{280}^{1\%}$ 12.5 was obtained for rat muscle phosphorylase *b*. This is slightly higher than that determined by Appleman *et al.* (1963) for the rabbit enzyme (11.9). Recently, a high absorbancy index of 13.2 (Buc and Buc 1967) was reported for the *rabbit* muscle enzyme. This value could not be confirmed here; upon redetermination, no value greater than 12.0 could be obtained. The reason for this discrepancy is unknown.

Using the absorbancy index of 12.5 to calculate the concentration of the rat enzyme, 1.0 ± 0.1 mole of PLP/92,500 g of protein (molecular weight for the enzyme monomer) was obtained both by direct determination of PLP by the phenylhydrazine method of Wada and Snell (1961) and restoration of enzymatic activity following titration of apophosphorylase with the cofactor. Identical results were obtained with the rabbit enzyme when the absorbancy index of 11.9 was used. On the other hand, incorporation of phosphate during phosphorylase *b* into *a* conversion in the presence of γ-labeled [^{32}P]ATP consistently gave low values corresponding to the uptake of only three phosphates per enzyme tetramer for both the rat and the rabbit enzyme. Once more, no explanation can be offered as yet for this discrepancy.

Close chemical and physical relationships between the rat and the rabbit muscle enzymes were shown by their amino acid analysis, the amino acid sequence of the site phosphorylated in the phosphorylase *b* into *a* conversion, their pyridoxal 5′-phosphate content, and molecular weights.

Because of the considerable size of the phosphorylase subunit (molecular weight 92,500) amino acid analyses were carried out at five times of hydrolysis on three different crystalline preparations of the enzyme, in order to reduce as much as possible the margins of error. Very good agreement was obtained between the triplicate analyses (Table II) indicating excellent constancy in the composition of the purified material. Also, to allow for a better comparison with the rabbit enzyme,

amino acid analyses of the latter were repeated under precisely the same conditions. As expected, and in spite of these precautions, the limits of error of the method are such that for several amino acids, assignment of definite integral numbers of residues per mole of enzyme could not be made. Nonetheless, comparison of the closest integral values listed in Table II for the rat and the rabbit enzyme shows that two-thirds of the amino acids are either present in identical number or differ by only a single residue. The remaining six amino acids differ by only 19 residues out of a total of 345.

Obvious structural similarity is also underlined by the amino acid sequence of the site phosphorylated during the $b \rightarrow a$ conversion: it shows only one conservative substitution (an aspartyl residue in the rat replacing a glutamyl residue in the rabbit) over a sequence of fourteen amino acids. By contrast, the phosphopeptide isolated from the rabbit liver enzyme displays two conservative substitutions over a sequence of only six amino acids surrounding the phosphoseryl residue (Appleman *et al.*, 1966; Wolf, 1967). It is interesting to note that evolution has found it nesessary to preserve more structural similarities in an enzyme from the same tissue in two separate species than in two organs from the same animal.

One difference, however, is that, for the first time in any phosphorylase so far examined, an end group was found. Carboxypeptidase A attack of rat phosphorylase released one isoleucyl residue per monomer unit and subsequent treatment with carboxypeptidase B on the denatured enzyme suggested the presence of a lysyl residue in the penultimate position. Release of the isoleucyl residue was accompanied by no loss of enzymatic activity; however, since removal of the lysyl residue required prior denaturation of the protein, no conclusion as to its necessity for enzymatic activity could be drawn. The finding of a single carboxyl terminal, of course, does not allow any conclusion as to the subunit structure of the enzyme; at least, it is not inconsistent with all the physical and chemical data now on hand indicating that the rabbit enzyme monomer is made up of a single chain. Search for blocked amino-terminal groups in both the rat and the rabbit enzyme is being further investigated.

The present report has been limited to a chemical and physical characterization of rat muscle phosphorylase; no attempt has yet been made to investigate the mechanism by which the activity of this enzyme may be physiologically controlled. However, the interconversions of the *a* and *b* forms of the rat enzyme proceeded identically with those of the rabbit enzyme when both purified rabbit phosphorylase kinase and phosphatase were used to drive the reactions. This suggests that the same complex regulatory mechanism, involving a succession of activating and inactivating steps as established for the rabbit muscle enzyme (Krebs and Fischer, 1962), will be found.

Acknowledgments

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Conformational Changes in the Molecular Control of Muscle Contraction*

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ABSTRACT: The McConnell "spin-label" technique (mainly SH-directed labels) has been used to examine whether a structural effect is transmitted across the protein system which controls the contraction of muscle: troponin–tropomyosin–actin. It was found that a spin label attached to tropomyosin responds to Ca^{2+} fluctuations in the micromolar range only when troponin is present, and that a spin label attached to actin responds only when troponin–tropomyosin are present. Thus some structural effect appears to be communicated to actin across this control system.

The contractile system of muscle is thought to be controlled by changes in intrafibrial [Ca^{2+}], and this control to be exerted through tropomyosin and troponin, proteins which are attached probably at regular intervals to the F-actin of the thin filaments. In the absence of Ca^{2+} these attachments prevent the force-generating interactions between F-actin and the myosin of the thick filaments. Approximately micromolar Ca^{2+} is thought to nullify this prevention, thus restoring force generation; in this sense Ca^{2+} is said to "activate" muscle. Since the tropomyosin–F-actin complex is stable and the troponin–F-actin complex is not, and since troponin–tropomyosin is stable, it is thought that the control arrangement is troponin–tropomyosin–F-actin. Moreover, Ca^{2+} has a high affinity for troponin, or for the mixture troponin–tropomyosin, a mixture which when unresolved has been called "relaxing protein," but myosin reacts directly with F-actin; therefore, it seems that there is some form of communication between one end and the other of Ca^{2+}–troponin–tropomyosin–F-actin–myosin. The foregoing ideas are largely the fruit of elegant work by Ebashi and his associates (Ebashi and Ebashi, 1964; Ebashi and Kodama, 1966; Endo *et al.*, 1966; Ebashi *et al.*, 1967; Ohtsuki *et al.*, 1967). In this paper we examine whether some effect is indeed transmitted across the system troponin–tropomyosin–F-actin when [Ca^{2+}] is varied in the micromolar range. For this purpose we employed the "spin-label" technique of McConnell and coworkers (Hamilton and McConnell, 1968), *i.e.*, the attaching to the protein under study of stable, environment-sensing free radicals whose electron paramagnetic resonance spectrum can be followed as the labeled protein participates in its reactions. Mainly we employed spin labels directed to the SH groups of the proteins; of the two employed, the analog of *N*-ethylmaleimide was more useful than the analog of iodoacetamide. In one instance we used a spin label directed to NH_2 groups, but perhaps also reactive toward SH groups, *viz.*, the analog of isothiocyanate. Our strategy of labeling was to label in

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