Phosphorylase from Dogfish Skeletal Muscle. Purification and a Comparison of Its Physical Properties to Those of Rabbit Muscle Phosphorylase*

Philip Cohen,† Theresa Duewer, and Edmond H. Fischer‡

ABSTRACT: Large amounts of glycogen phosphorylases *b* and *a* (EC 2.4.1.1) have been purified from skeletal muscles of the Pacific dogfish (*Squalus sucklii*) to a state of homogeneity as judged by the criteria of ultracentrifuge analysis and polyacrylamide gel electrophoresis. An investigation of the molecular properties showed many similarities and certain distinct differences when compared to those of rabbit skeletal muscle phosphorylases *b* and *a*. Specific activities of dogfish phosphorylase *b* (62 units/mg with AMP, <1 without AMP) and *a* (62 and 48 units per mg with and without AMP) were comparable to those of the rabbit enzymes (88 and <1 units per mg for *b*, and 80 and 54 for *a* when measured with and without AMP, respectively). Other closely similar properties for dogfish and rabbit phosphorylase *b*, respectively, include the absorbancy indices $A_{280}^{1\%}$ (12.9 *vs.* 13.1), 260/280 nm ratios (0.56 *vs.* 0.53), the apparent specific volumes (0.746 *vs.* 0.746 ± 0.002), sedimentation coefficients (8.9 S *vs.* 8.8 S), subunit size, and molecular weights (100,000 ± 1000 and 200,000 ± 2000 for both enzymes). Dogfish and rabbit phosphorylase *b* both incorporated one molecule of phosphate per subunit in the *b* into *a* conversion, and dogfish phosphorylases *b* and *a* were as good substrates for rabbit muscle phosphorylase kinase and phosphatase, respectively, as were the rabbit muscle phosphorylases themselves. Each enzyme contained one molecule of pyridoxal 5′-phosphate per subunit and interactions between this cofactor and the proteins were very similar. Dogfish phosphorylase *a* was only 20–25% associated from dimer to tetramer under conditions where rabbit phosphorylase *a* was completely in a tetrameric form. Addition of NaCl to 0.5 M promoted further tetramerization (to *ca.* 40%), but had no effect on the subunit interactions of rabbit phosphorylase *a*, although dissociation of the latter toward dimer occurred above 1.5 M NaCl. Likewise, 2 mM AMP had no effect on the $s_{20,w}$ or molecular weights of dogfish phosphorylase *b*, whereas rabbit phosphorylase *b* was 75–80% associated to tetramer at 10° under these conditions. In contrast to the rabbit enzymes, neither *a* nor *b* (in the presence of Mg^{2+} and AMP) crystallized at 0°, suggesting an inverse correlation between solubility at low temperature and tetramerization. The K_m for glycogen as primer also differed markedly for dogfish and rabbit phosphorylase *b* (0.12 and 0.018%, respectively). The subunit molecular weight obtained for phosphorylases from both species is 8% higher than had previously been reported for the rabbit muscle enzyme. The reasons for a revision in both the size of the polypeptide chain and the nature of the interactions between subunits is discussed, together with possible implications of the differences observed between the rabbit and dogfish enzymes.

Phosphorylases have now been isolated in a homogeneous form from many sources: from the skeletal muscles of rabbit (Green and Cori, 1943; Fischer and Krebs, 1958), man (Yunis *et al.*, 1960), frog (Metzger *et al.*, 1968), rat (Sevilla and Fischer, 1969), and also from rabbit heart muscle (Yunis *et al.*, 1962; Davis *et al.*, 1967), lobster muscle (Cowgill, 1959; Assaf and Graves, 1969), and insect flight muscle (Childress and Sacktor, 1970). From mammalian tissues other than muscle, pure phosphorylase preparations have been obtained from canine, porcine, and rabbit livers (Sutherland and Wosilait, 1956; Appleman *et al.*, 1966) and bovine corpus luteum (Yunis and Assaf, 1970). Potato phosphorylase (Lee, 1960; Kamogawa *et al.*, 1968), *Escherichia coli* phosphorylase (Schwartz and Hofnung, 1967), and the baker's yeast enzyme (M. Fosset *et al.*, in preparation) have also been completely purified.

There is, however, very little data available on the chemical homology between these enzymes. Apart from rabbit skeletal muscle phosphorylase, sequence analysis has been limited to the phosphopeptides of the mammalian phosphorylases from human muscle (Hughes *et al.*, 1962), rat muscle (Sevilla and Fischer, 1969), and rabbit liver (Wolf *et al.*, 1970).

All muscle phosphorylases studied thus far can exist in either an inactive *b* or active *a* form. The control of the *b* to *a* conversion by regulation of phosphorylase kinase activity is particularly well documented in rabbit skeletal muscle. This enzyme can be reversibly activated by cAMP (DeLange *et al.*, 1968; Riley *et al.*, 1968; Walsh *et al.*, 1968) and also by calcium ions (Brostrom and Krebs, 1970; Ebashi *et al.*, 1969; Meyer *et al.*, 1970; Heilmeyer *et al.*, 1970), the latter effector probably providing the link between muscle contraction and glycogen degradation. However, apart from a preliminary report on insect muscle phosphorylase kinase (Hansford and Sacktor, 1970) extremely little is known about the structure and molecular control of phosphorylase kinase and phosphatase from nonmammalian sources.

The complex regulatory mechanism of glycogen degradation in rabbit skeletal muscle would appear to make it ideally suited for studies concerned with the evolution of a control

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† Recipient of a North Atlantic Treaty Organization Postdoctoral Fellowship from the Science Research Council, London, England.

‡ To whom requests for reprints should be sent.

mechanism. For this purpose it was necessary to select sources which not only diverged from the main lines leading to mammals a long time ago, but which would also yield the large amounts of phosphorylase required for this kind of investigation, including sequence homology. We have chosen skeletal muscle from the Pacific dogfish (*Squalus sucklii*) as one source. This fish represents one of the most primitive of vertebrates having separated from the main vertebrate line over 400 million years ago.

As the first phase of these projects, this manuscript describes the purification of the two forms of dogfish muscle phosphorylase (*a* and *b*) and a detailed characterization of their physical properties as compared to those of the rabbit muscle enzymes.

Materials and Methods

Bovine serum albumin (Pentex Biochemicals, Ill.), ovalbumin (Mann Research Laboratories, New York), and γ-globulin (Sigma Chemical Company, St. Louis) were the best available grades. *E. coli* alkaline phosphatase was obtained from Worthington Biochemicals Corp., Freehold, N. J. Crystalline rabbit muscle glyceraldehyde 3-phosphate dehydrogenase and yeast phosphoglycerate kinase for the preparation of γ-labeled [^{32}P]ATP were purchased from C. F. Boehringer und Sons, Mannheim, Germany. *E. coli* arginine decarboxylase was kindly provided by Dr. Elizabeth Boeker from this laboratory. Glycogen and glucose 1-phosphate were purified as previously described (Sevilla and Fischer, 1969). Whatman DE-52 cellulose ion exchanger was obtained from H. Reeve Angel, Inc., Clifton, N. J., and G-200 and G-25 Sephadexes from Pharmacia Fine Chemicals, Piscataway, N. J.

Enzyme Preparations. Rabbit phosphorylase *b* was prepared by the method of Fischer and Krebs (1958); the third crystals were dissolved in 50 mM glycerophosphate–50 mM mercaptoethanol (pH 7.0) and all experiments were carried out after passing the enzyme through a charcoal column to remove AMP. Charcoal was also used to remove AMP from dogfish phosphorylase *b*, but a number of experiments were carried out with the freshly purified enzyme, which as prepared contains no bound nucleotide. The 260/280 ratios for the AMP-free forms of phosphorylase *b* were 0.56–57 for the dogfish and 0.53–54 for the rabbit enzyme.

Rabbit muscle phosphorylase kinase was prepared according to DeLange *et al.* (1968). Rabbit muscle and liver phosphorylase phosphatases were partially purified preparations obtained from Dr. Richard Haschke and Mr. Guy Pocinwong from this laboratory.

Rabbit muscle phosphorylase *a* was prepared from phosphorylase *b* at pH 8.2 according to Krebs (1966). The enzyme was crystallized at 0° at pH 7.0, and recrystallized several times by dissolving the crystals at 30° with a few drops of 2 M NaCl, followed by dialysis against 50 mM glycerophosphate–50 mM mercaptoethanol (pH 7.0) at 0–4°.

Assay. Both dogfish and rabbit phosphorylases were assayed by the method of Hedrick and Fischer (1965), in the direction of glycogen synthesis; the same definition of units (micromoles of P_i released per minute) and specific activities were used.

Absorbancy Index and Protein Concentration. Because of discrepancies in the absorbancy indices used for phosphorylase solutions among various groups, the $A_{280}^{1\%}$ of dogfish and rabbit phosphorylase *b* (freed of AMP) was carefully measured after dialysis of the enzymes against 50 mM glycerophosphate, 1.0 mM EDTA, and 1.5 mM mercaptoethanol at pH 7.0. Dilutions for 280 nm absorbance measurements were made in triplicate with agreement better than ±1%.

Protein concentrations of rabbit phosphorylase solutions were measured both from synthetic boundary experiments made at 10° in a Spinco Model E analytical ultracentrifuge and from amino acid analysis. Using Rayleigh interference optics and a titanium AN-H rotor, with speed maintained at 5200 rpm, the difference in fringes between protein solution and buffer, *J*, was measured. Taking the refractive index increment, dn/dc, of the protein as 0.00185, the concentration of the protein, *c*, was determined from the equation $dn/dc = J\lambda/ac$, where λ, the wavelength of light used, is 5.46×10^{-5} cm, and *a*, the optical path length, is 1.20 cm. Substituting these values gave 4.05 fringes/mg of protein. The general validity of this method has been recently demonstrated by Babul and Stellwagen (1969). For measurements of protein concentration from amino acid analyses, aliquots of phosphorylase *b* solution, with a norleucine standard included (Walsh and Brown, 1962) were hydrolyzed *in vacuo* with 6 N HCl for 24 hr at 110° following repeated flushing with nitrogen (Moore and Stein, 1963). Micromoles of aspartic acid, alanine, leucine, and phenylalanine were converted into the phosphorylase concentration in milligrams using the composition reported by Sevilla and Fischer (1969) and the results obtained from these four amino acids were averaged.

The $A_{280}^{1\%}$ for rabbit phosphorylase *b* was 13.1 ± 0.2 from the synthetic boundary experiments and 13.0 ± 0.3 from amino acid analysis. These numbers are the averages of duplicate determinations on three enzyme preparations; they are in excellent agreement with the values of 13.2 (Buc and Buc, 1967) and 13.1 (Gold, 1968), but higher than the value of 11.9 based on dry weight measurements (Appleman *et al.*, 1963) previously used in this laboratory. In view of the agreement between the two methods, only the synthetic boundary procedure was used for dogfish phosphorylase *b* and yielded 12.9 ± 0.2. This value and 13.1 for rabbit phosphorylase were used in all other experiments reported here. For determination of phosphate incorporated during the *b* to *a* conversion, phosphorylase *a* was assumed to have the same absorbancy index as phosphorylase *b*.

Polyacrylamide gel electrophoresis of native dogfish phosphorylase was carried out as described by Ornstein and Davis (1963) with 7.5% gels; activity stains were performed according to Davis *et al.* (1967). For determinations of subunit size, gel electrophoresis following sodium dodecyl sulfate treatments (Shapiro and Maizel, 1969) was carried out as described by Weber and Osborn (1969) with the following modifications. The acrylamide, methylenebisacrylamide, and *N,N,N'N'*-tetramethylenediamine concentrations were halved to give a 5% gel. With this system, a completely linear calibration graph was obtained with proteins ranging in subunit size from 36,000 to 136,000, which corresponded to a relative migration range of 84–29% with the batch of acrylamide used. The following markers were included: bovine serum albumin, mol wt 68,000 and 136,000 for monomer and dimer, respectively (Tanford *et al.*, 1967); ovalbumin, 43,000 and 86,000 for monomer and dimer, respectively (Castellino and Barker, 1968); and glyceraldehyde 3-phosphate dehydrogenase, 36,000 (Harrington and Karr, 1965; Harris and Perham, 1968). Arginine decarboxylase (82,000) (Boeker *et al.*, 1969) was also used as a marker in the molecular weight range close to that of the phosphorylase subunit. Incubation of the proteins in sodium dodecyl sulfate prior to electrophoresis was for 15 min at 70°; 5 μg of protein was used in the analytical runs, and

10 μg for purity checks on dogfish phosphorylases. All gels were stained with coomassie brilliant blue.

Partial Specific Volumes. Since the $A_{280}^{1\%}$ of 13.1 found for rabbit muscle phosphorylase agreed to within 1% with the values obtained by two other laboratories, it was possible to measure the apparent specific volume of the protein with some confidence.

Phosphorylase solutions were dialyzed at 4° against 50 mM glycerophosphate–1.0 mM EDTA–1.5 mM mercaptoethanol (pH 7.0); experiments were carried out in a water bath regulated at 20 ± 0.001° (Hvidt *et al.*, 1954) according to two procedures.

METHOD 1. According to Hvidt *et al.* (1954) with the following modifications devised by Dr. David Teller in this Department. Two miscible oils, dodecane (Baker Chemical Co., Phillipsburg, N. J., density 0.7487 g/cm³) and KEL-F (3M Company, Minnesota, density 1.877 g/cm³), were mixed to form two solutions with densities close to 1.00 and 1.01, and each solution was shaken with water to remove water-soluble impurities. After filling the lower bulb with the heavier solution, the density column was established from equal volumes of the two solutions and a linear gradient maker. Extremely good linearity of the gradient was demonstrated on calibration with six standard KCl solutions. Because of the low viscosities of dodecane–KEL-F mixtures, the drops of protein and salt solutions reached their final position well within 1 hr.[1] About 10 μl of protein solution at 10–15 mg/ml was required for each determination.

METHOD 2. By direct pycnometric measurement of the densities of protein and buffer solutions using a 2.3-ml capillary pycnometer, weighed to within 0.001 mg on a Mettler M5 balance. Corrections were made for the weight of air displaced from the pycnometer; the protein concentration was 45 ± 5 mg per ml.

Using two preparations of rabbit phosphorylase *b*, values of 0.748, 0.745, and 0.744 were obtained by method 1, and 0.749 and 0.746 by method 2. In view of this good agreement, only method 1 was used for dogfish phosphorylase *b*, giving a value of 0.746. Averaging these results gives 0.746 ± 0.002 as the apparent specific volume in 50 mM sodium glycerophosphate (pH 7.0) and 20°. This is just over 1% higher than the partial specific volume calculated from the amino acid compositions of the proteins, 0.737 for rabbit phosphorylase (Seery *et al.*, 1967) and 0.736 for dogfish phosphorylase (P. Cohen, J. C. Saari, and E. H. Fischer, in preparation).

Ultracentrifugation. BUFFERS. All solutions contained 50 mM glycerophosphate, 1.0 mM EDTA, 50 mM mercaptoethanol (pH 7.0) (designated as buffer A), and NaCl concentrations ranging from 0 to 2.0 M. A number of solutions also included 2.0 mM AMP. Each buffer was adjusted to pH 7.0 at 20° with either 6 N HCl or NaOH. The density of buffer A (pH 7.0) was 1.0046 at 20° and 1.0054 at 10°; its relative viscosity, measured with an Ostwald viscometer, and a Hewlett-Packard Autoviscometer timer, was 1.037 ± 0.002 at 20.0° and 10.0°.[2] Densities and relative viscosities of NaCl solutions and the viscosity of water at 10° relative to 20° (1.298) were taken from Washburn (1928).

Protein solutions were dialyzed 24 hr at 4° against 200 volumes of the desired buffer with one buffer change. Ultracentrifugation of dogfish phosphorylases *b* and *a*, and rabbit phosphorylase *b* was performed at 10.0°, and with rabbit phosphorylase *a* at 20.0° because of solubility problems. The apparent specific volume at 20° was taken as 0.746 (see above) and at 10° as 0.741, applying the correction factor $d\bar{v}/dt$ = 0.0005 ml/g per deg (Svedberg and Pedersen, 1940).

A Spinco Model E analytical ultracentrifuge, equipped with an RTIC unit and electronic speed control, was used in all experiments. Photographic plates were read on a Nikon microcomparator.

Sedimentation velocity experiments were carried out with schlieren optics, using double-sector cells, in either an AN-D rotor (at 52,000 rpm) or a four-hole AN-F rotor (at 48,000 rpm). The initial protein concentration was maintained at 4 ± 1 mg per ml. While in most runs single symmetrical boundaries were obtained, in experiments with dogfish phosphorylase *a*, rabbit phosphorylase *b* in the presence of AMP, and rabbit phosphorylase *a* above 1.5 M NaCl, skewed schlieren peaks were observed. In these cases, the sedimentation coefficients were calculated from the migration of the second moment (Goldberg, 1953).

Sedimentation Equilibrium. In low-speed sedimentation equilibrium runs, the schlieren pattern at equilibrium was used to calculate M_Z (van Holde and Baldwin, 1958) and Rayleigh interference optics to calculate M_w (Richards and Schachman, 1959). Double-sector cells and a titanium AN-H rotor were employed in all equilibrium experiments and the protein concentration was maintained at 4.5 ± 0.5 mg/ml. Fluorocarbon FC43 was omitted from all runs after an initial experiment showed that dogfish phosphorylase *b* interacted strongly with this oil.

Samples of dogfish phosphorylase *b* (freed of AMP) in buffer A plus 0.1 M NaCl, form small amounts of high molecular weight material which become visible near the bottom of the cell after *ca.* 36 hr when viewed under schlieren optics, *i.e.*, before equilibrium is reached in 3-mm solution columns (*ca.* 48 hr). These high molecular weight species could not be seen by sedimentation velocity carried out immediately after the equilibrium run, but appeared on storage of the sample for 2 weeks; their $s_{20,w}$ ranged from 8.9 S given by the native enzyme to *ca.* 80 S, suggesting that the few per cent of aggregate which formed was the sum of trace amounts of a number of species which are in too low a concentration individually to be observed by sedimentation velocity. These quantities were sufficient, however, to cause an increase in the molecular weight averages (particularly M_Z) and the M_Z/M_w ratio, which becomes higher than expected for a homogeneous protein. This problem could be overcome in buffer A with or without 0.1 M NaCl by reducing the solution columns from 3 to 2 mm, which reduced the time required to reach equilibrium to *ca.* 20 hr: no aggregation was then seen and the values calculated for M_w and M_Z almost coincided.

Further improvement of the molecular weight measurements of dogfish phosphorylase *b* became possible when it was found that inclusion of 2 mM AMP completely abolished the time-dependent formation of soluble aggregates (even after prolonged storage), although this nucleotide had no effect on the $s_{20,w}$ or the molecular weight of this enzyme. This very high stability with respect to size in the presence of AMP allowed further measurements to be made using the high-speed sedimentation equilibrium technique of Yphantis (1964) carried out with six-channel cells.

Rabbit phosphorylase *b* freed of AMP also showed a time-

[1] In view of the very high density of Kel-F, different mixtures of these two oils would also be suitable for apparent specific volume measurements in buffers of widely differing concentration.

[2] There is uncertainty as to the degree of hydration of glycerophosphate; the sodium glycerophosphate used in these studies was a sample of the monohydrate (molecular weight taken as 234) from Nutritional Biochemicals Corp., Cleveland.

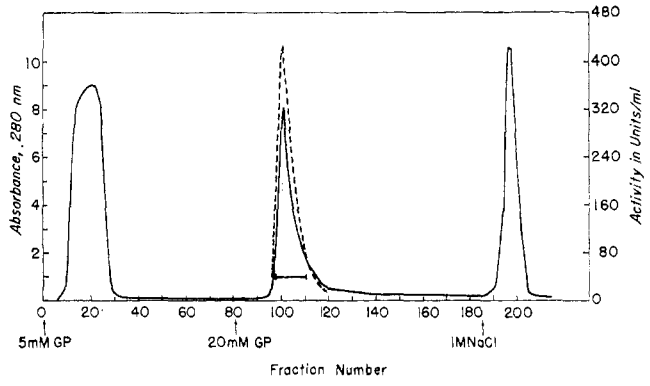

FIGURE 1: Elution profile from DEAE-cellulose chromatography (step 4). The full line represents the absorbance at 280 nm and the broken line, activity in units per milliliter. The horizontal line indicates the fractions pooled after elution. GP = sodium glycerophosphate buffer. Each fraction was 20 ml.

dependent aggregation at both 10 and 20°. With both the dogfish and the rabbit enzyme, the rate of aggregation increased markedly as the NaCl concentration was raised. It is unlikely that this aggregation resulted from sulfhydryl oxidation since all solutions contained 50 mM mercaptoethanol. These problems were not encountered with either dogfish or rabbit phosphorylase *a*.

Pyridoxal phosphate content of dogfish phosphorylases was measured both by (a) the method of Wada and Snell (1961) after release of pyridoxal phosphate from the protein with perchloric acid (Sevilla and Fischer, 1969), and (b) by titration of the apoenzyme with pyridoxal phosphate. In this latter procedure, pyridoxal phosphate was first resolved from phosphorylase *b* with imidazole–citrate–cysteine (Shaltiel *et al.*, 1966) and phosphorylase activity was then determined after incubation (30 min at 30°) of the apoprotein with different amounts of the cofactor, to achieve complete reactivation at each pyridoxal phosphate concentration. Values were corrected for residual activity remaining after resolution, which was assumed to equate with residual pyridoxal phosphate as found for the rabbit muscle enzyme (Shaltiel *et al.*, 1966).

Measurement of Phosphate Incorporated into Phosphorylase a. γ-Labeled [^{32}P]ATP was prepared by the method of Glynn and Chappell (1964). Inorganic phosphate was eliminated through charcoal, and the [^{32}P]ATP, eluted with 0.5% ammonium hydroxide in 50% ethanol, was measured both spectrophotometrically at 260 nm and colorimetrically according to Fiske and Subbarow (1925) after hydrolysis with sulfuric acid. The [^{32}P]ATP specific radioactivity determined by the two procedures agreed to within 1%; contaminating inorganic phosphate was below 1%, as estimated by the latter method.

Phosphorylase *b* (from dogfish or rabbit muscle) was incubated with the γ-labeled [^{32}P]ATP of known specific radioactivity (780,000 cpm/μmole), Mg^{2+} and rabbit muscle phosphorylase kinase at 20°, for four times the period required to reach constant specific enzymatic activity measured in the absence of AMP. The phosphorylase kinase added was less than 1% of the phosphorylase in the reaction. Saturated ammonium sulfate (1.5 volume) was then added, and the precipitate collected by centrifugation, washed twice with 50% ammonium sulfate, redissolved in 50 mM glycerophosphate–10 mM mercaptoethanol (pH 7.0), and this solution was passed through a Sephadex G-25 column equilibrated in the same buffer. Protein concentration was determined on an aliquot of the eluate from the 280-nm absorbancy; the 260/280 ratio was identical with that found for the enzymes freed of AMP which was taken to indicate complete removal of ATP. Further aliquots were suitably diluted with 88% formic acid and counted for ^{32}P in a Nuclear-Chicago scintillation counter using a dioxane-based scintillant.

Results

Purification of Dogfish Phosphorylase b. The enzyme prepared by the following procedure was in a form completely dependent on AMP for activity and designated phosphorylase *b* by analogy with the rabbit muscle enzyme.

Dogfish, weighing 5–10 lb each, were netted in the waters of Puget Sound and killed by severing the spinal cord below the head. The back and tail muscles were removed rapidly, freed from skin and cartilage, and frozen. The frozen muscle was used within 3 months. Purification of the enzyme was carried out at 0–4° unless otherwise stated.

Muscle (1000 g) was thawed for 1 hr under 20° running tap water, minced, extracted with 1000 ml of water for 15 min at room temperature, and the solution collected by straining through cheesecloth and squeezing. The muscle was extracted two more times, first with 1000 ml of water for 10 min then with 500 ml for 5 min. The combined extracts (step 1) were pooled, adjusted from pH 6.0 to pH 5.4 with 1 M acetic acid, and centrifuged for 20 min at 10,000*g*. The supernatant was filtered through fluted filter paper to remove lipid and titrated to pH 6.8 with solid potassium bicarbonate (step 2). To each liter of protein solution, 1250 ml of 3.6 M ammonium sulfate (475 g/l.), containing 0.6 ml of ammonium hydroxide/l., was added making the final concentration 2.0 M (50%); 2-mercaptoethanol (7.5 mM) and EDTA (0.5 mM) were included and also added to all buffers after this stage. After leaving at 4° overnight, the precipitate was collected by centrifugation at 10,000*g* for 20 min, resuspended in 5 mM glycerophosphate (pH 7.0), and dialyzed for 30 hr against two changes of 50 volumes of the same buffer. The enzyme solution was then centrifuged for 1 hr at 80,000*g* to remove a fine precipitate of denatured protein (step 3), and layered on a 30 × 4 cm DEAE-cellulose column equilibrated with the dialysis buffer. The column was washed extensively with this buffer, and the enzyme eluted with 20 mM glycerophosphate (pH 7.0). At this salt concentration dogfish phosphorylase was essentially the only protein eluted, and began to emerge after four to five void volumes. The elution profile is shown in Figure 1. The active fractions were pooled (step 4) and the solution taken to 40% saturation in ammonium sulfate. Very slight turbidity was removed by centrifugation and the ammonium sulfate increased to 50%. After standing at 4° for 2 hr, the precipitate was collected and dissolved in buffer A.

A summary of a typical purification is shown in Table I. It can be seen that there is approximately 1.25 g of phosphorylase per 1000 g of dogfish muscle, *i.e.*, slightly less than half the amount present in rabbit skeletal muscle (Fischer and Krebs, 1958) but as much as found in human muscle (Yunis *et al.*, 1960). From 2000 g of muscle, approximately 1 g of purified enzyme can be obtained (40% yield) in 3–4 days.

Stability. To store the enzyme, the redissolved precipitate from step 5 was dialyzed against buffer A containing 2 mM AMP, and the dialyzed solution was then frozen after addition of glycerol to 20%. When kept in this way, there was no loss of enzyme activity in 4 months. If AMP and glycerol were omitted, there was an activity loss of 20–30% a week at 4°.

TABLE I: Summary of a Typical Purification of Dogfish Skeletal Muscle Glycogen Phosphorylase.[a]

Step	Volume (ml)	Protein (mg)	Units	Sp Act.	Purification	Yield (%)
1. Extract	5750	46,000	153,000	3.3	1.0	100
2. Acid (pH 5.4) supernatant	5350	40,000	149,000	3.7	1.1	97
3. 50% $(NH_4)_2SO_4$ precipitate after dialysis and centrifugation	240	8,160	100,000	12.3	3.7	67
4. DEAE-cellulose chromatography (pH 7.0)	300	1,080	65,000	60.0	18.2	43
5. 50% $(NH_4)_2SO_4$	30	1,000	62,000	62.0	18.8	41

[a] At steps 1, 2, and 3 the protein was measured by the procedure of Lowry *et al.* (1951). At steps 4 and 5 the $A_{280}^{1\%}$ of 12.9 found for the pure dogfish phosphorylase was used. 2000 g of muscle was used in this preparation.

Preparation of Dogfish Phosphorylase a and Interconversion of the b and a Forms. Dogfish phosphorylase *b* could be converted to the *a* form (almost fully active in the absence of AMP) using rabbit muscle phosphorylase kinase, Mg^{2+}, and ATP. The rate of this reaction was identical with that obtained for the conversion of rabbit muscle phosphorylase *b* under the same experimental conditions (Figure 2). Likewise, dogfish phosphorylase *a* could be converted back to *b* by incubation with either rabbit muscle or rabbit liver phosphorylase phosphatase; again, the rate of conversion was identical for the two enzymes (Figure 2) stressing once more the lack of species specificity for these interconverting enzymes.

Homogeneity. Purified dogfish phosphorylase *b* migrated as a single band on polyacrylamide gel electrophoresis at pH 8.3 as judged by both protein (Figure 3A) and enzyme activity (Figure 3B) stains. When phosphorylase *b* was converted to phosphorylase *a* (prepared as indicated under Methods), a band migrating faster toward the anode appeared (Figure 3D,E) while no material remained in the original phosphorylase *b* position (Figure 3C). Polyacrylamide gel electrophoresis in sodium dodecyl sulfate at pH 7.2 also yielded a single protein band (Figure 3F). Sedimentation velocity of phosphorylase *b* in buffer A gave a single symmetrical peak in the ultracentrifuge (Figure 4) and sedimentation equilibrium experiments to be described later likewise indicated that the preparations were homogeneous.

Both rabbit skeletal muscle phosphorylases *a* (Green and Cori, 1943) and *b* (Fischer and Krebs, 1958) have been crystallized at 0° in the presence or absence of AMP and Mg^{2+}; these methods did not lead to the crystallization of either dogfish phosphorylase *b* or *a*.

Activities of Dogfish Phosphorylases b and a. Dogfish phosphorylases *b* and *a* had identical specific activities in the presence of AMP (62 units/mg). In the absence of AMP, phosphorylase *b* was almost completely inactive while phosphorylase *a* retained 80% of the activity measured in the presence of this nucleotide. The specific activity of dogfish phosphorylase *b* was 70% that of rabbit phosphorylase *b* when measured in the standard assay (Hedrick and Fischer, 1965). Michaelis constants showed that the substrate concentrations were quite close to saturating levels for dogfish phosphorylase *b*. The

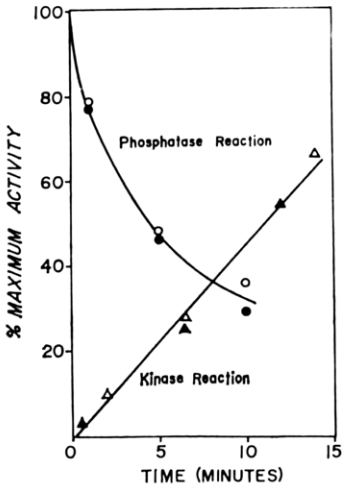

FIGURE 2: Conversion of dogfish (△) and rabbit (▲) muscle phosphorylase *b* into phosphorylase *a* by rabbit muscle phosphorylase kinase at pH 8.6 and conversion of dogfish (○) and rabbit (●) muscle phosphorylase *a* into *b* by rabbit muscle phoshorylase phosphatase at pH 7.0. Reaction mixtures contained 3.0 mg/ml of phosphorylase; all assays were carried out at 30° in the absence of AMP.

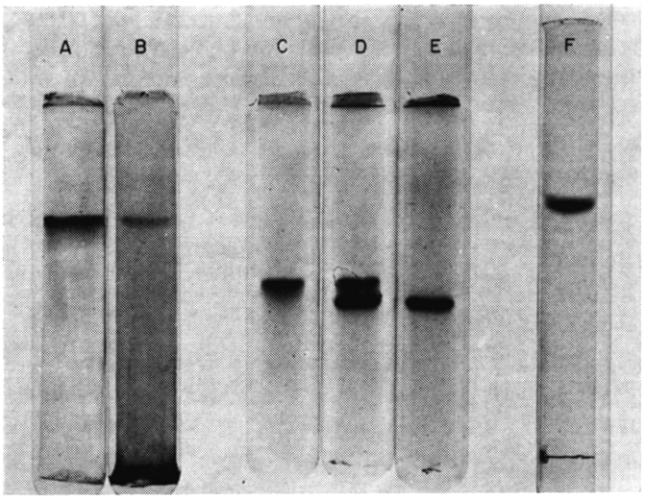

FIGURE 3: Polyacrylamide gel electrophoresis of dogfish phosphorylase preparations were carried out at pH 8.3 (A to E) and at pH 7.2 (F). A and B show dogfish phosphorylase *b* stained for protein (coomassie blue) and activity, respectively. Phosphorylase *a* (E) migrates faster than phosphorylase *b* (C) as shown by separation of mixtures of the two forms (D); these electrophoreses were carried out for a longer period than in A and B to afford a better separation of the *b* and *a* forms. F shows electrophoresis of phosphorylase *b* in sodium dodecyl sulfate. In all instances, 10 μg of protein was applied to the gel. Migrations are from top to bottom.

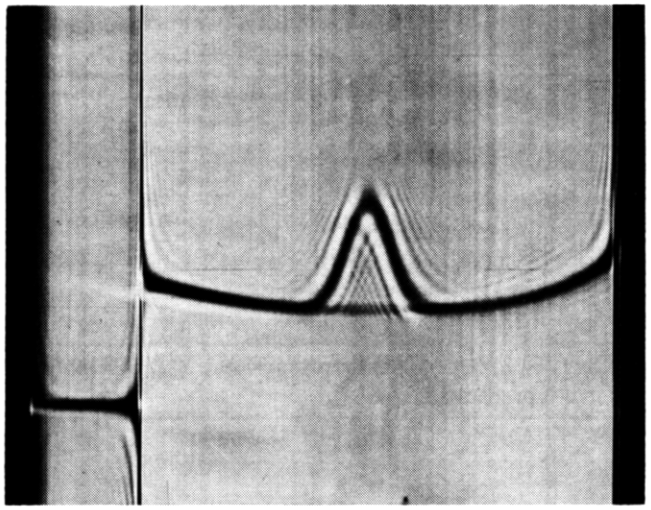

FIGURE 4: Sedimentation pattern of purified dogfish phosphorylase *b* in buffer A. The picture was taken 64 min after maximum speed of 52,000 rpm was reached. Migration is from left to right; the temperature was 10.0°.

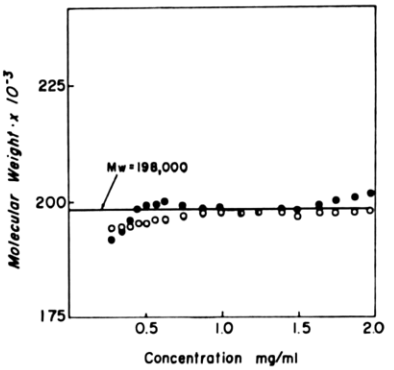

FIGURE 6: Distribution of molecular weights of dogfish phosphorylase *b* in a high-speed sedimentation equilibrium experiment in buffer A + 2 mM AMP. Open circles show M_n and closed circles, M_w. The whole cell average M_w value is shown by the horizontal line. The initial protein concentration was 0.35 mg/ml, rotor speed 12,000 rpm and temperatue 10°.

slightly higher K_m of 24 mM for glucose 1-phosphate (*vs.* 15 mM for rabbit phosphorylase) and the higher K_m for glycogen as primer (0.12% *vs.* 0.018%) only account for *ca.* 10% of the 30% difference in specific activity from the rabbit enzyme. Reciprocal plots of $1/V$ *vs.* 1/(S) were linear for glucose 1-phosphate with 1.0 mM AMP and 1% glycogen, or for glycogen with 1.0 mM AMP and 75 mM glucose 1-phosphate, but the corresponding plots for AMP were nonlinear. The K_m for AMP (0.15 mM) was calculated from Hill plots of log $V/(V_{max} - V)$ *vs.* log (AMP). The average of n calculated for experimental points between 0.5 and 0.05 mM AMP was 1.5 for both dogfish and rabbit muscle phosphorylase *b*. The specific activity for 62 units/mg in the standard assay corresponds to a turnover number of 6200 moles of glucose 1-phosphate consumed per min per mole of enzyme monomer (mol wt 100,000). By extrapolation of the reciprocal plots for each reaction component to obtain a V_{max} value, a turnover number of 8700 may be estimated for dogfish phosphorylase *b* compared to 11,000 for rabbit phosphorylase *b* treated in the same fashion. A summary of these data will be found in Table III.

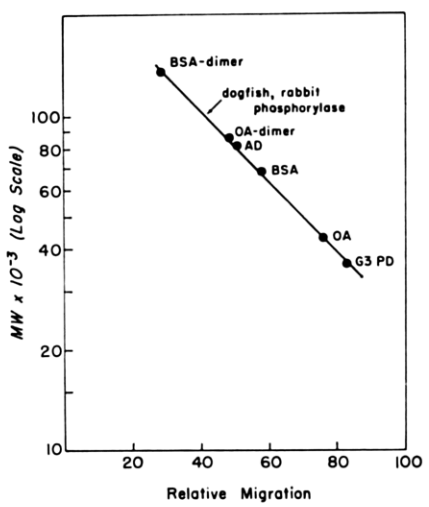

FIGURE 5: Calibration of sodium dodecyl sulfate gels with marker proteins. Abbreviations: BSA, bovine serum albumin; OA, ovalbumin; AD, arginine decarboxylase; G3PD, glyceraldehyde 3-phosphate dehydrogenase. Further details are given in the text.

Molecular Weight and Subunit Structure. An investigation of the dependence of molecular size on pH or salt concentration or both can be a useful method of elucidating the subunit equilibria existing in native proteins, when coupled with a reliable value for the subunit size (Cohen and Rosemeyer, 1969). In the present work we have investigated the molecular weight and subunit interactions of dogfish phosphorylases *b* and *a* by sedimentation equilibrium experiments and from the variation of the sedimentation coefficients of the proteins with increasing NaCl concentrations. The subunit molecular weights were measured by acrylamide gel electrophoresis in sodium dodecyl sulfate. For comparison purposes, parallel experiments were also carried out with rabbit phosphorylases *b* and *a*.

Subunit Molecular Weights by Sodium Dodecyl Sulfate Gel Electrophoresis. As described under Methods, slight modification of the published procedure of Weber and Osborn (1969) yielded a linear calibration graph with markers ranging from 36,000 to 136,000 (Figure 5). Single protein bands were obtained with both dogfish phosphorylase (Figure 3F) and rabbit phosphorylase, showing that each was composed of subunits of uniform size. The subunit molecular weights obtained were 99,000 for dogfish phosphorylase and 100,000 for the rabbit enzyme. The deviation from these values was less than ±2% in ten separate experiments.

Sedimentation Equilibrium Studies. PHOSPHORYLASE *b*. In low-speed sedimentation equilibrium experiments with dogfish phosphorylase *b*, M_z, calculated directly from the schlieren patterns, gave extremely reproducible values (±2%) in duplicate determinations with different enzyme preparations (Table II). M_w, for which both equilibrium and synthetic boundary runs are required, showed rather more scatter (±4–5%), probably because of small and variable losses of material in the equilibrium runs, for reasons discussed by Yphantis (1964).

Following the precautions described under Methods, the M_z/M_w ratio was very close to unity in all experiments with dogfish phosphorylase *b* (Table II). The distribution of molecular weights within a single cell at equilibrium were homogeneous; a high-speed sedimentation equilibrium experiment is illustrated in Figure 6. The agreement between the results of low- and high-speed sedimentation equilibrium was extremely

TABLE II: Summary of Molecular Weight Determinations of Dogfish and Rabbit Muscle Phosphorylases by Sedimentation Equilibrium.[a]

Phosphorylase	M_w	M_Z	Method	Rotor Speed
1. Dogfish *b* − AMP	194,000	203,000	L.S.	5,200
2. Dogfish *b* + AMP	196,000 ± 4%	202,000 ± 2%	L.S.	5,200
	198,000	201,000	H.S.	12,000
3. Dogfish *a*	249,000	280,000	L.S.	5,200
4. Rabbit *a*		402,000 ± 2%	L.S.	4,400

[a] Sedimentation equilibrium of dogfish phosphorylases *b* and *a* was carried out at 10° in buffer A. Runs with rabbit phosphorylase *a*, carried out at 20°, also included 0.2 M NaCl. H.S., high-speed and L.S., low-speed sedimentation equilibrium. Values for dogfish phosphorylase *b* + AMP and rabbit phosphorylse *a* were averages from experiments with three and two different enzyme preparations, respectively. Protein concentrations were 4.5 ± 1 mg/ml; $\bar{v}$ of 0.746 at 20° and 0.741 at 10° were used (see Methods).

good, although they covered a 20-fold range in protein concentration. These results, together with the measured molecular weight of exactly twice the subunit size of 99,000 within experimental error, show that dogfish phosphorylase *b* has a dimeric structure in this solvent.

PHOSPHORYLASE *a*. Sedimentation equilibrium experiments with rabbit phosphorylase *a* were previously carried out at high speed using initial concentrations of *ca.* 0.5 mg/ml (Seery *et al.*, 1967). However in view of the report that rabbit phosphorylase *a* starts dissociating from tetramer to dimer below 3 mg/ml (Chignell *et al.*, 1968) and the present work which suggested a higher value for the phosphorylase subunit, it was thought desirable to carry out sedimentation equilibrium experiments with rabbit phosphorylase *a* at low speed using high initial concentrations (*ca.* 5–6 mg/ml); NaCl (0.2 M) was included to maintain the solubility of the protein. Values of M_Z = 402,000 ± 2% were obtained, exactly four times the subunit size of 100,000 found for rabbit phosphorylase. The equilibrium plots were completely homogeneous (not illustrated); likewise, sedimentation velocity experiments carried out with the solutions both before and after the equilibrium run revealed no trace of either heavy or light component.

The molecular weight averages found for dogfish phosphorylase *a* in buffer A were M_w = 249,000 and M_Z = 280,000, indicating that dogfish phosphorylase *a* is about 25% associated from dimer to tetramer in this solvent, a conclusion already suggested by the weight-average sedimentation coefficients and further supported by boundary patterns of the protein obtained in sedimentation velocity experiments (see below). The M_Z/M_w ratio of 1.12 was also consistent with a partially associated molecule. The sedimentation equilibrium data are summarized in Table II.

Sedimentation Velocity of Phosphorylases b and a. In order to look for possible differences existing in the dissociation equilibria between the native dogfish and rabbit enzymes, solutions of increasing NaCl concentration were used to perturb the interactions between the subunits. Results from sedimentation velocity experiments for both dogfish and rabbit phosphorylase *b* (with or without AMP) as well as for dogfish and rabbit phosphorylase *a*, are illustrated in Figure 7.[3] In the absence of AMP, dogfish and rabbit phosphorylase *b* had closely similar sedimentation coefficients of 8.9 and 8.8 S,[4] respectively. On increasing the NaCl concentration, both enzymes showed an identical decrease in sedimentation coefficient, reaching 7.6 S in 2.0 M NaCl.

These data were analyzed in order to determine whether the decrease represented partial dissociation of the proteins to monomer, or whether it was caused by an increase in the apparent specific volume, resulting from preferential binding of water with increasing salt concentration. Assuming no change in the size, or in the frictional ratio (f/f_0) of the enzymes, the increase in apparent specific volume needed to account for the observed decrease in $s_{20,w}$ in 2.0 M NaCl was calculated from the equation (Aune and Timasheff, 1970)

$$S\eta = k\frac{(1 - \phi'_2\rho)}{(\phi'_2)^{1/3}}$$

where $S\eta$ is the sedimentation coefficient corrected only for viscosity, ϕ'_2 is the apparent specific volume, and p is the density. The constant, k, was evaluated from the measured values of $S\eta$, ϕ'_2, and p in buffer A. Substitution of the data obtained in 2 M NaCl gave ϕ'_2 = 0.769 at 20°.

The apparent specific volume of *rabbit* phosphorylase *b* in buffer A + 2.0 M NaCl (pH 7.0) was also determined by direct pycnometric measurement as described under Methods (aliquots diluted with or without 2.0 M NaCl gave identical 280-nm readings). Four separate determinations gave ϕ'_2 = 0.765 ± 0.003 at 20° (as compared to 0.746 in the absence of NaCl) in close agreement with the value of 0.769 calculated theoretically. This proves that the decrease in $s_{20,w}$ of phosphorylase *b* solutions with increasing NaCl concentration is almost entirely due to preferential solvation. This conclusion correlates with the observation that the schlieren patterns were symmetrical at all NaCl concentrations up to 2.0 M: if substantial dissociation had occurred, either a single asymmetric boundary, or two separate boundaries corresponding to dimer and monomer would have been expected, depending on whether the dissociation equilibrium was fast or slow (Gilbert, 1959).

In view of the difficulties in measuring apparent specific volumes to better than ±0.5%, and the assumptions made in the theoretical calculation, 0.765 cannot be regarded as significantly less than 0.769. On the other hand, if slight dissociation

[3] Tables listing all experimental conditions and results of sedimentation velocity experiments can be obtained by writing directly to the authors.

[4] The $s_{20,w}$ of 8.4 S reported previously (Seery *et al.*, 1967) was an observed sedimentation coefficient and not corrected for the relative viscosity and density of "buffer A" (this factor being 1.040 at 20°).

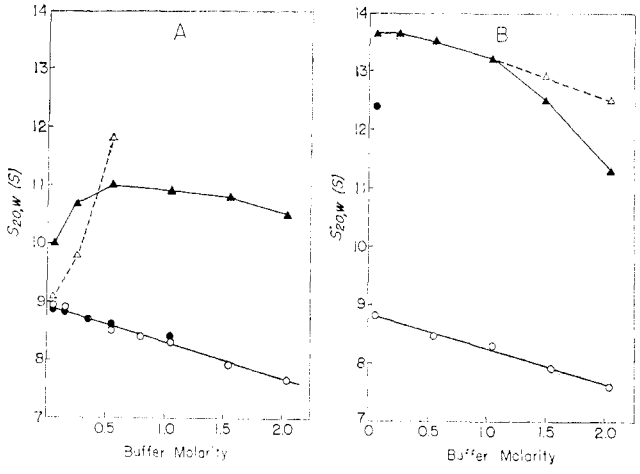

FIGURE 7: Dependence of s_{20}, of phosphorylases on NaCl concentration at pH 7.0. (A) Dogfish phosphorylases *b* and *a*. (B) Rabbit phosphorylases *b* and *a*. Symbols refer to: phosphorylase *b* without AMP (○) or with 2 mM AMP (●), and phosphorylase *a* (▲). The open triangles and broken lines show the sedimentation coefficient of phosphorylase *a* estimated from the peak height positions in experiments where asymmetric schlieren patterns were obtained. A temperature of 10° was used except with rabbit phosphorylase *a*, when experiments were carried out at 20°. All runs contained buffer A plus NaCl to the final molarity indicated in the figure. For further details, see Methods.

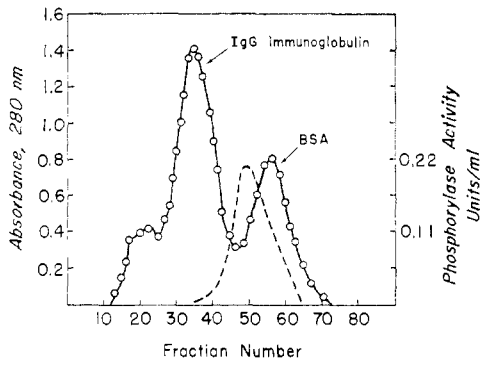

FIGURE 8: Gel filtration of rabbit phosphorylase *b* on Sephadex G-200 equilibrated in 2 M NaCl. Full line and open circles, absorbance at 280 nm; broken line, phosphorylase activity in units per milliliter. BSA, bovine serum albumin. The column was 110 × 1.5 cm and 1-ml fractions were collected; fraction 1 was collected 35 ml after the sample was applied. Phosphorylase was located by mixing the eluate with an equal volume of standard substrate mixture.

from dimer to monomer in 2 M salt had occurred, it could not be detected by the above treatment. Accordingly, rabbit phosphorylase *b* was subjected to gel filtration on Sephadex G-200 equilibrated in 2 M NaCl, at very low (less than 10 μg/ml) enzyme concentration. The reference markers were bovine γ-globulin (mol wt 150,000) and bovine serum albumin (68,000), which do not undergo alteration in size in this solvent. Figure 8 shows that rabbit phosphorylase *b* elutes in a position much closer to serum albumin that to γ-globulin, suggesting almost complete dissociation to monomer. From mass action considerations this would imply a degree of dissociation of *ca.* ≥3% in 2 M NaCl at the concentration used in sedimentation velocity experiments (4 mg/ml).

The sedimentation coefficient of dogfish phosphorylase *b* was identical in the presence or absence of 2 mM AMP in buffer A (8.9 S). In contrast, the sedimentation coefficient of rabbit phosphorylase *b* increased in the presence of 2 mM AMP and showed an asymmetric pattern from which a value of 13.3 S was calculated from the peak height position, and a value of 12.4 S from the migration of the second moment (see single point in Figure 7B).

Phosphorylase a. The sedimentation coefficient of rabbit phosphorylase *a* also decreased from 13.6 to 13.2 S on increasing the NaCl concentration to 1.0 M, as shown in Figure 7B. The percentage decrease was, however, smaller than with phosphorylase *b*, suggesting less preferential hydration. A plot of the sedimentation coefficient corrected only for viscosity as a function of solution density (Katz and Schachman, 1955; Cox and Schumaker, 1961) was linear, and extrapolation to the density corresponding to zero sedimentation gave 1.28 for phosphorylase *a* and 1.24 for phosphorylase *b*. Using the apparent specific volumes measured in dilute buffer (0.746 at 20° and 0.741 at 10°), it was calculated that rabbit phosphorylase *a* can preferentially bind 0.19 g of water/g of protein at 20° as compared to 0.34 g/g at 10° for both dogfish and rabbit phosphorylase *b*.

Dissociation of rabbit phosphorylase *a* from tetramer to dimer at 4 ± 1 mg/ml starts to occur only at NaCl concentrations of 1.5 M and above, as shown by the replacement of symmetrical peaks with asymmetric boundaries and by sharper decrease in the weight-average sedimentation coefficient (Figure 7B). It has been previously shown by Wang and Graves (1963) that dissociation of phosphorylase *a* from tetramer to dimer is almost complete at 2.5 M NaCl.

In contrast to both rabbit phosphorylase *a* and to dogfish and rabbit phosphorylase *b*, NaCl produced an *increase* in the sedimentation coefficient of dogfish phosphorylase *a* as the concentration was raised to 0.5 M. The schlieren peaks were not symmetrical, showing forward skewing in the absence of NaCl and backward skewing at 0.5 M or higher concentrations of this salt (Figure 9). This is also shown in Figure 7A, where the sedimentation coefficients calculated from the peak height positions are an underestimate of the weight-average sedimentation coefficient at zero NaCl, and an overestimate at 0.5 M NaCl.

The weight-average sedimentation coefficient of dogfish phosphorylase *a* in buffer A was 10.0 S. Assuming that molecular weight is directly related to $S^{3/2}$ and calculating the proportionality constant from the $s_{20,w}$ of 8.9 S and the molecular weight of 200,000 found for dogfish phosphorylase *b*, a molecular weight of 240,000 was estimated. This agrees closely with the value of 249,000 measured by sedimentation equilibrium under the same conditions (Table II).

These experiments therefore support the contention that dogfish phosphorylase *a* is 20–25% associated from dimer to tetramer in buffer A and demonstrate that the equilibrium can be displaced to some extent in favor of the tetramer by addition of NaCl. This influence of NaCl suggests that there may be ionic forces preventing the association of dimeric dogfish phosphorylase *a* molecules. Addition of 2 mM AMP did not alter the sedimentation coefficient of dogfish phosphorylase *a* in buffer A.

Pyridoxal 5-Phosphate Content. The spectrum of dogfish phosphorylase *b* at pH 7.0 closely resembles that of the rabbit muscle enzyme, with an absorption peak at 330 nm in addition to that at 280 nm (Kent *et al.*, 1958), suggesting the presence of pyridoxal 5′-phosphate in the dogfish enzyme.

Treatment of dogfish phosphorylase *b* with imidazole–citrate–cysteine (pH 6.1) which resolves pyridoxal 5′-phosphate from rabbit muscle phosphorylase *b* (Shaltiel *et al.*, 1966) led to virtually complete loss of enzyme activity, which could be

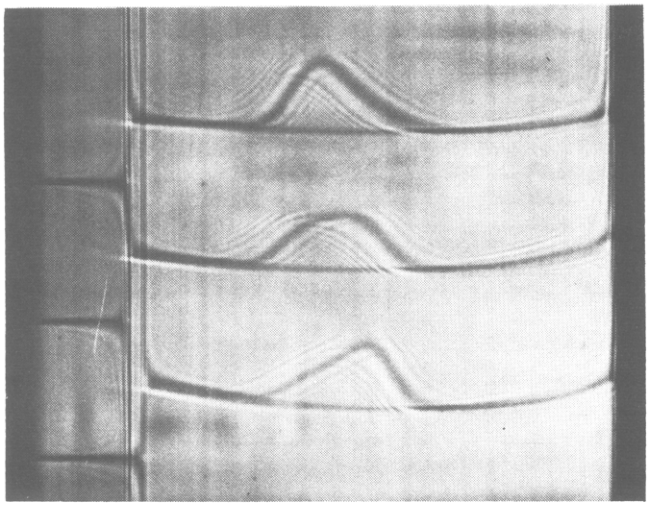

FIGURE 9: Sedimentation velocity of dogfish phosphorylase *a* at 10°. Experiments were carried out in buffer A with zero NaCl (upper), 0.2 M NaCl (middle), and 0.5 M NaCl added (lower pattern). The photograph was taken 64 min after reaching the maximum speed of 48,000 rpm. Migration is from left to right. Note the change in boundary shape with increasing NaCl concentration; above 0.5 M there was no further alteration in the appearance of the schlieren peak.

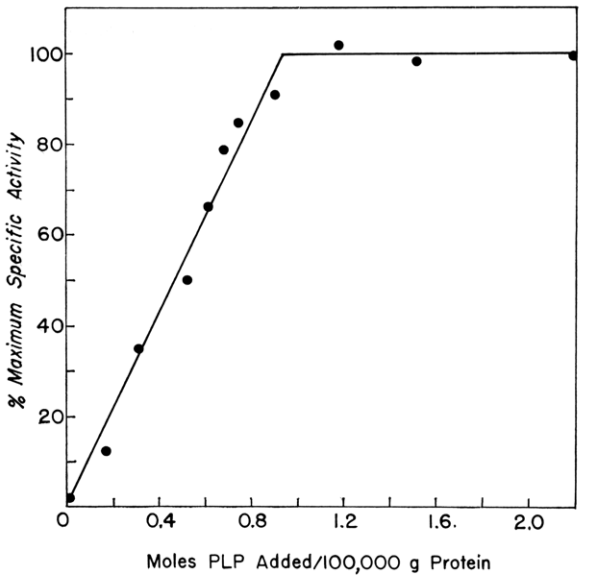

FIGURE 10: Titration of dogfish apophosphorylase *b* with pyridoxal 5-phosphate. Details are given in text.

almost fully restored by readdition of pyridoxal 5′-phosphate, but not pyridoxal.

The presence of pyridoxal 5′-phosphate was further confirmed as follows: addition of a drop of glacial acetic acid to dogfish phosphorylase *b* produced a bright yellow coloration which faded within 1 hr; the solution was neutralized with NaOH, boiled for a few seconds, and centrifuged to remove denatured protein. The absorption spectrum of the supernatant was characteristic of pyridoxal 5′-phosphate with a maximum at 390 nm both in 0.1 N NaOH and at pH 7.0. The neutralized supernatant reactivated *rabbit* apophosphorylase *b* to exactly the same extent as occurred with pyridoxal 5′-phosphate (usually to *ca.* 90%). Prior incubation of the supernatant with alkaline phosphatase abolished this reactivation.

The stoichiometry of pyridoxal 5′-phosphate binding to dogfish phosphorylase was determined both by the phenylhydrazine method of Wada and Snell (1961) and by direct titration of the apoenzyme with the cofactor as described under Methods (Figure 10). The average of several determinations on different enzyme preparations by each procedure showed an uptake of one molecule of pyridoxal 5′-phosphate per monomer within experimental error (Table III).

Pyridoxal 5′-phosphate could be reduced onto dogfish phosphorylase *b* with sodium borohydride, after formation of the yellow Schiff base by the high salt method of Strausbauch *et al.* (1967). This was confirmed by the isolation and sequence determination of a unique peptide containing the phosphopyridoxyllysyl residue following cyanogen bromide cleavage of the reduced enzyme (P. Cohen, J. C. Saari, and E. H. Fischer, in preparation). Reduced dogfish phosphorylase *b* was 65% as active as the native enzyme under the normal assay conditions (Table III).

Stoichiometry of Phosphate Incorporation in the Conversion of Phosphorylase b into a. One phosphate was incorporated per monomer in the *b* to *a* conversion within experimental error for both dogfish and rabbit enzymes. The results summarized in Table III are the averages of three separate determinations for each enzyme. The specificity of phosphate incorporation was confirmed by the isolation and sequence determination of a single peptide containing the phosphate group bound to a unique seryl residue (P. Cohen, J. C. Saari, and E. H. Fischer, in preparation).[5]

Discussion

The properties of dogfish and rabbit skeletal muscle phosphorylases, summarized in Table III, show many similarities. The near identity of properties 1, 2, 3, 5, 6, 9, 10, and 11 are suggestive of structural homology and this has been confirmed by sequence studies on the two proteins (P. Cohen, J. C. Saari, and E. H. Fischer, in preparation). It is of interest that the level of phosphorylase in dogfish muscle is similar to that found in mammalian skeletal muscles, while the existence of two forms of dogfish phosphorylase, *b* and *a*, dependent and independent of AMP for activity, and their interconversion by rabbit phosphorylase kinase and phosphatase suggests that several of the factors important to the control of glycogen degradation in rabbit skeletal muscle are also present in dogfish muscle. Indeed, both a phorphorylase kinase and phosphatase have been identified in dogfish muscle and their purification and furtherc haracterization is presently being investigated.

From the results given here, a subunit size of 100,000 ± 1000 has been proposed for both dogfish and rabbit skeletal muscle phosphorylase which is 8% higher than that reported previously for the rabbit enzyme (Seery *et al.*, 1967). Several factors have contributed to this upward revision: high-speed sedimentation equilibrium experiments with rabbit phosphorylase *b* previously gave M_w = 191,000 and M_Z = 195,000 and these values were extrapolated downward to 185,000 for a discrete species of the enzyme, because it was believed that the enzyme was slightly associated from dimer to tetramer (Seery *et al.*, 1967). This assumption was based on low-speed

[5] The lower values for phosphate stoichiometry reported previously (Sevilla and Fischer, 1969) for the rabbit enzyme are almost completely explained by a combination of low molecular weight and absorbancy index used.

TABLE III: Summary of Properties Found for Dogfish and Rabbit Skeletal Muscle Phosphorylases.[a]

Property	*b* or *a* Form of the Enzyme	Dogfish	Rabbit
1. 260/280 nm absorbance ratio	*b*	0.56	0.53
2. $A_{280}^{1\%}$	*b*	12.9 ± 0.2	13.1 ± 0.2
3. Specific activities (units/mg)[b]	*b* − AMP	<1	<1
	b + AMP	62	88
	a − AMP	48	54
	a + AMP	62	80
	$NaBH_4$-reduced *b*	38	50 ± 5[c]
4. K_m glucose 1-phosphate (mM)	*b*	24	15
K_m AMP (mM)	*b*	0.15	0.14
K_m glycogen (%)	*b*	0.12	0.018
5. Apparent specific volume	*b*	0.746	0.746 ± 0.002
6. Subunit molecular weight		99,000	100,000
7. Molecular weight of native enzyme	*b* − AMP	M_w = 194,000 M_Z = 203,000 (L.S.)	M_w = 198,000 ± 3%[d] M_Z = 202,000 ± 3% (H.S.)
	b + AMP	M_w = 196,000 ± 4% (L.S.) M_Z = 202,000 ± 2% M_w = 198,000 M_Z = 201,000 (H.S.)	Dimers associate
	a	M_w = 249,000 M_Z = 280,000 (L.S.)	M_Z = 402,000 ± 2% (L.S.)
8. $s_{20,w}$ (S)	*b* − AMP	8.9	8.8
	b + AMP	8.9	12.4
	a	10.0	13.7
9. PLP stoichiometry (moles/100,000 g)			
(1) Phenylhydrazine method	*b*	1.03 ± 0.05	1.0[e]
(2) Titration of apoenzyme	*b*	0.96	1.03
10. Moles of phosphate incorporated per 100,000 g in *b* → *a* conversion		1.04 ± 0.06	1.08 ± 0.09
11. Effectiveness as substrates for interconverting enzymes			
(a) Rabbit muscle phosphorylase kinase	*b*	++	++
(b) Rabbit muscle phosphorylase phosphatase	*a*	++	++
(c) Rabbit liver phosphorylase phosphatase	*a*	++	++
12. Crystallization	*a* (pH 7.0, 0°)	−	+
	b (±AMP, Mg^{2+}, 0°)	−	+

[a] Activity measurements were carried out in 0.1 M maleate buffer (pH 6.5). Other experiments were carried out in 50 mM glycerophosphate–1.0 mM EDTA (pH 7.0) with mercaptoethanol varying from 1 to 50 mM. For sedimentation velocity and sedimentation equilibrium of rabbit phosphorylase *a*, 0.2 M NaCl was also included. H.S., high-speed; L.S., low-speed sedimentation equilibrium. [b] A unit of activity is expressed as μmoles of P_i released/min at 30°. [c] Recalculated from Strausbauch *et al.* (1967) using $A_{280}^{1\%}$ = 13.1. [d] Recalculated from Table I of Seery *et al.* (1967) using $\bar{v}$ = 0.746. [e] Recalculated from Kent *et al.* (1958) for 100,000 molecular weight.

sedimentation equilibrium at higher initial protein concentrations which gave increased molecular weight averages, and on a slight inhomogeneity of the distribution of molecular weights observed in the high-speed experiments. However, in low-speed experiments the increases were almost entirely reflected by changes in M_Z and not M_w (Seery *et al.*, 1967) and the-present work now suggests that the above observations sulted not from the existence of a dimer–tetramer equilibrium, but from the formation of small amounts of soluble aggregates during these runs, a problem encountered with both rabbit and dogfish phosphorylase *b* freed of AMP. This, therefore, invalidates the extrapolation used previously, and after substituting the corrected apparent specific volume of 0.746 values of M_w = 198,000 and M_Z = 202,000 were recalculated from the data of Seery *et al.* (1967) (Table III). These are identical with the values measured for dogfish phosphorylase *b* by both high- and low-speed sedimentation equilibrium in the present work. The dogfish enzyme was particularly suitable for molecular weight measurements since, in contrast to rabbit phosphorylase *b*, AMP provided complete stabilization against time-dependent aggregation, while having no effect on molecular size. Confirming these results, sedimenta-

tion equilibrium of rabbit phosphorylase *a* gave 402,000 ± 2% using conditions where no dissociation to dimers occurred, while acrylamide gel electrophoresis in sodium dodecyl sulfate gave 100,000 ± 1000 for the subunits of both dogfish and rabbit phosphorylase.

The latter procedure is perhaps the most reliable measurement of the subunit size which has been made with phosphorylase and any difference greater than 2% between the dogfish and rabbit enzymes would have been detected upon running mixtures of the two proteins. Yeast phosphorylase which could just be separated from dogfish phosphorylase by gel electrophoresis in sodium dodecyl sulfate was assigned a molecular weight of 103,000 from the calibration graph (M. Fosset *et al.*, in preparation).

The previous view that rabbit phosphorylase *b* is slightly associated from dimer to tetramer is therefore not supported by the present evidence; on the contrary, other data suggest that these molecules are actually very slightly dissociated from dimer to monomer. Thus Davis *et al.* (1967) found that dimeric rabbit skeletal muscle phosphorylase *b* isozymes will hybridize simply on mixing in glycerophosphate buffer (pH 7.0) and that the rate of hybridization was increased by raising the salt concentration. This correlates with the present finding that on gel filtration with G-200 equilibrated in 2 M NaCl, rabbit phosphorylase *b* at very low concentration elutes essentially as the monomer. Davis *et al.* (1967) also showed that hybridization was blocked by glucose 6-phosphate, an effector previously found to block resolution of the pyridoxal 5′-phosphate from the enzyme which also proceeds through monomer formation (Shaltiel *et al.*, 1966). Glucose 6-phosphate was, however, found to have no observable effect on either the molecular weight (Seery *et al.*, 1967) or the sedimentation coefficient (P. Cohen, unpublished experiments) of rabbit muscle phosphorylase *b*. This and other sedimentation equilibrium data given here indicate that the degree of dissociation of phosphorylase *b* to monomers is below the level of detection by the ultracentrifuge techniques employed and probably much less than 1%. It does not show, however, that no equilibirum with monomer exists as was concluded previously (Seery *et al.*, 1967). The rabbit phosphorylase *b* monomer produced by gel filtration at very low enzyme concentration in 2 M NaCl was active when mixed with an equal volume of the standard substrate mixture (Figure 8) and no lag period was observed before return to full activity. However, it is not yet possible to conclude that this monomeric species is active since one or more of the components of the assay (AMP, glucose 1-phosphate, and glycogen) may have produced an instantaneous reassociation of the enzyme.

Marked differences between the dogfish and rabbit phosphorylases were found in several properties. It has been demonstrated that dogfish phosphorylase *a* is *ca.* 25% associated from dimer to tetramer in solvents where rabbit phosphorylase *a* exists as a tetramer. The presence of a dimer–tetramer equilibrium in phosphorylase *a* is a property common to all the vertebrate skeletal muscle enzymes; with the species examined so far, namely, dogfish, frog (Metzger *et al.*, 1968), rabbit, rat (Sevilla and Fischer, 1969), and man (Yunis *et al.*, 1960), the extent to which tetramerization can occur increases progressively through vertebrate evolution. In contrast, lobster muscle (Assaf and Graves, 1969) and insect muscle phosphorylase *a* (Childress and Sacktor, 1970), the only invertebrate species studied, apparently show no tendency to tetramerization (insect muscle phosphorylase *a* was in fact reported to be monomeric). The relevance of the tetrameric state of rabbit skeletal muscle phosphorylase *a in vivo* is questionable since it is only partially active (Huang and Graves, 1970), while dilution, high pH, incubation with glucose, and in particular, incubation with glycogen, and temperatures above 30°, all promote dissociation to the dimeric state and markedly increase activity (Wang *et al.*, 1965a,b; DeVincenzi and Hedrick, 1970). One can, however, speculate that the presence of a dimer–tetramer equilibrium creates the potential for phosphorylases *a* and *b* dimers to interact and form hybrids with susceptibilities to metabolites different from those displayed by the *a* and *b* forms themselves (Hurd *et al.*, 1966). If such a hypothesis were correct, it might imply that there has been an increased importance of these hybrid molecules to the control mechanism in skeletal muscle during the course of evolution.

Among all phosphorylases so far investigated, there seems to be a distinct correlation between tetramerization and crystallization in the cold. Thus, only mammalian phosphorylase *a* from rabbit (Green and Cori, 1943), human (Yunis *et al.*, 1960), and rat (Sevilla and Fischer, 1969) skeletal muscle which associate essentially completely will crystallize at 0° while the enzymes from frog (Metzger *et al.*, 1968), dogfish, and lobster (Assaf and Graves, 1969) muscle, as well as those from rabbit heart muscle (isozyme 1, Davis *et al.*, 1967) and liver (Appleman *et al.*, 1966) which all show reduced or lack of association to tetramers, fail to crystallize. The same holds true for the *b* forms of the enzymes in the presence of Mg^{2+} and AMP. Enzymes that tetramerize upon addition of the nucleotide, from rabbit (Kent *et al.*, 1958), human, and rat skeletal muscle crystallize readily at 0° whereas phosphorylase *b* from the dogfish, lobster muscle, and rabbit liver whose size remains unchanged under these conditions will not crystallize. These striking correlations suggest that insolubility at low temperature and tetramerization are linked processes and that the peptide sequence(s) responsible for these properties are common only to mammalian skeletal muscle phosphorylases. In the case of the Pacific dogfish, a cold water animal which lives at 4 ± 1°, this increased solubility of phosphorylase at low temperature is of physiological importance.

A further point of general interest is illustrated by the association–dissociation properties of dogfish phosphorylase *a*. The experimental conditions in Figure 9 (upper pattern) were selected to show the skew boundary optimally, *i.e.*, high protein concentrations (5 mg/ml) and centrifugation for a long time period; at lower concentration (2–3 mg/ml) and shorter centrifugation times the asymmetry might be difficult to observe and conceivably be missed. If the $s_{20,w}$ were then calculated from the peak height position rather than from the migration of the second moment, a value of 9.1 S (*vs.* 10.0 S) would be obtained, only 0.2 S higher than found for phosphorylase *b* (Figure 7). Therefore, where phosphorylases *b* and *a* are thought to have the same sedimentation coefficient or molecular weight [*e.g.*, mammalian liver phosphorylases (Sutherland and Wosilait, 1956; Appleman *et al.*, 1966) and rabbit heart muscle isozyme 1 (Davis *et al.*, 1967)], considerable care must be taken before reaching this conclusion: small differences will be important in deciding whether or not any interaction between dimers exists. With the dogfish enzyme the different subunit equilibria in the *a* and *b* forms were clearly distinguished by varying the NaCl concentration; it remains to be seen whether this test is applicable to other systems where the interaction between phosphorylase *a* dimers is slight. This experiment and other data presented here also demonstrate that phosphorylation of phosphorylase *b* from vertebrate skeletal muscles does not simply shift the position of an equilibrium which already exists but gives rise to inter-

actions between the dimers which are quite absent in the original *b* form of the enzyme.

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