Purification and Properties of Inactive Liver Phosphorylase*

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ABSTRACT: The purification of the inactive form of glycogen phosphorylase from pig liver is described. The procedure involves differential centrifugation of the enzyme bound to endogenous glycogen particles, solubilization of the glycogen complex by α-amylase, ammonium sulfate precipitation, and DEAE-cellulose column chromatography. The purified enzyme appeared to be homogeneous in the ultracentrifuge ($s_{20,w}$ 8.4). The inactive enzyme displayed catalytic activity only at high salt concentration. It could be assayed in 0.7 M Na_2SO_4 and 10^{-3} M adenosine monophosphate (AMP) where it displayed *ca.* 20% of the activity obtained after enzymatic conversion to the active form. Pig liver phosphorylase contained 1.2 moles of pyridoxal 5′-phosphate/100,000 g of protein and incorporated 2.0 moles of radioactive phosphate/100,000 g when converted to the active form by muscle phosphorylase *b* kinase and ^{32}P-labeled adenosine triphosphate (ATP).

Tryptic attack of the phosphorylated site yielded a series of related phosphopeptides, very similar in charge to those obtained from rabbit and human muscle phosphorylases, but differing in amino acid composition. Similar results were obtained with the inactive form of rabbit liver phosphorylase which was purified according to the same procedure.

The regulation of glycogenolysis in liver has been the subject of extensive physiological and biochemical research (*cf.* Niemeyer *et al.*, 1962; Sutherland and Rall, 1962). The factors involved in the activation of liver phosphorylase, and in particular the role of epinephrine, glucagon, and adenosine 3′,5′-phosphate have been reviewed in detail (Sutherland and Rall, 1960).

Essentially all the work on liver phosphorylase and the related kinase and phosphatase has been carried out on dog liver by Sutherland and co-workers (Sutherland, 1951; Sutherland and Wosilait, 1956; Wosilait and Sutherland, 1956; Rall *et al.*, 1956; Henion and Sutherland, 1957; Wosilait, 1958). It was shown that liver phosphorylase, like the enzyme from striated muscle, exists in both phosphorylated (also referred to as "active") and dephosphorylated ("inactive") forms. Unlike the muscle enzymes, however, inactive liver phosphorylase displays little or no activity in the presence of AMP[1] (Sutherland and Wosilait, 1956). Furthermore, both the active and inactive forms of the liver enzyme have identical sedimentation constants of 8.4.

In the course of a comparative study of mammalian phosphorylases undertaken in this laboratory, it appeared of interest to investigate further the structure of the liver enzyme. The work reported here concerns the purification of phosphorylase from pig and rabbit liver and a study of the properties of the purified proteins as compared to those of the rabbit muscle enzyme.

Materials and Methods

Muscle phosphorylase *b* kinase was prepared according to the method of Krebs *et al.* (1964). ^{32}P-labeled ATP was prepared by the method of Kielley and Kielley (1951) and isolated by chromatography (Hurlbert *et al.*, 1954) in a procedure slightly modified to allow elution of ATP with a volatile buffer (Cohn and Ballum, 1961). Human salivary α-amylase (Fischer and Stein, 1961) as fourth crystals was a generous gift from Dr. Stein. Twice-crystallized trypsin (Worthington) was dialyzed *vs.* 10^{-3} M HCl before use. DEAE-cellulose (Brown and Co., exchange capacity 0.9 mequiv/g) was thoroughly washed with 1 N NaOH and distilled water; fines were removed by decantation. Saturated ammonium sulfate (Baker's reagent grade) solutions were prepared and kept at room temperature. All other chemicals were reagent grade commercial products used without further purification. Phosphorylase activity was measured in the direction of glycogen synthesis. Active liver phosphorylase was assayed at pH 6.8 in the presence of 10^{-3} M AMP according to the standard procedure of Illingworth and Cori (1953). Inactive liver phosphorylase was also measured by this method, but sufficient Na_2SO_4 was added to the substrate to bring the final concentration to 0.7 M in the assay.

Protein determinations on crude preparations were carried out by the biuret procedure of Weichselbaum (1946). On purified preparations, protein concentra-

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[1] Abbreviations used: AMP, adenosine monophosphate; ATP, adenosine triphosphate; UDPG, uridine diphosphate glucose.

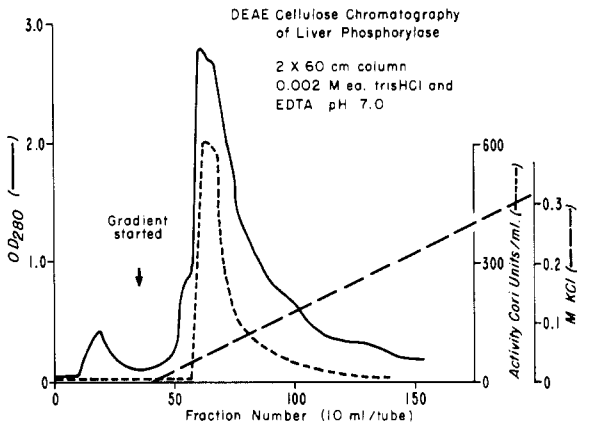

FIGURE 1: DEAE-cellulose chromatography of inactive pig liver phosphorylase. Conditions as indicated in text.

tions were measured by optical density at 278 mμ using the absorbancy index $A^{1\%}_{1cm}$ 11.9 found for muscle phosphorylase (Appleman *et al.*, 1963). Good agreement was obtained between the two methods. Pyridoxal 5′-phosphate was determined spectrophotometrically on supernatant solutions obtained after precipitating the enzyme with 0.3 N perchloric acid, using the molar absorbancy index A_M (275 mμ) 6770 (Peterson and Sober, 1954). The samples were previously treated with charcoal (Norit) to remove bound nucleotides. Radioactivity of [^{32}P] was measured in a thin-window gas-flow counter (Model D-47, Nuclear Chicago). Protein-bound ^{32}P was determined after precipitating the samples with an equal volume of 10% trichloroacetic acid, washing the precipitate three times with 5% trichloroacetic acid, and dissolving the final pellets in 88% formic acid for plating.

Isolation and purification procedures for peptides from tryptic digests were carried out as reported by Fischer *et al.* (1959). Preliminary separation of the three peptides (see Results) was achieved on Dowex 50×2 columns using, sequentially, 10^{-3} M acetic acid, 0.2 M citrate buffer, pH 3.1, and a gradient from the pH 3.1 buffer to 5.1 citrate–acetate 1 M (Hirs *et al.*, 1956). The fractions in each radioactive peak were pooled, lyophilized, and, when necessary, desalted on Dowex 50 columns (Haugaard and Haugaard, 1955). Purification of the separate peptides was carried out by paper electrophoresis in pyridine acetate buffers pH 3.6 and 6.5 (Ryle *et al.*, 1955), and paper chromatography in butanol–acetic acid–water, 4:1:5 (Slotta and Primosign, 1955). Radioactive peptides were located on the papers by radioautography with Eastman Kodak "no screen" X-ray film. For analyses, the purified peptides were hydrolyzed for 20 hr at 107° in sealed, evacuated tubes containing redistilled 5.7 N HCl. The hydrolysates were freed of acid *in vacuo* over NaOH pellets. Amino acid analyses were carried out on a Technicon autoanalyzer.

Results

Preparation of the Tissue Extracts. Pigs, killed at the slaughterhouse by electric shock, were immediately bled by cutting the jugular vein. The liver was removed and immersed in chopped ice after separation of the gall bladder. All subsequent steps were carried out at ≤4°. The chilled livers were rapidly freed of foreign tissue, cut into small portions, and homogenized for 1.5 min in a Waring blendor with 4 volumes of cold, neutral 10^{-3} M EDTA. The homogenate was centrifuged for 30 min at 3000*g*, and the supernatant solution, about two-thirds of the initial volume, was passed through two layers of cheese cloth to remove fat and tissue particles. The extract obtained was pink and quite turbid.

High-Speed Centrifugation. If the volume of the extract was <2 l., it was centrifuged directly for 2 hr at 40,000*g* in a Spinco Model L ultracentrifuge. In preparations involving kilogram quantities of liver, the extract was first subjected to centrifugation at 60,000*g* in a Sharples continuous-flow centrifuge. The resulting precipitate was suspended in distilled water and recentrifuged as indicated above in the Spinco ultracentrifuge. Three phases appeared in the tubes following this procedure: a clear red supernatant which was visible on top, a "fluffy" red precipitate of subcellular particles which could be washed out with a stream of water and a glassy, gray pellet remaining in the bottom of the tube. The two upper fractions were discarded and the dense precipitate containing the inactive phosphorylase bound to particulate glycogen was removed with a spatula. This preparation can be stored at −20° for 2 months without affecting subsequent procedures.

Amylase Treatment. The glycogen precipitate was suspended in 4 volumes of distilled water. To the milky white suspension was added 10 μg/ml of crystalline human salivary α-amylase. The material was transferred to Visking casings and dialyzed for 14 hr at 4° *vs.* two 6-l. portions of a 0.02 M NaCl–0.002 M Ca EDTA solution, adjusted to pH 6.6 by addition of 2 M Tris. The resulting solution was turbid; the particles remaining in suspension could not be cleanly removed by direct centrifugation, perhaps because of low salt concentration. The dialyzed material was brought to 33% saturation with neutral saturated ammonium sulfate. After 30 min at 0°, the suspension was centrifuged at 3000*g*; the supernatant solution was collected and the precipitate was discarded.

Ammonium Sulfate Fractionation. The supernatant solution was brought to 50% saturation by further addition of neutral saturated ammonium sulfate solution. After 1 hr at −10° the suspension was centrifuged for 1 hr at 3000*g*. The supernatant was discarded; the precipitate was taken up in a minimum amount of water and dialyzed extensively *vs.* 0.002 M Tris HCl–0.002 M EDTA buffer, pH 7.0.

DEAE-Cellulose Chromatography. This operation was carried out in the cold room. Well-washed DEAE-cellulose was equilibrated with 0.002 M EDTA pH 7.0

(initial buffer). The dialyzed ammonium sulfate precipitate, in portions containing *ca.* 1.0 g of protein, was introduced onto a 2 × 60 cm column of the ion exchanger, which was then washed with 250 ml of the initial buffer. A linear gradient of 1 l. of this buffer to 1 l. of the same buffer containing 0.3 M KCl was then applied. The column was eluted at a constant flow rate of 1.5 ml/min regulated by a Sigma pump. Inactive liver phosphorylase appeared in those fractions collected between 600 and 900 ml of effluent (Figure 1). Fractions 58–70, containing the enzyme at high specific activity, were pooled and dialyzed *vs.* neutral saturated ammonium sulfate until precipitation occurred. The precipitate was collected by centrifugation, suspended in distilled water, and redialyzed, this time *vs.* the initial buffer to remove residual ammonium sulfate.

Table 1 gives a summary of the steps in a typical preparation of inactive phosphorylase from pig liver. The preliminary continuous-flow (Sharples) centrifugation described above was included because of the large volume of extract. The yield from the DEAE-cellulose column represents only those fractions of highest specific activity.

TABLE 1: Purification of Inactive Pig Liver Phosphorylase.[a]

Fraction	Total Units[b]	Total Protein (mg)	Sp. Act. (units/mg)	Yield (%)
Crude extract	1,900,000	670,000	2.8	100
1st glycogen ppt (continuous-flow centrifugation)	600,000	91,000	6.6	32
2nd glycogen ppt (ultracentrifugation)	300,000	9,100	33	16
Amylase digest supernatant	305,000	4,270	71	16
Ammonium sulfate ppt	345,000	2,530	136	18
DEAE-chromatography peak fractions	110,000	495	222	6

[a] Starting material, 5 kg of fresh pig liver. [b] Phosphorylase units measured at pH 6.8, 30°, in the presence of 10^{-3} M AMP and 0.7 M Na_2SO_4.

Activity Measurements on Inactive Liver Phosphorylase. When assayed under the standard conditions for muscle phosphorylase *b* (Illingworth and Cori, 1953) the purified inactive liver phosphorylase showed a specific activity of <4 units/mg, or *ca.* 0.2% of that of the active enzyme. Inactive liver phosphorylase was, however, reversibly activated by high concentrations of sodium sulfate (Figure 2). Although higher concentrations of this salt resulted in even greater activation, 0.7 M was chosen for the standard assay to keep the salt in solution prior to the incubation. The highest specific activity obtained for the inactive enzyme under these conditions was 350 units/mg of protein. Inactive liver phosphorylase activity was not stimulated by cysteine, and nucleotide requirements were not absolute. When assayed in the presence of 0.7 M sodium sulfate, the ratio of activity without AMP to that in the presence of 10^{-3} M AMP was 0.45.

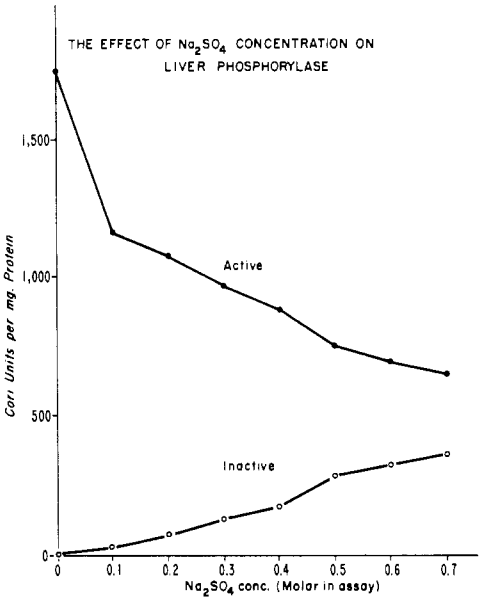

FIGURE 2: Effect of Na_2SO_4 concentration on the activity of purified inactive and active liver phosphorylase. The standard assay was used: 5 min at 30° in 0.02 M glycerophosphate–0.015 M cysteine buffer, pH 6.8, in the presence of 10^{-3} M AMP. The substrate contained 0.016 M α-glucose 1-phosphate and 1% glycogen as primer.

The pH optimum of inactive pig liver phosphorylase was 6.5 when assayed in the presence of Na_2SO_4 and 10^{-3} M AMP. The enzyme did not release inorganic phosphate from glucose 1-phosphate in the absence of added glycogen under the assay conditions. The purified enzyme from the DEAE column had no UDPG–glycogen transglucosylase activity (measured under the conditions of Kornfeld and Brown, 1962) and no hexokinase activity (method of Crane and Sols, 1953). It retained some residual amylase activity (introduced during the purification) which could be removed by passage of 10 ml of the enzyme through a 2 × 60 cm column of Sephadex G-100 equilibrated with 0.04 M glycerophosphate, pH 6.8. This treatment did not produce an increase in the specific activity of the phosphorylase.

Purified preparations of liver phosphorylase contained no phosphorylase kinase or ATPase activity; incubation of the enzyme with 0.01 M Mg^{2+}, 0.001 M

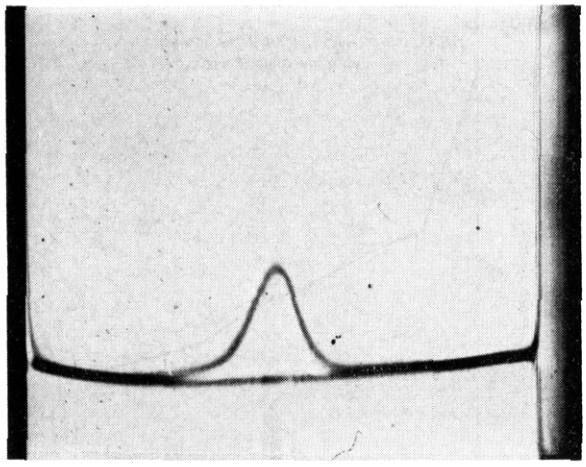

FIGURE 3: Sedimentation pattern of purified inactive pig liver phosphorylase in 0.05 M sodium glycerophosphate buffer, pH 6.8, at 5.0 mg/ml. Sedimentation from right to left. The photograph was taken 72 min after a speed of 59,780 rpm was reached.

ATP, pH 8.6, for 30 min at 30°, produced neither a conversion to the active form nor a release of inorganic phosphate from the ATP. When phosphorylase *b* kinase purified from rabbit muscle (Krebs *et al.*, 1964) was added to this incubation reaction mixture, there was a rapid activation of the liver phosphorylase. The enzyme remained in its active form during concentration with ammonium sulfate and dialysis even though this latter operation was carried out in the absence of fluoride ions. This indicated no significant contamination by phosphorylase phosphatase (inactivating enzyme).

When AT^{32}P was used in the activation reaction catalyzed by the addition of muscle phosphorylase *b* kinase, there was an uptake of 0.82 mole of phosphate/100,000 g of pig liver phosphorylase. The active liver phosphorylase produced in this reaction was strongly inhibited by high concentrations of sodium sulfate (Figure 2). The highest specific activity obtained for the purified active enzyme without sodium sulfate was 1760 units/mg of protein in the presence of AMP and 1260 units/mg of protein in the absence of this nucleotide.

Chemical and Physical Characteristics. Neutral concentrated solutions of liver phosphorylase were quite yellow. Spectrophotometric examination showed an identical spectrum with that obtained with muscle phosphorylase (Kent *et al.*, 1958), namely a major protein absorbtion maximum at 278 mμ and a secondary maximum at 333 mμ. This was shown to be due to the presence of pyridoxal 5′-phosphate in the protein. The identity of this cofactor released by perchloric acid was confirmed by comparison with standards on paper electrophoresis and chromatography (Peterson and Sober, 1954). Quantitative analysis indicated the presence of 1.2 moles of pyridoxal 5′-phosphate/100,000 g of pig liver phosphorylase. Partial resolution of the cofactor was also possible under the same conditions as were used for muscle phosphorylase *a* (Cori and Illingworth, 1957; Illingworth *et al.*, 1958); a 4-min treatment at 0° in an 0.08 M cysteine solution, pH 3.5, followed by ammonium sulfate precipitation, led to the loss of >75% of the initial activity in a sample of inactive pig liver phosphorylase. Incubation with 10^{-4} M pyridoxal 5′-phosphate restored 60% of the activity initially present. No resolution was obtained under the conditions used for resolving muscle phosphorylase *b* (14 hr at 0° in 0.08 M cysteine, pH 6.0) (Illingworth *et al.*, 1958).

Purified inactive pig liver phosphorylase obtained from the peak fractions of the DEAE-cellulose columns behaved as a single component in the analytical ultracentrifuge (Figure 3). The sedimentation coefficients for the active and inactive forms of the enzyme were identical, with an $s_{20,w}$ value of 8.4 in 0.04 M glycerophosphate–0.03 M cysteine buffer, pH 6.0, as was already shown by Sutherland and Wosilait (1956) for dog liver phosphorylase. The sedimentation behavior of both forms of liver phosphorylase was unaffected by 10^{-3} M AMP in the presence or absence of 0.01 M Mg^{2+}. Upon exposure of the inactive form of the enzyme to 5×10^{-3} M *p*-mercuribenzoate, components of both lower ($s_{20,w}$ 3.7) and higher ($s_{20,w}$ 30) sedimentation were observed, indicating both dissociation and formation of higher aggregates; the enzyme lost all phosphorylase activity under these conditions. A similar observation was made with rabbit muscle phosphorylase (Madsen and Cori, 1956; Madsen and Gurd, 1956). The properties of the inactive form of liver phosphorylase are summarized in Table II and compared to those of rabbit muscle phosphorylase *b*.

Properties of Rabbit Liver Phosphorylase. A number of preparations of inactive phosphorylase have also been made from fresh rabbit liver. The method employed for the purification of the pig enzyme has been found to apply equally well to that of the rabbit, and some properties of purified rabbit liver phosphorylase have been investigated. The effects of AMP and of high sodium sulfate concentrations on the activity of rabbit liver phosphorylase were essentially identical with those for the pig enzyme but the pH optimum (6.2) was somewhat lower. The rabbit liver enzyme contained 1 mole of pyridoxal 5′-phosphate/100,000 g of protein. Sedimentation coefficients of 8.4 and 9.8 were obtained for the inactive and active forms, respectively, in the same glycerophosphate–cysteine buffer, pH 6.8, used for the pig liver enzyme.

Isolation of Phosphopeptides from Rabbit and Pig Liver Phosphorylase. The phosphorylation of rabbit muscle phosphorylase, the release of phosphopeptides, and the separation and characterization of these peptides has been described (Fischer *et al.*, 1959). It was shown that the phosphopeptides are almost quantitatively released by trypsin without extensive proteolysis and that three peptides are detectable by paper electrophoresis and separable on Dowex 50 anion-exchange columns. The major basic peptide (peptide C) (Fischer *et al.*, 1959) was found to have the sequence Lys-Glu-NH_2-Ileu-SerP-Val-Arg. A second peptide, B, was a

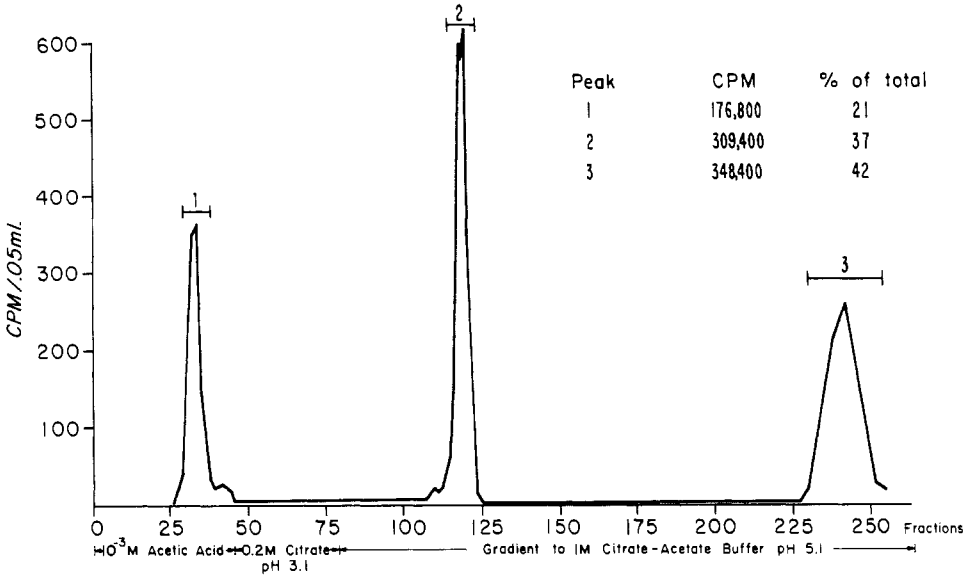

FIGURE 4: Ion-exchange chromatography of the peptides released by brief tryptic attack on pig liver phosphorylase activated with ^{32}P-labeled ATD and muscle phosphorylase *b* kinase.

TABLE II: A Comparison of the Properties of Phosphorylase from Muscle and Liver.

Properties	Muscle Phosphorylase *b*[a]	Inactive Liver Phosphorylase
Activation by 0.7 M Na_2SO_4	−	+
Activation by sulfhydryl	+	−
p-CMB inhibition	+	+
AMP activation	+	±
Optimum pH	6.7	6.6
Pyridoxal 5′-phosphate (moles/100,000)	0.95	1.2
^{32}P incorporated during enzymatic activation (moles/100,000 g)	0.80	0.82
$s_{20,w}$	8.2	8.4
$s_{20,w}$ (10^{-2} M Mg^{2+}, 10^{-3} M AMP)	13.2	8.6
$s_{20,w}$ (enzymatically activated form)	13.2	8.4

[a] From Kent *et al.* (1958).

pentapeptide resulting from tryptic release of the N-terminal lysyl residue and a third peptide, A, was derived from B through heat-induced deamination and cyclization of the newly exposed N-terminal glutaminyl residue.

The amino acid composition of a peptide isolated from the site which is phosphorylated during the activation of rabbit and pig liver phosphorylase was determined using the methods of Fischer *et al.* (1959) as a guide. Both proteins (250 mg) were incubated with 10^{-3} M ^{32}P-labeled ATP, 10^{-2} M Mg^{2+}, and 4 mg of purified muscle phosphorylase *b* kinase at pH 8.6, under the conditions described by Krebs *et al.* (1958) for the conversion of rabbit muscle phosphorylase *b* to *a*. Activation was followed by the increase in activity measured in the absence of added Na_2SO_4. When maximum activation was achieved, the protein samples were precipitated with an equal volume of saturated ammonium sulfate. Residual nucleotides were removed by washing the precipitates once with 50% saturated ammonium sulfate followed by 8 hr of dialysis *vs.* distilled water. Crystalline trypsin was added to the dialyzed protein solutions, pH 7.8, to give final trypsin:phosphorylase molar ratios of 1:10. The release of radioactivity from the proteins was essentially quantitative after 30 min at 25°. The residual proteins were precipitated by the addition of 0.1 volume of 50% trichloroacetic acid; the supernatant solutions were lyophilized after extraction with ether to remove the trichloroacetic acid.

The methods used for purifying the phosphopeptides from rabbit muscle phosphorylase were found to apply equally well to the separation of peptides released from the rabbit and pig liver enzymes; therefore, the procedure will not be reported in detail. Column chromatography on Dowex 50 (Hirs *et al.*, 1956) of both tryptic hydrolysates yielded three radioactive peaks (Figure 4); upon further purification by paper electrophoresis and paper chromatography, these three fractions behaved similarly to phosphopeptides A–C isolated from rabbit muscle phosphorylase *a*.

The major basic peptide (corresponding to peptide C

from the muscle enzyme) was converted to a neutral peptide (similar to B) by trypsin and further converted to an acidic peptide (corresponding to A) by heat. However, the amino acid composition of the major basic peptide from rabbit liver phosphorylase differed in two respects from that of the muscle enzyme: it contained (in addition to glutamine and serine) two arginyl residues instead of one lysine and one arginine, and two isoleucyl residues instead of isoleucine and valine. The radioactive peptides corresponding to B and A from the muscle yielded after purification and hydrolysis the following composition: Arg-Glu-$Ileu_2$-Ser. Scarcity of material prevented an exact determination of the sequence of these phosphopeptides. The only amino acid data obtained from *pig liver* phosphorylase was for the pentapeptide; it was Arg-Glu-$Ileu_2$-Ser, identical with that from the rabbit liver enzyme.

Discussion

The purification method described herein differs markedly from the procedures reported for the purification of other phosphorylases. Liver contains too many soluble proteins to permit the use of the method of Fischer *et al.* (1958) for rabbit muscle phosphorylase *b*. High glycogen levels in liver prevent the application of starch columns to the purification (de la Haba, 1962). The procedure described for the purification of active phosphorylase from canine liver (Sutherland and Wosilait, 1956) involves a large number of steps designed to maintain the enzyme in its active form, or to destroy the potent liver phosphorylase phosphatase. Purification of the much more stable inactive form of the enzyme was made possible by the fact that it displays enzymatic activity when tested in concentrated salt solutions.

Particulate glycogen and its presence in tissue extracts (initially noted by Lazarow, 1942) have been used recently as a source of UDPG–glycogen transglucosylase from rat liver (Leloir and Goldemberg, 1960). The purification of liver phosphorylase reported here, like that of Leloir's enzyme, depends on the binding of the enzyme to its polysaccharide substrate. Indeed, the distribution of phosphorylase in the early steps parallels that of glycogen, and purification of *ca.* 10-fold has been achieved through these stages. Particulate glycogen is a very labile metabolite (Bueding and Orrell, 1961) and since the nutritional condition of the source animal cannot always be controlled, occasional preparations have failed in these steps. Attempts to replace endogenous glycogen with added polysaccharides (potato starch, glycogen) have not been successful.

The phosphorylase–glycogen complex is not suitable for further purification by salt fractionation or other classical methods. It was found, however, that glycogen could be almost completely removed without loss of phosphorylase activity by α-amylase digestion and dialysis. Crystalline human salivary α-amylase was used as it is not likely to be contaminated by proteolytic enzymes. The subsequent ammonium sulfate fractionation gave a good purification of the phosphorylase. An apparent increase in total activity in these two steps probably resulted from the removal of some inhibitory material. DEAE-cellulose column chromatography removed some inactive protein as well as large amounts of pigmented material and nucleic acids. The specific activity obtained (100–350 units/mg of protein) varied from preparation to preparation and depended to some extent on the number of effluent fractions which were pooled. Although inactive liver phosphorylase was not crystallized, the homogeneity of the protein was adequate to permit investigation of its enzymatic, chemical, and physical properties.

The activation of inactive liver phosphorylase by high concentrations of sodium sulfate permits the determination of the total level of enzymatic activity as well as the percentage of enzyme in the active form. This is similar to what has been done for many years with muscle phosphorylase, when it was assayed in the presence and absence of AMP.

The specific activities obtained for the two forms, assayed in the presence of AMP, can be summarized as follows: active form, normal assay 1760 units/mg (100%), high salt 650 units/mg (37%); inactive form, normal assay 4 units/mg (0%), high salt 350 units/mg (20%). A much higher concentration of salt is required here than in the case of lobster muscle phosphorylase *b*, where sulfate ions have been shown to lower the K_m for glucose 1-phosphate (Cowgill, 1959). The activation of phosphorylase by high concentrations of sulfate salts was first noted by Riley and Haynes (1963) for the enzyme in beef adrenal preparations.

The identification of pyridoxal 5′-phosphate as the prosthetic group of liver phosphorylase is not surprising, since the vitamin B_6 derivative has been found in all polysaccharide phosphorylases so far investigated. Sedimentation and ^{32}P-incorporation data reported here serve to confirm the earlier findings on active dog liver phosphorylase (Sutherland and Wosilait, 1956; Wosilait and Sutherland, 1956). Both muscle and liver phosphorylase appear to have a similar oligomeric type of basic structure; however, whereas the former undergoes aggregation to a tetramer during enzymatic conversion to the active form, the latter does not.

It is of some interest to compare the phosphopeptides derived from the sites involved in the enzymatic activation of muscle and liver phosphorylase. Hughes *et al.* (1962) have shown that the phosphopeptides obtained from human and rabbit muscle phosphorylases are identical. The phosphopeptides obtained from rabbit and pig liver phosphorylases also appear to be very similar to one another, at least insofar as their amino acid composition is concerned, but differ somewhat from the muscle phosphopeptides. Similarity in behavior between the liver and the muscle phosphopeptides might be taken as an indication that their difference in composition results only from a "conventional" or "conservative" substitution of arginine for lysine and isoleucine for valine, as discussed by Smith and Margoliash (1964) in reference to the evolution of cytochrome *c*. These substitutions would not be expected to affect the charge or chemical properties of the phosphopep-

tides under physiological conditions. An indication that this modification is not radical can be seen in the fact that the specific muscle phosphorylase kinase will readily act on the liver enzyme.

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