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Purification and Partial Characterization of Glucose 6-Phosphate Dehydrogenase from Cow Adrenal Cortex*

Wayne E. Criss† and Kenneth W. McKerns

ABSTRACT: Bovine adrenal glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate:$NADP^+$ oxidoreductase, EC 1.1.1.49) was purified by isoelectric precipitation, calcium phosphate gel adsorption, ammonium sulfate precipitation, and column chromatography on diethylaminoethyl Sephadex, carboxymethyl Sephadex, and Sephadex G-200. The enzyme was purified 2500-fold with a 20% yield. The average specific activity at pH 8.0 and 37° of the crystalline enzyme was 340 μmoles of oxidized nicotinamide–adenine dinucleotide phosphate ($NADP^+$) reduced/min per mg of protein. The preparation was demonstrated to be homogeneous by diethylaminoethylcellulose column chromatography, electrophoresis on cellulose acetate and polyacrylamide gel, velocity ultracentrifugation, and three consecutive crystallizations to a constant specific activity. The molecular weight was calculated to be 235,000, derived from a V_e/V_0 ratio of 1.58 on a calibrated Sephadex G-200 column and to be 238,700 by a sedimentation constant ($s_{20,w}$) of 9.8 S on the ultracentrifuge. The optimum pH in the absence of Mg^{2+} was 8.5; with Mg^{2+} it was the same but with a 20% greater activity. The K_m for $NADP^+$ at pH 8.0 and 25° in the presence of Mg^{2+} was 5.6×10^{-6} M as compared to a K_m of 4.7×10^{-2} M for NAD^+. The K_m for glucose 6-phosphate with $NADP^+$ at pH 8.0 and 25° in the presence of Mg^{2+} was 4.2×10^{-5} M compared to 1.9×10^{-4} M with NAD^+.

The activity of the pentose phosphate pathway is very high in endocrine tissues (Glock and McLean, 1954; McKerns, 1962a, 1965a,b). The first enzyme in this pathway is glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate:$NADP^+$ oxidoreductase,[1] EC 1.1.1.49) which catalyzes the reaction: glucose 6-phosphate + $NADP^+$ ⇌ 6-phosphate glucolactone + NADPH + H^+.

The specific activity and the amount of glucose 6-phosphate dehydrogenase can vary. Striking increases of this enzyme were observed in the mammary gland during lactation (Glock and McLean, 1954), in the ovary during estrus and after the administration of gonadotropin (McKerns, 1965b; McKerns and Nordstrand, 1965), in the liver upon refeeding a high carbohydrate diet following fasting (Tepperman and Tepperman, 1958), and in the adrenal upon administration of ACTH (McKerns, 1964b). Interest in glucose 6-phosphate dehydrogenase also derives from differences of the enzyme found in erythrocytes of people suffering from various congenital anemias (Kirkman *et al.*, 1964).

Stimulation of the activity of glucose 6-phosphate dehydrogenase would seem to be of major importance in the control of function and cell replication in many tissues regulated by hormones (McKerns, 1964b, 1967). There is evidence for unique species of the enzyme, each having binding sites for its activating hormone (McKerns, 1964b, 1966, 1967, 1968). Glucose 6-phosphate dehydrogenase controls the rate of entry of glu-

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[1] Abbreviations used: $NADP^+$ and NADPH, oxidized and reduced nicotinamide–adenine dinucleotide phosphates; ACTH, corticotropin; NAD^+ and NADH, oxidized and reduced nicotinamide–adenine dinucleotide.

cose 6-phosphate into the pentose phosphate pathway, and thus regulates the production of ribose sugars for RNA and other nucleotide syntheses. Excess ribose sugars could form glyceraldehyde 3-phosphate by the transketolase reaction and provide other metabolites. Glucose 6-phosphate dehydrogenase is also of major importance in controlling the rate of reduction of $NADP^+$. NADPH is required for steroid hydroxylations and cholesterol synthesis in endocrine tissues, in reduction reactions, and for fatty acid synthesis. In addition, many steroids inhibit glucose 6-phosphate dehydrogenase in the adrenal cortex (McKerns and Bell, 1960; McKerns and Kaleita, 1960; Marks and Banks, 1960; McKerns, 1963), but by a different mechanism from the activation effect of ACTH (McKerns, 1963, 1964b). This paper presents the preparation and partial characterization of glucose 6-phosphate dehydrogenase from the adrenal cortex of the cow.

Materials and Methods

Calcium phosphate gel was prepared according to the method of Tsuboi and Hudson (1957). This consisted of adding 6 ml of concentrated ammonium hydroxide (29% NH_3) to 200 ml of 0.5 M Na_2HPO_4 solution. This was immediately added to 1500 ml of 0.1 M $CaCl_2$. The suspension was allowed to stand for 30 min. The precipitate was centrifuged down and washed three times in deionized water and resuspended in 1 l. of 0.005 M phosphate buffer at pH 7.8 containing the three buffer additives listed below.

Ion Exchange and Molecular Sieving Elements. DEAE Sephadex A50 (3.5 mequiv/g, 40–120-μ particle size), CM Sephadex C-50 (4.5 mequiv/g, 40–120-μ particle size), and Sephadex G-25 were purchased from Pharmacia Fine Chemicals, Piscataway, N. J. DEAE-cellulose (DE fibrous) was obtained from Whatman, H. Reeve Angel & Co., Clifton, N. J. All materials were treated according to the manufacturer's instructions.

Substrates and Buffers. D-Glucose 6-phosphate and 6-phosphate D-gluconate were obtained from Sigma Chemical Co., St. Louis, Mo.; $NADP^+$ and NAD^+ were from P-L Biochemicals, Inc., Milwaukee, Wis. Phosphate buffer was Na_2HPO_4–NaH_2PO_5; Tris-HCl buffer was Tris (trizma base) plus HCl added to obtain required pH; acetate buffer was sodium acetate plus acetic acid added to desired pH; Tris–EDTA–boric acid buffer was Tris (trizma base), 0.005 M EDTA, plus boric acid to obtain required pH; amine buffer was triethanolamine and 0.005 M EDTA. Deionized water was used in all buffers. Chemicals used in the buffers were purchased from Fisher Scientific Co., Fair Lawn, N. J.; J. T. Baker Chemical Co., Phillipsburg, N. J.; W. H. Curtin & Co., Jacksonville, Fla.; E. H. Sargent & Co., Springfield, N. J.

Buffer Additives and Other Materials. Many of the buffers contained additives: 1 was glycerol at 5%, 2 was β-mercaptoethanol at 2.7×10^{-3} M, and 3 was EDTA at 2×10^{-4} M. The additives were reagent grade and were purchased from Fisher Scientific Co., Fair Lawn, N. J., and Mann Research Laboratories, Inc., New York, N. Y. The ammonium sulfate used was enzyme grade, from Nutritional Biochemicals Inc., Cleveland, Ohio. A saturated solution of ammonium sulfate was taken as 767 g/l. at 25°. Percentage saturations were derived from this amount. A kit containing proteins of known molecular weight was acquired from Mann Research Laboratories, Inc., New York, N. Y. Muscle pyruvate kinase (EC 2.7.1.40) was purchased from Worthington Biochemical Corp., Freehold, N. J. Materials and equipment for cellulose acetate electrophoresis was purchased from Gelman Instrument Co., Ann Arbor, Mich. Materials for polyacrylamide gel electrophoresis were obtained from Canalco (Canal Industrial Corp.), Bethesda, Md., and the equipment was made locally.

Enzyme Assay. Dehydrogenase activity was measured by monitoring the initial rate of reduction of $NADP^+$ or NAD^+. The increase in absorbance at 340 mμ was followed using a Beckman Model DU spectrophotometer with a Minneapolis-Honeywell 10-ma recorder coupled directly to the photomultiplier tube. A bucking voltage attachment (equivalent to scale expansion) constructed in our electronic shop, was also coupled to the photomultiplier tube (McKerns, 1962b). With this arrangement, the reduction of levels of NADPH as low as 10^{-3} μmole/3 ml could be measured easily. The routine assay mixture contained the following components at 37°: 0.1 M Tris-HCl buffer (pH 8.0), 10^{-5} M $NADP^+$ or 10^{-2} M NAD^+, 10^{-4} M glucose 6-phosphate, 6×10^{-3} M $MgCl_2$, and enzyme. During early stages (I–IV only) of the enzyme purification, corrections for 6-phosphate gluconic dehydrogenase were made (by noting the difference in the reaction rate obtained with $NADP^+$, 10^{-4} M 6-phosphate gluconate, and 10^{-4} M glucose 6-phosphate, and the reaction rate obtained with $NADP^+$ and 10^{-4} M 6-phosphate gluconate). The reactions were initiated with glucose 6-phosphate or 6-phosphate gluconate. All assays were made with the enzyme diluted to 1–20 μg of protein/3 ml and were performed under conditions of zero-order kinetics for substrates. Enzymatic activity is defined as micromoles of $NADP^+$ reduced per minute at 37°. Specific activity is the number of enzymatic activity units per milligram of protein. All values for enzyme activity given in this paper are the mean of three or more closely agreeing determinations.

Protein Assay. Protein was assayed by the method of Lowry *et al.* (1961), using crystalline bovine serum albumin as a standard.

Experiments

Purification of Adrenal Glucose 6-Phosphate Dehydrogenase. I. HOMOGENATE. The adrenal glands were taken directly from freshly slaughtered cows, placed in ice, and processed within 1 hr. All subsequent procedures were carried out in ice baths or in a cold room (5 ± 1°). The adrenal cortex was separated from the adrenal medulla after cutting thick slices of the gland

with a Stadie-Riggs slicer. The cortex was minced with scissors and homogenized in a stainless-steel Sorvall Mini-Mixer with speed control. The homogenate was made up 1 g/2 ml in 0.01 M phosphate buffer at pH 6.7 with all three buffer additives.

II. HIGH-SPEED CENTRIFUGATION. The adrenal homogenate was centrifuged at 2000*g* for 15 min in a Model V International centrifuge to remove cell debris and unbroken cells. The supernatant was centrifuged at 105,000*g* for 1 hr in a Spinco preparative ultracentrifuge. The high-speed supernatant was carefully removed with a syringe fitted with a long needle, to avoid disturbing the large compact layer of lipid at the top of the tubes.

III. ISOELECTRIC PRECIPITATION. The enzyme was precipitated from high-speed supernatant (fraction II) while mixing rapidly, by adding an equimolar amount of 0.1 M acetate buffer at pH 4.5, containing 15% ethanol. Rapid mixing was accomplished with the aid of a Fisher Vibro-Mixer and by adding the acetate buffer rapidly through a syringe equipped with a 25-gauge needle. The enzyme precipitate was centrifuged at 25,000*g* for 15 min in a Sorvall automatic centrifuge. The precipitate was redissolved in 0.005 M phosphate buffer at pH 7.8 with all three additives.

IV. FREEZING AND THAWING. Fraction III was frozen at −30°. The enzyme was stable for up to 3 months at this stage of preparation. It was essential to obtain an extract which would remain stable for short periods because only a limited amount (usually 100–200 g) of adrenal cortical tissue could be obtained and processed daily. Subsequent purification was carried out with several batches of fraction IV which were prepared from adrenal tissue collected over a 2–3-week period. The frozen fractions were thawed, centrifuged at 25,000*g* for 20 min, and pooled for subsequent purification.

V. CALCIUM PHOSPHATE GEL ADSORPTION. An equal volume of calcium phosphate gel suspension (see Materials) was added to batches of fraction IV containing approximately 7 mg of protein/ml. The suspensions were thoroughly mixed and stirred intermittently for 0.5 hr. The gel was centrifuged at 2000*g* for 15 min and the supernatant was discarded. The gel was resuspended in 0.005 M phosphate buffer at pH 7.8 containing ammonium sulfate at 20% saturation and all three buffer additives. This volume of phosphate buffer was equal to approximately one-half the original volume of fraction IV. The mixture was allowed to remain, with intermittent stirring, for 0.5 hr. The gel was centrifuged as before, and the supernatant containing the enzyme was poured off.

VI. FIRST AMMONIUM SULFATE PRECIPITATION. The ammonium sulfate concentration was increased to 40% saturation by the slow addition of ammonium sulfate crystals to fraction IV, which was stirred constantly with a magnetic stirrer. Stirring was continued for 1 hr. The precipitate was centrifuged at 25,000*g* for 15 min and discarded. Ammonium sulfate was added (as before) to the supernatant up to a concentration of 55% saturation. The solution was stirred continuously for 1 hr and then centrifuged at 25,000*g* for 15 min. This supernatant was discarded and the precipitated enzyme was redissolved in a small volume of 0.02 M phosphate buffer at pH 7.8 with all three additives.

VII. DEAE SEPHADEX COLUMN CHROMATOGRAPHY. Fraction VI was desalted by passing it through an 0.8 × 20 cm column of Sephadex G-25 equilibrated in 0.02 M phosphate buffer at pH 7.8 and containing all three buffer additives. $NADP^+$ was added to the desalted fraction VI to give a final concentration of 10^{-4} M. This enzyme solution was applied to the top of a 2.5 × 45 cm Pharmacia column containing DEAE Sephadex A-50 suspended in the phosphate buffer. A volume equal to one-half the column volume was allowed to flow through the column. A NaCl gradient from 0 to 0.2 M was then passed through the column. This gradient was achieved by running 0.4 M NaCl in phosphate buffer into a mixing chamber containing 500 ml of phosphate buffer. Flow rate was 18 ml/hr. Fractions of approximately 7 ml/tube were collected throughout the eluting gradient and analyzed for protein (optical density at 280 $m\mu$) and enzymatic activity. The first tube to contain measurable protein was called tube 1. Tubes 48–62, containing 90% of the glucose 6-phosphate dehydrogenase activity, were pooled.

VIII. CM SEPHADEX COLUMN CHROMATOGRAPHY. Ammonium sulfate was slowly added to a final concentration of 60% saturation to the pooled fractions of step VII. The solution was stirred continuously for 1 hr. The precipitated enzyme was centrifuged at 25,000*g* for 15 min and resuspended in a small volume of 0.02 M phosphate buffer at pH 6.0 with buffer additives 2 and 3. The enzyme solution was desalted on an 0.8 × 20 cm column of Sephadex G-25 equilibrated with the phosphate buffer. The desalted glucose 6-phosphate dehydrogenase solution was allowed to flow into a Pharmacia reverse flow 2.5 × 45 cm column containing CM Sephadex C50 suspended in the phosphate buffer. One-half column volume was allowed to flow up through the column. A gradient from 0 to 0.5 M NaCl was then passed up through the column. The gradient was established similar to the gradient for fraction VII. Flow rate was 18 ml/hr. Fractions of approximately 8 ml/tube were collected throughout the eluting gradient and analyzed for protein (optical density at 280 $m\mu$) and glucose 6-phosphate dehydrogenase activity. The first tube to contain measurable protein was called tube 1. Tubes 58–75, containing 95% of the glucose 6-phosphate dehydrogenase activity, were pooled.

IX. SECOND AMMONIUM SULFATE PRECIPITATION. A second ammonium sulfate fractionation was carried out similar to that for fraction VI. The fractionation was performed with the pooled enzyme from fraction VIII. All of the glucose 6-phospahte dehydrogenase activity was located between 40 and 55% saturation. The precipitated enzyme was redissolved in a 1–2-ml volume of 0.01 M phosphate buffer at pH 7.8 with buffer additives 2 and 3.

X. SEPHADEX G-200 COLUMN CHROMATOGRAPHY. $NADP^+$ was added to fraction IX to give a concentration of 2×10^{-5} M. This enzyme solution was placed

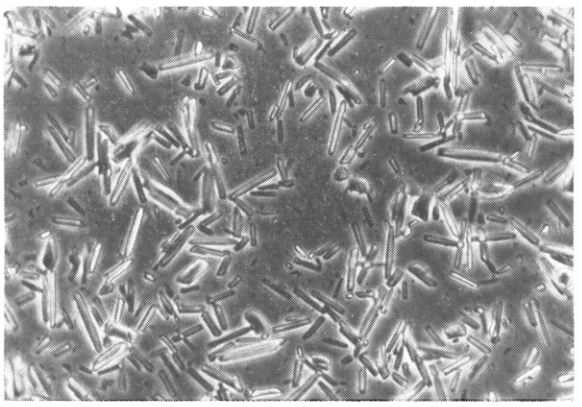

FIGURE 1: Photomicrograph (×185) of adrenal glucose 6-phosphate dehydrogenase crystals (crystallization II, see Properties).

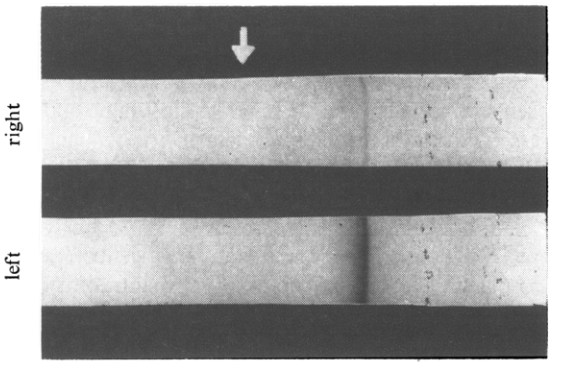

FIGURE 3: Electrophoresis of crystallized glucose 6-phosphate dehydrogenase on cellulose acetate in 0.1 M Tris–EDTA–boric acid buffer at pH 9.0. A current was applied for 2 hr at 4 ma/strip. The strip on the right was stained for protein and the strip on the left was stained for glucose 6-phosphate dehydrogenase activity (see Properties for procedure). The arrow marks the location where the protein was mounted.

on a 2.5 × 100 cm column containing Sephadex G-200 suspended in 0.01 M phosphate buffer at pH 7.8 containing $NADP^+$ at a concentration of 2×10^{-5} M and additives 2 and 3. Fractions of 8 ml/tube were collected as the buffer was allowed to flow through the column at a rate of 12 ml/hr. The first tube to contain Blue Dextran was called tube 1. The fractions were assayed for protein (optical density at 280 mμ) and enzymatic activity. Tubes 6–15, containing 95% of the glucose 6-phosphate dehydrogenase activity, were pooled.

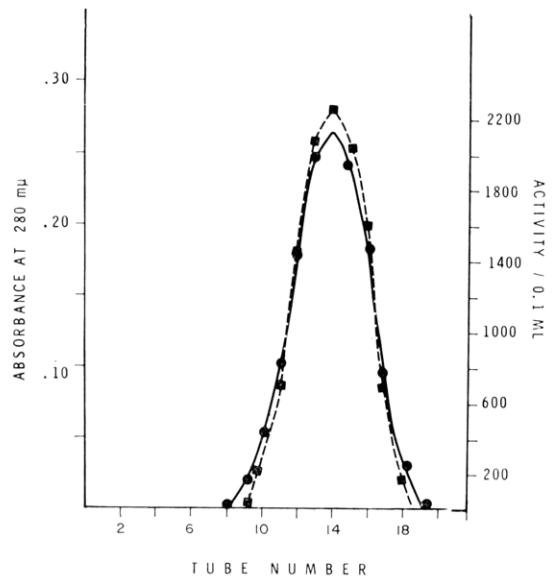

FIGURE 2: Elution pattern from a DEAE-cellulose column. A solution of crystallized glucose 6-phosphate dehydrogenase was placed on a DEAE-cellulose column (1.5 × 30 cm) and eluted with a gradient of 0–0.2 M NaCl in 0.01 M phosphate buffer at pH 7.8 with EDTA and β-mercaptoethanol. (●————●) Absorbance and (■— — — —■) glucose 6-phosphate dehydrogenase activity.

XI. THIRD AMMONIUM SULFATE PRECIPITATION. Crystalline ammonium sulfate was added to fraction X to give a 60% saturation with respect to ammonium sulfate. The solution was continuously stirred for 2 hr and then centrifuged at 25,000*g* for 15 min. The precipitate was resuspended in a small volume of 0.01 M phosphate buffer at pH 7.8 containing ammonium sulfate at 35% saturation and with buffer additives 2 and 3. This suspension was allowed to stand overnight. It was then centrifuged at 25,000*g* for 30 min. The supernatant containing the enzyme was retained.

XII. CRYSTALLIZATION. Fraction XI was dialyzed against 0.01 M phosphate buffer at pH 7.8 with buffer additives 2 and 3. Crystals appeared within 24 hr and continued to come out of solution for several days. The crystals were collected by centrifugation at 25,000*g* for 15 min, washed (with the dialysis buffer), and recentrifuged several times. The crystals were redissolved in 0.01 M phosphate buffer at pH 4.0 or in 0.01 M phosphate buffer at pH 6.7 including 10^{-4} M $NADP^+$ (both buffers contained additives 2 and 3). Recrystallizations were accomplished by adding ammonium sulfate to 35% saturation, followed by dialysis as described for the first crystallization. A photograph of the crystals (from a second crystallization) illuminated by polarized light is shown in Figure 1.

Comments. Purification of fresh adrenal tissue was carried through to fraction IV, since this fraction could be stored frozen for several weeks with minimum loss of enzyme activity. The pH must be kept between pH 6 and 7 with the early fractions, possibly because of proteases which may be less active at a lower pH. Glycerol helped to stabilize the enzyme at earlier stages, but was of little value in the partially purified preparations. Adrenal glucose 6-phosphate dehydrogenase

TABLE I: Purification of Adrenal Glucose 6-Phosphate Dehydrogenase.

Fraction No.	Purification Steps	Total Vol (ml)	Total Protein (mg)	Total Act. (units)	Sp Act.[a] (units/mg)	Accumulative Purifcn	% Yield
I	Whole homogenate[b]	1,227	80,013	9,988	0.13		100.0
II	High-speed centrifugation[b]	780	12,437	9,879	0.80	6.1	98.9
III	Isoelectric precipitation[b]	493	3,441	8,743	2.54	19.5	87.6
IV	Freezing and thawing[b]	487	3,412	8,691	2.55	19.6	87.0
V	CaP gel adsorption	280	1,334	8,428	6.27	48.3	84.4
VI	First ammonium sulfate precipitation	14	532	7,075	13.30	102.3	70.9
VII	DEAE Sephadex column chromatography	140	140	5,726	40.90	314.8	57.3
VIII	CM Sephadex column chromatography	145	70.1	5,146	73.42	564.8	51.5
IX	Second ammonium sulfate precipitation	1.5	39.8	3,958	99.45	765.3	39.6
X	Sephadex G-200 column chromatography	130	28.6	3,425	119.77	921.3	34.3
XI	Third ammonium sulfate precipitation	1.6	17.1	3,001	175.48	1348.3	30.0
XII	Crystallization I	2.1	6.6	2,271	344.09	2,646.8	22.7
	Crystallization II	2.0	6.3	2,090	331.70	2,551.5	20.9
	Crystallization III	2.0	6.1	1,989	326.07	2,508.2	19.9

[a] All activity measurements and therefore specific activity calculations were performed with protein concentrations at 1–20 μg/3 ml, in 0.1 M Tris-HCl buffer at pH 8.0, with 10^{-3} M $MgCl_2$, 10^{-5} M $NADP^+$, and 10^{-4} M glucose 6-phosphate. [b] Values represent pooled assay values of 13 daily collections of adrenal glands which were processed up to fraction IV.

was most stable in the presence of either $NADP^+$ or glucose 6-phosphate. Since it was least stable in Tris and acetate buffers, phosphate buffers were used during purification.

Purification Summary. A summary of a typical purification of adrenal glucose 6-phosphate dehydrogenase is given in Table I. The ten fractionation steps (IV was a storage step) produced a 2500-fold purification with a 20% yield. The entire procedure usually required a 3-week period of collecting and processing to the fraction IV stage and 1 week between fraction IV and preparation of crystals. The final specific activity was 340 μmoles of $NAPD^+$ reduced/min per mg of protein at 37° in 0.1 M Tris buffer at pH 8.0 with Mg^{2+}.

Properties of the Crystallized Enzyme. DEAE CELLULOSE COLUMN CHROMATOGRAPHY. A solution of twice-crystallized glucose 6-phosphate dehydrogenase was added to the top of a 1.5 × 30 cm column containing DEAE-cellulose equilibrated in 0.01 M phosphate buffer at pH 7.8 with buffer additives 2 and 3. A gradient of NaCl from 0 to 0.2 M in the phosphate buffer was allowed to flow through the column. The gradient was established as described in the preparation of fraction VII of the purification procedure. The flow rate was 10 ml/hr. Fractions of 8 ml/tube were collected throughout and analyzed for protein (optical density at 280 mμ) and glucose 6-phosphate dehydrogenase activity (Figure 2). It was observed that all of the protein and enzymatic activity occurred under one peak.

CELLULOSE ACETATE ELECTROPHORESIS. A solution of crystallized glucose 6-phosphate dehydrogenase was placed on a cellulose acetate strip (Sephaphore III) which had been equilibrated for 1 hr in 0.1 M Tris–EDTA–boric acid buffer at pH 9.0 in a cold room. Current was applied to the solution at 4 ma/strip for 80 min. Ambient temperature was 5°. The protein was located by staining the strips for 3 min in 0.01% Amido Schwartz and destaining in 3% acetic acid. Glucose 6-phosphate dehydrogenase activity was located by staining the strips for 12 hr in darkness in 0.1 M Tris-HCl buffer at pH 8.0 containing 20 μmoles of $MgCl_2$, 0.08 mg of sodium 2,6-dichloroindophenol, 0.1 mg of phenazine methosulfate, 8 μmoles of glucose 6-phosphate, and 1 μmole of $NADP^+$ in 5 ml of buffer. Only one enzyme band and one protein band were located. These bands had identical electrophoretic mobilities as shown in Figure 3.

VERTICAL DISC ELECTROPHORESIS IN POLYACRYLAMIDE GEL. Proteins from various purified fractions of glucose 6-phosphate dehydrogenase were separated by vertical

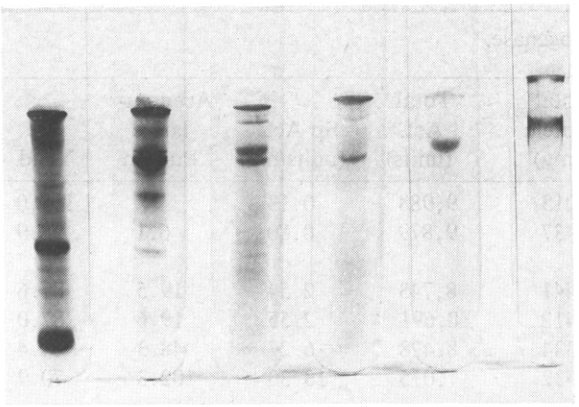

FIGURE 4: Electrophoresis on vertical gels of polyacrylamide of several fractions obtained during the purification of glucose 6-phosphate dehydrogenase. A current was applied for 2 hr at 6 ma/gel. Gels 1–5 were stained for protein; they are from left to right, fractions II, IV, VI, IX, and XII. Gel 6 was fraction XII stained for glucose 6-phosphate dehydrogenase activity.

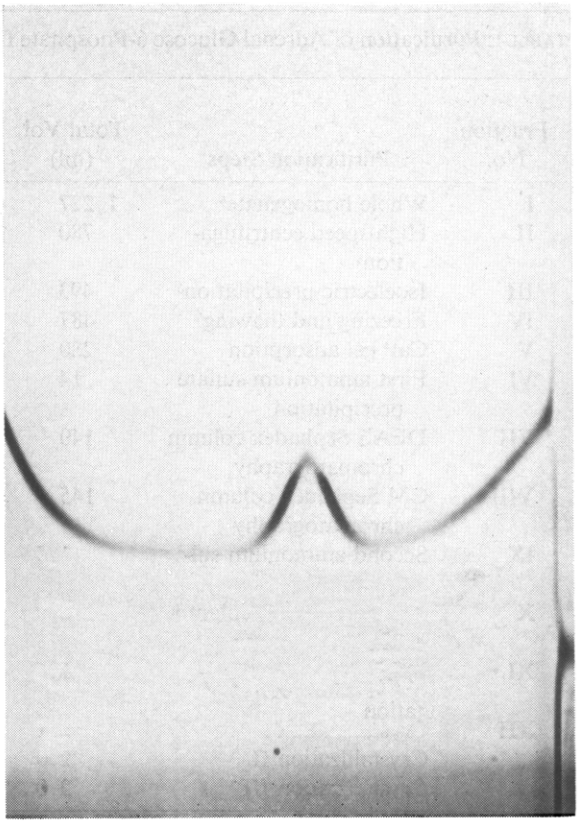

FIGURE 5: Schlieren ultracentrifuge pattern of a preparation of crystalline glucose 6-phosphate in a standard centrifuge cell taken 40 min after reaching maximal speed of 56,100 rpm. The protein concentration was 0.01% in 0.05 M phosphate buffer at pH 6.7 containing EDTA, β-mercaptoethanol, and 10^{-5} M $NADP^+$.

disc electrophoresis in polyacrylamide using the method of Davis (1964) with modifications. Modifications included using a 10% "hard" gel and a 4% "spacer" gel; sucrose was dissolved in the protein solutions to allow layering of the sample under the buffer and directly on top of the "spacer" gel; no "sample" gel was used; the buffer solution in the reservoirs was 0.1 M Tris–EDTA–boric acid at pH 9.0. Current was applied to 6 ma/gel for 2 hr. Room temperature was 5°. Proteins were located by standing for 5 min in 0.01% Amido Schwarz and destaining in 3% acetic acid. Glucose 6-phosphate dehydrogenase activity was located by staining the gels for 30 min in the dark in 0.06 M amine buffer at pH 8.0 containing 0.1 mg of phenazine methosulfate, 0.05 mg of nitro blue tetrazolium, 1 μmole of $NADP^+$, 8 μmoles of glucose 6-phosphate in 5 ml of buffer. The results from various stages of purification are shown in Figure 4. The results indicated that the crystallized enzyme (fraction XII) showed only one protein band and one enzyme band. The bands were of identical electrophoretic mobility.

VELOCITY ULTRACENTRIFUGATION. Ultracentrifugation of the crystallized enzyme was carried out in a Spinco Model E ultracentrifuge. The enzyme was dissolved in 0.05 M phosphate buffer at pH 6.7 containing 10^{-5} M $NADP^+$ with buffer additives 2 and 3. When a standard centrifuge cell was used the schlieren patterns revealed a single, symmetrical peak (Figure 5).

The molecular weight was calculated from an $s_{20,w}$ of 9.8 S to be 238,700 using the formula

$$\bar{M}_i = \left[\frac{S_i K_s}{1 - \bar{V}_i \rho}\right]^{3/2} \bar{V}_{i^{1/2}}$$

assuming viscosity (V_i) = 0.726, density (ρ) = 1.006, and K_s = 1.2(10^{15}). This was assumed to be approximately correct since pyruvate kinase with a known molecular weight of 237,000 was determined to have an $s_{20,w}$ of 9.7 S.

MOLECULAR SIEVE ANALYSIS. The rechromatography of the crystallized fraction on a Sephadex G-200 column showed one protein peak corresponding to the enzyme. The ratio of the glucose 6-phosphate dehydrogenase elution volume to the elution volume of Blue Dextran (V_e/V_0 ratio described by Andrews, 1965) was 1.58. The column was calibrated using proteins of the following molecular weights: 237,000 for muscle pyruvate kinase, 160,000 for γ-globulin, 67,000 for bovine serum albumin, and 25,000 for chymotrypsin. Blue Dextran (approximately 2,000,000) was used to determine the void volume. The flow rate was maintained at 12 ml/hr. The molecular weight of glucose 6-phosphate dehydrogenase from the molecular sieve data was calculated to be 235,000 (Figure 6).

CRYSTALLIZATION TO A CONSTANT SPECIFIC ACTIVITY. Adrenal glucose 6-phosphate was crystallized three consecutive times. Crystallization I yielded only a

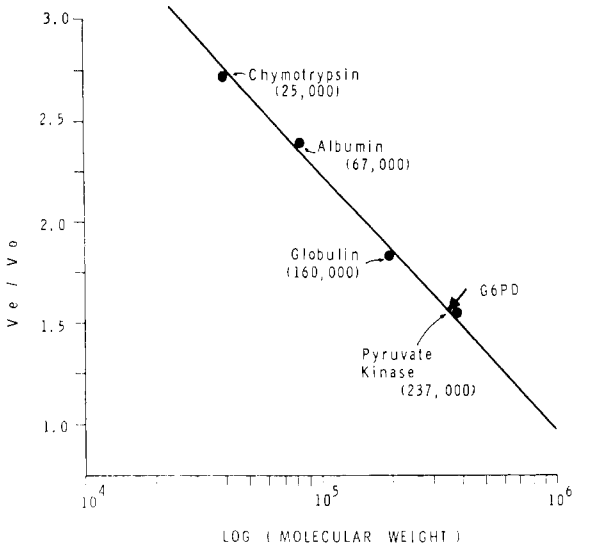

FIGURE 6: Calibration curve for Sephadex G-200 column and location of glucose 6-phosphate dehydrogenase elution ratio. A 1.5-ml solution containing 1% Blue Dextran. 5 mg of chymotrypsin, 4 mg of albumin, 5 mg of γ-globulin, and 4 mg of pyruvate kinase was mounted on a Sephadex G-200 column (2.5 × 100 cm) equilibrated with 0.01 M phosphate buffer with $NADP^+$, EDTA, and β-mercaptoethanol. Elution was accomplished using the same buffer. Assays were made according to Andrews (1965). Similar runs were made using only crystallized glucose 6-phosphate dehydrogenase and Blue Dextran.

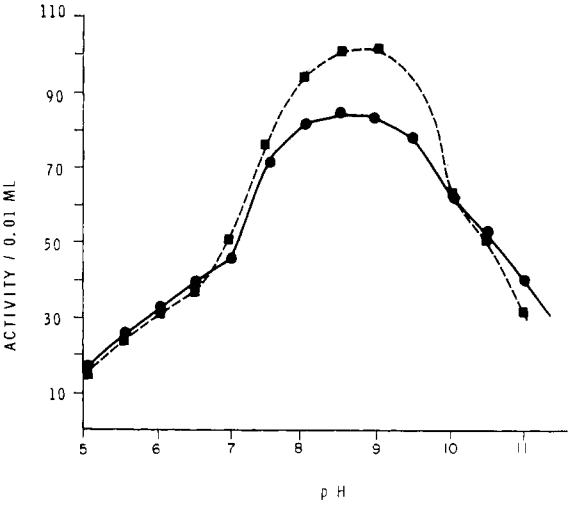

FIGURE 7: Effect of pH and Mg^{2+} on glucose 6-phosphate dehydrogenase activity. The assay mixture contained 10^{-5} M $NADP^+$, 10^{-4} M glucose 6-phosphate (6 mM $MgCl_2$ where noted), enzyme, and either 0.1 M acetate buffer (pH 5.0–7.0) or 0.1 M Tris buffer (pH 7.0–11.0). (●—●) Without $MgCl_2$ and (■— —■) with $MgCl_2$.

few large rod-shaped crystals and many small crystals. These dissolved easily at pH 4.0 in acetate or phosphate buffer at 0.01 M. The second and third crystallizations, which were carried out as described in Experiments, yielded successively more of the larger crystals (Figure 1) which dissolved more slowly. During three consecutive crystallizations the specific activity remained constant within ±9.1 std dev (Table I). The mother liquor had the same specific activity as the crystals by the second crystallization.

OPTIMAL PH (Figure 7). The effect of pH on enzyme activity was examined using acetate buffer (pH 5.0–7.0) and Tris buffer (pH 7.0–11.0) with and without 6 mM Mg^{2+}; however, Mg^{2+} increased the activity about 20% in this pH range. It was also observed that an approximate doubling of activity occurred between pH 7.0 and 8.0, both with and without Mg^{2+}.

KINETIC CONSTANTS. The Lineweaver–Burk plots of the reaction of various substrates with the crystalline enzymes are shown in Figure 8. The K_m for $NADP^+$ was 5.6×10^{-6} M and 4.7×10^{-2} M for NAD^+. Glucose 6-phosphate yielded a K_m for the $NADP^+$-linked reaction of 4.2×10^{-5} M compared to 1.9×10^{-4} M for the NAD^+-linked reaction. These agree closely with the previously determined K_m for glucose 6-phosphate and $NADP^+$ with the partially purified enzyme (McKerns, 1962b), in 0.1 M Tris-HCl buffer at pH 8.0 with 3.3 mM $MgCl_2$. However, the physiological activity of the enzyme is considerably less at the pH of intracellular fluid. In addition, the maximum potential activity would never be approached since cellular levels of glucose 6-phosphate and $NADP^+$ are far lower than those used for the assays above.

Discussion

With the purification of glucose 6-phosphate dehydrogenase from the adrenal cortex, three mammalian glucose 6-phosphate dehydrogenases have been crystallized. They all have similar molecular weights. The enzyme from the mammary gland has a molecular weight of 241,000 in the presence of $NADP^+$ and 130,000 in the absence of $NADP^+$, as estimated by Sephadex column chromatography (Levy *et al.*, 1966). Erythrocyte glucose 6-phosphate dehydrogenase was observed to have a molecular weight of 240,000 with $NADP^+$ and a range from 123,000 to 180,000 without $NADP^+$ as calculated from analytical ultracentrifugation (Yoshida, 1966). These molecular weights agree closely with the molecular weight of 236,000 for adrenal glucose 6-phosphate dehydrogenase in the presence of $NADP^+$ as estimated from both Sephadex column chromatography and analytical ultracentrifugation.

The enzymes from the mammary gland and erythrocytes apparently consist of two monomeric units (Nevaldine and Levy, 1965; Levy *et al.*, 1966). It has been postulated that these enzymes are capable of a reversible association of units caused by the presence of $NADP^+$ and high protein concentration (Nevaldine and Levy, 1965; Yoshida, 1966). Our ultracentrifugation pattern of

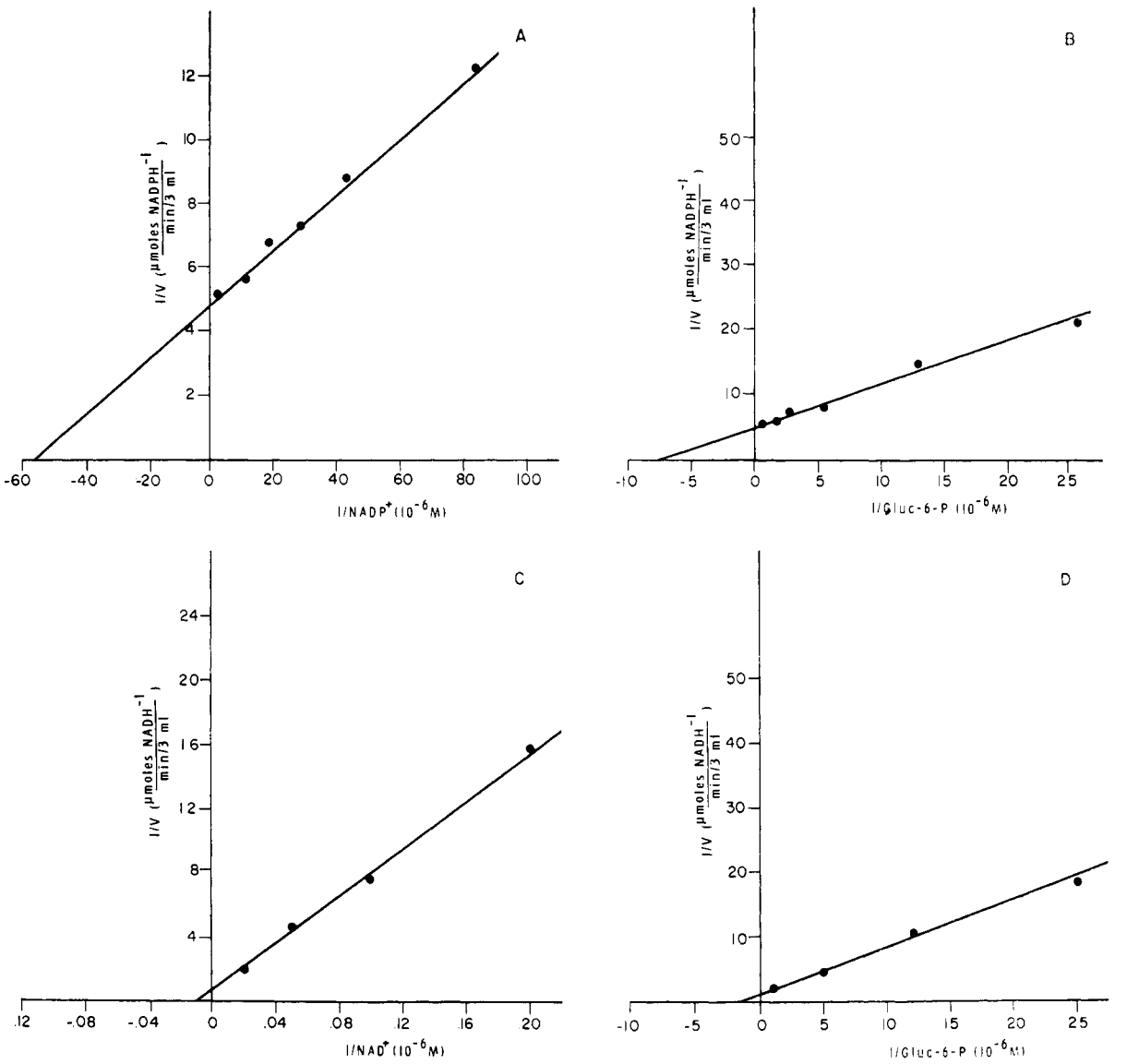

FIGURE 8: Lineweaver–Burk plots of solutions of crystalline glucose 6-phosphate dehydrogenase at 25° with various substrates. The assay mixture contained 0.1 M Tris-HCl buffer at pH 8.0, 6 μmoles of $MgCl_2$, enzyme, and various concentrations of substrates to a total of 3 ml. Each point represents the mean of two or more closely agreeing determinations. Michaelis constants were calculated from an equation which gives K_m as the negative reciprocal of the intercept of the line with the abscissa, according to the method of McKerns (1962b). (A) Glucose 6-phosphate concentration was 10^{-4} M with various concentrations of $NADP^+$. The K_m for $NADP^+$ was calculated as 5.6×10^{-6} M. (B) $NADP^+$ concentration was 10^{-5} M with varying concentrations of glucose 6-phosphate. The K_m for glucose 6-phosphate for the $NADP^+$-linked reaction was calculated as 4.2×10^{-5} M. (C) Glucose 6-phosphate concentration was 10^{-4} M with various concentrations of NAD^+. The K_m for NAD^+ was calculated as 4.7×10^{-2} M. (D) NAD^+ concentration was 10^{-2} M with various concentrations of glucose 6-phosphate. The K_m for glucose 6-phosphate for the NAD^+-linked reaction was calculated as 1.9×10^{-4} M.

adrenal glucose 6-phosphate dehydrogenase showed a single peak. It was obtained at high protein concentration in the presence of 10^{-5} M $NADP^+$. It is not known at present whether dissociation will occur at lower protein concentration or in the absence of $NADP^+$. This is under study.

There are also similarities between the three mammalian glucose 6-phosphate dehydrogenases when compared to the crystallized yeast and bacterial enzymes. Yeast glucose 6-phosphate dehydrogenase has a $s_{20,w}$ of 9.6 S in the presence of $NADP^+$ (Noltmann and Kuby, 1963) and 6.1 S (with an average molecular weight of 101,700) in the absence of $NADP^+$ (Yue *et al.*, 1967). The enzyme from bacteria has a molecular

weight of 135,000 in the absence of $NADP^+$ as determined by Sephadex column chromatography (Olive and Levy, 1967); it has not been examined with $NADP^+$. Therefore, all of the glucose 6-phosphate dehydrogenases which have been crystallized appear to have similar molecular weights and possibly a similar mode of unit association.

Even though the molecular weights of the mammalian and nonmammalian glucose 6-phosphate dehydrogenases appear to agree, there is considerable evidence that there are differences between enzymes and that molecular species may exist. Palmityl coenzyme A inhibits the yeast and mammary enzymes but does not affect the bacterial enzyme (Olive and Levy, 1967). Low concentrations of phosphate stimulate the yeast and bacterial enzymes, but inhibit the mammary and erythrocyte glucose 6-phosphate dehydrogenases (Levy *et al.*, 1966; Yoshida, 1966; Olive and Levy, 1967). Concerning nucleotide specificity, yeast glucose 6-phosphate dehydrogenase reduces only $NADP^+$ (Warburg and Christian, 1936), the bacterial enzyme reduces NAD^+ as well as $NADP^+$ (DeMoss *et al.*, 1953), while the three mammalian enzymes reduce $NADP^+$ at a rate about 1000 times greater than NAD^+ (Levy, 1963; Yoshida, 1966).

Individual molecular species of the mammalian glucose 6-phosphate dehydrogenases may also exist. The optimum pH for all three mammalian enzymes is about pH 8.5, but the optimum pH range and the shape of the pH *vs.* activity curves vary considerably (Levy, 1963; Yoshida, 1966). The Michaelis constants are for the mammary enzyme, K_m ($NADP^+$) ($-Mg^{2+}$) = 8.9×10^{-6} M and K_m (NAD^+) ($-Mg^{2+}$) = 1.5×10^{-2} M (Levy, 1963); for the erythrocyte enzyme, K_m ($NADP^+$) ($+Mg^{2+}$) = 4.4×10^{-6} M and K_m (NAD^+) ($+Mg^{2+}$) = 4×10^{-3} M (Yoshida, 1966); for the adrenal enzyme, K_m ($NADP^+$) ($+Mg$) = 5.6×10^{-6} M and K_m (NAD^+) ($+Mg$) = 4.7×10^{-2} M. Stilbenoid compounds inhibit adrenal glucose 6-phosphate dehydrogenase (McKerns and Kaleita, 1960; McKerns, 1963), but do not affect the erythrocyte enzyme (Marks and Banks, 1960); dehydroisoandrosterone inhibits all three enzymes, but a different percentage of inhibition is observed for all three glucose 6-phosphate dehydrogenases at the same concentration of inhibitor (Marks and Banks, 1960; McKerns and Kaleita, 1960; Levy, 1963). Recently, two immunologically distinct glucose 6-phosphate dehydrogenases have been isolated from mammalian liver; one enzyme was purified from the supernatant fraction and another was prepared from disrupted mitochondria (Zaheer *et al.*, 1967). Therefore, similar to that observed for creatine kinases (Eppenberger *et al.*, 1967) and malic dehydrogenases (Kitto and Kaplan, 1966), individual species of glucose 6-phosphate dehydrogenases probably exist which have their individual specificities for substrates, cofactors, ions, activators, and inhibitors.

It was postulated previously that a mechanism of action of many hormones in regulating cell function and replication in their target organs may depend on activation or inhibition of unique species of glucose 6-phosphate dehydrogenases (McKerns, 1964a,b, 1966, 1967). Evidence was presented that ACTH activated adrenal glucose 6-phosphate dehydrogenase (McKerns, 1964a,b, 1967), and that follicle stimulating and luteinizing hormones activated ovarian glucose 6-phosphate dehydrogenase (McKerns, 1965b). In addition, estrogens stimulated lipid synthesis in adipose tissue from glucose-1-H^3 (Gilmour and McKerns, 1966) and estrogen increased many parameters of function of the uterus in the immature rat that associate with an activation of uterine glucose 6-phosphate dehydrogenase (McKerns, 1967). The purification of cow adrenal glucose 6-phosphate dehydrogenase will enable this concept of direct activation of an enzyme by a hormone to be tested further.

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Chicken Heart Soluble Aspartate Aminotransferase. Purification and Properties*

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ABSTRACT: Chicken heart soluble aspartate aminotransferase has been purified, and both the holo- and apoenzymes have been crystallized. Pyridoxal phosphate stabilizes the apoenzyme against denaturation by heat, urea, and extremes of pH. The binding of the coenzyme to the apoenzyme decreases the fluorescence of the tryptophan residues in the enzyme and increases the protein polarization of fluorescence.

The minimum weight of enzyme per mole of bound coenzyme has been determined by fluorescence titrations to be 52,000 ± 5000 for pyridoxal phosphate and 48,900 ± 4000 for pyridoxamine phosphate and by polarization of fluorescence measurements to be 48,700 ± 4000 for pyridoxal phosphate.

The native enzyme has a sedimentation coefficient of 5.5 and a sedimentation equilibrium molecular weight of 100,000. This 5.5S enzyme is dissociated at pH 3.0 into a 2.45S component. Sedimentation equilibrium ultracentrifugation of dilute holoenzyme solutions at pH 9 and 10 demonstrates an equilibrium between the monomer and dimer. An enzymatically active tetramer (8.1 S; molecular weight, 190,000) has been isolated which can be dissociated in 1×10^{-2} M 2-mercaptoethanol to the 5.5S protein.

The existence of two electrophoretically different forms of aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase (2.6.1.1) were observed in dog heart extracts by Fleisher (1960). Differential centrifugation of rat liver extracts, also containing these two forms, demonstrated that the anionic protein was associated with the supernatant fraction of the cell, while the cationic form was associated with the mitochondria (Boyd, 1961, 1962).

The mitochondrial and soluble forms of the aspartate aminotransferase have been purified and characterized from a number of mammalian tissues: beef heart (Morino *et al.*, 1963), pig heart (Nisselbaum and Bodansky, 1966; Martinez-Carrion and Tiemeier, 1967), rat liver (Hook and Vestling, 1962; Harpring, 1965), and human tissues (Bodansky *et al.*, 1966).

We have observed that both the anionic and cationic forms of aspartate aminotransferase are also present in chicken heart extracts. This paper reports the purification and crystallization of the soluble, anionic form of chicken heart AAT[1] and presents an investigation of the

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[1] Abbreviations used: AAT, aspartate aminotransferase; DPNH, reduced diphosphopyridine nucleotide.