Purification and Properties of Bovine Prothrombin*

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ABSTRACT: A rapid, simple, and reproducible method for the purification of bovine prothrombin, starting with a commercial preparation, is reported. By carrying out isoelectric precipitation, chromatography on DEAE-Sephadex, and ultracentrifugation, a product in 18% yield, free of contaminants as evaluated by the sensitive criterion of disc gel electrophoresis, was obtained. Chemical and physiocochemical characterization studies were performed to determine the size and shape of the molecule. Prothrombin in phosphate buffer has a sedimentation equilibrium molecular weight of 74,000 ± 4100, a sedimentation coefficient of 4.80 S, intrinsic viscosity of 3.4 cc/g, a value for β of 2.07×10^6, possesses low helix content, and contains 2.3% carbohydrate as hexose. In the denaturing solvents 6 M guanidine hydrochloride and 6 M guanidine hydrochloride plus 0.1 M β-mercaptoethanol, prothrombin has essentially the same molecular weight as in buffer, indicating that the protein molecule consists of a single chain. Values for s and $[\eta]$ in these solvents are also consistent with those for proteins known to be made up of one chain in these solvents. We conclude that prothrombin is a globular compact protein molecule of one chain, possessing little helix content. Preliminary activation studies are also reported, and indicate that pure prothrombin does not activate autocatalytically.

The critical step in the blood clotting process is the formation of the fibrin clot by the action of the enzyme thrombin on fibrinogen. Generation of thrombin from its inactive plasma precursor prothrombin has been the subject of much investigation for decades.

At present there are two conflicting schools of thought regarding the role which the molecule prothrombin plays in the blood clotting scheme. One of them, led by Seegers and coworkers, suggests that prothrombin is able to generate thrombin autocatalytically in a suitable environment such as 25% sodium citrate (Seegers, 1965). In addition, one of several prothrombin derivatives, depending upon the accelerators supplied to the system, is also generated. One of these additional products is an enzyme (autoprothrombin C); the others are reported to be derivatives of the parent prothrombin molecule and can be activated to thrombin under certain conditions. In this view, prothrombin is a multienzyme system that is able to produce several different product enzymes, depending upon the system used to accelerate the activation of prothrombin.

The other approach, suggested independently by Macfarlane (1964) and by Davie and Ratnoff (1964), postulates that the traditional clotting factors present in plasma (factors XII, XI, IX, VIII, X, V, and phospholipid from the platelets) participate in a cascade of proenzyme–enzyme conversions leading to the formation of an enzyme or enzyme complex which is capable of activating prothrombin to thrombin. The prothrombin derivatives of Seegers are identified with these traditional clotting factors (*e.g.*, autoprothrombin C is reported to be the same as activated factor X; Spaet, 1964). Proponents of the cascade theory, therefore, suggest that activation of prothrombin in citrate is caused by the presence of contaminating clotting factors in the prothrombin preparations. The conflicts and the similarities involved in these two approaches have been summarized (Davie and Ratnoff, 1965; Kline, 1965; Macfarlane, 1966, 1968). It is our view that resolution of these conflicts will be possible only when each of the blood clotting factors is obtained free from other contaminating clotting factors and is chemically and physicochemically well characterized (Scheraga, 1962).

Because of the prominent position of prothrombin in the clotting scheme, many workers have attempted to purify and characterize prothrombin from several species, such as man (Lanchantin *et al.*, 1963), cow (Moore *et al.*, 1965;[1] Malhotra and Carter, 1968; Magnusson, 1965b), horse (Miller and Phelan, 1967), and rat (Li and Olson, 1967). In general, these different groups report conflicting results on the characteristics of prothrombin. Some preparations have not been demonstrated to be pure, and/or physicochemical characterization has not been reported. In general, then, these studies have achieved only partial success.

This project was undertaken several years ago to develop a reproducible purification method for bovine prothrombin and to characterize this product by chemical and physicochemical techniques, in preparation for well-controlled activation studies. The starting material was a partially purified sample of prothrombin pre-

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[1] We are indebted to Dr. John R. Carter for sending us a copy of this paper prior to publication.

pared by the Sigma Chemical Co. (St. Louis, Mo.) using a method essentially the same as that of Moore *et al.* (1965). The Sigma material was then subjected to isoelectric precipitation and chromatography on DEAE-Sephadex, A-50, to yield a prothrombin product free from any contaminants, as determined by polyacrylamide disc gel electrophoresis. This material was characterized as to molecular weight, sedimentation coefficient, and intrinsic viscosity in phosphate buffer and in denaturing solvents, optical rotatory dispersion, amino acid analysis, and per cent carbohydrate as hexose. Preliminary activation studies are also reported.

During the preparation of this manuscript, the work of Tishkoff *et al.* (1968) on the purification and characterization of bovine prothrombin, using techniques similar to ours, was published. A comparison of the results presented here and those obtained by Tishkoff *et al.* (1968) is included in the Discussion.

Experimental Procedures

Purification of Bovine Prothrombin. Bovine prothrombin was prepared using a modification and extension of the method described by Moore *et al.* (1965). Sigma Chemical Co. performed the initial steps in the purification procedure involving large volumes. These steps consisted of the adsorption of prothrombin from plasma onto barium citrate, elution of prothrombin from the barium citrate–prothrombin complex with EDTA, dialysis of the prothrombin solution against deionized distilled water, and ammonium sulfate fractionation (retaining the 0.5–0.67 M fraction). The prothrombin was then dissolved in 0.05 M phosphate buffer (pH 7.0) and frozen for shipment. Approximately 1 l. of frozen prothrombin solution, containing $\sim 1 \times 10^6$ *p*-tosyl-L-arginine methyl ester units (Ehrenpreis and Scheraga, 1957), from each of three preparations (lots 86B-8550, 87B-8010, and 78B-8200), was received. The solution was thawed, divided into portions of 100–150 ml, and stored at −70°.

The aliquots of the Sigma preparations were thawed and dialyzed with frequent changes of the dialysate against deionized distilled water for 12–36 hr at 2–4° in cellophane dialysis bags. The dialysis tubing had been treated at 70° for 15 min in EDTA, rinsed, and stored in the cold in deionized distilled water. After dialysis, the pH of the solution (~pH 6.8) was adjusted to 5.4 with dilute HCl. A precipitate was removed by centrifugation at 7500 rpm for 30 min at 4–6°. The pH of the supernatant was lowered to 4.1–4.2 with 0.25% HCl, usually in steps of 0.4 pH unit, for isoelectric precipitation of prothrombin. The prothrombin precipitates which formed were collected by centrifugation at 3500 rpm for 20 min at 4–6°, and then dissolved in 0.1 M phosphate buffer (pH 6.0). The solution was usually clarified by ultracentrifugation at 30,000 rpm for 5 hr at 4–6° and then concentrated to 2–10 ml (3–13 mg/ml) in a Diaflo Model 50 ultrafiltration cell using UM-1 membranes at 2–4°.

The above solution was then chromatographed on DEAE-Sephadex, A-50 fine (either in the phosphate or chloride ion form; Pharmacia, Uppsala, Sweden), at 2–4°. The Sephadex had been equilibrated with 0.1 M phosphate buffer (pH 6.0) at 2–4° before application of the prothrombin solution. A linear salt gradient (0.0–1.0 M NaCl in 0.1 M phosphate buffer, pH 6.0) was used to elute the protein. Flow rates of 6–10 ml/hr were obtained for 3.5 × 5–10 cm columns. Fractions (1.5 ml) were collected using a Technicon time-flow automatic fraction collector, and the eluted protein was detected by reading the optical density of the fractions at 280 mμ. All fractions common to the prothrombin peak were pooled and concentrated to ~3.3 mg/ml on the Diaflo apparatus. The extruded buffer from the Diaflo unit was used in subsequent experiments to dilute the stock prothrombin solutions and served as a reference solvent. Occasionally, ultracentrifugation at 30,000 rpm for 5 hr at 4–6° was performed after the chromatography. Stock solutions of prothrombin were stored at 4° and used within 1 week.

Assay for Prothrombin. The method used to assay for prothrombin was essentially that described by Ware and Seegers (1949). Prothrombin solutions (200 μl; optical density of 0.3–1.5) was incubated in 2.3 ml of Bacto Ac-Globulin (Difco, Detroit, Mich.) for 30 min at 28°; 1.0 ml of this solution was then added to 3.0 ml of Bacto-Prothrombin II-stage reagent (Difco) to begin the incubation. At regular intervals over a time span of 60–80 min, 0.4-ml aliquots of the above incubation mixture were added to 0.4 ml of fibrinogen solution (see below), and the time required for the appearance of a visible clot was measured. These assays were continued until the clotting time became independent of the incubation time.

The Bacto products were dissolved according to manufacturer's directions. The fibrinogen solution was prepared as follows. Armour bovine fibrinogen (1.0 g) (lot U4704, containing ~50% citrate) was stirred into 50 ml of 5.0% imidazole buffer (pH 7.2) for 15 min. The solution was filtered and allowed to stand for 30 min. The solution became cloudy and was again filtered. The optical density of fibrinogen solutions prepared in this manner did not change for 60–80 min, indicating that these solutions were stable throughout the clotting assay. The solution was approximately 0.35% fibrinogen, calculated using the factor 0.625 mg/ml per optical density unit at 280 mμ (Ehrenpreis and Scheraga, 1957).

The number of clotting units per milliliter detected in the assay was taken to be $t_c^{-1} \times 10^2 \times$ dilution factor of assay, where t_c is the clotting time in seconds. Similarly, the specific activity was set equal to the inverse clotting time $\times 10^2$ divided by the optical density of the solution assayed.

Activation of Prothrombin. A *preliminary* study of the conversion of prothrombin to thrombin was carried out by incubating 100 μl of chromatographically purified prothrombin (2.74 mg/ml) separately with each of the following reported activators for 48 hr at 28°: 0.1 M phosphate buffer (pH 6.0) as a control; 25.0% sodium citrate; Bacto-Prothrombin II-stage reagent (Difco); Bacto Ac-Globulin (*viz.*, factor V) (Difco); Bacto-Prothrombin II-stage reagent plus Bacto Ac-Globulin; activated factor X (factor X*), 2.0 units/ml (Sigma); purified factor V, 2.0 units/ml (Sigma); factors X* + V

(Sigma); and thrombin, 1.41 mg/ml (Upjohn, Kalamazoo, Mich.), respectively. The Bacto-Prothrombin II-stage reagent and Bacto Ac-Globulin were dissolved as directed by the manufacturer.

The assay for the thrombin which was generated in these prothrombin activation studies was carried out by diluting an aliquot of the activation mixture with 0.4 ml of fibrinogen solution. The number of clotting units generated per milligram of prothrombin originally present in the aliquot assayed was computed from the reciprocal of the clotting time.

Polyacrylamide Gel Electrophoresis. Disc electrophoresis was performed with a Buchler Polyanalyst with Buchler power supply type 3-1014A. Polyacrylamide gels (7.5%) were used according to Buchler formulations for anionic and cationic systems, running pH 9.3 and 4.3, respectively (see Buchler Instruments Instruction Folder).

Acrylamide (American Cyanamid, New York, N. Y.) was recrystallized from acetone–benzene at 2–4°. Buffalo Black NBR (Naphthol Blue Black) (Allied Chemical, New York, N. Y.) was used as the staining agent, and bromophenol blue and pyronin B as tracker dyes for the anionic and cationic systems, respectively. Samples were applied in 40% sucrose.

Densitometer tracings of the gels were made with a Canalco Model E microdensitometer. We are indebted to Professor F. C. Steward for the use of his microdensitometer.

Amino Acid Analysis. The amino acid analyses of prothrombin were carried out with a Technicon amino acid analyzer. Lyophilized salt-free samples were hydrolyzed in constant-boiling HCl (6.0 M) at 110° for 15, 25, and 35 hr in evacuated, sealed ampules. Corrections for destruction of amino acids during hydrolysis were made by extrapolation to zero hydrolysis time; in the cases of Ile and Val, which are released very slowly during hydrolysis, the data were extrapolated to 70-hr hydrolysis time. Tryptophan was determined spectrophotometrically in 0.1 N NaOH as described by Goodwin and Morton (1946). We are indebted to Mr. Hua Tjan for running the Technicon amino acid analyzer.

Other Chemical Analyses. Total carbohydrate was determined as hexose by the anthrone method of Scott and Melvin (1953) using anhydrous dextrose as the standard. Nitrogen was determined by the Kjeldahl method as described by Lang (1958).

Extinction Coefficients. The extinction coefficient, $\epsilon^{1\%}_{1\,cm}$, for purified prothrombin at 280 mμ was determined by two methods. In the first, ϵ was calculated from the number of Tyr, Trp, and Cys residues together with their molar extinction coefficients given by Wetlaufer (1962). ϵ was calculated for both the maximum and minimum values for Cys, because separate assays for cysteine and cystine were not carried out. In the second, ϵ was calculated from the amino acid composition and the amount of ammonia liberated on hydrolysis (both corrected to zero time), together with the Kjeldahl nitrogen content. This extinction coefficient was used for the calculations of protein concentrations, where required.

Partial Specific Volume. The partial specific volume, $\bar{v}$, for prothrombin *in water and in buffers* was calculated from the amino acid composition and the values for the partial molal volumes of the amino acid residues as discussed by Cohn and Edsall (1943). This value was used to calculate all molecular weights and corrected sedimentation coefficients.

As discussed by Castellino and Barker (1968), $\bar{v}$ of proteins in guanidine hydrochloride solutions may be decreased by 0.01 because of binding of guanidine hydrochloride to the protein. Therefore, the molecular weights and sedimentation coefficients of prothrombin in guanidine hydrochloride and guanidine hydrochloride plus β-mercaptoethanol solutions were also calculated with the value of $\bar{v}$ in water decreased by 0.01 cc/g.

Sedimentation Equilibrium. Molecular weights were determined by the high-speed sedimentation equilibrium method described by Yphantis (1964) using a Spinco Model E ultracentrifuge equipped with Rayleigh interference optics and a temperature control unit. An AN-J rotor was used at speeds below 19,000 rpm, and an AN-D rotor at higher speeds. The cell consisted of the Yphantis six-chambered centerpiece and sapphire windows. Fluorocarbon FC-43 was used as base fluid in the chambers where protein was present; 3-mm column heights were used. All experiments were performed at 23°. Rotor speeds were chosen so that the concentration of sedimenting material at the meniscus was essentially zero. All runs were allowed to proceed until there was no further increase in fringe number across the cell.

Solutions were prepared by diluting stock solutions of protein with the appropriate dialysate. Prothrombin solutions in phosphate buffer were diluted with the extruded buffer from the Diaflo unit, and this was also used as the reference solvent. The prothrombin–guanidine hydrochloride and prothrombin–(guanidine hydrochloride plus β-mercaptoethanol) solutions were prepared in the following way. Guanidine hydrochloride (Eastman) was decolorized with charcoal and recrystallized from methanol–absolute ether. Guanidine hydrochloride solutions (6 M) were made by adding buffer solution, adjusted to pH 7.4, to the solid guanidine hydrochloride. The buffer composition was 0.021 M Tris, 0.01 M NaCl, 0.01 M EDTA, and 0.1 M β-mercaptoethanol (the latter being omitted in some cases) (Ullmann *et al.*, 1968). Concentrated prothrombin solutions in buffer were diluted with the appropriate guanidine hydrochloride solution and dialyzed against that solution at room temperature for 48–72 hr with frequent changes of the dialysate. The final dialysates were used to dilute the prothrombin–guanidine hydrochloride solutions further, and also as reference solvents.

The Rayleigh interference patterns were photographed on Kodak spectroscopic plates, type II-G backed, and analyzed on a Gaertner two-dimensional comparator. After alignment along the y coordinate, any one fringe at the meniscus was selected as the starting point for counting half-fringes, $J/2$. The x coordinate at each half-fringe was measured. Since blank runs showed that deviations from linear parallel fringes were less than 0.057 fringe, no corrections for sedimen-

tation of buffer salts were made. Weight-average molecular weights, $\bar{M}_w$, were determined from the slopes of plots of ln J *vs.* x^2, according to eq 1, where x is the

$$\bar{M}_w = \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \frac{d \ln J}{dx^2} \quad (1)$$

distance from the center of rotation of the rotor to the position of the half-fringe, R is the gas constant, T is the absolute temperature, $\bar{v}$ is the partial specific volume, ρ is the solvent density, and ω is the angular velocity of the rotor.

Sedimentation Velocity. Sedimentation velocities were measured in a Spinco Model E ultracentrifuge using schlieren optics at 23°. Double-sector cells with quartz windows were used, and Kodak metallographic plates were used for photography and analyzed on a Gaertner two-dimensional comparator. Sedimentation coefficients were calculated from the rate of movement of the peak and corrected to standard conditions, taken to be 23° in the buffer system 0.1 M phosphate buffer plus 0.65 M NaCl. Sedimentation coefficients for prothrombin in guanidine hydrochloride and in guanidine hydrochloride with β-mercaptoethanol were corrected to standard conditions according to eq 2,

$$s_{cor} = s_{obsd} \frac{\eta_{Gu}}{\eta_{std}} \frac{(1 - \bar{v}\rho)_{std}}{(1 - \bar{v}\rho)_{Gu}} \quad (2)$$

where η_{Gu}/η_{std} is the ratio of the viscosity of the guanidine hydrochloride solvent to that of standard buffer, and $(1 - \bar{v}\rho)_{std}/(1 - \bar{v}\rho)_{Gu}$ corrects for the effect of guanidine hydrochloride on $\bar{v}$ and differences in the densities of the reference solvents (Schachman, 1959).

Optical Rotation. Optical rotatory dispersion measurements were made with a Cary Model 60 spectropolarimeter, using quartz, water-jacketed cells of path lengths 0.01 and 0.10 dm. An Osram xenon arc lamp (450 W) was used as the light source, and measurements were made in the wavelength range 195–500 mμ. The temperature in the cell was controlled with flowing water from a Haake constant-temperature bath. The solutions used were prepared by diluting stock solutions of prothrombin with appropriate solvents.

The optical rotatory dispersion data were expressed in terms of the reduced mean residue rotation, $[m']_\lambda$ (in deg cm²/dmole), defined by eq 3, where MRW, the

$$[m']_\lambda = \frac{3}{n^2 + 2} \frac{MRW}{100} [\alpha]_\lambda \quad (3)$$

mean residue molecular weight, was taken to be 115, $[\alpha]_\lambda$ is the specific optical rotation calculated from the concentration of protein in solution, and n is the refractive index of the solution. Appropriate refractive indices were taken from Fasman (1963).

The parameters a_0 and b_0 of the Moffit–Yang equation (Fasman, 1963)

$$[m']_\lambda = \frac{a_0\lambda_0^2}{(\lambda^2 - \lambda_0^2)} + \frac{b_0\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2} \quad (4)$$

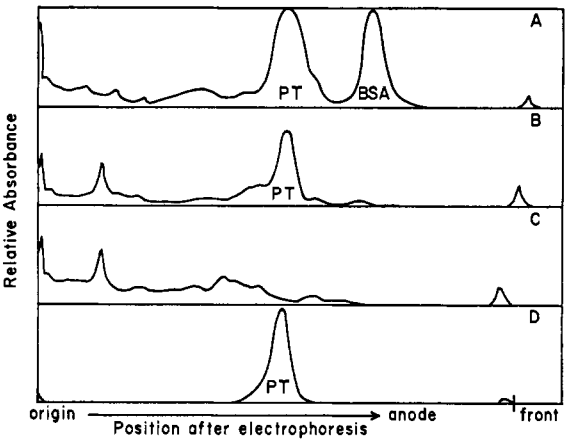

FIGURE 1: Densitometer traces of patterns obtained from disc electrophoresis of prothrombin (PT) solutions on 7.5% polyacrylamide gels. (A) Sigma PT, (B) isoelectric product, (C) "non-PT" peak from DEAE-Sephadex chromatography, (D) PT peak from DEAE-Sephadex chromatography, after preparative ultracentrifugation at 30,000 rpm for 5 hr.

were obtained by plotting the quantity $[m']_\lambda((\lambda^2 - \lambda_0^2)/\lambda_0^2)$ against $\lambda_0^2/(\lambda^2 - \lambda_0^2)$, using $\lambda_0 = 212$ mμ.

Viscosity measurements were made using a Cannon-Ubbelohde viscometer at 25.00 ± 0.05°. Outflow times for water were approximately 102 sec. Intrinsic viscosities were obtained by extrapolating the reduced viscosity to zero protein concentration.

Calculation of β. The hydrodynamic parameter, β (Scheraga and Mandelkern, 1953), was computed from the sedimentation coefficient and intrinsic viscosity by means of

$$\beta = \frac{Ns[\eta]^{1/3}\eta_0}{M^{2/3}(1 - \bar{v}\rho)} \quad (5)$$

pH measurements were made with a Radiometer type TTT1a instrument equipped with a scale expander, type PHA 630 Ta. A Radiometer combined electrode, type GK2021C, was used and the system was standardized with Fisher Certified buffer solutions.

Preparative ultracentrifugations were performed on a Beckman Model L-2 preparative ultracentrifuge using polypropylene centrifuge tubes. Rotors type 19 and 30 were used, depending upon the volume of solution to be centrifuged and upon the desired rotational speed. Rotor type 19 was used for large volumes and for speeds below 19,000 rpm, and rotor type 30 for higher speeds.

Optical density measurements were made with a Zeiss spectrophotometer, M4QII, using 1.0-cm cells.

Phosphate buffer (0.1 M) was prepared by mixing 0.1 M disodium phosphate and 0.1 M monosodium phosphate to the desired pH.

Results

Purification. Further purification of Sigma prothrombin by isoelectric precipitation and chromatography on DEAE-Sephadex led to a pure preparation of prothrombin, as indicated by gel electrophoresis patterns. Figure 1 shows the electrophoresis patterns for the starting ma-

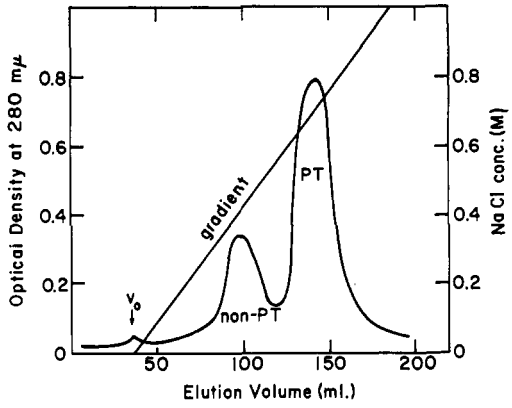

FIGURE 2: Chromatographic pattern of the isoelectric product on a 3.5 × 10 cm column of DEAE-Sephadex, A-50, using a linear NaCl gradient (0.0–1.0 M) in 0.1 M phosphate buffer (pH 6.0) at a flow rate of ~6 ml/hr. The arrow indicates the position of the void volume, v_0. Approximately 18 mg of isoelectric product was applied in sucrose, and ~12 mg of prothrombin was recovered.

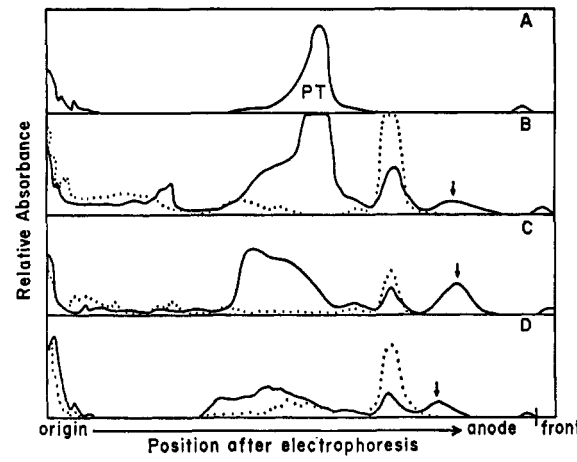

FIGURE 3: Densitometer traces of patterns obtained from disc electrophoresis of prothrombin solutions incubated for 24 hr at 28° with 25% sodium citrate (A), Bacto Ac-Globulin (B), thrombin (C), and Bacto-Prothrombin II-stage reagent plus Bacto Ac-Globulin (D) on 7.5% polyacrylamide gels. Solid lines represent mixtures of prothrombin and activator, and dotted lines represent the activator alone. Patterns for the 48-hr incubation mixtures were different in only two respects, *viz.*, those fast-moving peaks designated by an arrow disappeared and the prothrombin peaks in B and C were diminished.

terial (Figure 1A) and for the products obtained at various stages of purification. It can be seen from Figure 1A that the starting material contained ~40% prothrombin, 30–40% of another component which appeared to be bovine serum albumin, and at least five to six other components present in small amounts. Isoelectric precipitation removed the bovine serum albumin and some of the other impurities (see Figure 1B).

Chromatography of this product on DEAE-Sephadex (see Figure 2) resulted in the final purification of prothrombin. The chromatography was developed with a linear gradient of 0.0–1.0 M NaCl in phosphate buffer as described in the Experimental Section. The elution profile shown in Figure 2 was independent of the protein concentration range used (3–13 mg/ml), and the prothrombin was consistently eluted at a NaCl concentration of 0.58–0.72 M. As a result of the chromatography, prothrombin was eluted after the nonprothrombin impurities, and could therefore be separated from them. Figure 1C,D shows the electrophoresis patterns of the nonprothrombin impurities and prothrombin, respectively. From gel electrophoresis, it was observed that a material, too large to enter the separating gel, appeared in the products of both isoelectric precipitation and chromatography. However, this material could be removed by preparative ultracentrifugation at 30,000 rpm for 5 hr either before or after the chromatography step. Figure 1D, which shows the electrophoresis pattern for prothrombin which had been eluted from DEAE-Sephadex and centrifuged, indicates that the protein is pure by this criterion.

The peak in Figure 1 labeled prothrombin was found to contain prothrombin by means of the assay described in the Experimental Section. This assay was performed on (nondyed) material obtained from polyacrylamide gels in the following way. After the usual required time for electrophoresis had elapsed, the gel was cut into three to five sections, each of which was placed in an electrophoresis tube fitted with a cellophane bag secured with a sleeve of tygon tubing. The material in each section was eluted from the gel electrophoretically, collected in the cellophane bag, and assayed for prothrombin. On comparison with a duplicate gel dyed and destained in the usual way, it was found that the gel section which yielded prothrombin as detected by the assay was the one which contained the so-called prothrombin band. Furthermore, it was this band, defined by an R_F value, which became more and more dominant during the various purification steps.

From an analysis of eight preparations, we find that the over-all yield of protein was 18%, and the over-all yield of prothrombin was 45%, with an increase in specific activity of a factor of 2.5.

Activation. Preliminary activation studies of chromatographically purified prothrombin using various activators yielded the results indicated below and in Figure 3. Solid lines in Figure 3 show the densitometer traces of polyacrylamide gel electrophoresis for prothrombin and the activator after 24-hr incubation, and the dotted lines for the activator alone. Densitometer traces for prothrombin incubated in buffer and in 25% sodium citrate were identical (Figure 3A). No change was observed in the patterns obtained at 24- and 48-hr incubation times, and no clot formed in the thrombin assay described in the Experimental Section even after 15-min incubation time with fibrinogen (a blank of fibrinogen in buffer did not clot for >15 min). These results indicate that pure prothrombin is *not* activated in 25% sodium citrate under these conditions. An example of those activators [Bacto Ac-Globulin, factor X*, factor V, and factors (X* + V)] which generated ≤1–2 clotting units/mg is given in Figure 3B for prothrombin incubated with Bacto Ac-Globulin. After 24-hr incubation, several new diffuse bands were observed in addition to the dominant prothrombin band. By 48 hr, however, the band designated by the arrow disappeared and the amount of prothrombin was sig-

nificantly decreased. Densitometer traces of those activators which demonstrated significant thrombin-generating ability, *viz.*, thrombin (~10 clotting units/mg, Figure 3C), Bacto-Prothrombin II-stage reagent (≥215 clotting units/mg), and Bacto-Prothrombin II-stage reagent + Bacto Ac-Globulin (≥260 clotting units/mg, Figure 3D), all indicated that the prothrombin band was greatly diminished or absent by 24 hr. However, the fast-migrating band designated by the arrow was present at 24 hr and absent at 48 hr, in common with the activators represented by prothrombin with Bacto Ac-Globulin.

Chemical Analyses. Some properties of purified prothrombin determined from chemical analyses are summarized in Table I. It is to be noted that the values of

TABLE I: Amino Acid Analysis.[a,b]

Amino Acid	Moles/ 74,000 g of Protein	Amino Acid	Moles/ 74,000 g of Protein
Asp	67.7	Met	4.5
Thr	31.7	Ile	23.8
Ser	41.1	Leu	43.7
Glu	83.7	Tyr[c]	17.9
Pro	36.9	Phe	22.1
Gly	57.0	Lys	34.0
Ala	38.0	His	9.7
Val	38.4	Arg	41.3
Cys (1/2)	20.1	Trp[c]	17.6

[a] Based on a molecular weight of 74,000 (see Table II). The data in this table correspond to 17.4% total nitrogen. [b] The carbohydrate content (as hexose) was 2.3%, the extinction coefficient, $\epsilon^{1\%}_{1\,cm}$ at 280 mμ, was 16.5 (from Trp, Tyr, and Cys content and from Kjeldahl and amino acid analyses), and the partial specific volume, $\bar{v}$, was 0.721. [c] Trp was obtained from the Tyr/Trp ratio, which was determined spectrophotometrically in 0.1 N NaOH (Goodwin and Morton, 1946) and found to be 1.02.

$\epsilon^{1\%}_{1\,cm}$, calculated from the Trp, Tyr, and Cys content, agree with that obtained from the micro-Kjeldahl nitrogen determination and the per cent total nitrogen determined from the amino acid analysis.

Sedimentation Equilibrium. Plots of ln J *vs.* x^2 for prothrombin in buffer and in denaturing solvents for both high (1.0–2.0 mg/ml) and low (0.4 mg/ml) concentrations showed linearity throughout the length of the cell. Quantities used to determine the molecular weights of native and denatured prothrombin are summarized in Table II. Plots of $10^5/\bar{M}_w$ *vs.* optical density at 280 mμ, showing the dependence of apparent molecular weight upon concentration, are illustrated in Figure 4. Figure 4A gives the plot for prothrombin in buffer; Figure 4B,C for prothrombin in 6 M guanidine hydrochloride and in 6 M guanidine hydrochloride plus 0.1 M β-mercaptoethanol, respectively. Filled symbols represent values obtained for the calculated $\bar{v}$, open circles for a value of $\bar{v}$ decreased by 0.01 cc/g as discussed by Castellino and Barker (1968). All lines were determined by a least-squares fit.

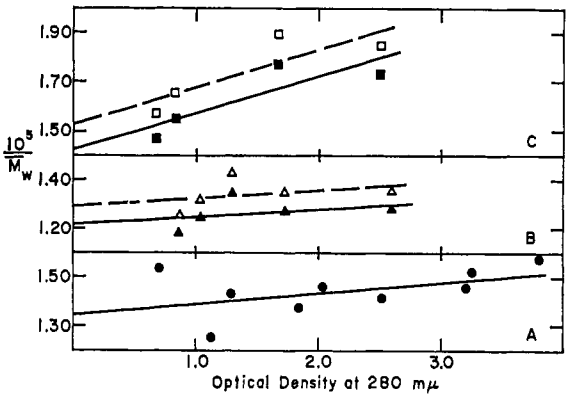

FIGURE 4: Plots of $10^5/\bar{M}_w$ *vs.* optical density at 280 mμ. (A) Prothrombin in 0.1 M phosphate buffer (pH 6.0) plus ~0.65 M NaCl (●). (B) prothrombin in 6 M guanidine hydrochloride. ▲ represents values calculated using $\bar{v}$ = 0.721, △, for $\bar{v}$ = 0.711. (C) prothrombin in 6 M guanidine hydrochloride plus 0.1 M β-mercaptoethanol. ■ represents values calculated for $\bar{v}$ = 0.721; □, for $\bar{v}$ = 0.711.

Sedimentation velocity experiments are summarized in Table III and Figure 5. In all cases, *i.e.*, for both buffer and denaturing solvents, a single symmetrical peak was observed. The value of s for prothrombin in 0.1 M phosphate buffer (pH 6.0) plus ~0.65 M NaCl at 23° extrapolated to zero concentration (Figure 5) was found to be 4.80 S. Using 0.1 M phosphate buffer (pH 6.0) plus ~0.65 M NaCl at 23° as the standard conditions, sedimentation coefficients for prothrombin in the denaturing solvents were corrected for differences in the viscosities and densities of the solvents and for $\bar{v}$ calculated and decreased by 0.01 cc/g according to eq 3. At a concentration of ~3.2 mg/ml, s values for native and denatured prothrombin are compared in Table III.

Intrinsic Viscosity and β. Intrinsic viscosities, $[\eta]$, of prothrombin in buffer and in the denaturing solvents are also given in Table III. Outflow times were reproduced to within 0.3%. The quantity, β, computed from eq 5, has the value 2.07×10^6 for prothrombin in buffer, and 1.58×10^6 and 2.34×10^6 for prothrombin in guanidine hydrochloride and in guanidine hydrochloride plus β-mercaptoethanol, respectively. In the denaturing solvents, the extrapolation of s to zero concentration was not carried out.

Optical Rotation. Figure 6 shows the optical rotatory dispersion data in the ultraviolet for prothrombin in 0.1 M phosphate buffer (pH 6.0) plus ~0.65 M NaCl at 25°. There is a trough near 232 mμ, a zero value of $[m']_\lambda$ near 217 mμ, a shoulder at 213 mμ, and a positive peak near 198 mμ. Curves for the data obtained for prothrombin at 75 and 6°, and for the solutions restored to 25°, were essentially the same as the one shown. However, gel electrophoresis of a sample which had been heated showed a dominant band at the inter-

TABLE II: The Molecular Weights of Native and Denatured Prothrombin as Determined by Sedimentation Equilibrium.

Buffer System	0.1 M Phosphate Buffer (pH 6.0) Plus ~0.65 M NaCl	6 M Guanidine Hydrochloride	6 M Guanidine Hydrochloride plus 0.1 M β-Mercaptoethanol
Solvent density, g/cc	1.020	1.140	1.141
$\bar{v}$, cc/g	0.721	0.721–0.711[a]	0.721–0.711[a]
Molecular weight, $\bar{M}_w$[b]	74,000 ± 4,100[c]	82,000 ± 3,200–77,300 ± 3,100[c,d]	70,000 ± 3,000–65,400 ± 2,700[c,d]
$\bar{M}_w$ denatured/$\bar{M}_w$ native		1.10–1.04	0.95–0.88

[a] As discussed in text. [b] Calculated from eq 1, and extrapolated to zero concentration. [c] The standard deviation was computed by the usual least-squares procedure. [d] The range in $\bar{M}_w$ corresponds to the range in $\bar{v}$.

TABLE III: Sedimentation Coefficients and Intrinsic Viscosities of Native and Denatured Prothrombin.

Buffer System	0.1 M Phosphate Buffer (pH 6.0) Plus ~0.65 M NaCl	6 M Guanidine Hydrochloride	6 M Guanidine Hydrochloride Plus 0.1 M β-Mercaptoethanol
Concentration, mg/ml	3.2[a]	3.2	3.1
Slope of ln x *vs.* t, sec^{-1}	1.59×10^{-5}	0.479×10^{-5}	0.361×10^{-5}
Viscosity of solvent/viscosity, standard[b]	1.0	1.34	1.37
$(1 - \bar{v}\rho)_{std}/(1 - \bar{v}\rho)$	1.0	1.49–1.40[c]	1.50–1.40[c]
s, exptl,[d] S	4.05	1.22	0.921
s, cor,[e] S	4.05	2.44–2.30[c]	1.88–1.77[c]
$[\eta]$, cc/g	3.4	12–14	≤74

[a] This is the highest concentration shown in Figure 5. [b] Standard conditions are taken to be values for 0.1 M phosphate buffer (pH 6.0) plus ~0.65 M NaCl at 23°. [c] The range corresponds to the range in $\bar{v}$. [d] Determined at ω = 59,780 rpm and 23°. [e] From eq 2.

face of the stacking and separating gels and a reduced prothrombin band, indicating the presence of aggregates. A sedimentation velocity experiment with this material yielded a value of ~21 S for the "aggregate."

Optical rotatory dispersion data for prothrombin in buffer in the wavelength range 275–500 mμ were plotted according to eq 4, and yielded values of −35 for b_0 and −197 for a_0. Similar plots for prothrombin heated or cooled gave essentially the same b_0; a_0 varied with changes in temperature and concentration.

Discussion

Purification. In this paper, a rapid reproducible purification method, starting with a commercial preparation of bovine prothrombin, has been described. Electrophoretically pure prothrombin, obtained as a result of isoelectric precipitation, DEAE-Sephadex chromatography, and ultracentrifugation of Sigma prothrombin, has been prepared in a yield of ~18% protein (or ~45% of the prothrombin available in the starting material) and ~47% clotting units. All steps in the purification were monitored using both gel electrophoresis and assays for prothrombin. Using these monitoring tools, it was found that, to obtain a final product of maximum purity, without traces of *any* contaminating bands upon gel electrophoresis, only the purest prothrombin solutions prepared from the isoelectric precipitation could be used. For this reason, only the precipitate which formed as the pH was varied from <5.4 to ≥4.6 was used for chromatography. Using these monitoring tools, it was also found that loss of prothrombin occurred in three stages, *viz.*, in the pH 5.4 nonprothrombin precipitate (specific activity ratio 0.2–0.4), in the final isoelectric supernatant (specific activity ratio 0.0–0.5), and during ultrafiltration in the Diaflow apparatus.

The quality of prothrombin prepared in this way, as evaluated by disc gel electrophoresis, appears to be superior to other *bovine* prothrombin preparations reported in the literature. Of these reports, the preparation of Malhotra and Carter (1968) seems to be the purest, with ≤3% contamination as detected by gel electrophoresis. Tishkoff *et al.* (1968), using DEAE-

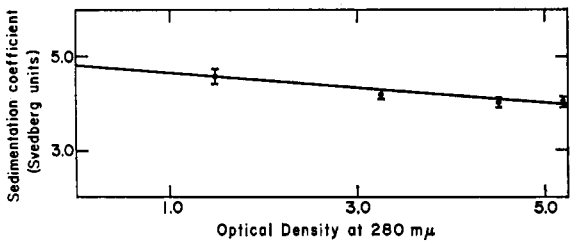

FIGURE 5: Plot of observed sedimentation coefficient, *s*, *vs.* optical density at 280 mμ for prothrombin in 0.1 M phosphate buffer plus ~0.65 M NaCl.

Sephadex, and Magnusson (1965b), using TEAE- and DEAE-cellulose columns, report a fractionation of prothrombin into two types. The one eluted first required additional clotting factors for activation, while the second and usually dominant type could be activated in the usual prothrombin II-stage assay. Gel electrophoresis of both of these types of prothrombin obtained by Tishkoff *et al.* (1968) showed that the first type had several bands and was similar to his starting material; the second had two major and two minor bands. We did not observe such a chromatographic pattern, regardless of the concentration of protein applied to the column, nor could we detect any prothrombin in the fast eluting peak either by gel electrophoresis or by assay for prothrombin. The difference in behavior may be due to differences in composition of the starting material. Lanchantin *et al.* (1968) did not observe two prothrombin peaks from DEAE-cellulose chromatography of *human* prothrombin unless the column was improperly equilibrated.

Activation. The activation studies, although preliminary in nature and performed on only ~96% pure prothrombin, confirm the findings of Malhotra and Carter (1968) and Tishkoff *et al.* (1968) that purified prothrombin does not activate, or at least activates very slowly, in 25% sodium citrate, nor in buffer, *i.e.*, autocatalytically. This is in contrast to the work of Seegers (1962) and Lanchantin *et al.* (1968). Incubating prothrombin with factor V (Sigma, or as supplied by Bacto Ac-Globulin), factor X* (Sigma), or factors X* plus V (Sigma) was not sufficient to generate significant levels of thrombin in 48 hr, even though the amount of prothrombin as detected by gel electrophoresis was greatly diminished by that time. On the other hand, the components of Bacto-Prothrombin II-stage reagent (a source of phospholipid and Ca^{2+}, as well as unidentified components) and to a much lesser extent, thrombin, did generate thrombin from prothrombin. In the cases where Bacto-Prothrombin II-stage reagent was used, prothrombin was completely consumed by 24-hr incubation time, again as detected by gel electrophoresis. The role of phospholipid appears to be very important for rapid conversion of prothrombin into thrombin. An extended activation study of pure prothrombin must be carried out before any definite conclusions as to the requirements for activation and identity of the apparent transition components, detected by electrophoresis, can be made.

Chemical Composition. A comparison of the amino acid analysis of bovine prothrombin, reported in this paper, with literature values, as summarized by Lanchantin *et al.* (1968), supports the conclusion of Lanchantin *et al.* that there are few if any differences between human and bovine prothrombins. By recalculating the amino acid analyses reported in Table I on a g of amino acid residue per 100 g of protein basis (not shown here) for detailed comparison with the summary given by Lanchantin *et al.* we find that the values in Table I represent higher values for Asp, Glu, Gly, Ile, and Trp and a lower value for Met than other bovine analyses. The value reported for Trp, the amino acid cited by Lanchantin *et al.* to be found in significantly higher levels in human prothrombin than in bovine prothrombin, falls intermediate. The per cent carbohydrate determined as hexose, reported in Table I, is about one-half the value reported elsewhere (Lanchantin *et al.*, 1968; Tishkoff *et al.*, 1968; Magnusson, 1965a,b). Analyses for hexosamine and *N*-acetylneuraminic acid were not performed.

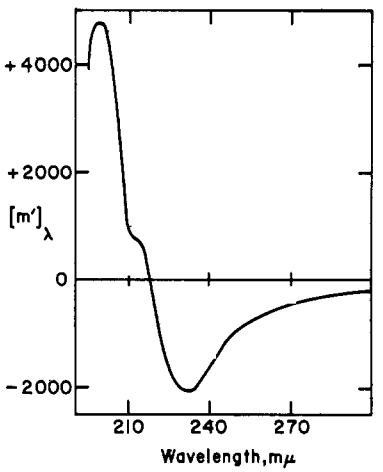

FIGURE 6: Plot of reduced mean residue rotation, $[m']_\lambda$, *vs.* wavelength in millimicrons at 25°.

Physical Properties. Physicochemical studies were undertaken to determine the size and shape of the prothrombin molecule. In buffer, the sedimentation equilibrium molecular weight of prothrombin, found by extrapolation to zero concentration, was 74,000 ± 4,100. The value for $\bar{v}$ used here was calculated from the amino acid analysis, as described in the Experimental Section, and was found to be 0.721. This value is higher than the value $\bar{v}$ = 0.70 (Lamy and Waugh, 1954) used by other workers. The value $\bar{v}$ = 0.721 seems to lie in the range observed for other proteins and was, therefore, used. For comparison, the data obtained here, recalculated using $\bar{v}$ = 0.70, gives a molecular weight of 68,700, which is the value usually quoted in the literature (Lamy and Waugh, 1954; Seegers, 1962).

The value for the sedimentation coefficient in 0.1 M phosphate buffer (pH 6.0) plus ~0.65 M NaCl, extrapolated to zero concentration, was 4.80 S, in good agreement with those of Tishkoff *et al.* (1968) and Lamy and Waugh (1954). The quantity β, calculated from eq 5, has the value 2.07×10^6. This would imply that the equivalent hydrodynamic ellipsoid is probably spher-

ical and compact. In addition, from the optical rotatory dispersion data, native prothrombin seems to have a low helix content.

The effect of denaturing solvents, 6 M guanidine hydrochloride and 6 M guanidine hydrochloride plus 0.1 M β-mercaptoethanol, on the sedimentation equilibrium molecular weight, sedimentation coefficient, and intrinsic viscosity was studied to determine the possibility of the existence of subunits in prothrombin. The sedimentation equilibrium molecular weights for prothrombin in the denaturing solvents, extrapolated to zero concentration, are essentially the same as that of prothrombin in buffer (see Table II). Values for the sedimentation coefficients, s, corrected to standard conditions (see Table III), demonstrate the increasing random coil character of prothrombin as it is partially unfolded in 6 M guanidine hydrochloride (s = 4.05 to 2.44) to being more fully extended after the disulfide bonds are ruptured in 6 M guanidine hydrochloride plus 0.1 M β-mercaptoethanol (s = 2.44 to 1.88). Despite the experimental uncertainties in the values used to compute β, the β values are consistent with this conclusion.

Gel electrophoresis of prothrombin in the denaturing solvents was performed. The precaution of diluting the sample with electrophoresis buffer prior to application to the gels was taken in order to reduce any inhibition of migration due to salt effects. For prothrombin in 6 M guanidine hydrochloride, gel electrophoresis showed one band at the expected R_F value for prothrombin. For prothrombin in 6 M guanidine hydrochloride plus 0.1 M β-mercaptoethanol, there was one band at the interface of the stacking and separating gels and no observable bands in the separating gel. We conclude from this that the prothrombin molecule is sufficiently extended when the disulfide bonds are ruptured that it cannot enter a 7.5% polyacrylamide gel of pore size ~50 Å, and that neither aggregation nor dissociation of the prothrombin molecule occurs in denaturing solvents.

As further evidence of the one-chain character of prothrombin, the intrinsic viscosity, $[\eta]$, and the sedimentation coefficient, s, of prothrombin in 6 M guanidine hydrochloride plus 0.1 M β-mercaptoethanol have values compatible with those discussed by Tanford *et al.* (1967) for proteins known to be single chains in this solvent system. Using a value of 647 for the number of amino acid residues/chain, obtained from the amino acid analysis, and eq 2 of Tanford *et al.* (using the upper limit of the exponent, *viz.*, 0.70), we compute a value of 1.823 for log $[\eta]$ of prothrombin in 6 M guanidine hydrochloride plus 0.1 M β-mercaptoethanol; this compares favorably with the measured value of 1.869. Similarly, we calculated a value of log $s^0/(1 - \bar{v}\rho) = 0.785$ from their eq 5; this compares reasonably well with the experimental value of 0.716. If the extrapolation to zero concentration had been carried out, a larger value for log $s^0/(1 - \bar{v}\rho)$ would have been calculated, and therefore better agreement would have been obtained.

It is clear that the denaturing solvents 6 M guanidine hydrochloride and 6 M guanidine hydrochloride plus 0.1 M β-mercaptoethanol do not dissociate the prothrombin molecule. We, therefore, conclude that prothrombin is a globular compact molecule of one chain, possessing little helix content.

Added in Proof

A preliminary experiment, carried out by Karen Platzer, indicates that the N-terminal amino acid of prothrombin is threonine.

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Studies on the Mechanism of Rat Liver Nicotinamide Mononucleotide Pyrophosphorylase*

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ABSTRACT: Preparations of rat liver have been shown to possess a nicotinamide mononucleotide: pyrophosphate phosphoribosyl transferase (EC 2.4.2.12) which specifically requiresadenosine triphosphate, as well as 5-phosphoribosyl 1-pyrophosphate. These same preparations will synthesize nicotinamide mononucleotide in the absence of adenosine triphosphate if the concentration of both 5-phosphoribosyl 1-pyrophosphate and magnesium is greatly increased. Similar heat labilities, identical gel electrophoretic patterns, and molecular weights indicate that nicotinamide mononucleotide synthesis stimulated by adenosine triphosphate or high levels of 5-phosphoribosyl 1-pyrophosphate and magnesium is probably catalyzed by the same enzyme. These data, together with an analysis of the kinetics of cotinamide mononucleotide formation in the presence and in the absence of adenosine triphosphate, lead to the suggestion that adenosine triphosphate is a positive allosteric effector of nicotinamide mononucleotide pyrophosphorylase. Analysis of the product inhibition data for nicotinamide mononucleotide synthesis in the presence of adenosine triphosphate and initial velocity patterns of the reaction in the absence of adenosine triphosphate indicate that the mechanism for the enzymatic formation of nicotinamide mononucleotide is either an ordered sequence beginning with the binding of 5-phosphoribosyl 1-pyrophosphate and culminating in the release of nicotinamide mononucleotide, or an Iso–Theorell–Chance mechanism, in which case the order of substrate binding cannot be predicted from the data.

Rat liver NMN pyrophosphorylase requires ATP as well as nicotinamide and PRPP (Dietrich *et al.*, 1966), and is markedly inhibited by physiological levels of NAD (Dietrich and Muniz, 1966). The enzyme fraction which synthesizes NMN in the presence of ATP will also form NMN in the absence of ATP if the levels of both PRPP and magnesium are greatly increased (Powanda *et al.*, 1968). In addition, the activity of this enzyme is decreased some 40% by adrenalectomy or hypophysectomy (Dietrich *et al.*, 1967). These data suggested that nicotinamide mononucleotide pyrophosphorylase is a likely point of control in pyridine nucleotide metabolism and that ATP may be a positive agent of control. The following presents a probable mechanism for enzymatic NMN formation together with the evidence that ATP does act as a positive allosteric effector.

Methods and Materials

Enzymatic Material. The enzyme was purified as previously reported (Dietrich *et al.*, 1966). This preparation (fraction A), or a more purified fraction (fraction B), was used throughout as indicated. The latter preparation, which has a specific activity of *ca.* 200,000 cpm/mg of protein and is free of inorganic pyrophosphatase, was prepared by heating fraction A at 55° for 5 min and centrifuging at 20,000*g* for 15 min. The resultant supernatant material was used and represents a 300-fold purification over the homogenate. Neither fraction A nor B contains any detectable NAD glycohydrolase, nicotinate phosphoribosyl transferase, NAD pyrophosphorylase, nicotinamide deamidase, or NAD kinase. Enzymatic destruction of NMN and PRPP cannot be observed. ATP degradation measured by luciferin–luciferinase assay (Strehler and McElroy, 1957) is, under the assay conditions, less than 4%.

Enzymatic Assay. This is the same as previously de-

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