Purification and Some Characteristics of an α,β-Unsaturated Ketone Reductase from Dog Erythrocytes and Human Liver

I. M. Fraser, M. A. Peters, and M. G. Hardinge

Department of Pharmacology, School of Medicine, Loma Linda University, Loma Linda, California 92354

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SUMMARY

An enzyme which catalyzes the reduction of α,β-unsaturated ketones by NADPH has been purified from dog erythrocytes and human liver. The enzyme was relatively stable at low temperatures but rapidly inactivated above 50°. The pH optimum was broad with a range of 5.5–7.5. Among the possible substrates tested most, but not all, unsaturated ketones were attacked by the enzyme; none of the saturated aldehydes and ketones were attacked, however, nor were any of the unsaturated compounds which lacked a keto group. Michaelis constants for active substrates were in the vicinity of 10^{-4} M. Approximately one molecule of NADPH was oxidized for each molecule of unsaturated ketone reduced. The activity of the enzyme was decreased by the sulfhydryl inhibitors *p*-chloromercuribenzoate and *N*-ethylmaleimide. The enzyme has been differentiated from a variety of other enzymes including methemoglobin reductase, glutathione reductase, aldehyde-ketone reductase, aromatic α-keto acid reductase, alcohol dehydrogenase, lactic dehydrogenase, NADPH-cytochrome *c* reductase and fatty acid synthetase, but not from an enzyme previously described as crotonyl CoA reductase.

INTRODUCTION

Williams (1) has reviewed the metabolism of various ketones in animals and indicates that reduction of the keto group is the major process. Reduction of the double bond may also occur with α,β-unsaturated ketones. When Fischer and Bielig (2) administered benzylidene acetone (BA) and some related unsaturated ketones orally to rabbits and examined the urine, they obtained metabolites in which both the double bond and keto group or the keto group alone were reduced. Fischer and Wiedemann (3) found that BA, furfurylidene acetone (FA), and related unsaturated ketones were metabolized by fermenting yeast with reduction of either the keto group or the double bond or both.

Several enzymes are known which attack various ketones. Culp and McMahon (4) reported an enzyme from rabbit kidney which catalyzed the reduction of a variety of aldehydes and ketones by reduced nicotinamide adenine dinucleotide phosphate (NADPH). Recently Zannoni and Weber (5) reported the presence of an aromatic keto reductase in a variety of tissues. Other widely distributed enzymes which reduce ketones or aldehydes are lactic dehydrogenase and alcohol dehydrogenase.

Reduction of double bonds is also catalyzed by several enzymes. The azoreductase of liver microsomes is involved in such a reaction and is probably identical with NADPH-cytochrome *c* reductase (6). Several steps in fatty acid synthesis involve the reduction of α,β-unsaturated

ketones by NADPH (7). Langdon (8) and Seubert *et al.* (9) have described an enzyme which catalyzes the reduction of crotonyl CoA and related compounds by NADPH.

In connection with studies (10, 11) of the pharmacology of FA related to its anticancer activity (12), it was found that FA added to heparinized dog blood rapidly disappeared and could not be recovered (13). Further work, previously described in preliminary reports (14–16), showed that an enzyme present in erythrocytes was responsible for the disappearance of FA and BA in the blood and that this enzyme also occurred in the liver. In this paper studies on the purification and characterization of the enzyme are reported.

MATERIALS AND METHODS

Chemicals

Furfurylidene acetone, Eastman commercial grade, was recrystallized from aqueous alcohol to obtain white to pale yellow crystals (melting point 37.5–39°). Benzylidene acetone, Eastman chemical grade, was used as supplied or after recrystallization from aqueous alcohol to provide yellow crystals (melting point 38.5–39.5°). 2-Butanone, phenyl-1-propenal (cinnamaldehyde), 3-phenyl-2-propen-1-phenyl-1-one (chalcone), 5-phenyl-2,4-pentadienephenone, 3-(2-furyl)-2-propen-1-phenyl-1-one (β-2-furylacrylophenone), 1,2-diphenylethane-1,2-dione (benzil), and 1-propene-3-urea (allylurea), were all obtained from Eastman Organic Chemicals. The compounds 3-buten-2-ol, 3-buten-2-one, 2,4-pentanedione, phenyl ethyne, phenyl ethan-1-one (acetophenone), 3-phenyl-2-propanone, and 4-chlorobenzaldehyde were obtained from J. T. Baker Chemical Company. The chemicals furyl-1-propenoic acid (furanacrylic acid), 1-ethyl-2-furanacrolein (α-ethyl β-furyl acrolein), propyl furyl acrylate (*n*-propyl furyl-1-acrylate) were a gift from F. Ritter and Company, Los Angeles, California. The 4-phenyl-2-butanone and 2-cyclo-hexenone-1 were obtained from K & K Laboratories of California Inc. Calbiochem supplied 3,5-diiodo-*p*-hydroxyphenylpyruvic acid. Ethacrynic acid ((2,3-dichloro-4-(2-methylene butyryl)phenoxy)acetic acid) was provided through the courtesy of Merck, Sharp and Dohme. Griseofulvin was donated through the courtesy of Schering Corporation. Sephadex G-100, G-150, and G-200, DEAE-Sephadex A-50 (non-bead form) and CM-Sephadex A-50 (non-bead form) were obtained from Pharmacia Fine Chemicals Inc. Nicotinamide adenine dinucleotide phosphate ($NADP^+$), NADPH, nicotinamide adenine dinucleotide (NAD^+), NADH, glucose 6-phosphate (G-6-P), glucose 6-phosphate dehydrogenase (G-6-PD) (type V, 130 units per milligram of protein), coenzyme A, acetyl CoA, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), lactic dehydrogenase (type II, from rabbit muscle), *p*-chloromercuribenzoic acid, and *N*-ethyl maleimide were supplied by Sigma Chemical Company. Alcohol dehydrogenase, from yeast (2 × cryst.) and from horse liver (1 × cryst.) was purchased from Worthington Biochemical Corporation. Crotonic anhydride was obtained from K & K Laboratories and used to prepare crotonyl CoA by the method of Stern (17). Mann Laboratories special enzyme grade ammonium sulfate was employed for protein fractionation. All other chemicals were reagent grade.

Enzyme Assays

Flask assay. This assay utilized an absorbance peak at 287 mμ shown by solutions of BA in alcohol and water. The absorbance at this wavelength progressively decreased when BA was incubated with the enzyme preparations. A similar relationship was found for FA with an absorbance peak at 317 mμ. The basic reaction mixture contained 17 μmoles of BA, 0.25 μmoles of $NADP^+$, 30 μmoles of G-6-P, and 30 μmoles of nicotinamide dissolved in Krebs-Ringer phosphate buffer (pH 7.4) (18), together with the enzyme preparation (usually 3 ml), in a total volume of 5 ml. The $NADP^+$ and G-6-P, together with G-6-PD present in the crude enzyme preparations, provided a system

for generation of NADPH. When preliminary tests indicated loss of G-6-PD after certain enzyme purification steps, 2 units of G-6-PD were added to the reaction mixture. Nicotinamide was included to protect the $NADP^+$ against destruction by NADPase possibly present in the tissue preparations (19). The concentrations of $NADP^+$, G-6-P, and nicotinamide were chosen on the basis of the work of Huennekens *et al.* (19) with human erythrocyte hemolysates. These concentrations of $NADP^+$, G-6-P, nicotinamide, and BA were found to be adequate to provide a linear relationship between amount of enzyme and disappearances of BA over a considerable range of the former except with liver homogenates, which required 5 μmoles of $NADP^+$ for maximum activity. The solubility of BA in water imposed an upper limit on its concentration in the reaction mixture, which was only slightly above that employed in this assay. The enzyme assays were conducted in 25-ml Erlenmeyer flasks shaken in a water bath at 37°. Immediately after addition of BA to the otherwise complete system and again after incubation for 30 min, 0.2-ml aliquots were withdrawn in triplicate and added to 9.8 ml of 95% ethanol in glass-stoppered centrifuge tubes. This procedure precipitated the tissue protein, stopped the reaction, and extracted the BA into the ethanol. In some experiments samples were also taken after 60 and 120 min incubation. In order to provide a blank for the subsequent absorbance measurements, a 0.2-ml aliquot was also taken from a reaction flask which was identical with the above except for the omission of BA. Centrifugation at 500 *g* for 10 min removed the precipitated protein. Measurements of the absorbance of the supernatant ethanol solution were made at 287 mμ with either a Beckman DU spectrophotometer or a Perkin-Elmer 202 recording spectrophotometer. A milliunit of enzyme assayed in this system was defined as the amount of enzyme which catalyzed the disappearance of 1 mμmole of BA per minute as measured by the decrease in absorbance at 287 mμ. The usefulness of this assay was limited by the minimum time interval (30 min) required for sufficient BA to be metabolized to provide reproducible measurements with preparations of low activity.

Cuvette assay. The decrease of absorbance of BA at 287 mμ could also be observed in a cuvette containing the enzyme preparations and NADPH. However, the decrease in the absorbance of NADPH at 340 mμ which occurred as it was oxidized to $NADP^+$ was more suitable for use in the cuvette assay since the NADPH had a lower molar absorbance than BA and could thus be employed at higher concentrations without exceeding the upper limit of accurate absorbance measurement on the spectrophotometer. A reaction system was developed which contained 0.68 μmole of BA, 0.68 μmole of NADPH, 100 μmoles of pH 7.4 potassium phosphate buffer, and appropriate volumes of enzyme solution and distilled water to make a total volume of 3.0 ml. The components were added to a 1-cm cuvette, NADPH being added last to start the reaction. The decrease of absorbance at 340 mμ was followed for up to 5 min at 30° using a Perkin-Elmer 202 spectrophotometer with an automatic enzyme assay attachment. The initial rates were obtained from the graphical record and used in the calculations. A linear relation between amount of enzyme and rate of NADPH oxidation was observed over a considerable range, and all assays were conducted in this range. A similar reaction mixture without BA was used to correct for any oxidation or destruction of NADPH due to other reactions. A milliunit of enzyme was defined as the amount which catalyzed the oxidation of 1 mμmole of NADPH per minute at 30°.

The flask assay was employed with human liver homogenate and its initial fractions (fractions 1–3, Table 3A), since oxidation of NADPH due to reactions other than reduction of BA was so rapid that it was very difficult to obtain satisfactory enzyme assays with the cuvette assay. As early as possible in the human liver homogenate fractionation (fraction 4, Table 3A and 3B) and throughout the dog erythrocyte sonicate fractionation (Table

2) the cuvette assay was employed because it used much less enzyme and was rapid and highly reproducible. Thus it was particularly suitable for assays on the fractions from the column chromatography employed extensively in the purification procedure.

Tissue Sources, Tissue Preparation, and Enzyme Purification

Blood was obtained from 10–30 kg healthy mongrel dogs of both sexes under ether anesthesia by venipuncture or heart puncture, depending on the volume required. Heparin was used as the anticoagulant. Human blood was obtained from laboratory volunteers by sterile venipuncture or in the form of pooled excess samples from the Clinical Laboratory. Livers or kidneys were obtained from 200–400 g Sprague Dawley rats, 200–300 g pigeons, and 2–3 kg New Zealand rabbits under ether anesthesia. Samples of healthy human liver were obtained from two autopsies within 6 hours of death with the cooperation of the Department of Pathology and kept frozen at —20° until used.

All steps in the preparation of the tissues and the purification of the enzyme were conducted at 2–5°. Erythrocytes were washed three times and suspended in Krebs-Ringer phosphate buffer pH 7.4 (18) with 0.4% dextrose added. Sonicates of erythrocytes were prepared with a Bronwill Biosonik disintegrator operating at about 72 watts average output for 5–10 sec with batches of up to 50 ml of resuspended cells. Twenty per cent homogenates of small quantities (5–20 g) of rat, pigeon, or human liver and rabbit kidney were prepared in 0.25 M sucrose with a motor driven Teflon-glass homogenizer. For larger-scale preparations of human liver homogenate it was necessary to use a Servall Omni-Mixer although this resulted in less active homogenates. Subcellular fractions of liver and kidney homogenates were prepared and suspended in 0.25 M sucrose according to the methods described by Umbreit *et al.* (18) with the aid of a Spinco Model L ultracentrifuge.

The sonicated cell preparations were used as the starting point (fraction 1, Table 2) in the purification of the enzyme from dog erythrocytes. Hemoglobin was removed by the method of Huennekens *et al.* (19) to provide a dialyzed hemoglobin-free supernatant (Fraction 2, Table 2). Ammonium sulfate was added to 50% saturation (20), and the pH was readjusted to 7.4. The precipitate which formed after standing for 15 min was removed by centrifugation at 16,000 *g* for 10 min and discarded. Additional ammonium sulfate was added to 80% saturation, and the pH was adjusted to 7.4. An active precipitate (fraction 3, Table 2) was obtained after the sample had stood for 15 min and was separated by a similar centrifugation. It was dissolved in minimum volume of deionized distilled water and frozen until further use in chromatograhic purification procedures. In early runs dialysis was carried out at this point, but it was omitted later without undesirable effects and with some increase in yield. DEAE-Sephadex A-50 was prepared for chromatography of the enzyme by swelling in and equilibration with 0.03 M Tris HCl buffer, pH 7.9, containing 100 mg/l dithiothreitol (DTT) and 10^{-5} M disodium ethylenediaminetetraacetic acid (EDTA). Although a buffer of pH 7.4 was employed for elution, initial equilibration with the more alkaline buffer provided better separation of the enzyme from other proteins. Addition of DTT and EDTA, particularly the former, was found in preliminary runs to reduce some losses of enzyme activity during the column chromatography, presumably by protection of sulfhydryl groups (21). Sephadex columns, 2.5 cm diameter, were packed to a height of 40 cm, and 5-ml aliquots of the 50–80% $(NH_4)_2SO_4$ fraction were applied to them. The protein was eluted with a linear concentration gradient of pH 7.4 Tris HCl buffer from 0.03 M to 0.15 M (containing 100 mg/l DTT and 10^{-5} M EDTA). The effluent was monitored for protein absorbance at 280 mμ, and 5-ml fractions were collected with a Gilson fraction collector and ultraviolet absorption meter. The cuvette assay, with 0.5 ml of eluate, was used to test the fractions for

enzyme activity. The active fractions were pooled and concentrated (fraction 4, Table 2) in dialysis sacs covered with dry Sephadex G-200 (22). Sephadex G-150 was equilibrated with 0.03 M pH 7.4 Tris HCl buffer containing 100 mg/l DTT and 10^{-5} M EDTA and packed in a Sephadex column, 1.5 cm diameter, to a height of 85 cm. Aliquots (2-ml) of the concentrated fractions were applied and eluted with the same buffer. The effluent was monitored, and fractions were collected and assayed as previously (fraction 5, Table 2).

More extensive purification procedures were necessary with human liver. Weighed amounts of frozen human liver were homogenized in the cold with 4 volumes of 0.25 M cold sucrose in a Servall Omni-Mixer at 80% top speed for 1 min, filtered through cheesecloth, and made up with 0.25 M sucrose to a total volume 5 times that of the liver. The homogenate (fraction 1, Table 3) was centrifuged in the cold at 105,000 *g* for 60 min; the supernatant (fraction 2, Table 3) was withdrawn, and its volume was restored to that of the homogenate with 0.25 M sucrose. The pH of the supernatant was adjusted to 5.0, and the resulting solution was allowed to stand at 2° for 0.5 hr. The precipitate obtained by centrifuging at 4000 *g* for 10 min was discarded, and the pH of the supernatant was adjusted back to 7.4. The pH 5 supernatant (fraction 3, Table 3) was brought to 50% saturation with ammonium sulfate (20), and the pH was readjusted to 7.4. After standing in the cold for 1 hr with occasional stirring, the solution was centrifuged at 16,000 *g* for 10 min. The supernatant containing the enzyme was brought to 85% saturation with ammonium sulfate. The pH was readjusted to 7.4, and the solution was centrifuged at 16,000 *g* for 10 min after standing for 1 hour. The supernatant was discarded, and the precipitate (fraction 4, Table 3) containing the enzyme was resuspended in an equal volume of 0.01 M potassium phosphate buffer, pH 7.4 and frozen until further use for chromatographic separations. To detect the enzyme up to this point it was necessary to use the flask assay, but this 50–85% $(NH_4)_2SO_4$-precipitated fraction (fraction 4, Table 3) and subsequent chromatographic fractions could be assayed by the standard cuvette method. Columns of Sephadex G-200 (40 × 2.5 cm) were packed in equilibrium with 0.03 M Tris HCl buffer, pH 7.4, containing 100 mg/l of DTT. The 50–85% ammonium sulfate-precipitated fraction (fraction 4, Table 3) was added to the column in amounts of 5.0–7.5 ml and eluted with the same Tris HCl buffer. Fractions of 5 ml each were collected, and the active fractions were pooled. The pooled active fractions (fraction 5, Table 3) were brought to 85% saturation with ammonium sulfate, the pH was readjusted to 7.4, and after standing and centrifugation, the precipitate containing the enzyme was resuspended in 0.1 M potassium phosphate buffer, pH 7.4. The resulting solution was frozen until further use. Columns of DEAE-Sephadex A-50 (40 × 2.5 cm) were prepared, and chromatography of the active fraction from Sephadex G-200 was carried out as in the purification of the dog erythrocyte enzyme. The active fractions (fraction 6, Table 3) were pooled and reprecipitated with ammonium sulfate as described above in the Sephadex G-200 step. The precipitate was resuspended in 0.01 M potassium phosphate buffer, pH 7.4, and the resulting solution was frozen until further use. Columns of CM-Sephadex A-50 (40 × 2.5 cm) were packed in equilibrium with 0.05 M potassium phosphate buffer, pH 6.5, containing 100 mg/l of DTT. Up to 5 ml of the enzyme fraction concentrated from DEAE-Sephadex chromatography (fraction 6, Table 3) was placed on the column and elution carried out with an increasing linear concentration gradient of potassium phosphate buffer, pH 6.5, from 0.05 M to 0.20 M (containing 100 mg DTT/l); 5-ml fractions were collected, and the activity was pooled and frozen (fraction 7, Table 3). The purified enzyme was used in this form (fraction 7, Table 3) for further characterization or reprecipitated with ammonium sulfate as above when greater concentration was desired.

The protein content of the various frac-

tions was determined by the method of Lowry *et al.* (23).

Enzyme Characterization

Studies on the effect of temperature, pH, and inhibitors on enzyme activity employed the standard cuvette assay system. In the investigations of substrate specificity and the determination of Michaelis constants (K_m) for various substrates, the cuvette assay was also used. The values for K_m with the dog erythrocyte enzyme were derived from duplicate determinations of reaction rate over an appropriate range of substrate concentrations. Only a single determination of rate at each substrate concentration was made with the human enzyme because of the restricted supply of this. To improve the precision of the values of K_m obtained by plotting the reciprocals of the rates against the reciprocals of the substrate concentrations in the standard manner (24), the equations developed by Wilkinson (25) were employed. A program for the IBM-1620 computer for solution of the equations was developed, similar to that reported by Cleland (26).

Methemoglobin reductase was assayed by the method of Huennekens *et al.* (27) while glutathione reductase activity was measured by the system described by Racker (28). In assaying for the aldehyde-ketone reductase in rabbit kidney described by Culp and McMahon (4), *p*-chlorobenzaldehyde (PCB) was used as the substrate since they reported it to be one of the most readily attacked of those tested. It was simply substituted for BA in the cuvette assay system. The same system was also used to test for aromatic α-keto acid reductase activity with 3,5-diiodo-*p*-hydroxyphenylpyruvic acid as substrate (5). The assay for lactic dehydrogenase followed Kornberg's (29) method. Alcohol dehydrogenase activity was tested in a system containing pH 7.4 phosphate buffer, 200 μmoles; acetaldehyde, 75 μmoles; NADH or NADPH, 0.34 μmole; EDTA, 13 μmoles; and enzyme fraction and water to 3 ml total volume. NADPH–cytochrome *c* reductase was assayed by the method described by Ciotti and Kaplan (30).

Fatty acid synthetase was partially purified from pigeon liver homogenate by following the procedures of Hsu *et al.* (7) up to the DEAE-cellulose chromatography. At this point chromatography on DEAE-Sephadex A-50 (bead form) was carried out as described previously. The fraction R_{1g} from pigeon liver, which contains acetyl CoA carboxylase, was prepared according to the method of Gibson *et al.* (31). The system of Wakil *et al.* (32), with R_{1g} added to ensure adequate malonyl CoA synthesis, was used to assay fatty acid synthetase by following the oxidation of NADPH. Crotonyl CoA reductase activity was followed using the system described by Langdon (8). Crotonyl CoA was also added to the cuvette assay system instead of BA with equally satisfactory results.

RESULTS

Selection of Enzyme Sources

In preliminary work (15), comparison of the rate of disappearance of BA when added to suspensions of erythrocytes of several mammalian species revealed that dog erythrocytes were the most active and human erythrocytes were the least active of those tested. Evidence for the participation of an enzyme in the disappearance of the BA was obtained with dog erythrocyte sonicate (Fig. 1). Little activity was observed with the dog erythrocyte sonicate unless a system for the generation of NADPH, based on the work of Huennekens *et al.* (19), was added. Heat treatment (56° for 1 hr) abolished most of the activity observed when NADPH was supplied. Similar enzyme activity was minimal, if not absent, in a human erythrocyte sonicate (Fig. 1). Erythrocyte sonicates (supplied with NADPH) from five dogs uniformly showed much more enzyme activity than did those from five humans (Table 1). Dog erythrocyte sonicates were therefore chosen as the source for purification of the enzyme from erythrocytes.

Both human and rat liver homogenates, when supplied with an appropriate

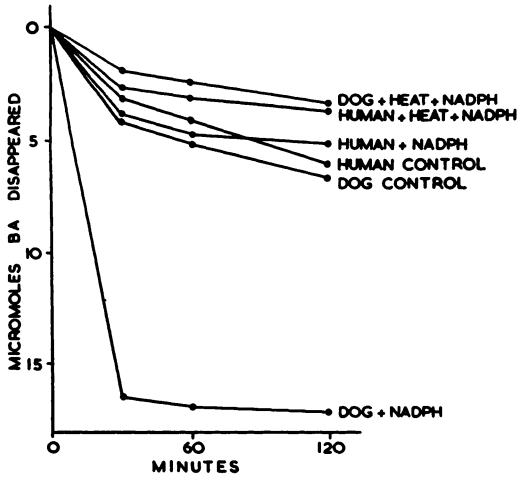

FIG. 1. *Effect of NADPH and heat pretreatment (56° for 1 hr) on the metabolism of BA by sonicates of dog and human erythrocytes*

The flask assay procedure was employed with 3 ml of sonicate and 17 μmoles of BA in Krebs-Ringer phosphate buffer (pH 7.4). NADPH was supplied by adding 30 μmoles of G-6-P, 0.25 μmole of NADP, and 30 μmoles of nicotinamide in Krebs-Ringer phosphate buffer (pH 7.4). The final volume was made up to 5 ml with Krebs-Ringer phosphate buffer (pH 7.4) in all cases.

NADPH-generating system, metabolized BA more rapidly than did the erythrocyte sonicates (Table 1). The relatively high activity of human liver homogenate and the metabolic significance of this organ led to the choice of this preparation as a secondary source for the purification of the enzyme.

Purification of Enzyme

The results obtained for the dog erythrocyte enzyme with the purification procedures employed are shown in Table 2. A typical elution pattern produced by chromatography of the 50–80% $(NH_4)_2SO_4$ precipitate (fraction 3, Table 2) on DEAE-Sephadex is shown in Fig. 2. Further chromatography on Sephadex G-150 did not result in significant further purification, and instability of the enzyme resulted in loss of about half the units of activity. The DEAE-Sephadex eluted preparation was therefore used as the source of enzyme for most further studies.

The results of the more extensive purification procedures employed with the human liver enzyme are shown in Table 3. The overall purification of over 150-fold was not nearly as great as that achieved with dog erythrocytes. Significant increases

TABLE 1

Comparison of rates of metabolism of BA by human and dog erythrocyte sonicates and human and rat liver homogenates

Metabolism of BA was measured by the flask assay procedure using 3 ml of tissue preparation; 17 μmoles of BA, 30 μmoles of G-6-P, 30 μmoles of nicotinamide, and either 0.25 μmole (with sonicates) or 5 μmoles (with homogenates) of $NADP^+$ were added in 2 ml of pH 7.4 Krebs-Ringer phosphate buffer. Values shown are the means and standard deviations.

Tissue preparation	Number tested	Mμmoles BA metabolized per mg protein per min
Dog erythrocyte sonicate	5	0.90 ± 0.07
Human erythrocyte sonicate	5	0.17 ± 0.03
Rat liver homogenate	5	1.9 ± 0.4
Human liver homogenate	2	1.7

TABLE 2

Purification of BA reductase from dog erythrocyte sonicate

Fraction	Total volume	Total munits	Yield (%)	Total protein (mg)	Specific activity (munits/mg)	Purification factor
1. Sonicate	500	43,000	100	87,500	0.49	1
2. Hemoglobin-free	1800	23,250	54	1,008	23.1	47
3. 50–80% $(NH_4)_2SO_4$ precipitate	16	21,200	49	408	52.0	106
4. DEAE Sephadex A-50 eluate	100	10,600	25	9.5	1115.0	2275
5. Sephadex G-150 eluate	210	5,060	12	8.8	575.0	1170

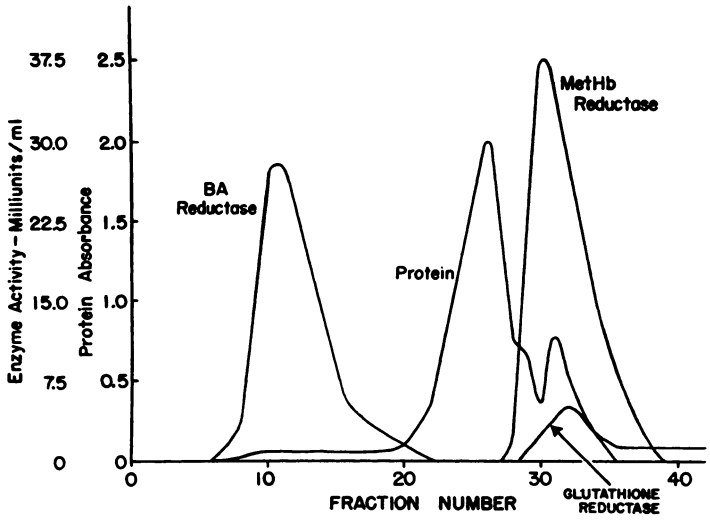

FIG. 2. *Chromatographic separation of BA reductase, methemoglobin reductase, and glutathione reductase in protein precipitated between 50 and 80% ammonium sulfate saturation from hemoglobin-free dog erythrocyte sonicate (fraction 3, Table 2)*

A DEAE-Sephadex A-50 column was used, and the 5-ml fractions eluted by a 0.03–0.15 *M* gradient of pH 7.4 Tris HCl buffer were assayed for enzyme activity.

in specific activity without loss of units and some actual increase in units of enzyme activity occurred in the last step of part A (fraction 4, Table 3A) and the first two steps of part B (fractions 5 and 6, Table 3B) of the purification. The removal of some inhibitor at these points was suggested by the observation that the ad-

TABLE 3
Purification of BA Reductase from Human Liver Homogenate

A. Initial purification employing flask assay

Fraction	Total volume (ml)	Total munits	Yield (%)	Total protein (mg)	Specific activity (munits/mg)	Purification factor
1. Homogenate	500	10,100	100	11,250	0.90	1.00
2. Supernatant (105,000 *g*)	500	5,470	54	6,020	0.91	1.01
3. pH 5 supernatant	486	5,340	53	3,900	1.4	1.5
4. 50–85% $(NH_4)_2SO_4$ precipitate	35	5,370	53	1,820	2.9	3.3

B. Further purification employing cuvette assay

Fraction	Total volume (ml)	Total munits	Yield (%)	Total protein (mg)	Specific activity (munits/mg)	Purification factor
4. 50–85% $(NH_4)_2SO_4$ precipitate	77	8,460	100	1,780	4.8	1.0
5. Sephadex G-200 eluate, concentrated	46	10,600	125	1,260	8.4	1.8
6. DEAE Sephadex A-50 eluate, concentrated	10	8,830	104	77	115	24.2
7. CM Sephadex A-50 eluate	120	4,880	58	21	236	49.7

dition of pH 5 supernatant (fraction 3, Table 3A) (after readjustment to pH 7.4) produced a significant decrease of activity of subsequent fractions such as the Sephadex G-200 eluate (fraction 5, Table 3B).

Stability and Heat Denaturation of the Enzyme

The enzyme from both sources, in crude or partially purified form, was fairly stable at low temperatures. Preparations ranging from the initial sonicate to the DEAE-Sephadex eluate could be held at 2–5° for up to a week without loss of more than 20% of the initial activity. Similar preparations kept frozen at —20° retained at least 80% of the initial activity for a month or even several months in some instances. Only after further chromatography on Sephadex G-150 or CM-Sephadex was a rapid loss of activity found to occur, as noted previously.

At the temperature of 30° employed for assays the enzyme was stable for periods of several hours with virtually no loss of activity. Thermal denaturation at higher temperatures was studied by heating 1 ml of dog erythrocyte enzyme in an 18 × 150-mm test tube for 2 min at various temperatures and then assaying the remaining activity. The results are shown in Fig. 3. The limited amount of human liver enzyme available precluded similar extensive studies with it, but it was shown to be inactivated after 2 min at 60°.

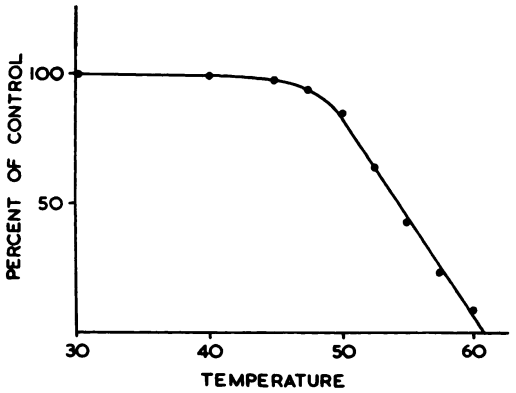

FIG. 3. *Effect of temperature on the activity of dog erythrocyte BA reductase*

One-milliliter aliquots of enzyme solution were exposed to the temperature indicated for 2 min, chilled, and assayed by the cuvette assay. Points shown are the mean of duplicate determinations.

Effect of pH on the Rate of the Enzyme Reaction

The effect of pH on the rate of the reaction with enzyme from dog erythrocytes was studied using three overlapping 0.066 M buffer systems: citrate-phosphate for pH 3.85–7.10, phosphate for pH 5.80–7.90 and Tris HCl for pH 7.10–8.90. The rates in the three different buffers used were virtually identical at pH 7.10. A composite curve for activity at different pH values is shown in Fig. 4. The limited amount of human liver enzyme available precluded similar extensive studies with it but tests at a few selected pH values in the range studied with dog erythrocyte enzyme showed quite similar effects of pH.

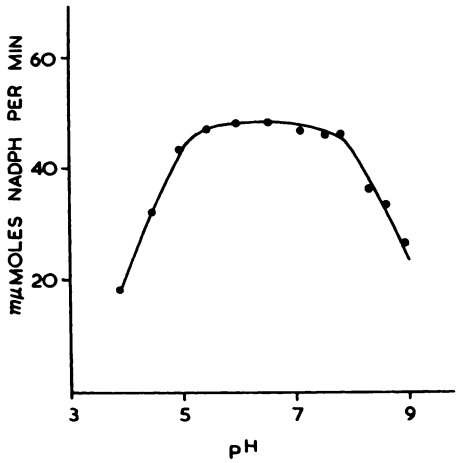

FIG. 4. *Effect of pH on the activity of dog erythrocyte BA reductase*

Overlapping ranges of 0.066 M citrate-phosphate, phosphate, and Tris HCl buffers were employed with no significant differences at equivalent pH values. Enzyme activity in the buffer was measured in the cuvette assay. Points shown are the mean of triplicate determinations.

Substrate Specificity

Fairly extensive studies on substrate specificity were undertaken with the dog erythrocyte enzyme since it was readily

TABLE 4

Michaelis constants and maximum initial velocities for different substrates with α,β-unsaturated ketone reductase from dog erythrocytes and human liver

Substrate	Enzyme source	K_m ± SE (mM)	V_{max} (Calc.) ± SE μmoles/min/mg	V_{max} (Expt.) μmoles/min/mg	V_{max} (Expt.) Substrate concn. (mM)
3-Buten-2-one	Dog	0.78 ± 0.12	2.7 ± 0.3	1.1	0.57
	Human	0.28 ± 0.02	0.24 ± 0.02	0.08	0.34
4-Phenyl-3-buten-2-one	Dog	0.20 ± 0.02	1.5 ± 0.05	1.2	0.90
(benzylidene acetone, BA)	Human	0.14 ± 0.03	0.16 ± 0.02	0.10	0.23
3-Phenyl-2-propen-1-phenyl-one	Dog	0.09 ± 0.02	6.9 ± 1.2	2.6	0.057
(chalcone, benzylidene acetophenone)	Human	0.13 ± 0.03	1.1 ± 0.2	0.3	0.057
4-(2-Furyl)-3-buten-2-one	Dog	0.13 ± 0.04	0.45 ± 0.09	0.2	0.11
(furfurylidene acetone, FA)					
3-(2-Furyl)-2-propen-1-phenyl-1-one	Dog	0.03 ± 0.005	2.6 ± 0.2	1.7	0.057
(furfurylidene acetophenone)					
Crotonyl CoA	Dog	0.05 ± 0.01	2.8 ± 0.3	0.8	0.20

prepared in large quantity. Only limited tests to obtain Michaelis constants (K_m) for active substrates were undertaken with the small quantities of the human liver enzyme available. In addition to the two unsaturated ketones originally studied (BA and FA), a number of other substances containing keto groups and/or double bonds were investigated as possible substrates with the results shown in Tables 4 and 5. The only other compounds which served as substrates were related α,β-unsaturated ketones and crotonyl CoA.

Michaelis constants (K_m) for the active substrates are tabulated with their standard errors in Table 4. With FA and chalcone the reciprocal plots departed from linearity with the higher concentrations tested so that these points were excluded in making the K_m calculations. Values for the maximum velocity obtained from the calculations for each active substrate are also shown in Table 4. The maximum velocities observed experimentally in the course of studying the rate of the reaction with different concentrations are also tabulated, together with the concentration with which this was achieved. These probably represent a closer approximation to the maximum velocities attainable experimentally with the enzyme since above these values rates with chalcone and FA decreased (possibly substrate inhibition), and this may also occur with concentrations of the other ketones higher than those tested. Since the human enzyme was much less active, maximum velocities with it were significantly lower.

TABLE 5

Various ketones, aldehydes, and unsaturated compounds tested as substrates for α,β-unsaturated ketone reductase but found inactive

Ketones	*Other unsaturated compounds*	*Aldehydes*
2-Butanone	3-Buten-2-ol	4-Chlorobenzaldehyde
Pentene-2,4-dione	Phenyl-1-ethyne	Phenyl-1-propenal
Phenyl ethan-1-one	Furyl-1-propenoic acid	1-Ethyl-2-furanacrolein
3-Phenyl-2-propanone	Propyl furyl acrylate	
4-Phenyl-2-butanone	Allylurea	
1,2-Diphenyl ethane-1,2-dione	Griseofulvin	
5-Phenyl-2,4-pentadienephenone	Ethacrynic acid	
2-Cyclohexen-1-one		

TABLE 6

Stoichiometry of the reaction catalyzed by the purified BA reductase of dog erythrocytes

Purified enzyme eluted from Sephadex G-150 (fraction 5, Table 2) was employed in the cuvette assay procedure with 0.34 μmole of NADPH and 0.17 μmole of BA. The oxidation of NADPH was measured by the decrease in absorbance at 340 mμ and the reduction of BA by the decrease in absorbance at 287 mμ during a 5-min reaction period. No absorbance change occurred in control cuvettes lacking either NADPH or BA.

Expt. No.	Mμmoles NADPH oxidized	Mμmoles BA reduced	Ratio NADPH:BA
1	55	55	1.00
2	46	44	1.05
3	41	41	1.00
4	49	38	1.29
		Mean ratio	1.09 ± 0.04

The requirement for NADPH was quite specific since only a trace of activity could be detected with NADH. A value of 0.25 ± 0.075 mM for the K_m of NADPH was obtained with the dog erythrocyte enzyme in the presence of 0.23 mM BA. The effect of possible variables such as enzyme and substrate concentration was not explored.

Stoichiometry and Nature of the Reduction

Studies on the stoichiometry of the reaction were possible, utilizing the decrease of the absorbance peak of BA at 287 mμ and of the absorbance peak of NADPH at 340 mμ. With purified dog erythrocyte enzyme obtained by elution from Sephadex G-150, an average of 1.09 molecules of NADPH was oxidized for every molecule of BA reduced (as measured by the decrease of the absorbance peak at 287 mμ) (Table 6). With cruder enzyme preparations higher ratios were obtained due to unidentified reactions. These were not attributable to oxidation of NADPH in the absence of BA since this was small and was corrected for in making the calculations. Addition of FMN or FAD enhanced the oxidation of NADPH in either the presence or absence of BA but did not significantly change the ratio of NADPH oxidation to BA disappearance when the former was corrected for the enhanced oxidation of NADPH in the absence of BA.

Effect of Inhibitors

The results of some limited tests of the effect of several common enzyme inhibitors on the rate of the enzyme reaction are shown in Table 7.

Differentiation from Other Enzymes

The results of various tests made to differentiate BA reductase from a number of other enzymes are shown in summary form in Table 8.

TABLE 7

Effect of inhibitors on the purified BA reductase of dog erythrocytes and human liver

Purified enzyme (fraction 4, Table 2 or fraction 7, Table 3) was incubated for 30 min with the inhibitor in the assay mixture prior to the measurement of its activity in the standard cuvette assay. Rates shown are the mean of duplicate determinations with dog erythrocyte enzyme and single determinations with human erythrocyte enzyme.

Inhibitor	Enzyme source	Mμmoles NADPH oxidized/min	Percent inhibition
Control	Dog	33	—
Control	Human	49	—
10^{-4} M *p*-Chloromercuribenzoate	Dog	1	97
10^{-4} M *p*-Chloromercuribenzoate	Human	0	100
5×10^{-3} M *N*-Ethylmaleimide	Dog	11	67
5×10^{-3} M *N*-Ethylmaleimide	Human	0	100
10^{-2} M Potassium fluoride	Dog	34	−3
10^{-2} M Sodium arsenate	Dog	36	−9
10^{-2} M EDTA	Dog	37	−12

TABLE 8
α,β-Unsaturated ketone reductase activity of various enzymes

Enzyme	Activity with BA		Activity with normal substrate
	+ NADPH	+ NADH	
Methemoglobin reductase (dog erythrocyte)	Inactive	Inactive	Active with methylene blue and NADPH
Glutathione reductase (dog erythrocyte)	Inactive	Inactive	Active with glutathione and NADPH
Aldehyde-ketone reductase (rabbit kidney)	Slightly active	—	Active with PCB and NADPH
Alcohol dehydrogenase (yeast and horse liver)	Inactive	Inactive	Active with acetaldehyde and NADH
Lactic dehydrogenase (rabbit muscle)	Inactive	Inactive	Active with pyruvate and NADH
NADPH-cytochrome *c* reductase (rat liver microsomes)	Inactive	Inactive	Active with cytochrome *c* and NADPH
Fatty acid synthetase (pigeon liver)	Inactive	—	Active with acetyl CoA, malonyl CoA, and NADPH
Crotonyl CoA reductase (rat liver)	Active	—	Active with crotonyl CoA and NADPH

When the protein from hemoglobin-free dog erythrocyte sonicate precipitated between 50 and 80% ammonium sulfate saturation (fraction 3, Table 2) was chromatographed on DEAE-Sephadex A-50, it was possible to show a clear separation of BA reductase from both methemoglobin reductase and glutathione reductase (Fig. 2).

When a protein fraction precipitated from rabbit kidney supernatant (105,000 *g*) between 50 and 70% saturation with ammonium sulfate was chromatographed on DEAE-Sephadex A-50, only a limited separation of the peak activity for reduc-

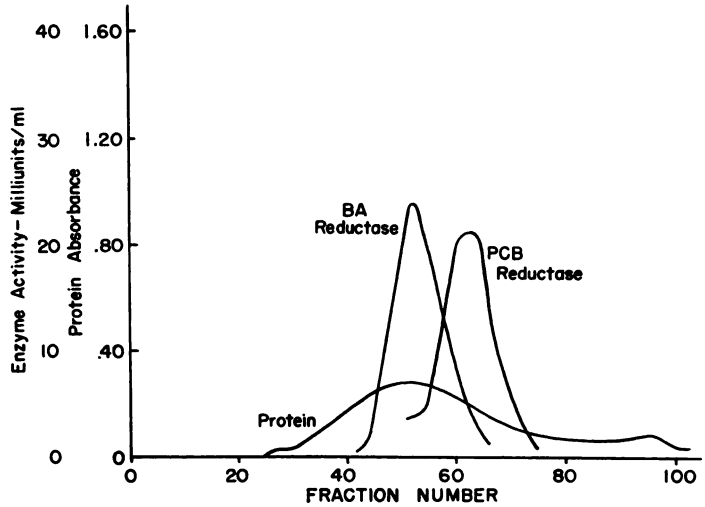

FIG. 5. *Chromatographic separation of BA reductase from PCB reductase in rabbit kidney supernatant (105,000 g) protein precipitated between 50 and 70% ammonium sulfate saturation*

A Sephadex G-150 column was used, and the 3-ml fractions eluted by 0.03 M Tris HCl buffer, pH 7.4, were assayed for enzyme activity.

tion of BA and PCB occurred. However, as shown in Fig. 5, fairly good separation was achieved on a Sephadex G-150 column. It seemed clear that there were two distinct enzymes and that BA was poorly metabolized by the PCB reductase while the BA reductase probably did not attack PCB.

The preparation and partial purification of the recently described (5) aromatic α-keto acid reductase was not attempted. However, the substrate with which this enzyme is most active, 3,5-diiodo-*p*-hydroxyphenylpyruvic acid, was tested with in an attempt to dissociate it into fragments (33), showed no BA reductase activity following this treatment.

Rat liver supernatant (105,000 *g*) contained BA reductase and also crotonyl CoA reductase activity as reported by Langdon (8). Fractionation of the supernatant with ammonium sulfate revealed that both enzyme activities were present in the protein precipitated between 40 and 70% saturation. Chromatography on DEAE-Sephadex A-50, as previously with other preparations, resulted in elution of

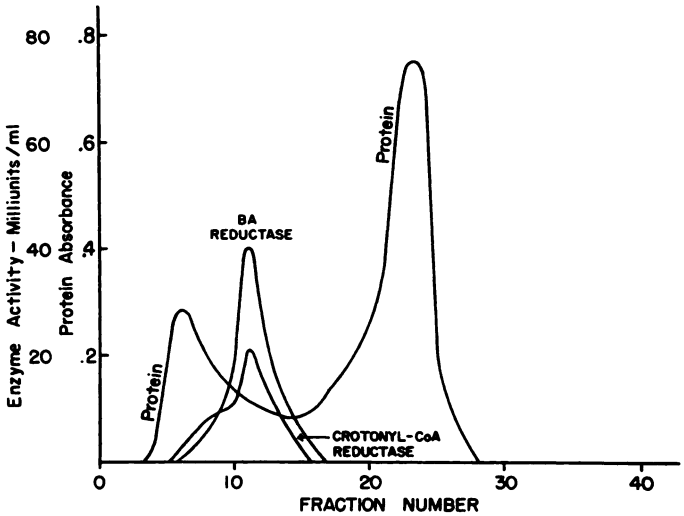

FIG. 6. *Chromatographic distribution of BA reductase and crotonyl CoA reductase in rat liver supernatant (105,000 g) protein precipitated between 40 and 70% ammonium sulfate saturation*

A DEAE-Sephadex A-50 column was used, and the 5-ml fractions eluted by a 0.03–0.15 M gradient of pH 7.4 Tris HCl buffer were assayed for enzyme activity.

the purified dog erythrocyte enzyme instead of BA in the standard cuvette assay. No activity was detected with this substrate.

Fatty acid synthetase and BA reductase activity was found in pigeon liver supernatant (105,000 *g*) and the R_{2g} fraction. However, chromatography on DEAE-Sephadex A-50 resulted in complete separation of the two enzymic activities. Fatty acid synthetase was eluted in the earliest large protein peak and was free of BA reductase activity. BA reductase was eluted much later in a small peak which did not possess any fatty acid synthetase activity. Fatty acid synthetase which was allowed to stand in 8 M urea for 24 hr the two enzyme activities in the same fractions (Fig. 6). Purified BA reductase from both dog erythrocyte and human liver was active in attacking crotonyl CoA. The K_m and V_{max} for crotonyl CoA with the dog erythrocyte enzyme are shown in Table 4.

DISCUSSION

The results obtained indicate that dog erythrocytes and human liver contain a protein with characteristic enzymic properties which render it capable of catalyzing the reduction of α,β-unsaturated ketones by NADPH. The inhibitory effects of *p*-chloromercuribenzoate and *N*-ethylmaleim-

ide can be interpreted as indicating a requirement for free sulfhydryl groups on the protein in order for it to show enzymic activity (24). The kinetic studies provide evidence of considerable affinity of the enzyme for several α,β-unsaturated ketones as substrates, while the absence of reactions with compounds containing only a keto group or double bond suggests a high degree of specificity for enzymic attack on the unsaturated keto group. The stoichiometry of the reaction (Table 6) is compatible with reduction of either the double bond or the keto group, but not both. Further elucidation of the exact nature of the reaction must await the results of studies in progress on the product of the reaction.

The partial purification of the BA reductase from rat liver, rabbit kidney, and pigeon liver in connection with the experiments designed to differentiate it from other enzymes suggests that it is widely distributed in different tissues and species. A number of other widely distributed enzymes are clearly differentiated from BA reductase by the data presented in Table 8. The absence of glutathione reductase from the purified dog erythrocyte BA reductase preparation (Fig. 2) appears to exclude the possibility of nonenzymic oxidation of glutathione by BA followed by its reduction by NADPH and glutathione reductase as intermediate steps in the overall reaction. The evidence obtained to date suggests that the α,β-unsaturated ketone reductase described in this paper may be identical with the crotonyl CoA reductase described by Langdon (8). Several groups of investigators (34–37) have reported crotonyl CoA reductase activity of various degrees in the microsomal and mitochondrial fractions and the supernatant of rat liver and lactating rabbit mammary gland homogenates but further purification of the enzyme does not appear to have been attempted. Further purification and characterization of both BA reductase and crotonyl CoA reductase activity will be necessary to establish their relationship to each other.

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