Presence of Rhodanese in the Cytosolic Fraction of the Fruit Bat (*Eidolon helvum*) Liver

Femi Kayode Agboola* and Raphael Emuebie Okonji

Department of Biochemistry, Obafemi Awolowo University, Ile-Ife, Nigeria

Rhodanese was isolated and purified from the cytosolic fraction of liver tissue homogenate of the fruit bat, *Eidolon helvum*, by using ammonium sulphate precipitation and CM-Sephadex C-50 ion exchange chromatography. The specific activity was increased 130-fold with a 53% recovery. The K_m values for KCN and $Na_2S_2O_3$ as substrates were 13.5 ± 2.2 mM and 19.5 ± 0.7 mM, respectively. The apparent molecular weight was estimated by gel filtration on a Sephadex G-100 column to be 36,000 Da. The optimal activity was found at a high pH (pH 9.0) and the temperature optimum was 35°C. An Arrhenius plot of the heat stability data consisted of two linear segments with a break occurring at 35°C. The apparent activation energy values from these slopes were 11.5 kcal/mol and 76.6 kcal/mol. Inhibition studies on the enzyme with a number of cations showed that Mg^{2+}, Mn^{2+}, Ca^{2+}, and Co^{2+} did not affect the activity of the enzyme, but Hg^{2+} and Ba^{2+} inhibited the enzyme.

Keywords: Cyanide, Detoxication, Fruit bat, Liver, Rhodanese

Introduction

Cyanide is known to be one of the most toxic substances present in a wide variety of food materials that are consumed by mammals (Montgomery, 1965). Many are known to contain cyanogenic glucosides, which upon hydrolysis, release cyanide (Wokes and Willimott, 1951).

Rhodanese (thiosulphate: cyanide sulphur transferase, E.C. 2.8.1.1) catalyses the formation of thiocyanate from free cyanide and a sulphur donor. It has been studied from variety of sources, which include bacteria, yeast, plants, and animals (Cosby and Summer, 1945; Himwich and Saunders, 1948;

Sorbo, 1951; Jarabak and Westley, 1974; Anosike and Ugochukwu, 1981; Lee *et al.*, 1995). It is a heat labile enzyme with an optimum pH of 8.0 and an average molecular weight of about 37,000. The enzyme represents the chief site of detoxication of cyanide and occurs in all parts of the body with the highest concentration in the mitochondrial fraction of the liver of mammals. The main physiological function of rhodanese in mammalian tissues, where it is localized in the mitochondria, is the supply of sulphide for the formation of an iron sulphur centres for the election transport chain. The ability to detoxify cyanide may be only a secondary physiological benefit (Oke, 1973; White *et al.*, 1981).

Fruit bat, *Eidolon helvum*, feeds basically on fruits, which may include cyanogenic fruits. It is plausible to assume that it possesses an efficient mechanism for cyanide detoxication. This paper describes the isolation and characterization of rhodanese from the cytosolic fraction of a fruit bat liver.

Materials and Methods

Sodium chloride, sodium thiosulphate (pentahydrate), nitric acid, ammonium sulphate (enzyme grade), glycine, sodium acetate, and ferric nitrate (nonahydrate) were obtained from BDH Chemical Limited (Poole, England). The β-mercaptoethanol, urea, ethanol, methanol, and phosphoric acid were also products of BDH Chemicals Limited. Ethylenediamine tetraacetic acid (EDTA), citric acid, ε-amino-n-caproic acid, sodium borate, boric acid, bovine serum albumin (BSA), and Coomassie Brilliant-Blue G-250 were obtained from Sigma Chemical Company (St. Louis, USA). CM-Sephadex C-50, and Sephadex G-100, Sephadex G-25 were obtained from Pharmacia Fine Chemical (Uppsala, Sweden). All of the other reagents were of analytical grade and obtained from either Sigma or BDH.

Bats were collected at the Botanical Garden of the Obafemi Awolowo University, Ile-Ife, Nigeria.

Enzyme and protein assays Rhodanese activity was measured routinely according to the method of Lee *et al.* (1995). The reaction mixture consisted of a 25 mM borate buffer (pH 9.4), 0.5 M KCN,

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*To whom correspondence should be addressed.

Tel: Fax: 08034738078

E-mail: fkagboola@yahoo.co.uk

0.5 M $Na_2S_2O_3$, and 20 μl and of appropriately diluted enzyme solution in a total volume of 1.0 ml. The mixture was incubated for 1 min at room temperature and the reaction was stopped by the addition of 0.5 ml of 15% formaldehyde, followed by the addition of 1.5 ml of Sorbo reagent (containing 10 g Fe $(NO_3)_2$ $9H_2O$, and 20 ml HNO_3 (sp.g 1.40) and distilled water to 100 ml) (Sorbo, 1951). The absorbance was then read at 460 nm. The activity was expressed in rhodanese unit (RU). One rhodanese unit was taken as the amount of enzyme, which under the given condition, produced an optical density reading of 1.08 at 460 nm per min (Sorbo, 1951). Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin (BSA) as the standard.

Enzyme isolation and purification   All operations were carried out at temperatures between 0-10ºC either in a cold room or ice bucket for the entire time. All of the buffers contained 10 mM sodium thiosulphate to stabilize the enzyme. The pH measurement was made at 25ºC using a Kenway Model 3015 pH meter.

Crude extract   The animals were knocked on the back of their heads to unconsciousness and the liver quickly excised and stored in the refrigerator until required. The frozen liver was thawed at room temperature and rinsed several times with cold saline (0.9% NaCl at pH 7.4) to remove blood and other impurities, and then weighed. In a typical extraction process, approximately 95 g of tissue (collected from twelve bats) was used for the preparation. The liver was minced with scissors into smaller pieces and homogenized in 2 volumes of 0.1 M acetate glycine buffer, pH 7.8 containing 1 mM ε-amino-n-caprioc acid, and 10 mM sodium thiosulphate (Buffer A) in a blender. The homogenate was centrifuged for 15 min at 10,000 rpm at 6ºC. The supernatant was filtered through a double layer of cheesecloth. The cellular debris was resuspended in one volume of the homogenization buffer, homogenized in a blender, and centrifuged under the same condition. The obtained supernatant was combined with that from the first centrifugation step.

Ammonium sulphate fractionation   The combined supernatant was brought to 65% ammonium sulphate saturation (430 g/l) by the addition of solid ammonium sulphate over a period of 1 hour with continuous stirring, and then left overnight. The resulting precipitate was collected by centrifugation at 6,000 rpm for 30 min and immediately dialyzed against several changes of a 50 mM citrate buffer, pH 5.0 containing 10 mM $Na_2S_2O_3$ (buffer B). The dialysate was centrifuged at 6,000 rpm for 30 min to remove insoluble materials.

CM-Sephadex ion exchange chromatography   CM-Sephadex C-50 was pretreated by first swelling 10 g of the resin in 1 l of distilled water for 3 d. The resin was then washed with 1 M KCl (2 l) in a series of stirring and decantation processes. It was later washed with 0.1 M KOH (2 l) and 0.1 M HCL (2 l). Distilled water was used to rinse the resin between each washing.

The resin was packed into a 2.5 × 40 column and equilibrated with buffer B. The dialyzed protein from the preceding step was layered on the column. The column was first washed with 200 ml buffer B to wash the unbound protein, followed by elution with a 250 ml linear gradient of 0-0.5 M KCl in buffer B. Fractions of 5 ml

were collected at a rate of 36 ml/h. The protein profile was monitored spectrophotometrically at 280 nm. The fractions were also assayed for enzyme activity. The active fractions were pooled and brought to 70% ammonium sulphate saturation.

Polyacrylamide gel electrophoresis (PAGE)   Polyacrylamide gel electrophoresis in the absence of sodium dodecyl sulphate (SDS) was carried out according to the method described in the Pharmacia's Manual (Polyacrylamide Gel Electrophoresis, Laboratory Techniques) on 7.5% rod gel. The protein sample was first desalted on a Bio-Gel P-10 column using a 10 mM phosphate buffer, pH 7.0, containing 10 mM sodium thiosulphate (buffer D) as the eluent. To 50 μl of the sample was added 50 μl bromophenol blue (0.05% bromophenol blue in 0.01 M sodium phosphate buffer, pH 7.0). An aliquot of 10 μl of the mixture was applied on a gel. The gels were run at 8 mA per gel and stained for the protein after electrophoresis. Also, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed for the purpose of ascertaining purity of the preparation according to the procedure of Weber and Osborn (1975) on 10% rod gels using the phosphate buffer system. The protein sample was prepared by the addition of a 0.2 ml sample buffer (containing phosphate buffer, pH 7.0, 1% SDS, and 2-mercaptoethanol) to 10 μl of the desalted enzyme preparation and heated in a boiling water bath for 2 min. After cooling, 10 μl aliquot was applied on a rod gel. Electrophoresis was run at 8 mA per gel for 5 h.

Molecular weight determination   The native molecular weight was determined on a calibrated Sephadex G-100 column (1.5 × 40 cm). The standard proteins were bovine serum albumin (M_r 66,000), ovine albumin (M_r 45,000), thermolysin (M_r 37,500) and α-chymotrypsinogen A (M_r 25,000). To calibrate the column, 3 ml of each standard of 3 mg/ml was applied to the column and run separately. The column was eluted at a flow rate of 8.4 ml/h. The eluent was a 10 mM phosphate buffer, pH 7.0. Fractions of 2 ml were collected and monitored for protein. The void volume (V_o) of the column was determined by the elution volume (V_e) of Blue Dextran. A plot of V_e/V_o against logarithm of the molecular weight of the marker proteins was made on a semilogarithmic paper.

Determination of kinetic parameters   The kinetic parameters (K_m and V_{max}) of the enzyme were determined according to Lee *et al.* (1995). The K_m^{KCN} was determined by varying the concentration of KCN between 10 mM and 100 mM at 50 mM $Na_2S_2O_3$. Also, K_m for sodium thiosulphate was determined by varying the $Na_2S_2O_3$ concentration from 50 mM to 500 mM at 50 mM of KCN. The ammonium sulphate precipitate of the enzyme was first desalted on a Bio-gel P10 column using a 10 mM phosphate buffer, pH 7.0 containing 10 mM sodium thiosulphate (buffer D). The kinetic parameters were estimated from the double reciprocal plot (Lineweaver and Burk, 1934).

Effect of temperature on the enzyme   To investigate the effect of temperature on the enzyme activity, 20 μl of the enzyme (1.15 mg/ml) was assayed at temperatures between 0º and 70ºC. The assay mixture was first incubated at the indicated temperature for 10 min before initiating the reaction by the addition of the enzyme that had been equilibrated at the same temperature.

For the effect of heat on enzyme stability, a 1 ml aliquot of the

Table 1. Purification of rhodanese from bat liver

Step/Fraction	Total activity (RU)	Total protein (mg)	Specific activity (RU/mg)	Yield (%)
Crude extract	29,800	6,320	4.71	100
65% Ammonium sulphate precipitation	24,600	522	47.1	83
CM-Sephadex ion exchange	15,700	115	137	53

Each step was carried out as described in the text. Activity was measured by the rate of formation of thiocyanate. Protein concentration was determined by the method of Bradford. One unit of activity was taken as the amount of enzyme, which under the given condition, produced an OD reading of 1.08 at 460 nm per min (Sorbo, 1951).
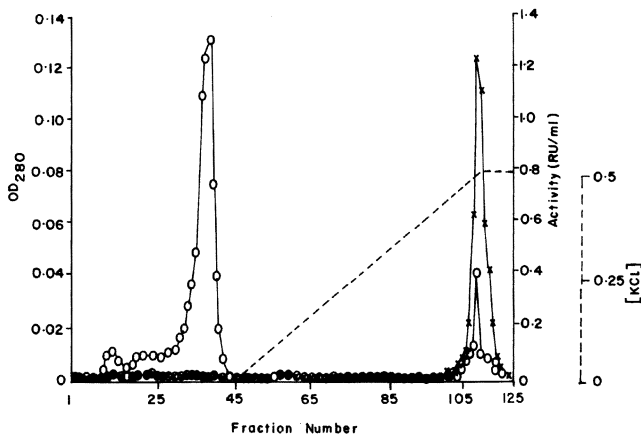
Fig. 1. CM-Sephadex C-50 ion exchange chromatography of fruit bat liver rhodanese. The column was first washed with 200ml of citrate buffer, pH 5.0, containing 10 mM sodium thiosulphate. The column was further eluted with 250 ml linear salt gradient of 0-0.5 M KCl at a flow rate of 36 ml/hr. Fractions of 5 ml were collected. OD$_{280}$ (O-O), Enzyme activity ($\times$-$\times$), Linear KCl gradient (---------), Pooled fractions (——).

enzyme solution (previously desalted on a Biogel P10 column) was incubated at the temperature. Next, 20 μl aliquot were withdrawn and assayed for residual activity at 10 min intervals.

Effect of pH on enzyme activity   The effect of pH on the fruit bat liver rhodanese was carried out by assaying the enzyme at different pH using the following buffers: 0.2 M citrate buffer (pH 5.0-6.5), 0.2 M phosphate buffer (pH 6.5-8.5), and 0.2 M borate buffer (pH 8.5-11.0).

Effect of cations on enzyme activity   The effect of various metal ions on the activity of rhodanese was also studied. The salts included mercuric chloride (HgCl$_2$), barium chloride (BaCl$_2$), calcium chloride (CaCl$_2$), borate chloride (BoCl$_2$), magnesium chloride (MgCl$_2$), and manganese chloride (MnCl$_2$) at 0.5 mM and 1.0 mM. The final concentration of the salt in the assay mixture was either 250 μM or 500 μM.

Results

The results of the purification procedures are summarized in
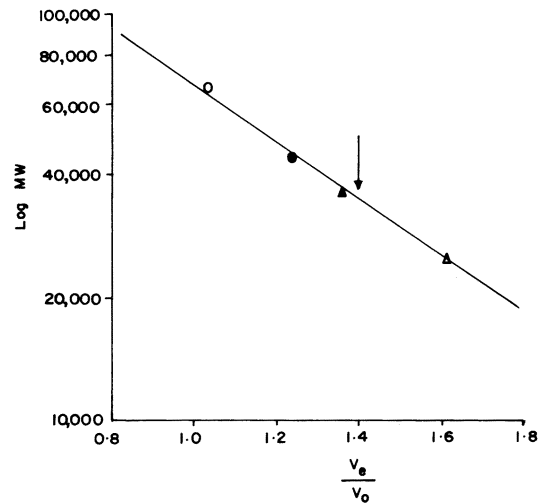
Fig. 2. Calibration curve on Sephadex G-100 for molecular weight determination. 3 ml of the maker proteins was applied to a Sephadex G-100 column (2.5 × 100 cm) and eluted by 10 mM phosphate buffer, pH 7.0, at a flow rate of 8.4 ml/h. The V$_o$ was determined by the elution of 2 mg/ml solution of Blue Dextran eluted under the same condition. Marker proteins are bovine serum albumin (M_r 66,000), ovine albumin (M_r 45,000), thermolysin (M_r 37,500), and chymotrypsinogen A (M_r 25,000). The arrow indicates the position of the fruit bat liver rhodanese.

Table 1. During the purification, the enzyme was stabilized by the presence of sodium thiosulphate. Figure 1 shows the elution profile after the CM-Sephadex C-50 ion exchange chromatography. The specific activity of the pure enzyme was 137 RU/mg.

Only one protein band was observed after gel electrophoresis of the purified rhodanese, either in the presence or absence of sodium dodecyl sulphate (Figures not shown). Gel filtration on the Sephadex G-100 column resulted in an apparent molecular weight of about 36,000 Da (Fig. 2).

The Lineweaver-Burk plots for the determination of kinetic parameters (K_m and V_{max}) of the fruit bat liver rhodanese are shown in Figures 3a and 3b. Table 2 shows the summary of the K_m and V_{max} values for the substrates. A comparison of fruit bat rhodanese with some other mammalian enzymes is shown in Table 3.

The optimum temperature for rhodanese activity was 35ºC.

(A)

(B)
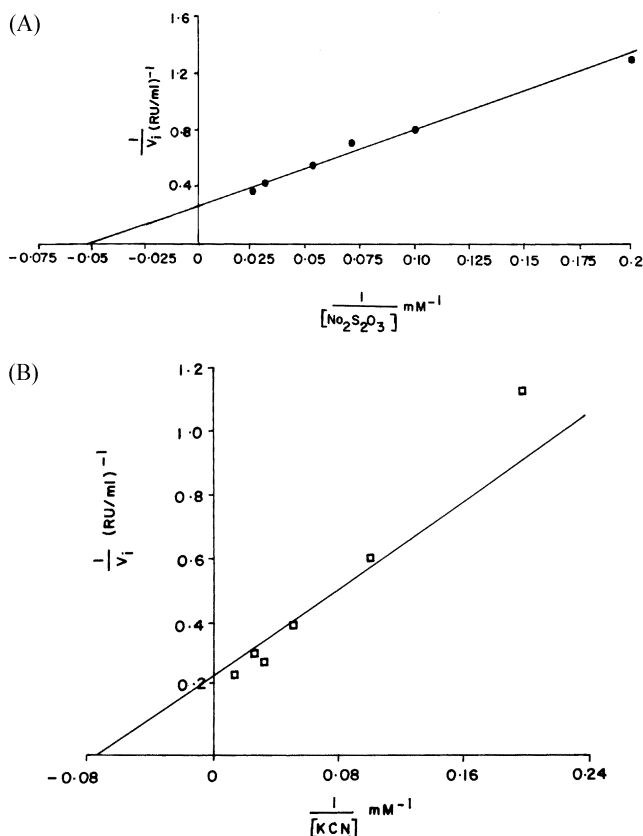
Fig. 3. Lineweaver-Burk plot for the determination of the kinetic parameters. Enzyme was assayed at varying concentrations of $Na_2S_2O_3$ at 50 mM KCN (A) and varying concentrations of KCN at 50 mM $Na_2S_2O_3$ (B). The reaction mixture also contained 25 mM borate buffer, pH 9.4, and 20 µl of the enzyme in a total volume of 1.0 ml at 25°C.

An Arrhenius plot of the effect of temperature on enzyme activity at pH 9.4 is biphasic (Fig. 4). The apparent activation energy values estimated from the two slopes were 11.5 kcal/mol and 76.6 kcal/mol. Figure 5 shows the effect of temperature on enzyme stability. The influence of pH on the rate of rhodanese activity is shown in Fig. 6. An optimum pH of 9 was obtained. The result of the effect of various salts on the activity of rhodanese is presented in Table 4.

Table 2. Kinetic Parameters of fruit bat liver rhodanese

Substrate	K_m (mM)	V_{max} (RU/mL)
KCN	13.5 ± 2.2	6.5 ± 0.5
Na_2S_2O3	19.5 ± 0.7	3.7 ± 0.2

The K_m and V_{max} values are means of five determinations. The kinetic parameters (K_m and V_{max}) for the two substrates were determined by varying the concentrations of KCN (between 10 mM and 100 mM) at a fixed 50 mM $Na_2S_2O_3$ and also varying the concentrations of $Na_2S_2O_3$ (between 50 mM and 500 mM) at a fixed KCN concentration of 50 mM. The kinetic parameters were determined from the double reciprocal plots.

Discussion

Several animals are able to thrive on cyanogenic plants, due primarily to inherent cyanide detoxifying mechanisms of the organisms. Rhodanese and 3-mercaptopyruvate sulphurtransferase (3-MST) represent the chief enzymes of cyanide detoxification (Westley, 1980; Nagahara et al., 1999). This research shows the existence of rhodanese in the cytosolic fractions of the fruit bat liver homogenate. The enzyme was purified further to apparent homogeneity by ammonium sulphate precipitation and ion-exchange chromatography on CM-Sephadex. Fruit bats feed on a wide variety of ripe fruits (Mutere, 1965; Halstead and Segun, 1975; Okon et al., 1976). Many fruits contain prussic acid (hydrogen cyanide) (Montgomery, 1965). Since hydrogen cyanide spray was unsuccessful in controlling fruit bats in East Africa (Constantine, 1970), this provides further evidence of the presence of a powerful mechanism for the detoxication of cyanide in this animal. In another set of experiments that are ongoing in our laboratory, it has been shown that 3-MST is also abundant in the cytosolic fraction of the fruit bat liver.

The specific activity of this preparation was 137 RU/mg with a 53% yield. Sorbo (1953a, 1953b) obtained 256 RU/mg for bovine liver rhodanese. Lee et al. (1995) reported a value of 1,076 RU/mg for the mitochondrial enzyme from mouse liver. From human liver, 1240 RU/mg was obtained (Jarabak and Westley, 1974).

Table 3. Comparison of K_m values for fruit bat liver and other mammalian liver rhodaneses

Substrate	K_m (mM)				
	Fruit bat[a]	Mouse[b] liver	Bovine[c] liver	Human[d] liver	Rat[e] liver
KCN	13.5	12.5	19.0	9.5	NA
$Na_2S_2O_3$	19.5	8.3	6.7	4.5	4.4

The K_m values of fruit bat liver rhodanese are compared with those of the enzyme from other mammalian sources. (a) This work in 50 mM borate, pH 9.4, 25°C (b) Lee et al. (1995) in 50 mM borate, pH 9.4, 25°C (c) Sorbo (1953a) in 40 mM phosphate, pH 8.6, 25°C (d) Jarabak and Westley (1974) in 0.1 M glycine, pH 9.1, 25°C (e) Nagahara et al. (1996) in 40 mM phosphate, pH 8.6, 25°C. NA means not available.
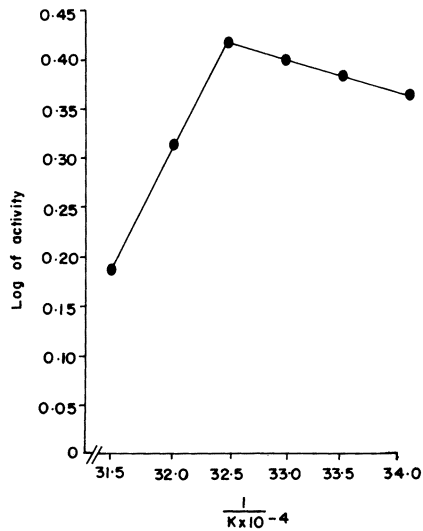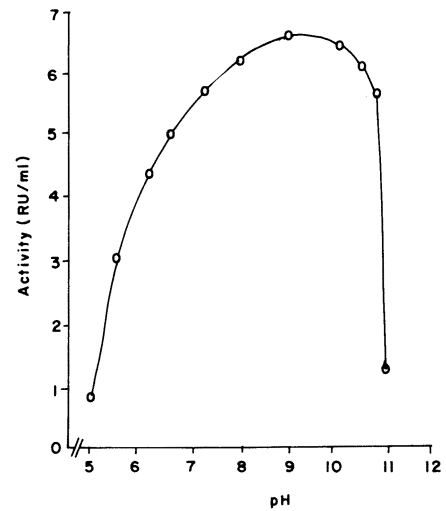
Fig. 4. Effect of temperature on the activity of rhodanese. Arrhenius plot (logarithm of activity (RU/ml)) against the reciprocal of the incubation temperature in Kelvin. 20 ml aliquot of the enzyme was assayed at temperatures between 0ºC and 70ºC. The assay mixture was first incubated at the indicated temperature for 10 mins before initiating the reaction by the addition of the enzyme. Assays were in a 25 mM borate buffer, pH 9.4.
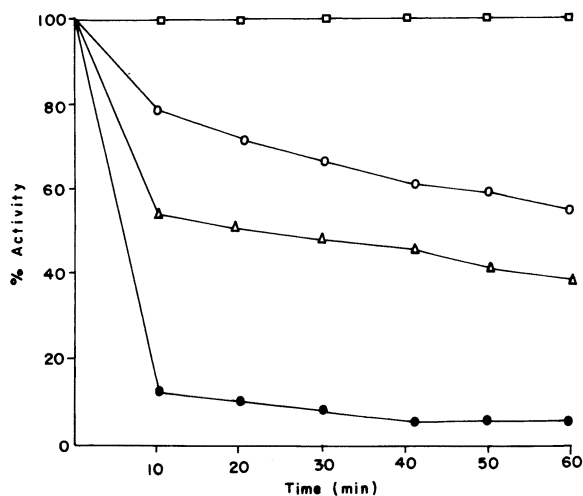
Fig. 5. Effect of temperature on the stability of fruit bat rhodanese. An enzyme solution of 1.0 ml (1.15 mg/ml) was incubated at the indicated temperature and aliquots were withdrawn at various times (as indicated) and assayed for residual enzyme activity. The activity at 30ºC (□), 40ºC (○), 50ºC (△), and 60ºC (●) was expressed as a percentage of the activity of the enzyme at 30ºC (control). Assays were in 25 mM borate buffer, pH 9.4.

Fig. 6. Effect of pH on fruit bat liver rhodanese. The assay mixture contained 0.1 M of the appropriate buffer, 0.05 M KCN, 0.05 M $Na_2S_2O_3$, and 0.02 ml of the enzyme solution in a final volume of 1.0 ml. The buffers and their respective pH ranges were: 0.2 M citrate buffer, (pH 5.0-6.5), 0.2 M phosphate buffer (pH 7.0-8.5), and 0.2 M borate buffer, (pH 9.0-11).

Table 4. Effect of salt's metal ions on bat liver rhodanese

Salt	Enzyme activity (%)	
	250 μM	500 μM
None	100 ± 0	100 ± 0
MgCl$_2$	88 ± 4	90 ± 5
MnCl$_2$	86 ± 5	86 ± 5
CoCl$_2$	85 ± 2	85 ± 2
CaCl$_2$	78 ± 3	72 ± 3
HgCl$_2$	49 ± 2	44 ± 2
BaCl$_2$	46 ± 2	42 ± 4

Enzyme assay was carried out using the standard assay mixture containing each salt at final concentrations of 250 μm and 500 μm. The values are the means of triplicate determinations.

thiosulphate of the enzymes from the liver of this fruit bat and that of bovine liver i.e. 19.5 mM and 6.7 mM respectively (see Table 3) are different. This may indicate that the affinity of the enzyme from a fruit bat liver for thiosulphate is less than that of the bovine liver enzyme. It has been observed that ruminants were more susceptible to the effect of cyanogenic plants than monogastric animals (Kingsbury, 1964). A number of roles other than detoxication of cyanide have been assigned to the enzyme, depending on the tissue/organ concerned. White *et al.* (1981) reported that the main physiological role of rhodanese is the supply of sulphide for the formation of an iron sulphur center for the electron transport chain in mammalian tissues, while the ability to detoxify cyanide may be only a secondary benefit. Tomati *et al.* (1974) suggested the reactivation of ferredoxin from apoferredoxin by rhodanese. Ali *et al.* (2001) suggested that the function of

The apparent relative molecular weight of 36,000 Da compares very well with that reported for the enzyme from other sources (Sorbo, 1953a, 1953b; Jarabak and Westley, 1974; Lee *et al.*, 1995). The results of the kinetic studies are similar to that of the enzyme from some mammalian sources (Table 3). It is noted, however, that the K_m values for

rhodanese in cattle is the protection of the respiratory chain in the case of cyanide toxicity.

The pH optimum is in good agreement with that from bovine liver, which is between 8.0 and 9.0 (Sorbo, 1953a, 1953b). Chew and Boey (1972), working on the tapioca leaf, obtained a high pH value of 10.2-11, while Lee *et al.* (1995) reported a pH of 9.4 for mouse liver rhodanese. Lang (1933a) showed the presence of heat labile enzyme of optimum temperature of 38ºC in a rat liver. Himwich and Saunders (1948), working on bovine liver rhodanese, obtained an optimum temperature between 38ºC and 40ºC. Sorbo (1953a, 1953b) also reported an optimum temperature of 50ºC from bovine liver. Chew and Boey (1972) obtained an optimum temperature between 57ºC and 59ºC from the rhodanese of tapioca leaves. Vaughan (1972) and Halstead and Segun (1975) showed that fruit bats do not hibernate, but maintain their body temperature within narrow limits by physiological and behavioral means; they are homoiothermic. Thus the optimum temperature of 35ºC obtained for the fruit bat liver rhodanese, which is the same temperature suggesting a conformational change in the enzyme (i.e. the point of break in the Arrhenius plot), is an indication of the effect of structural change due to temperature change. The apparent activation energy value of 11.5 kcal/mol from the Arrhenius plot was a little higher than the 7.5 kcal/mol that was reported for bovine liver rhodanese (Sorbo, 1953a). This value, however, falls within the physiological activation energy range (1-25 kcal/mol) for physiological processes in living organisms (Fruton and Summonds, 1963). The second value of 76.6 kcal/mol lies within the range (40 kcal/mol to 100 kcal/mol) for protein denaturation. These results, combined with the result that the enzyme was stable up to 45ºC, suggests that rhodanese from fruit bat will be most active under the body temperature because there will be no time when the fruit bat will be exposed to a temperature that is higher than 45ºC, since *E. helvum* is a tropical animal (Mutere, 1965; Vaughan, 1972).

Metal ions, such as Cu^{2+} and Fe^{2+}, have been reported to significantly inhibit rhodanese (Lang, 1933a, 1933b). Metal ions showing inhibitions are those that have strong affinity for ligands, such as the phosphate, cysteinyl, and the histidyl side chain of protein (Vallee and Ulmer, 1972; Stokinger, 1984). The inhibition of fruit bat liver rhodanese by Hg^{2+} and Ba^{2+} is probably due to the interaction of these metal ions with sulphydryl groups at the enzyme catalytic site (Vallee and Ulmer, 1972; Lee *et al.*, 1995; Nagahara and Nishino,1996).

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