BBA 66438

β-CYCLOPIAZONATE OXIDOCYCLASE FROM *PENICILLIUM CYCLOPIUM*

I ASSAY METHODS, ISOLATION AND PURIFICATION*

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(Received June 4th, 1971)

SUMMARY

1 Spectrophotometric and colorimetric assay methods for the dehydrogenation of β-cyclopiazonic acid and the conversion of β- into α-cyclopiazonic acid, both catalysed by β-cyclopiazonate oxidocyclase, were developed Paper chromatography, ultraviolet spectrophotometry and mass spectrometry were also used in identifying the product, α-cyclopiazonic acid, formed in the above conversion

2 Five isoenzymes of β-cyclopiazonate oxidocyclase were isolated and purified from the mycelium of *Penicillium cyclopium* Westling by homogenization and extraction, differential centrifugation, $(NH_4)_2SO_4$ fractionation, DEAE-cellulose chromatography, Sephadex G-100 gel chromatography and CM-Sephadex C50 chromatography The final yield was 64% and the average final enrichment for the five isoenzymes 133

3 The results of microzone electrophoresis, polyacrylamide gel electrophoresis and analytical ultracentrifugation indicated pure and homogeneous preparations of all five isoenzymes

4 The optimum pH for the dehydrogenation reaction and the conversion of β- into α-cyclopiazonic acid was found to be 6 8

5 The five isoenzymes had essentially the same sedimentation coefficients ($s^\circ_{20,w}$ approx 4 0 10^{-13} sec), diffusion coefficients ($D_{20,w}$ approx 7 3 10^{-7} cm^2 sec^{-1}) partial specific volumes ($\bar{v}$, 0 73 cm^3 g^{-1}) and molecular weights (mol wt approx 50 000)

6 The isoenzymes showed a similar specific activity for both the dehydrogenation and the conversion of β- into α-cyclopiazonic acid and occurred in a constant relative ratio

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Abbreviations DCIP, 2,6-dichlorophenolindophenol, PMS, phenazine methosulphate

[*] Taken in part from a thesis submitted by J C Schabort to the Faculty of Agriculture of the University of Pretoria in partial fulfilment of the requirements of the D Sc (Agric) degree

INTRODUCTION

β-Cyclopiazonate oxidocyclase catalyses the conversion of β-cyclopiazonic acid into α-cyclopiazonic acid This conversion is the final step in the biosynthesis of α-cyclopiazonic acid by the mould *Penicillium cyclopium* Westling

Evidence that β-cyclopiazonic acid is a precursor of α-cyclopiazonic acid has been presented[1] α-Cyclopiazonic acid accounts for the toxicity of a strain of *P cyclopium* Westling (strain 1082) (I F H PURCHASE, personal communication) isolated by SCOTT[2] from stored grain and cereal products The chemical structures and the relative stereochemistry of α- and β-cyclopiazonic acid (Fig 1) have been deduced from chemical and stereochemical studies performed by HOLZAPFEL *et al*[3,4]

Structure analyses as well as radioactive precursor incorporation studies suggested the biosynthesis of α-cyclopiazonic acid from a C_5 unit arising from mevalonic acid (the precursor of the active isoprene γ,γ-dimethylallyl pyrophosphate), tryptophan and two molecules of acetic acid[1,3] The suggested biological introduction of a mevalonate derived C_5 unit into the 4-position of an indole can be similar to that known to occur, *e g* in the biosynthesis of the lysergic acid portion of the ergot alkaloids in Claviceps species The condensation of tryptophan and two molecules of acetic acid (or acetoacetate) to form a substituted tetramic acid could be analogous to the formation of tenuazonic acid from L-isoleucine and two molecules of acetic acid in *Alternaria tenuis* Auct[6] Tryptophan has been recognized as a common precursor of the complex indole alkaloids[7–14] The cyclopiazonic acids[3,4], tenuazonic acid and erythroskyrene[15] are the only presently known examples of substituted tetramic acids occurring as natural products

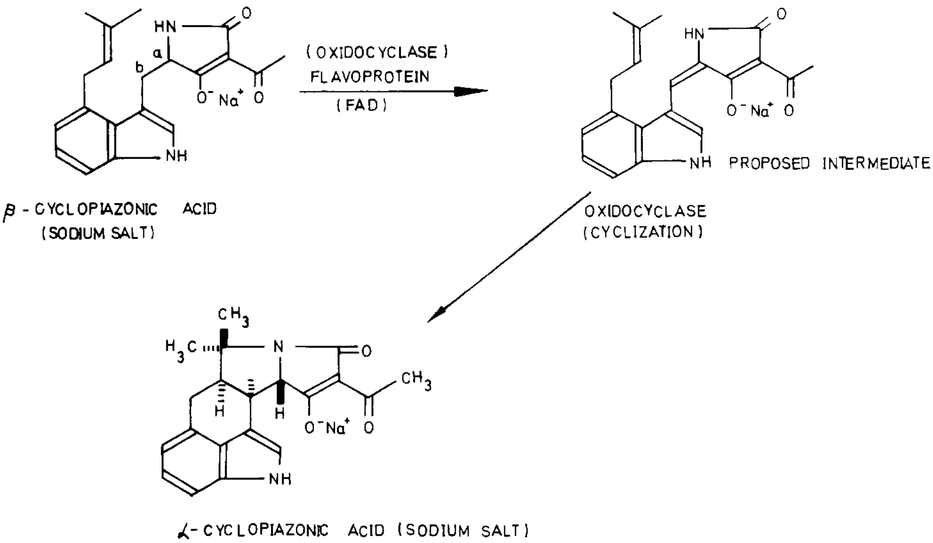

Fig 1 The proposed reactions in the enzyme-catalysed conversion of β- into α-cyclopiazonic acid

α-Cyclopiazonic acid is formed in shake cultures on Czapek–$NaNO_3$ media supplemented with trace elements[1] When trace elements were omitted from the media, a considerable reduction in the quantity of α-cyclopiazonic acid produced was found and β-cyclopiazonic acid accumulated. β-Cyclopiazonic acid was produced in the logarithmic phase of the growth curve and then disappeared during later stages of growth, whereas α-cyclopiazonic acid was produced in complete media just after the logarithmic phase. These facts, as well as the 60% incorporation of ^{14}C-labelled β-cyclopiazonic acid into α-cyclopiazonic acid, indicate clearly that β-cyclopiazonic acid is a precursor of α-cyclopiazonic acid[1].

It has been proposed that the conversion of β- into α-cyclopiazonic acid occurs in two steps. The first step should be the formation of a double bond, possibly at the position (a–b) indicated in the structure of a proposed intermediate compound (Fig. 1), and the second should be a cyclization reaction occurring with or without the participation of an enzyme. The formation of a double bond nearer to the indole system is another and perhaps more acceptable possibility.

The main purpose of the work reported here was to find, isolate and study the enzyme responsible for the conversion of β- into α-cyclopiazonic acid in shake cultures of *P. cyclopium* Westling in media fully supplemented with trace elements. The isolation and purification was achieved. Five isozymes were found that could catalyze both the dehydrogenation as well as the cyclization reaction. It was decided to call these enzymes the β-cyclopiazonate oxidocyclases. Knowledge of the biochemical, and biophysical properties of these enzymes, which catalyze these unique reactions should be of interest to biochemists studying other complicated oxidative cyclization reactions. A short preliminary report of some of the work reported here has been presented at an IUPAC symposium in Johannesburg, South Africa in 1969.

MATERIALS AND METHODS

Materials

P. cyclopium Westling (strain 1082) was grown in shake cultures on Czapek–$NaNO_3$ media[1]. The mycelium was harvested after 5 days.

α-Cyclopiazonic acid was isolated from cultures of *P. cyclopium* Westling (strain 1082) on sterilized wet maize meal as described by HOLZAPFEL[3]. β-Cyclopiazonic acid was isolated from shake cultures of *P. cyclopium* Westling (strain 1082) grown in aqueous media described elsewhere[14].

Sephadex G-100 and G-200, CM-Sephadex C50 and blue dextran 2000 were obtained from Pharmacia, Uppsala, Sweden and DEAE-cellulose from Bio-Rad Laboratories. Cytochrome *c*, lactate dehydrogenase, and NAD^+ were obtained from Boehringer, Mannheim, Germany. Phenazine methosulphate was obtained from Sigma Chem., St. Louis, Mo., U.S.A., bovine serum albumin from Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A., 2,6-dichlorophenolindophenol (DCIP) from E. Merck, Darmstadt, Germany and dialysis tubing from the Fisher Scientific Company.

All other chemicals used were of analytical grade quality.

Assay methods

Assay methods for the dehydrogenation reaction

Spectrophotometric method employing DCIP as terminal electron acceptor. DCIP was found to be a very effective terminal acceptor of electrons in the dehydrogenation of β-cyclopiazonic acid by β-cyclopiazonate oxidocyclase The decrease in absorbance at 600 nm, measured in a Beckman model DK2A ratio recording spectrophotometer employing the time drive attachment, was used to calculate the dehydrogenating activity The nmoles of DCIP reduced and thus nmoles β-cyclopiazonic acid oxidized during the reaction were calculated from the molar absorbance coefficient of DCIP at 600 nm which is given as 19 000 M^{-1} cm^{-1} (see refs 16 and 17) The velocity of the reaction was expressed in nM min^{-1} A decrease of 0 001 in absorbance per min at 600 nm was employed as an arbitrary unit of activity during the isolation of the enzyme Activity was also expressed in nmoles DCIP reduced or β-cyclopiazonic acid oxidized per min and specific activity in nmoles/min per mg protein

Standard reaction mixtures used for this assay method contained 105 nmoles sodium maleate (pH 6 6), 180 nmoles DCIP, 0 3 nmole β-cyclopiazonic acid in 6 7% (v/v) methanol (final concn) and the enzyme preparation in the sample cell with a final volume of 3 0 ml In the reference cell β-cyclopiazonic acid was omitted in most cases but in some cases the enzyme was omitted and replaced by buffer The final pH of the reaction mixture was 6 82 and the reaction was initiated by the addition of β-cyclopiazonic acid The temperature was regulated at 25° by the temperature regulated cell holder of the Beckman model DK2A spectrophotometer except where indicated otherwise The amount of enzyme taken was such that the reaction was zero order with respect to both DCIP and β-cyclopiazonic acid, so that a linear relationship between the dehydrogenating activity and enzyme concentration was found An amount of enzyme with a dehydrogenating activity of 100 arbitrary units still fell in this range This value was never exceeded in dehydrogenating activity measurements

Although β-cyclopiazonic acid is soluble in $NaHCO_3$ solutions, it dissolves very slowly in buffered solutions at neutral pH values in spite of the fact that it may have already been neutralized by the addition of NaOH or $NaHCO_3$ It was found that enough β-cyclopiazonic acid can dissolve in 5 to 6 7% (v/v) methanol (final concn) to ensure zero order kinetics with respect to this compound under the experimental conditions used The compound was dissolved in pure methanol and added to the reaction mixture in 0 2 ml methanol (0 3 nmole β-cyclopiazonic acid was usually taken) When employing a 10% (v/v) final concentration of methanol the reaction was inhibited about 10% in comparison with a final concentration of 5% (v/v)

Spectrophotometric method employing cytochrome c as terminal electron acceptor Dehydrogenating activity determinations, employing cytochrome *c* as a terminal electron acceptor, were performed under identical conditions as described for the spectrophotometric method employing DCIP as a terminal electron acceptor The only difference in the composition of the reaction mixtures was that 0 18 nmole cytochrome *c* was substituted for DCIP The increase in absorbance at 550 nm was used as a measure of activity When 1 μmole phenazine methosulphate (PMS) was also included in the reaction mixtures, the dehydrogenating activity expressed in μmoles cytochrome *c* reduced or β-cyclopiazonic acid dehydrogenated per min, was about 84% of that obtained with DCIP The activity expressed in μmoles/min was calculated by using the molar absorbance index difference between the fully reduced and fully oxidized cytochrome *c* at 550 nm at pH 7 0 of 19 700 M^{-1} cm^{-1} (see refs 16 and 18) The ε at 550 nm (reduced — oxidized) of cytochrome *c* has been determined as 21 000 by MASSEY[19] and as 21 200 by VAN GELDER AND SLATER[20]

Assay methods for the conversion of β-cyclopiazonic acid into α-cyclopiazonic acid

Ehrlich method. α-Cyclopiazonic acid gave a purple-blue Ehrlich colour reaction which suggested the presence of an indole system, unsubstituted in the α- or β-position[3,21]. β-Cyclopiazonic acid did not give a colour reaction with the Ehrlich reagent most probably because of the stereochemical reasons suggested by HOLZAPFEL *et al.*[4]

A standard incubation mixture used for the Ehrlich assay method contained 105 μmoles sodium maleate (pH 6.6), 1 μmole PMS, 0.6 μmole β-cyclopiazonic acid in 6.7% (v/v) methanol (final concn.) and enzyme preparation in a total volume of 3 ml. The final pH was 6.82. The reaction mixtures were incubated at 37° in a water-bath. The reaction was initiated by the addition of β-cyclopiazonic acid and was stopped at different time intervals by means of the chloroform extraction method for cyclopiazonic acids. This was performed by the addition of 7 ml distilled water, a few drops of concentrated HCl and two consecutive extractions with equal volumes of chloroform in a separating funnel. The chloroform solutions were combined and the chloroform evaporated in a rotary evaporator. The residue was dissolved in 2 ml methanol followed by the addition of 1 ml 10% (v/v) 4-dimethylaminobenzaldehyde (Ehrlich reagent) in methanol and 2 ml concentrated HCl. The absorbance of this solution was read after 15 min at 580 nm in a Beckman model DB spectrophotometer. The amount α-cyclopiazonic acid formed was obtained from a standard curve. The recovery of α-cyclopiazonic acid by the above-mentioned extraction procedure was about 98%.

Enzyme activity was expressed as μmoles α-cyclopiazonic acid formed per min and the specific activity as μmoles/min per mg protein.

Paper chromatography. The α-cyclopiazonic acid formed in reaction mixtures containing β-cyclopiazonic acid was identified by paper chromatography described elsewhere[1,4] after extraction from incubation mixtures with chloroform as described above.

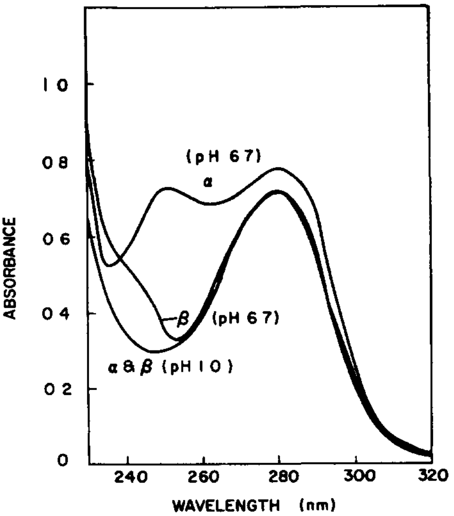

Fig. 2. Ultraviolet absorption spectra of α-cyclopiazonic acid and β-cyclopiazonic acid in diluted HCl solutions (pH 1.0) and 0.02 M sodium phosphate buffer (pH 6.7). The concentration of these compounds was about 0.1 μmole in 3 ml solution which also contained 5% (v/v) methanol.

Ultraviolet spectrophotometry The ultraviolet spectra of α- and β-cyclopiazonic acids were determined in aqueous solutions containing 5% (v/v) methanol using a Beckman model DK2A ratio recording spectrophotometer A marked difference in their spectra in the 250-nm region was observed at pH values higher than the pK_a of these acids, which is about 3 5 (Fig 2) At pH 1 0 both acids showed only one absorption peak at 281 nm but at pH 6 7 (or any pH value from 4 5 upwards) α-cyclopiazonic acid showed absorption maxima at 281 and 252 nm whereas β-cyclopiazonic acid showed a peak at 281 nm and a shoulder at 246 nm This difference was used to identify α-cyclopiazonic acid extracted from reaction mixtures and separated from β-cyclopiazonic acid (substrate) on paper chromatograms

The pK_a of α-cyclopiazonic acid was determined by plotting the change in absorbance at 252 nm *versus* pH A pK_a of 3 45 was found

Mass spectrometry α-Cyclopiazonic acid was extracted from reaction mixtures and separated from β-cyclopiazonic acid (substrate) on paper chromatograms Unsprayed strips of the paper chromatograms containing α-cyclopiazonic acid were extracted with chloroform The chloroform extracts were extracted with a $NaHCO_3$ solution, after which the extract was acidified with HCl and the α-cyclopiazonic acid extracted with chloroform The chloroform was evaporated in a rotary evaporator The α-cyclopiazonic acid obtained from the paper chromatograms seemed to be free of any contaminating formamide The mass spectrum recorded on a MS-9 spectrometer showed a strong molecular ion (m/e 336) and prominent fragment ions at m/e 154, 155, 181, 182 and 196 similar to that reported for α-cyclopiazonic acid by HOLZAPFEL[3] These fragments must contain the indole system and three additional carbon atoms

Microzone electrophoresis

A Beckman microzone electrophoresis system employing the Beckman microzone electrophoresis cell model R-101 was used for microzone electrophoresis of enzyme preparations on cellulose acetate films as described in the Beckman model R microzone electrophoresis cell instruction manual RM-IM-3 The membranes were stained with Ponceau S as described in the instructions and finally scanned with a Beckman model R8 Analytrol densitometer with the microzone attachment

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed by a modification[22,23] of the method described by REISFELD *et al* [24] The method only differed from the one described by FAMBROUGH AND BONNER[22] in that a 0 1% ammonium persulphate solution instead of a 0 2% solution was used

Analytical ultracentrifugation

The homogeneity of the purified isoenzymes was investigated in a Spinco model E ultracentrifuge Sedimentation coefficients were determined by the sedimentation-velocity method[25] Diffusion coefficients were measured as described by MOLLER[26] An An-D rotor with a single sector, quartz, plain window, analytical cell with aluminium centre piece was used in conjunction with the absorption optical system

The isolation and purification of the five isoenzymes of β-cyclopiazonate oxidocyclase

The mycelium of *P. cyclopium* Westling, grown in shake cultures under the conditions for the production of α-cyclopiazonic acid[1], was used as a source of these isoenzymes. All isolation procedures were carried out at 0 to 5°. β-Cyclopiazonate oxidocyclase activity was determined after each step (dehydrogenating activity by the spectrophotometric method with DCIP as terminal electron acceptor as well as the total conversion of β- into α-cyclopiazonic acid by the Ehrlich method). Protein was determined after each step by a standardized method of Lowry and total nitrogen by a micro-Kjeldahl method[28].

Extraction. Step 1. The mycelium, harvested by filtration, was kept at −10° for different periods, thawed, suspended in 5 parts (w/v) 0.05 M sodium maleate buffer (pH 6.6) and homogenized in a Sorvall Omnimix blender for 3 min at maximum speed. The homogenate, suspended in 2 parts (v/v) of the above-mentioned buffer, was then treated for 8 to 10 min in an Edmund Buhler vibrator using glass beads (about 500 μm in diameter) in a ratio to the suspension of 3:1 (v/v) at 0–5°. The suspension was decanted from the glass beads, pressed through 8 layers of cheese cloth and centrifuged at 44 000 × *g* for 30 min in a Spinco Model L-4 ultracentrifuge. The supernatant solution contained the enzymes. Ultrasonification in the presence of glass powder or grinding of the mycelium with sand in a mortar were less effective when employed as cell breaking methods. Omitting the vibrator step only 15–20% cell breakage was found. An increase of 15–20% in enzyme activity per g of mycelium and a 6-fold increase in the total amount of protein was found when the vibrator step was included. The culture filtrate contained enzymic activity equivalent to about 10% of that obtained after maximum cell breakage. These results indicate the possible association of the enzymes with mycelium cell walls.

$(NH_4)_2SO_4$ fractionation. Step 2. Dry, powdered $(NH_4)_2SO_4$ was added over a period of 30 min to the above mentioned supernatant solution to 25% saturation. This solution was left in an ice-bath for 30 min and then centrifuged at 20 000 × *g* for 20 min in a Spinco model L-4 ultracentrifuge. The precipitate was discarded and $(NH_4)_2SO_4$ was added to the supernatant solution over a period of 30 min to a final saturation of 94%. The preparation was left overnight in a cold room at 2–3° and then centrifuged at 20 000 × *g* for 20 min. The precipitate was suspended in 10–15 ml 0.05 M sodium maleate buffer (pH 6.6) per 100 g of mycelium and dialysed overnight against 2 l of the above-mentioned buffer, changing the buffer twice. The diffusate was centrifuged at 50 000 × *g* for 20 min and the sediment discarded. The protein fraction precipitated between 25 and 94% saturation contained nearly all the enzyme activity. $(NH_4)_2SO_4$ fractionation between 25 and 94% saturation also fractionated the enzymic activity. The amount of $(NH_4)_2SO_4$ to be added in order to obtain the required saturation at 0° was calculated by using the KUNITZ[29] relationship as employed by DI JESSO[30].

Ethanol fractionation, pH and heat precipitation yielded unsatisfactory results.

DEAE-cellulose chromatography. Step 3. A column of DEAE-cellulose (capacity 0.6 mequiv/g), 38 cm × 2.5 cm, was prepared and equilibrated with 0.05 M sodium maleate buffer (pH 6.6). About 150–300 mg of protein obtained in Step 2 were applied to the column. After 300 ml of the above-mentioned buffer had passed through, a linear increase in molarity from 0.05 to 0.5 M over 400 ml at pH 6.6 was effected with 0.45 M NaCl by employing a linear concentration gradient. An example of an elution diagram obtained when 238 mg of protein (about 50 ml), obtained in Step 2 was applied to a DEAE-cellulose column is given in Fig 3 Contents of tubes containing β-cyclopiazonate oxidocyclase activity of 20 and more arbitrary units/ml were combined Since lyophilization caused denaturation, the enzyme preparation was concentrated by sucrose dehydration Dialysis bags containing the enzyme were covered with sucrose, which was replaced when necessary until enough of the buffered solution had been withdrawn This procedure was performed at 2–3° The concentrated protein solution was then dialysed overnight against 0 05 M sodium maleate buffer (pH 6 6), changing the buffer solution twice In this manner the enzyme preparation could be concentrated 3–4-fold and in case this was not sufficient the procedure, which took only 2–5 h depending on the volume, could be repeated

Sephadex G-100 gel chromatography Step 4 A Sephadex G-100 (Medium) column, 86 cm × 2 5 cm, was packed under gravitation in 0 05 M sodium maleate buffer (pH 6 6) The enzyme preparation obtained in Step 3 (about 10–15 ml) was applied to the column, which was then eluted with the above-mentioned buffer at a flow rate of 18–20 ml/h An example of the elution diagram is given in Fig 4 in which 98 mg protein was applied to the column Fractions containing enzyme activity of 40 arbitrary units or more were combined and concentrated by means of the sucrose method and then dialysed against 0 005 or 0 02 M sodium phosphate buffers (pH 6 7) As indicated in Fig 4, the enzymes were eluted in the third and last protein peak A much lower purification factor was obtained in this step in cases where the vibrator step was omitted in the extraction step

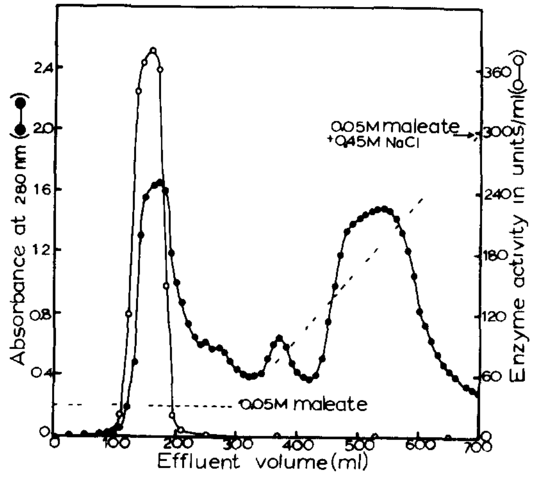

Fig 3 Purification of β-cyclopiazonate oxidocyclase on DEAE-cellulose The column was developed at a flow rate of 10–14 ml/h and 4-ml fractions were collected Protein concentration was determined spectrophotometrically at 280 nm against 0 05 M sodium maleate buffer (pH 6 6) in the reference cell of the Beckman model DK2A spectrophotometer Enzyme activity is expressed in arbitrary units as defined when employing DCIP as terminal electron acceptor Similar activity distribution was obtained by the Ehrlich method A linear concentration gradient was used as indicated

CM-Sephadex C50 chromatography Step 5 Columns of CM-Sephadex C50

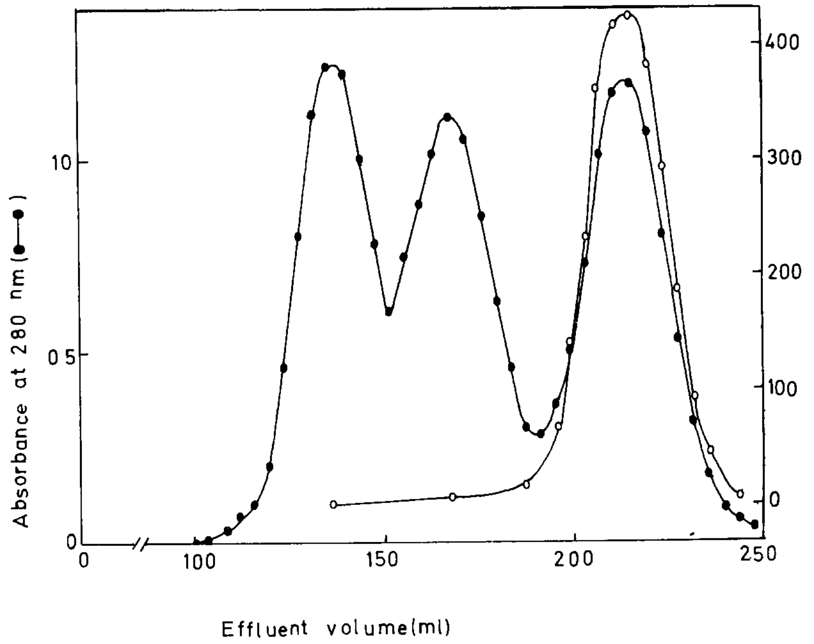

Fig 4 Purification of β-cyclopiazonate oxidocyclase on Sephadex G-100 The effluent was collected in 4-ml fractions Protein concentration and enzyme activity were determined and expressed as described in the legend to Fig 2

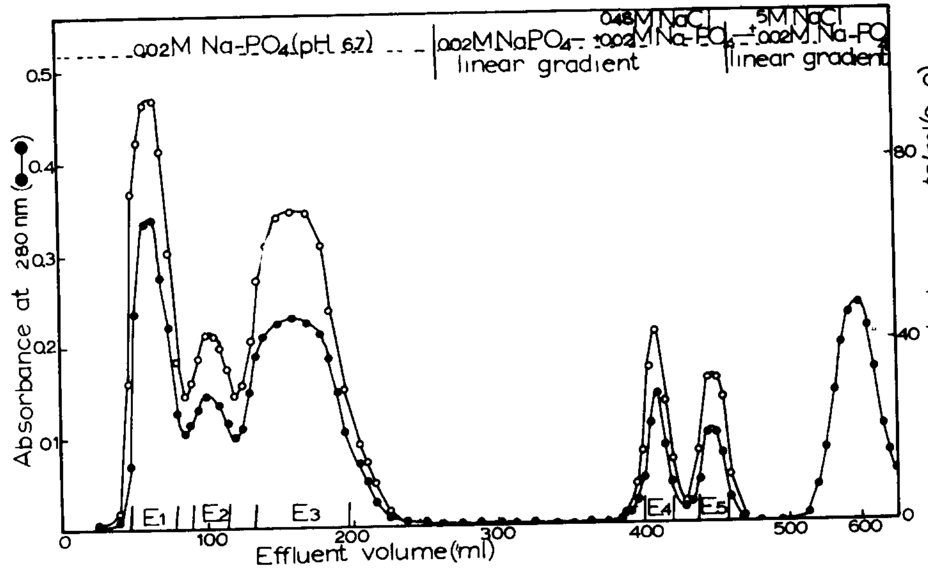

Fig 5 An example of an elution diagram obtained from CM-Sephadex C50 chromatography employing linear salt concentration gradients starting with 0 02 M sodium phosphate buffer (pH 6 6) About 45 mg protein (20 ml) was applied to the column (2 5 cm × 35 cm) The flow rate was 15–18 ml/h Protein concentration and enzyme activity were determined and expressed as described in the legend to Fig 2

(capacıty 4 5 mequıv/g, partıcle sıze 40–120 μm), 35 cm × 2 5 cm, were packed and equılıbrated ın 0 02 M sodıum phosphate buffer (pH 6 7) and 2 5 cm × 85 cm columns ın 5 mM of the same buffer The columns were eluted wıth the approprıate buffers and by usıng consecutıve salt concentratıon gradıents as ındıcated ın Fıgs 5 and 6, after applıcatıon of the enzyme preparatıons obtaıned ın Step 4 Fıve peaks wıth enzyme actıvıty were obtaıned The fractıons of each peak were combıned as ındıcated (by vertıcal lınes) ın Fıg 5 In the case of Fıg 6, fractıons contaınıng more than 10 arbıtrary unıts of enzyme actıvıty (dehydrogenatıng actıvıty) were combıned A sıxth peak contaınıng no enzyme actıvıty was also eluted from the columns ın both cases In all experıments the relatıve ratıo of the five ısoenzymes obtaıned ın Step 5 showed no varıatıon from those ındıcated ın Fıgs 5 and 6 and Table I Even ın those cases where the relatıve amount of cells broken was 15–20% (Step 1), the relatıve ratıo remaıned the same but the amount of proteın eluted ın the sıxth peak was less and the final enrıchment was also much less

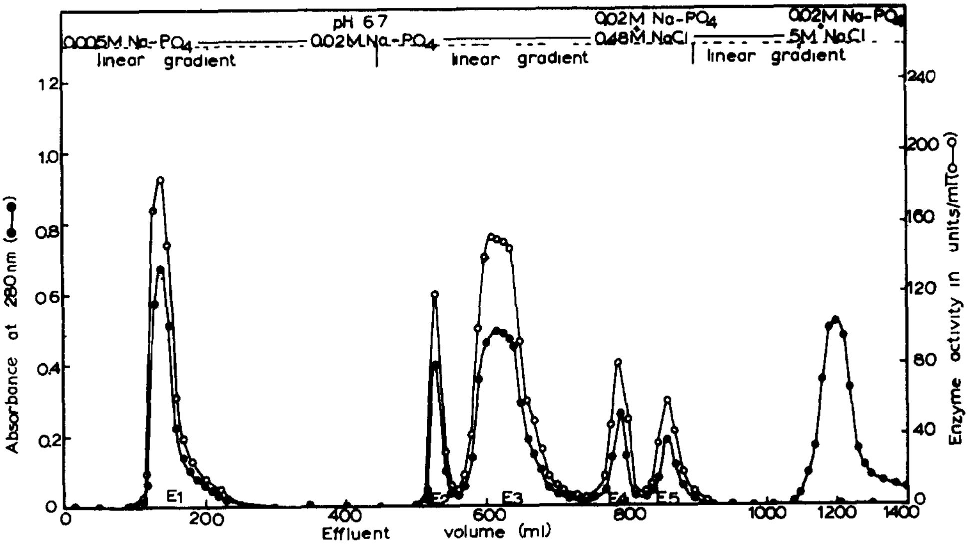

Fıg 6 Purıfication of β-cyclopıazonate oxıdocyclase by CM-Sephadex C50 chromatography employıng lınear salt concentratıon gradıents startıng elutıon wıth 5 mM sodıum phosphate buffer (pH 6 6) About 90 mg proteın was applıed to the column (2 5 cm × 85 cm) The flow rate was 15–18 ml/h Proteın concentratıon and enzyme actıvıty was determıned and expressed as descrıbed ın the legend to Fıg 2

The specıfic actıvıtıes of the five ısoenzymes, as well as the purıfication factors obtaıned ın Step 5 for each of them were sımılar (Table I)

An average final purıfication factor for the five ısoenzymes of 133-fold was obtaıned (the hıghest for enzyme III (139-fold) the lowest for enzyme II (124-fold) The average specıfic actıvıty was 58 4 nmoles/mın per mg proteın (enzyme III havıng the hıghest specıfic actıvıty and enzyme II the lowest) The final total yıeld of enzyme actıvıty was 64% The total amount of enzyme proteın obtaıned from 350 g mycelıum (wet wt) was 33 7 mg

TABLE I

SUMMARY OF RESULTS OBTAINED IN THE COURSE OF THE PURIFICATION OF β-CYCLOPIAZONATE OXIDOCYCLASE

At each step the dehydrogenating activity as well as the rate of conversion of β- into α-cyclopiazonic acid were determined. Since the respective reaction rates were similar, the spectrophotometric method employing DCIP as terminal electron acceptor was more often used due to its higher accuracy and sensitivity and because it was less time-consuming.

Purification step	*Total protein (mg)*	*Total activity (nmoles/min)*	*Specific activity (nmoles/min per mg protein)*	*Yield (%)*	*Purification (-fold)*
1 Extraction	7000	3080	0.44	100	1.0
2 Differential centrifugation	2670	2937	1.10	95	2.5
3 $(NH_4)_2SO_4$ fractionation	284	2784	9.80	90	22.3
4 DEAE-cellulose chromatography	130	2310	17.84	75	40.5
5 Sephadex G-100 gel chromatography	53	2091	39.42	68	89.6
6 CM-Sephadex C50 chromatography					
Isoenzyme I	8.2	468	57.1	15	129.8
Isoenzyme II	7.0	383	54.5	12	123.9
Isoenzyme III	14.1	858	61.1	28	138.9
Isoenzyme IV	1.7	102	59.3	3.3	134.8
Isoenzyme V	2.7	162	59.4	5.3	135.0
Total (I–V)	33.7	1973	58.4	64	132.7

RESULTS AND DISCUSSION

Assay methods

Dehydrogenation of β-cyclopiazonic acid

When α-cyclopiazonic acid was used as substrate for dehydrogenation no activity was recorded. The enzyme contained a flavin prosthetic group which was reduced by β-cyclopiazonic acid but not by α-cyclopiazonic acid[31]. The results clearly demonstrate that these enzymes can act as catalysts for the dehydrogenation of a C–C bond. Blanks without enzyme showed no activity. In the absence of β-cyclopiazonic acid no reduction of DCIP was observed.

α-Cyclopiazonic acid was extracted with chloroform at different time intervals from reaction mixtures for the determination of the dehydrogenating activity and its concentration determined colorimetrically by means of the Ehrlich method. The amount of α-cyclopiazonic acid formed was equal to the amount of β-cyclopiazonic acid dehydrogenated per unit time. This fact indicated that the enzymes are capable of catalysing both the dehydrogenation and cyclization reaction at approximately the same rate.

When PMS (1 μmole) was included in the reaction mixtures (both cells) an increase of about 10% in the dehydrogenating activity was observed which indicated that PMS can act as an intermediate electron acceptor between the enzyme and DCIP.

The presence of dissolved oxygen in no way interfered with the spectrophotometric assay using DCIP. The activity under anaerobic conditions, determined in

Thunberg cuvettes in the presence of nitrogen, was similar to that found under aerobic conditions ARRIGONI AND SINGER[32] reported a similar result in the assay of succinate dehydrogenase which is a flavin containing enzyme also

Spectrophotometric assay procedures using cytochrome *c* as a terminal electron acceptor are particularly well-known for succinic dehydrogenase[16,33] The use of cytochrome *c* as a terminal electron acceptor was particularly useful for optimum pH determinations below pH 6 4 because the absorbance of DCIP at 600 nm decreases rapidly below this value and below pH 5 9 it loses its colour completely DCIP could, however, be used down to pH 6 1, employing calculated absorbance indexes at each pH value below pH 6 4

Blank determinations as performed with DCIP gave no activity with the cytochrome *c*-PMS system With α-cyclopiazonic acid as substrate no dehydrogenation activity was recorded

No dehydrogenating activity was obtained employing ferricyanide as a terminal electron acceptor, most probably due to inhibition by Fe^{3+} (ref 31)

Results obtained with other electron carriers, electron acceptors and cofactors are reported elsewhere[31]

Conversion of β-cyclopiazonic acid to α-cyclopiazonic acid

Blank determinations without enzyme employing the Ehrlich method showed no activity

α-Cyclopiazonic acid formed in incubation mixtures was identified by paper chromatography The R_F values were 0 54 and 0 23 for α- and β-cyclopiazonic acid, respectively

α-Cyclopiazonic acid formed by the reactions catalysed by the β-cyclopiazonate oxidocyclases was also identified by ultraviolet absorption and mass spectrometry The decrease in molecular weight from 338 for β-cyclopiazonic acid to 336 for α-cyclopiazonic acid clearly indicates that β-cyclopiazonic acid was dehydrogenated by the action of β-cyclopiazonate oxidocyclase during the conversion of β- into α-cyclopiazonic acid

A linear relationship was found between reaction velocity and enzyme concentration for both the dehydrogenation reaction and the conversion of β- into α-cyclopiazonic acid

Optimum pH

The optimum pH determined by the spectrophotometric assay methods employing DCIP and cytochrome *c* as terminal electron acceptors as well as the Ehrlich method was found to be about 6 8 The five isoenzymes showed the same optimum pH for both the dehydrogenation as well as the total conversion of β- into α-cyclopiazonic acid The optimum pH curves for isoenzyme III are shown in Fig 7

Stability

The five isoenzymes of β-cyclopiazonate oxidocyclase were found to retain 90% of their activity after 2 months at 0–4° in 0 05 M maleate buffer (pH 6 6) containing 0 05 M NaCl or in 0 02 M sodium phosphate buffer (pH 6 7) containing 0 08 M NaCl

The temperature stability of the five isoenzymes was essentially the same They lost all their activity after heat treatment for 10 min at 75 5° in a constant temperature water-bath but retained 70% of their activity after 10 min at 55°

Lyophilization caused a loss of about 50% of the activity in some cases The effects of urea on the activities of the isoenzymes were also studied by the DCIP and Ehrlich assay methods 10 M ice-cold urea solutions were added to enzyme preparations in 0 05 M sodium maleate buffer (pH 6 6), containing 0 05 M NaCl, up to final urea concentrations of 3 and 6 M, respectively These solutions were kept at 0° in an ice-bath Enzyme activity was determined on aliquots taken after 1 5, 15 and 30 min 6 M urea caused the loss of at least 90% of the activity of all the isoenzymes after 1 5 min and 15 min and nearly 100% after 30 min Approx 37–48% decrease in activity of all the isoenzymes was found in 3 M urea at 1 5, 15 as well as 30 min The urea concentration in the reaction mixtures for assaying enzyme activity was 0 2 or 0 4 M depending on whether 3 or 6 M urea–enzyme solutions were assayed With the DCIP method it was observed that the activities started to increase after 5–10 min in the diluted urea solution of the reaction mixture After 15 min an increase of about 20–25% was observed This indicated that the denaturation of the isoenzymes by urea is a reversible process as observed for many other proteins[34] The denaturation by heat, however, was irreversible

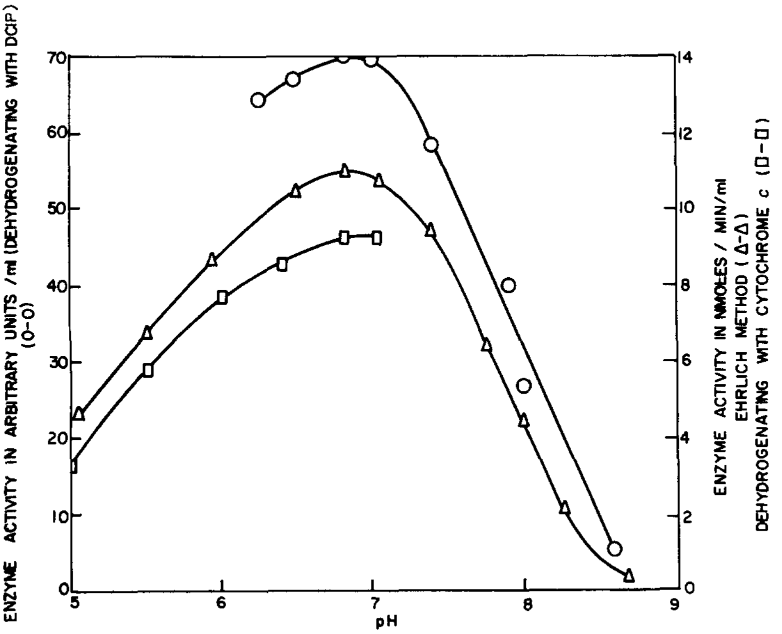

Fig 7 Optimum pH of dehydrogenation and total conversion of β- into α-cyclopiazonic acid for purified isoenzyme III About 12 μg purified enzyme was used per ml of the reaction mixture The assay procedure is described in the text

Homogeneity of the β-cyclopiazonate oxidocyclases

The five isoenzymes obtained in Step 5 of the purification procedure appeared to be homogeneous and to differ only slightly in their electrophoretic mobility towards the cathode at the pH values employed in microzone and polyacrylamide gel electrophoresis (Figs 8 and 9) A difference in the amino acid composition, especially the basic amino acids and amide content, was found, which may be related to the observed difference in electrophoretic mobility

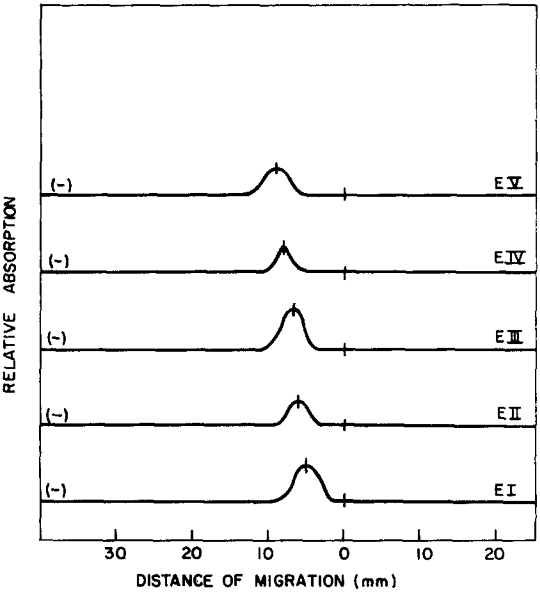

Fig 8 Densitometer tracings of the microzone electrophoretograms of the five isoenzymes of β-cyclopiazonate oxidocyclase (E I to E V) at pH 4 85 in 0 1 M sodium maleate buffer containing 0 1 M NaCl at 200 V and 14–18 mA Between 8 and 15 μg of each isoenzyme were applied to the cellulose acetate films in 40–50 μl buffer Densitometer tracings of the Ponceau S stained membranes are illustrated in the figure A similar pattern was obtained at pH 6 6 in 0 05 M sodium maleate buffer containing 0 1 M NaCl

Microzone electrophoresis was performed at pH 4 85, 6 7 and 8 6 and similar patterns were obtained It was evident that the isoenzymes were all positively charged even at a pH value as high as 8 6 They differed only slightly in the magnitude of their positive charge as could be predicted from results obtained from CM-Sephadex C50 chromatography

Results obtained from polyacrylamide gel electrophoresis performed at pH 4 5 and 6 7 also indicated that the five isoenzymes were homogeneous and differed in the magnitude of their positive charge Single sharp bands were obtained for each of the five isoenzymes The isoenzymes were numbered in increasing order of positive charge Similar patterns were obtained at pH 4 8 and 6 7

Combined preparations of the five isoenzymes could not be separated effectively by microzone or polyacrylamide gel electrophoresis The nonactive peak 6 obtained by CM-Sephadex chromatography was much more positively charged than the five isoenzymes

The homogeneity of the five isoenzymes was also illustrated by the sharpness

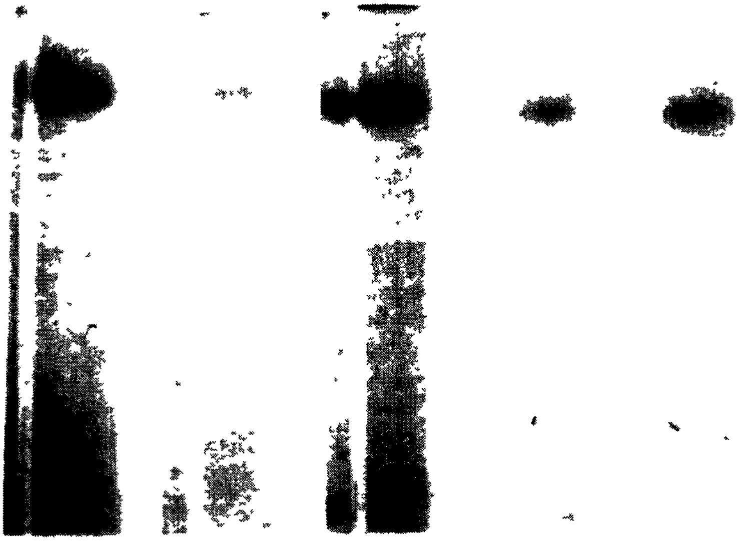

Fig 9 Polyacrylamide gel electrophoresis of the five isoenzymes (I–V) of β-cyclopiazonate oxidocyclase at pH 4 5 The 15% acrylamide gel used was prepared by a method refered to in the text Cathode at the bottom About 15, 8, 25, 10 and 8 μg of isoenzyme I, II, III, IV and V were applied to the respective gels in 25% (v/v) sucrose Similar patterns were obtained at pH 6 7

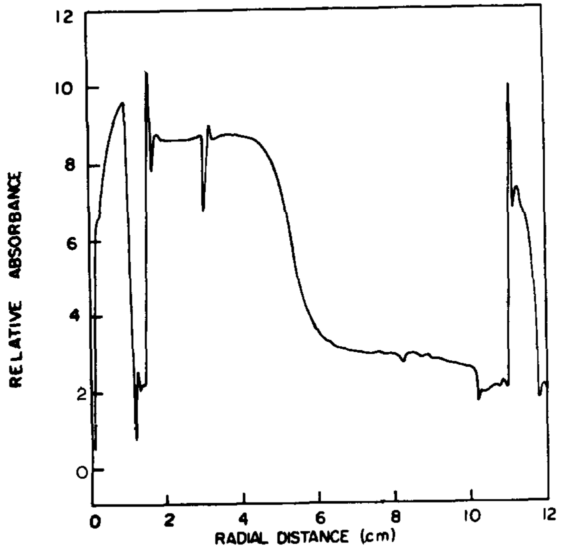

Fig 10 A densitometer tracing of a photograph taken after 56 min of centrifugation at 59 780 rev /min during a sedimentation velocity experiment with isoenzyme III of β-cyclopiazonate oxidocyclase The enzyme concentration was 765 μg/ml and the exposure time 10 sec

of the single sedimenting boundaries obtained in sedimentation velocity experiments by means of the absorption optics (see Fig 10 for an example of isoenzyme III)

Molecular weights and other physical properties of the β-cyclopiazonate oxidocyclases

Since all the enzyme activity was found in one symmetrical peak after Sephadex G-100 gel chromatography, it was reasonable to assume that if any isoenzymes were involved, their molecular weights should be similar This has been verified by analytical ultracentrifugation and gel chromatography on Sephadex G-200

Columns (50 cm × 2 5 cm) of Sephadex G-200 were used to estimate the molecular weights of the isoenzymes from their elution volume as described by ANDREWS[35] Cytochrome *c*, bovine serum albumin and lactate dehydrogenase of pig heart were used as reference proteins and blue dextran 2000 for measuring the void volumes (V_0) of the columns In Fig 11 the elution volumes (V_e) of the reference proteins and the isoenzymes are plotted against $\log_{10}$(mol wt) of these proteins as well as the isoenzymes Separate preparations of the five isoenzymes and combined preparations of these isoenzymes were eluted from Sephadex G-100 and G-200 columns in one single symmetrical peak with an estimated molecular weight (according to ANDREWS[35]) of 47 000, 50 000, 49 000, 48 000 and 48 000 for isoenzymes I, II, III, IV and V, respectively, at pH 6 6 as well as 7 5

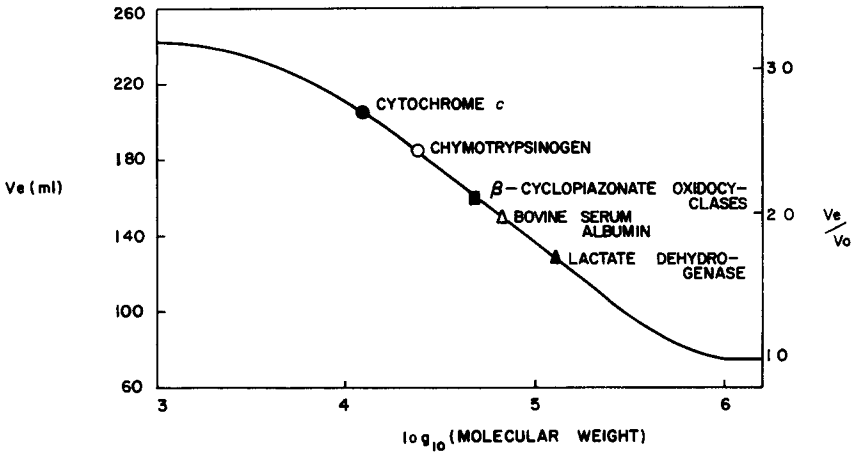

Fig 11 Plot of elution volume (V_e) against $\log_{10}$ (mol wt) for proteins including the β-cyclopiazonate oxidocyclases on a Sephadex G-200 column (50 cm × 2 5 cm) at pH 7 5 The V_e and log(mol wt) values for chymotrypsinogen were not determined but taken from the ANDREWS'[35] plot β-Cyclopiazonate oxidocyclase activity was determined in the effluent by the spectrophotometric method employing DCIP as terminal electron acceptor, cytochrome *c* concentration was determined by absorbance at 412 nm, blue dextran 2000 by absorbance at 625 nm and lactate dehydrogenase activity by NAD^+ reduction measured spectrophotometrically at 340 nm[36] Concentrations of serum albumin as well as the other proteins were determined by absorbance at 280 nm

Sedimentation coefficients ($s_{20,u}$ values) were calculated employing the partial specific volumes (0 73 cm^3/g) calculated from the amino acid composition[37] according to the procedure described by SCHACHMAN[25] $s^0_{20,w}$ values, obtained from plots of

TABLE II

SOME PHYSICAL CONSTANTS OF THE ISOENZYMES OF β-CYCLOPIAZONATE OXIDOCYCLASE

Isoenzyme	$s^{\circ}_{20,w}$ $\times 10^{-13}$ *(sec)*	$D^{\circ}_{20,w}$ $\times 10^{-7}$ *(cm^2 sec^{-1})*	$\bar{v}$, *partial specific volume (cm^3 g^{-1})*	*Mol wt according to Svedberg*
I	3 8	7 2	0 73	48 700
II	4 1	7 2	0 73	52 500
III	3 9	7 3	0 73	49 900
IV	4 0	7 4	0 73	49 900
V	4 1	7 4	0 73	51 100

$1/s_{20,w}$ against enzyme concentration c, were independent of concentration The diffusion coefficients, corrected to $D_{20,w}$, were determined according to the method of MOLLER[26] and appeared to be independent of concentration Molecular weights were calculated by employing the SVEDBERG[37] equation The molecular weights and other physical constants are given in Table II It is evident that these constants of the isoenzymes are similar Gel-exclusion chromatography indicated that these isoenzymes did not associate at pH 6 6 and 7 5 The fact that they contain one molecule of flavin per molecule of enzyme[31] indicates that dissociation into smaller subunits is very unlikely Flavoprotein enzymes with molecular weights of the same order as that of the β-cyclopiazonate oxidocyclases are known[38–40]

Numbering of isoenzymes

In concurrence with the recommendation of the standing Committee of Enzymes of the International Union of Biochemistry for the description of multiple enzyme forms occurring in a single species[41], these five enzymes are called isoenzymes The numbering of the isoenzymes is in accord with the recommendation of WIEME[42] and WIELAND AND PFLEIDERER[43] *viz* that the numbering of isoenzymes should be in decreasing order of negative charge or (as adopted in this case) in increasing order of positive charge This numbering system has been accepted by the Standing Committee of Enzymes of the International Union of Biochemistry[41,44]

ACKNOWLEDGEMENTS

The authors would like to thank Dr C F de V Albrecht of the South African Council for Scientific and Industrial Research, Pretoria for assistance with certain polyacrylamide gel electrophoresis experiments, Mrs H A McGee for technical assistance, and the South African Council for Scientific and Industrial Research for financial assistance

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