Biochem. J. (1969) **114**, 463
Printed in Great Britain

Carbonic Anhydrase Isoenzymes in the Erythrocytes and Dorsolateral Prostate of the Rat

By J. E. A. McINTOSH*
Agricultural Research Council, Unit of Reproductive Physiology and Biochemistry, University of Cambridge

(*Received* 21 *April* 1969)

1. Three forms of the zinc-containing enzyme carbonic anhydrase (EC 4.2.1.1) were isolated from the erythrocytes of the rat and two forms from the dorsolateral prostate of the rat. Several additional minor components were observed but not isolated. Separation of the isoenzymes was achieved by ion-exchange chromatography, polyacrylamide-gel electrophoresis and isoelectric focusing. 2. The general properties of the isolated isoenzymes, their molecular weights and their contents of zinc were closely similar. As catalysts of the hydration of carbon dioxide, however, they were distinctly different. The two most abundant isoenzymes of the erythrocytes, which were found in equal proportions, differed 70-fold in specific activity, whereas the isoenzymes of the dorsolateral prostate were similar to one another and resembled the high-activity component of the erythrocytes. The inhibition of the latter by acetazolamide (5-acetamido-1-thia-3,4-diazole-2-sulphonamide) was mainly competitive, whereas in identical conditions the low-activity erythrocyte component and the dorsolateral prostate isoenzymes were non-competitively inhibited. 3. The use of chloroform–ethanol to remove haemoglobin from the rat haemolysate was found (*a*) to bring about changes in the kinetic properties of the soluble isoenzymes and (*b*) to cause the appearance of an additional isoenzyme. 4. The actions were compared of the inhibitors acetazolamide, 1,1-dimethylaminonaphthalene-5-sulphonamide and ethoxzolamide (6-ethoxybenzothiazole-2-sulphonamide) on the hydrolysis of *p*-nitrophenyl acetate catalysed by the isoenzymes. 5. The low-activity erythrocyte isoenzyme was an efficient catalyst of the hydrolysis of β-naphthyl acetate whereas the high-activity forms were much less active towards this ester. Neither of the isoenzymes present in the dorsolateral prostate catalysed this reaction. 6. Carbonic anhydrase in the rat dorsolateral prostate accounts for no more than 5% of the unusually high content of zinc in this organ.

Erythrocyte CA† has been found to occur in several species in the form of two or more isoenzymes (Lindskog, 1960; Nyman, 1961; Rickli & Edsall, 1962; Laurent, Marriq, Nahon, Charrel & Derrien, 1962; Tashian, 1965; Duff & Coleman, 1966; Furth, 1968). The human erythrocyte enzyme is particularly noteworthy because it can be resolved into two isoenzymes with markedly different catalytic efficiencies (Gibbons & Edsall, 1964). In addition to catalysing the reversible hydration of carbon dioxide, the erythrocyte enzyme accelerates the hydration of other carbonyl compounds (Pocker & Meany, 1965; Pocker & Dickerson, 1968), the hydrolysis of several types of ester (Pocker & Stone, 1965; Pocker & Stone, 1968*a*) and the hydrolysis of a sultone (Lo & Kaiser, 1966). CA has also been detected in tissues other than the erythrocytes, notably the gastric mucosa (Davenport, 1939), the central nervous system (Ashby & Chan, 1943), the kidney (Ashby, 1943) and both the male and the female reproductive tracts (Mawson & Fischer, 1952; Lutwak-Mann, 1955). Investigations in this laboratory are concerned with CA in certain tissues of the male and female reproductive organs, notably the prostate, uterus and placenta (McIntosh & Lutwak-Mann, 1967; Lutwak-Mann & McIntosh, 1969).

In the rat the prostate has a complex structure in which two major parts can be distinguished, the so-called ventral and dorsolateral lobes. The ventral prostate secretes a remarkably large quantity of citrate but no fructose, whereas the dorsolateral

* Postal address: Animal Research Station, 307 Huntingdon Road, Cambridge CB3 0JQ.

† Abbreviations: CA, carbonic anhydrase (EC 4.2.1.1); DNSA, 1,1-dimethylaminonaphthalene-5-sulphonamide.

prostate secretes both citrate and fructose (Humphrey & Mann, 1949). The dorsolateral prostate itself, however, consists of three parts, a dorsal part and two lateral lobes (Mann, 1964, p. 44). Striking biochemical characteristics of these lateral lobes are an unusually large content of zinc, that exceeds the concentration in all other soft tissues of the rat, and a high activity of CA equal to that in the erythrocytes (Mawson & Fischer, 1952).

The present paper compares the properties of CA occurring in the erythrocytes and dorsolateral prostate of the rat.

MATERIALS AND METHODS

Reagents. Tris (reagent grade), *p*-nitrophenyl acetate, β-naphthyl acetate, Fast Blue RR and soya-bean trypsin inhibitor were all obtained in a satisfactory state of purity from the Sigma (London) Chemical Co., London S.W.6. Diethylmalonic acid was prepared by the hydrolysis of diethyl diethylmalonate (Kodak Ltd., Liverpool). The method of Weber (1952) was used to prepare DNSA from the sulphonyl chloride (British Drug Houses Ltd., Poole, Dorset). DEAE-Sephadex A-50, Sephadex G-100 and Blue Dextran were all obtained from Pharmacia (G.B.) Ltd., London W.13. Coomassie Blue (Brilliant Blue R 250) was supplied by George T. Gurr Ltd., London S.W.6. Diamox (the sodium salt of acetazolamide, 5-acetamido-1-thia-3,4-diazole-2-sulphonamide, assumed to have an equivalent weight of 270; Whitney, Fölsch, Nyman & Malmström, 1967) was the product of the Cyanamid Co., Pearl River, N.Y., U.S.A., and ethoxzolamide (6-ethoxybenzothiazole-2-sulphonamide) was a gift from the Upjohn Co., Kalamazoo, Mich., U.S.A.; both inhibitors were used without further purification. Myoglobin and ovalbumin were generously given by Seravac Laboratories Ltd., Maidenhead, Berks., and Dr E. F. Hartree respectively. Human erythrocyte CA isoenzymes B and C were prepared by the method of Armstrong, Myers, Verpoorte & Edsall (1966). Most other chemicals were of A.R. grade and the water used was deionized or glass-distilled.

Assay of hydratase activity towards carbon dioxide. Precise measurement of the initial hydration rate was carried out by titration at constant pH (McIntosh, 1968). The assay mixture (18·0 ml.), maintained at 0°, contained 5 mM-sodium phosphate, 45 mM-NaCl, 0·01% peptone and enzyme, at pH 7·00. Reaction was inititated by the addition of CO_2, as an aqueous solution of the gas saturated at 0°, and the hydration rate recorded. The hydration rate in the absence of enzyme was subtracted from the observed velocity. In the experiments with acetazolamide, the enzyme and inhibitor were mixed 5 min. before the addition of substrate. A unit of CA was defined as that amount which catalysed the hydration of 1 μmole of CO_2/min. under the assay conditions stated above, with a concentration of 1 mM-CO_2. Because of the limited solubility of CO_2 in water it was impossible to use a concentration sufficient to saturate the enzyme.

CA activity was determined in the effluents from column chromatography by the method of Philpot & Philpot (1936). This assay gives an approximate measure only of CO_2 hydratase activity, and is unsuitable for the determination of kinetic constants.

Assay of esterase activity towards p-*nitrophenyl acetate.* This convenient and rapid spectrophotometric assay, which measures the rate of hydrolysis of *p*-nitrophenyl acetate, has been fully described by Armstrong *et al.* (1966), Thorslund & Lindskog (1967) and Verpoorte, Mehta & Edsall (1967). The increase in the E_{348} (the isosbestic point of *p*-nitrophenol and the *p*-nitrophenolate ion) of the assay solution in a 1 cm. cuvette, divided by 0·005 (Bergmann, Rimon & Segal, 1958; Armstrong *et al.* 1966), gave the concentration (μM) of total hydrolysis product independently of pH. The observed rate of hydrolysis included a contribution from the non-enzymically catalysed reaction, but this was minimized by using a buffer prepared from diethylmalonic acid (Pocker & Meany, 1965).

A 3 mM stock solution of substrate was prepared by dissolving 27·2 mg. of *p*-nitrophenyl acetate in 1 ml. of acetone and diluting this rapidly with water to 50 ml. Acetone was used because of several organic solvents examined by Verpoorte *et al.* (1967) it was found to be the weakest inhibitor of the esterase reaction. Generally, it was impossible to prepare solutions of the substrate more concentrated than 3 mM. Measurement of the initial rate of hydrolysis was carried out with a Unicam SP. 800 double-beam spectrophotometer, in a cell compartment maintained at 25°.

In assays in which acetazolamide was included, enzyme and inhibitor were mixed 5 min. before the addition of the substrate.

Spectrophotometric assay of esterase activity towards β-naphthyl acetate. β-Naphthol has an E_{330} $1{\cdot}70 \times 10^3$ M^{-1} $cm.^{-1}$, in a solution containing 5% acetone at pH 8·0 (10 mM-diethylmalonic acid buffer); the extinction due to β-naphthyl acetate is very low. A stock solution of the substrate was prepared by dissolving 18·6 mg. of β-naphthyl acetate in 5·0 ml. of acetone.

An assay solution was prepared by mixing, in a 1 ml. semi-micro spectrophotometer cuvette, 0·1 ml. of 0·1 M-diethylmalonic acid buffer, pH 8·0, a suitable volume of enzyme solution, and water such that addition of substrate solution (50 μl. to give 1 mM-β-naphthyl acetate) gave a total volume of 1·0 ml. The assay temperature was 25°.

DEAE-Sephadex ion-exchange chromatography. DEAE-Sephadex A-50 was swollen in water and washed with dilute acid and alkali, according to the manufacturer's instructions, before equilibration with the desired buffer at 4°. Three buffers were used: 0·1 M-tris–0·024 M-HCl (pH 9·3 at 4°), and the same buffer diluted twofold and fourfold with water. These buffers were named I, II and III respectively. The prepared gel was packed to form a column measuring 35 cm. × 1·35 cm. and was washed with at least 200 ml. of the appropriate buffer to complete the equilibration. CA was eluted with the buffer with which the particular column had been equilibrated, according to the method of Armstrong *et al.* (1966). All chromatography was performed at 4°. As a routine pooled fractions of effluent were concentrated by freeze-drying after exhaustive dialysis against deionized water.

Isoelectric focusing. Electrolysis in stable pH gradients was carried out as described by Svensson (1962) and Vesterberg & Svensson (1966). The 110 ml.-capacity apparatus and the low-molecular-weight aliphatic polyamino-polycarboxylic acid carrier ampholyte mixtures were manufactured by LKB-Produkter AB, Stockholm, Sweden. Stabilization of the electrolytic column was achieved with a 0–50% (w/v) stepwise gradient of sucrose and the anode (top) and

cathode (bottom) solutions were composed of dil. H_3PO_4 and ethanolamine respectively. The apparatus was maintained at 0°. With a potential difference of 400 v, equilibrium fractionation was attained after 3–4 days. The column contents were displaced by pumping water on to the top of the gradient. Fractions (1–2 ml.) were collected and the pH of each at 0° was determined immediately with a Pye–Ingold type 401-M5 combination electrode (W. G. Pye and Co., Cambridge) attached to a Radiometer Titrator type TTT1C pH-meter compensated for operation at 0°. Absorption of CO_2 by the fractions was considered to be negligible at the pH of the fractions in which the isoenzymes of CA were found (Vesterberg & Svensson, 1966). No correction was made for the effect of sucrose on the measured pH, since this is small at neutral pH (Flatmark & Vesterberg, 1966).

Polyacrylamide-gel electrophoresis. The method of Davis (1964) was used except that sample and spacer gels were omitted. The running gel was polymerized with riboflavin in daylight, after layering with water. The protein, in a volume of 5–30 μl. containing both Naphthalene Blue (to indicate the progress of electrophoresis) and sucrose, was layered directly on to the upper surface of the gel. The current was 1 mA/tube until the samples had entered the gel, whereupon it was increased to 3 mA/tube. The temperature of the gel did not then exceed 35°. The region of the discontinuous buffer system in which the proteins separated is reported to have a pH of 9·5 (Ornstein, 1964). Protein was detected with Coomassie Blue (Chrambach, Reisfeld, Wyckoff & Zaccari, 1967) and esterase activity towards β-naphthyl acetate at pH 7·0 by coupling Fast Blue RR with released β-naphthol (Tashian & Shaw, 1962). Acetazolamide was added to control gels to inhibit CA esterase activity.

Determination of molecular weight by gel filtration. The method of Andrews (1964, 1965) was used. Sephadex G-100 was packed in a column measuring 50 cm. × 1·5 cm. The elution volumes of bovine plasma albumin, ovalbumin, myoglobin and soya-bean trypsin inhibitor, used to calibrate the column, were measured by monitoring the effluent at 280 nm. and those of most of the CA isoenzymes found by measurement of activity. Human erythrocyte CA isoenzymes B and C (Rickli, Ghazanfar, Gibbons & Edsall, 1964), of known molecular weight, were also used for calibration purposes. The quantities of the isoenzymes of high specific activity applied to the column were in the range 10–50 μg., whereas for the human and rat erythrocyte isoenzymes that have a low specific activity 1–2 mg. was used. Filtration was carried out at 4° with buffer III containing 0·05 M-NaCl. A Blue Dextran high-molecular-weight reference was included in every experiment.

Determination of zinc. Effluents from column chromatography were analysed directly to find the elution pattern of zinc by using a Perkin–Elmer model 303 atomic-absorption spectrophotometer. The apparent zinc concentration measured in this way, without preliminary digestion of the protein, was inaccurate owing to a viscosity effect in the instrument. The zinc contents of small samples of the purified isoenzymes were determined after oxidation (with A.R. fuming HNO_3) of the protein that had previously been exhaustively dialysed against glass-distilled water. The samples were evaporated to dryness before addition of the HNO_3 and the digestion was then allowed to proceed for 24 hr. Most of the acid was removed by gentle heating and the digested residues were then diluted and analysed with the atomic absorption spectrophotometer. All glassware used in these experiments was soaked in 6 M-HCl for 24 hr. and rinsed well with glass-distilled water before use.

Determination of protein. The effluents from column chromatography were monitored at 280 nm. with a Gilson u.v. meter. Specific extinction coefficients of the purified isoenzymes at 280 nm. were determined by weighing, under atmospheric conditions (with a Cahn microbalance), the dried residues from weighed portions of protein solutions that had previously been exhaustively dialysed against glass-distilled water. $E^{1cm.}_{280}$ of each solution was measured before drying and allowance for light-scattering at 280 nm. was made by linear extrapolation from higher wavelengths. It was assumed that approx. 10% of the measured dry weight was contributed by absorbed water, because when a sample of the rat erythrocyte high-activity isoenzyme was weighed after equilibration with an atmosphere having a relative humidity of 52%, and after drying over P_2O_5, the amount of adsorbed water was 9·6% of the total mass.

RESULTS

Preparation of the isoenzymes

Erythrocyte isoenzymes. Male white rats were anaesthetized with ether, blood was collected into an excess of chilled glucose–citrate anticoagulant (Loutit & Mollison, 1943) and the erythrocytes were washed four times with cold 0·9% sodium chloride by centrifugation. The erythrocytes were then haemolysed by the addition of a volume of water equal to 1·1 times the volume of the packed cells. Haemoglobin was removed from the haemolysate by treatment with chloroform–ethanol by the method of Armstrong *et al.* (1966) and the CA-containing extract was dialysed against water and freeze-dried. The residue, contaminated by only a little haemoglobin, was dissolved in buffer I, dialysed exhaustively against the buffer and applied to a column of DEAE-Sephadex equilibrated with the same buffer.

Fig. 1 shows the result of the chromatography. Each peak of carbon dioxide hydratase activity represents a distinct form of CA; rechromatography of certain of the peaks yielded CA isoenzymes. The solid bars (close to the horizontal axes) show which fractions were pooled for freeze-drying and rechromatography; in general, rechromatography in a buffer of lower ionic strength increased the resolution of the peaks. For example, Fig. 2 shows the result of rechromatographing peak 1 (Fig. 1) in buffer III; separation of the major and the minor peaks of activity was thus achieved. The isoenzymes were all identified by number, corresponding to their position on elution from the chromatographic column. [The CA isoenzymes are referred to below as follows: erythrocyte isoenzymes, Eryth. (number); dorsolateral prostate isoenzymes, Prost. (number). (CEP) after an isoenzyme indicates purification from a chloroform–ethanol extract.]

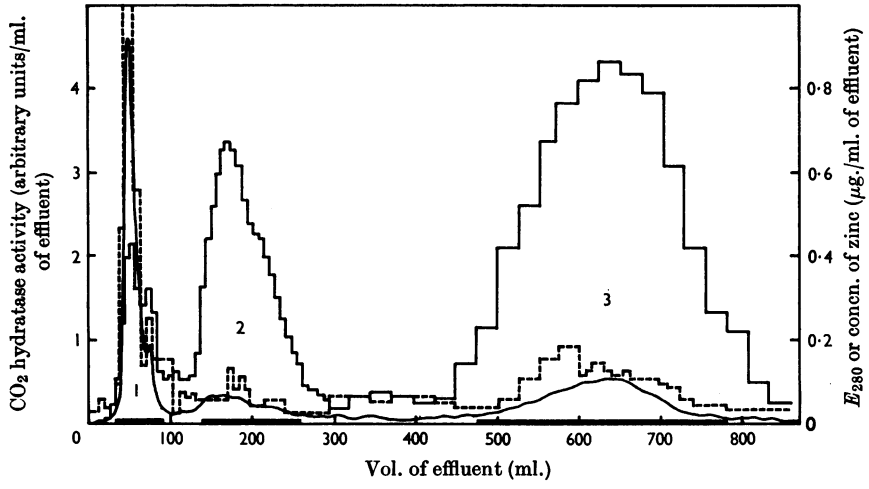

Fig. 1. Purification of erythrocyte CA and resolution into isoenzymes, by column chromatography on DEAE-Sephadex. The starting material was a chloroform–ethanol extract of washed erythrocytes. The buffer used for equilibrating the column and eluting the isoenzymes was 0·1M-tris–0·024M-HCl, pH 9·3 at 4°. —— (continuous), E_{280}; —— (stepped), CO_2 hydratase activity (Philpot assay); ----, concn. of zinc. The solid bars near the horizontal axis show which fractions were combined for further purification. The three major peaks are numbered 1, 2 and 3.

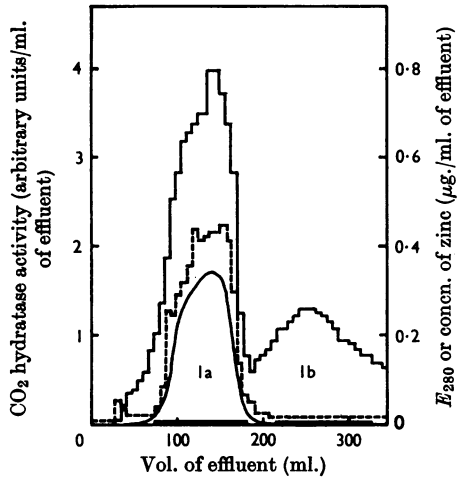

Fig. 2. Rechromatography of peak 1 (Fig. 1) on DEAE-Sephadex. The equilibrating and eluting buffer was 0·025M-tris–0·006M-HCl, pH 9·3 at 4°. —— (continuous), E_{280}; —— (stepped), CO_2 hydratase activity (Philpot assay); ----, concn. of zinc. The solid bars near the horizontal axis show which fractions were pooled for further purification. The two peaks are numbered 1*a* and 1*b*.

Three isoenzymes were isolated: Eryth. 1*a* (CEP), Eryth. 2 (CEP) and Eryth. 3 (CEP). Fig. 3 depicts the results of polyacrylamide-gel electrophoresis performed at successive stages of purification and stained for protein and β-naphthyl acetate esterase activity in the presence and absence of acetazolamide.

To determine the effect of organic solvents on the erythrocyte CA an alternative method for removal of haemoglobin was used. The haemolysate, dialysed against buffer II, was applied directly to a half-length column of DEAE-Sephadex equilibrated with buffer II. Buffer I was used to elute the isoenzymes, which were slightly contaminated by haemoglobin (Armstrong *et al.* 1966). When the active effluent was concentrated in the usual manner and rechromatographed under the same conditions as were used for the chloroform–ethanol extract, a different elution pattern was obtained. A very small amount only of activity, and no protein, was detected in the position of peak 2, and peak 3 was eluted more rapidly than with the chloroform–ethanol extract. However, the behaviour of peak 1*a* appeared to be unchanged. The results of electrophoresis are shown in Fig. 4, which shows that the band corresponding to peak 2 (Fig. 1) was missing, suggesting that peak 2 appeared as a result of the treatment with chloroform–ethanol. Rechromatography produced isoenzymes Eryth. 1*a* and Eryth. 3.

Electrophoresis showed that peak 1*a* (Fig. 2), Eryth. 1*a* (CEP) (which resulted from the repeated rechromatography of peak 1*a*) and Eryth. 1*a* all contained a minor component. The ratio of protein to acetazolamide-inhibitable esterase activity in

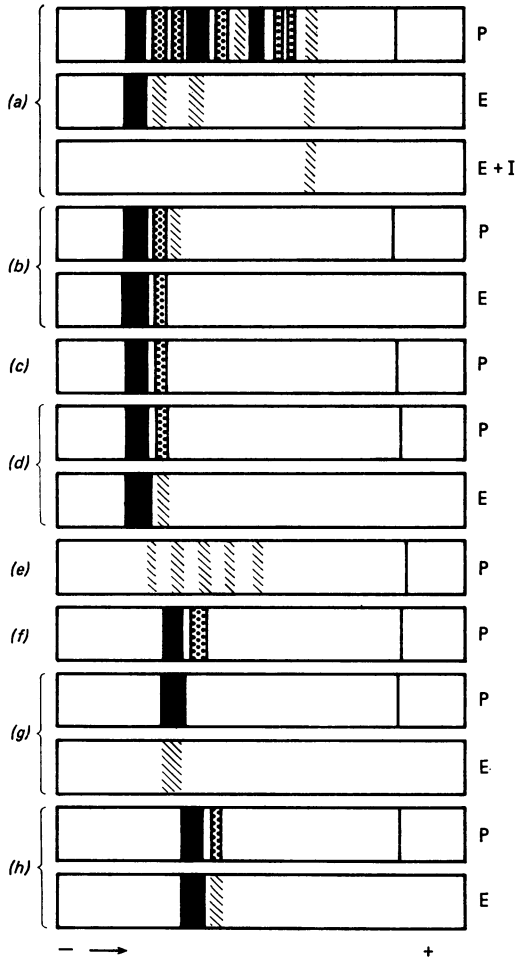

Fig. 3. Polyacrylamide-gel electrophoretograms obtained at several stages in the isolation of CA isoenzymes from a chloroform–ethanol extract of washed erythrocytes. The direction of migration of protein is indicated by the arrow. P, protein stained with Coomassie Blue; E, esterase activity towards β-naphthyl acetate at pH 7; E+I, esterase activity in the presence of acetazolamide. Materials used: (*a*) chloroform–ethanol extract of erythrocytes; (*b*) peak 1 (Fig. 1); (*c*) peak 1*a* (Fig. 2); (*d*) Eryth. 1*a* (CEP), from rechromatography of peak 1*a* with buffer III; (*e*) peak 1*b* (Fig. 2); (*f*) peak 2 (Fig. 1); (*g*) Eryth. 2 (CEP), from rechromatography of peak 2 with buffer II; (*h*) peak 3 (Fig. 1); Eryth. 3 (CEP).

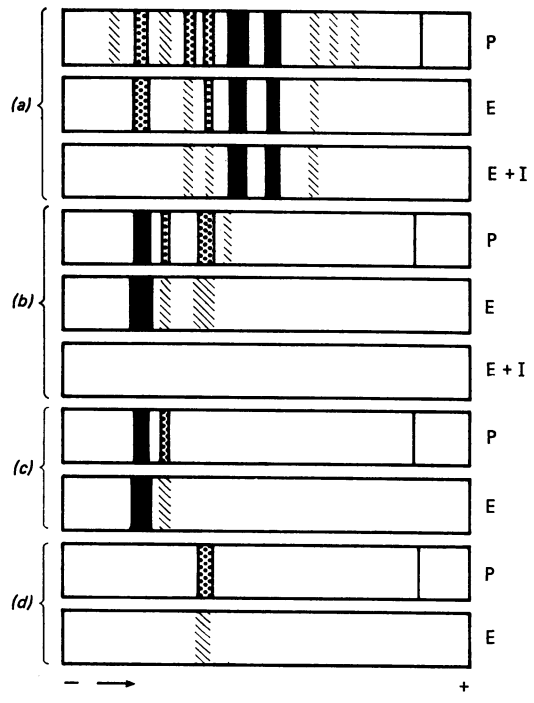

Fig. 4. Polyacrylamide-gel electrophoretograms obtained at several stages in the isolation of CA isoenzymes from washed erythrocytes that were not treated with chloroform–ethanol. The direction of migration of protein is indicated by the arrow. P, protein stained with Coomassie Blue; E, esterase activity towards β-naphthyl acetate at pH 7; E+I, esterase activity in the presence of acetazolamide. Materials used: (*a*) whole centrifuged haemolysate of washed erythrocytes; (*b*) pooled fractions containing CA activity after DEAE-Sephadex chromatography for the removal of haemoglobin; (*c*) Eryth. 1*a*; (*d*) Eryth. 3.

both the major and the minor bands was almost constant throughout the purification procedure. Contamination of peak 1*a* would not be expected to arise from peaks 1*b* or 2 for the following reasons. Peak 1*b* was clearly separated from peak 1*a* by chromatography and in addition appeared to be of low activity towards the ester, whereas peak 2 was also clearly separated and was completely absent from the preparations in which chloroform–ethanol was not used. A minor CA component was observed similarly on electrophoresis of peak 3 (Fig. 1) and again this secondary band possessed the same ratio of protein to acetazolamide-inhibitable esterase activity as Eryth. 3 (CEP) itself. The possibility that the minor components were artifacts produced by electrophoresis was ruled out by the following experiment.

A chloroform–ethanol extract was subjected to isoelectric focusing in a gradient of pH 3–10 with the result shown in Fig. 5. Electrophoresis of the peaks clearly established their identity, and Eryth. 1*a* (CEP) and Eryth. 3 (CEP) were revealed to be free of minor components (Fig. 6), demonstrating that the latter did not appear as a result of electrophoresis. Electrophoresis of fractions 24, 26 and 29

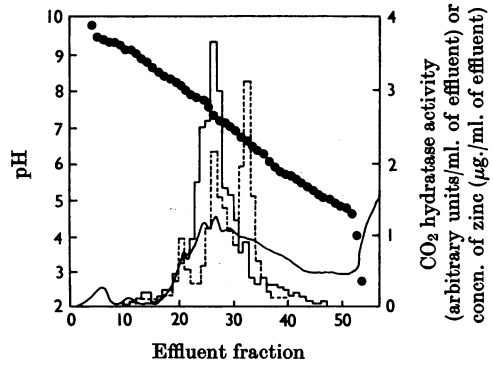

Fig. 5. Results of isoelectric focusing of a chloroform–ethanol extract of washed erythrocytes. The fraction volume was 2 ml. ●, pH; —— (continuous), E_{254} (no units given; the measurement has little quantitative meaning because the carrier ampholytes absorb strongly at this wavelength); —— (stepped), CO_2 hydratase activity (Philpot assay); ----, concn. of zinc.

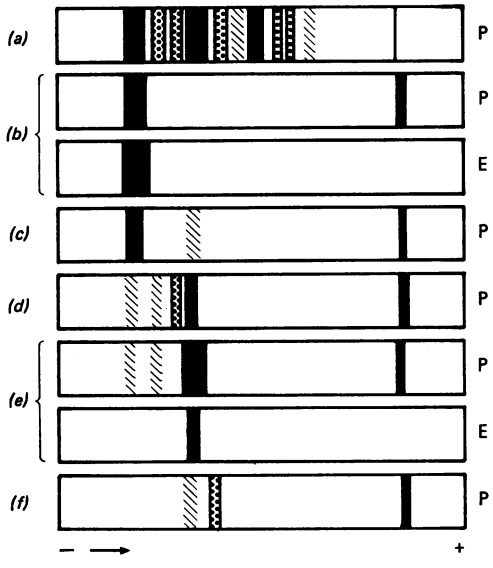

Fig. 6. Polyacrylamide-gel electrophoretograms of fractions from the isoelectric focusing experiment (Fig. 5). The heavy staining at the positive end of the gels is caused by the carrier ampholytes. The direction of migration of protein is indicated by the arrow. P, protein stained with Coomassie Blue; E, esterase activity towards β-naphthyl acetate at pH 7; E+I, esterase activity in the presence of acetazolamide. (*a*) Chloroform–ethanol extract of erythrocytes; (*b*)–(*f*), fractions 20, 22, 24, 26 and 29 respectively.

did show, however, the presence of bands that were tentatively identified, on the basis of their mobilities, as the minor components.

Isoenzymes of the dorsolateral prostate. Prostatic tissue dissected from rats that had been thoroughly bled (used for the collection of erythrocytes) showed negligible contamination by blood. From 20 mature rats about 10 g. of dorsolateral prostates could be collected; no attempt was made to separate the lateral lobes from the dorsal region. The tissue was ground with 0·2 part by weight of acid-washed sand to disintegrate the fibrous structure. When the thick mass had been reduced to a paste, 9 vol. of buffer II was added slowly while the grinding was continued. The homogenate was then centrifuged at 10000*g* for 30 min. at 4°. The precipitate was not re-extracted as only a further 10% of the activity in the first supernatant could be thus recovered. This supernatant, opalescent and only faintly pink, was centrifuged at 108000*g* for 1 hr. at 4°. A clear solution was obtained, and the slight viscous precipitate was discarded. The extract of soluble CA, which had an activity corresponding to 56000 units/g. wet wt. of dorsolateral prostate, was freeze-dried, the residue dissolved in buffer I and this solution exhaustively dialysed against the same buffer.

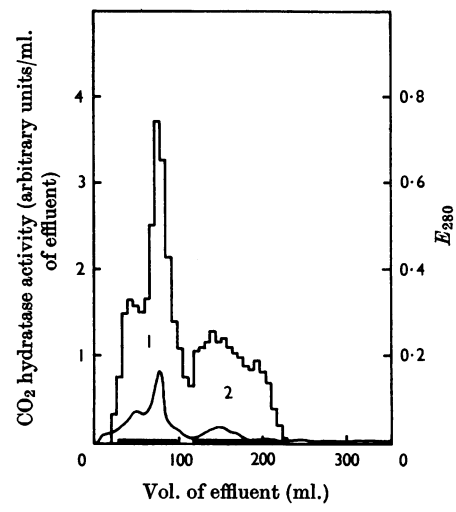

Fig. 7. Purification of dorsolateral prostate CA and its resolution into isoenzymes by column chromatography on DEAE-Sephadex. The buffer used for equilibrating the column and eluting the isoenzymes was 0·1 M-tris–0·024 M-HCl, pH 9·3 at 4°. —— (continuous), E_{280}; —— (stepped), CO_2 hydratase activity (Philpot assay). The solid bars show which fractions were combined for further purification. The two major peaks are numbered 1 and 2.

Chromatography with DEAE-Sephadex was performed under conditions identical with those used in the isolation of the erythrocyte isoenzymes. The dialysed extract was centrifuged briefly to remove a slight precipitate before being loaded on to a column

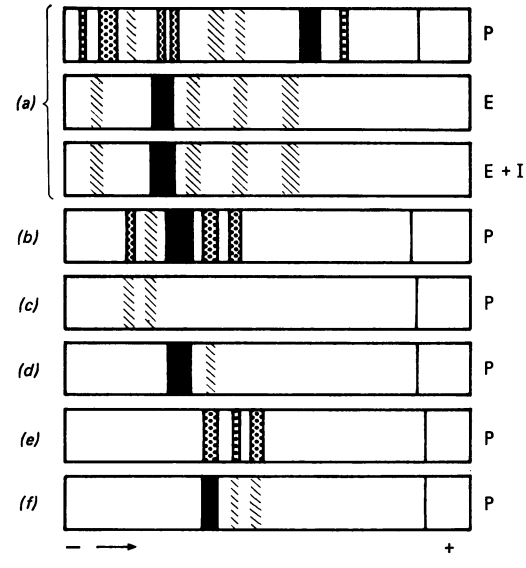

Fig. 8. Polyacrylamide-gel electrophoretograms obtained at several stages during the isolation of CA isoenzymes from a soluble extract of dorsolateral prostate. The direction of migration of protein is indicated by the arrow. P, protein stained with Coomassie Blue; E, esterase activity towards β-naphthyl acetate at pH 7; E+I, esterase activity in the presence of acetazolamide. (*a*) Whole soluble extract; (*b*) peak 1 (Fig. 7); (*c*) peak 1*a*, from rechromatography (in buffer II) of peak 1; (*d*) Prost. 1*b*, from rechromatography (in buffer II) of peak 1; (*e*) peak 2 (Fig. 7); (*f*) Prost. 2, from rechromatography (in buffer I) of peak 2.

equilibrated with buffer I. The results of chromatography are shown in Fig. 7, where three isoenzymes can be identified. Rechromatography (in buffer II) of peak 1 yielded two forms of CA, Prost. 1*a* and Prost. 1*b*; rechromatography (in buffer I) of peak 2 gave Prost. 2. Prost. 1*a* was recovered in very small amounts and electrophoresis showed it to be non-homogeneous; its properties were not investigated. Prost. 1*b* and Prost. 2 were judged to be fairly pure by electrophoresis at a single pH (Fig. 8).

Another purification procedure was tested, involving fractionation both by acetone and by ammonium sulphate. These were performed on a dilute extract of the prostatic tissue and the active fraction was freeze-dried and dialysed against buffer I before being chromatographed in the usual way. As a result an identical elution pattern was obtained. This finding, together with the fact that an average of 86% of the activity applied to the chromatography column was recovered, support the conclusion that the simple extraction and chromatography schemes adopted were adequate and that no major soluble CA isoenzyme with a high specific activity towards carbon dioxide hydration escaped detection.

No esterase activity that could be inhibited by acetazolamide was detected in the gels after electrophoresis of either the extract of prostate or the isolated isoenzymes. When the soluble extract of dorsolateral prostate was assayed for β-naphthyl acetate esterase activity by the spectrophotometric method, a powerful esterase that was insensitive to acetazolamide (Fig. 8*a*) masked any activity that might have been exhibited by the CA.

Properties of the isoenzymes

The properties of the isoenzymes isolated from the erythrocytes (Eryth.) and the prostate (Prost.) are listed in Tables 1 and 2.

Molecular weights. The isoenzymes isolated from both the erythrocytes and the prostate were found to have molecular weights in the range 26 000–32 000, and this variation must be considered to be within the limits of experimental error (Fig. 9).

Carbon dioxide hydratase activities of the isoenzymes and their inhibition by acetazolamide. Measurements were made of the dependence of the initial velocity of the hydration of carbon dioxide on the initial substrate concentration in the reactions catalysed by Eryth. 1*a*, Eryth. 1*a* (CEP), Eryth. 2 (CEP), Eryth. 3, Eryth. 3 (CEP), Prost. 1*b* and Prost. 2, after verification that the initial velocity of hydration was in each case proportional to the concentration of the enzyme. Assays were also carried out in the presence of a constant concentration of acetazolamide. The concentrations of the isoenzymes (5–10 μM) in stock solutions in distilled water were estimated by using the measured values of $E^{1\%}_{1cm.}$ at 280nm. and the measured molecular weights. The results of the kinetic experiments are shown in Fig. 10.

It was possible to determine Michaelis constants and maximum velocities for all the isoenzymes of the erythrocytes and the prostate except Eryth. 1*a* (CEP). The initial velocity of the reaction catalysed by Eryth. 1*a* (CEP) was proportional to the initial substrate concentration in both the presence and the absence of acetazolamide, showing that this isoenzyme was far from saturated with substrate at the maximum experimental concentration of carbon dioxide (16mM).

The isoenzymes Eryth. 1*a*, Prost. 1*b* and Prost. 2 were inhibited non-competitively by acetazolamide, whereas Eryth. 2 (CEP), Eryth. 3 (CEP) and Eryth. 3 were inhibited in a mixed, but predominantly competitive, manner. It was not possible to determine the type of inhibition of Eryth. 1*a* (CEP) (Fig. 10*a*). Values of K_m and $V/[E_0]$, and approximate estimates of K_i, are listed in Table 1.

p-Nitrophenyl acetate hydrolase (esterase) activities

Table 1. *Summary of certain properties of the CA isoenzymes*

The kinetic experiments were carried out at 0° at pH 7·0, with 45 mM-NaCl, 5 mM-sodium phosphate and 0·01% peptone. The kinetic constants were evaluated from the results in Fig. 10; the maximum errors in the values of K_m and V are about ±10% and ±20% respectively (McIntosh, 1968). No values of the constants could be determined for Eryth. 1*a* (CEP) (see Fig. 10*a*). Acetazolamide is a type B inhibitor and so the estimates of K_i are approximate only.

	Erythrocyte isoenzymes					Dorsolateral prostate isoenzymes	
	Eryth. 1*a* (CEP)	Eryth. 1*a*	Eryth. 2 (CEP)	Eryth. 3 (CEP)	Eryth. 3	Prost. 1*b*	Prost. 2
General properties							
Mol. wt. (±3000)	29000	29000	29000	29000	29000	29000	29000
$E^{1\%}_{1cm.}$ at 280 nm. (approx.)	18	18	18	17	17	18	—
g.atom of Zn/mole (nearest whole number)	1	1	1	1	1	1	—
Isoelectric point (approx., from isoelectric focusing)	8·1	—	7·7	7·3	—	—	—
Kinetic properties as CO_2 hydratases							
K_m (mM)	—	8·6	8·6	5·7	11	17	9·6
$10^{-4}V/[E_0]$ (sec.$^{-1}$)	—	0·13	4·2	7·4	8·7	18	3 (approx.)
Type of inhibition caused by acetazolamide	—	Non-competitive	Mixed	Mixed, mainly competitive	Mixed, mainly competitive	Non-competitive	Non-competitive
K_i (acetazolamide) (nM)	—	66	4·9	1·5	4·8	4·6	2·6
$[E_0]/K_i$ (acetazolamide) (approx.)	—	1·8	0·84	1·2	0·56	0·61	—

Table 2. *Properties of the CA isoenzymes as catalysts of ester hydrolysis*

The experiments were carried out at 25° at pH 8·0, with 10 mM-diethylmalonate and either 1·7% (v/v) or 5% (v/v) acetone for Expts. 1 and 2 respectively. The maximum error in $v/[E_0]$ was estimated as ±10%. In Expt. 1 the values of K_i for Eryth. 3 (CEP) and Prost. 1*b* and their standard errors were determined by least-squares regression analysis of the results shown in Fig. 13.

	Erythrocyte isoenzymes				Dorsolateral prostate isoenzymes	
	Eryth. 1*a* (CEP)	Eryth. 1*a*	Eryth. 3 (CEP)	Eryth. 3	Prost. 1*b*	Prost. 2
Expt. 1. Hydrolysis of 1 mM-*p*-nitrophenyl acetate						
$v/[E_0]$ (min.$^{-1}$)	11	9·7	44	30	57	16 (approx.)
K_i (μM) and type of inhibition caused by DNSA	—	—	2·2±0·2 partially competitive	—	2·1±0·1 non-competitive	—
$[E_0]/K_i$	—	—	0·19	—	0·12	—
Classification of sulphonamide inhibitors by zone of inhibition:						
DNSA	B	B	A	A	A	—
Acetazolamide	B	B	B	B	B	—
Ethoxzolamide	C	C	C	C	C	—
Expt. 2. Hydrolysis of 1 mM-β-naphthyl acetate						
$v/[E_0]$ (min.$^{-1}$)	1·5	1·3	0·12	0·13	0	0

of the isoenzymes. The initial velocities of the reactions catalysed by Eryth. 1*a* (CEP), Eryth. 1*a*, Eryth. 3 and Prost. 1*b* were almost proportional to the substrate concentration (Fig. 11). These isoenzymes were not saturated with substrate at the maximum concentration of *p*-nitrophenyl acetate that could be attained. Similar behaviour was noted by Thorslund & Lindskog (1967) in their study of

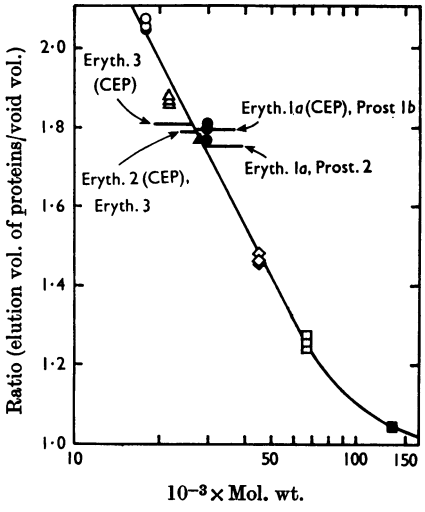

Fig. 9. Molecular weights of the CA isoenzymes, determined by gel filtration on Sephadex G-100. The eluting buffer was 0·025 M-tris–0·006 M-HCl, pH 9·3 at 4°, containing 0·05 M-NaCl. ○, Myoglobin; △, soya-bean trypsin inhibitor; ●, human CA isoenzyme C; ▲, human CA isoenzyme B; ◇, ovalbumin; □, bovine plasma albumin monomer; ■, bovine plasma albumin dimer.

the bovine erythrocyte enzyme and by Verpoorte *et al.* (1967) for human erythrocyte isoenzyme C.

Nature of the inhibition of the isoenzymes by acetazolamide, DNSA and ethoxzolamide. The values of K_i (Table 1) for the isoenzymes interacting with acetazolamide were calculated from the results shown in Fig. 10 by the customary method, in which it is assumed that the concentration of free inhibitor is equal to the total concentration of inhibitor. These values are unlikely to have proper physical meaning, for the following reason. When the initial velocity of carbon dioxide hydration catalysed by Eryth. 3 (CEP) was measured at constant substrate concentration in the presence of various amounts of acetazolamide, a Dixon (1953) plot of the results (Fig. 12) was not linear but curved. This is an example of the behaviour typical of a 'mutual depletion system', which is characterized by strong association between inhibitor and enzyme (Webb, 1963, p. 187). A similar curved plot was obtained when the same isoenzyme catalysed the hydrolysis of *p*-nitrophenyl acetate in the presence of the same inhibitor (Fig. 13). It is clear that in the present conditions of assay, and with the nomenclature of Webb (1963), inhibition by acetazolamide of Eryth. 3 (CEP) lay within zone B of inhibition. The binding of inhibitor to enzyme was not complete because activity remained when the concentration of acetazolamide was greater than that of the enzyme (Fig. 12). In fact, the approximate value of $[E_0]/K_i$ (Table 1) indicates that the system varied between zones A and B of inhibition (Webb, 1963, p. 70), which meant that for Eryth. 3 (CEP) the value calculated for K_i was a good approximation.

Results presented in Fig. 13 and Table 2 show that Eryth. 1*a* (CEP) and Eryth. 1*a* were more powerfully inhibited by acetazolamide than were Eryth. 3 (CEP), Eryth. 3 or the other high-activity isoenzymes. Therefore the Eryth. 1*a*–acetazolamide system was certainly in zone B of inhibition, and the calculated value of K_i was only approximate.

The results of inhibition experiments with the three sulphonamides DNSA, acetazolamide and ethoxzolamide, in the hydrolysis of *p*-nitrophenyl acetate catalysed by the isoenzymes, clearly illustrate zones A, B and C of inhibition respectively. DNSA, which has been shown to combine with bovine erythrocyte CA, causing inhibition of the enzyme and giving rise to a notable fluorescent system (Chen & Kernohan, 1967), is a relatively weak inhibitor of Eryth. 3, Eryth. 3 (CEP) and Prost. 1*b*. With this inhibitor and Eryth. 3 (CEP) a linear Dixon (1953) plot was obtained (Fig. 13) showing that the system was in zone A of inhibition. The results of inhibiting Eryth. 3 (CEP) with DNSA at two substrate concentrations are shown in Fig. 13, from which it is concluded that the inhibition is probably partially competitive because the lines at the two substrate concentrations do not meet on the abscissa (Dixon, 1953). (The intercepts with the abscissa have been shown to be significantly different at the 1% level.) The inhibition can only be partially competitive because, when K_m (*p*-nitrophenyl acetate) was calculated from this result assuming totally competitive inhibition (Dixon, 1953), its magnitude was low in comparison with the value of K_m indicated by the experiment with Eryth. 3 (CEP) illustrated in Fig. 11.

Acetazolamide was a stronger inhibitor of Eryth. 3 (CEP) than was DNSA, giving rise to a curved Dixon plot (Fig. 13); the system lay in zones A and B. Ethoxzolamide, however, had a very great affinity for Eryth. 3 (CEP) and stoicheiometrically titrated the activity; in other words, this was zone C mutual-depletion inhibition (not shown).

In comparison with Eryth. 3 (CEP) and Eryth. 3, Eryth. 1*a* (CEP) and Eryth. 1*a* were more powerfully inhibited by the three sulphonamides, although the relative efficiencies of the inhibitors were maintained (Table 2), and even DNSA yielded a zone B system (Fig. 13). The prostate isoenzyme Prost. 1*b* was clearly non-competitively inhibited by DNSA (Fig. 13), although generally it resembled Eryth. 3 in its properties as a catalyst of hydrolysis of *p*-nitrophenyl acetate.

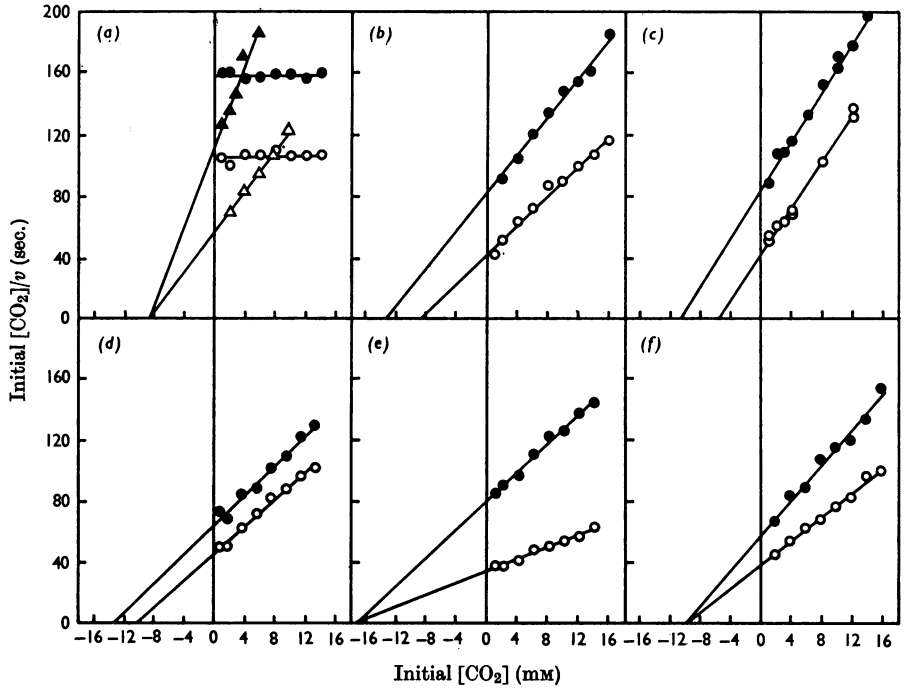

Fig. 10. Relationship between $[CO_2]$ and initial velocity of hydration of CO_2 catalysed by the CA isoenzymes, illustrated by plotting [initial substrate]/(initial rate) against [initial substrate]. The experiments were carried out at 0° at pH 7·0, with 45mM-NaCl, 5mM-sodium phosphate and 0·01% peptone. ○, Uninhibited; ●, in the presence of acetazolamide, at the concentrations given in parentheses. (*a*) 120nM-Eryth. 1*a* (CEP) (64nM); △ and ▲, 120nM-Eryth. 1*a*. (63nM); (*b*) 4·1nM-Eryth. 2 (CEP) (2·6nM); (*c*) 1·8nM-Eryth. 3 (CEP) (1·3nM); (*d*) 2·7nM-Eryth. 3 (1·3nM); (*e*) 2·8nM-Prost. 1*b* (6·4nM); (*f*) approx. 8nM-Prost. 2 (1·3nM). Constants derived from these results are presented in Table 1. Each point is the result of a single assay. The lines were fitted by least-squares regression analysis.

β-Naphthyl acetate hydrolase (esterase) activities of the isoenzymes. The results of measurements of initial velocities by the spectrophotometric assay fully confirmed the earlier observation, made after electrophoresis, namely that at a substrate concentration of 1mM both preparations of the low-activity erythrocyte isoenzyme Eryth. 1*a* were superior to both preparations of the high-activity form Eryth. 3 as catalysts of the hydrolysis of β-naphthyl acetate. Neither dorsolateral prostate isoenzyme could be shown to act as a catalyst of this reaction (Table 2).

Zinc content of the rat dorsolateral prostate and of the prostate isoenzymes. The concentration of zinc in the dorsolateral prostate varied in the range 160–190μg. of zinc/g. wet wt., with an average value of 180μg./g. wet wt. This result is in agreement with the findings of Mawson & Fischer (1952). Since a homogenate of the tissue contained an average of 56000 units of carbon dioxide hydratase activity/g. wet wt., the average value for the ratio of CA activity to zinc content in Prost. 1*b* and Prost. 2 was 6200 units/μg. of zinc, assuming approximately equal quantities of the two isoenzymes in the intact tissue. Therefore in the dorsolateral prostate CA accounts for only 5% of the total zinc found in this organ. CA in the endometrium of the rabbit represents only 1–2% of the total zinc content of that tissue (Lutwak-Mann & McIntosh, 1969).

DISCUSSION

The two major isoenzymes of rat erythrocytes, which are present in haemolysates in almost equal amounts, differ markedly in their efficiencies as catalysts. This conclusion is admissible even though the purity of the isolated isoenzymes has not been rigorously established. Comparison of values of $V/[E_0]$ shows that as a catalyst of carbon dioxide hydration Eryth. 3 was 70-fold more effective than Eryth. 1*a*. Isoenzymes of CA exhibiting different activities have been found previously in the erythrocytes of humans (Nyman, 1961; Rickli & Edsall, 1962; Laurent *et al.* 1962), rhesus monkeys

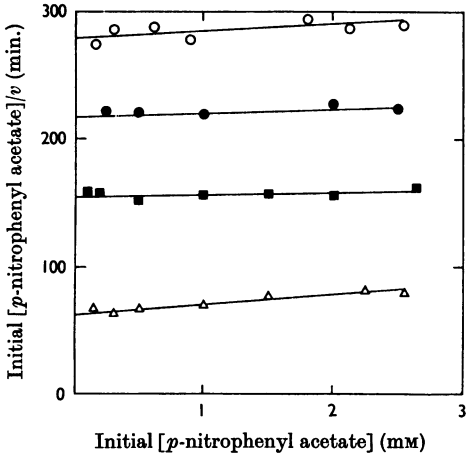

Fig. 11. Relationship between [*p*-nitrophenyl acetate] and initial velocity of the esterase reaction catalysed by several CA isoenzymes. The results are illustrated by plotting [initial substrate]/(initial rate) against [initial substrate]. The experiments were carried out at 25° at pH 8·0, with 10 mM-diethylmalonate and 1·7% (v/v) acetone. ○, 0·35 μM-Eryth. 1*a*; ●, 0·55 μM-Eryth. 1*a* (CEP); ■, 0·11 μM-Eryth. 3 (CEP); △, 0·26 μM-Prost. 1*b*. Each point is the average of two assays.

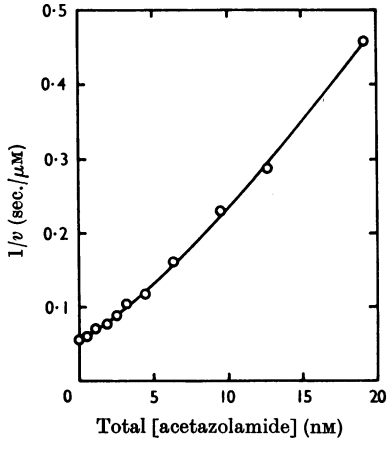

Fig. 12. Relationship between the reciprocal of the initial rate of CO_2 hydration catalysed by Eryth. 3 (CEP) and the total concentration of acetazolamide [Dixon (1953) plot]. The experiments were carried out at 0° at pH 7·0, with 45 mM-NaCl, 5 mM-sodium phosphate and 0·01% peptone, and the initial [CO_2] was 1·0 mM; the enzyme concentration was 1·8 nM. Each point is the result of a single assay.

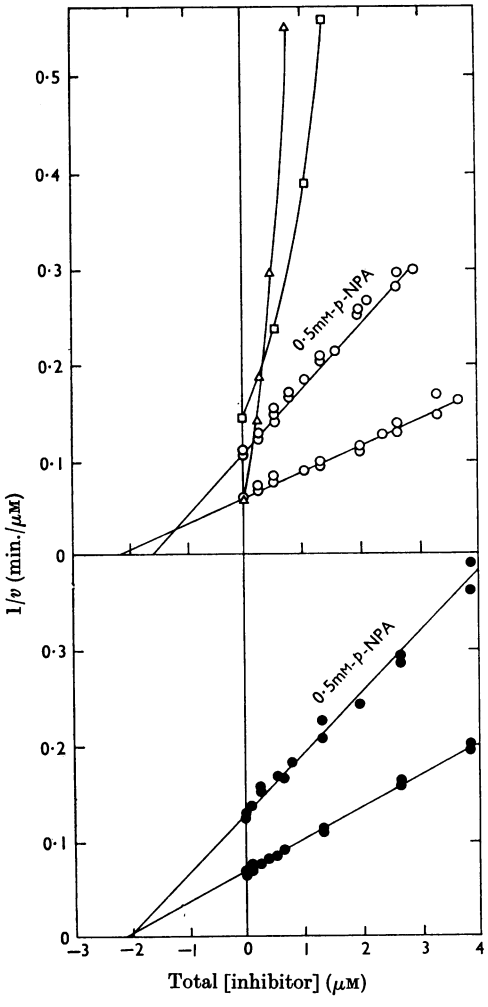

Fig. 13. Relationship between the reciprocal of the initial rate of *p*-nitrophenyl acetate hydrolysis catalysed by several CA isoenzymes and the total concentration of sulphonamide inhibitor [Dixon (1953) plots]. The experiments were carried out at 25° at pH 8·0, with 10 mM-diethylmalonate and 1·7% (v/v) acetone, and the initial [*p*-nitrophenyl acetate] was 1·0 mM (except where indicated as being 0·5 mM). △, 0·42 μM-Eryth. 3 (CEP) inhibited by acetazolamide; □, 0·55 μM-Eryth. 1*a* (CEP) inhibited by DNSA; ○, 0·42 μM-Eryth. 3 (CEP) inhibited by DNSA; ●, 0·26 μM-Prost. 1*b* inhibited by DNSA. Each point is the result of a single assay. The straight lines were fitted to the experimental points by least-squares regression analysis; the derived constants are to be found in Table 2. *p*-NPA, *p*-nitrophenyl acetate.

(Duff & Coleman, 1966) and certain other monkeys and lower animals (Tashian, Shreffler & Shows, 1968), and horses (Furth, 1968).

Removal of haemoglobin from the rat haemolysate by denaturation with chloroform induced distinct changes in certain kinetic properties of the CA isoenzymes. For example, K_m (carbon dioxide hydration) of Eryth. 1*a* (CEP) was so large as to be immeasurable and yet, when the isoenzyme had not been exposed to the organic solvents, its K_m was

lower and could be measured without difficulty. In the same conditions, K_m of Eryth. 3 (CEP) was lower than K_m of Eryth. 3. The specific activities, however, of these pairs of isoenzymes were indistinguishable. It is conceivable that isoenzymes of rat erythrocyte CA are unusually susceptible to organic solvents, because with the two human erythrocyte isoenzymes Gibbons & Edsall (1964) found no significant differences in the values of K_m (CO_2 hydration) when they compared samples that had been extracted into chloroform–ethanol with those from the alternative preparation. Moreover, Riddiford (1964) obtained identical protein titration curves from samples of the human B isoenzyme prepared by both procedures.

The appearance of Eryth. 2 (CEP) in the chloroform–ethanol extract may have been caused by the organic solvents facilitating its release from the erythrocytes. Alternatively the chloroform–ethanol may have modified one of the other isoenzymes, probably Eryth. 3, to form this component. Since some of the kinetic properties of the other isoenzymes are altered by treatment with chloroform–ethanol, it seems possible that changes might similarly occur in those parts of an isoenzyme molecule that are responsible for interaction with DEAE-Sephadex.

In contrast with the erythrocyte isoenzymes, the two major forms of CA isolated from the dorsolateral prostate had similar catalytic efficiencies. Like the erythrocyte isoenzyme Eryth. 3 they had high specific activities. There were, however, notable differences in the kinetic behaviour of, for example, Prost. 1*b* and Eryth. 3. The values of K_m and $V/[E_0]$ for Prost. 1*b* were two to three times as great as for Eryth. 3 prepared by either method of purification. Moreover inhibition by acetazolamide of Prost. 1*b* was non-competitive, whereas Eryth. 3 was inhibited competitively in identical conditions.

Furth (1968) proposed that CA isoenzymes should be classified according to specific activity, rather than on the basis of chromatographic behaviour as was suggested by Rickli *et al.* (1964). Furth's (1968) nomenclature is certainly more appropriate than the latter for the rat isoenzymes, which accordingly are listed thus: Eryth. 1*a*, as type B; Eryth. 2, Eryth. 3, Prost. 1*b* and Prost. 2, as type C.

The observation that the low-activity erythrocyte isoenzyme Eryth. 1*a* was considerably more effective than the high-activity form Eryth. 3 as a catalyst of 1mM-β-naphthyl acetate hydrolysis illustrates a remarkable change in the relative catalytic efficiency of two isoenzymes with a change in substrate. In the same conditions the high-activity form was, however, the more efficient catalyst of the hydrolysis of *p*-nitrophenyl acetate. A similar erythrocyte CA isoenzyme with an unusually high hydrolytic activity towards β-naphthyl acetate has been detected by Tashian *et al.* (1968). The surprising observation that neither dorsolateral prostate isoenzyme catalysed the hydrolysis of the naphthyl ester emphasizes the need for caution when attempting to detect, with this substance, CA activity after electrophoresis.

Acetazolamide caused both a competitive and a non-competitive type of inhibition with the different isoenzymes. Whereas the high-activity erythrocyte isoenzyme was inhibited in a mixed but mainly competitive manner, the low-activity erythrocyte form and the prostate isoenzymes were non-competitively inhibited in identical conditions. The results of earlier quantitative experiments suggested that sulphonamides inhibit CA only non-competitively (Davis, 1959; Leibman, Alford & Boudet, 1961). It must be noted, however, that these experiments were done with a human erythrocyte enzyme preparation, which presumably consisted of a mixture of the two isoenzymes now known to differ considerably in their kinetic properties (Gibbons & Edsall, 1964). Under certain conditions inhibition by benzenesulphonamide of bovine erythrocyte CA has been shown to be competitive (Kernohan, 1966). In recent similar experiments the inhibition by sulphanilamide of carbon dioxide hydration catalysed by the cobalt form of bovine erythrocyte CA was examined by using a stopped-flow apparatus (Lindskog & Thorslund, 1968). Two consecutive types of kinetic behaviour were observed when a solution of carbon dioxide was mixed rapidly with a solution containing both enzyme and inhibitor. The rates of the reaction at the instant of mixing yielded apparent non-competitive inhibition but the rates measured after a short time indicated competition between carbon dioxide and inhibitor. When the sulphanilamide was mixed, before reaction, with the substrate solution rather than the enzyme, the velocity on mixing was equal to the uninhibited rate but decreased progressively over a short period, after which the degree of inhibition was constant. These observations were explained by postulating slow interactions between enzyme and inhibitor.

Any rapid initial phase of reaction, which might have occurred in the present experiments with the rat CA isoenzymes, would not have been detected because of the relatively slow response of the titrator in comparison with the stopped-flow device. Therefore the competitive inhibition observed with Eryth. 2 and both preparations of Eryth. 3 was entirely compatible with the above-mentioned findings for the bovine enzyme, and it was surprising to see the non-competitive inhibition displayed by Eryth. 1*a* and the prostate isoenzymes. Either the nature of the interaction between these

isoenzymes and the inhibitor was different or the dissociation of isoenzyme and inhibitor was extremely slow.

A great deal of evidence has been accumulated showing that those compounds that inhibit the carbon dioxide hydratase activities of the human and bovine CA isoenzymes also inhibit their esterase activities (Pocker & Stone, 1965, 1967, 1968*a*; Verpoorte *et al.* 1967; Thorslund & Lindskog, 1967; Pocker & Storm, 1968). Further, there is universal agreement that acetazolamide, the sulphonamide most commonly studied, causes reversible non-competitive inhibition of the hydrolysis of several esters, particularly *p*-nitrophenyl acetate, when catalysed by these forms of CA. Competitive inhibition has been described, but only in connexion with certain substrate analogues, all of them carbonyl compounds (Pocker & Stone, 1968*b*). The present observation that the esterase activity of Eryth. 3 (CEP) was partially competitively inhibited by DNSA is not incompatible with the previous findings because, not only is it possible that the rat isoenzyme is unlike the bovine isoenzyme in the nature of its inhibition by sulphonamides, but also DNSA has a much lower affinity for the enzyme than does acetazolamide. Similarly, in the carbon dioxide reaction it is possible that the mixed, rather than purely competitive, inhibition noted for certain of the rat erythrocyte isoenzymes resulted from the greater affinity of acetazolamide for the isoenzymes, and not from a change in the mechanism of inhibition, since it is clear that purely competitive inhibition cannot occur in a system in which there is strong binding between inhibitor and enzyme (Webb, 1963, p. 74). Clearly it is preferable to base classification of competition type on results from experiments with inhibitors of relatively low affinity for the enzyme.

It is difficult to judge the significance of the minor CA isoenzymes. Furth (1968) found minor components in various quantities when she chromatographed CA from horse erythrocytes and suggested that these components, which sometimes occurred in surprisingly large amounts, were artifacts produced during purification. They all appeared to be closely similar to the high-activity horse isoenzyme. Rabbit erythrocytes yield several minor, high-activity, isoenzymes of CA (J. E. A. McIntosh, unpublished work). In a review Edsall (1968) refers to unpublished observations made in his laboratory where as many as five CA components were detected in rabbit erythrocytes. The present paper describes several minor isoenzymes, in addition to the two major forms, in rat erythrocytes. Although it is possible that Eryth. 2 (CEP) resulted from treatment of Eryth. 3 with chloroform–ethanol, there were several additional minor components that were identified in preparations that had not been exposed to organic solvents. Moreover, the presence of a minor isoenzyme was detected by chromatography in the soluble extract of the rat dorsolateral prostate. The mild preparative steps used in the latter case make it unlikely that this polymorphism resulted from changes occurring during isolation and purification, and there is no evidence that any of the dorsolateral prostate isoenzymes is unstable.

The extraordinarily large quantity of CA in the rat dorsolateral prostate, demonstrated in this paper, appears to represent only a small fraction of the unusually high concentration of zinc in the same region of that organ (Gunn, Gould, Ginori & Morse, 1955). Evidence of a different kind also suggests that much of the prostatic zinc is not bound to CA. When zinc-chelating agents were administered to rats, large quantities of the zinc–chelate complexes were found in the dorsolateral prostates (Sternberg, Cronin & Philips, 1965), but, since CA is itself a powerful chelator of zinc, it is most unlikely that the compounds that were injected could remove the metal from the enzyme. Indeed it has proved impossible to extract zinc from the bovine erythrocyte enzyme at neutral pH with even the most effective chelating compounds (Lindskog & Malmström, 1960). Further, examination of the subcellular distribution of zinc in the rat dorsolateral prostate indicates that only about half of the total zinc is to be found in the soluble fraction of the homogenate (Kar & Chowdhury, 1966). Experiments have been carried out to examine the intracellular distribution of CA in several tissues, and the conclusion in each case was that at least as much as 80% of the activity was recoverable in the supernatant fraction (Datta & Shepard, 1959; Karler & Woodbury, 1960).

It is difficult to assign to CA a definite function in the biochemistry and physiology of the rat prostate. The prostate is an accessory organ, the metabolism of which is dependent on the male sex hormone (Mann, 1964, p. 45). It would undoubtedly be desirable to investigate the relationship between testosterone and the amounts of the individual prostatic CA isoenzymes. Two approaches to this are possible. Rats could be castrated, thus directly depriving the prostatic tissue of the influence of testosterone, or alternatively the action of testosterone could be indirectly counteracted by administering to rats anti-androgenic compounds such as chlormadinone acetate (McIntosh & Lutwak-Mann, 1967) or cyproterone acetate. Either procedure, however, would induce regression of the prostate tissue, resulting in less material for the identification of the isoenzymes. The other approach might be to attempt to synthesize CA *in vitro* in cultures of prostatic tissue and to study in this way the type of isoenzyme formed in response to

testosterone itself and also to the antagonistic hormonal agents.

I am grateful to Professor T. R. R. Mann, C.B.E., F.R.S., and Dr C. Lutwak-Mann for their interest and criticism, to Dr M. Webb, Strangeways Laboratory, Cambridge, for permitting me to use the atomic-absorption spectrophotometer and to Mr J. C. Rowell, Agricultural Research Council Statistics Group, Cambridge, for a statistical analysis.

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