

Ph.D. Dissertation 6809

CARBONIC ANHYDRASE:  
ISOENZYMES IN BLOOD AND REPRODUCTIVE ORGANS

By

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A dissertation submitted to the University  
of Cambridge for the degree of  
Doctor of Philosophy

Corpus Christi College, Cambridge

May, 1969

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## PREFACE

The experiments described in this dissertation were performed at the Agricultural Research Council Unit of Reproductive Physiology and Biochemistry, 307 Huntingdon Road, Cambridge, from Lent, 1966, to Michaelmas, 1968. My research was supervised by the Director of the Unit, Professor T. R. R. Mann, C.B.E., F.R.S.

These studies are the original work of the author. Any assistance from others is specifically acknowledged. The experiments described in Chapter 5 were done in collaboration with Dr C. Lutwak-Mann.

No part of the dissertation has been submitted to any other university for any degree or diploma.

Some parts of the dissertation have already been published and reprints of communications appearing in the Journal of Reproduction and Fertility, The Biochemical Journal, and Nature are attached.

### ACKNOWLEDGEMENTS

I am much indebted to Professor Mann and the Agricultural Research Council for their hospitality and generous provision of facilities. It has been a great privilege to be supervised by Professor Mann and I have enjoyed a happy association with Professor and Mrs Mann; their guidance and enthusiasm, advice and encouragement, have contributed greatly to my research.

I take this opportunity to express gratitude to my colleagues in the Unit for their assistance and advice; in particular I wish to thank Dr J. C. Bournsnel, Dr E. F. Hartree, Dr A. G. C. Benwick, and Mr R. A. P. Harrison. I am grateful to Mr R. J. Patman for his excellent photography of my drawings.

I am indebted also to: Professor M. Dixon for a very helpful discussion; Dr E. F. Hartree for lending me a microspectroscope; Dr J. C. Kernohan for providing a sample of bovine erythrocyte carbonic anhydrase, isoenzyme B; Dr C. Lutwak-Mann for carrying out many dissections; Dr R. M. Moor for collecting sheep uteri; Mr J. G. Rowell for performing a statistical analysis; Dr D. F. Sharman for advice, and help in operating the fluorometer; and Dr M. Webb for allowing me the use of his atomic absorption spectrophotometer. Human blood was kindly provided by Professor H. Lehmann and a sample of sheep haemolysate by Mr M. Perrella.

### GENERAL INTRODUCTION

The enzyme carbonic anhydrase (EC 4.2.1.1.) was first isolated, from mammalian erythrocytes, by Meldrum & Roughton (1933). The isolation of this enzyme provided conclusive evidence for a catalyst in blood that facilitated the reversible hydration of carbon dioxide; earlier experiments had suggested the existence of such a catalyst (Van Slyke & Hawkins, 1930; Dirken & Mook, 1930; Brinkman & Margaria, 1931), and it had been clear, since the work of Faurholt (1924), that the spontaneous reaction of carbon dioxide was far too slow to account for the observed composition of air expired from the lungs. The development of the understanding of carbon dioxide transport in blood, and the events which led up to the discovery of carbonic anhydrase, have been described by Roughton (1944). Several years later Keilin & Mann (1940) achieved nearly complete purification of the enzyme from bovine erythrocytes and showed the presence of zinc as an essential constituent, the first demonstration of a role for zinc in biochemistry. Mann & Keilin (1940) also made the important and interesting discovery that the activity of carbonic anhydrase is powerfully and specifically inhibited by aromatic unsubstituted sulphonamides.

For a period of twenty years few important developments occurred in the chemical characterization of red-cell carbonic anhydrase. Renewed interest in the enzyme was stimulated however by the discovery by Lindskog (1960) that bovine erythrocyte carbonic anhydrase could be separated into two forms, or isoenzymes. Reports followed rapidly from three laboratories of the separation of human erythrocyte carbonic anhydrase into three isoenzymes



(Nyman, 1961; Rickli & Edsall, 1962; Laurent et al., 1962). The first step in the purification of the erythrocyte enzyme is its separation from the great excess of haemoglobin in the red-cell. In the early investigations haemoglobin was removed by selective denaturation with chloroform and ethanol (Tsuchihashi, 1923). Though the isoenzymes appeared to be largely unchanged by this treatment (Gibbons & Edsall, 1964) considerable effort was made in Edsall's laboratory to devise more gentle methods for the removal of haemoglobin. A series of procedures was developed but by far the most successful of these was a technique involving repeated ion-exchange chromatography (Armstrong et al., 1966).

All the isoenzymes of carbonic anhydrase that have been examined carefully were found to be composed of single peptide chains having molecular weights of about 30,000. There was no indication of the presence of subunits, nor has aggregation of the native protein been observed (Rickli et al., 1964; Nyman & Lindskog, 1964; Armstrong et al., 1966). None of the isoenzymes contains a disulphide bond though the native molecules appear to be compact and almost spherical (Rickli et al., 1964). Each molecule of the enzyme contains one atom of zinc and this is tightly bound; it can be removed by prolonged dialysis in the presence of chelating agents at low pH (Lindskog & Malmström, 1960; 1962). Enzyme activity is lost on removal of the zinc but is regained on adding  $\text{Zn}^{++}$ . Other metallic ions can also be added to the apoenzyme but of these only  $\text{Co}^{++}$  restores the activity, and then only partially (Lindskog, 1963; 1966a & b, Lindskog & Nyman, 1964; Thorslund & Lindskog, 1967). X-ray diffraction studies of the human high activity component have revealed the crystalline isoenzyme to be compact, with the zinc atom lying at the centre of the molecule at the bottom of a deep cleft

(Fridborg et al., 1967).

Of great interest is the discovery that the two major isoenzymes of human erythrocytes differ markedly in specific activity (Gibbons & Edsall, 1964). The high activity component is some 30 times more effective than the low activity form as a catalyst of the reversible hydration of carbon dioxide. The third, minor, isoenzyme is of the high activity type. Carbonic anhydrase from the erythrocytes of the rhesus monkey also has been separated into high and low activity components, very similar in all respects to the human isoenzymes (Duff & Coleman, 1966). This similarity extends even to the marked differences in amino acid composition that are found between the two isoenzymes in both species (Armstrong et al., 1966; Duff & Coleman, 1966). Horse erythrocyte carbonic anhydrase has been resolved into several isoenzymes, of which the two major components are low and high activity forms; the high activity isoenzyme is unusual in having an isoelectric point greater than pH 10 (Furth, 1968).

In contrast to these findings, there is only one major isoenzyme in bovine erythrocytes and this is of the high activity type (Lindskog, 1960). A similar situation appears to exist in the case of dog erythrocytes, where there is evidence for only a single high activity isoenzyme (Byvoet & Gotti, 1967). Apart from comparative electrophoretic studies on a wide range of primates carried out by Tashian et al. (1968) no other species have been examined. A preliminary report has appeared recently describing the isolation of a single high activity isoenzyme from a microorganism; this appears to have all the general properties of carbonic anhydrase from the higher animals (Brundell et al., 1969).



Until very recently it was believed that carbonic anhydrase had an absolute specificity for carbon dioxide and hydrogen carbonate. However it has been shown that the enzyme also catalyses the hydrolysis of p-nitrophenyl acetate (Schneider & Liefländer, 1963; Pocker & Stone, 1965; 1967; 1968b; Verpoorte et al., (1967), naphthyl esters (Tashian & Shaw, 1962), and a sultone (Lo & Kaiser, 1966), as well as the hydration of carbonyl compounds other than carbon dioxide such as acetaldehyde (Pocker & Meany, 1965), other aliphatic aldehydes (Pocker & Dickerson, 1968), and pyridine aldehydes (Pocker & Meany, 1967). The catalytic activity of bovine carbonic anhydrase for the hydration of acetaldehyde is several hundred times less than its activity as a catalyst of carbon dioxide hydration, and the hydrolytic activity with the pyridine aldehydes is much lower again; with the esters it is even less. These findings apply generally also to the human erythrocyte isoenzymes. It is interesting that the high and low activity human components are comparably active in ester hydrolysis.

Carbonic anhydrase is found in tissues other than blood, though in most cases in very much smaller amounts. Typically, it is present in cells having secretory functions; mention may be made of its occurrence in the gastric mucosa (Davenport, 1939), the central nervous system (Ashby & Chan, 1943), the kidney (Ashby, 1943), the avian shell gland (Bernstein et al., 1968) and the reproductive tracts of both male and female animals (Mawson & Fischer, 1952; Lutwak-Mann, 1955). Of these tissues the dorsolateral prostate of the rat is distinguished by its extremely high content of the enzyme, at a concentration equal to that in rat blood (Mawson & Fischer, 1952). Probably because of the relatively small

quantities of the enzyme in most tissues other than blood, no detailed examination of carbonic anhydrase from these sources had been carried out before the present work was undertaken.

Many excellent accounts of the progress of research into carbonic anhydrase have been published over the years; a complete list of reviews up to about 1966 has been given by Maren (1967). In fact Maren's review (Maren, 1967), while maintaining a physiological viewpoint, is very comprehensive; the author provides an excellent bibliography. Other, more recent, reviews have been presented by Edsall (1968a & b). Roughton (1944) surveyed the whole subject of carbon dioxide transport in blood as well as describing his early work with the enzyme, while Roughton & Clark (1951), and Davis (1961), outlined methods of determining the activity of the enzyme and described its properties, prior to the discovery of the isoenzymes.

The aim of the present work was to compare, in the light of the recent intensive investigations concentrated on the erythrocytes, carbonic anhydrase occurring in the reproductive tracts of several mammals with the enzyme found in the erythrocytes of the same species. None of the species that form the subject of the present work have been examined previously for the presence of isoenzymes of carbonic anhydrase. It was hoped that these preliminary investigations might lead the way to the discovery of interesting and enlightening relationships between the forms of carbonic anhydrase existing in the different tissues of the body.

The dissertation is divided into five chapters. Chapter 1 is concerned with the development of a precise method for the assay of carbonic anhydrase by titration at constant pH. This method has been shown to attain the same



level of precision as the stopped-flow spectrophotometric technique and, apart from being easy to assemble from standard equipment and straightforward in use, has several advantages in comparison with the stopped-flow method.

Chapters 2, 3, and 4 deal with comparative investigations made of carbonic anhydrase occurring in the erythrocytes and certain organs of the reproductive tracts of rats, sheep, and rabbits. Chapter 2, concerned with the enzyme in the erythrocytes and dorsolateral prostate of the rat, contains details of the techniques that were later applied to the study of the enzyme in the tissues of the other species; a great part of the time was devoted to rat carbonic anhydrase because the level of the enzyme in the prostatic tissue is very high and it can be isolated and purified relatively easily. Apart from investigations of the general properties of the isoenzymes, special attention was given to the kinetics of the hydration of carbon dioxide in the presence and absence of inhibitors, the hydrolysis of p-nitrophenyl acetate and  $\beta$ -naphthyl acetate, and the variation in behaviour of different sulphonamide inhibitors.

Chapter 3 describes the results of experiments in which the properties were compared of carbonic anhydrase isoenzymes isolated from the erythrocytes and uterus of the sheep. Most of the comparative data are concerned with the kinetics of the hydration of carbon dioxide.

Chapter 4, concerned with a comparison of the properties of the enzyme in the erythrocytes and uterus of the rabbit, is closely similar to Chapter 3.

Chapter 5 deals with an investigation of the relationship between the concentrations of carbonic anhydrase and zinc in the uterus of the rabbit.

In addition, the results of a previous study on the connection between carbonic anhydrase activity in this tissue and the ovarian hormones in pregnancy were confirmed using the more precise method of assay.

Points of general interest arising from the experiments are considered in the GENERAL DISCUSSION.

## MATERIALS AND METHODS

Specialized procedures are described in the appropriate chapters. Materials and methods of general application are presented here.

Reagents. Tris (Reagent Grade), p-nitrophenyl acetate,  $\beta$ -naphthyl acetate, Fast Blue RR (4<sup>1</sup>-amino-2<sup>1</sup>,5<sup>1</sup>-dimethoxy-benzanilide), and soya bean trypsin inhibitor were all obtained in a satisfactory state of purity from the Sigma Chemical Co., London. DEAE-Sephadex (A-50), Sephadex G-100, and Blue Dextran were obtained from Pharmacia Ltd., London. Coomassie Blue (Brilliant Blue R250) was supplied by George T. Gurr Ltd., London. Diamox<sup>1</sup> was the product of the Cyanamid Co., Pearl River, New York, and ethoxzolamide<sup>2</sup> a gift of the Upjohn Co., Kalamazoo, Mich.; both inhibitors were used without further purification. N, N, N<sup>1</sup>, N<sup>1</sup>-tetramethylethylenediamine, acrylamide, N, N<sup>1</sup>-methylenebisacrylamide, and acetonitrile (anhydrous grade) were supplied by Kodak Ltd., Liverpool. Myoglobin and ovalbumin were generously given by Seravac Laboratories, Maidenhead, and Dr E. F. Hartree respectively.

DNSA<sup>3</sup> was prepared by dissolving the sulphonyl chloride (British Drug Houses Ltd., Poole, Dorset) in acetone and adding strong ammonia; the

- 
1. Diamox, the sodium salt of acetazolamide, 5-acetamido-1,3,4-thiadiazole-2-sulphonamide, was assumed to have an equivalent weight of 270 (Whitney, Folsch, Nyman & Malmström, 1967).
  2. Ethoxzolamide, 6-ethoxybenzothiazole-2-sulphonamide.
  3. DNSA, 1,1-dimethylaminonaphthalene-5-sulphonamide.

sulphonamide crystallized rapidly. Re-crystallization was carried out using ethanol, yielding pale lemon needles (Weber, 1952). Diethyl malonic acid was prepared by the hydrolysis of diethyl-diethylmalonate (Kodak Ltd., Liverpool). The ester was refluxed with ethanol, water and KOH; the ethanol was distilled off, and the residue was dissolved in water and acidified with conc.-HCl. The free diethyl malonic acid was extracted with ether and re-crystallized from petroleum ether (m.p. 125°, Lange (1952) quotes 126°). Most other chemicals were of A.R. grade and the water used was deionized or glass-distilled.

Assay of hydratase activity toward CO<sub>2</sub>. Precise measurement of the initial hydration rate was carried out by titration at constant pH using the procedure described in Chapter 1. In the experiments with acetazolamide, the enzyme and inhibitor were mixed 5 min. before the addition of substrate. A unit of carbonic anhydrase was defined as that amount which catalysed the hydration of 1  $\mu\text{mole-CO}_2 \text{ min.}^{-1}$  under the assay conditions state above, with a substrate concentration of 1mM-CO<sub>2</sub>. Because of the limited solubility of CO<sub>2</sub> in water it was impossible to use a concentration of this substrate sufficient to saturate the enzyme.

Carbonic anhydrase was determined in the effluents from column chromatography by a modification of the method of Philpot & Philpot (1936). In this modified procedure buffering was provided by tris-HCl, pH 9.3 (4°). The apparatus of Philpot & Philpot was used, with a floatmeter to regulate the flow of CO<sub>2</sub>. A volume of cold (4°) CO<sub>2</sub>-saturated water (5ml.), containing a little phenol red, was added to a boiling tube standing in ice-water, followed by an aliquot (a few  $\mu\text{l.}$ ) of enzyme solution. CO<sub>2</sub> was then bubbled

through the assay solution (about 0.5l/min.) and immediately 2ml. of 0.1M-tris-HCl buffer was added. The uncatalysed reaction was completed (colour change) in 60-70 sec. It was found that under these conditions the logarithm of the fraction (catalysed time)/(uncatalysed time) was inversely proportional to the enzyme concentration for values of the fraction between 1.0 and 0.4 (Fig. 1). This observation facilitated rapid, approximate quantitation of enzyme activity when analysing column effluents. The Philpot assay is discussed in the INTRODUCTION, Chapter 1.

Assay of esterase activity toward p-nitrophenyl acetate. The convenient and rapid spectrophotometric assay used was a combination of procedures described by Armstrong et al. (1966), Thorslund & Lindskog (1967), and Verpoorte et al. (1967). At the wavelength of the isosbestic point of p-nitrophenol and the p-nitrophenolate ion, 348 mμ,  $\epsilon = 5.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  for both species, and at the same wavelength  $\epsilon = 0.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  for p-nitrophenyl acetate (Bergmann et al., 1958; Armstrong et al., 1966). These values were not changed significantly by the concentration of acetone used as a solvent in these experiments (Verpoorte et al., 1967). Therefore, if measurements were made in a 1 cm. cuvette, the increase in extinction at 348 mμ divided by 0.005 gave the concentration (μM) of total hydrolysis product, independently of pH. The observed rate of hydrolysis included a contribution from the non-enzyme-catalysed reaction, and this was reduced to a minimum 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. This solution was stable for several hours. Acetone



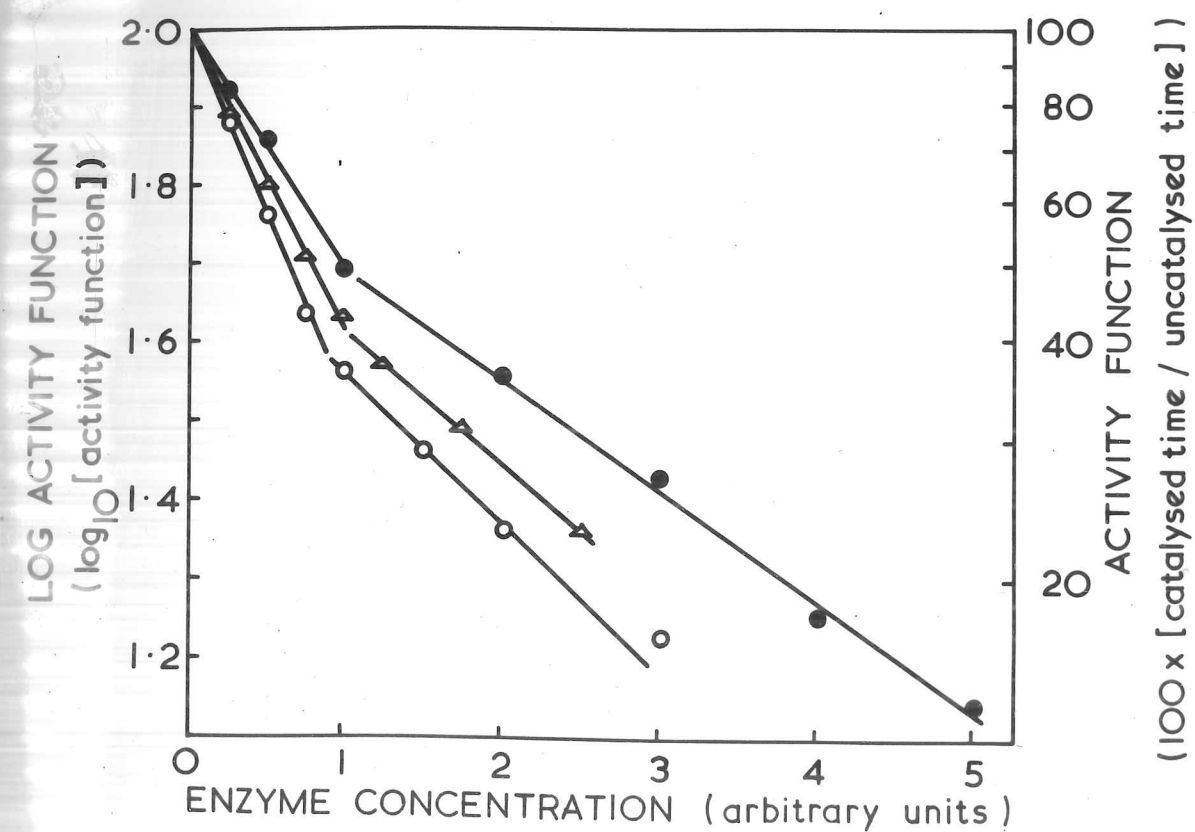
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A 3 mM stock solution of substrate was prepared by dissolving 27.2 mg of p-nitrophenyl acetate in 1 ml. of acetone and diluting this rapidly with water to 50 ml. This solution was stable for several hours. Acetone

Figure 1. The relationship between concentration of carbonic anhydrase and the logarithm of the activity function in the modified Philpot assay. The activity function was defined as the ratio (time for the enzyme-catalysed reaction)/(time for the uncatalysed reaction).  $\circ$ , Rat erythrocyte isoenzyme Eryth. 3 (CEP);  $\bullet$ , rat prostate isoenzyme Post. 1b (the nomenclature is explained in Chapter 2, EXPERIMENTAL AND RESULTS);  $\triangle$ , human erythrocyte isoenzyme C (see Chapter 1, EXPERIMENTAL).

Figure 1.



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 3mM. Measurement of the initial rate of hydrolysis was carried out with an SP 800 double beam spectrophotometer, in a cell compartment maintained at 25°. The use of the automatic sample changer allowed the simultaneous examination of four assay solutions.

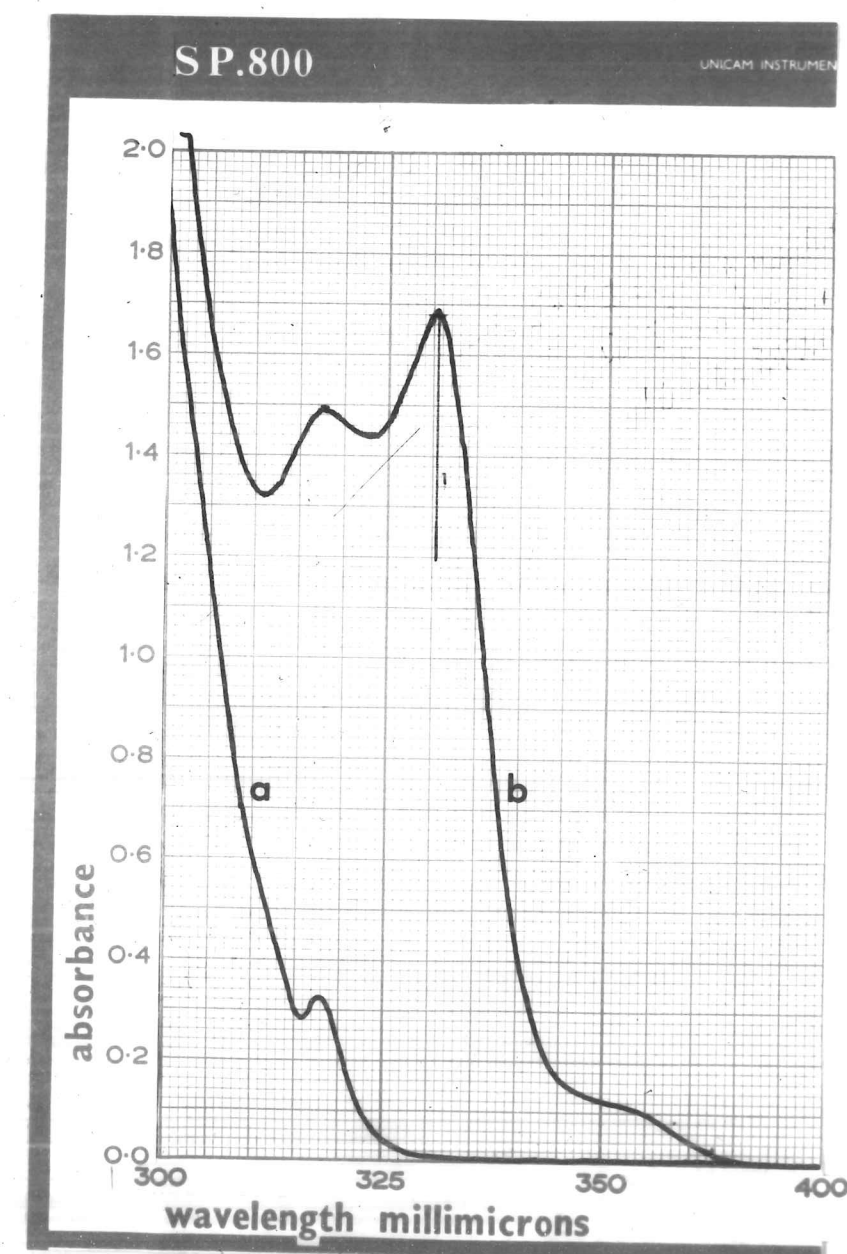
An assay solution was prepared by mixing, in a 1 ml. semi-micro spectrophotometer cuvette, 0.1 ml of a 0.1 M-diethylmalonate buffer, pH 8.0, a suitable volume of enzyme solution (a few  $\mu$ l.) and finally a volume of 2% (v/v) acetone in water, such that after addition of an appropriate quantity of substrate solution, the total volume of the assay mixture would be 1.0 ml. The reaction was started by the addition of substrate and the initial rate was determined. In assays in which acetazolamide was included, enzyme and inhibitor were mixed 5 min. before the addition of the substrate.

Assay of esterase activity toward  $\beta$ -naphthyl acetate. This spectrophotometric assay depended upon the fact that whereas a solution of  $\beta$ -naphthol, which is the product of hydrolysis, has an absorption maximum at 330 m $\mu$  a solution of the unhydrolysed ester makes no significant contribution to the extinction at this wavelength (Fig. 2). For  $\beta$ -naphthol,  $\epsilon = 1.70 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  at 330 m $\mu$ , in a solution containing 5% acetone at pH 8.0 (10mM-diethylmalonic acid buffer). When measurements were made in a 1 cm. cuvette, the increase in extinction at 330 m $\mu$  divided by 0.0017 gave the concentration ( $\mu$ M) of the hydrolysis product. A stock



Figure 2. A portion of the absorption spectra of  $\beta$ -naphthyl acetate and  $\beta$ -naphthol. a, 1.0 mM- $\beta$ -naphthyl acetate and b, 1.0 mM- $\beta$ -naphthol, both in a solvent consisting of 5% (v/v) acetone in an aqueous solution of 10 mM-diethylmalonate, pH 8.0. It can be seen that  $\beta$ -naphthyl acetate makes no significant contribution to the extinction at a wavelength of 330m $\mu$ .

Figure 2. ABSORPTION SPECTRA OF  $\beta$ -NAPHTHYL ACETATE &  $\beta$ -NAPHTHOL



solution of the substrate was prepared by dissolving 18.6 mg.  $\beta$ -naphthyl acetate in 5.0 ml. acetone.

An assay solution was prepared by mixing, in a 1 ml. semi-micro spectrophotometer cuvette, 0.1 ml. of a 0.1 M-diethylmalonic acid buffer, pH 8.0, a suitable volume of enzyme solution, and finally a volume of water such that after addition of an appropriate quantity of substrate solution (50  $\mu$ l to give 1mM- $\beta$ -naphthyl acetate) the total volume of the assay solution would be 1.0 ml. The reaction was started by the addition of substrate and the assay temperature was 25°. Allowance was made for the non-enzyme-catalysed reaction.

Assay of hydratase activity toward 4-pyridine aldehyde. The spectrophotometric assay used, which is summarised below, has been described fully by Pocker & Meany (1967). Measurement of reaction rate was carried out at 3° in the SP 800 spectrophotometer, the cell compartment of which was fitted with tubing to allow a stream of N<sub>2</sub> to be passed continuously over the outer surfaces of the cuvettes thus preventing condensation. Hydration of 4-pyridine aldehyde was measured by recording the decrease in extinction of the assay solution at a wavelength of 320m $\mu$ . From the progress curve of the reaction was calculated, by the usual method, a pseudo-first-order rate constant and multiplication of this by the approximate value of the fraction of hydration under the present conditions (0.66), yielded the rate constant for the hydration reaction. The initial rate of the enzyme-catalysed reaction was readily obtained after subtracting the contribution of the uncatalysed rate.

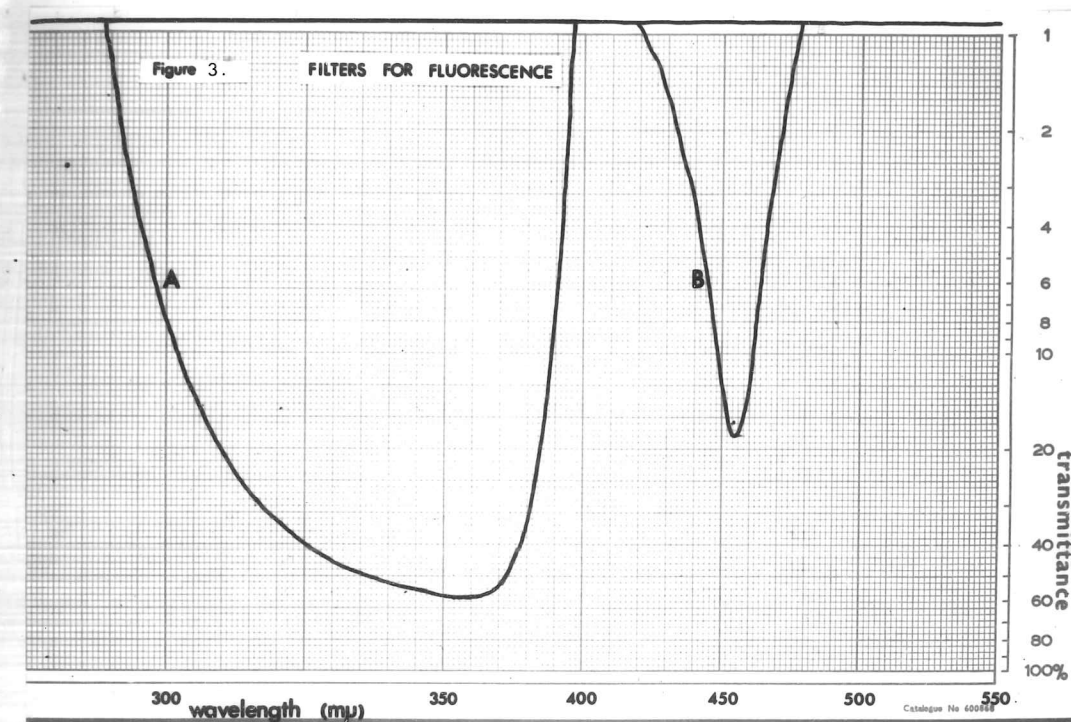
An assay solution was prepared by mixing, in a 1ml. semi-micro spectrophotometer cuvette, 0.1ml. of a 0.1M-dithyl malonate buffer, pH 7.0, a

suitable volume of enzyme solution, and finally a volume of water such that after addition of substrate the total volume of the assay would be 1.0ml. The substrate solution was prepared by diluting 4-pyridine aldehyde 10 times with anhydrous acetonitrile; 1.2 $\mu$ l. of this solution, when diluted to a volume of 1.0ml., gave a substrate concentration of approximately 1mM. A typical progress curve is shown in Fig. 28, Chapter 2.

The 4-pyridine aldehyde (L. Light & Co., Colnbrook) was distilled twice under N<sub>2</sub> at reduced pressure before use. N<sub>2</sub> was introduced through a bubbler in the distillation flask, heated on a water bath at 80°. The bulk of the liquid distilled at a temperature of 77° (uncorrected) and a pressure of 13mm.Hg leaving a dark tar. After re-distillation the product, which was colourless, was stored at 4° under nitrogen. The sample of 4-pyridine aldehyde used by Pocker & Meany (1967) distilled at a temperature of 76 - 77° under nitrogen at 12mm.Hg. The temperature of distillation, together with the rate of the uncatalysed hydration of the 4-pyridine aldehyde (see Chapter 2, EXPERIMENTAL AND RESULTS), constitute the only criteria of purity of my sample of the compound.

Measurement of fluorescence. A Farrand flurometer, model A, was used to measure fluorescence. The light source was a General Electric ultra-violet lamp (type H3FG) with a hole cut in the outer envelope to increase the output of short-wavelength ultraviolet radiation. The excitation filter was Wood's glass and the fluorescence filter was a Farrand interference filter, type 442. The sample cuvettes were made from "Spectrosil" tubing, and required a sample volume of 1ml. The methods of measurement used, described fully by Chen & Kernohan (1967),

Figure 3. The transmission characteristics of the filters used in the measurement of the fluorescence displayed by carbonic anhydrase in the presence of DNSA, illustrated as a graph of per cent transmittance against wavelength. A, excitation filter (Wood's glass); B, fluorescence filter (Farrand interference filter, type 442). Neither filter transmitted a significant amount of radiation in the range of wavelength at which the other was transmitting.





are summarised here.

Binding of DNSA to carbonic anhydrase is accompanied by great enhancement of ligand fluorescence. Moreover, while the wavelength of maximum fluorescence of DNSA alone at neutral pH is 580m $\mu$ , maximum fluorescence of the combination of ligand and enzyme occurs at wavelengths from 460 to 470m $\mu$ . Consequently it is a simple matter to follow the progress of the interaction between DNSA and carbonic anhydrase. Because DNSA is bound relatively weakly to the enzyme ( $K_D$  about 0.2 $\mu$ M), it is displaced quantitatively by the addition of ethoxzolamide ( $K_D$  about 0.2m $\mu$ M).

Experiments were carried out by adding aliquots of a solution of DNSA to a solution of carbonic anhydrase contained in the fluorometer cuvette, and plotting the progressive increase in fluorescence. When a considerable excess of DNSA had been added, in order to obtain an estimate of the maximum fluorescence, titration with ethoxzolamide was carried out and the decrease in fluorescence was recorded. A stock solution of DNSA was prepared by dissolving 3.33mg. (Cahn microbalance) of dried sulphonamide in 10ml. of 0.02M-HCl, giving a 1.33mM solution which was stable at 4° for several weeks. A stock solution of ethoxzolamide was prepared by dissolving 3.04mg. of the sulphonamide in 10ml. of 50% (v/v) acetone, giving a 1.18mM solution which was also stable. The stock solutions of inhibitor were added to the assay solutions (containing approximately 0.5 $\mu$ M-carbonic anhydrase in 0.02M-sodium phosphate buffer, pH 7) with a 10 $\mu$ l. Hamilton syringe. Results were expressed in the form of graphs of fluorescence against the molar ratio of inhibitor to enzyme.

DEAE-Sephadex ion-exchange chromatography. DEAE-Sephadex (A-50) was swollen in water and washed with diluted acid and alkali, according

to the manufacturer's instructions. Finally, the gel was equilibrated with the desired buffer at 4°. Three buffers were used; 0.1M-tris-0.024M-HCl (pH 9.3 at 4°), and the same buffer diluted twice and four times with water. These buffers were named I, II, and III respectively. The prepared gel was packed to form a column measuring (35 x 1.35)cm. and was washed with at least 200 ml. of the appropriate buffer to complete the equilibration. Carbonic anhydrase was eluted using 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°. Routinely, pooled fractions of effluent were concentrated by lyophilization 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 poly-amino-polycarboxylic acid carrier ampholyte mixtures were manufactured by LKB Produkter AB, 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 dilute orthophosphoric acid and ethanolamine, respectively. The apparatus was maintained at 0°. Employing a potential difference of 400 v., equilibrium fractionation was attained after 3-4 days. The column contents were displaced by pumping water onto the top of the gradient. 1 - 2 ml. fractions were collected and the pH of each at 0° was determined immediately with a Pye-Ingold combination electrode, type 401-M5 (W. G. Pye & Co., Cambridge) attached to a Radiometer Titrator pH meter, type TTT1C, compensated for operation at 0°.

Absorption of  $\text{CO}_2$  by the fractions was considered to be negligible at the pH of the fractions in which the isoenzymes of carbonic anhydrase 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 stock solutions used to prepare the gel had the following compositions:

Solution A.

1 N-HCl	48ml.
Tris	36.6g.
N,N,N <sup>1</sup> ,N <sup>1</sup> -tetramethylethylenediamine	0.23ml.
Water	to 100ml.

Solution B.

Acrylamide	28.0g.
N,N <sup>1</sup> -methylenebisacrylamide	0.735g.
Water	to 100ml.

Solution C.

Riboflavin	4mg.
Water	to 100ml.

A gel solution was prepared by mixing 1 vol.-A + 2 vol.-B + 1 vol.-C + 4 vol.-water. This mixture was poured into tubes standing on a pad of "Plasticine", water was layered onto the surface and polymerization was carried out in strong light. The protein, in a volume of 5 - 30  $\mu\text{l}$ . containing both naphthalene blue (to indicate the progress of electro-

phoresis) and sucrose, was layered directly onto the upper surface of the gel. The current was 1mA/tube until the samples had entered the gel, whereupon it was increased to 3mA/tube. The temperature of the gel did not then exceed 35°. That 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 the dye Coomassie Blue dissolved in 12.5% trichloroacetic acid (Chrambach *et al.*, 1967) and esterase activity toward p-naphthyl acetate at pH 7.0 was demonstrated by the appearance of claret coloured bands resulting from the coupling of the diazotised salt Fast Blue RR with released p-naphthol (Tashian & Shaw, 1962). Acetazolamide was added to control gels to inhibit esterase activity of carbonic anhydrase.

Determination of molecular weight by gel filtration. The method of Andrews (1964, 1965) was used and the gel employed was Sephadex G-100, packed in a column measuring (50 x 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 mμ, and those of the majority of the carbonic anhydrase isoenzymes found by measurement of activity. Human erythrocyte carbonic anhydrase 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 ranged from 10 - 50 μg whereas in the cases of the human and the rat erythrocyte isoenzymes that have a low specific activity, 1 - 2 mg was used. Filtration was carried out at 4° with



Buffer III containing 0.05M-NaCl. A Blue Dextran high molecular weight reference was included in every experiment. Activity was assayed approximately using an automatic device (constructed by the author) incorporating a pH meter and potentiometric recorder, the operating principle of which was based on a changing pH method similar to the modified Philpot assay described above.

Estimation of haemoglobin in solutions that were not optically clear.

The apparatus used for the quantitative comparison of spectra of haemoglobin has been described by Keilin & Wang (1946) and Hartree (1955). It consisted of a Zeiss microspectroscope fitted with a wedge trough. The concentration of oxyhaemoglobin in an optically unclear tissue homogenate was determined by comparison of the major absorption band of oxyhaemoglobin in the homogenate with the same band produced by diluted whole blood, contained in the wedge trough. From a knowledge of the depth of the sample solution, together with the width of the trough at the point of equal absorption and the dilution of the blood, it was possible to calculate the degree of dilution of the blood contaminating the sample. In this way estimates could be made of the degree of contamination of the homogenate (such as a centrifuged extract of rabbit endometrium) by erythrocyte carbonic anhydrase; the true contamination was probably less than that estimated because no correction was made for the contribution by other haematin to the observed absorption.

Determination of zinc. Effluents from column chromatography were analysed directly to find the elution pattern of zinc, using a Perkin Elmer atomic absorption spectrophotometer, model 303. The apparent zinc concentration measured in this way, without first digesting 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 AR fuming  $\text{HNO}_3$ ) of the protein which had previously been exhaustively dialysed against glass-distilled water. The samples were evaporated to dryness before adding the  $\text{HNO}_3$  and the digestion was then allowed to proceed for 24 hr. The bulk of the acid was removed by gentle heating and the digested residues were then diluted, and analysed using the atomic absorption spectrophotometer.

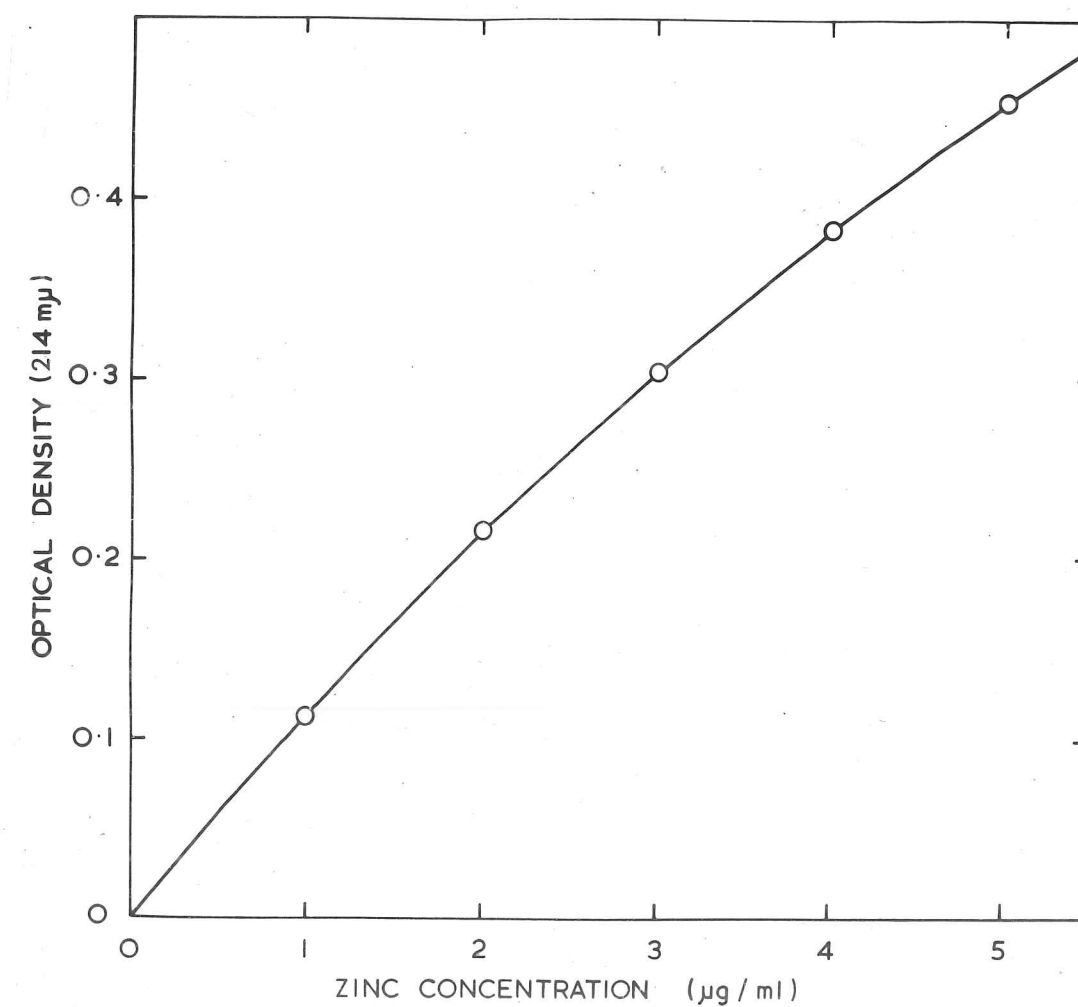
Zinc in samples of tissue was determined in much the same way. Tissue (100-200mg. wet weight) was dissected with appropriate safeguards and transferred to weighed micro-Kjeldahl flasks. After reweighing, 1ml. of fuming  $\text{HNO}_3$  was added to each flask and digestion allowed to proceed to 24 hr. at room temperature. Acid was evaporated until approximately 0.1ml. remained, care being taken to avoid the loss of volatile zinc compounds. The contents of each flask were made up with glass distilled water to 2ml.

Blanks and standard solutions were similarly treated. A standard curve is shown in Fig. 4. Standard solutions were prepared by dissolving a weighed amount of AR-grade zinc in 6N-HCl (re-distilled), and diluting to a known volume. When estimating zinc in samples of tissue no correction was made for light scatter caused by the presence of other salts because the effect is unlikely to have been significantly great (Willis, 1963). Zinc could be determined with an average reproducibility of  $\pm 5\%$ . All glassware used in these experiments was soaked in 6N-HCl for 24 hr. and rinsed well with glass-distilled water before use.

Determination of protein. The effluents from column chromatography

Figure 4. A standard curve for the determination of zinc by atomic absorption spectrophotometry. The results are illustrated by a graph of extinction at about 213.9m $\mu$  against the concentration of zinc in a standard solution of zinc chloride. 213.9m $\mu$  is the wavelength corresponding to the energy required to excite atomic zinc in the ground state to its lowest excited state.

Figure 4. DETERMINATION OF ZINC



were monitored at 280 m $\mu$  with a Gilson UV meter. Estimates were made of concentration of protein in certain of the purification procedures using the method of Lowry et al., (1951), standardized with bovine plasma albumin. Allowance was made for water adsorbed on the albumin (10.1% of the total mass). Specific extinction coefficients of the purified isoenzymes at 280 m $\mu$  were determined by weighing, under atmospheric conditions (Cahn microbalance), the dried residues from weighed aliquots of protein solutions, which had previously been exhaustively dialysed against glass-distilled water.  $E_{280}^{1 \text{ cm.}}$  of each solution was measured prior to drying and allowance for light scatter at 280 m $\mu$  was made by linear extrapolation from higher wavelengths. It was assumed that approximately 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.

## CHAPTER 1

### ASSAY OF CARBONIC ANHYDRASE BY TITRATION AT CONSTANT pH

#### INTRODUCTION

Unlike many enzymes, carbonic anhydrase catalyses reactions which themselves proceed at an appreciable rate in the absence of any catalyst. These reactions may be expressed, in a simplified form, as:



At or near neutral pH the equilibrium constant allows the reversible reaction to be conveniently examined when proceeding in either direction. In the past the study of carbonic anhydrase has been considerably impeded by difficulties in estimating precisely its activity, and the majority of the methods that have been evolved require important assumptions.

Manometric methods. The first successful technique for assaying carbonic anhydrase was the manometric method developed by Meldrum & Roughton (1933) and elaborated by Roughton & Booth (1946a). The rate of uptake of  $\text{CO}_2$  was measured manometrically in a specially designed vessel that contained the assay mixture and was shaken vigorously at  $0^\circ$ . Several corrections were applied to the results in order to derive kinetic information: (a) allowance was made for the limiting effect of diffusion using a complex formula. (b) An estimate was made of the rate of enzyme inactivation caused by the extremely vigorous agitation of the reaction vessel. (c) A correction was made for the solubility of the  $\text{CO}_2$  in the assay solution. (d) Care was taken to check that with any given enzyme preparation, the rate of the reaction was proportional to the enzyme concentration. In some cases this



proportionality was not obtained; the reason for this is considered in the section entitled DISCUSSION, below. The method was subsequently adapted for use with the standard Warburg apparatus; in this case pairs of flasks having equal volumes were required (Krebs & Roughton, 1948). A simple though less precise manometric method, together with an ingenious way of recording photographically the results, have been described by Van Goor (1940).

Indicator methods. The simple procedures described by Brinkman (1934) and Philpot & Philpot (1936) depend on measurement of the time required for the pH of a reaction solution to fall by several units. In the original Philpot method an aliquot of carbonate buffer (pH 10.5) was added rapidly to ice-cold water, through which was bubbled continuously a stream of  $\text{CO}_2$ . Unfortunately carbonate ion, at the concentration used, is an inhibitor of carbonic anhydrase (Roughton & Booth, 1946b) and therefore it is preferable to substitute another type of buffer. Roughton & Booth (1946b) suggested both the use of a veronal buffer (pH 8) and the addition of the  $\text{CO}_2$  in the form of an aliquote of a saturated aqueous solution. A modification of the Philpot method that was used in the course of the present work is fully described in the section of the dissertation entitled MATERIALS AND ANALYTICAL METHODS. These simple methods are suitable only for approximate determination of enzyme activity; I have found that the present modification of Philpot's procedure is particularly convenient for detecting the presence of carbonic anhydrase activity in effluents from column chromatography. The chief disadvantages of these technique are: (a) over the wide range of pH studied the mechanism of the uncatalysed reaction changes from



to



(b) No allowance can be made for any progressive inactivation of the enzyme that might occur since only one observation is made in each test. (c) No correction is made for the limiting effects of the rate of solution of  $\text{CO}_2$ . (d) The effect of extraneous buffers is considerable. (e) The carbonate ion at these concentrations exercises a serious inhibitory effect on the enzyme which varies during the course of the reaction. Further limitations are imposed by the relatively high concentration of indicator which is probably inhibitory (Wilbur & Anderson, 1948), and the broad range of pH over which the reaction is carried out. The procedure described by Wilbur & Anderson (1948) is probably the most precise version of the indicator techniques.

Electrometric methods. Certain of the difficulties of the indicator methods are avoided by the continuous registration of pH in the electrometric procedures. These were developed by Brinkman & Magaria (1931), using an antimony electrode; by Stadie & O'Brien (1933), using a quinhydrone electrode; and by Wilbur & Anderson (1948), using a glass electrode. The last of these methods employed a specially designed reaction vessel having permanently attached tubes for the delivery of reactants. The whole apparatus was submerged in a cooled bath. The course of the reaction was followed over a range of pH that was narrow in comparison with the range covered in the case of the indicator methods. This approach was also adopted by Davis (1958) who introduced the use of a sensitive pH meter to reduce to a minimum the change of pH required.

Miscellaneous methods. An indirect assay has been reported by Cutolo (1957) but not carefully examined. It depends on the sensitivity to pH of the alcohol dehydrogenase-catalysed oxidation of ethanol.

Assays in current use. Reliable assays in current use depend on rapid measurement of the change in extinction of a buffered reaction mixture containing an acid-base indicator, in a stopped-flow device (DeVoe & Kistiakowsky, 1961; Gibbons & Edsall, 1963, 1964; Ho & Sturtevant, 1963; Kernohan, Forrest & Roughton, 1963). A flow technique has been developed by Kernohan & Roughton (1966) that depends on the measurement of the heat of reaction and is particularly suitable for use with concentrated solutions of the enzyme. These methods all avoid the serious difficulties of the earlier procedures.

The method for the determination of carbonic anhydrase activity that was developed in the course of the present work depends on the titration at constant pH of  $H^+$  ions produced by the hydration of  $CO_2$ . Compared with the spectrophotometric procedures, the titration method offers several definite advantages. It makes possible the maintenance of constant pH; it eliminates the specific ion effects arising from the use of highly concentrated buffers; it dispenses with the need for the determination of the relationship between the  $H^+$  ion concentration change and the buffer-indicator ionization; it also can be simply assembled using commercially available standard equipment.

Previously reported titration assays. Several authors in the past have used titration for the purpose of studying catalysis by carbonic anhydrase, particularly with regard to the influence of inhibitors



(Holmgard, 1961; Leibman, Alford & Boudet, 1961; Leibman & Greene, 1967). However, none of these studies has been concerned primarily with the possibility of developing titration into a strictly quantitative and dependable method. The brief description of these earlier methods which is to be found at the end of the section entitled DISCUSSION, below, illustrates certain limitations and difficulties which I believe have been eliminated in the present technique. Nor has the claim been made previously that methods based on titration can achieve the same standard of precision as the stopped-flow spectrophotometric technique. The present study describes a titration method for the quantitative assay of carbonic anhydrase that offers a satisfactory alternative to the spectrophotometric method and achieves the same standard of precision. The method has been published (McIntosh, 1968).

### EXPERIMENTAL

Reagents. The chemicals used were all of A.R. grade unless otherwise stated. Bacteriological peptone was obtained from British Drug Houses Ltd., Poole, Dorset. The water was deionized.  $\text{CO}_2$  (purity > 99.5%) at atmospheric pressure was used to saturate water at  $0^\circ$  in a gas absorption bottle. When a sample of the  $\text{CO}_2$ -saturated water was added to  $\text{Ba}(\text{OH})_2$  solution back-titration indicated a concentration of  $\text{CO}_2$  within 1-2% of that predicted by calculation based on the Henry's law constant (Harned & Davis, 1943), when the atmospheric pressure and the vapour pressure of water at  $1^\circ$  were taken into account. Two NaOH solutions were prepared by diluting (with degassed water) the supernatant obtained on centrifugation of a saturated NaOH solution from which the carbonate had been precipitated. These solutions had concentrations of approx. 0.3N- and 2N-NaOH; the bottles in which they were kept were flushed with  $\text{N}_2$ . The 0.3N-NaOH solution was suitable for use when assays of enzyme activity in terms of units of enzyme were carried out, at a substrate concentration of  $1\text{mM}-\text{CO}_2$ . The 2N-NaOH solution was used in those experiments in which determinations were made of kinetic constants. The stock mixture of buffer and NaCl contained  $45\text{mM}-\text{NaH}_2\text{PO}_4-\text{Na}_2\text{HPO}_4$  and  $0.405\text{M}-\text{NaCl}$  at pH 6.89 ( $25^\circ$ ). At  $0^\circ$  the pH of this mixture was 6.99 and the ionic strength, after dilution in the assay, was 0.050 (5mM concn. of phosphate ions). Though it was possible to carry out titrations without

buffer, a low concentration of phosphate ions smoothed considerably the response of the titrator mechanism and improved the results.

Enzyme. Isoenzymes B and C (Rickli, Ghazanfar, Gibbons & Edsall, 1964) of human erythrocyte carbonic anhydrase was prepared from the erythrocytes of a single individual by the method of Armstrong, Myers, Verpoorte & Edsall (1966). This isolation entailed ion-exchange chromatography of the ethanol-chloroform extract from the haemolysate on DEAE-Sephadex (A-50) using the procedures described in detail in the section entitled EXPERIMENTAL AND RESULTS, Preparation of the erythrocyte isoenzymes of Chapter 2. Chromatography was performed twice to ensure that the preparation was homogeneous. The results of the chromatography are shown in Fig. 5. The kinetic properties of isoenzyme B were not determined. The concentration of the enzyme in the stock solution was determined by measurement of extinction at 280m $\mu$  (Gibbons & Edsall, 1964). There was no contamination by haemoglobin.

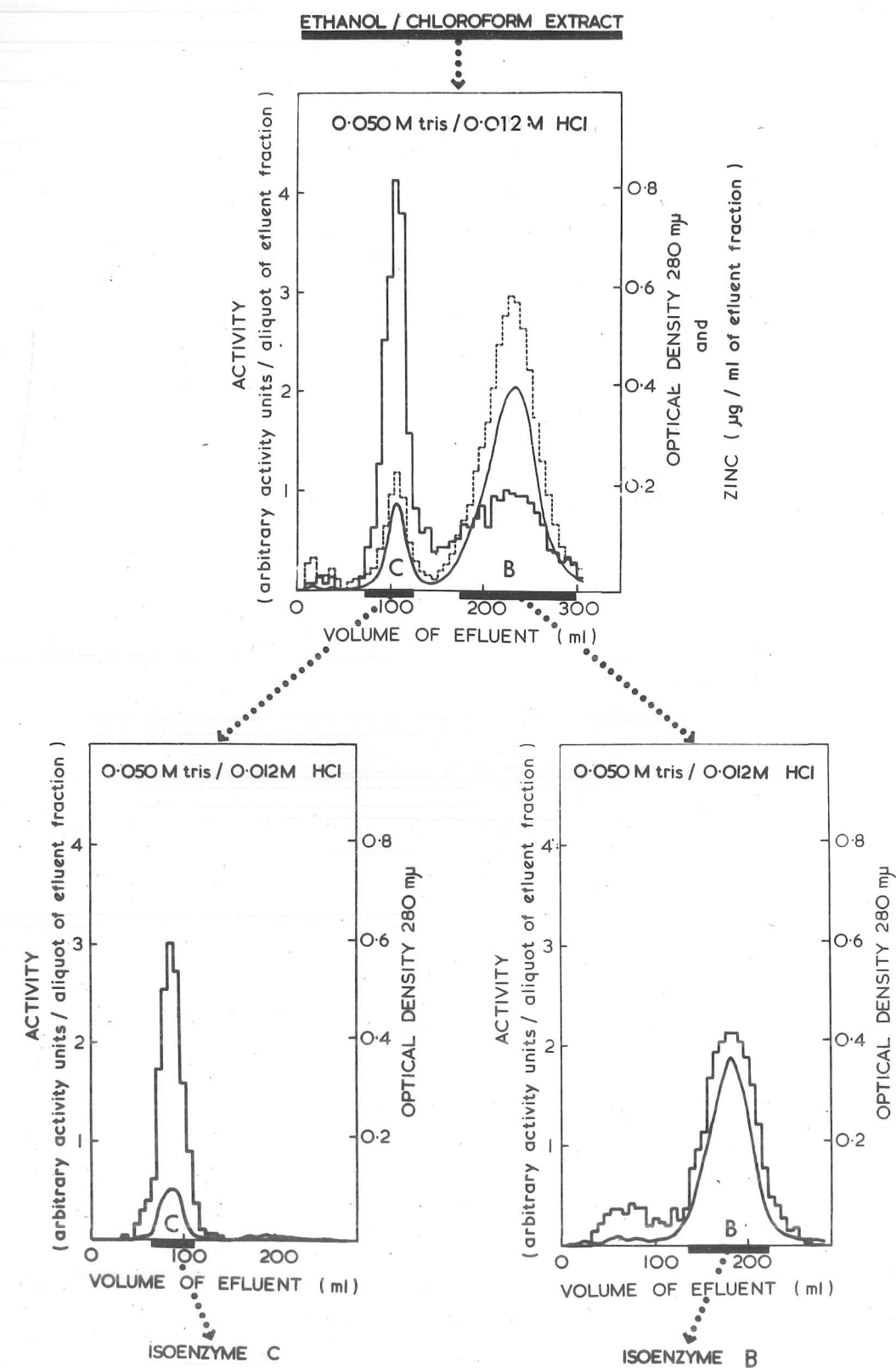
Titrator. The apparatus used for the continuous titration was a Radiometer Automatic Titrator TTT1c together with a Recorder SBR2c (V. A. Howe and Co. Ltd., London, W.11), with the controls set for pH-stat operation, and the fastest motor (30rev./min.) driving a syringe-burette of 0.5ml. capacity. The glass electrode was a Radiometer G202c type, recommended for use at 0°. A temperature compensator to correct the response of the instrument at 0° was supplied by the manufacturer of the titrator. pH calibration was performed by using a Beckman Instruments Inc. standard phosphate buffer.

The apparatus was connected to a second recorder that increased the speed of recording and amplified the movements of the syringe, thus making

**Figure 5.** A flow sheet summarizing the purification of human erythrocyte carbonic anhydrase and its resolution into isoenzymes, by column chromatography using DEAE-Sephadex. The starting material was a  $\text{CHCl}_3$ -EtOH extract of washed erythrocytes. The buffers had a pH of 9.3 at  $4^\circ$ . — (Continuous),  $E_{280}$ ; — (stepped),  $\text{CO}_2$  hydratase activity (modified Philpot assay); — — —, zinc. The heavy bars near the horizontal axes show which fractions were combined for further purification. The naming of the isoenzymes follows Rickli *et al.* (1964).

I apologise for the mis-spelling of the word "effluent" in many of the Figures.

**Figure 5. HUMAN ERYTHROCYTE DEAE-SEPHADEX CHROMATOGRAPHY**



it possible to use more concentrated solutions of titrant. This additional recorder was a Servoscribe potentiometric instrument (Kelvin Electronics Co., Wembley, Middx.) linked to a precision 10-turn potentiometer that, mounted below the syringe-burette of the titrator and operated by the drive, indicated the position of the piston, thus giving a direct measure of the volume added. Good results could also be obtained without this additional recorder, but only when a second 30 rev./min. motor was used to move the chart paper of the main SBR2c recorder.

The 20ml. titrator cell was maintained at a constant temperature ( $0^{\circ}$ ) by a circulating refrigerant, which was passed first through a copper coil immersed in an insulated ice-containing bath, next to the titrator cell, and finally back to a refrigerator. With the refrigerator set at  $-1.5^{\circ}$  and the insulated bath remaining at a temperature of  $0-0.5^{\circ}$ , the titrator cell had a temperature of  $0 \pm 0.1^{\circ}$ . The gas absorption bottle and all solutions, as well as two pre-set syringes, were kept in the ice bath. A burette was connected to a coiled pipe of large volume and cooled by immersion in the bath, which terminated in a narrow-bore polythene tube and syringe needle. This assembly enabled a measured volume of cold water to be passed into the cell.

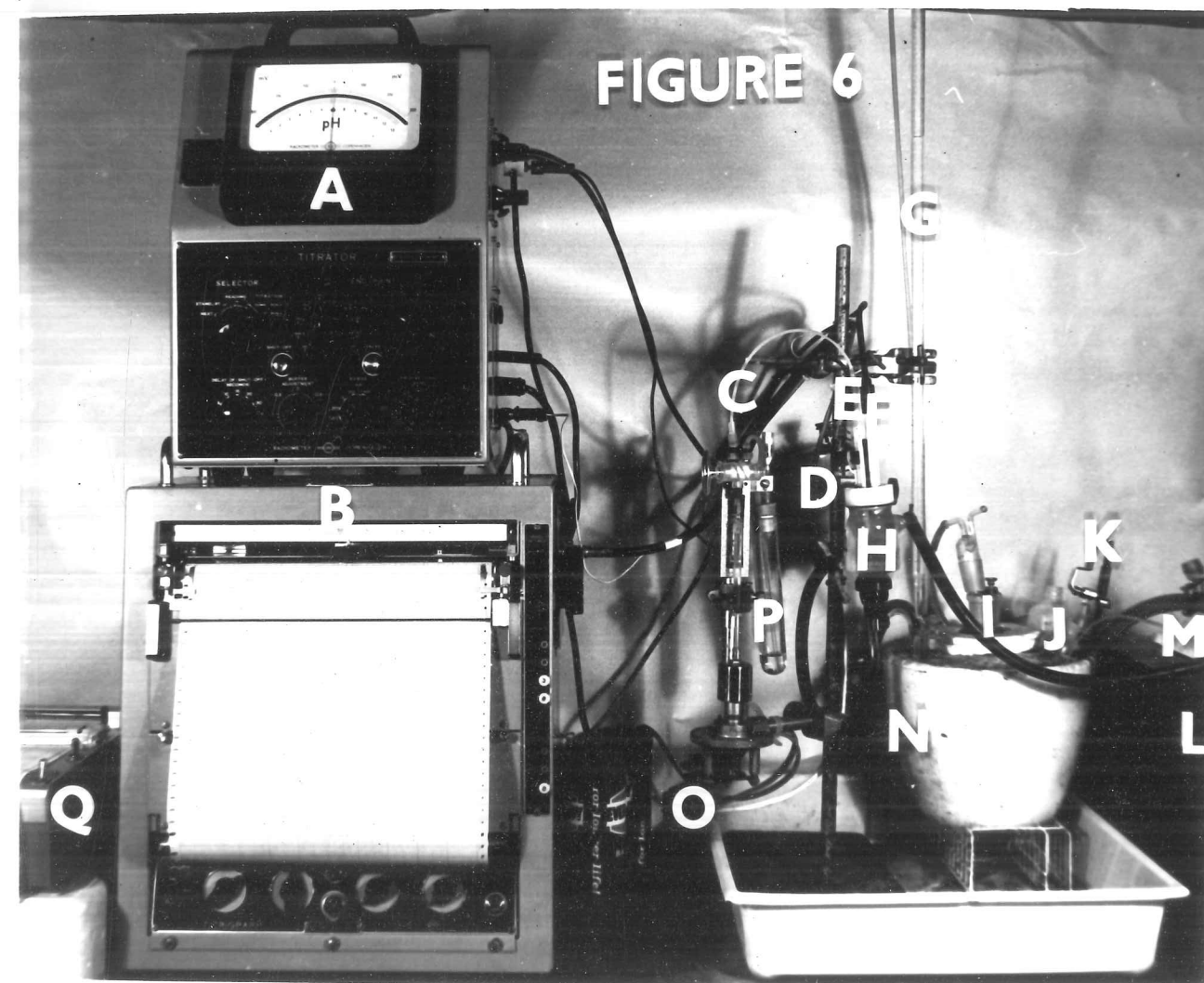
The temperature of the titrator cell was monitored with a calibrated miniature thermistor and bridge circuit whereby a temperature change of  $0.05^{\circ}$  could easily be detected. The volume of the cell, when positioned on the titrator, was adjusted by movement of the electrodes to exactly 18.0ml. of liquid. The only access to the cell was by way of a small hole through which additions of liquid were made. The mechanical stirrer was modified by shortening its shaft to accommodate the small reaction vessel, and the area of its blades was increased. The apparatus is illustrated in Fig. 6.



Figure 6 . Apparatus for assay of carbonic anhydrase by titration at constant pH.

- A. Radiometer Automatic Titrator TT11c.
- B. Radiometer Recorder SBR2c.
- C. Titrant delivery tube.
- D. Stirrer motor.
- E. Reference electrode.
- F. Glass electrode, Radiometer type G202c.
- G. Burette for admitting cooled water to the reaction vessel.
- H. Water-jacketed reaction vessel.
- I. Bottle containing  $\text{CO}_2$ -saturated water.
- J. Bottles containing peptone and buffer-salt solutions.
- K. Pre-set syringe.
- L. Circulating refrigerator.
- M. Tube for admitting cooled water to reaction vessel.
- N. Insulated bath maintained at about  $0.5^\circ$ .
- O. Beckman high-precision 10-turn potentiometer.
- P. Titrant reservoir.
- Q. Servoscribe potentiometric recorder.

The device for monitoring the temperature of the assay solution is not shown in this figure.

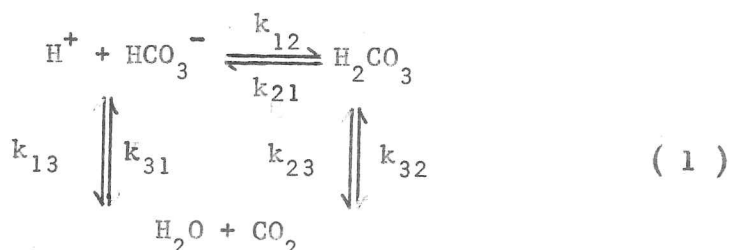


Assay procedure. The syringe-burette was filled with NaOH solution of the appropriate concentration and air bubbles were carefully removed from the delivery tube. Both recorders were set to zero. A quantity of degassed water, equal to 18.0 ml. less the volume of the reacting solutions, was admitted from the burette assembly into the cell, already in place on the titrator. A 2.0 ml. sample of the buffer-NaCl mixture was next added by means of the pre-set syringe, followed by 2.0 ml. of either 0.225mM-EDTA or 0.09% (w/v) peptone. When the contents of the cell reached the correct temperature (usually in 5 min.), the enzyme was injected and the pH adjusted by adding a small quantity of 0.01N-HCl and titration by the instrument to pH 7.0. The second pre-set syringe was then used to inject rapidly the required volume of CO<sub>2</sub>-saturated water, and at the moment of addition recording was begun. The initial part of the progress curve was recorded and after a suitable interval (approx. 10 half-lives of the reaction) the equilibrium value was obtained. At the end of the experiment the cell was emptied, rinsed and replaced rapidly so as not to alter the temperature of either the electrodes or of the alkali-delivery tube.

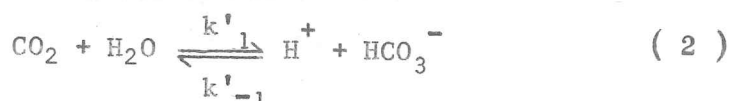
Treatment of experimental data. The rapid hydration reaction catalysed by carbonic anhydrase makes it convenient to determine a rate constant and calculate from it the initial rate, rather than to measure the rate directly as is usual in the study of enzymes. Though the uncatalysed reaction displays pseudo-first-order kinetics, it might be expected that in the presence of the enzyme zero-order behaviour would be observed, especially with substrate concentrations high in comparison with the Michaelis constant. However, under the conditions of the present study satisfactory results were obtained by treating the experimental data as though they obeyed first-order kinetics. A similar

treatment of data was adopted by Gibbons & Edsall (1964) in their experiments with the spectrophotometric technique.

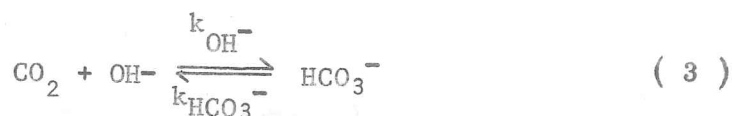
The following relationships were derived for the uncatalysed reaction, but were equally applicable in the presence of the enzyme, where  $k'_1$  becomes the rate constant of the step in which the enzyme-substrate complex reacts. By using the nomenclature of Gibbons & Edsall (1963), the equilibrium between the reactants in solution may be expressed as:



Since at 0° and pH 7 the ratio  $[\text{CO}_2] / [\text{H}_2\text{CO}_3] = 9.5 \times 10^2$  and the ratio  $a_{\text{HCO}_3^-} / [\text{H}_2\text{CO}_3] = 2.5 \times 10^3$  (Roughton, 1941),  $[\text{H}_2\text{CO}_3]$  is negligible. Therefore the expression can be simplified to:



where the primes denote values at a particular ionic strength. The experimentally observed rate includes a contribution from the reaction:



at pH values greater than 6-7. Pinsent, Pearson & Roughton (1956) report that  $k_{\text{OH}^-} = 1.1 \times 10^3 \text{ M}^{-1} \text{ sec}^{-1}$  at 0° and low ionic strength.

From eqn. (2) it is possible to derive the rate equation describing the

hydration of  $\text{CO}_2$ , as expressed in terms of concentration:

$$-da/dt = k'_1 a - k'_{-1} (\text{H}^+) x \quad (4)$$

where  $a = [\text{CO}_2]$  and  $x = [\text{HCO}_3^-]$  at any time. This reaction is seen to be of pseudo-first-order at constant pH if the activity coefficient of  $\text{H}^+$  remains unchanged. Such an assumption is justified by the fact that the ionic strength is approximately constant during the initial part of the reaction. Moreover, the rate constant is determined after extrapolation to zero time.

At equilibrium,  $-da/dt = 0$  and:

$$k'_{-1} = k'_1 a_{\text{eq.}} / (\text{H}^+)_{\text{eq.}} x_{\text{eq.}} \quad (5)$$

Substitution of this expression in eqn. (4) gives:

$$-da/dt = k'_1 a - k'_1 a_{\text{eq.}} (\text{H}^+) x / (\text{H}^+)_{\text{eq.}} x_{\text{eq.}} \quad (6)$$

Again assuming constant  $\text{H}^+$  activity coefficient, it follows by substitution of:

$$a_{\text{eq.}} = a_0 - x_{\text{eq.}} \quad (7)$$

where the subscripts indicate equilibrium and zero time, and rearrangement, that:

$$-da/dt = k'_{\text{app.}} (a - a_{\text{eq.}}) \quad (8)$$

Thus:

$$k'_1 = k'_{\text{app.}} (a_0 - a_{\text{eq.}}) / a_0 \quad (9)$$



where  $a_{eq.}$  and  $a_0$  represent the molar concentrations of  $CO_2$  at equilibrium and at zero time, respectively, and  $k'_{apparent}$  ( $k'_{app.}$ ) represents the rate constant of the pseudo-first-order reaction, as experimentally observed.

At the beginning of the reaction, assuming that there is no  $HCO_3^-$  present:

$$(-da/dt)_0 = k'_{app.} a_0 \quad (10)$$

This enables one to calculate the initial rate of the hydration reaction.

The value of  $(a_0 - a_{eq.})/a_0$  is best found by experiment, though an estimate of its magnitude can be made by introducing the equilibrium constant  $K = \frac{a_{H^+} a_{HCO_3^-}}{a_{CO_2}}$ . This constant has been measured by Harned & Davis (1943) under conditions such that simple interpolation gives the value  $2.7 \times 10^{-7} M$  for the present case. However, the use of this constant to calculate  $(a_0 - a_{eq.})/a_0$  involves the substitution of the activity coefficient of  $H^+$  and so the result, 0.78 at pH 7.0, is only approximately correct. The experimentally determined value at the same pH was 0.72.

The record of the volume of NaOH delivered against time was analysed by the usual method for a first-order reaction, which yielded a pseudo-first-order rate constant directly since the volume of NaOH titrated was proportional to the  $H^+$  ions formed which equalled  $CO_2$  hydrated. This rate constant was equal to the sum  $k'_{app.}$  (catalysed reaction) +  $k'_{app.}$  (uncatalysed reaction). The initial rate of the enzyme-catalysed hydration was calculated after subtracting  $k'_{app.}$  for the reaction in the absence of enzyme.

The assumption that the uncatalysed reaction followed first-order kinetics was justified by the linear form of the plots of  $\log(\text{titrant vol.}_{eq.} - \text{titrant vol.}_t)$  against time. In addition, it was found that the first third at least of the catalysed reaction closely followed this rate



law. There was no zero-order behaviour observed in the initial part of the progress curves, even at the highest substrate concentrations used. Therefore good estimates of the initial values of  $k'_{app}$  were obtained by extrapolation of the log plots to zero time. Approximate values of the initial velocity, made by examination of the progress curves at zero time, gave satisfactory agreement with those calculated as described (Fig. 7). Programmes were written for the Olivetti P101 computer to facilitate calculation of the kinetic constants. The equilibrium value for the volume of NaOH titrated, and several points on the progress curve, together with the volume of  $CO_2$  solution injected and the atmospheric pressure, were entered into the computer. The machine calculated and printed values for  $k_1$  ( $sec.^{-1}$ ), the initial velocity of the hydration reaction  $v$  ( $mM sec.^{-1}$ ), the initial substrate concentration  $s$  ( $mM$ ),  $s/v$  ( $sec.$ ), and the number of units of enzyme present in the assay (if the volume of  $CO_2$  solution added was 0.25 ml.). A typical progress curve is shown in Fig. 7 together with the computer calculations. The values read from the progress curve were always chosen near the beginning of the reaction to ensure that a good estimate was made of the initial rate. The computer found the natural logarithms of the differences between each point and the equilibrium value, calculated the best fitting regression line of the logarithms on time, and from the slope found the kinetic constants. The substrate concentration was calculated simultaneously.

Variation of initial velocity with enzyme concentration. Fig. 8 shows that the sum of the catalysed and uncatalysed rate constants of the hydration reaction, and hence the initial velocity, was a linear function

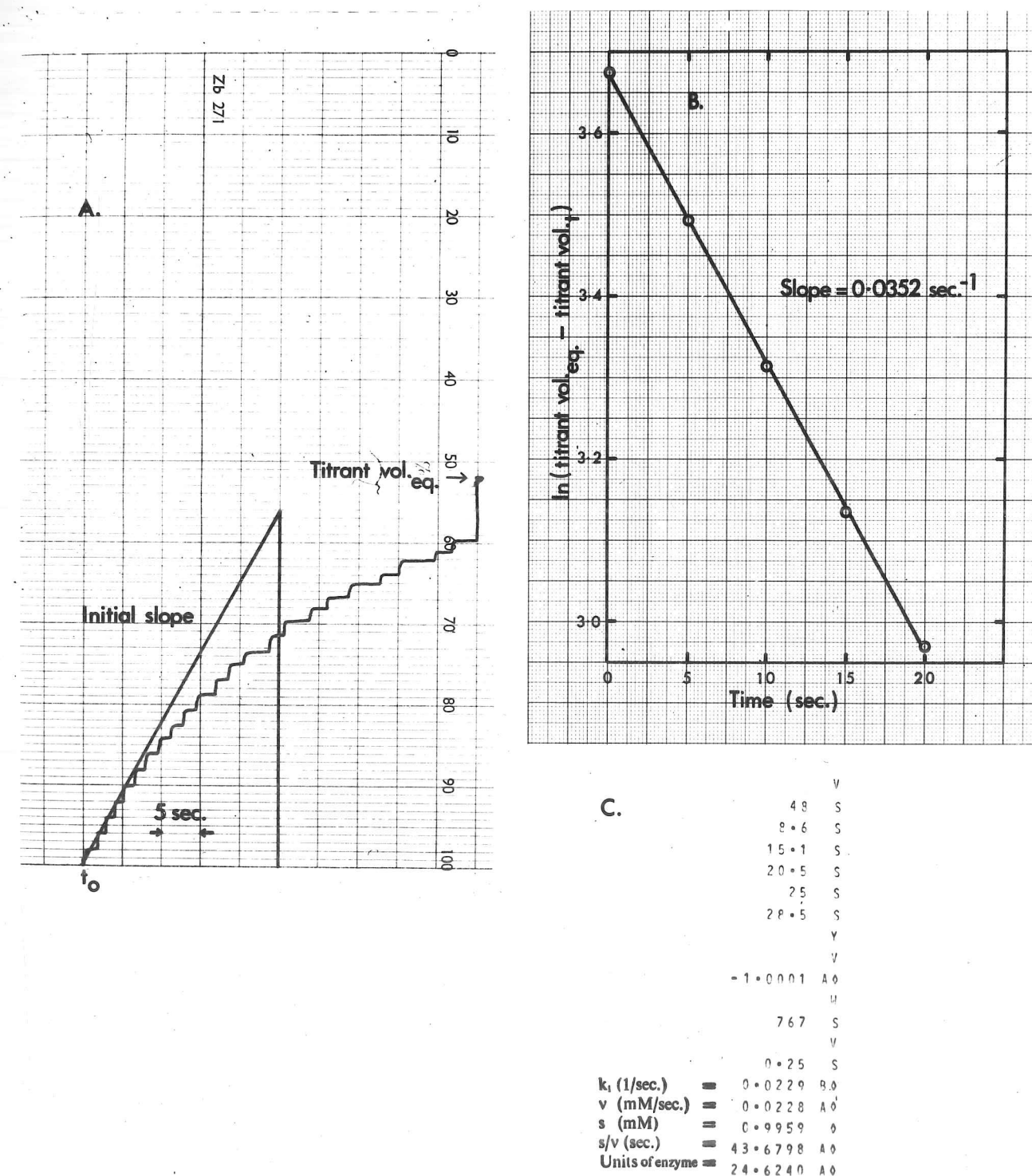
**Figure 7.** The results of a typical assay of  $\text{CO}_2$  hydratase activity by titration at constant pH. A, progress curve of the reaction, and an estimate of the initial slope; B, graph showing that the enzyme-catalysed reaction was pseudo-first-order to at least 50% completion; C, results produced by the computer. The first reliable value of the volume of titrant added to the reaction mixture was obtained 5sec. after the addition of substrate; zero time in B. refers to the time of this first observation ( $t_0 + 5\text{sec.}$ ). The values of the logarithms in B. were obtained from the computer for the purposes of illustration; normally the machine calculated the slope of the line shown in B. by the method of least squares. In C. the order of the figures is: volume of titrant added at equilibrium; five points on the progress curve, at 5sec. intervals (all volumes in arbitrary units); an approximate value of the correlation coefficient for the regression of the experimental points shown in B., printed as a check on the linearity of the points; atmospheric pressure (mm.Hg); the volume of  $\text{CO}_2$  solution added to the assay mixture. In this example, since the volume of substrate solution was 0.25ml., the computer calculated the number of units of enzyme activity corrected to a  $\text{CO}_2$  concentration of precisely 1.0mM.

Calculation of the initial rate of hydration of  $\text{CO}_2$  from the estimate of the initial slope of the progress curve.

Initial slope	$\div (44/25) \text{ div./sec.}$
$\text{CO}_2$ hydrated at equilibrium	$\equiv \text{NaOH added at equilibrium,}$
that is $(0.72 \times 0.996)\text{mM}$	$\equiv 48 \text{ div.}$
Therefore, the initial rate of the sum of the enzyme and non-enzyme-catalysed reactions	$\div (44/25) (0.72 \times 0.996)/48 \text{ mM/sec.}$
	$\div 0.026\text{mM/sec.}$
The rate of the non-enzyme-catalysed reaction	$= 0.0022 \times 0.996\text{mM/sec.}$
	$\div 0.002\text{mM/sec.}$
Therefore, the initial rate of the enzyme-catalysed reaction	$\div 0.024\text{mM/sec., in good agreement with the result shown in C.}$

No knowledge is required of the strength of the NaOH solution for calculation of the rate by either method once the fraction of hydration is known.

**Figure 7.** Calculation of kinetic constants



of the concentration of the enzyme. These assays were performed in the presence of  $25\mu\text{M}$ -EDTA or 0.01% (w/v) peptone, with a substrate concentration of  $2.0\text{mM}$ - $\text{CO}_2$ . Each point represents the results of a single determination. The useful upper limit of enzyme concentration was reached under the present experimental conditions when the total rate was 15 times the uncatalysed velocity. The initial velocity of the hydration reaction was not a linear function of the concentration of the enzyme if neither peptone nor EDTA was included in the assay solution (Fig. 8). The concentrations of these substances that were routinely used were sufficient to ensure a linear relationship between initial velocity and enzyme concentration (Fig. 9).

Rate constant of the uncatalysed reaction. Extrapolation to zero enzyme concentration (Fig. 8) yielded an uncorrected value of  $k_1'(\text{uncat-lysed}) = 0.0024 \text{ sec.}^{-1}$ . Subtraction of the contribution of  $\text{OH}^-$  catalysis mentioned above left  $0.0023 \text{ sec.}^{-1}$ . Finally, it was necessary to allow for the catalytic effect of phosphate ion. The data provided by Roughton & Booth (1938) and Gibbons & Edsall (1963) indicate that this correction amounts to a reduction of 4% and so the final value of  $k_1 = 0.0022 \text{ sec.}^{-1}$ .

Catalytic constants of human erythrocyte carbonic anhydrase isoenzyme C. The initial velocity of the hydration reaction catalysed by isoenzyme C at five initial substrate concentrations was measured, and the results are shown in Fig. 10 as a graph in which  $[\text{initial substrate}] / (\text{initial rate})$  is plotted against  $[\text{initial substrate}]$ . Each point represents the average of three determinations. The average deviation from the mean of the individual measurements was 2%. However, the error in the absolute value of each



**Figure 8.** Relationship between the concentration of human erythrocyte carbonic anhydrase isoenzyme C and the sum of the rate constants of the catalysed and uncatalysed reactions, which is proportional to the sum of the initial rates. The substrate concentration was  $2.0\text{mM-CO}_2$ . ●, In the presence of  $25\mu\text{M-EDTA}$ ; ○, in the presence of  $0.01\%$  peptone; △ in the absence of either EDTA or peptone.

**Figure 8.** VARIATION OF INITIAL RATE WITH ENZYME CONCENTRATION

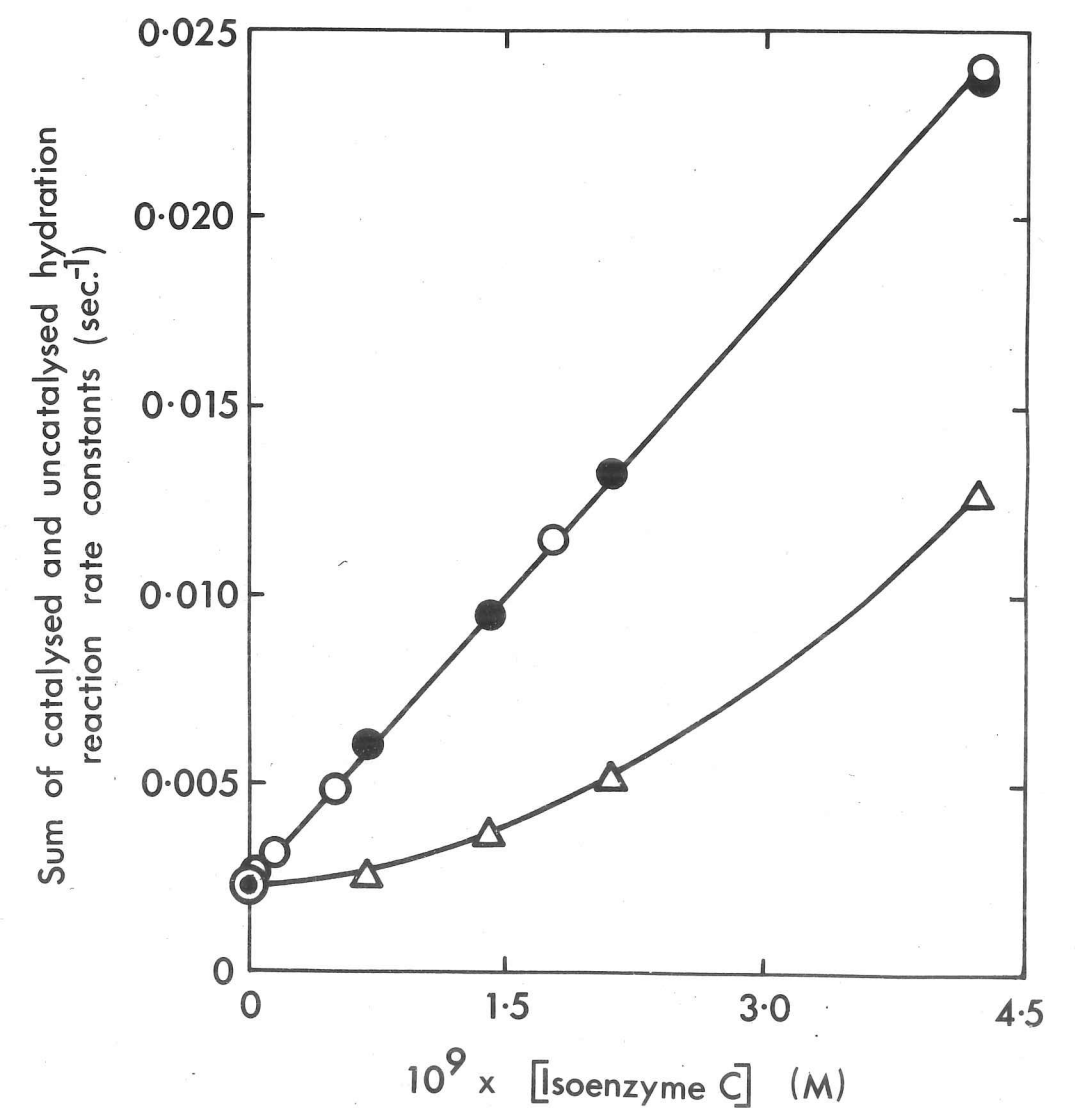


Figure 9. The effects of adding EDTA, peptone, or bovine plasma albumin to the  $\text{CO}_2$  hydratase assay, illustrated by plotting  $k'_{\text{app}}$  (catalysed reaction) +  $k'_{\text{app}}$  (uncatalysed reaction) against the concentration of the additive. The absence of any effect of the additives on the rate of the uncatalysed reaction is shown by the points near the horizontal axis. The enzyme was 3.6  $\mu\text{M}$ -human erythrocyte carbonic anhydrase isoenzyme C and the initial concentration of substrate was 2.0  $\text{mM}$ - $\text{CO}_2$ .  $\bigcirc$ , Peptone;  $\bullet$ , EDTA;  $\triangle$ , BPA.

Figure 9. Effects of additives on titrator assay

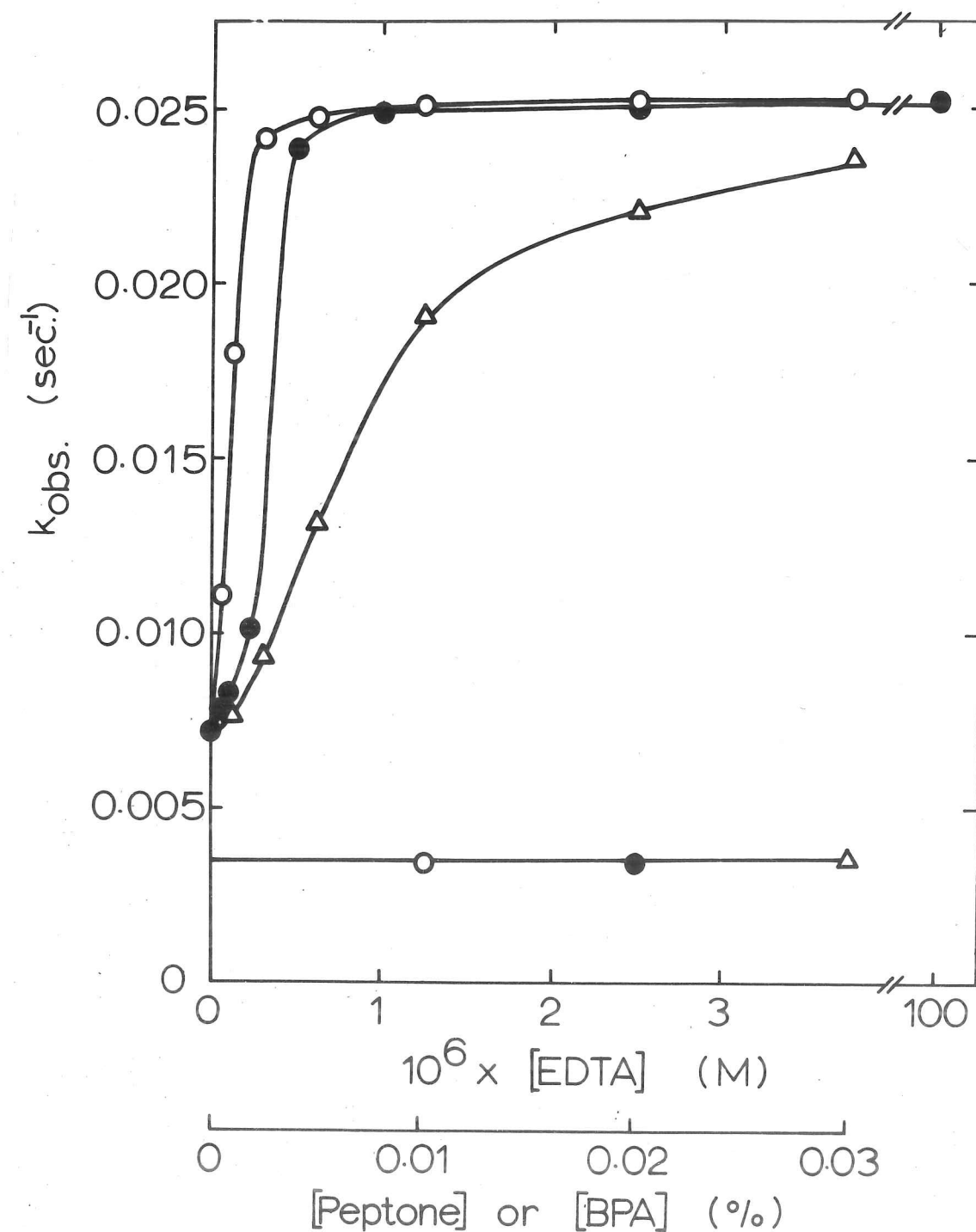
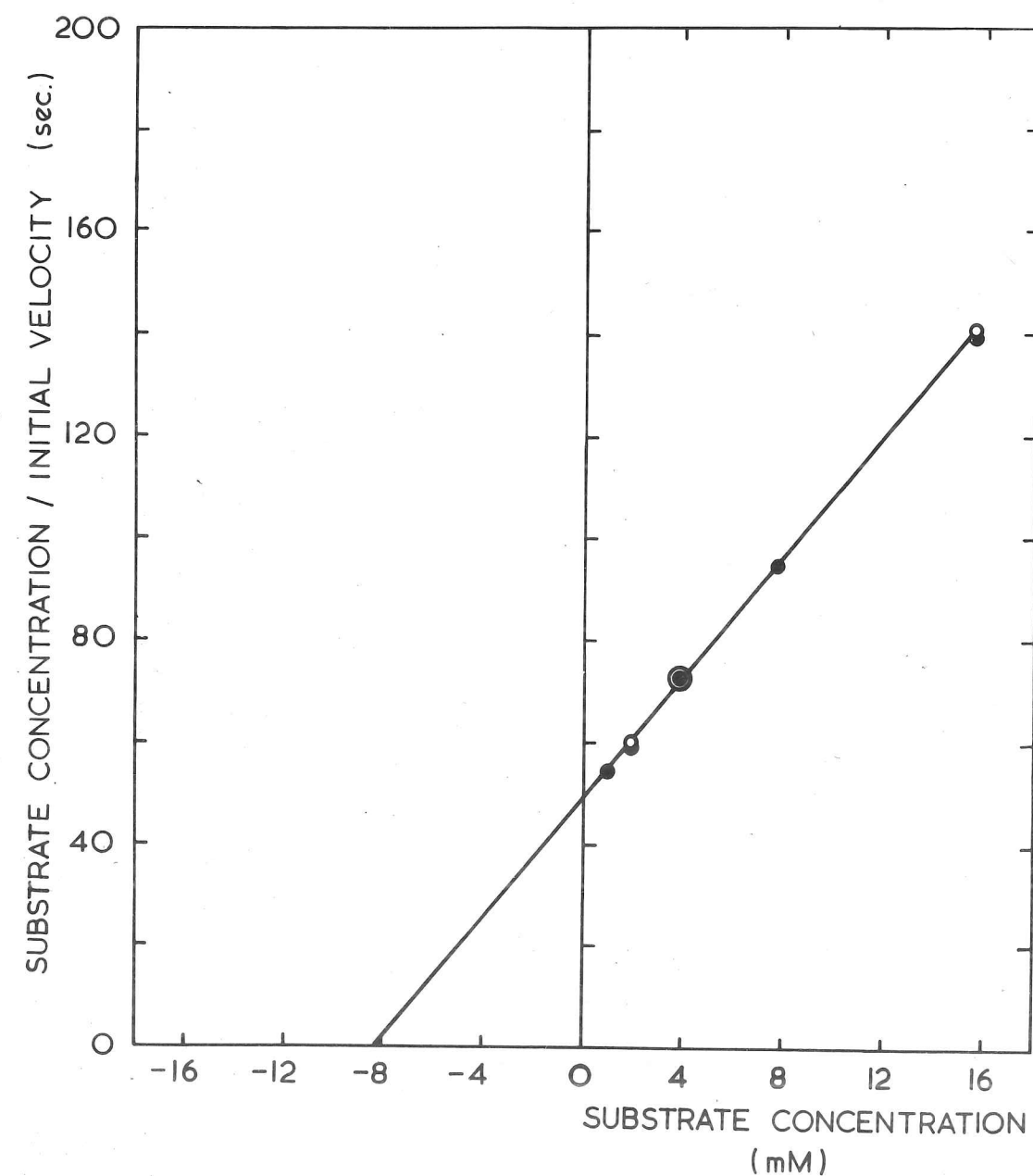




Figure 10. The relationship between substrate concentration and initial velocity of the hydration reaction catalysed by human erythrocyte carbonic anhydrase isoenzyme C, illustrated by plotting  $[\text{initial substrate}] / (\text{initial rate})$  against  $[\text{initial substrate}]$ . The enzyme concentration was  $3.6\mu\text{M}$  and the conditions were  $0^\circ$ , pH 7.0, 45 mM-NaCl and 5mM-sodium phosphate.  $\circ$ , In the presence of 25 $\mu\text{M}$ -EDTA;  $\bullet$ , in the presence of 0.01% peptone.

Figure 10. HUMAN ERYTHROCYTE ISOENZYME C.  
CO<sub>2</sub> HYDRATION



determination was probably greater than this, since the errors in  $\text{CO}_2$  concentration and in the volumes of the  $\text{CO}_2$  and enzyme solutions added were of the order of  $\pm 2\%$  and  $\pm 1\%$  respectively. Moreover, the temperature control of  $\pm 0.1^\circ$  would be expected to lead to an uncertainty in  $k'_{\text{app}}$  of  $\pm 2\%$  (Benson, 1960), and the accuracy of the pH-meter was probably not greater than  $\pm 0.05$  pH unit. It was concluded that an absolute error of  $\pm 10\%$  could reasonably be expected in the measured value of  $K_m$ , though the results obtained on separate occasions agreed within  $\pm 5\%$ . The uncertainty in  $V$  was likely to be greater owing to the difficulty of estimating  $[E_0]$  with accuracy and to systematic errors in the initial velocities and the determination of  $(a_0 - a_{\text{eq}})/a_0$ .

It can be seen that over the 16-fold range of initial  $\text{CO}_2$  concentration examined, in the presence of both peptone and EDTA, isoenzyme C closely obeyed the Michaelis equation. Consequently, it was possible to assign to it the values  $K_m = 8.2 \text{ mM}$ ,  $V/[E_0] = 5.0 \times 10^4 \text{ sec.}^{-1}$ , under the conditions of this experiment.

### DISCUSSION

In the present investigations only the hydration reaction catalysed by carbonic anhydrase was examined. The apparatus used for that purpose could equally well be employed to study the dehydration reaction, though in this case certain precautions would have to be observed for the following reasons. The  $K_m$  of dehydration appeared to be considerably greater than that of the hydration reaction when both were examined by Gibbons & Edsall (1964). Therefore, to make reliable measurements, higher concentration of the substrate, which is  $\text{HCO}_3^-$ , would have to be used and this would induce much variation in the initial ionic strength. Kernohan (1964) has suggested that results may be wrongly interpreted if insufficient attention is paid to the maintenance of constant ionic strength and anion concentration. In his kinetic study of the bovine erythrocyte enzyme, he found  $K_m$  of the dehydration to be dependent on the anion concentration, becoming very large concomitantly with an increase in the anion concentration. Moreover, he found that the initial velocity decreased as the anion concentration increased, and it was therefore presumably affected in this way by the substrate itself.

The value of  $k_1'$  for the uncatalysed reaction, found by the titration method used in the present study, is in good agreement with previous determinations at  $0^\circ$ , which averaged  $0.0021 \text{ sec.}^{-1}$  (Edsall & Wyman, 1958). Roughton & Booth (1938), Mills & Urey (1940) and Gibbons & Edsall (1963) have all shown that this rate constant is independent of ionic strength. In addition, Roughton & Booth (1938) have demonstrated that  $\text{HCO}_3^-$  ions have no significant catalytic action on the hydration rate.

On the other hand, the enzymic hydration rate may be influenced purely by ionic strength and there is no doubt that most anions exhibit

a specific inhibitory effect, as in the case of the reverse reaction. Evidence obtained with the use of the manometric assay, presented by Roughton & Booth (1946b), showed that the anions of many neutral salts were mildly inhibitory at pH 7.4 to the action of carbonic anhydrase. In his experiments with the bovine erythrocyte enzyme, Kernohan (1964) found anion inhibition also in the hydration reaction, though he presented no evidence that  $K_m$  was influenced by anions. For these reasons care was taken in the present work to minimize the increase in ionic strength during the reaction by adding sodium chloride; the added  $Cl^-$  ion caused a mild, but constant, inhibition of the enzyme.

With deionized water the initial velocity was not a function of the enzyme concentration unless peptone or EDTA was included in the assay. Instead, the slope of the plot of velocity against enzyme concentration increased with rising enzyme concentration and appeared to approach a maximum (Fig. 8). A similar effect was observed by Roughton & Booth (1946b) and also by Clark & Perrin (1951). The addition of peptone or EDTA completely eliminated this behaviour. It is assumed that some impurity, perhaps a heavy-metal ion, was responsible for causing an inhibition, and that when sufficient enzyme was available to chelate the inhibitor the effect was abolished. In the present work relatively small amounts of EDTA were required to produce a linear relationship;  $1\mu M$ -EDTA was found to be sufficient. A plot of initial velocity against the EDTA or peptone concentration indicated that the amounts used as a routine were ample and that neither substance led to inhibition when present in large quantities (Fig. 9). If peptone was replaced by EDTA in the assay there was no difference in the initial velocity and no change was detected under such conditions in the

kinetic constants of isoenzyme C (Fig.10). Gibbons & Edsall (1964) reported a similar finding and state that neither one nor the other substance affected the uncatalysed reaction. Davis (1959), too, showed that EDTA had no inhibitory action on the human erythrocyte enzyme.

In all routine experiments peptone, rather than EDTA was added to the assay. This procedure was followed because it has been observed by Ho & Sturtevant (1960) that EDTA causes a sixfold activation of the bovine erythrocyte enzyme.

Experimenting with an electrometric assay method in a flow system, DeVoe & Kistiakowsky (1961) obtained erratic results that they explained by postulating that the enzyme was being adsorbed on the surface of the glass electrode. No such effect was observed in the present work, but the presence of the peptone may have helped to prevent it.

When compared with the values of the kinetic constants of isoenzyme C obtained at 25° by Gibbons & Edsall (1964), the results found in the present work show that  $K_m$  decreased from 12mM at 25° to 8mM at 0°, and likewise  $V/[E_0]$  decreased from  $2.8 \times 10^5 \text{ sec.}^{-1}$  to  $5.0 \times 10^4 \text{ sec.}^{-1}$ . In both investigations the experiments were performed at approx. pH 7.0, but in the present one the ionic strength was twice as great and resulted mainly from the presence of sodium chloride, whereas the sodium phosphate concentration was fivefold lower. This difference in the composition of the assay mixture makes it difficult to compare the values of  $V/[E_0]$  found in the two investigations.



### A Criticism of Previously Reported Titration

#### Assays.

The method of Holmgard (1961). This brief report was the first to claim that the reaction in which  $\text{CO}_2$  is hydrated can conveniently be examined with the use of titration at constant pH. A pH-stat maintained constant pH in the reaction solution, which was a mixture of buffer,  $\text{CO}_2$ -saturated water, and enzyme. The initial rate of the hydration reaction was found to be constant for 1 min. and this rate was proportional to the amount of whole blood added to the assay solution. A modification was mentioned in which  $\text{CO}_2$  was bubbled continuously. No other data were provided.

The method of Leibman, Alford & Boudet (1961). The apparatus consisted of a sintered-glass funnel mounted upright, the bowl being the reaction vessel. This funnel was maintained at a constant temperature by partial immersion in a bath. A given substrate concentration was produced by bubbling a  $\text{N}_2$ - $\text{CO}_2$  mixture, of suitable composition, upwards through the reaction solution. A gas flow of 1 l/min. gave maximum rates of reaction, at the single enzyme concentration used.

The initial rate of  $\text{CO}_2$  hydration was measured and found to be constant for 15 to 30 sec. After this time the rate diminished. It was suggested that this could have resulted from an effect of changed ionic strength or from product inhibition. The possibility that it could have been caused by a decrease in the  $\text{CO}_2$  saturation with time was not considered.

The reaction was normally initiated by the flow of gas, though in some cases buffer was added to raise the pH. The former procedure does

not appear to me to be satisfactory since the rate of solution of  $\text{CO}_2$  in water at  $5^\circ$  (their experimental temperature) is not great. Gibbons & Edsall (1963) found that 20 min. of continuous bubbling was required to reliably saturate water at  $0^\circ$  with pure  $\text{CO}_2$ . Therefore it is unlikely that the  $\text{CO}_2$  concentration expected from application of Henry's law was rapidly attained in these experiments.

A graph illustrating the rate of addition of alkali against the  $\text{CO}_2$  concentration in the absence of enzyme was presented but no attempt was made to calculate from this a pseudo-first-order rate constant for comparison with accepted values.

No mention was made of the fractional contribution of the uncatalysed reaction to the total rate nor of variation of rate with enzyme concentration.

The method of Leibman & Greene (1967). This technique measured the dehydration reaction. In view of the comments made at the beginning of the DISCUSSION, above, there is some doubt that results obtained from a study of the dehydration reaction can be interpreted easily unless special precautions, not mentioned by the authors, are taken during the experiment. The apparatus was, in my opinion, complex in conception and design. There were several steps in the course of the reaction as it was observed; evolved  $\text{CO}_2$  was flushed from the reaction vessel by  $\text{N}_2$  and carried to the absorption vessel where the  $\text{CO}_2$  was re-hydrated rapidly in the presence of an excess of enzyme and the released  $\text{H}^+$  titrated. No evidence was presented to establish (a) that the flushing and absorption steps were quantitative, and (b) that the rates of these processes were sufficiently rapid as not to influence

the observed reaction rate.

The authors showed that in the absence of enzyme the rate at which the mechanism introduced alkali to the absorption vessel was proportional to the substrate concentration in the reaction vessel and concluded that the titrator record showed a rate proportional to the rate of dehydration of the  $\text{HCO}_3^-$ . However the rates were not equal because re-hydration of the  $\text{CO}_2$  proceeded to equilibrium only. Therefore, unless allowance was made for this (no mention of such an adjustment appears) the measured rates would be rather low. Also, unless the true rate was measured, in the case of the uncatalysed reaction, it would be impossible to determine the pseudo-first-order rate constant and hence check the method against this well-known value.

The pH of the reaction solution must have risen during the experiment, since for each equivalent of  $\text{HCO}_3^-$  dehydrated an equivalent of  $\text{H}^+$  vanished. The mixture was quite strongly buffered (43 mM-phosphate) and since an estimate was made of the initial rate, the change in pH possibly was not great. However no data were presented to show whether or not a significant change in pH did occur.

It is not clear why the initial rate was maintained for 30 sec. The rate would be expected to decrease from the start. The fact that it did not might be indicative of some anomaly.

The only experimental results presented were in the form of graphs showing the behaviour of the enzyme in the presence of two inhibitors. Except for one series, the points on the graphs deviated considerably from the least-squares best-fit line, also drawn. For example, the value of  $K_i$

for sulphanilamide calculated from these data varied from 1.0 to  $2.5 \times 10^{-5}$  M.

No mention was made of what fraction of the observed rate was caused by the uncatalysed reaction. No examination was made of the variation of the rate with variation of enzyme concentration so that it is not clear over what range of enzyme concentration the method was applicable, if indeed it was suitable for the assay of the enzyme.

The recent publication of an article by Magid (1968) brought my attention to another report (Hansen & Magid, 1966) of a titration method. This was similar to the procedure described by Leibman, Alford & Boudet (1961), referred to above, in which the reaction vessel was a sintered funnel. The method of Hansen & Magid is suited to the study of the dehydration rather than the hydration reaction and therefore suffered from the disadvantages discussed above. The rapid ( $\sim 1$ /min.) flow of  $\text{CO}_2$ -free air that was maintained through the assay solution was claimed to remove continuously  $\text{CO}_2$  as it was formed and not to cause deactivation of the enzyme. In this way it was possible to examine the dehydration reaction in the absence of any back reaction for up to 1 min., after which time the rate began to decrease. It is not clear why the rate did not decrease from the start. In the more recent article (Magid, 1968) a modification was described which was claimed to permit the determination of the dehydration rate at several different concentrations of the substrate,  $\text{HCO}_3^-$ , in a single experiment. In this modified procedure measurements were made of the rate at an unspecified number of 60sec. intervals. It was asserted that since the reaction product was continuously removed from the medium, each measurement represented an estimate of an initial rate at a new substrate

concentration. No account was taken however of the progressive change in the anion composition of the medium; in another section of the report the author showed that different anions inhibit differently the activity of carbonic anhydrase.



### SUMMARY

1. A method was described for measuring accurately the initial velocity of the hydration reaction catalysed by carbonic anhydrase; the method depends on the titration of  $H^+$  ions at constant pH.

2. Human erythrocyte carbonic anhydrase, isoenzyme C, was used to illustrate the method. Under the experimental conditions employed ( $0^\circ$ , pH 7.0, in the presence of 45mM-sodium chloride and 5mM-sodium phosphate) isoenzyme C obeyed the Michaelis equation over the range of substrate concentration 1 - 16mM-carbon dioxide. The kinetic constants found were  $K_m = 8.2\text{mM}$ ;  $V/[E_0] = 5.0 \times 10^4 \text{ sec.}^{-1}$ .

## CHAPTER 2

### CARBONIC ANHYDRASE ISOENZYMES IN THE ERYTHROCYTES AND DORSOLATERAL PROSTATE OF THE RAT

#### INTRODUCTION

Much study has been devoted to the prostate gland because in certain species, particularly in man and dog, it is a large organ, subject to various pathological conditions, and endowed with the property of producing a secretion which differs characteristically from other secretory fluids of the mammalian body. An account of experimental and clinical research relating to the prostate gland has been given by Jensen (1963). The state of knowledge concerning the biochemistry of the prostate has been reviewed by Mann (1964).

The prostate of the rat 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 citric acid but no fructose, while the dorsolateral prostate secretes both citric acid 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 of this element in all other soft tissues of the rat (Gunn *et al.*, 1955) and a high activity of carbonic anhydrase equal to that in the rat erythrocytes (Mawson & Fischer, 1952).

The aim of this investigation was to compare the properties of carbonic anhydrase occurring in the erythrocytes and dorsolateral prostate of the rat.

## EXPERIMENTAL AND RESULTS

### Preparation of the isoenzymes

Erythrocyte isoenzymes. Male white rats were anaesthetized with ether and bled. 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% saline, 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  $\text{CHCl}_3$  and EtOH using the method of Armstrong *et al.* (1966) and the carbonic anhydrase - containing extract was dialysed against water and lyophilized. 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.

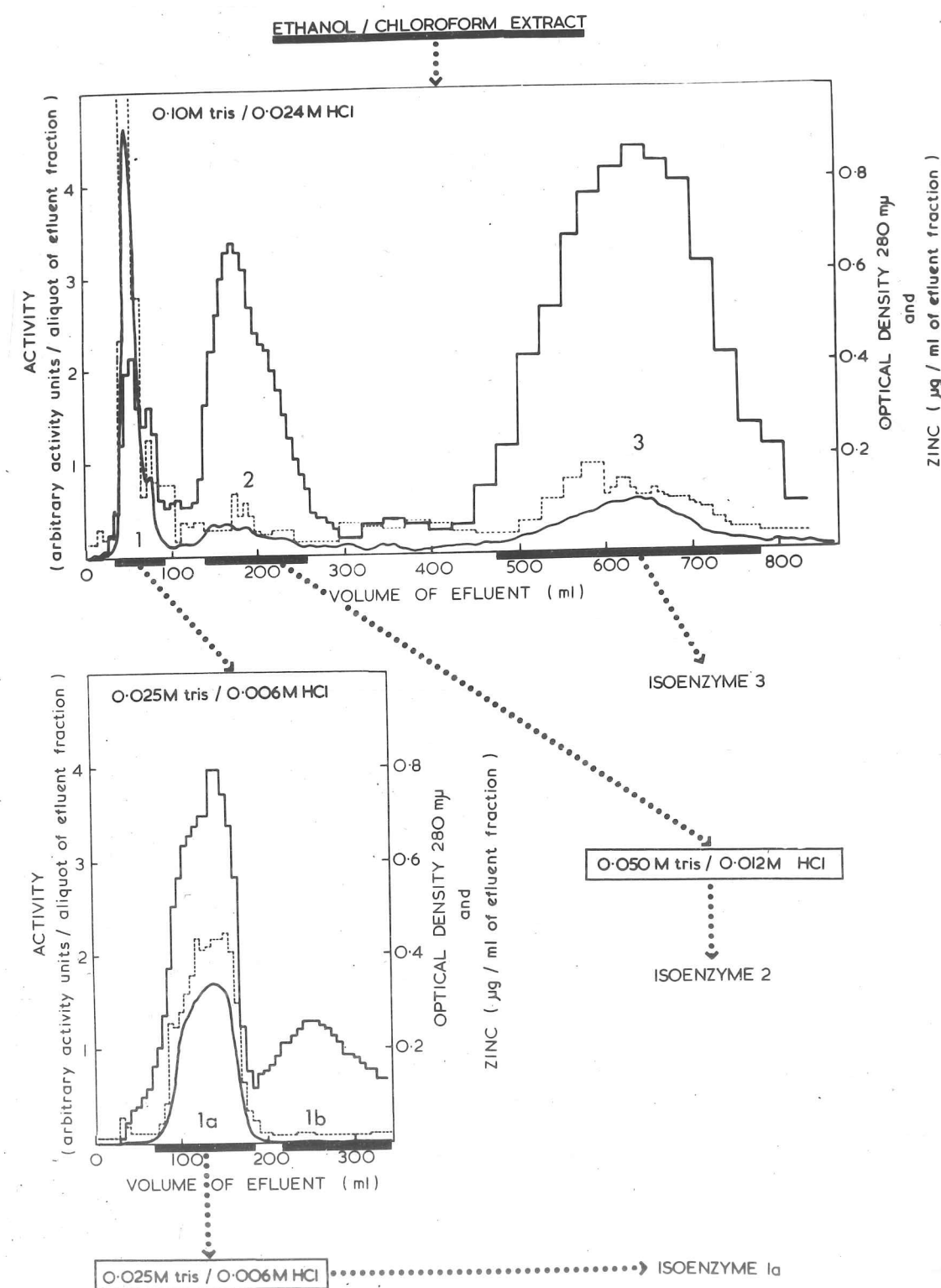
Fig. 11 shows the result of the chromatography and in addition indicates several subsequent steps in which rechromatography was performed. The heavy bars (close to the horizontal axes) show which fractions were pooled for lyophilisation and rechromatography; in general, rechromatography in a buffer of lower ionic strength increased the resolution of the peaks. Each peak of  $\text{CO}_2$  hydratase activity represents a distinct form of carbonic anhydrase. For example the result of rechromatographing Peak 1 in Buffer III is shown; resolution of the major and minor peaks of activity was thus achieved. The isoenzymes were all identified by number<sup>\*</sup>, corresponding to their position on elution from

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<sup>\*</sup>The CA isoenzymes will be referred to hereafter as follows: erythrocyte isoenzymes, Eryth. (number); dorsolateral prostate isoenzymes, Prost. (number). (CEP) after an isoenzyme indicates purification from a  $\text{CHCl}_3$ -EtOH extract.

**Figure 11.** A flow sheet summarizing the purification of rat erythrocyte carbonic anhydrase and its resolution into isoenzymes, by column chromatography using DEAE-Sephadex. The starting material was a  $\text{CHCl}_3$ -EtOH extract of washed erythrocytes. Each small box containing a description of a buffer indicates another chromatographic step with that buffer; the pH of each buffer was approximately 9.3 at  $4^\circ$ . — (Continuous),  $E_{280}$ ; — (stepped),  $\text{CO}_2$  hydratase activity (modified Philpot assay); — — —, zinc. The heavy bars near the horizontal axes show which fractions were combined for further purification. The numbering of the peaks corresponds to the numbering of the isoenzymes.

**Figure 11.** RAT ERYTHROCYTE DEAE-SEPHADEX CHROMATOGRAPHY



the chromatographic column. Three isoenzymes were isolated; Eryth. 1a (CEP), Eryth. 2 (CEP) and Eryth. 3 (CEP). Fig. 12 depicts the results of polyacrylamide gel electrophoresis performed at successive stages of the purification. The distribution is illustrated of protein bands and regions showing esterase activity toward  $\beta$ -naphthyl acetate in the presence and absence of acetazolamide.

To determine the effect of organic solvents on the erythrocyte carbonic anhydrase an alternative method for removal of haemoglobin was used as follows. The haemolysate was dialysed against Buffer II and 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  $\text{CHCl}_3$ -EtOH 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 in the case of the  $\text{CHCl}_3$ -EtOH extract. However the behaviour of Peak 1a appeared to be unchanged. The results of electrophoresis are shown in Fig. 13 where it can be seen that the band corresponding to Peak 2 was missing, suggesting that Peak 2 appeared as a result of the treatment with  $\text{CHCl}_3$ -EtOH. Rechromatography produced isoenzymes Eryth. 1a and Eryth. 3.

Electrophoresis showed that Peak 1a (Fig. 11), Eryth. 1a (CEP) (which resulted from the repeated rechromatography of Peak 1a) and Eryth. 1a all contained a minor component. The ratio of protein to acetazolamide-



**Figure 12.** Polyacrylamide gel electropherograms obtained at several stages in the isolation of carbonic anhydrase isoenzymes from the  $\text{CHCl}_3$ -EtOH extract of washed rat erythrocytes. The direction of protein migration is indicated by the arrow. Isoenzyme 1a = Eryth.1a (CEP); isoenzyme 2 = Eryth. 2 (CEP); isoenzyme 3 = Eryth. 3 (CEP). The numbering of the peaks refers to Fig. 11.

**Figure 12.** RAT ERYTHROCYTE ACRYLAMIDE -GEL ELECTROPHORESIS

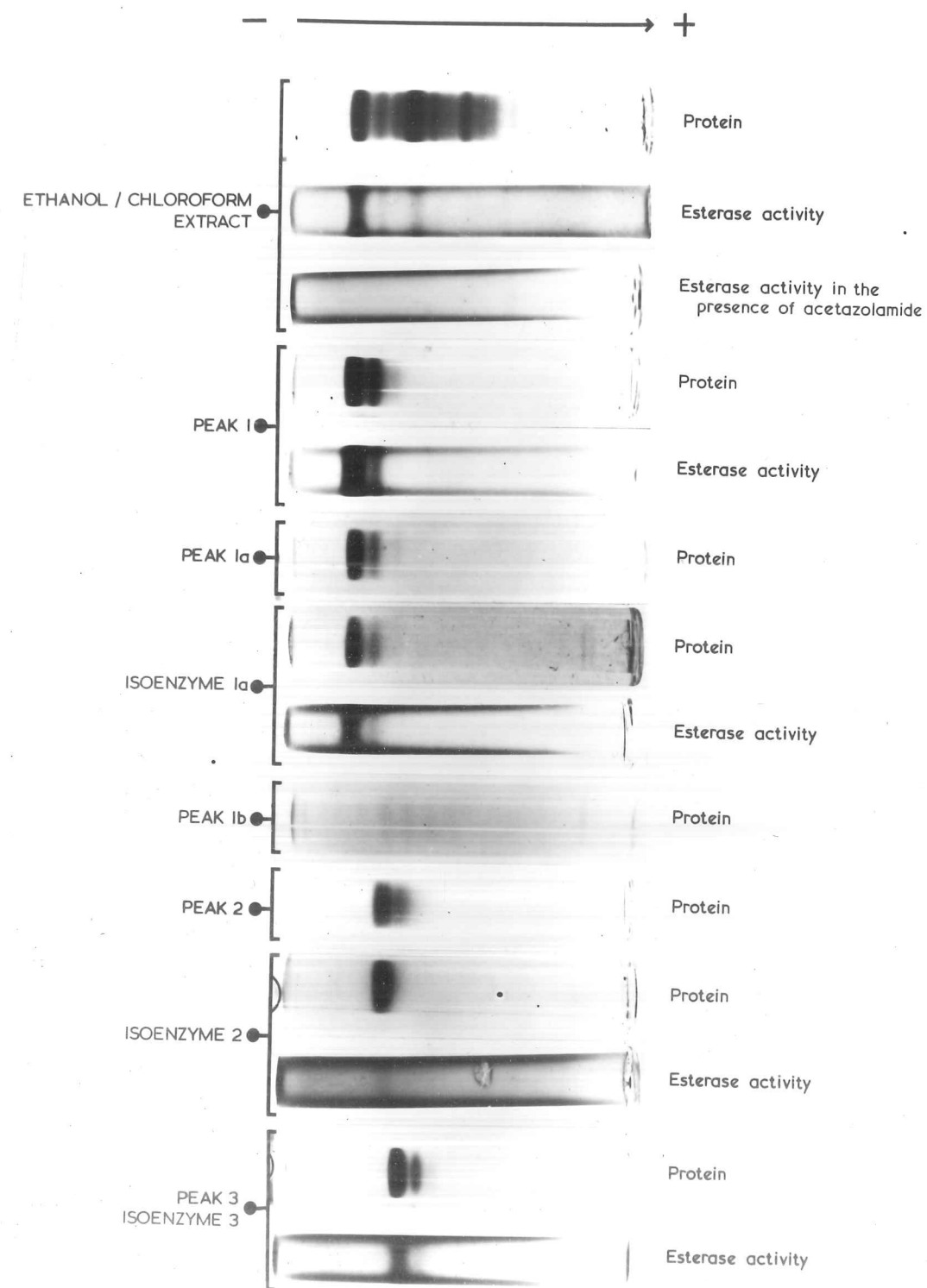
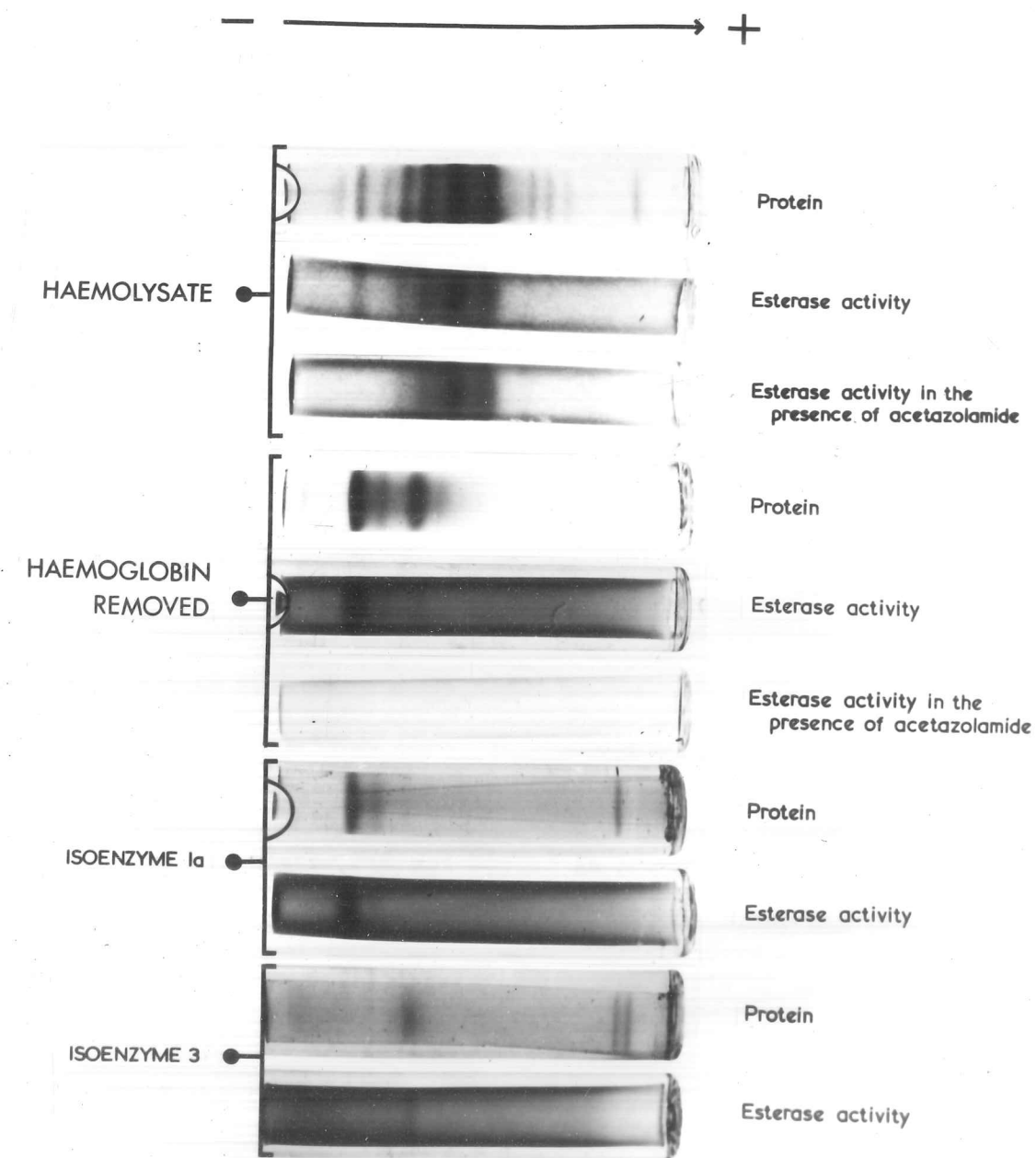


Figure 13. Polyacrylamide gel electropherograms obtained at several stages in the isolation of carbonic anhydrase isoenzymes from washed rat erythrocytes that were not treated with  $\text{CHCl}_3\text{-EtOH}$ . The direction of protein migration is indicated by the arrow. The label Haemoglobin Removed refers to the pooled fractions containing carbonic anhydrase activity after DEAE-Sephadex chromatography for the removal of haemoglobin.. Isoenzyme 1a = Eryth. 1a; isoenzyme 3 = Eryth. 3.

Figure 13. RAT ERYTHROCYTE ACRYLAMIDE -GEL ELECTROPHORESIS



-inhibitable esterase activity in both the major and minor bands was almost constant throughout the purification procedure. Contamination of Peak 1a would not be expected to arise from Peaks 1b or 2, for the following reasons. Peak 1b was clearly separated from Peak 1a by chromatography and in addition appeared to be of low activity toward the ester, while Peak 2 was also clearly separated and was completely absent from the preparations in which  $\text{CHCl}_3$ -EtOH was not used. A minor carbonic anhydrase component was observed similarly upon electrophoresis of Peak 3 (Fig. 12) 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.

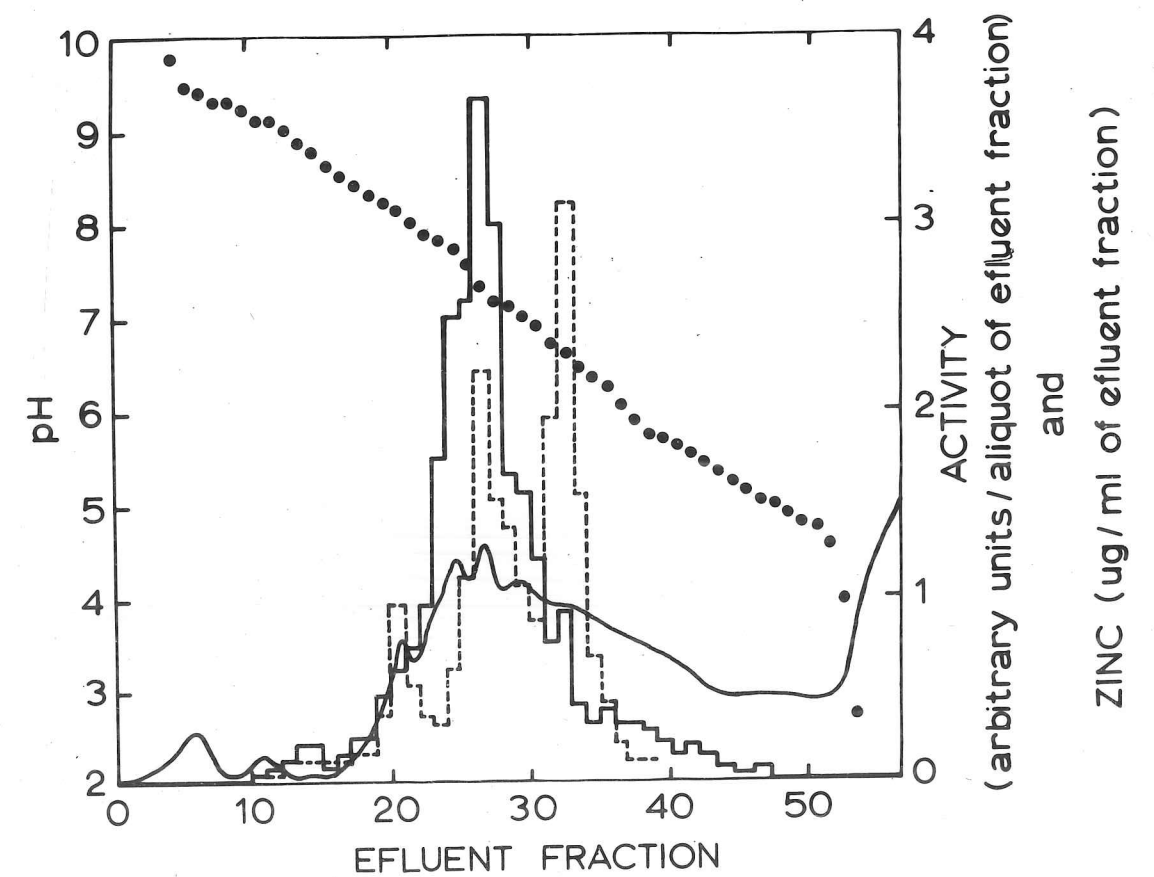
A  $\text{CHCl}_3$ -EtOH extract was subjected to isoelectric focusing in a gradient of pH 3 - 10 with the result shown in Fig. 14. Electrophoresis of the peaks clearly established their identity and Eryth. 1a (CEP) and Eryth. 3 (CEP) were revealed to be free of the minor components (Fig. 15), demonstrating that the latter did not appear as a result of electrophoresis. Electrophoresis of fractions 24, 26 and 29 does show however the presence of bands which 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

Figure 14. Results of the isoelectric focusing experiment on the  $\text{CHCl}_3$ -EtOH 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),  $\text{CO}_2$  hydratase activity (modified Philpot assay); ---, zinc.

Figure 14.

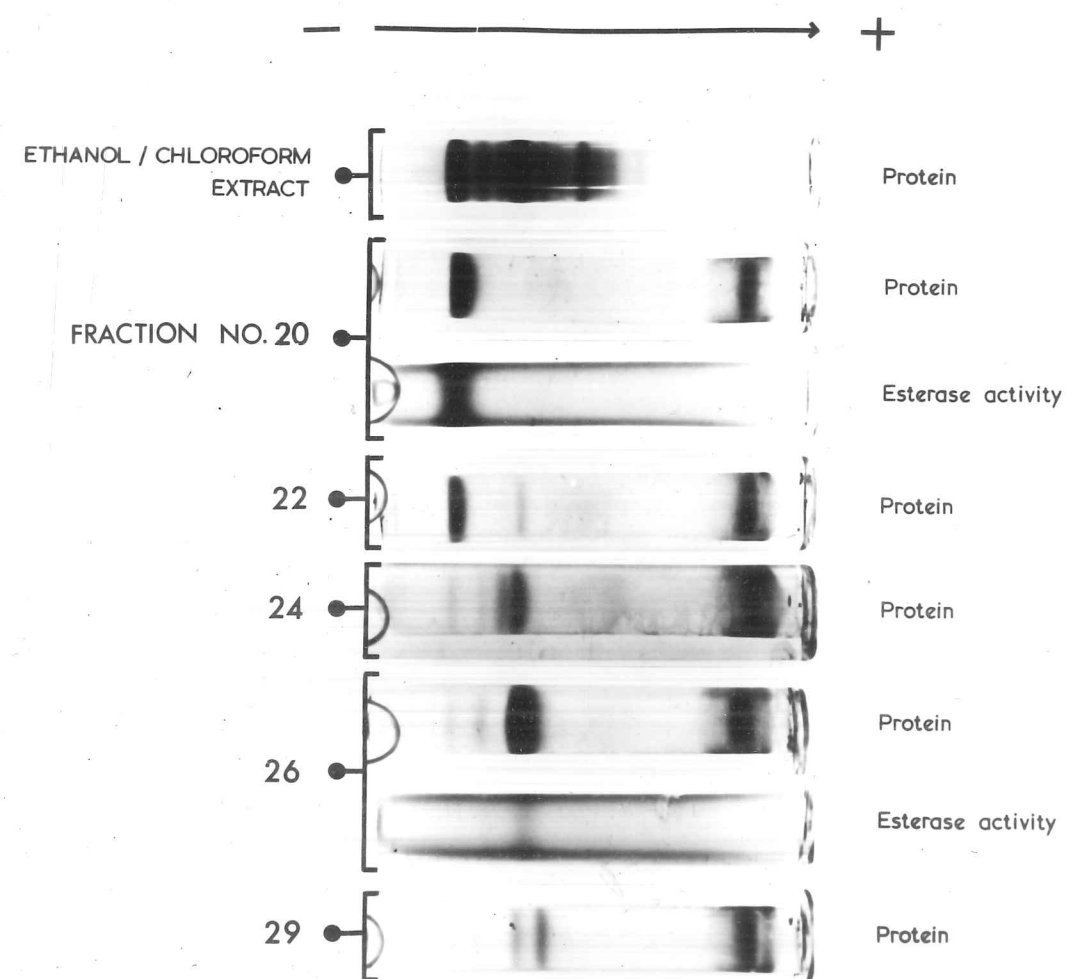
# ISOELECTRIC FOCUSING





**Figure 15.** Polyacrylamide gel electropherograms of fractions from the isoelectric focusing experiment illustrated in Fig. 14. The heavy staining at the positive ends of the gel is caused by the carrier ampholytes. The direction of protein migration is indicated by the arrow. The result of electrophoresis of the whole extract is included for reference.

**Figure 15.** RAT ERYTHROCYTE ACRYLAMIDE -GEL ELECTROPHORESIS





10 gm. 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 parts by weight of acid-washed sand to disintegrate the fibrous structure. When the thick mass had been reduced to a paste, 9 vol.-Buffer II was added slowly while the grinding was continued. The homogenate was then centrifuged at 10,000g. for 30 min. at 4°. The precipitate was not re-extracted; as only a further 1/10 of the activity in the first supernatant could be thus recovered. This supernatant, opalescent and faintly pink, was centrifuged at 108,000g. for 1 hr. at 4°. A perfectly clear solution was produced and the slight viscous precipitate was discarded. The extract of soluble carbonic anhydrase, which had an activity corresponding to 56,000 units/gm. wet wt. dorsolateral prostate, was lyophilised, the residue dissolved in Buffer I, and this solution exhaustively dialysed against the same buffer.

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 onto a column equilibrated with Buffer I. The results of chromatography are shown in Fig. 16 where subsequent steps in the purification are outlined. Three isoenzymes could be identified. Rechromatography (Buffer II) of Peak 1 yielded two forms of carbonic anhydrase, Prost. 1a and Prost. 1b; rechromatography (Buffer I) of Peak 2 gave Prost. 2. Prost. 1a was recovered in very small amounts and electrophoresis showed it to be non-homogeneous; its properties were not investigated. Prost. 1b and Prost. 2 were judged to be fairly pure by electrophoresis at a single pH (Fig. 17).

**Figure 16.** A flow sheet summarizing the purification of rat dorsolateral prostate carbonic anhydrase and its resolution into isoenzymes, by column chromatography using DEAE-Sephadex. The starting material was prepared as described in the text. The buffers used are described at the top of each box; the pH of each buffer was approximately 9.3 at 4°. — (Continuous),  $E_{280}$ ; — (stepped),  $\text{CO}_2$  hydratase activity (modified Philpot assay). The heavy bars near the horizontal axes show which fractions were combined for further purification. The numbering of the peaks corresponds to the numbering of the isoenzymes.

**Figure 16. RAT PROSTATE DEAE-SEPHADEX CHROMATOGRAPHY**

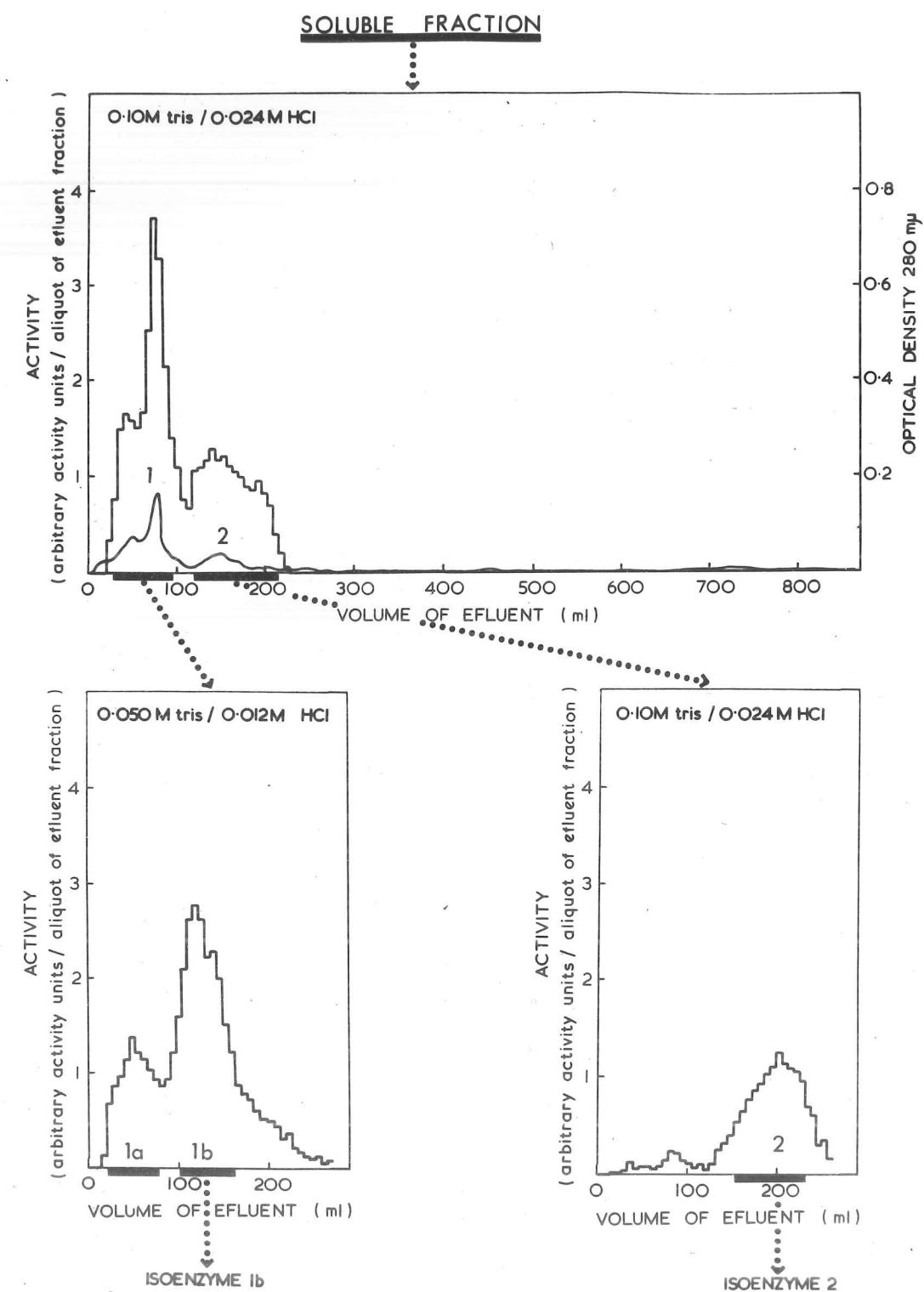
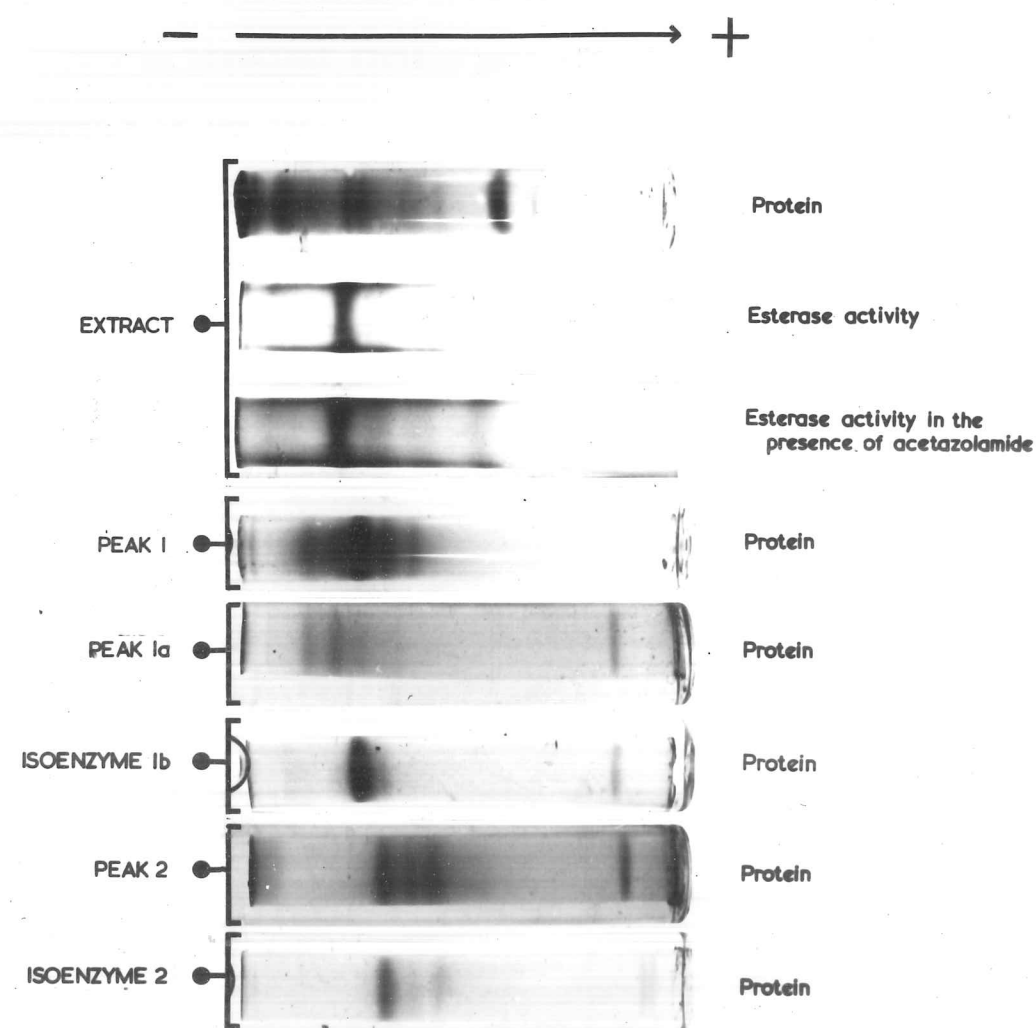


Figure 17. Polyacrylamide gel electropherograms obtained at several stages during the isolation of carbonic anhydrase isoenzymes from a soluble extract of rat dorsolateral prostate. The direction of protein migration is indicated by the arrow. It will be noted that there was no acetazolamide-inhibitable esterase activity in the extract. The resolution of the bands was considerably better than this photograph suggests. The numbering of the peaks refers to Fig. 16: isoenzyme 1b = Prost. 1b; isoenzyme 2 = Prost. 2.

Figure 17. RAT PROSTATE ACRYLAMIDE -GEL ELECTROPHORESIS



Another purification procedure was tested, involving both acetone and ammonium sulphate fractionations. These were performed on a dilute extract of the prostatic tissue and the active fraction was lyophilised 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 chromatographic column was recovered, support the conclusion that the simple extraction and chromatography schemes adopted were adequate and that no major soluble carbonic anhydrase isoenzyme with a high specific activity toward  $\text{CO}_2$  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 isoenzyme. When the soluble extract of dorsolateral prostate was assayed for  $\beta$ -naphthyl acetate esterase activity by the spectrophotometric method, a powerful esterase present that was insensitive to acetazolamide (Fig. 17) masked any activity which might have been exhibited by the carbonic anhydrase.

#### 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. 18).



Table 1

General properties of the rat carbonic anhydrase isoenzymes and their properties as catalysts of CO<sub>2</sub> hydration

The conditions in the kinetic experiments were 0°, pH 7.0, 45mM-NaCl, 5mM-sodium phosphate, and 0.01% peptone. The kinetic constants were evaluated from the results in Figs 19 and 20; the max. errors in the values of  $K_m$  and  $V$  are of the order of  $\pm 10\%$  and  $\pm 20\%$  respectively (McIntosh, 1968). No values of the constants could be determined for Eryth. 1a (CEP) (see Fig. 19a). Acetazolamide is a zone B inhibitor and so the estimates of  $K_i$  are approx. only.

	Erythrocyte isoenzymes					Dorsolateral prostate isoenzymes	
	Eryth.1a (CEP)	Eryth.1a	Eryth.2 (CEP)	Eryth.3 (CEP)	Eryth.3	Prost.1b	Prost.2
General properties							
Molecular weight ( $\pm 3,000$ )	29,000	29,000	29,000	29,000	29,000	29,000	29,000
$E_{280}^{1\%, 1 \text{ cm.}}$ (approx.)	18	18	18	17	17	18	-
g. atom 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 <sub>2</sub> hydratases							
$K_m$ (mM)	-	8.6	8.6	5.7	11	17	9.6
$10^{-4} \times V/[E_0]$ (sec. <sup>-1</sup> )	-	0.13	4.2	7.4	8.7	18	3 (approx.)
Type of inhibition caused by acetazolamide	-	non-comp.	mixed	mixed, mainly comp.	mixed, mainly comp.	non-comp.	non-comp.
$K_i$ (acetazolamide) ( $\mu\text{M}$ )	-	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

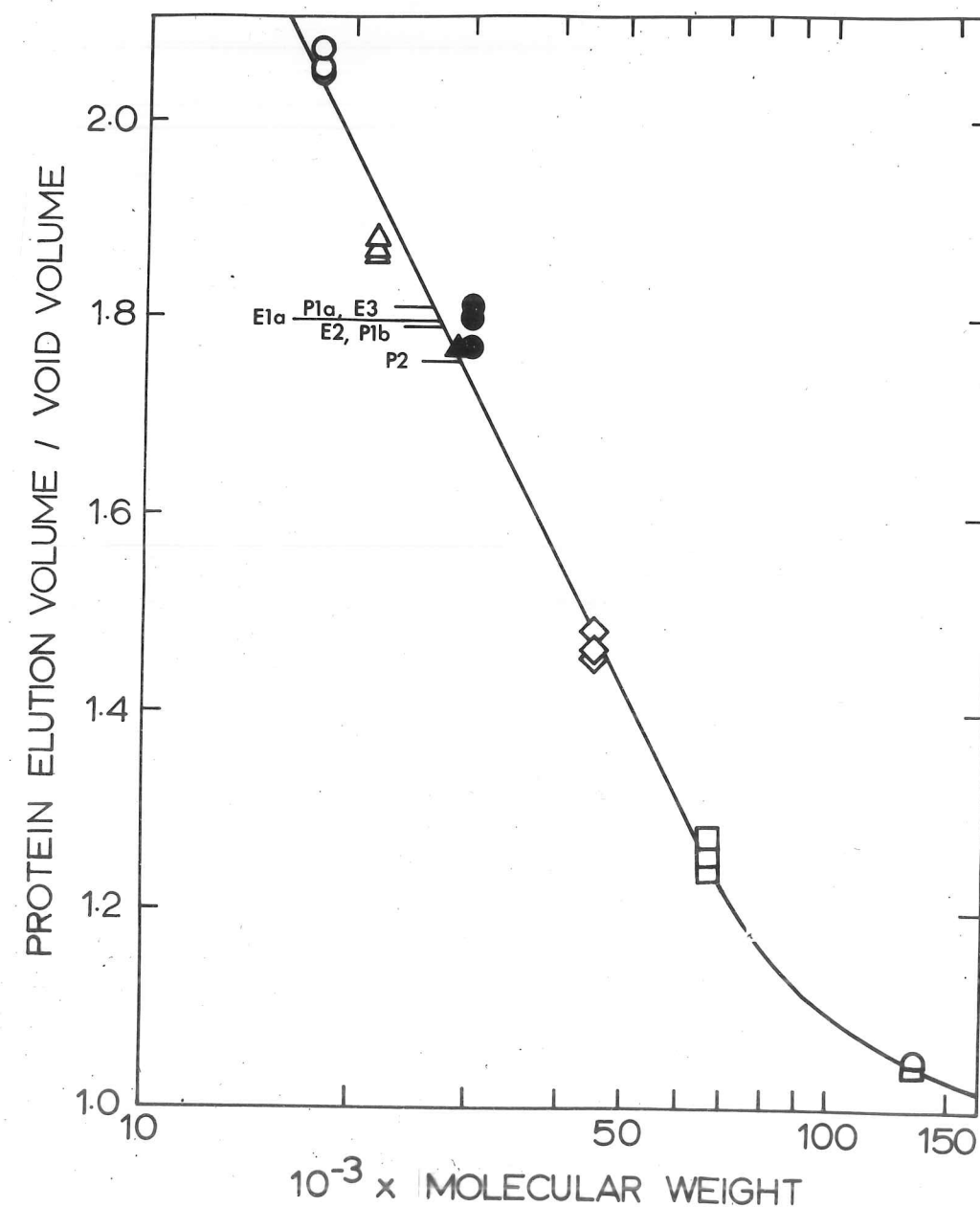
## Summary of the properties of the rat isoenzymes as catalysts of ester hydrolysis

The conditions were 25°, pH 8.0, 10mM-diethylmalonate and 1.7% (v/v) or 5% (v/v) acetone for (1) and (2) respectively. The maximum error in  $v/[E_0]$  was estimated as  $\pm 10\%$ . In 1., the values of  $K_i$  for Eryth.3 (CEP) and Prost. 1b and their standard errors were determined by least squares regression analysis of the results shown in Fig. 23.

	Erythrocyte isoenzymes				Dorsolateral prostate isoenzymes	
	Eryth.1a (CEP)	Eryth.1a	Eryth.3 (CEP)	Eryth.3	Prost.1b	Prost.2
1. Hydrolysis of 1mM-p-nitrophenyl acetate						
$v/[E_0]$ (min. <sup>-1</sup> )	11	9.7	44	30	57	16 (approx.)
$K_i$ ( $\mu$ M) and type of inhibition caused by DNSA	-	-	2.2 $\pm$ 0.2, partially comp.	-	2.1 $\pm$ 0.1, non-comp.	-
$[E_0]/K_i$	-	-	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	-
2. Hydrolysis of 1mM- $\beta$ -naphthyl acetate						
$v/[E_0]$ (min. <sup>-1</sup> )	1.5	1.3	0.12	0.13	0	0

**Figure 18.** Molecular weights of the rat erythrocyte and dorsolateral prostate isoenzymes, determined by gel-filtration on Sephadex G-100. The eluting buffer was 0.025M-tris-0.006M-HCl, pH 9.3. at 4°, containing 0.05M-NaCl. ○, myoglobin (mol.wt.17,800); △, soya bean trypsin inhibitor (21,500); ●, human erythrocyte carbonic anhydrase isoenzyme C (30,000); ▲, human erythrocyte carbonic anhydrase isoenzyme B (29,000); ◇, ovalbumin (45,000); □, bovine plasma albumin (67,000); △, bovin plasma albumin, dimer (134,000). Ela = Eryth. 1a (CEP); E2 = Eryth. 2 (CEP); E3 = Eryth. 3 (CEP); Pla = Prost. 1a; Plb = Prost. 1b; P2 = Prost. 2. The behaviour of the standards (except for the carbonic anhydrase isoenzymes) on filtration was precisely as described by Andrews (1964). It will be noted that the carbonic anhydrases are eluted rather more slowly than expected for a molecule of that mass. Slight variations of this kind were noted by Andrews (1964, 1965) and were explained by him as possibly arising from differences in shape. The ratios of the elution volumes of the rat isoenzymes to Blue Dextran (very high molecular weight) were indistinguishable from the human erythrocytes isoenzymes.

Figure 18. MOLECULAR WEIGHT BY GEL FILTRATION



The  $\text{CO}_2$  hydratase activities of the isoenzymes and their inhibition by acetazolamide. Measurements were made of the dependence of the initial velocity of the hydration of  $\text{CO}_2$  on the initial substrate concentration in the reactions catalysed by the isoenzymes of the erythrocytes and prostate after verifying 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 in stock solutions (5 - 10  $\mu\text{M}$ ) of distilled water were estimated using the measured values of  $E_{280}^{1\text{ cm.}, 1\%}$  and the measured molecular weights. The results of the kinetic experiments are shown in Figs. 19 and 20.

It was possible to determine Michaelis constants and maximum velocities for all the isoenzymes of the erythrocytes and the prostate except Eryth. 1a (CEP). The initial velocity of the reaction catalysed by Eryth. 1a (CEP) was proportional to the initial substrate concentration in both the presence and absence of acetazolamide, showing that this isoenzyme was far from saturated with substrate at the maximum experimental concentration of  $\text{CO}_2$  (16mM).

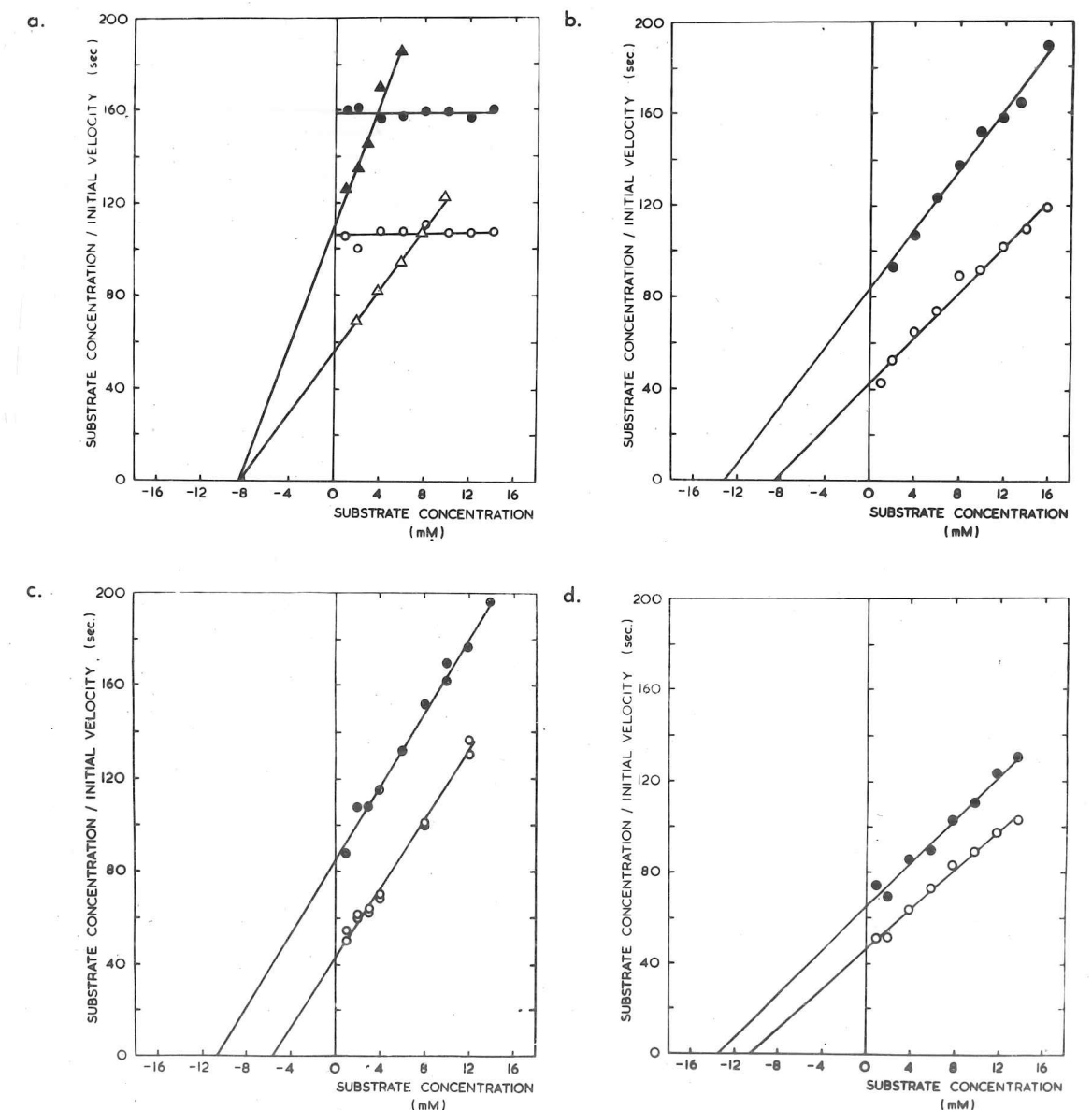
The isoenzymes Eryth. 1a, Prost. 1b, and Prost. 2 were inhibited non-competitively by acetazolamide, while Eryth. 2 (CEP), Eryth. 3 (CEP) and Eryth. 3 were inhibited in a mixed, but predominantly competitive, manner. It was not possible to determine the type of inhibition of Eryth. 1a (CEP) (Fig. 19a). Values of  $K_m$ ,  $V/[E_0]$  and approximate estimates of  $K_i$  are listed in Table 1.

The p-nitrophenyl acetate hydrolase (esterase) activities of the isoenzymes. The initial velocities of the reactions catalysed by Eryth. 1a



**Figure 19.** Relationship between  $[CO_2]$  and initial velocity of  $CO_2$  hydration catalysed by the rat erythrocyte isoenzymes, illustrated by plotting  $[initial\ substrate]/(initial\ rate)$  against  $[initial\ substrate]$ . The conditions were  $0^\circ$ , pH 7.0, 45mM-NaCl, 5mM-sodium phosphate, and 0.01% peptone.  $\circ$ , uninhibited;  $\bullet$  in the presence of acetazolamide, at the concn. given in brackets. (a) 120 $\mu$ M-Eryth.1a (CEP) (64 $\mu$ M);  $\triangle$  and  $\blacktriangle$ , 120 $\mu$ M-Eryth.1a (63 $\mu$ M); (b) 4.1 $\mu$ M-Eryth.2 (CEP) (2.6 $\mu$ M); (c) 1.8 $\mu$ M-Eryth.3 (CEP) (1.3 $\mu$ M); (d) 2.7 $\mu$ M-Eryth.3 (1.3 $\mu$ M). 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.

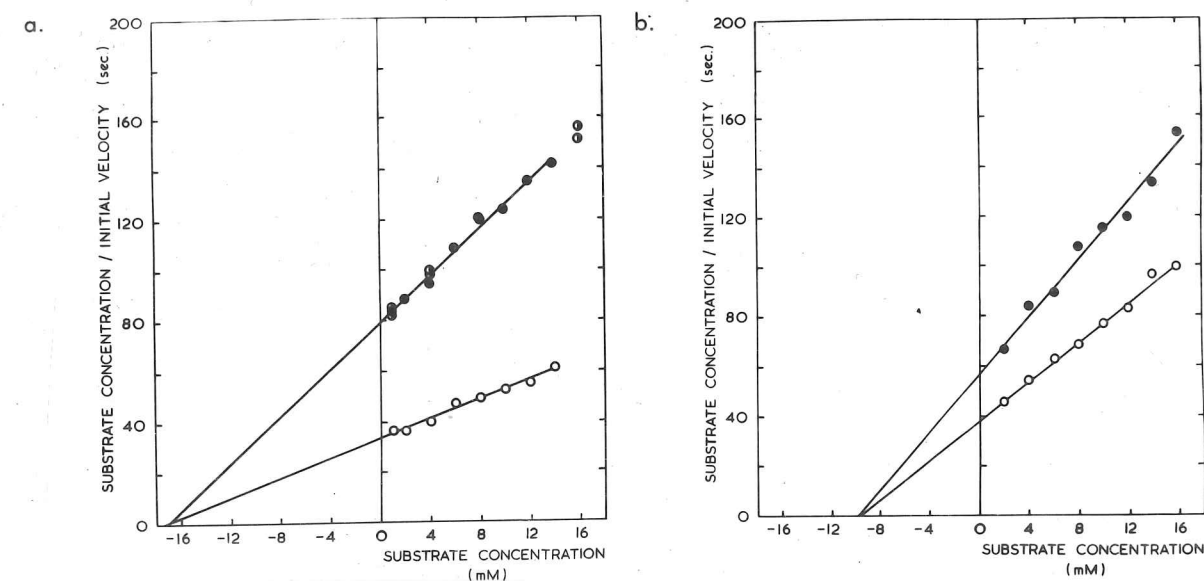
**Figure 19.** RAT ERYTHROCYTE ISOENZYMES.  $CO_2$  HYDRATION



**Figure 20.** Relationship between  $[CO_2]$  and initial velocity of  $CO_2$  hydration catalysed by the rat dorsolateral prostate isoenzymes, illustrated by plotting  $[initial\ substrate]/(initial\ rate)$  against  $[initial\ substrate]$ . The conditions were  $0^\circ$ , pH 7.0, 45mM-NaCl, 5mM-sodium phosphate, and 0.01% peptone.  $\bigcirc$ , Uninhibited;  $\bullet$  in the presence of acetazolamide, at the concn. given in brackets. (a) 2.8mM-Prost. 1b (6.4mM); (b) approx. 8mM-Prost. 2 (1.3mM). 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.

$\bullet$ , 1.3mM - Prost. 1b after storage (in water, at a concentration of 4.7 $\mu$ M) for a year at  $-20^\circ$ , illustrating absence of change in either V or  $K_m$ .

**Figure 20.** RAT PROSTATE ISOENZYMES.  $CO_2$  HYDRATION





(CEP), Eryth. 1a, Eryth. 3 and Prost. 1b were almost proportional to the substrate concentration (Fig. 21). 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 the bovine erythrocyte enzyme and by Verpoorte *et al.* (1967) for the case of human erythrocyte isoenzyme C.

The 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 Figs. 19 and 20 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  $\text{CO}_2$  hydration catalysed by Eryth. 3 (CEP) was measured at constant substrate concentration in the presence of varying amounts of acetazolamide, a Dixon plot (Dixon, 1953) of the results (Fig. 22) 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. 23). It is clear that in the present conditions of assay and with the nomenclature of Webb (1963), acetazolamide inhibition 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 (Figs. 22 and 23). In fact, the approximate value of

Figure 21. Relationship between [p-nitrophenyl acetate] and initial velocity of the esterase reaction catalysed by several rat carbonic anhydrase isoenzymes. The results are illustrated by plotting [initial substrate]/(initial rate) against [initial substrate]. The conditions were 25°, pH 8.0, 10mM-diethylmalonate, and 1.7% (v/v) acetone. ▲, 0.35μM-Eryth. 1a; ●, 0.55μM-Eryth. 1a (CEP); ○, 0.11μM-Eryth. 3 (CEP); △, 0.26μM-Prost. 1b. Each point is the average of two assays.

Figure 21. DEPENDENCE OF HYDROLASE ACTIVITY ON SUBSTRATE CONCENTRATION

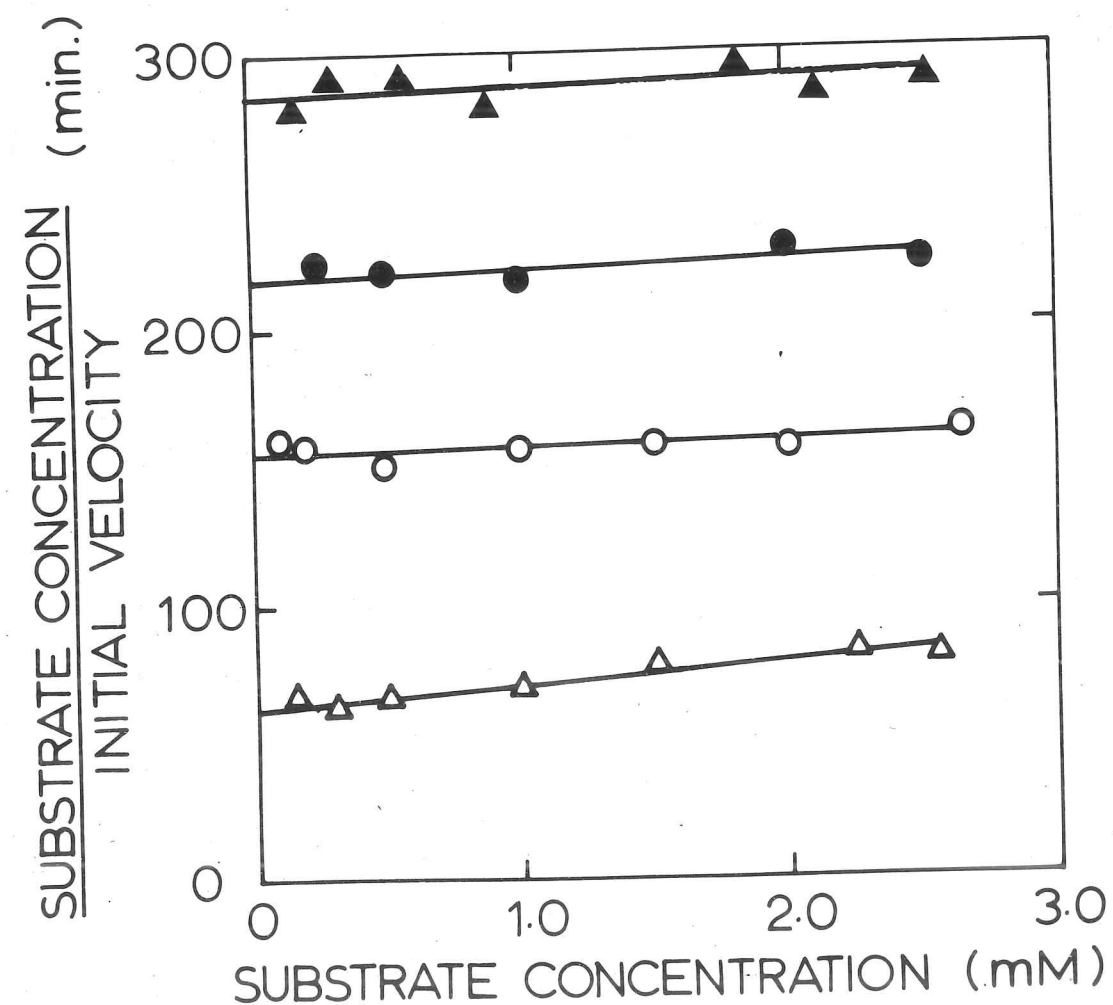
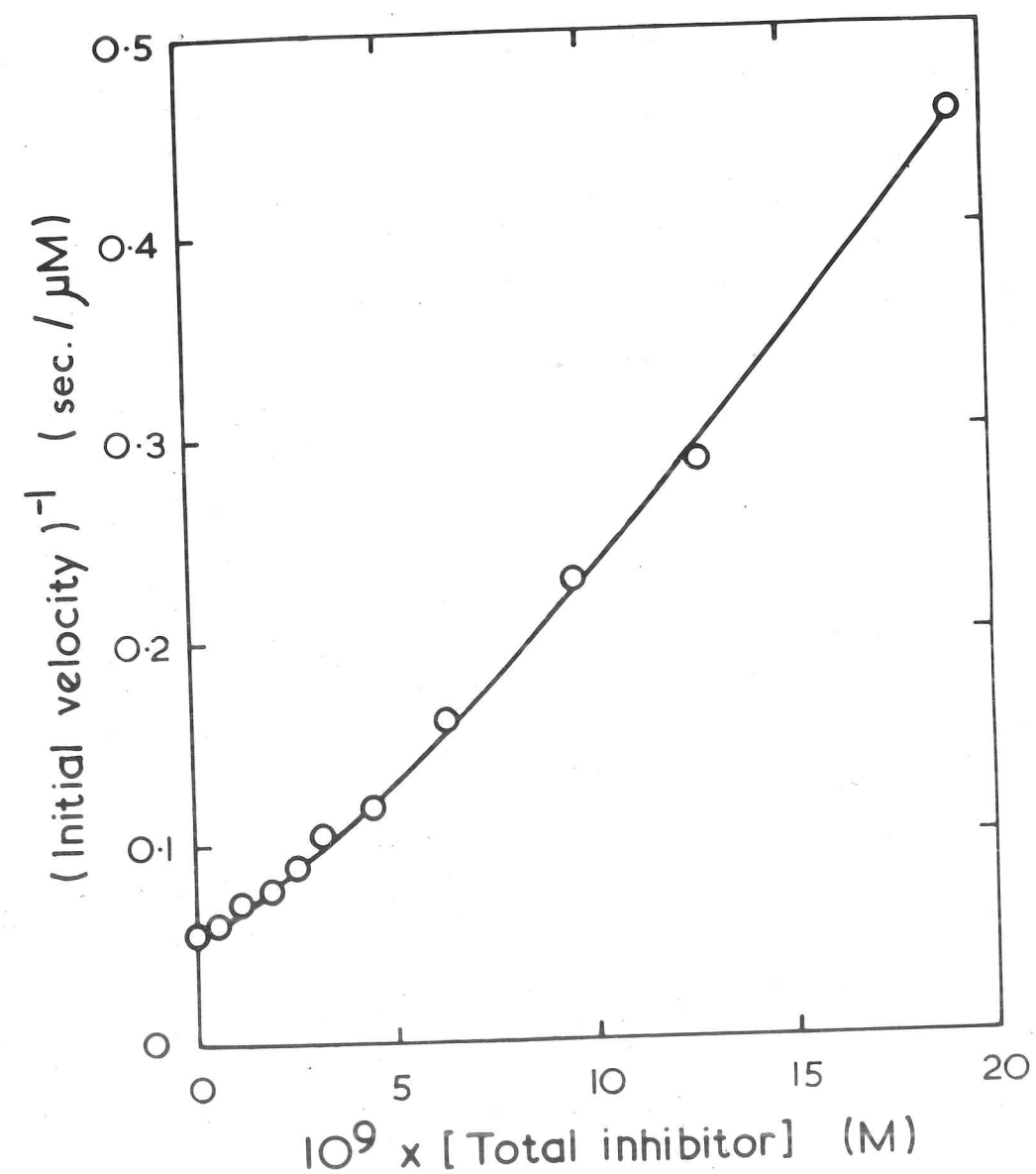


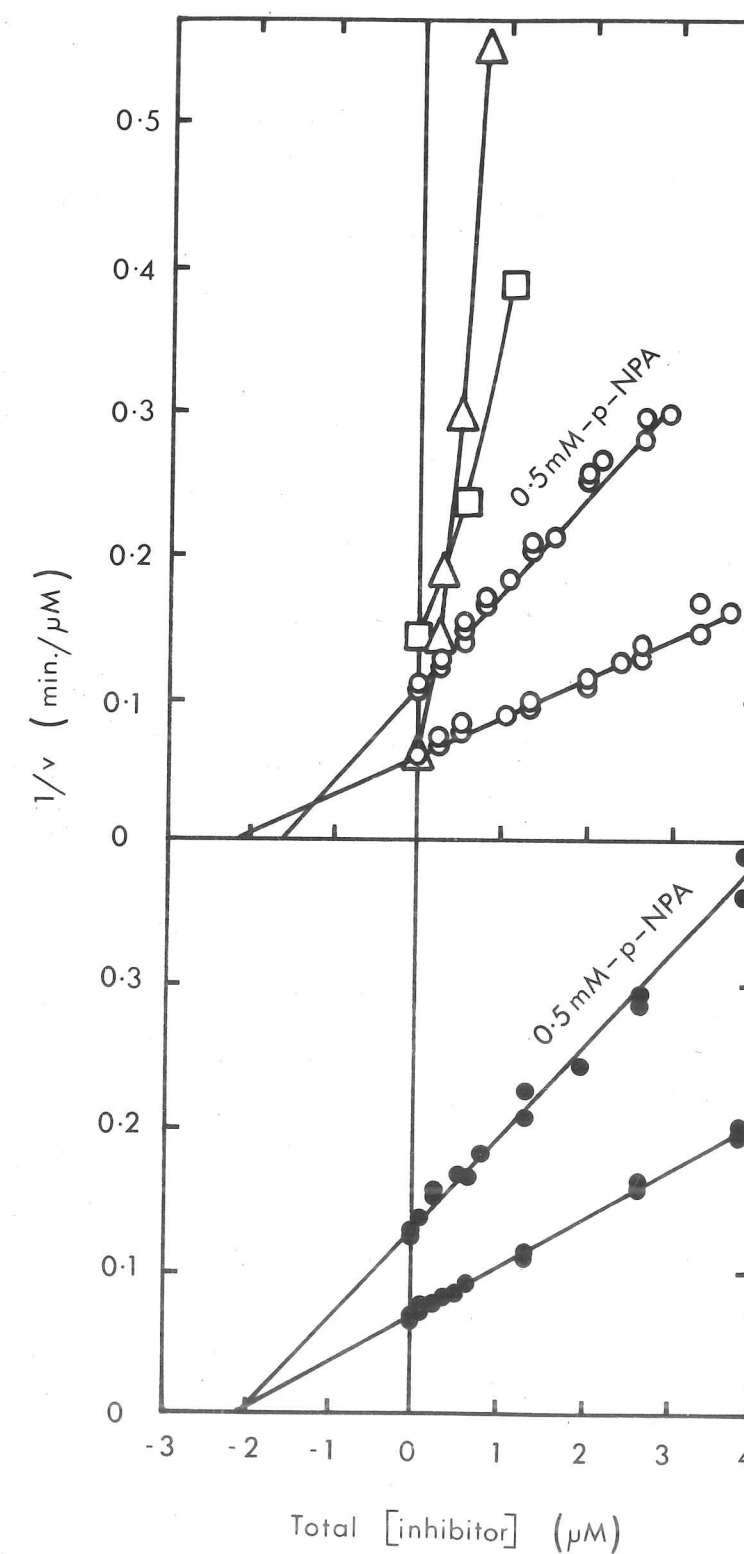
Figure 22. Relationship between the reciprocal of the initial rate of  $\text{CO}_2$  hydration catalysed by Eryth. 3 (CEP) and the total concn. of acetazolamide (Dixon plot). The conditions were  $0^\circ$ , pH 7.0, 45mM-NaCl, 5mM-sodium phosphate, 0.01% peptone, and the initial  $\text{CO}_2$  was 1.0mM. The enzyme concn. was 1.8 $\mu\text{M}$ . Each point is the result of a single assay.

Figure 22. ACETAZOLAMIDE INHIBITION OF HYDRATASE ACTIVITY



**Figure 23.** Relationship between the reciprocal of the initial rate of p-nitrophenyl acetate hydrolysis catalysed by several rat carbonic anhydrase isoenzymes and the total concn. of sulphonamide inhibitor (Dixon plots). The conditions were 25°, pH 8.0, 10mM-diethylmalonate, 1.7% (v/v) acetone, and the initial p-nitrophenyl acetate was 1.0mM (except where indicated as being 0.5mM).  $\Delta$ , 0.42 $\mu$ M-Eryth. 3 (CEP) inhibited by acetazolamide;  $\square$ , 0.55 $\mu$ M-Eryth. 1a (CEP) inhibited by DNSA;  $\circ$ , 0.42 $\mu$ M-Eryth. 3 (CEP) inhibited by DNSA;  $\bullet$ , 0.26 $\mu$ M-Prost. 1b 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.

**Figure 23.** p-NITROPHENYL ACETATE HYDROLYSIS





$[E_o]/K_i$  (Table 1) indicates that the system varied between zones A and B (Webb, 1963, p.70) which meant that in the case of Eryth.3 (CEP) the value calculated for  $K_i$  was a good approximation.

Results presented in Fig. 23 and Table 2 show that Eryth. 1a (CEP) and Eryth. 1a were more powerfully inhibited by acetazolamide than were Eryth. 3 (CEP), Eryth. 3, or the other high activity isoenzymes. Therefore the Eryth. 1a and 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 carbonic anhydrase causing inhibition of the enzyme and giving rise to an interesting fluorescent system (Chen & Kernohan, 1967), is a relatively weak inhibitor of Eryth. 3, Eryth. 3 (CEP) and Prost. 1b. With this inhibitor and Eryth. 3 (CEP) a linear Dixon plot was obtained 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. 23, 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 x-axis (Dixon, 1953). (The intercepts with the x-axis have been shown to be significantly different at the 1% level; Mr. J.G. Rowell kindly performed the rather complex calculation). 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. 21.

Acetazolamide was a stronger inhibitor of Eryth. 3 (CEP) than was DNSA, giving rise to a curved Dixon plot (Fig. 23); the system lay in zones A and B. Ethoxzolamide however had a very great affinity for Eryth.3 (CEP) and Prost. 1b and stoichiometrically titrated the activity; in other words, this was zone C mutual depletion inhibition (Fig. 24a and b).

In comparison with Eryth. 3 (CEP) and Eryth. 3, Eryth. 1a (CEP) and Eryth. 1a were more powerfully inhibited by the three sulphonamides, though the relative efficiencies of the inhibitors were maintained (Table 2), and even DNSA yielded a zone B system (Fig. 23). The prostate isoenzyme Prost. 1b was clearly non-competitively inhibited by DNSA (Fig. 23) though generally it resembled Eryth. 3 in its properties as a catalyst of p-nitrophenyl acetate hydrolysis.

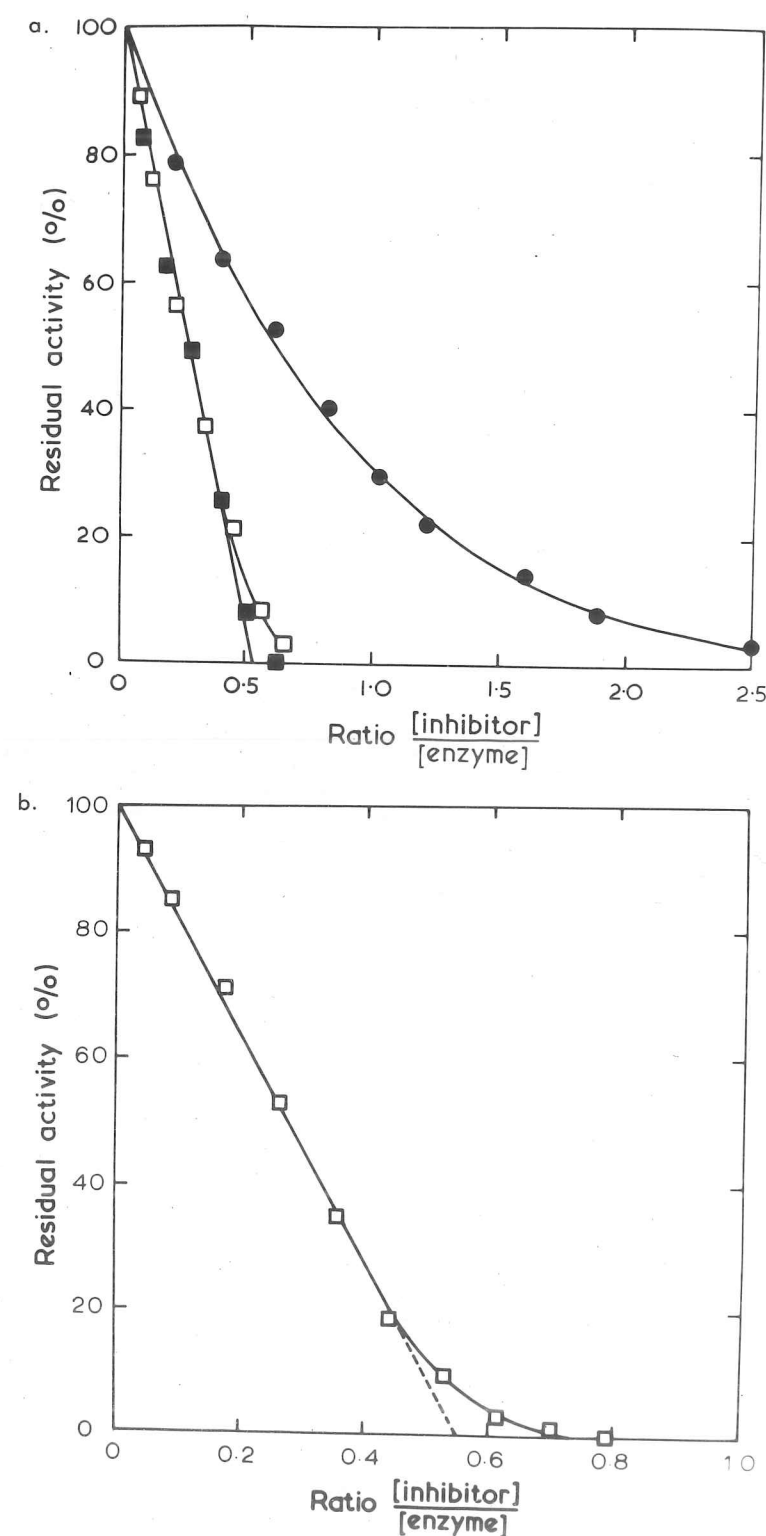
The interaction between sulphonamides and the isoenzymes. Inhibition of p-nitrophenyl acetate hydrolase activity, fluorescence quenching, and inhibition of  $\text{CO}_2$  hydratase activity. Most of the measurements presented in Fig. 24 indicate that the esterase activities of the isoenzymes were completely inhibited at a molar ratio of inhibitor to enzyme of about 0.5. The exception was Eryth. 1a (CEP) where the ratio was about 1. These ratios were calculated from the values of  $\epsilon_{280}^{1\%}$  and the molecular weights, determined in the course of the present investigation.

A series of experiments was carried out in an attempt to confirm these ratios using the method of Chen & Kernohan (1967), in which measurements were made of the fluorescence displayed by the fluorescent inhibitor DNSA in combination with carbonic anhydrase. This method had been devised for bovine erythrocyte carbonic anhydrase but experiment soon showed that the

**Figure 24a.** Relationship between the concentration of inhibitor in the assay for p-nitrophenyl acetate hydrolase activity and the fraction of uninhibited activity remaining, illustrated by plotting the residual activity against the molar ratio of total inhibitor to total enzyme. ●, 0.55 $\mu$ M-Eryth. 1a (CEP), inhibited by acetazolamide and ■ inhibited by ethoxzolamide; □, 0.42 $\mu$ M-Eryth. 3 (CEP), inhibited by ethoxzolamide. The conditions were 25°, pH 8.0, 10mM-diethylmalonate, 1.7% (v/v) acetone. Each point is the result of a single assay.

**Figure 24b.** As for Fig. 24a but the enzyme was 0.26 $\mu$ M-Prost. 1b, inhibited by ethoxzolamide.

**Figure 24.** INHIBITION OF HYDROLASE ACTIVITY



technique could be applied with equal success to the rat isoenzymes. The results illustrated in Fig. 25a, which were obtained with the use of a sample of the bovine isoenzyme (provided by Dr J.C.Kernohan), record the progressive increase in the blue fluorescence characteristic of DNSA in combination with carbonic anhydrase, as the concentration of the ligand was increased. Also illustrated is the progressive decrease in this fluorescence on titration of the DNSA-enzyme combination with ethoxzolamide. Both processes indicate that the ratio of binding of ligand to enzyme was about 1; this was the result reported by Chen & Kernohan and provides evidence for the correct functioning of the technique. However the other parts of Figs. 25 and 26 show that in most cases the rat isoenzymes appeared to interact with only half an equivalent of ligand, confirming the results of p-nitrophenyl acetate hydrolysis (Fig. 24). In the case of Eryth. 1a (CEP) however the ratio of DNSA to enzyme was about 1, while the amounts of either acetazolamide or ethoxzolamide required to replace the DNSA yielded a ratio of only 0.5 (Fig. 26b).

When the points of Fig. 22 were re-plotted in the form of a graph of per cent  $\text{CO}_2$  hydratase remaining against the molar ratio of inhibitor to enzyme, the result illustrated in Fig. 27 was obtained. This suggests that the  $\text{CO}_2$  hydratase activity of Eryth. 3 (CEP) required 3 or more equivalents of acetazolamide for complete inhibition.

Interpretation of these experiments is impossible at the present time. Careful repetition is required to confirm or disprove these preliminary findings, and particular attention must be paid to the stoichiometry of the inhibition of the catalysis of  $\text{CO}_2$  hydration by the rat carbonic anhydrase isoenzymes.

Figure 25. Results of fluorescence measurements on solutions of DNSA in combination with carbonic anhydrase. Fluorescence intensity is plotted against the molar ratio of DNSA or ethoxzolamide to enzyme. The procedure is described in MATERIALS AND METHODS.  $\circ$ , DNSA;  $\bullet$ , ethoxzolamide. (a) 0.88 $\mu$ M-Bovine erythrocyte carbonic anhydrase, isoenzyme B (provided by Dr J. C. Kernohan); (b) 0.55 $\mu$ M-Eryth. 3.

The sensitivity was increased before beginning titration with ethoxzolamide.

Figure 25. FLUORESCENCE MEASUREMENTS

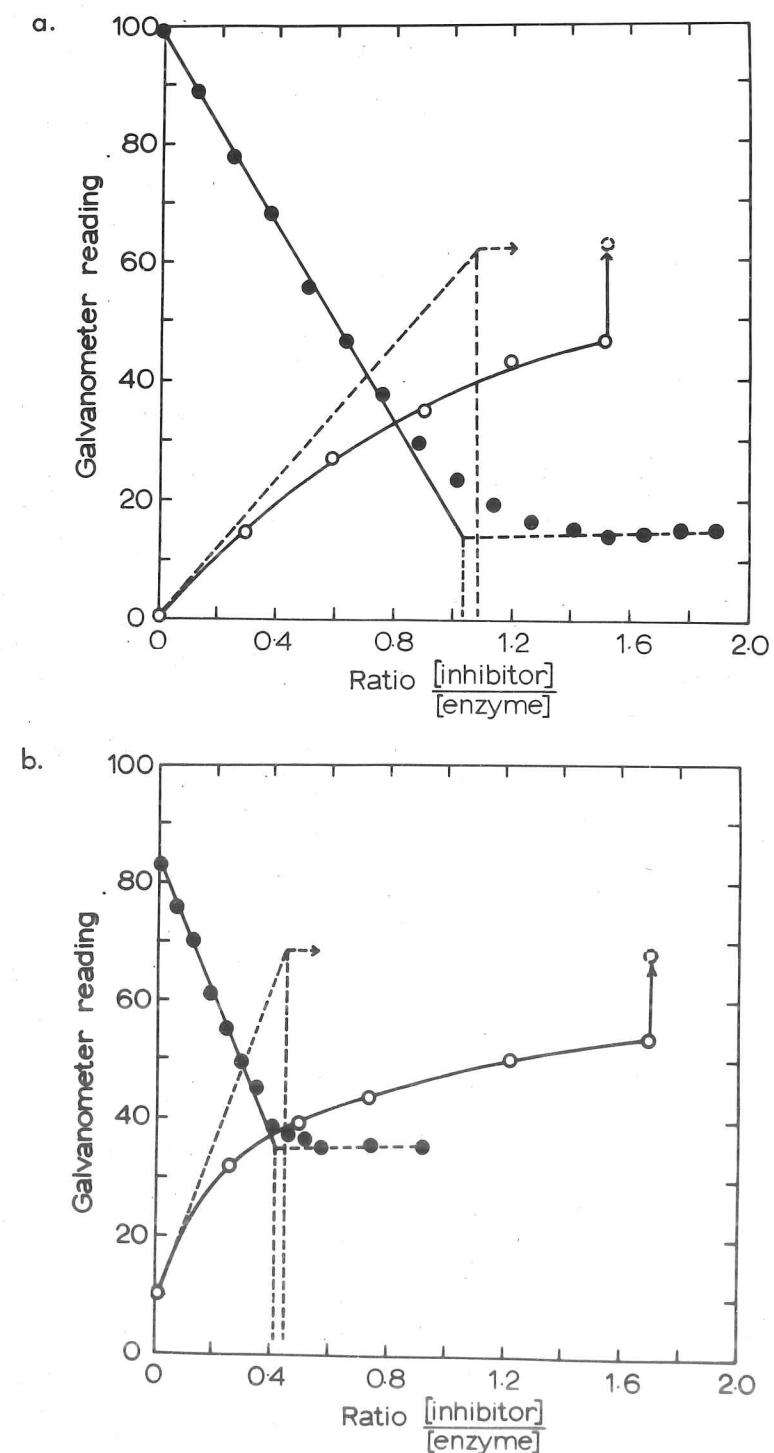
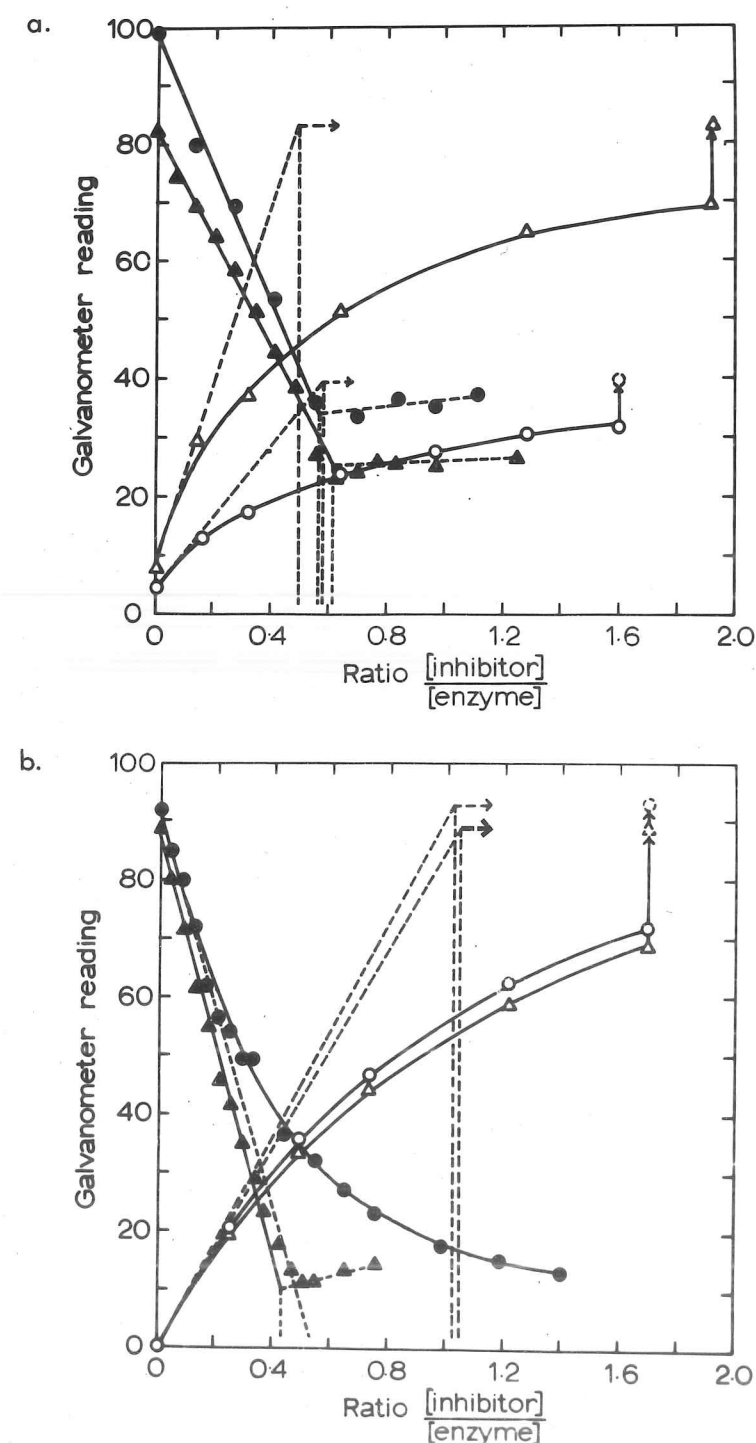




Figure 26. Results of fluorescence measurements on solutions of DNSA in combination with carbonic anhydrase. Fluorescence intensity is plotted against the molar ratio of DNSA, ethoxzolamide, or acetazolamide to enzyme. The procedure is described in MATERIALS AND METHODS. (a)  $\circ$  and  $\triangle$ , DNSA;  $\bullet$  and  $\blacktriangle$ , ethoxzolamide (  $\triangle$  and  $\blacktriangle$ , repetition with the initial sensitivity increased), 0.83 $\mu$ M-Eryth. 3 (CEP); (b)  $\circ$  and  $\triangle$ , DNSA;  $\bullet$ , acetazolamide;  $\blacktriangle$ , ethoxzolamide, 0.55 $\mu$ M-Eryth. 1a (CEP).

The sensitivity was increased before beginning titration with ethoxzolamide in ( a ) (  $\bullet$  ).

Figure 26. FLUORESCENCE MEASUREMENTS

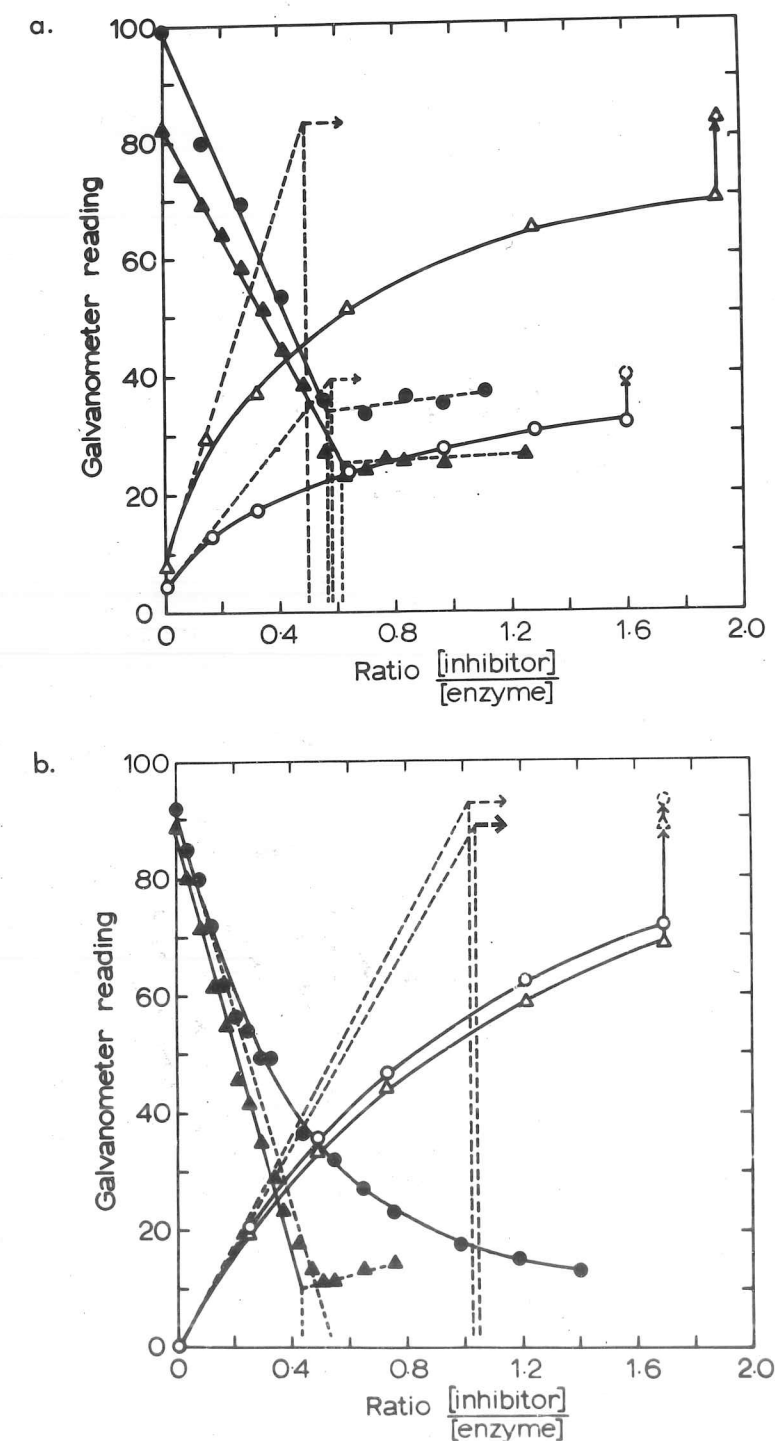




**Figure 26.** Results of fluorescence measurements on solutions of DNSA in combination with carbonic anhydrase. Fluorescence intensity is plotted against the molar ratio of DNSA, ethoxzolamide, or acetazolamide to enzyme. The procedure is described in MATERIALS AND METHODS. (a)  $\circ$  and  $\triangle$ , DNSA;  $\bullet$  and  $\blacktriangle$ , ethoxzolamide ( $\triangle$  and  $\blacktriangle$ , repetition with the initial sensitivity increased), 0.83 $\mu$ M-Eryth. 3 (CEP); (b)  $\circ$  and  $\triangle$ , DNSA;  $\bullet$ , acetazolamide;  $\blacktriangle$ , ethoxzolamide, 0.55 $\mu$ M-Eryth. 1a (CEP).

The sensitivity was increased before beginning titration with ethoxzolamide in (a) ( $\bullet$ ).

**Figure 26.** FLUORESCENCE MEASUREMENTS



The effect of p-nitrophenyl acetate on the  $\text{CO}_2$  hydration reaction.

An attempt was made to determine whether an ester, when added to the  $\text{CO}_2$  hydratase assay, would act as a competitive inhibitor of  $\text{CO}_2$ . p-Nitrophenyl acetate was included in the routine titration assay at concentrations up to 1mM, the maximum concentration attainable under the usual experimental conditions. The ester was added to the assay mixture 30 sec. before initiation of the reaction. The results of several experiments are summarised in Table 3, where it can be seen that the presence of the ester caused no significant change in the rate of  $\text{CO}_2$  hydration.

The effect of 4-pyridine aldehyde on the  $\text{CO}_2$  hydration reaction. The hydration of 4-pyridine aldehyde has been shown by Pocker & Meany (1967) to be catalysed effectively by bovine erythrocyte carbonic anhydrase. In the present investigation, an attempt was made to influence the rate of the  $\text{CO}_2$  reaction by the addition of this compound to the routine assay of  $\text{CO}_2$  hydratase activity. First however a cursory examination was made of both the enzyme-catalysed and uncatalysed rates of hydration of 4-pyridine aldehyde, in the absence of  $\text{CO}_2$ .

The progress curve of a typical experiment, shown in Fig. 28, illustrates the spontaneous hydration of 4-pyridine aldehyde\* at 3° and pH 7.0. This result yielded a value for the pseudo-first-order rate constant of the hydration reaction  $k_1 = 0.72 \text{ min.}^{-1}$ , in good agreement with the value of 0.63 quoted by Pocker & Meany (1967), obtained under identical conditions but at the slightly lower temperature of 0°. The results of similar

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\*Rigorous proof of the identity of my sample of 4-pyridine aldehyde is lacking (see MATERIALS AND ANALYTICAL METHODS).

**Table 3.** The effect of p-nitrophenyl acetate on the  $\text{CO}_2$  hydration reaction catalysed by several of the rat carbonic anhydrase isoenzymes. The conditions were  $0^\circ$ , pH 7.0, 45mM-NaCl and 5mM-sodium phosphate.

Isoenzyme	Initial concn. of $\text{CO}_2$ (mM) <sup>2</sup>	Initial concn. of p-NPA (mM)	$k'_1$ , the rate constant of the enzyme-catalysed $\text{CO}_2$ hydra- tion reaction, proportional to enzyme <sub>1</sub> activity. (sec <sup>-1</sup> )
1. Eryth. 1a(CEP) (0.2 4 $\mu\text{M}$ )	1.0	-	0.0188
	1.0	1.0	0.0192
2. Eryth. 3(CEP) (1.8 m $\mu\text{M}$ )	1.0	-	0.0202
	1.0	1.0	0.0205
	4.0	-	0.0145
	4.0	0.33	0.0140
	4.0	1.0	0.0147
3. Prost. 1b (2.8 m $\mu\text{M}$ )	1.0	-	0.0270
	1.0	1.0	0.0273

Figure 27. The results of inhibiting progressively with acetazolamide the  $\text{CO}_2$  hydratase activity of 1.8 $\mu\text{M}$ -Eryth. 3 (CEP), previously illustrated in Fig. 22 and replotted here as per cent activity remaining against the molar ratio of total inhibitor to enzyme (Dixon plot). The conditions were  $0^\circ$ , pH 7.0, 45mM-NaCl, 5mM-sodium phosphate, 0.01% peptone, and the initial  $\text{CO}_2$  concentration was 1.0mM. Each point is the result of a single assay.

Figure 27. ACETAZOLAMIDE INHIBITION OF  $\text{CO}_2$  HYDRATASE ACTIVITY

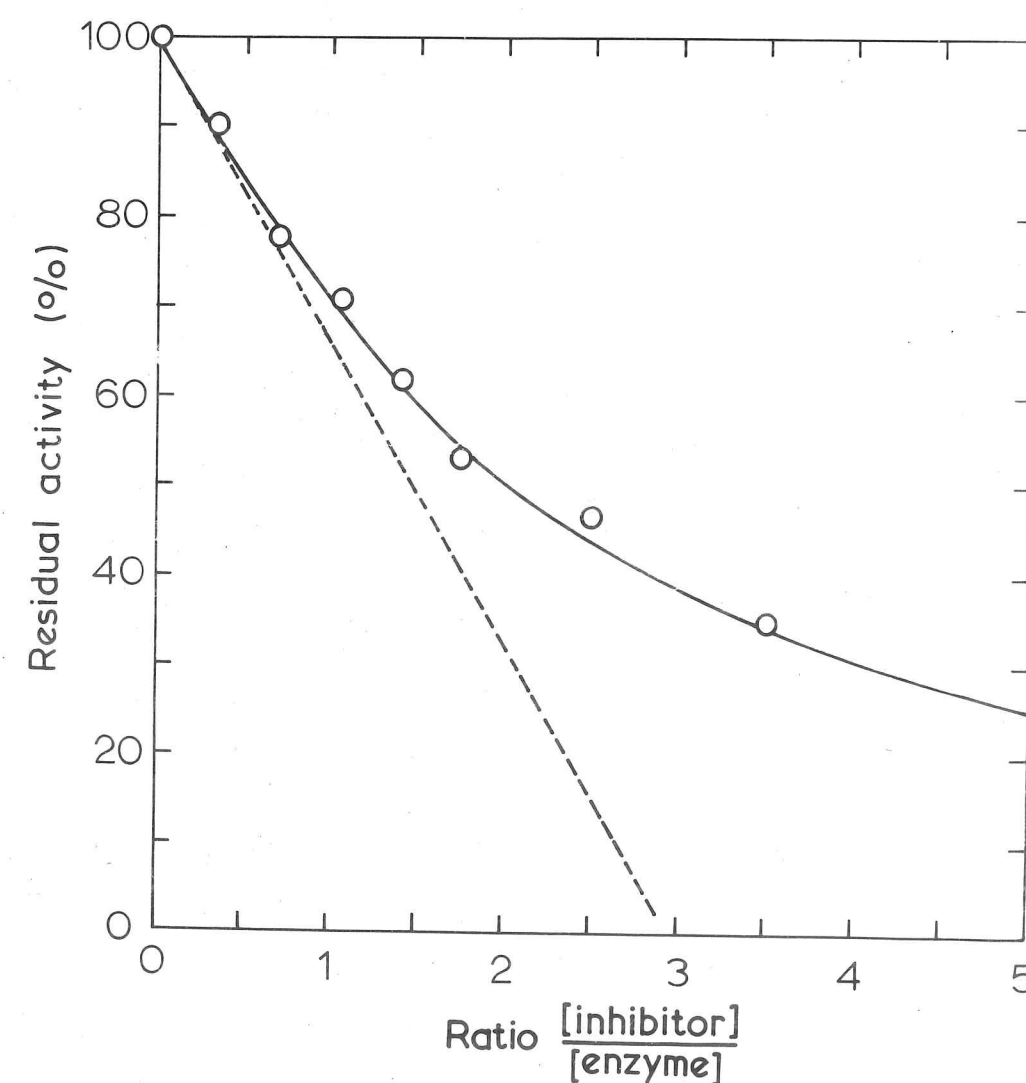




Figure 28. A recording of the decrease in extinction at a wavelength of 320m $\mu$  accompanying the spontaneous hydration of 4-pyridine aldehyde. The conditions were 3<sup>0</sup>, pH 7.0 and 10mM-diethylmalonate; the initial concentration of 4-pyridine aldehyde was 1mM. The pseudo-first-order reaction reaches equilibrium with the reverse reaction when the fraction of hydration of the 4-pyridine aldehyde equals approximately 0.66 under these conditions (Pocker & Meany, 1967). The equilibrium value of the extinction was measured 7min. after initiation of the reaction.

Figure 28. HYDRATION OF 4-PYRIDINE ALDEHYDE

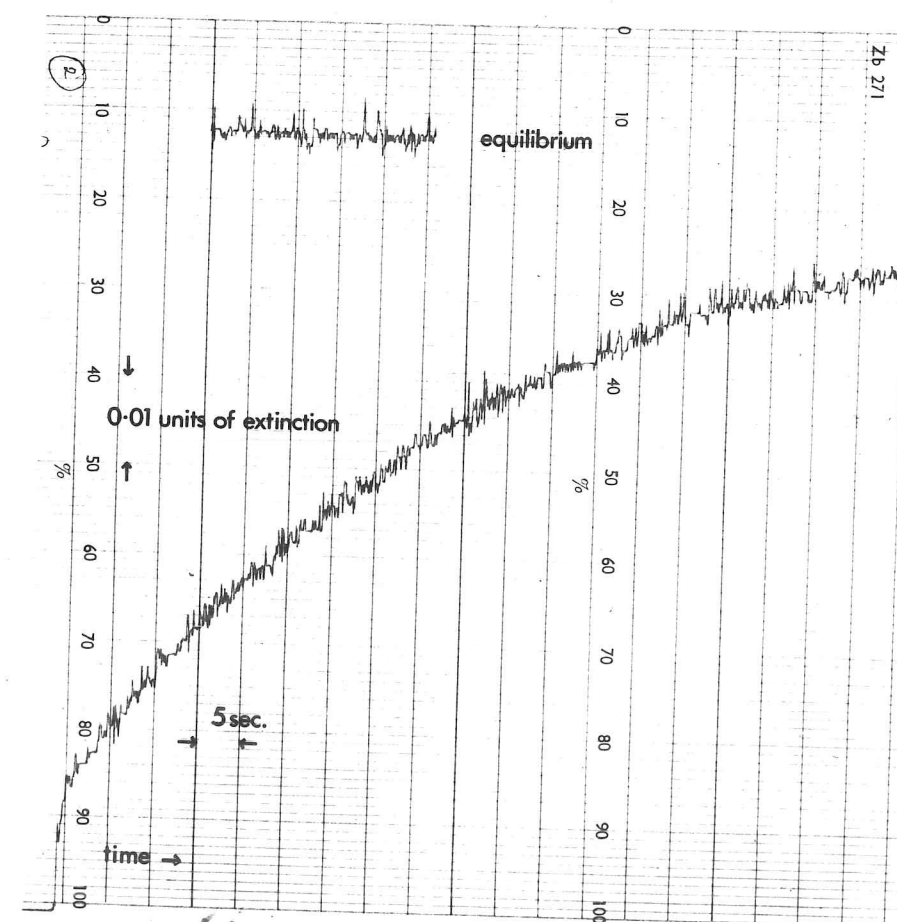




Table 4. The catalysis of the hydration of 1mM-4-pyridine aldehyde by several isoenzymes of carbonic anhydrase. The conditions were 3°, pH 7.0 and 10mM-diethylmalonate. The concentrations of the isoenzymes in the assays were such that the catalysed rates were about twice the uncatalysed rates. With the same conditions, and the same concentration of the substrate, Pocker & Meany (1967) obtained a value for  $v/[E_o] = 900 \text{ min}^{-1}$  with the bovine enzyme.

Isoenzyme	$v/[E_o]$ ( $\text{min}^{-1}$ )
1. Bovine erythrocyte carbonic anhydrase, isoenzyme B (3.8 $\mu$ M).	170
2. Eryth. 1a (4.2 $\mu$ M)	230
3. Eryth. 3 (2.8 $\mu$ M)	360

**Table 5.** The effect of 4-pyridine aldehyde on the  $\text{CO}_2$  hydration reaction catalysed by several rat carbonic anhydrase isoenzymes. The concentration of  $\text{CO}_2 = 1.0 \text{ mM}$  and the conditions were  $0^\circ$ , pH 7.0, 45mM-NaCl and 5mM-sodium phosphate.

Isoenzyme	Initial concn. of 4-PA(mM)	$k_1$ , the rate constant of the enzyme-catalysed $\text{CO}_2$ hydra- tion reaction, proportional to enzyme <sub>1</sub> activity (sec <sup>-1</sup> )
1. Eryth.1a(CEP)	(0.32 $\mu\text{M}$ )	0
	(0.63 $\mu\text{M}$ )	0
	(0.63 $\mu\text{M}$ )	23
	(0.95 $\mu\text{M}$ )	0
	(0.95 $\mu\text{M}$ )	0
	(0.95 $\mu\text{M}$ )	23
	(0.99 $\mu\text{M}$ )	23
2. Eryth. 1a	(0.99 $\mu\text{M}$ )	0
	(0.99 $\mu\text{M}$ )	0
	(0.99 $\mu\text{M}$ )	23
3. Eryth. 3	(3.1mpM)	0
	(3.1mpM)	4.6
	(3.1mpM)	23
4. Prost. 1b	(2.8mpM)	0
	(2.8mpM)	23

experiments conducted in the presence of Eryth. 1a, Eryth. 3, and also bovine erythrocyte carbonic anhydrase isoenzyme B, are summarised in Table 4. Though the value of  $V/[E_0]$  for the bovine isoenzyme found here was 5 times less than that reported by Pocker & Meany (1967), it was clear that both rat isoenzymes catalysed the hydration of 4-pyridine aldehyde.

The results of adding 4-pyridine aldehyde, at a final concentration of about 23mM, to the routine  $CO_2$  hydratase assay, in which the initial concentration of  $CO_2$  was 1.0mM, are shown in Table 5. The 4-pyridine aldehyde was added to the assay mixture (using a micro-syringe) 10 sec. before initiating the  $CO_2$  reaction. Allowance was made for the slight increase in pH occurring on addition of the pyridine derivative. The fact that the 4-pyridine aldehyde was hydrated at the same time as the  $CO_2$  did not lead to interference with the assay because the hydration of the aldehyde is not accompanied by release or absorption of  $H^+$  ions. It is clear from Table 5 that, apart from a slight inhibition of the  $CO_2$  reaction catalysed by Eryth. 1a (CEP), there was no difference between assays carried out in the presence and the absence of 4-pyridine aldehyde in the cases of the isoenzymes that were examined.

The  $\beta$ -naphthyl acetate hydrolase (esterase) activities of the isoenzymes.

The results of measurements of initial velocities using 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. 1a were superior to both preparations of the high activity form Eryth. 3 as catalysts of the

hydrolysis of  $\beta$ -naphthyl acetate. Neither dorsolateral prostate isoenzyme could be shown to act as a catalyst of this reaction (Table 2).

The zinc content of the rat dorsolateral prostate and of the prostate isoenzymes. The concentration of zinc in the dorsolateral prostate varied from 160 - 190  $\mu\text{g. zinc/g. wet weight}$ , with an average value of 180  $\mu\text{g./g. wet weight}$ . This result is in agreement with the findings of Mawson & Fischer (1952). Since an homogenate of the tissue contained an average of 56,000 units- $\text{CO}_2$  hydratase activity /g. wet weight, the average value for the ratio of carbonic anhydrase activity to zinc in Prost. 1b and Prost. 2 was 6,200 units/ $\mu\text{g. zinc}$ , assuming approximately equal quantities of the two isoenzymes in the intact tissue. Therefore in the dorsolateral prostate carbonic anhydrase accounts for only 5% of the total zinc found in this organ.

### DISCUSSION

The two major carbonic anhydrase 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  $\text{CO}_2$  hydration Eryth. 3 was more effective than Eryth. 1a by a factor of 70.

Removal of haemoglobin from the rat haemolysate by denaturation with  $\text{CHCl}_3$  induced distinct changes in certain kinetic properties of the carbonic anhydrase isoenzymes. For example,  $K_m$  ( $\text{CO}_2$  hydration) of Eryth. 1a (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 estimated 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 carbonic anhydrase are unusually susceptible to organic solvents, because in the case of the two human erythrocyte isoenzymes Gibbons & Edsall (1964) found no significant differences in the values of  $K_m$  ( $\text{CO}_2$  hydration) when they compared samples which had been extracted into  $\text{CHCl}_3$ -EtOH 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  $\text{CHCl}_3$ -EtOH extract may have been caused by the organic solvents facilitating its release from the erythrocyte. Alternatively the  $\text{CHCl}_3$ -EtOH 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  $\text{CHCl}_3$ -EtOH it seems possible that changes might similarly occur in these parts of an isoenzyme molecule which are responsible for interaction with DEAE-Sephadex.

In contrast to the erythrocyte isoenzymes, the two major forms of carbonic anhydrase 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. 1b and Eryth. 3. The values of  $K_m$  and  $V/[E_o]$  for Prost. 1b were 2 - 3 times greater than for Eryth. 3 prepared by either method of purification. Moreover acetazolamide inhibition of Prost. 1b was non-competitive while Eryth. 3 was inhibited competitively, in identical conditions.

The interesting observation that the low activity erythrocyte isoenzyme Eryth. 1a was considerably more effective than the high activity form Eryth. 3 as a catalyst of  $1\text{mM}$ - $\beta$ -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. 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, carbonic anhydrase 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 carbonic anhydrase 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). Certain recent findings that offer an explanation for these differences in inhibition type are summarised in the GENERAL DISCUSSION.

A great deal of evidence has been accumulated showing that these compounds which inhibit the  $\text{CO}_2$  hydratase activities of the human and bovine carbonic anhydrase isoenzymes also inhibit their esterase activities (Pocker & Stone, 1965; 1967; 1968a; Verpoorte et al., 1967; Thorslund & Lindskog, 1967; Pocker & Storm, 1968). Furthermore 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 carbonic anhydrase. Competitive inhibition has been described but only in connection with certain substrate analogues,

all of them carbonyl compounds (Pocker & Stone, 1968b). 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 in comparison with acetazolamide, DNSA has a much lower affinity for the enzyme. Similarly, in the case of the  $\text{CO}_2$  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.

The conflicting results of attempts to determine the number of molecules of the sulphonamide inhibitors that combine with the rat carbonic anhydrase isoenzymes, are inexplicable. It is clear that the experiments must be extended before any conclusions can be reached. Several previous reports involving bovine erythrocyte carbonic anhydrase have demonstrated clearly that one molecule of acetazolamide causes complete inhibition when bound to one molecule of this enzyme (Table 6). This is not to say that the present results are impossible; it is conceivable that the forms of carbonic anhydrase isolated from the tissues of the rat are inhibited in a different manner from the isoenzymes previously studied.

**Table 6.** Summary of investigations that have demonstrated that carbonic anhydrase from both human and bovine erythrocytes combines with inhibitors in the molar ratio of 1:1.

Isoenzyme	Inhibitor	Method	Reference
1. Cobalt form of bovine	Acetazolamide	Spectroscopy	Lindskog (1963)
2. Bovine	Acetazolamide	Hydrolysis of p-nitrophenyl acetate	Pocker & Stone (1967)
3. Bovine	Acetazolamide	Hydration of acetaldehyde	Pocker & Meany (1967)
4. Bovine	DNSA and ethoxzolamide	Fluorescence	Chen & Kernohan (1967)
5. Bovine	Several anions	Hydrolysis of p-nitrophenyl acetate	Pocker & Stone (1968a)
6. Human C	Acetoxymersulphanilamide	X-ray scattering of crystal	Fridborg <u>et al.</u> (1967)

It has been contended, partly on the basis of indirect evidence, that the hydration of  $\text{CO}_2$ , the hydration of acetaldehyde and other aldehydes, and the hydrolysis of p-nitrophenyl acetate and other esters, are all catalysed at a single active site on the molecule of carbonic anhydrase. For example, it has been reported that the zinc atom is essential for the hydration of both  $\text{CO}_2$  and aldehydes (Pocker & Stone, 1968a) and that the compounds that inhibit hydratase activity also inhibit esterase activity (Pocker & Stone, 1967; Verpoorte *et al.*, 1967; Pocker & Storm, 1968). What is more, the relative potencies of a series of anions as inhibitors of  $\text{CO}_2$  hydration exactly parallels their behaviour as inhibitors of the hydrolysis of p-nitrophenyl acetate (Roughton & Booth, 1946<sup>b</sup>; Kernohan, 1965; Pocker & Stone, 1968a). More direct evidence for a unique active site was provided by Pocker & Stone (1968a) when they showed that acetazolamide competed with the product of  $\text{CO}_2$  hydration,  $\text{HCO}_3^-$ , (as well as other anions) in experiments involving mutual inhibition of the hydrolysis of p-nitrophenyl acetate. However, the effect of ester or aldehyde substrates on the enzyme-catalysed hydration of  $\text{CO}_2$  has not been previously investigated.

In the present work the first attempt to inhibit competitively the  $\text{CO}_2$  reaction was made using p-nitrophenyl acetate. It was anticipated that if  $\text{CO}_2$  and p-nitrophenyl acetate interacted with the same active site, competition would be observed. In fact the rate of hydration of  $\text{CO}_2$  was not affected by the presence of the ester. Absence of competition cannot have been caused by the completion of ester hydrolysis before the addition of the  $\text{CO}_2$  because under the conditions of assay the hydrolysis of the ester was much slower than the hydration of the  $\text{CO}_2$ .



On the assumption that the lack of an effect might have been due to the great difference in the rates of the two reactions or to the possibility that the Michaelis constants for the interaction of the ester and the isoenzymes were much greater than those for  $\text{CO}_2$ , attention was turned to the compound 4-pyridine aldehyde, the hydration of which was known to be catalysed rapidly by carbonic anhydrase. Almost completely negative results were obtained, even though the concentration of the aldehyde exceeded that of the  $\text{CO}_2$  by a factor of 20 times.

These experiments indicate that, in the cases of the isoenzymes of carbonic anhydrase that were examined, either  $\text{CO}_2$  and p-nitrophenyl acetate or 4-pyridine aldehyde interact at different sites, or the conditions were not conducive to the occurrence of competition. While the latter explanation could conceivably have been true in the case of the experiments involving the ester it is less likely to have been so for the experiment with the aldehyde. One outstanding difference in the conditions of many of the experiments in which  $\text{CO}_2$  and either p-nitrophenyl acetate or 4-pyridine aldehyde are assayed is the concentration of the enzyme. In the case of  $\text{CO}_2$  hydration, the concentrations that are used of the high activity forms of the enzyme are in the region of 1-5  $\mu\text{M}$ , while in the assay of either the ester or the aldehyde the concentration of the same enzyme is between 0.5 and 5  $\mu\text{M}$ . It is perhaps significant that the only evidence for any competition was found in the case of the addition of the aldehyde to the assay of the low activity isoenzyme Eryth. 1a (CEP), where the concentration of the isoenzyme in the  $\text{CO}_2$  hydration assay (9.5  $\mu\text{M}$ ) was comparable with its concentration in the 4-pyridine aldehyde assay (about 4  $\mu\text{M}$ ).

The extraordinarily large quantity of carbonic anhydrase in the rat dorsolateral prostate, demonstrated in these experiments, appears to represent only a small fraction of the unusually high concentration of zinc in the same region of that organ (Gunn *et al.*, 1955). Evidence of a different kind also suggests that much of the prostatic zinc is not bound to carbonic anhydrase. When certain zinc-chelating agents were administered to rats, large quantities of the zinc-chelate complex were found in the dorsolateral prostates (Sternberg, Cronin & Philips, 1965). Since carbonic anhydrase is itself a powerful chelator of zinc, it is most unlikely that the compounds that were injected could have removed 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). Furthermore, examination of the subcellular distribution of zinc in the rat dorsolateral prostate indicates that about half only 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 carbonic anhydrase 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 carbonic anhydrase a definite function in the biochemistry and physiology of the rat prostate. The prostate is an accessory organ, the metabolism of which is dependent upon the male sex hormone (Mann, 1964; p. 45). It would undoubtedly be of interest to investigate the relationship between testosterone and the levels of the individual prostatic carbonic anhydrase 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 directly 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 carbonic anhydrase 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 the antagonistic hormonal agents.

### SUMMARY

1. Three forms of carbonic anhydrase 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 in specific activity by a factor of 70, 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 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  $\text{CHCl}_3$ -EtOH 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-dimethylaminonaphthlene-5-sulphonamide and ethoxzolamide on the hydrolysis of p-nitrophenyl acetate catalysed by the isoenzymes.

5. Several experiments suggested that the ratio of the sulphonamides to the isoenzymes causing complete inhibition were different in comparison with previously reported results obtained with the use of carbonic anhydrase from other species.

6. No significant inhibition of  $\text{CO}_2$  hydration was detected on the addition of either p-nitrophenyl acetate or 4-pyridine aldehyde to the  $\text{CO}_2$  assay.

7. The low activity erythrocyte isoenzyme was an efficient catalyst of the hydrolysis of  $\beta$ -naphthyl acetate whereas the high activity forms were much less active toward this ester. It is of interest that neither of the isoenzymes present in the dorsolateral prostate was found to catalyse this reaction.

8. Carbonic anhydrase in the rat dorsolateral prostate accounts for no more than 5% of the unusually high content of zinc in this organ.



### CHAPTER 3

#### CARBONIC ANHYDRASE ISOENZYMES IN THE ERYTHROCYTES AND UTERUS OF THE SHEEP

##### INTRODUCTION

Carbonic anhydrase has been shown to occur in the female reproductive tract of a variety of mammalian species, and the interior lining of the uterus, or endometrium, has been established as one of the main loci of activity (Lutwak-Mann, 1955). The endometrium of both the non-pregnant and pregnant sheep was found to be rich in carbonic anhydrase activity, the level of the activity being independent of ovarian function. Though a less attractive enzyme for study, perhaps, than rabbit endometrial carbonic anhydrase (the activity of which is dependent upon ovarian hormone control (Lutwak-Mann, 1955)), the sheep enzyme could be obtained in large amounts which greatly facilitated its purification and the examination of its properties.

The aim of this investigation was to characterize the endometrial enzyme and compare it with the forms of carbonic anhydrase isolated from the red blood cells of the sheep.

## EXPERIMENTAL AND RESULTS

### Preparation of the isoenzymes.

Erythrocyte isoenzymes. Blood was collected from a single female sheep and the erythrocytes were isolated, washed and haemolysed, and then treated with  $\text{CHCl}_3$ -EtOH. The details of these procedures have been described in Chapter 2. The lyophilized extract containing carbonic anhydrase was dissolved in Buffer I, dialysed exhaustively against the buffer, and applied to a column of DEAE-Sephadex equilibrated with the same buffer.

Fig. 29 shows the result of chromatography and in addition indicates subsequent steps in which rechromatography was performed. In the first chromatogram rather a large quantity of the initial extract was applied to the column causing overloading with the result that the isoenzymes emerged more rapidly than was usually observed and the major peak was skewed.

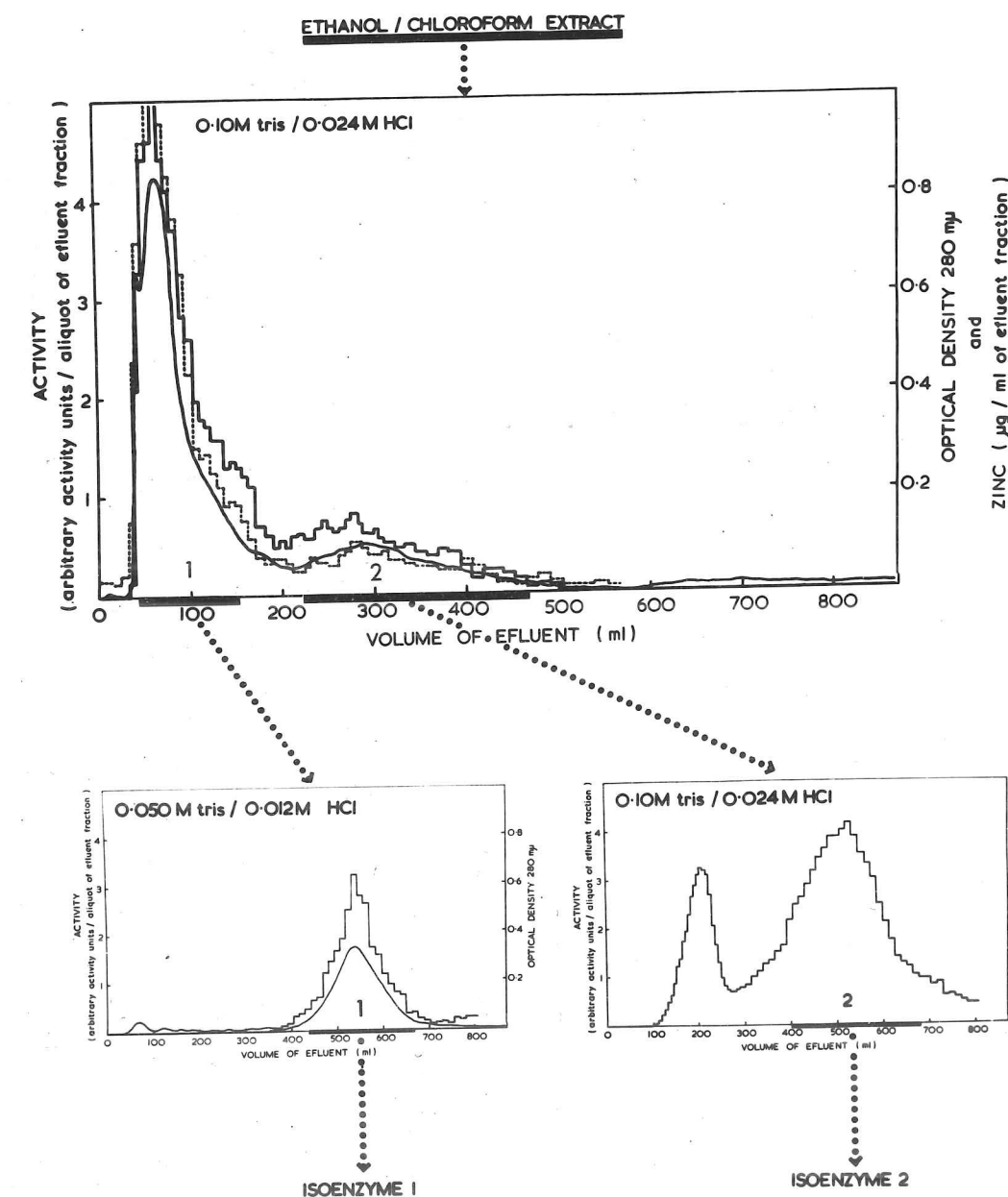
Two isoenzymes were found having similar specific activities. The first to emerge from the column represented the major part of both the carbonic anhydrase activity and protein. The two forms could be separated fairly efficiently by repeated chromatography. They were numbered\* in the usual manner according to the order of their elution from the column; Eryth. 1 (CEP) and Eryth. 2 (CEP). Fig. 30 depicts the results of polyacrylamide gel electrophoresis performed at successive stages of the purification. The distribution is illustrated of protein bands and regions showing esterase

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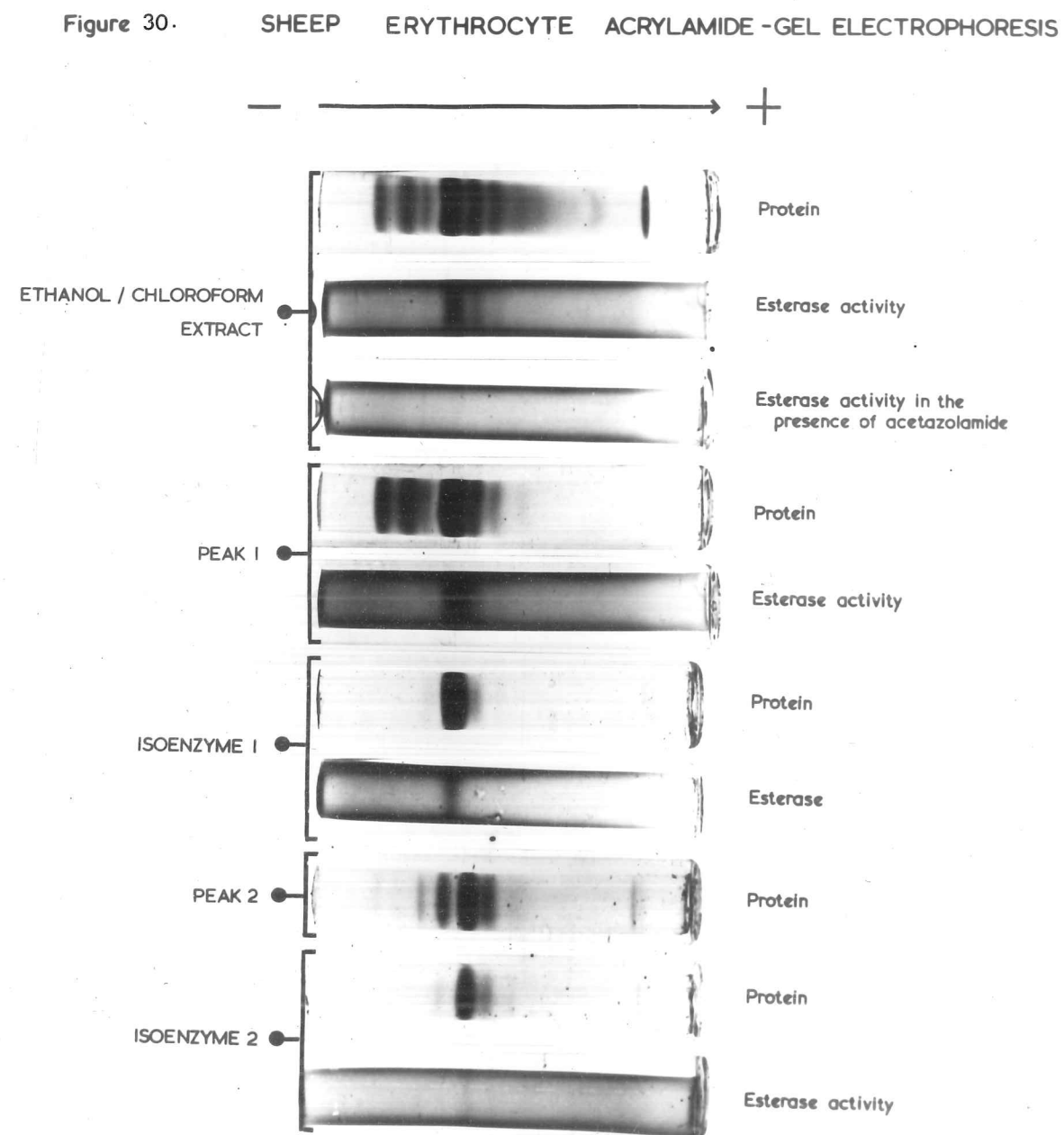
\* The carbonic anhydrase isoenzymes will be referred to hereafter as follows: erythrocyte isoenzymes, Eryth. (number); endometrial isoenzymes, Endo. (number). (CEP) after an isoenzyme indicates purification from a  $\text{CHCl}_3$ -EtOH extract.

Figure 29. A flow sheet summarizing the purification of sheep erythrocyte carbonic anhydrase and its resolution into isoenzymes, by column chromatography using DEAE-Sephadex. The starting material was a  $\text{CHCl}_3$ -EtOH extract of washed erythrocytes. Each box contains a description of the buffer used in that particular chromatographic step; the pH of each buffer was approximately 9.3 at  $4^\circ$ . (Continuous),  $E_{280}$ ; ——— (stepped),  $\text{CO}_2$  hydratase activity (modified Philpot assay); — — —, zinc. The heavy bars near the horizontal axes show which fractions were combined for further purification. The numbering of the peaks corresponds to the numbering of the isoenzymes.

Figure 29. SHEEP ERYTHROCYTE DEAE-SEPHADEX CHROMATOGRAPHY



**Figure 30.** Polyacrylamide gel electropherograms obtained at several stages in the isolation of carbonic anhydrase isoenzymes from the  $\text{CHCl}_3$ -EtOH extract of washed sheep erythrocytes. The direction of protein migration is indicated by the arrow. Isoenzyme 1 = Eryth. 1 (CEP); isoenzyme 2 = Eryth. 2 (CEP). The numbering of the peaks refers to Fig. 29.





activity toward  $\beta$ -naphthyl acetate in the presence and absence of acetazolamide. There appears to be a very minor carbonic anhydrase component moving slightly faster than Eryth. 2 (CEP). There is no evidence for this on chromatography, where the  $\text{CO}_2$  hydratase assay was used. It is possible that this minor component was a low activity form of the enzyme, only active in the catalysis of  $\beta$ -naphthyl acetate hydrolysis.

A sample of sheep haemolysate was subjected to isoelectric focusing with the result shown in Fig. 31. The colour photograph of the column, taken after equilibrium had been attained, shows the presence of several minor forms of haemoglobin. Before photography, a large part of the major band of haemoglobin was removed using a capillary tube. The discontinuity in the pH gradient thus produced is seen in the figure. The isoelectric point of the peak of carbonic anhydrase activity was about 6.6; Eryth. 2 (CEP) can perhaps be seen as a small shoulder on the high-pH side of the peak of activity. However, this result does not exclude the possibility that the activity peak might be a homogeneous single component and that one of the erythrocyte isoenzymes found on chromatography was produced by the  $\text{CHCl}_3$ -EtOH extraction procedure.

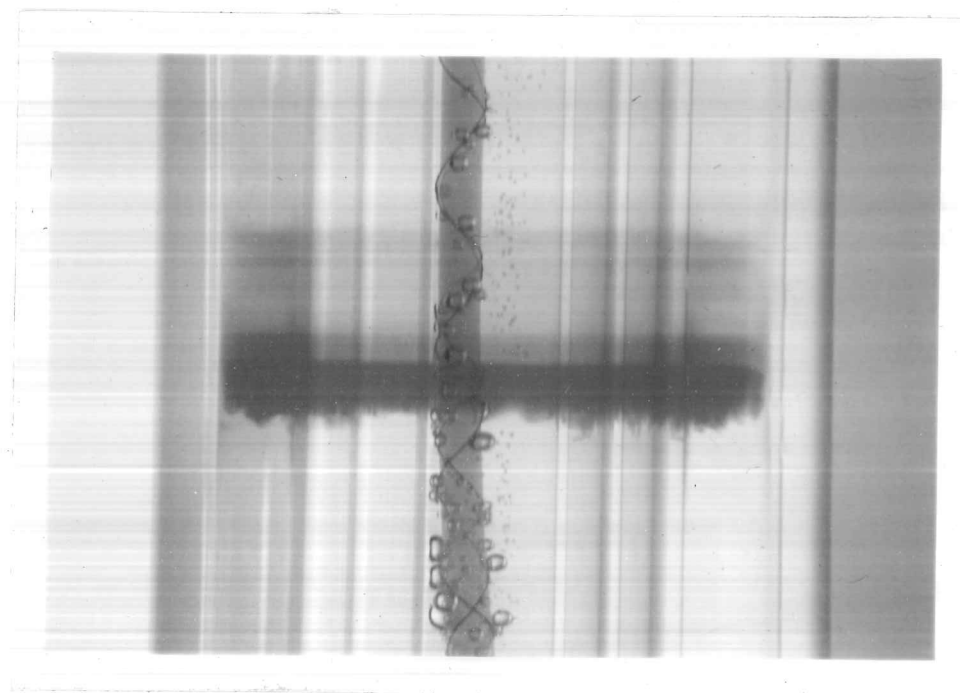
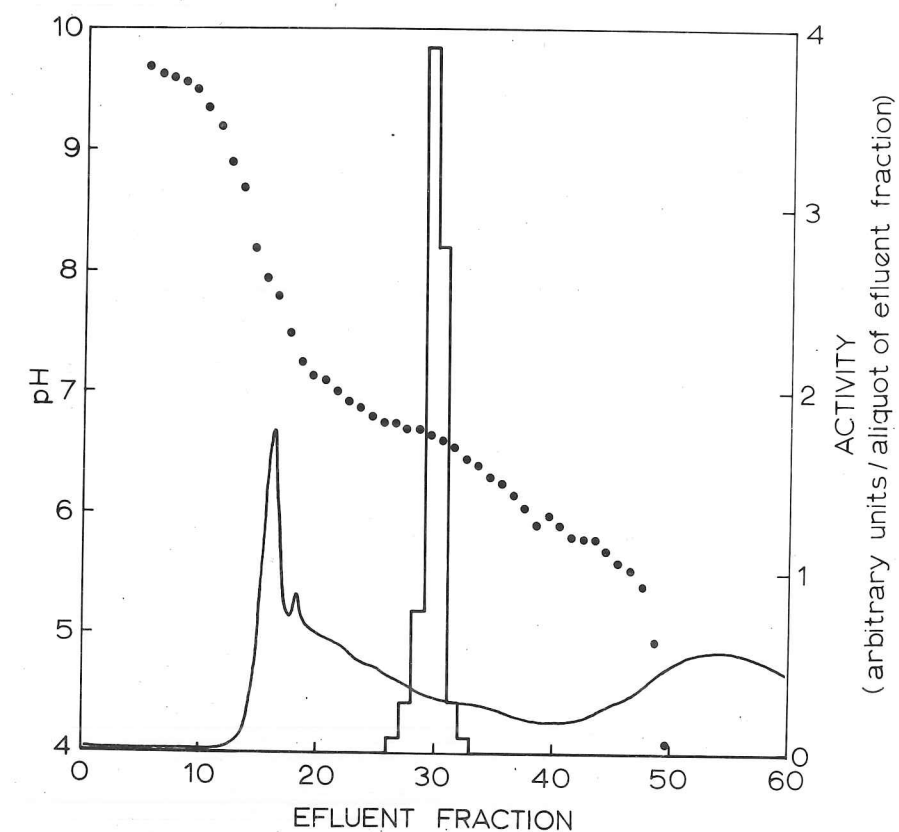
There was no opportunity to perform experiments similar to those described in Chapter 2 in which carbonic anhydrase was extracted without the use of  $\text{CHCl}_3$ -EtOH.

Endometrial isoenzyme. Endometrial tissue was dissected from the interior surfaces of uteri of non-pregnant sheep that were all in the luteal phase of the reproductive cycle. The sheep uterus is dotted with dark coloured protuberances known as cotyledons, and the endometrium covers the whole surface; ten uteri yielded a total of 97g. wet weight of this tissue.



Figure 31. Results of the isoelectric focusing experiment on a sheep haemolysate, not treated with  $\text{CHCl}_3\text{-EtOH}$ . The fraction volume was 2ml. ●, pH; — continuous,  $E_{254}$  (no units given; the measurement has little quantitative meaning because the carrier ampholytes absorb strongly at this wavelength); — (stepped),  $\text{CO}_2$  hydratase activity (modified Philpot assay).

Figure 31. SHEEP HAEMOLYSATE ISOELECTRIC FOCUSING



Contamination by haemoglobin was negligible.

The endometrial tissue was homogenized in a Waring blender with 2vol.-Buffer II and then centrifuged at 10,000g. for 30 min. An assay performed on the whole homogenate showed that this sample contained 1,500 units carbonic anhydrase activity /g. wet weight. (Other experiments showed that there was no significant difference between the specific activities of sheep endometrial tissue obtained from non-pregnant animals in the luteal and oestrus phases).

The 220ml. of slightly pink opalescent supernatant solution was dialysed against three changes of water, after which a pilot ammonium sulphate fractionation was carried out. It was found that almost all the carbonic anhydrase activity was precipitated between about 35% and 60% ammonium sulphate saturation (pH 7.7, 4°). Consequently, 45g. of ammonium sulphate (containing 1 part in 600 Tris to neutralize acidity) was dissolved in the dialysed extract, giving a volume of 250ml. This was equivalent to adding 242g. salt / litre extract. The pH of the extract before the addition of the ammonium sulphate was 7.7 and after, 7.8 (4°). The suspension was centrifuged after stirring for 15 min. at 4°. To the resulting pale pink supernatant solution was added a further 67g. of ammonium sulphate; this was equivalent to adding a total of 510g. salt / litre of the original extract. The precipitate from this step contained 90% of the enzyme activity and all the pink coloured material. It was dissolved in, and dialysed against, water to give a perfectly clear solution.

The dialysed material was freeze-dried and the residue dialysed against Buffer I. DEAE-Sephadex ion-exchange chromatography was carried out in the usual manner yielding a single, skewed, peak. The skewing is believed to have been caused by overloading. The active fractions were pooled, and

after freeze-drying, subjected to isoelectric focusing. The results of chromatography and isoelectric focusing are shown in Fig. 32. The carbonic anhydrase - containing fractions from the isoelectric focusing experiment were lyophilized, dissolved in the minimum volume of Buffer III and subjected to gel filtration, using the same column of Sephadex G-100 that was employed in determining molecular weights; the enzyme was thus separated from the low molecular weight ampholytes introduced during isoelectric focusing. The purification is summarized in Table 7, and results of electrophoresis performed at successive stages are shown in Fig. 33.

A single isoenzyme only of carbonic anhydrase was observed. The two strong bands of  $\beta$ -naphthyl acetate hydrolase activity, detected on electrophoresis, were undiminished in the control gels containing acetazolamide.

The good recovery of activity (Table 7) indicates that no major soluble carbonic anhydrase isoenzyme of sheep endometrium, with a high specific activity toward  $\text{CO}_2$  hydration, escaped detection.

#### Properties of the isoenzymes.

The properties of the isoenzymes isolated from the erythrocytes (Eryth.) and the endometrium (Endo.) are listed in Table 8.

Molecular weights. The isoenzymes from both the erythrocytes and the endometrium were found to have molecular weights in the range 28,000 - 31,000 (Fig. 34).

The  $\text{CO}_2$  hydratase activities of the isoenzymes. Experiments were carried out using methods identical to those already described in Chapter 2; an investigation was made of the effect on the initial rate of varying the initial substrate concentration in the presence and absence of acetazolamide. The concentrations of the erythrocyte isoenzymes in stock solutions (5-10 $\mu\text{M}$ )

Figure 32. A flow sheet summarizing the purification of sheep endometrial carbonic anhydrase by salt fractionation, column chromatography on DEAE-Sephadex and isoelectric focusing. — (Continuous),  $E_{280}$ ; — (stepped),  $\text{CO}_2$  hydratase activity (modified Philpot assay); ●, pH of each fraction. Details of the extraction and of the ammonium sulphate fractionation are given in the text. The heavy bars near the horizontal axes show which fractions were combined for further purification.

Figure 32. SHEEP ENDOMETRIUM DEAE-SEPHADEX CHROMATOGRAPHY AND ISOELECTRIC FOCUSING

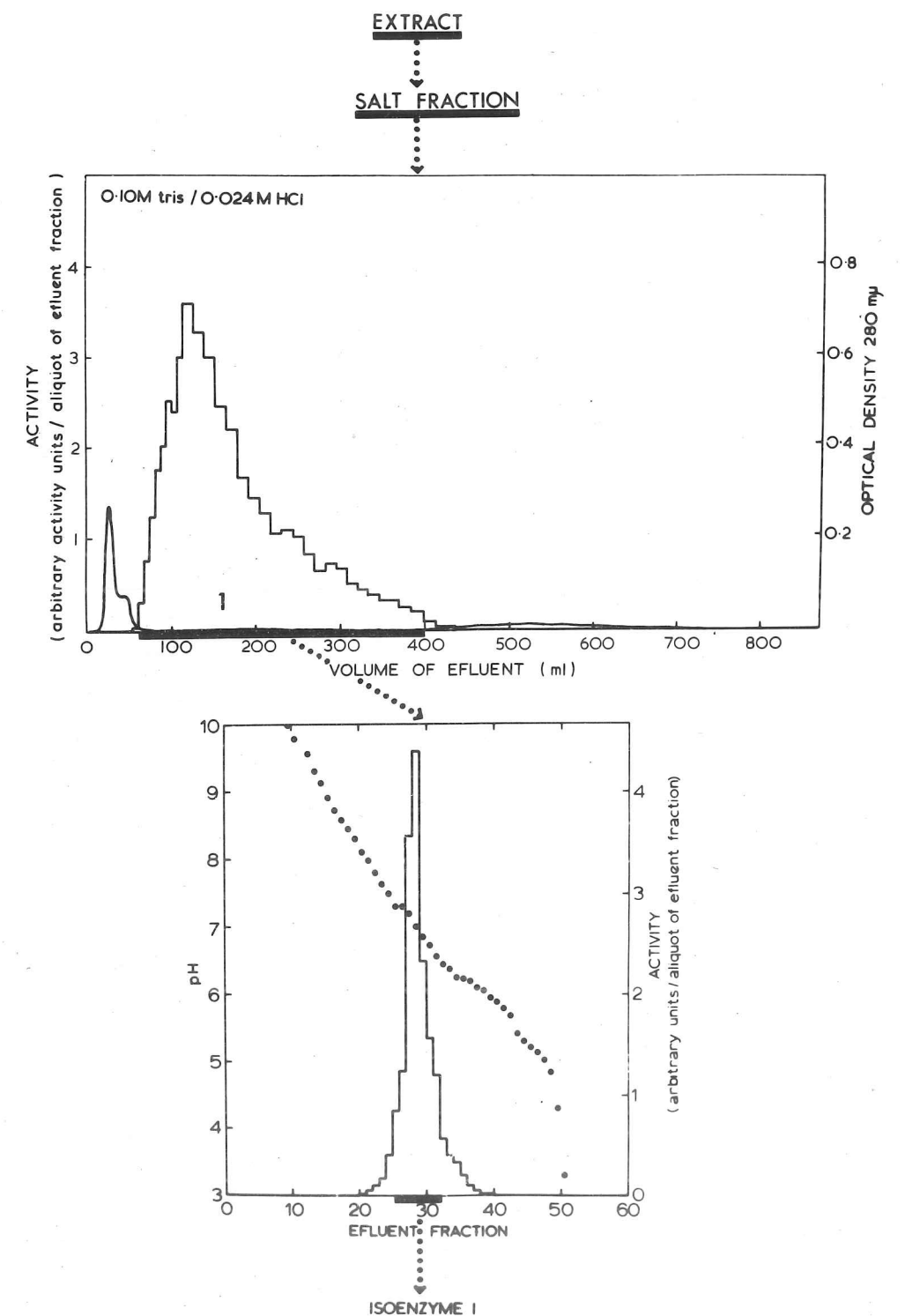


Table 7. Summary of the purification of carbonic anhydrase from 97g. of sheep endometrium.

	Volume	Units of activity/ ml	Total activity (units)	Yield of activity (%)
Supernatant of tissue homogenate	222	500	110,000	(100)
Active fraction from ammonium sulphate precipitation.	18.5	5,600	98,000	90
After ion-exchange chromatography.	335	280	94,000	85
After isoelectric focusing, gel filtration and lyophilization (dissolved in 2.0ml water).	2.0	44,500	89,000	81



Figure 33. Polyacrylamide gel electropherograms obtained at several stages in the isolation of carbonic anhydrase from the endometrium of the sheep. The direction of protein migration is indicated by the arrow. The preparation of the extract and the salt fraction is described in the text.

Figure 33. SHEEP ENDOMETRIUM ACRYLAMIDE -GEL ELECTROPHORESIS

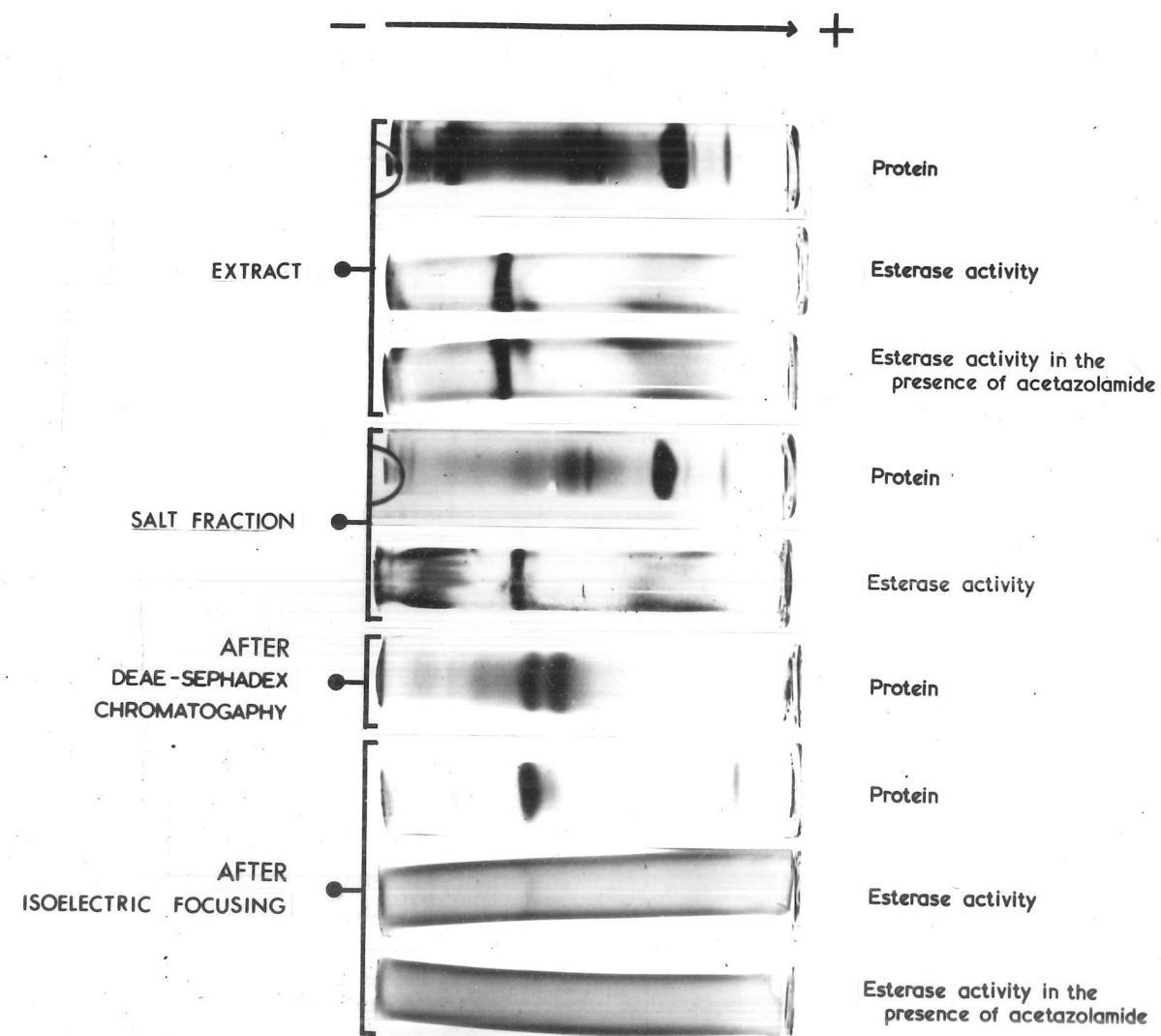


Table 8

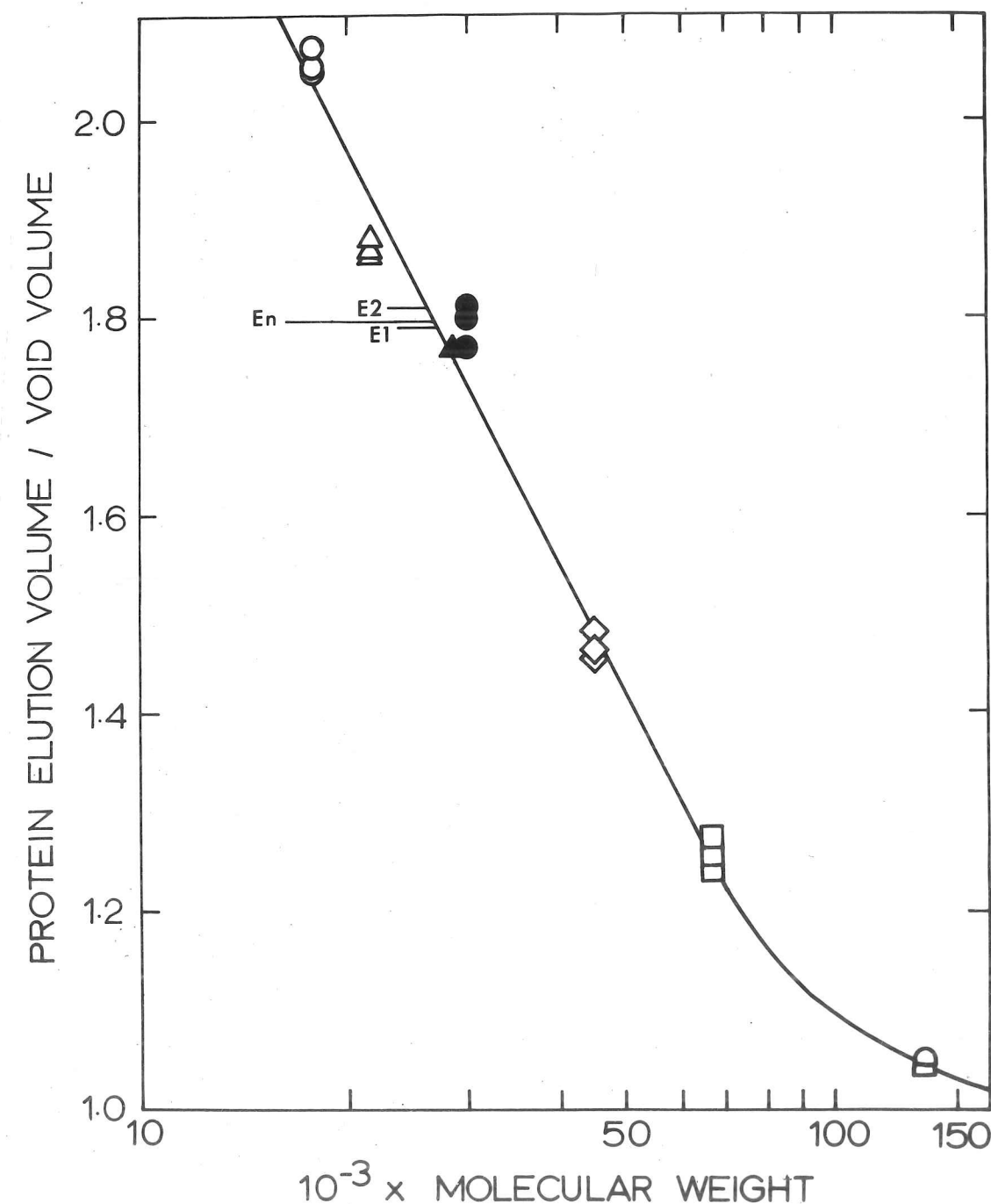
## Summary of the properties of the isoenzymes of carbonic anhydrase from the erythrocytes and endometrium of the sheep

The conditions in the kinetic experiments were: (1) 0°, pH 7.0, 45mM-NaCl, 5mM-sodium phosphate, and 0.01% peptone; (2) 25°, pH 8.0, 10mM-diethylmalonate, 1.7% (v/v)-acetone and the initial concn. of substrate was 1mM-p-nitrophenyl acetate; (3) 25°, pH 8.0, 10mM-diethylmalonate, 5% (v/v)-acetone and the initial concn. of substrate was 1mM- $\beta$ -naphthyl acetate. In (1) the kinetic constants were evaluated from the results in Fig. 35; the max. errors in the values of  $K_m$  and  $V$  are of the order of  $\pm 10\%$  and  $\pm 20\%$  respectively (Chapter 1). Acetazolamide is a type B inhibitor and so the estimates of  $K_i$  are approx. only.

	Erythrocyte isoenzymes		Endometrial isoenzyme
	Eryth. 1 (CEP)	Eryth. 2 (CEP)	Endo. 1
General properties			
Molecular weight ( $\pm 2,000$ )	29,000	29,000	29,000
$E_{280}^{1\%, 1 \text{ cm.}}$ (approx.)	17	15	-
g. atom Zn/mole (nearest whole number)	1	1	-
Isoelectric point (approx., from isoelectric focusing)	6.6	-	7.0
1. Kinetic properties as $\text{CO}_2$ hydratases			
$K_m$ (mM)	9.0	14	6.3
$10^{-4} \times V/[E_0]$ (sec. $^{-1}$ )	5.8	4.8	2.6
Type of inhibition caused by acetazolamide	mixed	mixed	non-comp.
$K_i$ (acetazolamide) (mM)	approx. 8	approx. 20	9
$[E_0]/K_i$ (acetazolamide)(approx.)	0.7	0.3	0.7
2. Kinetic properties as p-nitrophenyl acetate hydrolases			
$v/[E_0]$ (min. $^{-1}$ )	40	27	-
3. Kinetic properties as $\beta$ -naphthyl acetate hydrolases			
$v/[E_0]$ (min. $^{-1}$ )	0.58	0.37	-

**Figure 34.** Molecular weights of the sheep erythrocyte and endometrial isoenzymes determined by gel-filtration on Sephadex G-100. The eluting buffer was 0.025M-tris-0.006M-HCl, pH 9.3 at 4°, containing 0.05M-NaCl. ○, myoglobin (mol.wt.17,800); △, soya bean trypsin inhibitor (21,500); ●, human erythrocyte carbonic anhydrase isoenzyme C (30,000); ▲, human erythrocyte carbonic anhydrase isoenzyme B (29,000); ◇, ovalbumin (45,000); □, bovine plasma albumin (67,000); ◻, bovine plasma albumin, dimer (134,000). E1 = Eryth.1 (CEP); E2 = Eryth. 2 (CEP); En = Endo. 1. The behaviour of the standards (except for the carbonic anhydrase isoenzymes) on filtration was precisely as described by Andrews (1964). It will be noted that the carbonic anhydrases are eluted rather more slowly than expected for a molecule of that mass. Slight variations of this kind were noted by Andrews (1964, 1965) and were explained by him as possibly arising from differences in shape. The ratios of the elution volumes of the sheep isoenzymes to Blue Dextran (very high molecular weight) were indistinguishable from the human erythrocytes isoenzymes.

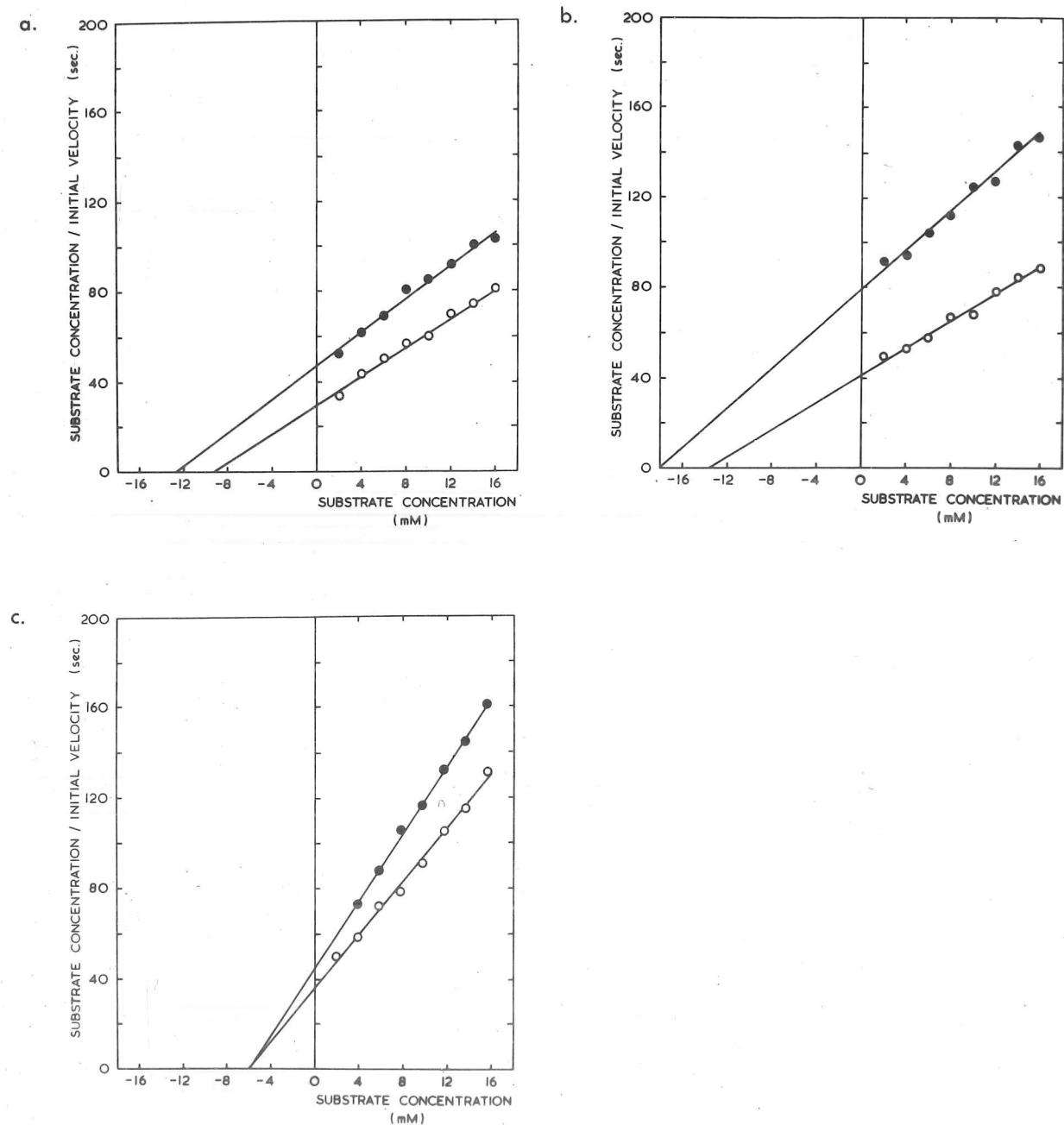
**Figure 34.** MOLECULAR WEIGHT BY GEL FILTRATION





**Figure 35.** Relationship between  $[CO_2]$  and initial velocity of  $CO_2$  hydration catalysed by the sheep erythrocyte and endometrial isoenzymes, illustrated by plotting  $[initial\ substrate]/(initial\ rate)$  against  $[initial\ substrate]$ . The conditions were  $0^\circ$ , pH 7.0, 45 mM-NaCl and 5 mM-sodium phosphate and 0.01% peptone.  $\circ$ , uninhibited;  $\bullet$ , in the presence of acetazolamide, at the concn. given in brackets. (a) 5.4 $\mu$ M-Eryth. 1 (CEP) (3.2 $\mu$ M); (b) 6.7 $\mu$ M-Eryth. 2 (CEP) (6.4 $\mu$ M); (c) 6.6 $\mu$ M Endo. 1 (2.5 $\mu$ M). Constants derived from these results are presented in Table 8. Each point is the result of a single assay. The lines were fitted by least squares regression analyses.

**Figure 35. SHEEP ERYTHROCYTE AND ENDOMETRIAL ISOENZYMES.  $CO_2$  HYDRATION**



of distilled water were estimated using the measured values of  $E_{280}^{1\text{cm.}, 1\%}$  and the measured molecular weights. The concentration of the endometrial isoenzyme was calculated assuming an average value for  $E_{280}^{1\text{cm.}, 1\%} = 17$ . The results of these kinetic experiments are shown in Fig. 35.

It was possible to determine Michaelis constants and maximum velocities in each case. The three isoenzymes behaved as typical high activity forms of carbonic anhydrase. Both erythrocyte components were inhibited by acetazolamide in a mixed manner while the endometrial isoenzyme was inhibited non-competitively in identical conditions. Values of  $K_m$ ,  $V/[E_o]$  and approximate estimates of  $K_i$  are listed in Table 8; the values of  $[E_o]/K_i$  show that the inhibitions of all the isoenzymes lay in zone B (Webb, 1963, p.70).

The p-nitrophenyl acetate and  $\beta$ -naphthyl acetate hydrolase (esterase) activities of the erythrocyte isoenzymes. Measurements of the velocities of both reactions were made under the conditions already described in detail for the case of the rat enzymes (Chapter 2). Both erythrocyte isoenzymes behaved as typical high activity forms of carbonic anhydrase at the single substrate concentration of 1mM. The results are shown in Table 8. Though there was no opportunity to perform experiments with the endometrial isoenzyme, results of  $\text{CO}_2$  hydration suggest that behaviour typical of a high activity component would have been observed.



### DISCUSSION

Sheep erythrocyte carbonic anhydrase is similar to the bovine enzyme in that both consist mainly of a single, high activity, component together with small amounts of a minor high activity isoenzyme. The sheep major and minor components were distinctly different from one another on the basis of their catalysis of  $\text{CO}_2$  hydration. Though the carbonic anhydrase was prepared from a  $\text{CHCl}_3$ -EtOH extract only, the results of the isoelectric focusing experiment done with an untreated haemolysate confirmed the presence of but a single major isoenzyme. There was no evidence for a low activity form of the enzyme, such as that found in rat blood, characterized by its high efficiency as a catalyst of  $\beta$ -naphthyl acetate hydrolysis. It would be most desirable, in the light of the findings with the rat erythrocyte isoenzymes, to compare kinetically the forms of carbonic anhydrase prepared from sheep erythrocytes with and without the use of organic solvents.

The single isoenzyme isolated from sheep endometrium was also of the high activity type, but it was distinguished from the erythrocyte components by its purely non-competitive, rather than mixed, inhibition by acetazolamide. There was no trace of an isoenzyme with high efficiency in the  $\beta$ -naphthyl acetate reaction, though it can be seen from the results of electrophoresis that Endo. 1 did catalyse the hydrolysis of this ester with moderate efficiency. Other comment is postponed until the GENERAL DISCUSSION.

### SUMMARY

1. Carbonic anhydrase extracted from sheep erythrocytes with  $\text{CHCl}_3$ -EtOH was resolved into two forms by ion-exchange chromatography and polyacrylamide gel electrophoresis. The isoenzyme which was eluted more rapidly from the column was present in great excess of the other.
2. Both forms, Eryth. 1 (CEP) and Eryth. 2 (CEP) were typical in most respects of the previously investigated high specific activity isoenzymes of carbonic anhydrase, and possessed relatively low activities as  $\beta$ -naphthyl acetate hydrolases. Their  $\text{CO}_2$  hydratase activities were inhibited in a mixed manner by acetazolamide.
3. Sheep endometrial carbonic anhydrase was isolated in very high yield in the form of one isoenzyme. None of the difficulties met with in the purification of the rabbit endometrial enzyme was encountered in this case. The isolation procedure involved an ammonium sulphate precipitation followed by ion-exchange chromatography and isoelectric focusing. The properties that were examined of the purified isoenzyme were typical of high activity forms of carbonic anhydrase; its  $\text{CO}_2$  hydratase activity was inhibited non-competitively by acetazolamide. There was no evidence for a component in the endometrium having a high efficiency as a  $\beta$ -naphthyl acetate hydrolase.
4. The molecular weights of the sheep isoenzymes were indistinguishable from those of the human erythrocytes, being approximately 30,000.

## CHAPTER 4

### CARBONIC ANHYDRASE ISOENZYMES IN THE ERYTHROCYTES AND UTERUS OF THE RABBIT

#### INTRODUCTION

The presence of carbonic anhydrase was detected in the endometrium of pregnant rabbits 6-8 days after mating by Lutwak-Mann (1954) who also demonstrated that, unlike the case of the sheep, the non-pregnant rabbit uterus was almost devoid of activity. These experiments were extended in later work (Lutwak-Mann, 1955) and the earlier observation, that the appearance of carbonic anhydrase activity in the endometrium could be stimulated by injection of gonadotrophins, was confirmed. Furthermore, progesterone was shown to be the principal ovarian hormone responsible for exerting this control (Lutwak-Mann & Adams, 1957).

An investigation of endometrial carbonic anhydrase was undertaken in conjunction with a parallel examination of the enzyme occurring in rabbit erythrocytes. The aim of this was to characterize the endometrial enzyme and to make comparison with the carbonic anhydrase of the rabbit red blood cell.

## EXPERIMENTAL AND RESULTS

### Preparation of the isoenzymes.

Erythrocyte isoenzymes. Blood was collected from female cross-bred rabbits and the erythrocytes were isolated, washed and haemolysed, and then treated with  $\text{CHCl}_3$ -EtOH. The details of these procedures have been described in Chapter 2. The lyophilized extract containing carbonic anhydrase was dissolved in Buffer II, dialysed exhaustively against the buffer, and applied to a column of DEAE-Sephadex equilibrated with same buffer. Initial chromatography was carried out in Buffer II rather than Buffer I because experiment showed that the rabbit extract did not contain any major slow-running isoenzyme of carbonic anhydrase.

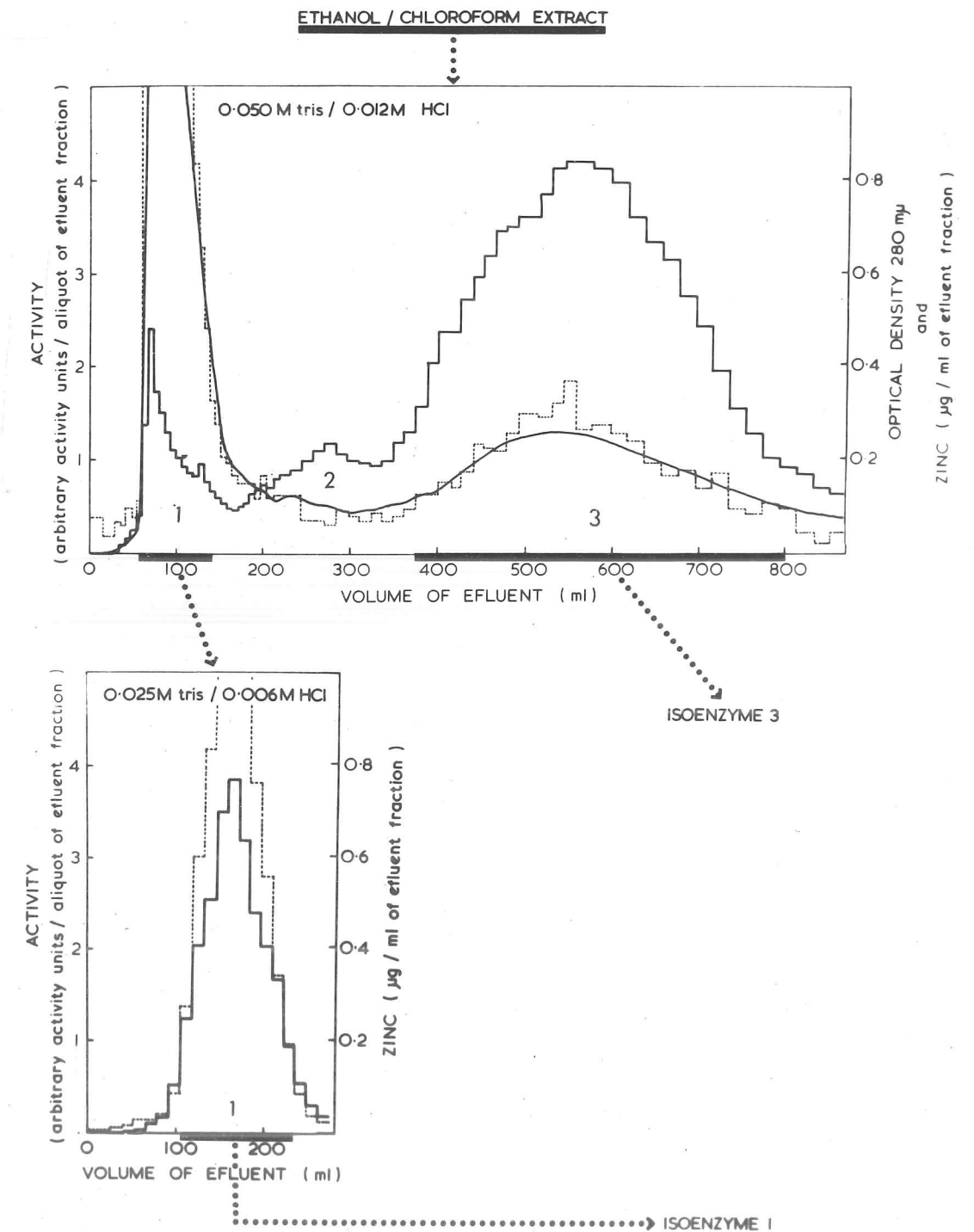
Fig. 36 depicts the result of chromatography and in addition indicates a step in which rechromatography was performed. The heavy bars show which fractions were pooled for lyophilization and rechromatography. Two major isoenzymes were found having very different specific activities as  $\text{CO}_2$  hydratases in the modified Philpot assay; this difference was later confirmed by kinetic experiments. A minor component of carbonic anhydrase was detected but not isolated. The two major isoenzymes were numbered\* in the usual

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\* The carbonic anhydrase isoenzymes will be referred to hereafter as follows: erythrocyte isoenzymes, Eryth. (number); endometrial isoenzymes, Endo. (number). (CEP) after an isoenzyme indicates purification from a  $\text{CHCl}_3$ -EtOH extract.

Figure 36. A flow sheet summarizing the purification of rabbit erythrocyte carbonic anhydrase and its resolution into isoenzymes, by column chromatography using DEAE-Sephadex. The starting material was a  $\text{CHCl}_3$ -EtOH extract of washed erythrocytes. Each box contains a description of the buffer used in that particular chromatographic step; the pH of each buffer was approximately 9.3 at  $4^\circ$ . — (Continuous),  $E_{280}$ ; — (stepped),  $\text{CO}_2$  hydratase activity (modified Philpot assay); — — —, zinc. The heavy bars near the horizontal axes show which fractions were combined for further purification. The numbering of the peaks corresponds to the numbering of the isoenzymes.

Figure 36. RABBIT ERYTHROCYTE DEAE-SEPHADEX CHROMATOGRAPHY





manner according to the order of their elution from the column; Eryth.1 (CEP) and Eryth. 3 (CEP). Fig. 37 shows the results of polyacrylamide gel electrophoresis performed at successive stages of the purification. The low activity isoenzyme Eryth. 1 (CEP) can be seen to be very active as a catalyst of the hydrolysis of  $\beta$ -naphthyl acetate. The high activity isoenzyme Eryth. 3 (CEP) was isolated in a somewhat impure condition; re-chromatography or isoelectric focusing would probably have improved its purity. It was interesting to note the presence in the  $\text{CHCl}_3$ -EtOH extract of several powerful esterases that were not inhibitable by acetazolamide.

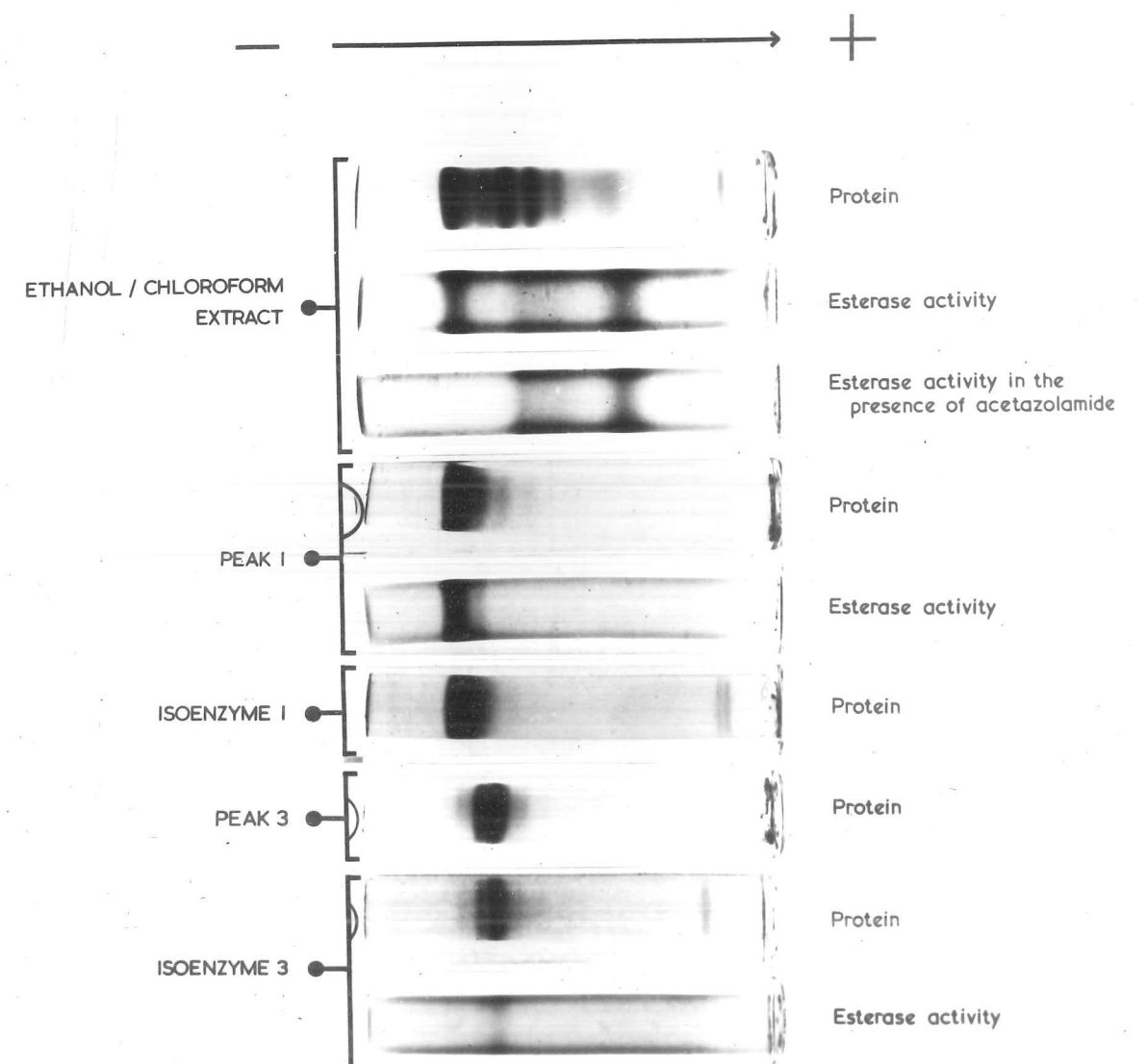
There was no opportunity to perform experiments similar to those described in Chapter 2 in which carbonic anhydrase was extracted without the use of  $\text{CHCl}_3$ -EtOH.

Endometrial isoenzymes. Attempts to chromatograph dilute, centrifuged homogenates of rabbit endometrium on DEAE-Sephadex columns of the usual dimensions were unsuccessful. (An experiment using a very short column is described below). Blocking of the column invariably occurred; this was believed to be caused by the large proportion of mucoprotein in this tissue. Several alternative procedures were examined for the extraction of carbonic anhydrase from the homogenate.

Treatment with  $\text{CHCl}_3$ -EtOH was tried initially. Endometrial tissue of 6-day pregnant cross-bred rabbits was carefully dissected from the uterine wall. A mass of 0.5-1.0g. of material could be collected from 1 animal. 20g. of tissue was ground in a mortar with sand at  $4^\circ$ . When the tissue had been broken up 5ml. (0.25vol.)-EtOH was added and then 5.9ml. (0.31vol.)- $\text{CHCl}_3$ , as the grinding was continued. Finally, after the mixture had been let stand for 15min. at  $4^\circ$ , 20ml. (1.1vol.) - 0.9% saline was stirred in and the homogenate

Figure 37. Polyacrylamide gel electropherograms obtained at several stages during the isolation of carbonic anhydrase isoenzymes from the  $\text{CHCl}_3$ -EtOH extract of washed rabbit erythrocytes. The direction of protein migration is indicated by the arrow. Isoenzyme 1 = Eryth. 1 (CEP); isoenzyme 3 = Eryth. 3 (CEP). The numbering of the peaks refers to Fig. 36.

Figure 37. RABBIT ERYTHROCYTE ACRYLAMIDE -GEL ELECTROPHORESIS



was centrifuged at 10,000g. for 30 min. The precipitate was re-extracted with the same volumes of EtOH,  $\text{CHCl}_3$ , and saline. The supernatant from this second extraction yielded a further 15% activity and had the same specific activity as the initial extract; the extracts were combined. After lyophilization the residue was dissolved in Buffer II and then dialysed against this buffer. The dialysed material was chromatographed on a column of DEAE-Sephadex equilibrated with Buffer II; the result of this is shown in Fig. 38. The material from the major peak of activity was dialysed against water and lyophilized. The residue was dissolved in Buffer II and passed through a column of Sephadex G-100. The fractions containing carbonic anhydrase activity were collected and lyophilized after dialysis against water. This material, when analysed by electrophoresis (Figure 39), was found to contain three protein components in approximately equal proportions and it was assumed therefore that the carbonic anhydrase was approximately 33% pure. The purification is summarized in Table 9. It is the properties of this preparation of the endometrial enzyme that are reported in Table 10.

Another procedure, in which an homogenate was extracted with n-butanol, following one of the methods devised by Morton (1955) for the isolation of lipo-proteins, has given promising results. A 10vol.-endometrial homogenate was prepared using Buffer II; to this was added, at  $4^{\circ}$ , 0.4vol. (with respect to the volume of the homogenate) - n-butanol and the mixture was let stand at  $4^{\circ}$ , with stirring. After  $\frac{1}{2}$ hr. the mixture was centrifuged ( $4^{\circ}$ ) for 30min. at 10,000g. The clear golden supernatant solution (recovered from beneath a pellet, and a layer of excess n-butanol) was dialysed against Buffer I, lyophilized, and

Figure 38. A flow sheet summarizing the purification of rabbit endometrial carbonic anhydrase, by column chromatography using DEAE-Sephadex and gel filtration using Sephadex G-100. The preparation of the  $\text{CHCl}_3$ -EtOH extract is described in the text. — (Continuous),  $E_{280}$ ; — (stepped),  $\text{CO}_2$  hydratase activity (modified Philpot assay). The heavy line near the horizontal axis shows which fractions were combined for chromatography.

Figure 38. RABBIT ENDOMETRIUM DEAE-SEPHADEX CHROMATOGRAPHY

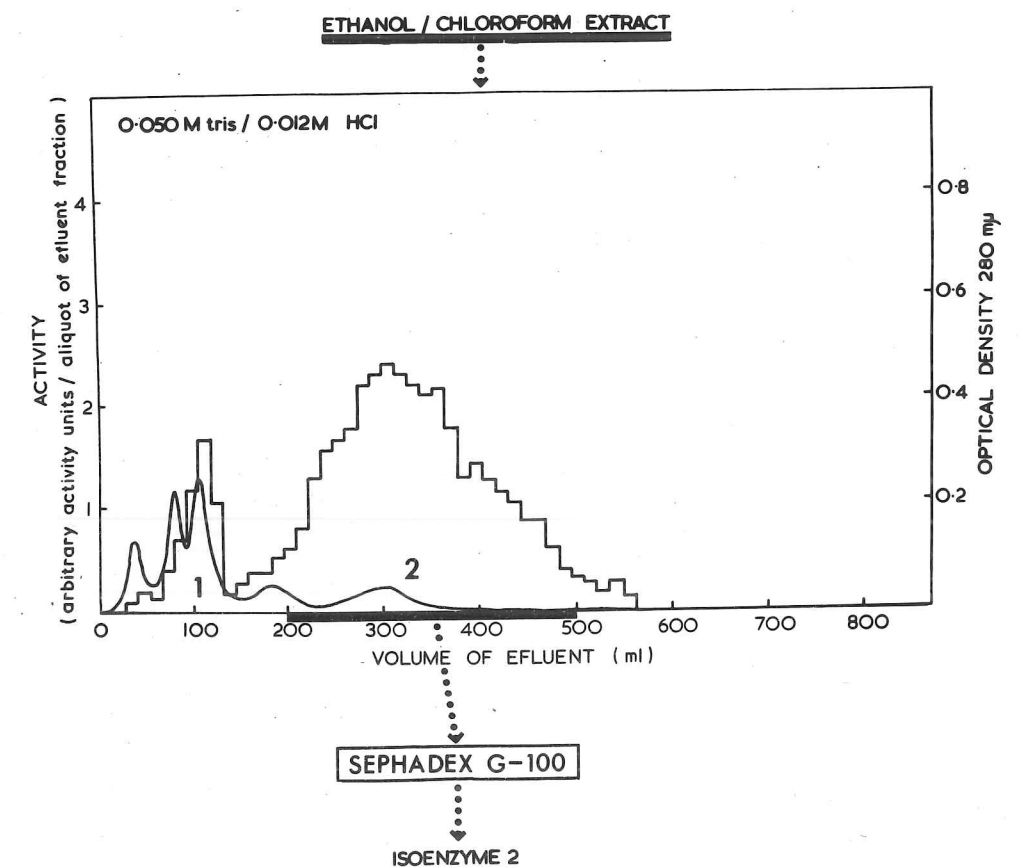
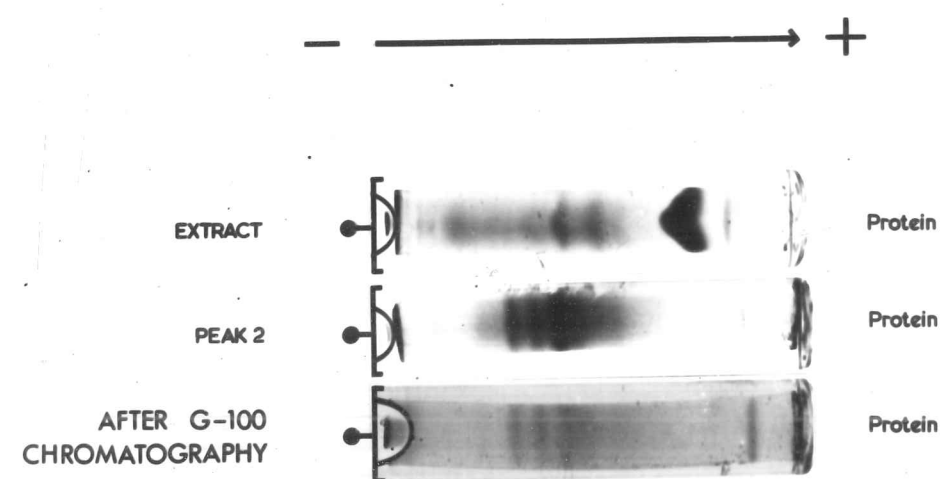




Figure 39. Polyacrylamide gel electropherograms obtained at several stages in the isolation of carbonic anhydrase from the  $\text{CHCl}_3$ -EtOH extract of rabbit endometrium. The direction of protein migration is indicated by the arrow. The numbering of the peak refers to Fig. 38.

Figure 39.

RABBIT ENDOMETRIUM ACRYLAMIDE -GEL ELECTROPHORESIS





**Table 9.** Results of three procedures for the extraction of carbonic anhydrase from rabbit endometrium, and a summary of the partial purification of an endometrial isoenzyme. The conditions in the assay (titration at constant pH; Chapter 1) were: 0°, pH 7.0, 45mM-NaCl, 5mM-sodium phosphate and 0.01% peptone, and the initial substrate concentration was 1.0mM-CO<sub>2</sub>. Protein was determined by the method of Lowry et al., (1951).

Treatment	Units extracted/ g. wet weight tissue	Specific activity (units/mg. protein)	Recovery of activity (%)
1. 10 vol-homogenate, made with Buffer II	2,500	29	(100)
After chromatography (7cm.column)	-	1,100	70
2. n-Butanol extract of Buffer II-homogenate	2,300	64	92
After chromatography (7cm.column)	-	1,500	80
3. CHCl <sub>3</sub> -EtOH extract	2,100	95	84
After chromatography (35cm.column)	-	2,600	70
After gel filtration	-	3,000	64

chromatographed on a column of DEAE-Sephadex 7cm. in length. The fractions containing carbonic anhydrase activity were pooled, dialysed, and lyophilized.

A portion of the homogenate was chromatographed directly on a 7cm. column of DEAE-Sephadex; in the case of this short column chromatography was successful, though the recovery of activity was low. The active fractions were pooled and lyophilized. These treatments are summarized in Table 9. There was no opportunity to continue further the purification of rabbit endometrial carbonic anhydrase.

#### Contamination of endometrial extracts with erythrocyte carbonic anhydrase.

When an homogenate of endometrial tissue was centrifuged, the supernatant solution was pink in colour. Assuming that this colour was due mainly to haemoglobin, an estimate of its concentration in the opalescent solution was made using the microspectroscope. The results of many such determinations showed that red blood cell carbonic anhydrase accounted for between 5% and 15% of the total activity this enzyme in the endometrial extract. Therefore it is unlikely that the carbonic anhydrase isolated from the endometrial homogenate was contaminated significantly by the erythrocyte isoenzymes. Nevertheless, in view of the close similarity in the properties of Eryth. 3 (CEP) and Endo. 2 (Table 10) the possibility of such a contamination cannot be excluded.

#### Properties of the isoenzymes.

The properties of the isoenzymes isolated from the erythrocytes (Eryth.) and the endometrium (Endo.) are listed in Table 10.

Molecular weights. The isoenzymes from both the erythrocytes and the endometrium were found to have molecular weights in the range 27,000 - 31,000 (Fig. 40).

Table 10

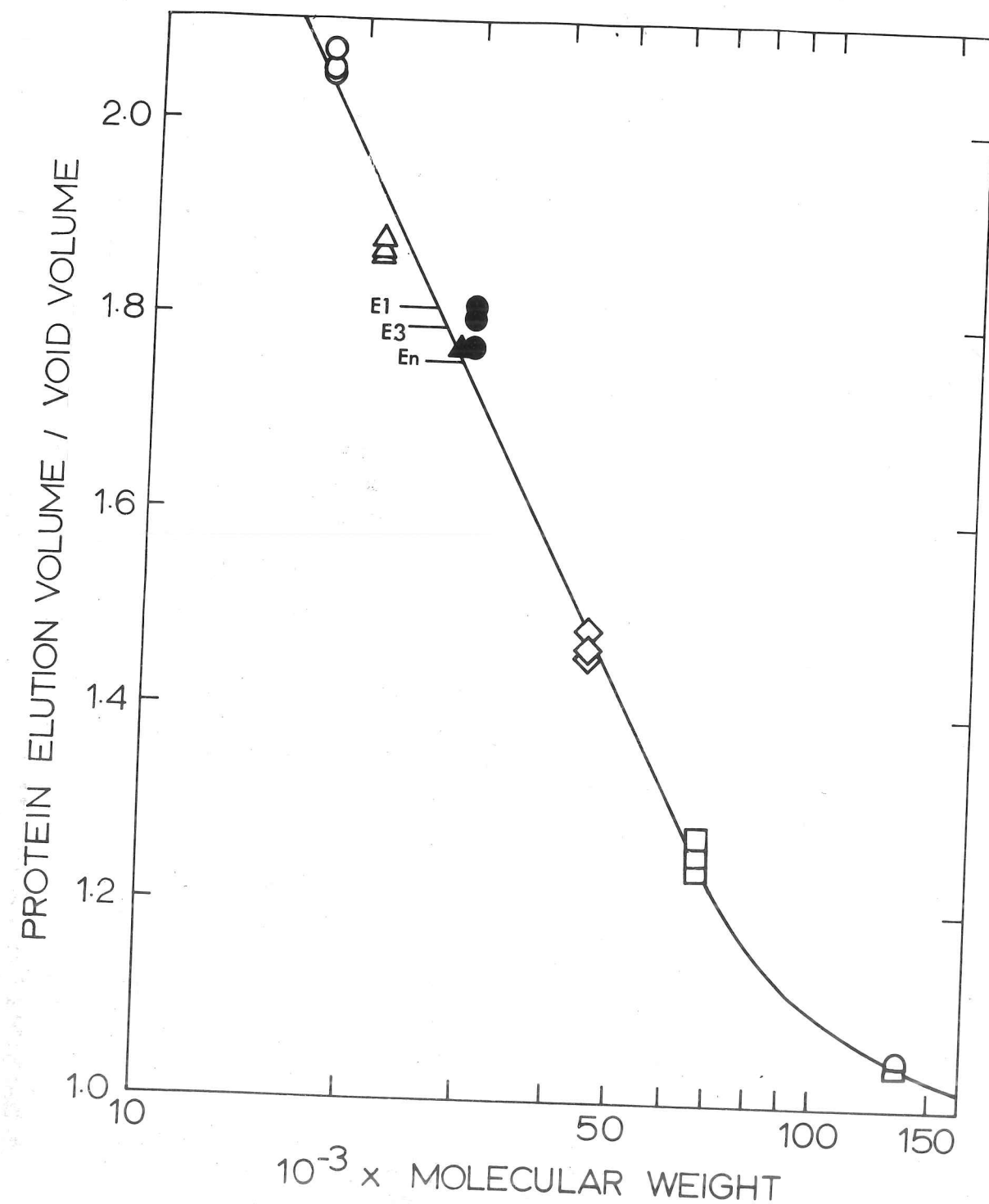
## Summary of the properties of the isoenzymes of carbonic anhydrase from the erythrocytes and endometrium of the rabbit

The endometrial isoenzyme was the semi-purified preparation isolated from a  $\text{CHCl}_3$ -EtOH extract (see text). The conditions in the kinetic experiments were: (1)  $0^\circ$ , pH 7.0, 45mM-NaCl, 5mM-sodium phosphate, and 0.01% peptone; (2)  $25^\circ$ , pH 8.0, 10mM-diethylmalonate, 1.7% (v/v)-acetone and the initial concn. of substrate was 1mM-p-nitrophenyl acetate; (3)  $25^\circ$ , pH 8.0, 10mM-diethylmalonate, 5% (v/v)-acetone and the initial concn. of substrate was 1mM- $\beta$ -naphthyl acetate. In (1) the kinetic constants were evaluated from the results in Fig. 41; the max. errors in the values of  $K_m$  and  $V$  are of the order of  $\pm 10\%$  and  $\pm 20\%$  respectively (Chapter 1). Acetazolamide is a type B inhibitor and so the estimates of  $K_i$  are approx. only.

	Erythrocyte isoenzymes		Endometrial isoenzyme
	Eryth. 1 (CEP)	Eryth. 3 (CEP)	Endo. 2 (CEP)
General properties			
Molecular weight ( $\pm 3,000$ )	29,000	29,000	29,000
$E_{280}^{1\%, 1 \text{ cm.}}$ (approx.)	17	16	-
g. atom Zn/mole (nearest whole number)	1	1	-
1. Kinetic properties as $\text{CO}_2$ hydratases			
$K_m$ (mM)	at least 40	6.5	(see Fig. 42)
$10^{-4} \times V/[E_0]$ (sec. $^{-1}$ )	-	4.4	(see Fig. 42)
Type of inhibition caused by acetazolamide	-	non-comp.	(see Fig. 42)
$K_i$ (acetazolamide) ( $\mu\text{M}$ )	-	8	(see Fig. 42)
$[E_0]/K_i$ (acetazolamide)(approx.)	-	0.6	-
2. Kinetic properties as p-nitrophenyl acetate hydrolases			
$v/[E_0]$ (min. $^{-1}$ )	8	48	23
3. Kinetic properties as $\beta$ -naphthyl acetate hydrolases			
$v/[E_0]$ (min. $^{-1}$ )	7.6	0.38	0.52

Figure 40. Molecular weights of the rabbit erythrocyte and endometrial isoenzymes determined by gel-filtration on Sephadex G-100. The eluting buffer was 0.025M-tris-0.006M-HCl, pH 9.3 at 4°, containing 0.05M-NaCl. ○, myoglobin (mol.wt.17,800); △, soya bean trypsin inhibitor (21,500); ●, human erythrocyte carbonic anhydrase isoenzyme C (30,000); ▲, human erythrocyte carbonic anhydrase isoenzyme B (29,000); ◇, ovalbumin (45,000); □, bovine plasma albumin (67,000); ◻, bovine plasma albumin, dimer (134,000). El = Eryth.1 (CEP); E3 = Eryth.3 (CEP); En = Endo. 2<sub>4</sub> (CEP). The behaviour of the standards (except for the carbonic anhydrase isoenzymes) on filtration was precisely as described by Andrews (1964). It will be noted that the carbonic anhydrases are eluted rather more slowly than expected for a molecule of that mass. Slight variations of this kind were noted by Andrews (1964, 1965) and were explained by him as possibly arising from differences in shape. The ratios of the elution volumes of the rabbit isoenzymes to Blue Dextran (very high molecular weight) were indistinguishable from the human erythrocytes isoenzymes.

Figure 40. MOLECULAR WEIGHT BY GEL FILTRATION





The  $\text{CO}_2$  hydratase activities of the isoenzymes. Experiments were carried out using methods identical to those already described in Chapter 2; an investigation was made of the effect on the initial rate of varying the initial substrate concentration in the presence and absence of acetazolamide. The concentrations of the erythrocyte isoenzymes in stock solutions (5-10 $\mu$ M) of distilled water were estimated using the measured values of  $E_{280}^{\text{lcm., 1\%}}$  and the measured molecular weights. The approximate concentration of the endometrial isoenzyme was calculated assuming (i) an average value for  $E_{280}^{\text{lcm., 1\%}} = 17$  and (ii) a purity of about 33% (Fig. 39).

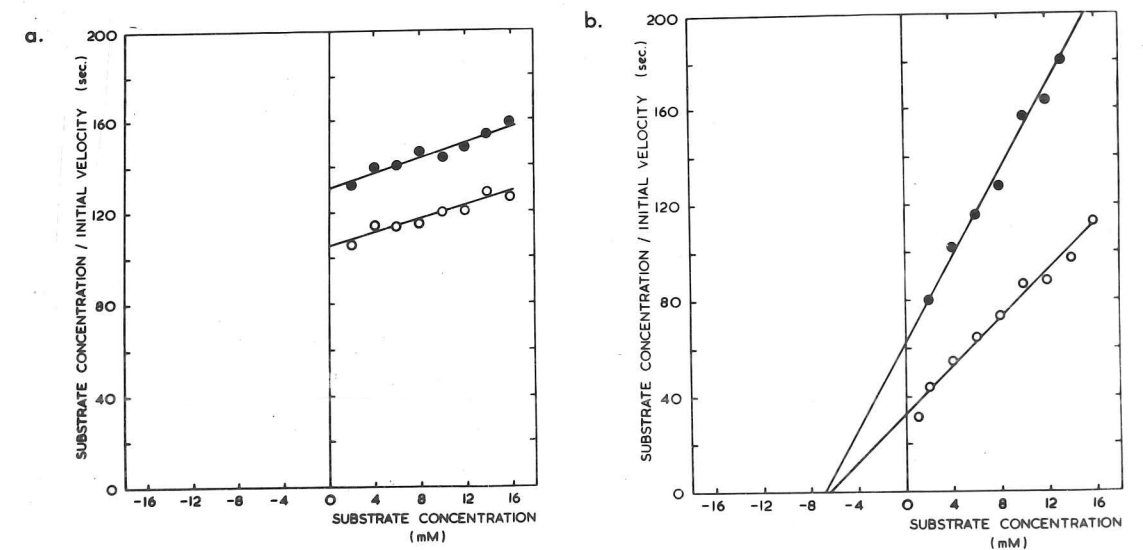
It was possible to determine the Michaelis constant and maximum velocity in the case of Eryth. 3 (CEP); the kinetic characteristics of this isoenzyme were typical of high activity forms of carbonic anhydrase. Constants could not be determined however in the case of Eryth. 1 (CEP), whose kinetic properties were very similar to those of the rat isoenzyme Eryth. 1a (CEP). The Michaelis constant was so high that it was not possible to make a reliable estimate of its value, though this was at least 40mM (Fig. 41). Kinetic constants are listed in Table 10.

The behaviour of the endometrial carbonic anhydrase was unusual. The results of experiments done with the isoenzyme isolated from a  $\text{CHCl}_3$ -EtOH extract, referred to as Endo. 2, and with the other partially purified preparations of the endometrial enzyme, namely the pooled fractions from DEAE-Sephadex chromatography described in Table 9, are shown in Fig. 42. Fig. 42a depicts the results in the form of the customary plot of  $s/v$  against  $s$ . In the presence of acetazolamide the behaviour of Endo. 2 was straightforward. In the absence of the sulphonamide however the result was complex; with low concentrations of  $\text{CO}_2$  (though not with high concentrations) the rate



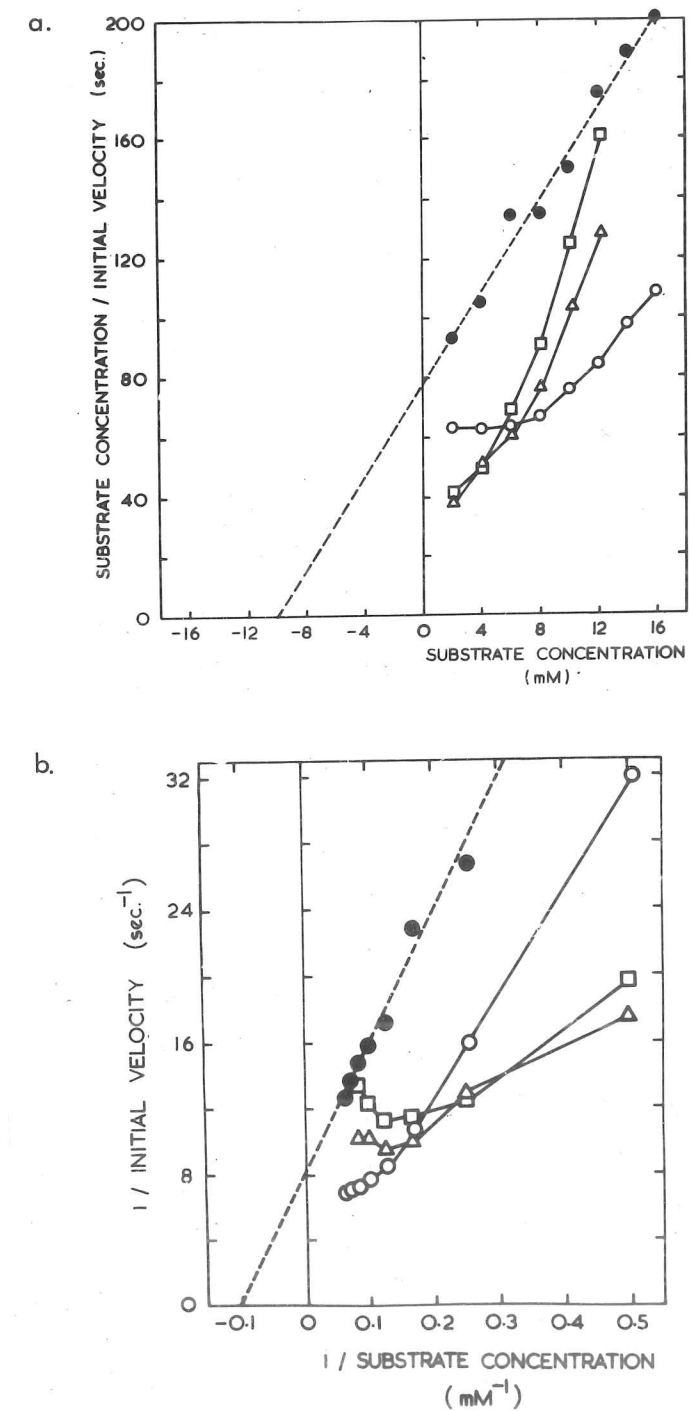
Figure 41. Relationship between  $[CO_2]$  concentration and initial velocity  $CO_2$  hydration catalysed by the rabbit erythrocyte isoenzymes, illustrated by plotting  $[initial\ substrate] / (initial\ rate)$  against  $[initial\ substrate]$ . The conditions were  $0^\circ$ , pH 7.0, 45mM-NaCl and 5mM-sodium phosphate and 0.01% peptone.  $\circ$ , uninhibited;  $\bullet$ , in the presence of acetazolamide at the concn. given in brackets. (a) 250 $\mu$ M-Eryth. 1 (CEP) (64 $\mu$ M); (b) 4.5 $\mu$ M-Eryth.3 (CEP) (6.4 $\mu$ M). Constants derived from the results are presented in Table 10. Each point is the result of a single assay. The lines were fitted by least squares regression analyses.

Figure 41. RABBIT ERYTHROCYTE ISOENZYMES.  $CO_2$  HYDRATION



**Figure 42.** Relationship between  $[CO_2]$  and initial velocity of  $CO_2$  hydration catalysed by rabbit endometrial carbonic anhydrase. The conditions were  $0^\circ$ , pH 7.0, 45mM-NaCl, 5mM-sodium phosphate and 0.01% peptone. Approximately 6mM-Endo.2 (CEP) (preparation 3, Table 9);  $\bigcirc$ , uninhibited;  $\bullet$ , inhibited by 6.4mM-acetazolamide;  $\triangle$ , Tris extract (1, Table 9);  $\square$ , n-butanol extract (2, Table 9). Each point is the result of a single assay.

**Figure 42.** RABBIT ENDOMETRIAL ISOENZYME.  $CO_2$  HYDRATION



of the reaction catalysed by the isoenzyme appeared to become proportional to the substrate concentration. A proportionality of this sort was seen in the case of the rat erythrocyte low activity form of the enzyme after treatment with  $\text{CHCl}_3$ -EtOH (Chapter 2), but the results were consistent over the range of substrate concentration. Turning attention to the preparations of the rabbit endometrial enzyme that were not treated with  $\text{CHCl}_3$ -EtOH, it can be seen that aberrant behaviour was again observed. When replotted in the form of a graph of  $1/v$  against  $1/s$ , the results were clearly suggestive of substrate inhibition (Fig. 42b).

The p-nitrophenyl acetate and  $\beta$ -naphthyl acetate hydrolase (esterase) activities of the erythrocyte and endometrial isoenzymes. Measurements were made of the velocities of both reactions; at initial substrate concentrations of 1.0mM, in the conditions already described in detail for the case of the rat isoenzymes (Chapter 2). The two rabbit erythrocyte isoenzymes were found to be very similar to the two rat erythrocyte isoenzymes; though the high activity component was slightly more effective than the low activity one in the catalysis of the hydrolysis of the phenyl derivative, the low activity form was clearly superior as a catalyst of the hydrolysis of  $\beta$ -naphthyl acetate (Table 10), confirming the observation made after electrophoresis (Fig. 37). The properties of the endometrial isoenzyme were very similar to those of the high activity erythrocyte component.

### DISCUSSION

The two major isoenzymes of carbonic anhydrase that were found to occur in  $\text{CHCl}_3$ -EtOH extracts of rabbit erythrocytes in approximately equal amounts, were markedly different in their efficiencies as catalysts; their properties were very similar to the high and low activity isoenzymes isolated from rat erythrocytes. Thus one of the rabbit isoenzymes (Eryth. 1 (CEP)), though much less effective as a catalyst of  $\text{CO}_2$  hydration than the other, was much superior in the  $\beta$ -naphthyl acetate reaction, more efficient even than the rat low activity component. Another point of similarity between the low activity isoenzymes from the two species was their rapid elution from DEAE-Sephadex, indicative of high isoelectric points.

Once again, it would be most desirable to compare kinetically the isoenzymes isolated with and without the use of organic solvents. For example, it would be interesting to observe whether the rabbit low activity component obtained here from a  $\text{CHCl}_3$ -EtOH extract would, if isolated without such treatment, exhibit changed  $\text{CO}_2$  hydration kinetics in the same way as did the analogous rat erythrocyte isoenzyme.

Several preparations of the rabbit endometrial enzyme, that had been extracted in different ways from the crude homogenate, all exhibited kinetic behaviour unusual for carbonic anhydrase. Though the endometrial enzyme was typical in many ways of high activity isoenzymes in other tissues, it appeared to be susceptible to inhibition by the substrate. While it is true that the kinetic experiments were carried out using only semi-purified preparations of the enzyme there is little reason, at present, to suppose that any impurity could have influenced the results. The only fact that throws doubt on this conclusion was the apparent restoration of

normal behaviour on the addition of acetazolamide, indicating perhaps the existence of some weakly-bound, extraneous, inhibitor. Confirmation and extension of these kinetic experiments would be of particular interest in view of the unprecedented nature of the findings.

There was no evidence for the existence of an isoenzyme of carbonic anhydrase in the rabbit endometrium comparable to the one found in the erythrocytes, with superior catalytic efficiency in the hydrolysis of  $\beta$ -naphthyl acetate.

A possible explanation for the lack of variation, in comparison with the rabbit, in the level of carbonic anhydrase activity in the uterus of the sheep, has been put forward by Lutwak-Mann (1955). She has suggested that the discrepancy might be explained by differences in the progesterone contents in the blood of these animals, and quotes the findings of Zarrow & Neher (1953) and Neher & Zarrow (1954). These authors showed that, whereas in rabbits the blood progestin concentration increases sharply during the first 4 days after mating and then slowly decreases and levels out, the blood progestin concentration in the sheep is fairly stable through the oestrous cycle and pregnancy.

Comment on certain other matters arising from the findings described in this chapter is postponed until the GENERAL DISCUSSION.



### SUMMARY

1. Carbonic anhydrase extracted from rabbit erythrocytes with  $\text{CHCl}_3$ -EtOH was resolved into two major isoenzymes by ion-exchange chromatography and polyacrylamide gel electrophoresis. The pattern of elution from the chromatographic column was similar to that observed in the case of the rat erythrocytes; the first isoenzyme to emerge from the column was a low activity form (Eryth. 1 (CEP)) while the second major isoenzyme was a typical high activity type (Eryth. 3 (CEP)).

2. The properties of Eryth. 1 (CEP) closely resembled those of the rat low activity form. In particular, it was superior to the high activity isoenzyme in its catalysis of the hydration of  $\beta$ -naphthyl acetate, and its catalysis of  $\text{CO}_2$  hydration was characterized by an unusually high Michaelis constant. The high activity rabbit isoenzyme was inhibited non-competitively by acetazolamide.

3. Partially purified rabbit endometrial carbonic anhydrase exhibited unusual kinetic behaviour in its catalysis of  $\text{CO}_2$  hydration, strongly suggestive of substrate inhibition. However it possessed the general properties of a high activity form of carbonic anhydrase. There was no evidence for more than one isoenzyme, or for a form of the enzyme with unusually high efficiency as a hydrolase of  $\beta$ -naphthyl, in the rabbit endometrium.

4. The molecular weights of the rabbit isoenzymes were indistinguishable from those of the human erythrocytes, being approximately 30,000.

### SUMMARY

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## CHAPTER 5

### ZINC AND CARBONIC ANHYDRASE IN THE UTERUS OF THE RABBIT

#### INTRODUCTION

Although there is abundant information on the presence, and some on the role, of zinc in the male reproductive tract (Mann, 1964), so far little is known about the relevance of this element to reproduction in the female. Serious interference with reproductive performance has recently been described (Hurley & Swenerton, 1966) in zinc deficient female rats. In marginally zinc deficient female rats matings still occurred, but foetal and neonate mortality greatly increased, and there was a high incidence of skeletal and soft tissue malformations. Moreover, the total zinc content of foetuses from the mildly deficient mothers was less than that of control foetuses. Skeletal defects have also been reported in chick embryos from zinc deficient hens (Kleinholz et al., 1961).

The aim of these experiments was to determine whether the zinc content of the endometrium, in comparison with the whole uterine wall tissue, shows any clearly recognizable relation to ovarian endocrine conditions, and to what extent fluctuations in the concentration of uterine zinc reflect those established for carbonic anhydrase activity (Lutwak-Mann, 1955). It seemed desirable to confirm at the same time the carbonic anhydrase activity of the endometrium in relation to hormonal conditions, by using for enzyme assays the method of titration at constant

pH, developed in the course of the present work (Chapter 1), in addition to the less precise method (Philpot & Philpot, 1936) used previously (Lutwak-Mann, 1955). Finally it was hoped to derive from these experiments, together with those reported in Chapter 4, an estimate of the proportion of uterine zinc that can be ascribed to the endometrial carbonic anhydrase. This work has been published (Lutwak-Mann & McIntosh, 1969).

### EXPERIMENTAL AND RESULTS

Endometrium was dissected from the uteri of rabbits at the following stages: (i) non-pregnant; (ii) pregnant, days 0-10; (iii) pseudopregnant (after intravenous inoculation of 25 IU of Luteohormone), days 6-16; (iv) superovulated (treatment schedules in Table 11), day 6.5; the day of mating or injection of gonadotrophin being designated day 0. Also used was whole uterine tissue (from which the endometrium had not been removed), of non-pregnant and pregnant (days 4-6) rabbits, early placental tissue (days 8 and 9), free-lying 6.5 day old blastocysts, and 7 day blastocyst fluid. All animals were multiparous does aged 1-2.5 yr, except two non-pregnant does aged 6.5 yr (Fig. 43a).

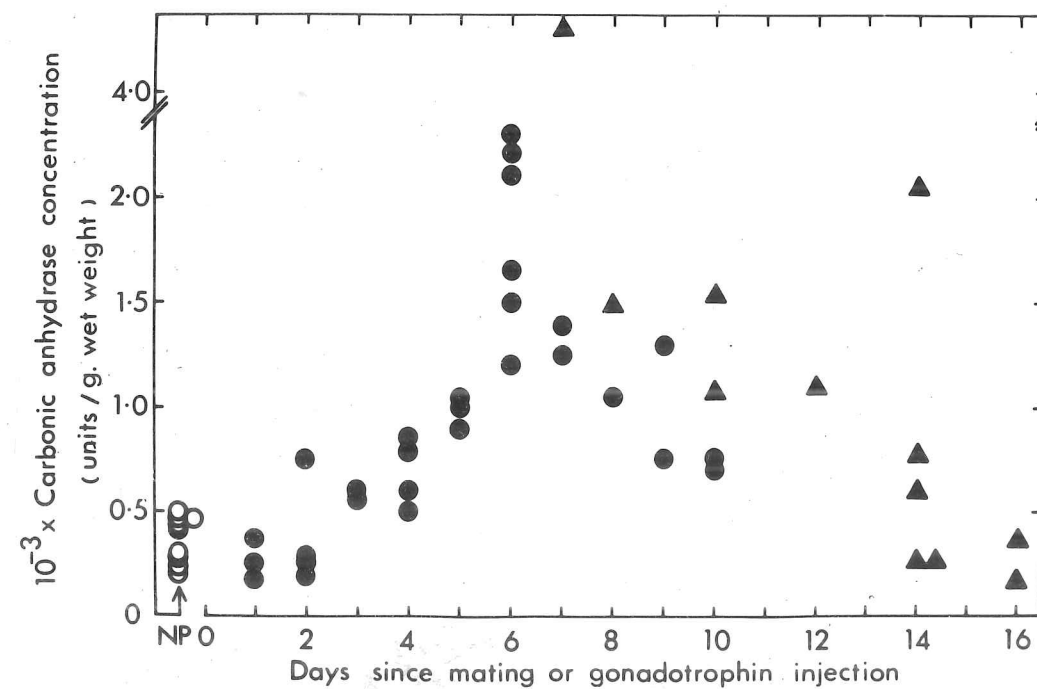
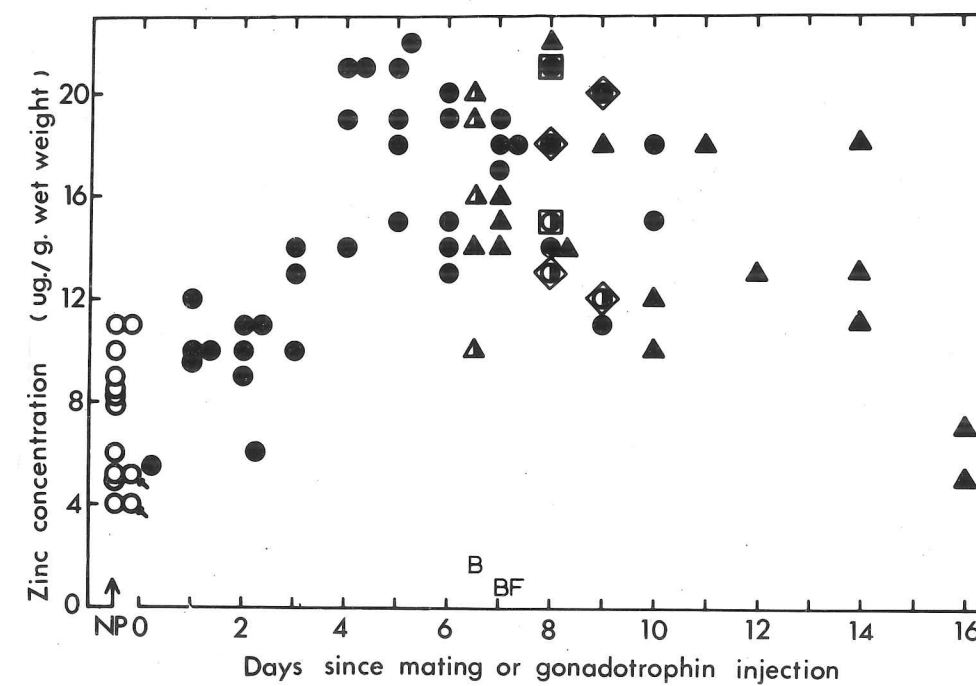
Zinc was determined in aliquots (100-200mg. wet weight) of tissue. The remaining tissue was ground with a volume of distilled water equal to four times the wet weight of the sample; the homogenates were centrifuged and the supernatants, which contained almost all the enzyme activity, were used for assay. Correction for blood contamination of the tissue was not routinely made, because it was established that this accounted for only a negligible proportion of the total carbonic anhydrase activity of the rabbit uterine tissue (see Chapter 4, EXPERIMENTAL AND RESULTS). Activity of carbonic anhydrase was determined by the original method of Philpot & Philpot (1936) as well as by titration at constant pH (Chapter 1). Between 20 and 200 $\mu$ l. of supernatant was necessary for each assay performed by the method of titration. The average reproducibility of determinations performed on any one supernatant was  $\pm 5$  per cent.



**Figure 43a.** Zinc concentration in the rabbit endometrium. Experimental animals: ○, non-pregnant (NP) ♀, senescent rabbits (6.5 yr. old); ●, pregnant; placental tissue and contiguous endometrium (days 8 and 9) are designated ◻◻, and ◻◻ respectively; ▲, pseudopregnant (25 IU Luteohormone intravenously); △, superovulated, listed 1-4 in Table 11. B, Whole blastocysts, 6.5 days old; BF, 7 day blastocyst fluid.

**Figure 43b.** Assay of carbonic anhydrase activity in rabbit endometrium by titration at constant pH (Chapter 1). The conditions were 0°, pH 7.0, 45mM-NaCl, 5mM-sodium phosphate, and 0.01% peptone. The initial [CO<sub>2</sub>] was 1.0mM. Experimental animals: ○, non-pregnant (NP); ●, pregnant; ▲, pseudopregnant.

**Figure 43.** ZINC AND CARBONIC ANHYDRASE IN RABBIT ENDOMETRIUM



Enzyme activity and the concentration of zinc were assayed, with minor exceptions, in samples of tissue recovered from the same animals thus making the results directly comparable.

Effect of pregnancy, pseudopregnancy and superovulation on the uterine concentration of zinc and carbonic anhydrase activity. Fig. 43a shows that in the rabbit endometrium the concentration of zinc was not constant but varied with the endocrine conditions prevailing during the period investigated. Moreover, at each point there was marked, presumably genetically conditioned, variability in concentration of zinc. For purposes of statistical appraisal the results were divided into four groups: I, non-pregnant, including two aged does, and groups II, III and IV, which corresponded to the following intervals from mating or gonadotrophin injection, respectively : 5 hr. after coitus - day 3; days 4-9; and days 10-16. Mean values ( $\mu\text{g. of Zn/g. wet weight}$ ) for each group are given in brackets.

Group II (10.1) representing pregnant animals in the post-ovulatory phase, was slightly but significantly ( $P = 0.04$ ) higher than the non-pregnant group I (8.2); values in the latter for the senescent rabbits with a pathologically altered cystic endometrium were within the "normal" range for reproductively active oestrous rabbits. The highest concentration of zinc (17.3) - double that of group I and highly statistically significant ( $P < 0.001$ ) - was attained by group III which comprised pregnant and pseudo-pregnant rabbits during days 4-9 (a period which is also characterized by high carbonic anhydrase activities). Placental tissue (days 8 and 9) had a distinctly lower concentration of zinc than the contiguous endometrium (again a trend in agreement with values for carbonic anhydrase activities). In group IV (12.7), consisting of two pregnant animals (day 10, the latest

stage in pregnancy suitable for dissection of the endometrium), all the rest being pseudopregnant rabbits, the values were significantly lower ( $P < 0.001$ ) than in the preceding group. The terminal values on day 16 clearly reverted to the non-pregnant range.

In comparison with endometrium, very low concentrations of zinc were found in unimplanted 6.5 day old blastocysts, and in 7 day blastocyst fluid. The slightly higher concentration of zinc in the entire blastocysts, compared with the cell-free fluid on day 7 probably arose from contamination of the embryos (which had not been rinsed) with endometrial secretion and epithelial cell debris. It may be added here that very low, but by no means negligible, values for carbonic anhydrase activity were also established in these embryonic materials.

Comparison of the concentration of zinc in the endometrium with that in the whole uterine wall. The following observations were made when zinc analyses were done using separately the dissected endometrium and the intact uterine wall of the same animals, at two different endocrine stages - the oestrous condition and the progestational phase of pregnancy (Fig. 44). The uterine wall had a markedly higher concentration of zinc than did the endometrium alone, and this was evident both in the non-pregnant and progestational uteri. Like the endometrium, the zinc values in the whole uterine tissue showed marked individual variation; this was least in the oestrous group, but was rather pronounced in the endometrium of the pregnant animals, especially when the low value for one of the superovulated animals is included (rabbit two, Table 11). The increase in the concentration of zinc between the oestrous and the progestational phase was roughly two-fold, both for the endometrium and the intact uterine tissue.

Figure 44. Comparison of zinc concentration in the endometrium alone (E) and in the intact uterine wall (U), in non-pregnant rabbits (NP) and in the progestational phase of pregnancy (PROG, days 4-6); average values and standard deviation, seven and eight animals, respectively. O, Superovulated rabbit listed 2, in Table 11.

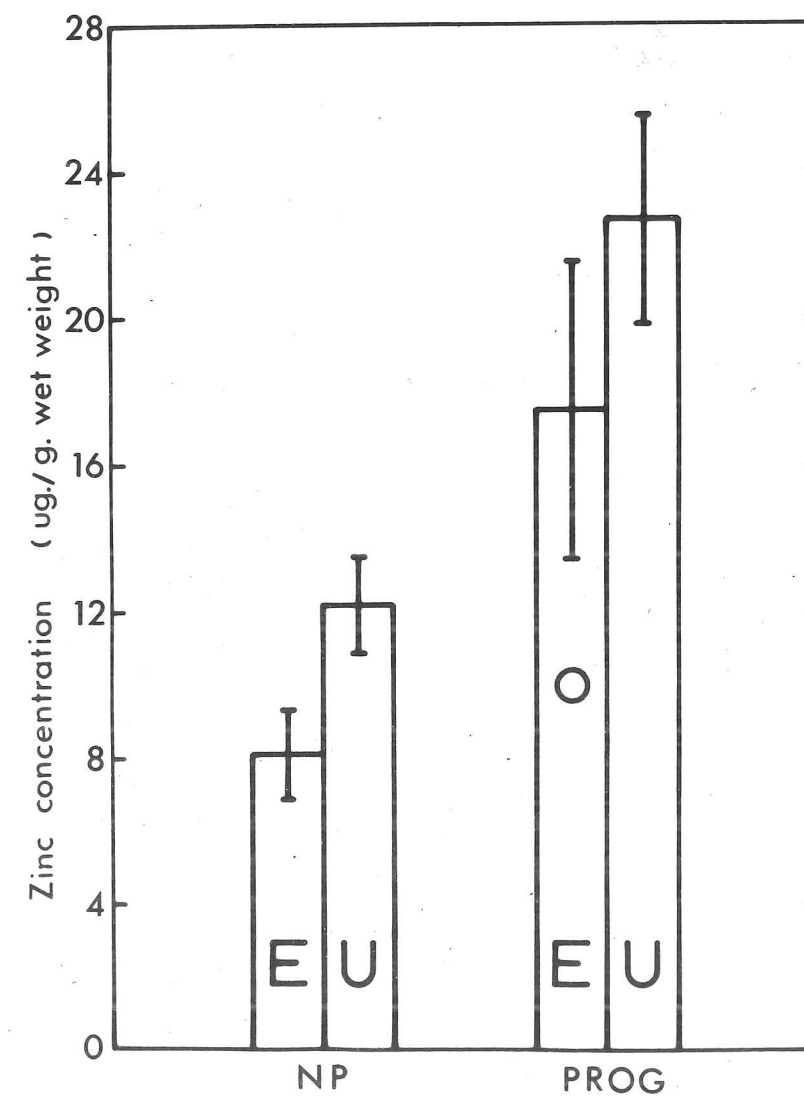


Figure 44. ZINC IN THE ENDOMETRIUM AND UTERINE WALL



Table 11

Effect of Superovulation on Endometrial Carbonic Anhydrase and Zinc Concentration and Blastocyst development 6.5 days after Coitus

Rabbit	Gonadotrophin injection (IU/female) (a) 72 h before and (b) 10 min after mating	Receptivity at mating	CA activity (titrator units/g wet weight)	Zn concentration ( $\mu\text{g/g}$ wet weight)	Blasto-cysts (No.)	Remarks	
						Corp. lutea (No.)	Endometrium
1	Gestyl (150) intramuscularly (a) and 'Luteohormone' (100) intravenously (b)	Poor	400	20	0	13*	Oedematous
2		Poor	400	10	0	15*	Oedematous
3	Gestyl (150) intramuscularly (a) and 'Pregnyl' (100) intravenously (b)	Poor	580	16	0	29*	Near normal
4		Poor	980	19	0	45*	Normal
5	Gestyl (75) intramuscularly (a) and 'Pregnyl' (25) intravenously (b)	Excellent	930	-	15	16	Normal
6		Excellent	1,400	-	18	21	Slight oedema
7		Excellent	890	-	31	32	Normal
8		Excellent	1,500	-	25	29	Normal

\* Two crops of corpora lutea were present, an indication that ovulation had occurred on at least two occasions; there were, in addition, numerous blood follicles.



Comparison of the results of the titration and Philpot assays.

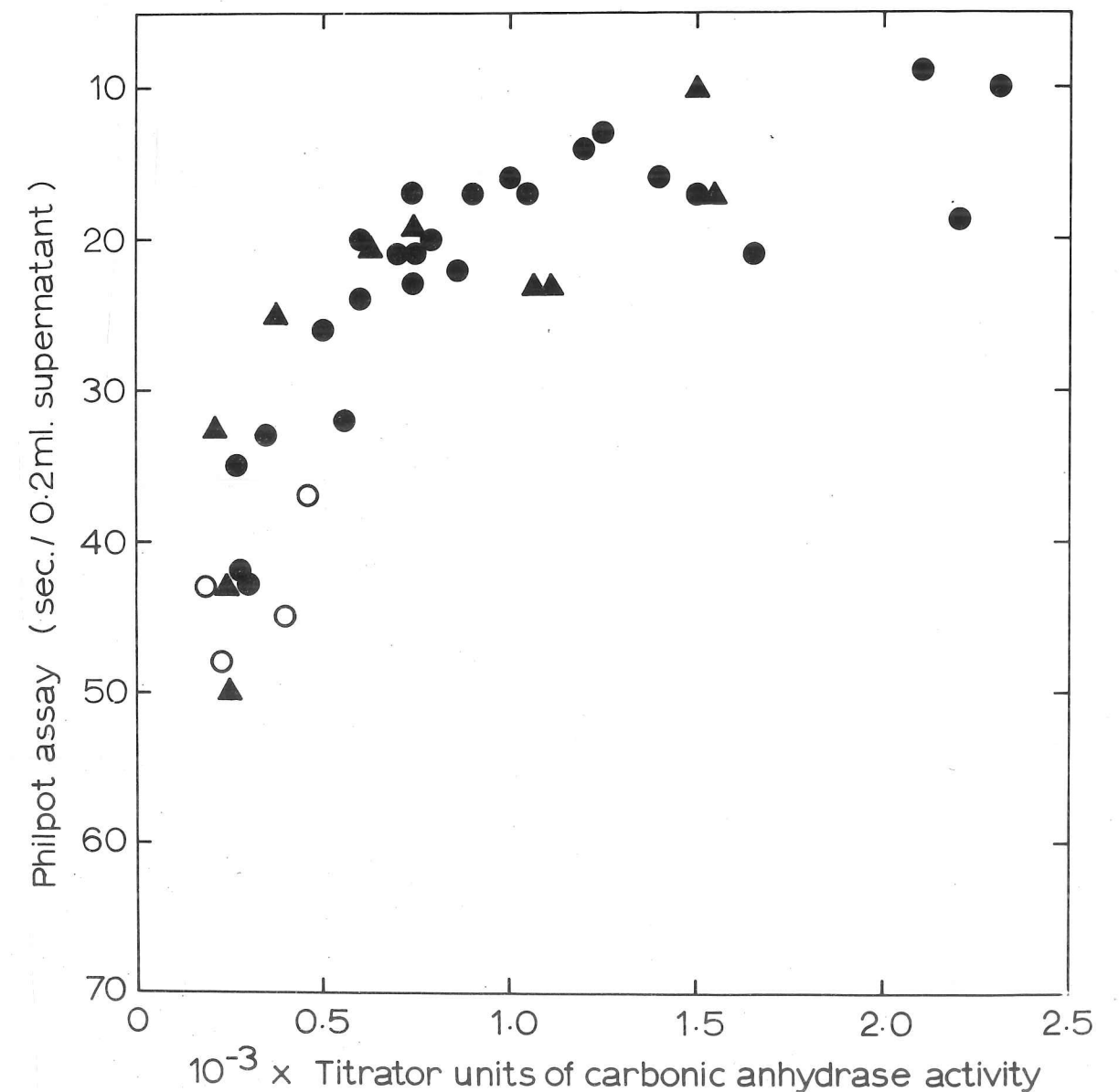
Parallel with determinations of zinc, carbonic anhydrase activity was assayed by the Philpot and titrator methods. Within the titrator range of 200-1,200 units of carbonic anhydrase activity, the correlation between the results was fairly consistent. The agreement was less close, however, with higher enzyme concentrations, corresponding to titrator units 1,200-2,400 (Fig. 45). No linear relationship can be expected between the enzyme added and the time recorded in the case of the Philpot assay, because of the way in which activity is estimated. It is possible that the results obtained by the two methods would begin to differ if another carbonic anhydrase isoenzyme, with a different  $K_m$ , appeared at stages characterized by high carbonic anhydrase activity, because the concentration of the substrate is different by a factor of 40-50 in these two methods of assay. As was shown in Chapter 4, however, there is no evidence for more than one major isoenzyme of carbonic anhydrase in endometrium of the rabbit.

Because the titrator method gives results which are proportional to the volume of endometrial supernatant added, however, and because its precision is relatively high, the discrepancies with the high enzyme concentrations probably arise from the generally acknowledged shortcomings of the Philpot technique (see Chapter 1, INTRODUCTION).

Fig. 43b shows that when the endometrial carbonic anhydrase activity was determined by the titrator method, the results confirmed those established earlier (Lutwak-Mann, 1955), in that an increase in activity from the preovulatory level was evident by about day 4, followed by a maximum at 6 - 7 days. The exceptionally high value (4,400 units) recorded for one rabbit endometrium on day 7, was fully

Figure 45. Comparison of the results of titration and Philpot assays performed on homogenates of rabbit endometrium, expressed as the time required for colour change of the indicator to occur in the Philpot assay against the carbonic anhydrase activity (units/g. wet weight tissue) found by titration at constant pH. The conditions in the titration assays were  $0^{\circ}$ , pH 7.0, 45mM-NaCl, 5mM-sodium phosphate, and 0.01% peptone; the initial  $[CO_2]$  was 1.0mM. Experimental animals:  $\circ$ , non-pregnant;  $\bullet$ , pregnant;  $\blacktriangle$ , pseudopregnant.

Figure 45. COMPARISON OF TITRATOR AND PHILPOT ASSAYS



substantiated by the Philpot test. Such unusually high values have been reported before (Lutwak-Mann & Adams, 1957), but are rare and have not been explained. It may be added that the zinc concentration of this particular endometrium was within the range typical of that endocrine stage.

Effect of large doses of gonadotrophins. Results for superovulated animals are summarized in Table 11. It can be seen that in rabbits 1-4 which received large amounts of gonadotrophins (following a recommended schedule of treatment (Brinster, 1968)), there was evidence of excessive stimulation of the ovaries, poor sexual receptivity (the animals had to be force-mated), pathologically altered uterine tissue, and a failure of embryonic development. In addition, there was a distinct decrease in the activity of endometrial carbonic anhydrase (rabbits one, two and three), and an unusually low concentration of zinc (rabbit two). In contrast, in rabbits five-eight, which were treated with smaller doses of gonadotrophins, mating proceeded very well, and there was good agreement between the number of corpora lutea and blastocysts. Zinc was not determined in this group, but values for carbonic anhydrase were all within the range established for day 6.

### DISCUSSION

Calculations based on the experiments with partially purified rabbit endometrial carbonic anhydrase described in Chapter 4 indicate that not more than 1-2% of the zinc present in the endometrium of the rabbit is associated with carbonic anhydrase. This disparity probably accounts for the fact that no quantitative relationship was observed between the changes in zinc and carbonic anhydrase concentrations in the endometrium.

A marked increase in the concentration of zinc occurred in the progestational phase of pregnancy in the rabbit, both in the endometrium and the uterine tissue as a whole. In effect this means that zinc must have been shifted from body depots to the uterus, ostensibly to meet the requirements of what is often referred to as the histiotrophic (endometrium-mediated) phase of embryonic development. How this mobilization of zinc, or for that matter, of other trace elements, is brought about in higher mammals is not well known. In the post-implantation or haemotrophic phase of gestation the placenta presumably takes over from the endometrium the supply of zinc to the foetus.

When fertilization failed to take place after ovulation, and pseudopregnancy resulted, the pattern of uterine zinc was essentially the same as in pregnancy, except that as pseudopregnancy drew to an end the concentration of zinc gradually reverted to preovulatory levels.

Determination of carbonic anhydrase activity by means of the titration procedure yielded results that confirmed satisfactorily the earlier observations of changes in the endometrial activity of this enzyme in pregnancy and pseudopregnancy.

Both pregnancy and pseudopregnancy have in common a striking but short lived preovulatory release of  $20\alpha$ -dihydroxypregn-4-en-3-one ( $20\alpha$ -OH) (Hilliard *et al.*, 1967; Hilliard *et al.*, 1968). During the next 16 days, the period studied in these experiments, control over uterine events in the pregnant and pseudopregnant animal is exercised by progesterone, the essential progestin of rabbit pregnancy, and  $20\alpha$ -OH, a gestagen of contributory role, thought to be implicated in the release of luteinizing hormone, but perhaps also responsible for the near-ovulatory formation of uterine fluid (Lutwak-Mann, 1962).

Probably one of the most significant metabolic events is the marked rise in endometrial RNA which starts on day 2 and extends up to at least day 8 Vittorelli *et al.* (1967). It is conceivable (although the proof is lacking) that this early increase in RNA is somehow associated with the preovulatory spurt of  $20\alpha$ -OH, and subsequently becomes responsible for stimulating the enzyme synthesis in the rabbit endometrium. This in turn might be responsible for the increase in carbonic anhydrase activity from day 4 onward, synchronously with the onset of the postovulatory release of the two progestins. The metabolic control mechanisms operative in respect of uterine zinc linked systems other than carbonic anhydrase have yet to be elucidated.

As to conditions arising from superovulation, an experimental procedure frequently resorted to in embryological research, much undoubtedly depends on the extent of the stimulus applied. In the strain of rabbits used in the present work the administration of massive amounts of gonadotrophins resulted in an unphysiological response, reflected in poor sexual receptivity, total embryonic failure at the blastocyst stage, as well as abnormally low endometrial carbonic anhydrase activity in three out of four and an



unusually low zinc concentration in one out of four of the treated animals. On the other hand, when the dose of gonadotrophin was adequately scaled down, reproductive performance and blastocyst development were entirely satisfactory, and carbonic anhydrase activity was in the physiological range of values.

### SUMMARY

1. The concentration of zinc in the rabbit endometrium was found to increase near the beginning of pregnancy and then to decrease again gradually as pregnancy progressed. The increase in zinc paralleled the increase in carbonic anhydrase activity in the endometrium. However the zinc increased by a factor of 2 while the activity of the enzyme increased by a factor of 10.
2. Zinc in the whole uterine wall also increased in pregnancy.
3. Overdoses of gonadotrophins followed by mating resulted in changes in the uterine tissue, absence of fertilization (though ovulation occurred) and lowered levels of carbonic anhydrase in the endometrium. Lesser doses, however, gave good fertilization and normal levels of carbonic anhydrase activity.
4. Carbonic anhydrase zinc was calculated to account for about 1-2% of the total endometrial content of this element.

### GENERAL DISCUSSION

The most striking feature of the isoenzymes of carbonic anhydrase is that while the general physical characteristics are invariant, the catalytic properties differ greatly. All isoenzymes of carbonic anhydrase that have been examined have molecular weights of about 30,000, all have one atom of zinc associated with each molecule, all are extremely efficiently inhibited by aromatic compounds having an unsubstituted sulphonamide group, and all are stable and water soluble. However a great difference is observed between the high and low activity forms of the enzyme in catalytic efficiency of  $\text{CO}_2$  hydration. Moreover, there is reversal of relative efficiency of the two forms of the enzyme in the catalysis of the hydrolysis of  $\beta$ -naphthyl acetate, and neither of the rat prostate isoenzymes catalyses the hydrolysis of this ester.

Species may be classified into two groups by the nature of the carbonic anhydrase isoenzymes found in their erythrocytes; those that have both high and low activity forms of the enzyme, and those that have only the high activity form. Furth (1968) proposed that carbonic anhydrase isoenzymes should be classified according to specific activity, rather than on the basis of chromatographic behaviour as suggested by Rickli *et al.* (1964). Furth's nomenclature is certainly more appropriate when applied to the isoenzymes examined in the present work. Accordingly, rat Eryth. 1a and rabbit Eryth. 1 are listed as type B, together with the low activity isoenzymes from the erythrocytes of humans, monkeys, and horses, while the high activity isoenzymes of rat, rabbit, and sheep are listed as type C, together with previously-reported high activity forms. It can be seen

that results to date group together human, monkey, horse, rat and rabbit (having both high and low activity erythrocyte isoenzymes) and cattle and sheep having one or more high, but no low, activity forms.

It is difficult to judge the significance of the minor carbonic anhydrase isoenzymes. Furth (1968) found lesser components in varying quantities when she chromatographed carbonic anhydrase from horse erythrocytes and suggested that these fractions, which sometimes occurred in surprisingly large amounts, were artifacts produced in the course of purification. They all appeared to be closely similar to the high activity horse isoenzyme. In a recent review, Edsall (1968a) refers to unpublished observations made in his laboratory where as many as five carbonic anhydrase components were detected in rabbit erythrocytes. The present report describes several minor isoenzymes, in addition to the two major forms, in both rat and rabbit erythrocytes. While it is possible that the rat isoenzyme Eryth. 2 (CEP) resulted from treatment of Eryth. 3 with  $\text{CHCl}_3$ -EtOH, there were several additional minor components which were identified in preparations that had not been exposed to organic solvents. Moreover, the presence of minor isoenzymes was detected by chromatography and electrophoresis in the soluble extract of the rat dorsolateral prostate; the mild preparative methods used were unlikely to cause this polymorphism, and there is no evidence that either dorsolateral prostate isoenzyme is unstable.

The variation in the nature of acetazolamide inhibition of  $\text{CO}_2$  hydration catalysed by rat, rabbit, and sheep isoenzymes is interesting. Similar variation in sulphonamide inhibition has been observed recently by others (Kernohan, 1966; Lindskog & Thorslund, 1968; S.Lindskog, personal communication, November, 1968). Kernohan found competitive

inhibition of bovine erythrocyte carbonic anhydrase under certain circumstances, and suggest that the reason for apparent non-competitive behaviour noted by others (Chapter 2, DISCUSSION) could be explained by slow dissociation of enzyme and inhibitor. In similar experiments the sulphanilamide inhibition of  $\text{CO}_2$  hydration catalysed by the cobalt form of bovine erythrocyte carbonic anhydrase was examined using a stopped-flow apparatus (Lindskog & Thorslund, 1968). Two consecutive types of kinetic behaviour were observed when a solution of  $\text{CO}_2$  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 interval indicated competition between  $\text{CO}_2$  and inhibitor. When the sulphanilamide was mixed prior to 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 interaction between enzyme and inhibitor.

Any rapid, initial phase of reaction that might have occurred in the present experiments 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 in the present work with certain of the isoenzymes was entirely compatible with the report of Lindskog & Thorslund. No conclusive explanation is provided, however, for the non-competitive inhibition found with the other isoenzymes. Either the nature of the interaction between the non-competitively inhibited isoenzymes and acetazolamide was different or the dissociation



of the inhibitor and enzyme was extremely slow.

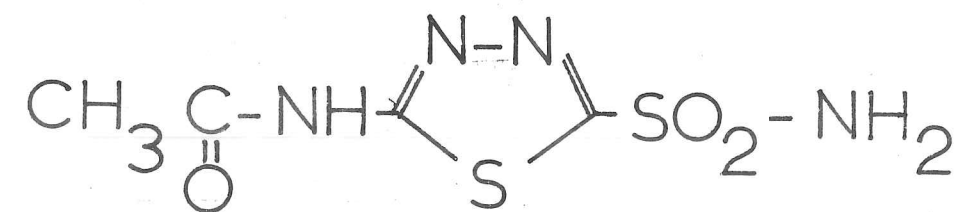
There is a good deal of information available describing the nature of the active site of carbonic anhydrase. The results of elegant infra-red spectrophotometric measurements performed by Riepe & Wang (1968) have shown that, under the conditions of their experiments with the bovine erythrocyte enzyme, ethoxzolamide and azide compete for binding to the zinc atom, and that both inhibitors interfere with the binding of  $\text{CO}_2$ . The  $\text{CO}_2$  molecule was shown, however, to bind at a hydrophobic site and not to the zinc atom. From these observations the authors inferred that the  $\text{CO}_2$  became bound to a site closely adjacent to the zinc, making the  $\text{CO}_2$  susceptible to steric hindrance by the inhibitors. These findings, together with the observation that anions (including  $\text{HCO}_3^-$ ) bind at the zinc, led Riepe & Wang (1968) to postulate a detailed mechanism of enzyme catalysis in which  $\text{CO}_2$  is hydrated by zinc-bound  $\text{OH}^-$ . Interference by sulphonamides with  $\text{CO}_2$  binding, revealed by the infra-red measurements, does not necessarily conflict with the many reports of non-competitive acetazolamide inhibition of p-nitrophenyl acetate hydrolysis (Chapter 2, DISCUSSION), because of the extremely strong binding that occurs between acetazolamide and the enzyme. For this reason it is surprising, perhaps, that competitive or even mixed inhibition of  $\text{CO}_2$  hydration is caused by acetazolamide and other sulphonamides.

An hypothesis which explains my results is as follows. The lack of inhibition of  $\text{CO}_2$  hydration by p-nitrophenyl acetate or 4-pyridine aldehyde, observed in the present work, suggests that there is more than one binding site for substrates. It is conceivable that the  $\text{CO}_2$  binding site is the "true" substrate binding site and that the other site, which is probably

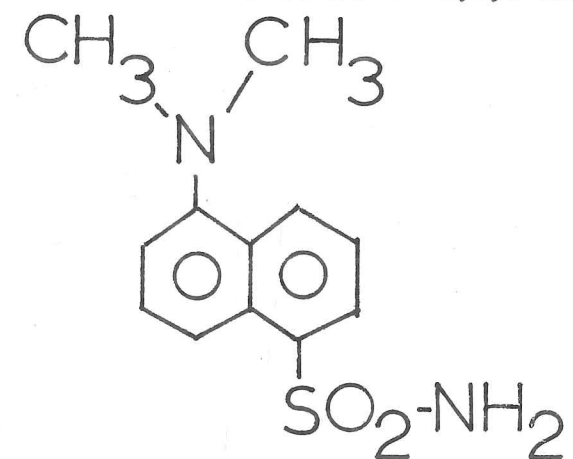
also adjacent to the zinc atom, binds the aromatic esters and pyridine derivatives in addition to being responsible for the binding of the aromatic parts of the sulphonamides. It is possible that the very strong binding of the aromatic sulphonamides results from a two-point attachment; for a sulphonamide to be a powerful inhibitor of carbonic anhydrase both the unsubstituted amino group, and an aromatic ring or pseudo-aromatic ring, are necessary (Mann & Keilin, 1940). The unsubstituted amino group is probably the way of attachment of the sulphonamide to the zinc (Mann & Keilin, 1940); certainly if the zinc is removed the apoenzyme binds the sulphonamides only about as strongly as it does the ester substrates (Lindskog, 1963). Therefore the weaker binding of the ester and pyridine substrates, in comparison with the sulphonamides, might simply be due to the fact that they lack the amino group; that these compounds bind in such a way that they can be hydrolysed by the enzyme may be coincidental to the "true" function of carbonic anhydrase.

This hypothesis suggests that competition should occur between aromatic ester substrates and aromatic esters that are not substrates, as well as between pyridine aldehydes and ester substrates. Moreover, competitive inhibition of esterase activity should be caused by sulphonamides that bind weakly. My observation of mixed inhibition of one of the rat isoenzymes by the less strongly bound sulphonamide DNSA supports this. The hypothesis does not necessarily exclude the possibility of interference in  $\text{CO}_2$  binding by sulphonamides; this could be caused by steric hindrance or by changes induced in the  $\text{CO}_2$  binding site (that is, changes in protein conformation) as a result of attachment of sulphonamides.

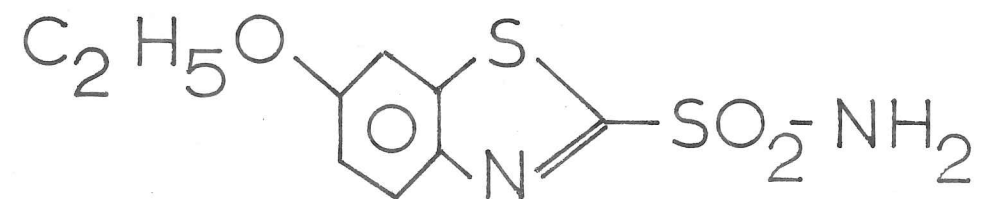
Figure 46. Comparison of the structures of certain sulphonamide inhibitors of carbonic anhydrase and the substrate 2-hydroxy-5- $\alpha$ -toluenesulphonic acid sultone.



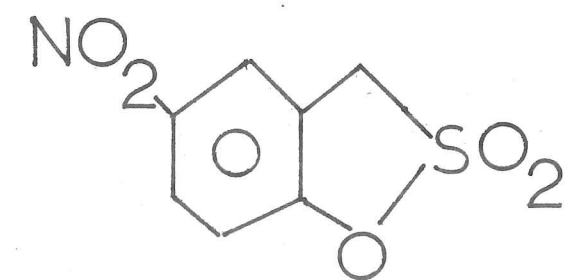
5-acetamido-1,3,4-thiadiazole-2-sulphonamide (acetazolamide)



5-dimethylaminonaphthalene-1-sulphonamide (DNSA)



6-ethoxybenzothiazole-2-sulphonamide (ethoxzolamide)



2-hydroxy-5-nitro- $\alpha$ -toluenesulphonic acid sultone

An example of a substrate of carbonic anhydrase that is even more similar to a sulphonamide than the esters or pyridine derivatives is 2-hydroxy-5-nitro- $\alpha$ -toluene sulphonic acid sultone (see Fig. 46) the hydrolysis of which is catalysed efficiently by the bovine erythrocyte enzyme Lo & Kaiser (1966). I have synthesized this compound from o-hydroxybenzyl alcohol (after conversion to the sulphonate, followed by cyclization with  $\text{POCl}_3$  (Zaborsky & Kaiser, 1966) and nitration (Marckwald & Frahne, 1898), and have found its hydration to be catalysed by the rat isoenzymes. This compound will provide a useful test of my hypothesis on the structure of the carbonic anhydrase active site.

### GENERAL SUMMARY

The dissertation describes the isolation and characterization of isoenzymes of carbonic anhydrase that were found to occur in the erythrocytes and certain organs of the reproductive tracts of the rat, the rabbit, and the sheep. In addition, the relationship has been examined between the concentration of zinc and the activity of carbonic anhydrase in the uterus of the rabbit during early pregnancy.

An assay for carbonic anhydrase, based on rapid titration of hydrogen ions produced by the hydration of the substrate, carbon dioxide, has been developed and adapted to the measurement of activity in both crude and purified enzyme preparations. The precision of the titration method has been shown to be equivalent to that of the spectrophotometric stopped-flow technique.

Carbonic anhydrase was resolved into isoenzymes by chromatography, electrophoresis, and isoelectric focusing. In rat erythrocytes the two major isoenzymes, referred to below as high and low activity forms, differed markedly in their efficiencies as catalysts of the hydration of carbon dioxide. The Michaelis constants and modes of inhibition by acetazolamide of the two forms were also different. Extraction of the enzyme from erythrocytes with chloroform and ethanol caused changes in the properties of the two major isoenzymes, and at the same time induced the appearance of an additional isoenzyme. The relatively weak esterase activity of the carbonic anhydrase isoenzymes has been investigated and it was observed



that the low activity form was the more efficient catalyst of the hydration of  $\beta$ -naphthyl acetate.

Carbonic anhydrase occurs in extraordinarily large quantities in the dorsolateral prostate of the rat. Three isoenzymes were isolated from this tissue, all of the high activity type. The two major isoenzymes were examined in detail. There were distinct differences in the kinetic behaviour of the isoenzymes from the erythrocytes and the prostate, and neither prostate isoenzyme catalysed the hydrolysis of  $\beta$ -naphthyl acetate. Zinc in the carbonic anhydrase of the dorsolateral rat prostate was shown to account for about five per cent of the unusually high concentration of zinc in this male accessory organ.

Sheep erythrocyte carbonic anhydrase was separated into a major and minor component, both of which were similar to the high activity forms found in the other species. Sheep uterine carbonic anhydrase was isolated in a high yield as a single component which had kinetic properties similar, but not identical, to those of the sheep erythrocyte isoenzymes.

Two major isoenzymes that have been isolated from the erythrocytes of the rabbit closely resembled those of the rat erythrocytes. Rabbit uterine carbonic anhydrase was found to consist of a single high activity isoenzyme. The level of the uterine carbonic anhydrase is known to be under the control of progesterone in the progestational phase of gestation, when the activity of the uterine enzyme increases by a factor of ten in two days. It was shown that the zinc concentration in the rabbit uterus increases at that stage of gestation by a factor of two. The content of zinc ascribable to the carbonic anhydrase was found to account for not more than one to two per cent of the total zinc in the rabbit uterus.

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