Biochem. J. (1970) **120**, 797–808
Printed in Great Britain

The Purification and Properties of Carbonic Anhydrases from Guinea-Pig Erythrocytes and Mucosae of the Gastrointestinal Tract

By M. J. CARTER and D. S. PARSONS
Department of Biochemistry, University of Oxford, Oxford OX1 3QU, U.K.

(*Received* 27 *July* 1970)

Procedures for isolating carbonic anhydrase (EC 4.2.1.1) enzymes from the erythrocytes and the mucosae of the gastrointestinal tract of guinea pigs are described. From a haemolysate, haemoglobin was removed by the addition of ammonium sulphate, and also by two other methods, namely by gel filtration or by adsorption on DEAE-Sephadex. The crude enzyme thus obtained was resolved into the different isoenzymes by chromatography with DEAE-cellulose. From particle-free supernatants of homogenates of some gastrointestinal tissues, carbonic anhydrases were purified by ammonium sulphate fractionation, gel filtration, and ion-exchange chromatography with DEAE-cellulose. The major isoenzymes from blood, stomach, proximal colonic mucosa and caecal mucosa were homogeneous during ion-exchange chromatography, acrylamide-gel electrophoresis, and centrifugal examination. From these tissues, carbonic anhydrase was isolated as two major isoenzymes. They resemble the pairs of isoenzymes discovered in the bloods of other species. The carbon dioxide hydratase activity of one isoenzyme ('high activity' carbonic anhydrase) was 40 times that of the other isoenzyme ('low activity' carbonic anhydrase), as measured at a single substrate concentration. Two other minor components of the enzyme are also found in guinea-pig erythrocytes. All of the enzymes isolated had molecular weights of nearly 30000 (sedimentation equilibrium). 'High activity' carbonic anhydrases from blood and gastrointestinal tissues were indistinguishable according to some chemical, physical and kinetic measurements; similarly 'low activity' carbonic anhydrases from those tissues were indistinguishable. 'High activity' carbonic anhydrase was markedly different from the 'low activity' carbonic anhydrase with respect to its amino acid composition, chromatographic behaviour and isoelectric pH value. Marked differences were also found in the tissue concentrations of the major isoenzymes. It is suggested that the characteristic and selective distribution of the different forms of carbonic anhydrase in the guinea-pig tissues is related to the specific and different physiological functions of the enzymes.

Meldrum & Roughton (1933) obtained from ox erythrocytes a preparation of carbonic anhydrase (EC 4.2.1.1) that was said to be 70% pure enzyme. To remove the large excess of haemoglobin, they treated an haemolysate with ethanol and chloroform. Further purification was effected by using classical techniques of treating protein mixtures with gel adsorbents and of salt precipitation. Similar procedures were described for purifying carbonic anhydrase from other mammalian erythrocytes during the 1930s and 1940s (see van Goor, 1948).

Technical advances subsequently provided the means to purify further the erythrocyte carbonic anhydrase. Thus Lindskog (1960) found two electrophoretically distinguishable carbonic anhydrases in ox erythrocytes. Although these two enzymes were kinetically and chemically very similar, this proved not to be the rule for erythrocyte carbonic anhydrase isoenzymes. In the erythrocytes of man, monkey, horse, rabbit (Edsall, 1968), guinea pig (Tappan, Jacey & Boyden, 1964) and rat (McIntosh, 1969) at least two major forms of carbonic anhydrase have been separated that differ in their turnover rates usually by an order of magnitude. Apparently a low-specific-activity carbonic anhydrase was also observed in ox erythrocytes by S. Lindskog (see Nyman, 1961), but it has not yet been fully described.

In the size of the difference in their catalytic activities, the two major sorts of carbonic anhydrase seem to be unique among enzymes. The possible

usefulness of the isoenzymes in studying the chemistry of the active site has been recognized (Furth, 1968), and the physicochemical aspects of their behaviour are being studied in detail (see Edsall, 1968). But the physiological significance of the isoenzymes is unknown, and the questions raised by their existence have received little attention. Although ultimately the answers may be obtained from studies on the erythrocyte, a relatively simple cell, none seem presently to be forthcoming. A comparative tissue study of the carbonic anhydrases was therefore thought likely to give ideas on the problem.

Few attempts have been made to isolate carbonic anhydrases from tissues other than blood. Keilin & Mann (1940) made an early attempt to purify pig stomach carbonic anhydrase, and succeeded in obtaining a preparation that was 30% pure enzyme. More recently two enzyme fractions have been obtained in a bovine lens extract, but they were not purified (Sen, Drance & Woodford, 1963). An enzyme has been partially purified from dog kidney (Byvoet & Gotti, 1967), and two forms of carbonic anhydrase have been obtained in a nearly homogeneous form from the dorsolateral prostate gland of the rat (McIntosh, 1969). In the present paper procedures are described for purifying carbonic anhydrases from some guinea-pig tissues, and the outstanding properties of the enzymes are described. A brief account of some of the findings has been given elsewhere (Carter & Parsons, 1970).

MATERIALS AND METHODS

Reagents. Tris [reagent grade, Sigma (London) Chemical Co., London S.W.6, U.K.], triethanolamine (reagent grade, British Drug Houses Ltd., Poole, Dorset, U.K.), DEAE-Sephadex and Sephadex G-75 [Pharmacia (G.B.) Ltd., London W.13, U.K.], Whatman DEAE-cellulose (H. Reeve Angel and Co. Ltd., London E.C.4, U.K.), bovine plasma albumin and Folin & Ciocalteu's reagent (British Drug Houses Ltd.) were obtained from the sources given. Other reagents were analytical grade from British Drug Houses Ltd., and water was de-ionized and glass-distilled.

Preliminary treatment of tissues. Guinea pigs, of about 300g and of both sexes, were decapitated, bled and the abdomens opened. Blood was collected in a vessel containing heparin and stored at 0–2°C. Segments of small intestine and colon were removed and their contents washed out with ice-cold NaCl (0.15mol/l). To obtain carbonic anhydrase isoenzymes in sufficient quantities for detailed study, samples of tissue from 6–12 guinea pigs were pooled. The segments were laid on absorbent paper, opened longitudinally and the mucosal tissue was taken with a glass slide. Tissues were homogenized in a 0.3mol/l solution of sucrose, containing sodium phosphate buffer (30mmol/l), pH7.2 at 0°C, with a Viridia glass tube (12cm × 2.5cm) (Chance Brothers Ltd., Smethwick, Staffs., U.K.) and a stainless-steel plunger (minimum clearance 0.1mm) of the Philpot & Stanier (1956) type; 50 return strokes were usually sufficient to break all but a small proportion of cells. Guinea-pig erythrocytes were not broken during this procedure (<0.5% broken). The caecum was removed, opened, the bulk of its contents scraped away, and the remaining matter washed away by agitating the tissue in a large volume of ice-cold NaCl solution (0.15mol/l). Mucosal tissue was obtained and treated as above. The stomach was cut into small pieces and a motor-driven homogenizer (Silverson Machines Ltd., London S.E.1, U.K.) was used to homogenize it.

Erythrocytes were harvested by centrifugation and washed three times with ice-cold NaCl (0.15mol/l). The washed erythrocytes were lysed with 2 vol. of ice-cold water. The stroma were not removed before the addition of $(NH_4)_2SO_4$, but were separated from the rest of an haemolysate by centrifugation when the haemolysate was to be fractionated by gel filtration.

Ammonium sulphate fractionation. Solid $(NH_4)_2SO_4$ was added to a protein solution and during the addition the pH was kept close to pH7.2, by titrating the mixture with NH_3 solution. The mixture was stirred continuously for 1–2h at 2–4°C. Haemolysates were made 45% saturated with respect to $(NH_4)_2SO_4$ [256g of $(NH_4)_2SO_4$/l of haemolysate]. High-speed supernatants obtained from homogenates of other tissues were made 55% saturated [326g of $(NH_4)_2SO_4$/l of supernatant]. Precipitated matter was separated by centrifugation at 50000*g* for 20 min (Spinco model L, type 30 rotor or SW39).

Gel filtration. Bead-form Sephadex G-75 [Pharmacia (G.B.) Ltd.] was allowed to swell in a solution containing Na_2SO_4 (0.1mol/l) and sodium phosphate buffer (0.05 mol/l), pH7.2, for 24h. A column measuring 4cm × 95cm was used for fractionating crude samples of carbonic anhydrase at 2–4°C. Sample volumes were 5–15ml, and the above buffer was used for elution (flow rate 30ml/h).

Ion-exchange chromatography. DEAE-cellulose (Whatman, microgranular pre-swollen) was used. For the earlier part of this work, the enzymes were eluted with tris–HCl buffers at pH8.2, but better separations were obtained with triethanolamine–HCl buffers. The sample and the ion-exchange material were equilibrated with triethanolamine (8mmol/l) titrated with HCl to pH7.8. By eluting with the same buffer, the 'high activity' carbonic anhydrases were appreciably retarded by the ion-exchange material and emerged in an apparently homogeneous form. The concentration of triethanolamine–HCl buffer was then changed to 25mmol/l, with respect to triethanolamine, and the 'secondary high activity' and 'low activity' isoenzymes eluted.

Buffer solutions were pumped through the column at 20ml/h and 2–5ml fractions were collected.

Stability of the isoenzymes. The isoenzymes were stored, at concentrations of at least 10g/l, in a 10mmol/l solution of sodium phosphate buffer, pH7.2 (0–2°C). Under these conditions enzyme activity was lost very slowly, and preparations were kept for a month or more. Enzyme preparations were neither precipitated nor dried, except where stated.

Measurement of carbon dioxide hydratase activity. The pH-stat method, described elsewhere (Carter & Parsons, 1968*a*), was used. The carbon dioxide concentration was 4mmol/l. The reaction mixture (5ml) contained sodium phosphate buffer (2mmol/l), bovine plasma albumin (25mg/l), EDTA (sodium salt) (1mmol/l). During a

measurement of enzymic activity, the reaction mixture was kept at pH7.4 (0°C) by continuous titration with a 0.5mol/l solution of NaOH. The rate of the reaction was deduced from the rate of addition of alkali. A correction was made for the spontaneous hydration of carbon dioxide. A unit of carbon dioxide hydratase activity was defined as the amount of enzymic activity that causes the hydration of 1μmol of carbon dioxide/min, under the conditions described above.

Concentration of large volumes of protein solutions. In the initial stages of this work, large volumes of crude and of purified enzymes were concentrated by packing solid sucrose around dialysis sacs containing these solutions (Hsiao & Putnam, 1961; Squire, Starman & Li, 1963), but for most of the work described the 'pressure dialysis' technique was used (Huehns & Shooter, 1961).

Amino acid analysis. A 150–200μg sample of enzyme in sodium phosphate buffer (10mmol/l) was taken to dryness in a test tube (1cm×12cm) by using a vacuum pump (room temperature). A 0.2ml portion of HCl (BDH Aristar, diluted 1:1 with water) was added to the dry sample and the tube was sealed as described by Moore & Stein (1963). The sample was hydrolysed by incubating the tube for 24–96h at 110°C, after which time the tube was cooled and centrifuged briefly at low speed. The sample was dried *in vacuo* (room temperature) and the dry hydrolysate analysed by a Locarte Mini Amino Acid Analyser.

Erythrocyte carbonic anhydrase isoenzymes were hydrolysed for 24, 48, 72 and 96h (duplicate or triplicate samples). Serine and threonine contents were determined by extrapolating the measurements obtained to zero hydrolysis time. Cysteine was measured as cysteic acid after performic acid oxidation of the unhydrolysed sample (Hirs, 1956). The tryptophan/tyrosine ratio was determined by the methods of Goodwin & Morton (1946) and Bencze & Schmid (1957), the results by the two methods being averaged. Ammonia was not determined.

Enzymes from tissues other than blood were hydrolysed for 24h. The correction factors that were found empirically for serine and threonine of erythrocyte 'low activity' carbonic anhydrase were used for colonic mucosal 'low activity' enzyme. Serine and threonine values for the 'high activity' carbonic anhydrases from stomach and from colonic mucosa were similarly corrected according to the data obtained with the erythrocyte 'high activity' enzyme. Cysteine and tryptophan were not determined in enzymes other than the major blood enzymes.

Isoelectric focusing. The principle of the method was described by Svensson (1962) and the equipment was obtained from LKB-Produkter AB, Stockholm, Sweden. The column (110ml) was maintained at 0°C and an ampholyte solution suitable for the pH range 3–10 was used. The cathode solution was ethanolamine and the anode solution dilute H_2SO_4. The potential difference between the electrodes was maintained at 400V until the current had remained stable for about 6h. Fractions of 2ml volume were collected. The pH of each fraction was measured immediately. The extinction of every other fraction was measured at 280nm. Enzyme activity was measured with the pH-stat method (see above).

Measurement of protein concentration. Concentrations of protein mixtures were determined by the method of Lowry, Rosebrough, Farr & Randall (1951), by using bovine serum albumin as standard. Reproducibility was about 10%.

Table 1. *Specific extinction coefficients of erythrocyte 'high activity' and 'low activity' carbonic anhydrases at* 280 nm

Specific extinction coefficients were determined experimentally by measuring the dry weights of dialysed enzyme samples of known extinction (dry weight), and by measuring the quantities of amino acid residues recovered during analysis of such enzyme samples (analysis). Values were also calculated from the amino acid compositions (Table 5) and specific extinction coefficients of tryptophan and tyrosine given by Wetlaufer (1962). The average of the two experimentally determined coefficients was used for estimating enzyme concentrations. Values are ±S.E.M. (number of observations).

Method used	$E^{1\%}_{1cm}$ at 280nm	
	HACA	LACA
Dry weight	17.1±0.1 (4)	16.4±0.1 (3)
Analysis	16.9±0.1 (5)	16.5±0.3 (8)
Calculated	16.7	16.1
Value used	17.0	16.5

Concentrations of solutions of purified enzymes were deduced from extinction measurements at 280nm (Zeiss PMQII spectrophotometer) at room temperature. The medium used was sodium phosphate buffer, pH6.0 (10 mmol/l). Specific extinction coefficients were obtained (*a*) by measuring the extinction of an enzyme solution and measuring the amounts of amino acids recovered during amino acid analysis of a known volume of that solution, and (*b*) by measuring the extinction and the dry weight (Mettler microbalance) of an enzyme solution that had been dialysed exhaustively against water. Extinction measurements were corrected for irrelevant absorption from values measured at 340 and 370nm. The extinction coefficients were also calculated from the extinction coefficients of tyrosine and tryptophan (Wetlaufer, 1962) by using the amino acid analysis data of Table 5. The results shown in Table 1 indicate that the calculated values are lower than the two experimentally obtained values for each enzyme. The discrepancy probably arises from the differing extinction values of the chromophores in protein and in the free amino acids (Wetlaufer, 1962).

In measuring the concentration of an enzyme solution, the average of the two experimentally determined values was used. The erythrocyte 'high activity' enzyme specific extinction coefficient was applied to the other 'high activity' enzymes and to the 'secondary high activity' enzyme too, because these enzymes had indistinguishable amino acid compositions. For the same reason, the specific extinction coefficient measured for the erythrocyte 'low activity' enzyme was used for the colonic and caecal mucosal 'low activity' carbonic anhydrases too.

Sedimentation equilibrium. Enzyme molecular weights were determined by sedimentation equilibrium, by using a Spinco model E analytical ultracentrifuge. Protein concentrations ranged between 1–6mg/ml and the solvent was sodium phosphate buffer, pH6.0 (0.1mol/l). The Rayleigh interference optical system was used and the

photographs were measured with a microcomparator (Projectorscope; Precision Grinding Instrument Co.). The results were calculated according to the method II of Van Holde & Baldwin (1958). Partial specific volumes were calculated from the data given by Cohn & Edsall (1943), by using the amino acid analysis data given in Table 5. Partial specific volumes thus calculated were 0.737 for 'high activity' carbonic anhydrase and 0.730 for the 'low activity' enzyme.

In some runs the schlieren optical system was used, results being treated according to both the method of Lamm (1929) and that of Van Holde & Baldwin (1958). Means of the values obtained by the two methods were used.

pH *measurements*. pH was measured at the appropriate temperature, with a glass combined electrode (Activion Glass Ltd., Kinglassie, Fife, U.K.) and a Vibret pH-meter model 3920 (Electronic Instruments Ltd., Richmond, Surrey, U.K.). Standard-pH buffer powder mixtures were obtained from Electronic Instruments Ltd.

RESULTS

Blood enzymes. The preparation of carbonic anhydrase from guinea-pig blood involves three steps. First, blood is collected from 6–12 guinea pigs and the erythrocytes are washed and lysed. Secondly, the bulk of the haemoglobin is removed from the haemolysate by the addition of solid ammonium sulphate till the haemolysate is 45% saturated. More than 99% of the haemoglobin is precipitated in this step, and 60–70% of the enzyme activity is recovered from the supernatants.

Thirdly, the crude enzyme in the supernatant obtained from the ammonium sulphate fractionation step is eluted from a column of DEAE-cellulose (see the Materials and Methods section). The crude erythrocyte enzyme is resolved into two major forms during ion-exchange chromatography (Fig. 1*a*). The first eluted peak of enzyme activity (HACA in Fig. 1*a*) is sufficiently retarded by the ion-exchange resin to give a highly purified preparation directly. Its specific activity [measured by the pH-stat method (Materials and Methods section)] is about 40 times that of the second eluted major form (LACA in Fig. 1*a*), which is eluted by raising the concentration of the buffer. The recovery of enzyme activity from ion-exchange chromatography was usually greater than 90%. The overall recovery was usually not less than 60% (Table 2).

The 'high activity' and the 'low activity' carbonic anhydrases obtained from guinea-pig erythrocytes are clearly the counterparts of the carbonic anhydrases 'C' and 'B' obtained from human erythrocytes by Rickli, Ghanzanfar, Gibbons & Edsall (1964). The same pattern of 'high activity' and 'low

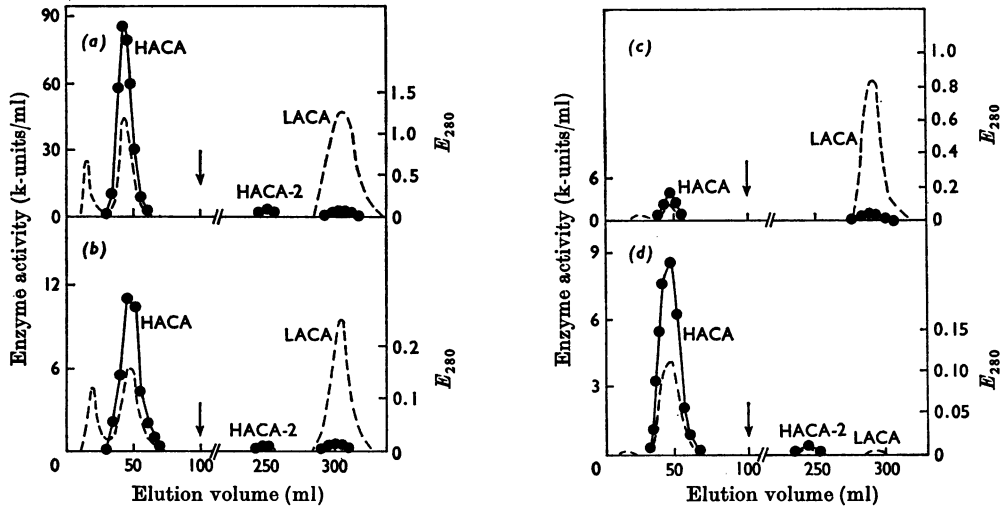

Fig. 1. Ion-exchange chromatography, with DEAE-cellulose, of (*a*) 200mg of crude erythrocyte carbonic anhydrase, (*b*) 15mg of crude proximal colonic mucosal carbonic anhydrase, (*c*) 10–15mg of crude caecal mucosal carbonic anhydrase, and (*d*) 5mg of crude stomach carbonic anhydrase. See Table 4 for pretreatment of starting material. The column was equilibrated, and elution started, with an 8mmol/l solution of triethanolamine titrated to pH7.8 with HCl (11mol/l) (4°C). The concentration of triethanolamine in the eluting buffer (adjusted with HCl to same pH) was subsequently raised to 25mmol/l (arrow). – – – –, Extinction at 280nm; –●–, carbon dioxide hydratase activity measured by the pH-stat method (see the Materials and Methods section). HACA, 'high activity' carbonic anhydrase; HACA-2, 'secondary high activity' carbonic anhydrase; LACA, 'low activity' carbonic anhydrase.

Table 2. *Purification of guinea-pig erythrocyte carbonic anhydrase*

Carbon dioxide hydratase activity was measured by the pH-stat method. Concentrations of protein mixtures were determined by the method of Lowry *et al.* (1951). Concentrations of purified enzymes were determined from extinction measurements at 280nm, with the $E^{1\%}_{1cm}$ values indicated in Table 1.

	Purification step	Total enzyme activity (M-units)	Recovered enzyme activity (%)	Specific activity (units/mg of protein)	Total protein (mg)
(*a*)	Haemolysate	4.23	100	400	11500
(*b*)	$(NH_4)_2SO_4$ spt.	2.89	69	9000	320
(*c*)	Ion-exchange chromatography				
	HACA	2.27		133500	17
	HACA-2	0.36	65	90300	4
	LACA	0.12		3100	40

activity' carbonic anhydrases has been found in the erythrocytes of horse, monkey and some other mammals and for this reason it seemed better to call the enzymes described here 'high activity' (HACA) and 'low activity' (LACA) carbonic anhydrases. In view of the findings to be described below, the same classification would seem adequately to describe the carbonic anhydrases of guinea-pig tissues generally.

In removing the large excess of haemoglobin by ammonium sulphate precipitation, selective loss of one or other of the major enzymes could occur. The measurement of recoveries of enzyme activity is of little value in this regard since the contribution of the 'low activity' carbonic anhydrase to the total carbon dioxide hydratase activity is only 5% (using the pH-stat method of assay). To ensure that selective loss of one or other enzyme does not occur during the ammonium sulphate step, two other completely different methods were used to remove haemoglobin, namely, gel filtration with Sephadex G-75, and the method of Armstrong, Myers, Verpoorte & Edsall (1966), using DEAE-Sephadex. Fig. 2 shows the elution pattern of a haemolysate from Sephadex G-75. In both of these methods more than 99% of the haemoglobin in the haemolysate was separated from the enzyme activity. In both methods the recovery of enzyme activity exceeded 90%. Crude enzyme obtained by these two methods gave the same two major forms of carbonic anhydrase, and they were obtained in the same proportions obtained by using the ammonium sulphate method, namely twice as much 'low activity' enzyme as of 'high activity' enzyme.

In addition to the two forms of carbonic anhydrase already described, guinea-pig erythrocytes contain two, possibly more, minor forms. Quantitatively the more important of these was eluted after the major 'high activity' carbonic anhydrase (Fig. 1*a*). This minor form possessed a specific activity which was approximately 70% that of the

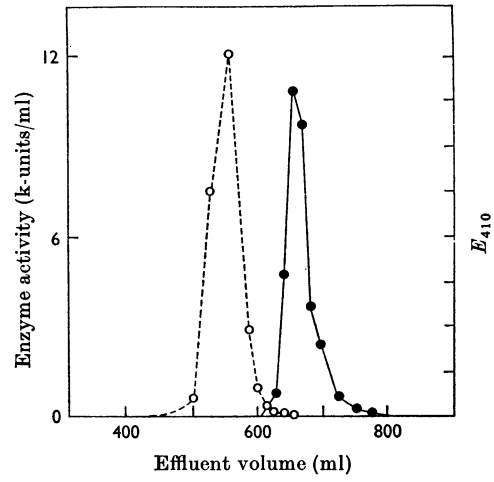

Fig. 2. Gel filtration of an haemolysate of guinea-pig erythrocytes with Sephadex G-75. Eluting buffer was sodium sulphate, (0.1mol/l)–sodium phosphate buffer (0.05mol/l), pH7.2. Haemolysate was about 2g of protein in 20ml. ○, Extinction at 410nm; ●, carbon dioxide hydratase activity, measured by the pH-stat method (see the Materials and Methods section).

major 'high activity' enzyme. Thus it was called 'secondary high activity' carbonic anhydrase (HACA-2 in Fig. 1*a*). Under the conditions of the pH-stat assay, the enzyme activity contributed by this minor form of carbonic anhydrase was greater than that contributed by the major 'low activity' enzyme. Another minor form of carbonic anhydrase was eluted before the 'high activity' enzyme (Fig. 3). It is called 'X' because it has not been obtained in sufficient quantity or in a sufficiently pure form for proper study.

Gastric mucosal enzymes. The carbonic anhydrase of the guinea-pig stomach were purified by using essentially the same procedure that was used for the

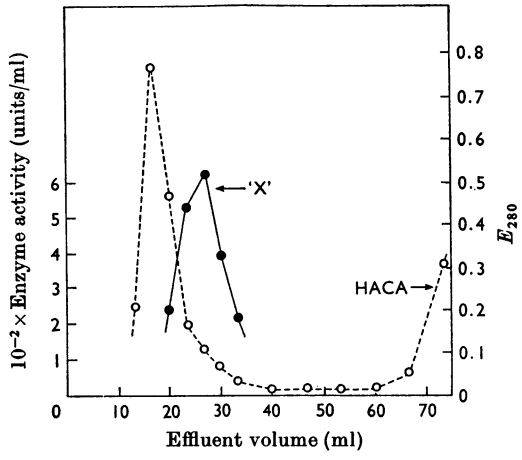

Fig. 3. Initial part of the elution pattern of guinea-pig erythrocyte carbonic anhydrases from DEAE-cellulose, to show a minor component, X. Note the difference in enzyme activity scale from Fig. 1. The equilibrating and starting buffer was a 10mmol/l solution of tris titrated with HCl (11mol/l) to pH8.1. ○, Extinction at 280nm; ●, carbon dioxide hydratase activity measured by the pH-stat method (see the Materials and Methods section).

erythrocyte enzymes. Stomachs were taken from 6–12 animals and homogenized in a 0.3mol/l solution of sucrose containing sodium phosphate buffer, pH7.2 (30mmol/l). Particulate matter was removed by centrifugation at 134000*g* for 60min, 0–2°C. The supernatant contained 97–98% of the enzyme activity of the whole homogenate. Secondly, the supernatant so obtained was made 55% saturated with ammonium sulphate, and the precipitate separated by centrifugation at 50000*g* for 20min, 0–2°C. The supernatant obtained from this step contained 80–90% of the enzyme activity. Thirdly, the supernatant was further fractionated by gel filtration with Sephadex G-75. Fig. 4(*b*) shows a representative elution curve. Eluted fractions were pooled in such a way that at least 90% of the enzyme activity was recovered from this step. Fourthly, the crude enzyme obtained by gel filtration was eluted from a column of DEAE-cellulose (Fig. 1*d*). Recovery of enzyme activity was greater than 90%. The overall recovery of enzyme activity was 50–60%. Fig. 1(*d*) shows that only a single major form of carbonic anhydrase is present in guinea-pig stomach. It is very similar in chromatographic behaviour and in its specific activity to the erythro-

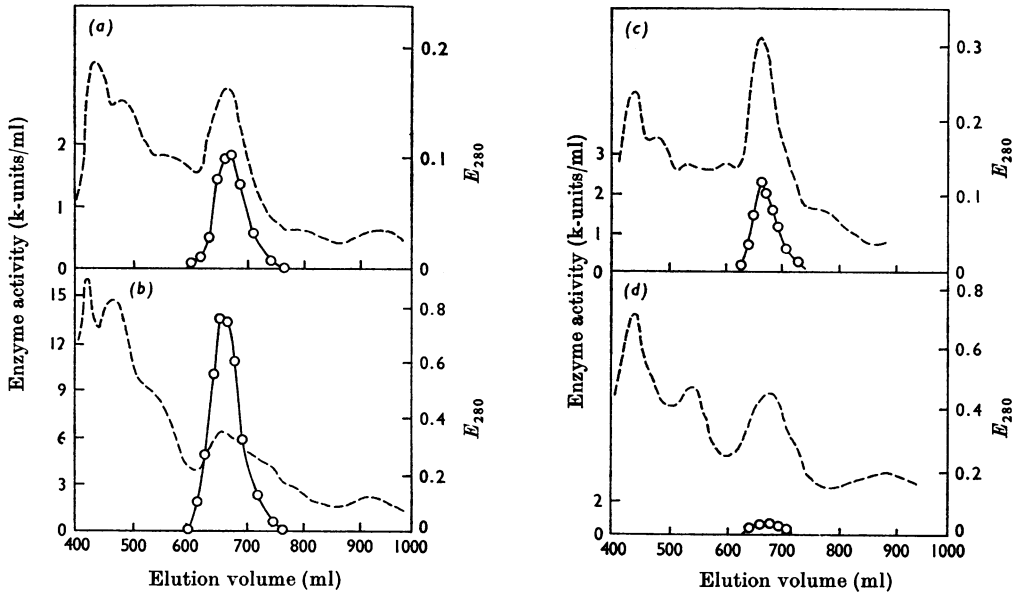

Fig. 4. Gel filtration with Sephadex G-75 of crude carbonic anhydrase extracted from (*a*) guinea-pig proximal colonic mucosal tissue, (*b*) guinea-pig stomach tissue, (*c*) guinea-pig caecal mucosal tissue, and (*d*) guinea-pig small-intestinal mucosal tissue. Pretreatment of the starting material is summarized in Table 4. The eluting buffer was a 0.1mol/l solution of Na_2SO_4, containing sodium phosphate buffer (0.05mol/l), pH7.2 (4°C). – – – –, Extinction at 280nm; ○, carbon dioxide hydratase activity measured by the pH-stat method (see the Materials and Methods section). Fractions were pooled so that at least 90% of the enzyme activity was recovered from this step in the purification procedure.

cyte 'high activity' carbonic anhydrase, and is therefore called stomach 'high activity' carbonic anhydrase (HACA). The trace amount of stomach 'low activity' carbonic anhydrase can probably be attributed to erythrocytes contaminating the tissue. A stomach 'secondary high activity' isoenzyme was found which closely resembled the erythrocyte 'secondary high activity' enzyme, in specific activity, chromatographic behaviour and in its quantity relative to the 'high activity' enzyme.

Colonic mucosal enzymes. The carbonic anhydrases of the proximal colonic mucosa were purified by using the procedures described for the stomach enzymes. Fig 4(*a*) shows the elution pattern obtained during gel filtration of the crude proximal colonic mucosal enzyme obtained from the ammonium sulphate step. The specific activity of the enzymically active material obtained after gel filtration is obviously less than that of the corresponding material obtained from the stomach (Fig. 4). The enzyme peak obtained by gel filtration is eluted from a column of DEAE-cellulose, and Fig. 1(*b*) shows that both a proximal colonic 'high activity' and a proximal colonic 'low activity' carbonic anhydrase are present, and that there is nearly twice as much of the 'low activity' as of the 'high activity' carbonic anhydrase. A 'secondary high activity' carbonic anhydrase was also detected. The recovery of enzyme activity was not less than 60% (Table 3).

Caecal mucosal enzymes. The carbonic anhydrases of the caecal mucosa were purified in the way already described for the stomach and proximal colonic mucosa. Fig. 4(*c*) shows the pattern of elution of the crude caecal enzyme from Sephadex G-75. Inspection of the peak of enzyme activity shows that the specific activity of that material is markedly lower than the specific activity of the corresponding material obtained from the proximal colonic mucosa (Fig. 4*a*). Fig. 1(*c*) shows that when crude caecal enzyme was eluted from DEAE-cellulose, the predominant form of carbonic anhydrase was of the 'low activity' kind. The quantity of 'high activity' carbonic anhydrase obtained from the caecal mucosal tissue was only about one-tenth the quantity of 'low activity' enzyme obtained. Yet the tissue concentration of the 'high activity' enzyme was itself at least half that obtained from the proximal colonic mucosa (Carter & Parsons, 1968*b*); thus the concentration of 'low activity' carbonic anhydrase in the caecal mucosa is extremely high.

Table 4 summarizes the purification procedures used. Occasionally, the blood 'secondary high activity' and 'low activity' carbonic anhydrases were slightly contaminated with haemoglobin. In such cases the haemoglobin was completely removed during re-chromatography with DEAE-cellulose as already described (Materials and Methods section). Final preparations of the isoenzymes from all tissues described above were homogeneous during acrylamide-gel electrophoresis (Carter & Parsons, 1970) and during ion-exchange chromatography. Sedimentation-equilibrium data, plotted according to the method of Van Holde & Baldwin (1958) (Fig. 5), also demonstrate the homogeneity of the preparations of the major isoenzymes obtained from the tissues referred to above. The amino acid analysis data in Tables 6 and 7 are also consistent with the view that the isoenzymes were isolated in a highly purified form.

The guinea-pig small-intestinal mucosa has very little carbonic anhydrase activity (measured with the pH-stat method) when compared with blood, gastric mucosal and proximal-colonic mucosal tissue (Carter & Parsons, 1968*b*). But in view of the very large difference between the specific activities of the two major isoenzymes, the possibility that

Table 3. *Purification of colonic mucosal carbonic anhydrase*

Carbon dioxide hydratase activity was measured by the pH-stat method. Concentrations of protein mixtures were determined by the method of Lowry *et al.* (1951). Concentrations of purified enzyme were determined from extinction measurements at 280nm, with the $E^{1\%}_{1cm}$ values indicated in Table 1.

	Purification step	Total enzyme activity (k-units)	Recovered enzyme activity (%)	Specific activity (units/mg of protein)	Total protein (mg)
(*a*)	Homogenate	312	100	600	510
(*b*)	High-speed spt.	306	98	1700	185
(*c*)	$(NH_4)_2SO_4$ spt.	276	89	4100	67
(*d*)	Gel filtration	258	83	18400	14
(*e*)	Ion-exchange chromatography				
	HACA	186	69	132900	1.4
	HACA-2	18		91500	0.2
	LACA	9		3100	3.0

Table 4. *Summary of procedures used for separating carbonic anhydrase isoenzymes*

Source of enzyme	Summary of purification procedures
Erythrocytes	(*a*) Cells washed with 0.9% NaCl and lysed with water (2 vol.) (*b*) Haemoglobin pptd. with $(NH_4)_2SO_4$ (45% saturation) (*c*) DEAE-cellulose ion-exchange chromatography
Gastrointestinal tissues	(*a*) Tissue homogenized in sucrose (0.3 mol/l)–sodium phosphate buffer (0.03 mol/l), pH 7.2 (*b*) Homogenate centrifuged $8 \times 10^6 g$-min (*c*) Supernatant from (*b*) fractionated with $(NH_4)_2SO_4$ (55% saturation) (*d*) Supernatant from (*c*) fractionated by gel filtration with Sephadex G-75 (*e*) DEAE-cellulose ion-exchange chromatography

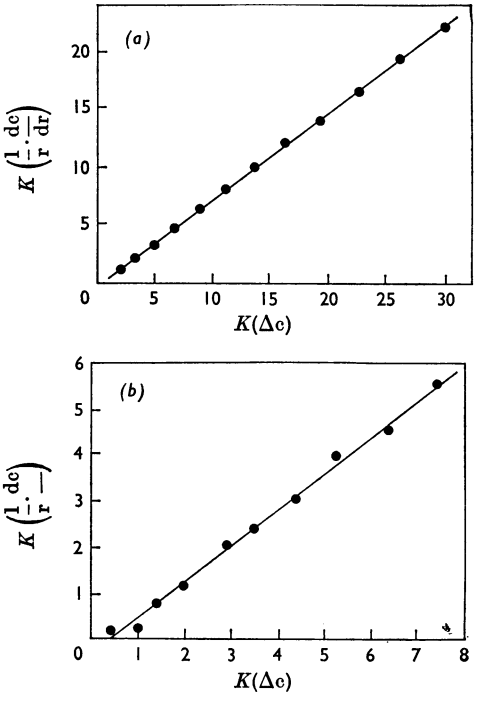

Fig. 5. Sedimentation-equilibrium results for (*a*) guinea-pig blood 'high activity' carbonic anhydrase, and (*b*) guinea-pig proximal-colonic-mucosal 'low activity' carbonic anhydrase, plotted according to the method of Van Holde & Baldwin (1958) (see the Materials and Methods section). Units are arbitrary.

small-intestinal mucosa contains an appreciable amount of a 'low activity' carbonic anhydrase was considered. By using acrylamide-gel electrophoresis, we have analysed the enzymically active material obtained from small-intestinal mucosal tissue during the gel-filtration step (Fig. 4*d*), and it appears that the 'high activity' carbonic anhydrase is the preponderant form (M. J. Carter & D. S. Parsons, unpublished work). Thus the small-intestinal mucosa (from the ligament of Treitz to the ileo-caecal valve) contains only small amounts of both 'high activity' and 'low activity' carbonic anhydrase isoenzymes.

Amino acid compositions. Table 5 shows the amino acid compositions of the guinea-pig erythrocyte 'high activity' and 'low activity' carbonic anhydrases prepared in the manner described above. The compositions were estimated on the basis that the molecular weights of both isoenzymes are about 30000, and assuming the presence of one atom of zinc per molecule and that the α-terminal amino group is acetylated (Marriq, Luccioni & Laurent, 1965). The values have been rounded to the nearest integer. The major guinea-pig erythrocyte isoenzymes show a broad similarity to the erythrocyte enzymes of other species (see the Discussion section).

Table 6 compares the amino acid compositions of the 'high activity' carbonic anhydrases that were obtained from erythrocytes, stomach and colonic mucosa. Also included in that table is the composition of the 'secondary high activity' carbonic anhydrase obtained from erythrocytes. All of these enzymes had very similar amino acid compositions. Table 7 compares the amino acid compositions of the 'low activity' carbonic anhydrases that were isolated from erythrocytes and colonic mucosa, and shows that these enzymes were also very similar.

Molecular weights. The molecular weights of some of the enzymes were determined by the method of sedimentation equilibrium. Table 8 shows that 'high activity' enzymes from erythrocytes and stomach had molecular weights that were not significantly different from one another. Similarly the molecular weights of erythrocyte 'low activity' carbonic anhydrase and of colonic mucosal 'low activity' carbonic anhydrase were identical by that method. But the 'high activity' enzymes studied showed molecular weights that were significantly higher than those of the two 'low activity' enzymes studied. Not included in Table 8

Table 5. *Amino acid compositions of guinea-pig erythrocyte 'high activity' and 'low activity' carbonic anhydrases*

The enzyme preparations were obtained as described in the text, and were homogeneous according to a number of criteria. Hydrolysis and analysis of enzyme samples was performed as described in the Materials and Methods section. The values are the results of 14 analyses of the blood 'high activity' isoenzyme (HACA) and of 19 analyses of the blood 'low activity' isoenzyme (LACA). Serine and threonine values were corrected for destruction during hydrolysis of the samples (see the Materials and Methods section). Cysteine was measured as cysteic acid. Tryptophan was deduced from the tryptophan/tyrosine ratio as measured by the methods of Goodwin & Morton (1946) and Bencze & Schmid (1957). It was assumed that the 'high-activity' isoenzyme contains 19 alanine residues/molecule, and that the 'low-activity' isoenzyme contains 17 alanine residues/molecule.

	Number of residues per molecule (nearest integer)	
Amino acid	HACA	LACA
Aspartate	25	25
Threonine	12	11
Serine	22	33
Glutamate	25	23
Proline	17	16
Glycine	20	18
Alanine	19	17
Cysteine	1	1
Valine	18	18
Methionine	3	1
Isoleucine	8	11
Leucine	25	20
Tyrosine	7	8
Tryptophan	7	6
Phenylalanine	11	9
Histidine	14	10
Lysine	23	19
Arginine	5	5
Total residues	262	251
Molecular weight	29049	27530

Table 6. *Amino acid compositions of 'high activity' carbonic anhydrases from some guinea-pig tissues*

Amino acid compositions of 'high activity' carbonic anhydrase isoenzymes from guinea-pig erythrocytes (eryth. HACA), stomach (stomach HACA), and proximal colonic mucosa (colonic HACA) are shown. Also shown is the amino acid composition of the erythrocyte 'secondary high-activity' isoenzyme (eryth. HACA-2). The enzymes were prepared as described in the text, and were homogeneous as judged by the usual criteria. In correcting for the loss of serine and threonine during hydrolysis of erythrocyte HACA-2, stomach HACA and colonic HACA, the correction factors found empirically for the erythrocyte HACA were used. A dash indicates that the quantity of a residue was not measured. The values are the averages of several analyses with a number of freshly prepared batches of enzymes, as indicated at the foot of the table (see the Materials and Methods section).

	Number of residues/molecule			
Amino acid	Eryth. HACA	Eryth. HACA-2	Stomach HACA	Colonic HACA
Aspartate	24.9	24.9	25.0	25.1
Threonine	11.6	12.3	11.5	11.3
Serine	21.6	24.4	23.8	21.3
Glutamate	24.8	25.2	24.8	25.0
Proline	17.2	17.0	17.7	17.4
Glycine	19.9	19.2	19.4	20.0
Alanine	19.0	19.0	19.0	19.0
Cysteine	0.6	—	—	—
Valine	17.6	17.6	17.2	16.8
Methionine	2.9	2.7	3.0	2.5
Isoleucine	8.1	8.3	7.8	7.7
Leucine	25.1	24.4	25.3	24.8
Tyrosine	7.1	7.2	7.4	7.2
Tryptophan	7.2	—	—	—
Phenylalanine	11.4	10.9	11.5	11.2
Histidine	14.2	13.5	14.5	13.9
Lysine	22.9	22.9	23.0	22.6
Arginine	5.3	5.6	5.0	5.2
Number of analyses	14	4	3	2
Number of preparations	5	2	2	1

is a value of 28600 for erythrocyte 'secondary high activity' carbonic anhydrase, that being the result of a single measurement.

Isoelectric pH. A few determinations of the isoelectric pH values of the two major isoenzymes were made by using the method of isoelectric focusing. Fig. 6 shows the result of such a determination with crude caecal carbonic anhydrase as the starting material. The measurements of isoelectric pH values were not very consistent with this method; the method did, however, have the advantage that it consumed only small amounts of enzymes. Table 9 shows the isoelectric pH values of the major types of carbonic anhydrase. The values represent the averaged results of experiments with crude large-intestinal mucosal enzyme and erythrocyte enzyme. For comparison, estimates of the isoelectric pH values for the enzymes of other species are also included.

DISCUSSION

We have isolated carbonic anhydrase isoenzymes from blood and some gastrointestinal tissues of guinea pigs. The procedures were designed to be as gentle as possible, and at no stage did the pH of an enzyme preparation exceed pH 7.8 or fall below pH 7.2 (0–4°C). From the tissues studied, two major

Table 7. *Amino acid compositions of 'low activity' carbonic anhydrases from guinea-pig blood and colonic mucosa*

'Low activity' carbonic anhydrases from erythrocytes (eryth. LACA) and colonic mucosal tissue (colonic LACA) were prepared as described in the text. They were homogeneous by the usual criteria. The correction factors for the loss of serine and threonine during hydrolysis that were found empirically for the erythrocyte LACA were also used to correct for the loss of those residues in colonic LACA during hydrolysis. A dash indicates that the quantity of a residue was not measured. The values are the averages of several analyses with a number of freshly prepared batches of enzymes, as indicated at the foot of the table (see the Materials and Methods section).

	Number of residues/molecule	
Amino acid	Eryth. LACA	Colonic LACA
Aspartate	25.4	24.8
Threonine	10.5	11.1
Serine	33.3	33.2
Glutamate	23.2	23.8
Proline	15.7	16.2
Glycine	18.4	18.7
Alanine	17.0	17.0
Cysteine	0.8	—
Valine	18.0	18.1
Methionine	0.6	0.3
Isoleucine	11.1	10.6
Leucine	19.9	20.1
Tyrosine	7.9	7.6
Tryptophan	5.8	—
Phenylalanine	8.5	8.6
Histidine	10.0	10.3
Lysine	18.5	18.7
Arginine	4.8	4.5
Number of analyses	19	4
Number of preparations	7	1

Table 8. *Molecular weights of 'high activity' and 'low activity' carbonic anhydrases as determined by the method of sedimentation equilibrium*

For details see the Materials and Methods section. Values are molecular weight ±S.E.M. (number of measurements, number of preparations used).

	Molecular weight	
Source of enzyme	HACA	LACA
Erythrocytes	30100±250 (15, 5)	27900±300 (15, 5)
Stomach	30250±600 (5, 2)	—
Colonic mucosa	—	28200±650 (3,1)

sorts of isoenzymes were isolated in a homogeneous form. The carbon dioxide hydratase activity of one kind ('high activity' carbonic anhydrase, HACA) was about 40 times that of the other kind ('low activity' carbonic anhydrase, LACA), when measured by the pH-stat method of Carter & Parsons (1968*a*). The two kinds of guinea-pig carbonic anhydrase are evidently the counterparts of the carbonic anhydrases 'C' (HACA) and 'B' (LACA) isolated from the bloods of man, monkey and horse (Furth, 1968).

The 'high activity' carbonic anhydrases from guinea-pig blood, stomach, and large-intestinal mucosa show no measurable differences in amino acid composition, chromatographic and electrophoretic behaviour, or in enzymic activities; the same is true for the 'low activity' isoenzymes from blood and large-intestinal mucosal tissue (see also Carter & Parsons, 1970). In contrast, the rat dorsolateral prostate is reported to contain two 'high activity' enzymes that differ from the rat blood isoenzymes in their enzymic specificities towards ester substrates (McIntosh, 1969).

The 'high activity' and 'low activity' carbonic anhydrases possess singular differences in their amino acid compositions and in some physical properties. In particular, there are two features in their contents of some amino acid residues that appear to be quite characteristic for each type of isoenzyme. First, blood 'low activity' carbonic anhydrases of man, monkey, horse and guinea pig contain about 1½ times as many serine residues as the blood 'high activity' carbonic anhydrases of those species. Secondly, the contents of basic residues (lysine, histidine, arginine) are markedly different (HACA 42; LACA 34) whereas the sum of the carboxylic residues (glutamate, aspartate) are more nearly equal (HACA 50; LACA 48) (see Table 5; cf. data of Table 4 in Furth, 1968).

Fig. 7 compares the amino acid compositions of the guinea-pig 'high activity' and 'low activity' carbonic anhydrases. It shows that 'high activity' carbonic anhydrase possesses a greater number of certain residues (notably lysine, leucine, histidine and methionine) than 'low activity' carbonic anhydrase, and a smaller number of other residues, the difference in their serine contents being outstanding. The differences are consistent with the view that the mammalian 'high activity' and 'low activity' carbonic anhydrases arose through a gene duplication (Nyman & Lindskog, 1964). Our finding that the guinea-pig isoenzymes are distributed very differently in the gastrointestinal tract implies the existence of independently controlled genetic loci for the two major isoenzymes.

The relative strength of retention on DEAE-cellulose of the two major types of isoenzyme, and their respective electrophoretic mobilities, are consistent with their respective isoelectric pH values. Whereas the isoelectric pH values observed here are in fairly close agreement with the values reported for the human blood enzymes, there seems to be no consistent relation between isoelectric pH and

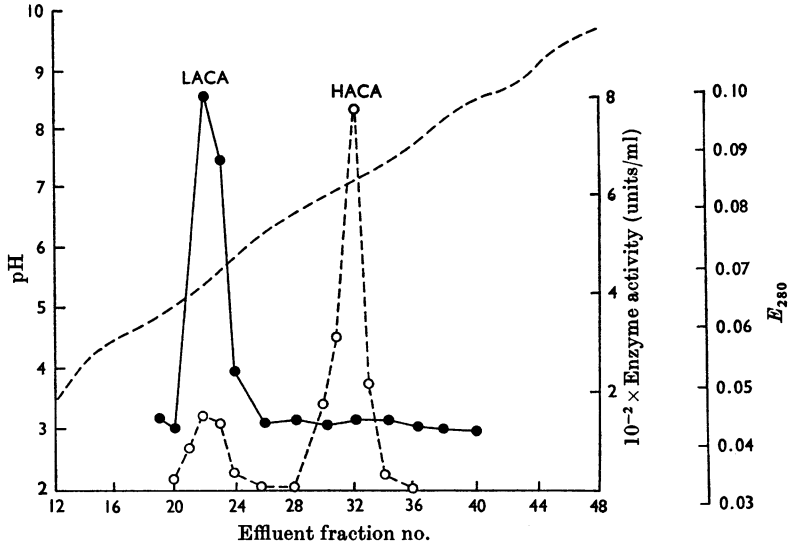

Fig. 6. Isoelectric focusing of caecal mucosal crude carbonic anhydrase. About 0.5mg of the enzymically active material obtained from gel filtration was the starting material (see Table 4); 2ml fractions were collected. ●, Extinction at 280nm; ○, carbon dioxide hydratase activity measured with the pH-stat method; – – – –, pH (see the Materials and Methods section).

Table 9. *Isoelectric* pH *values for erythrocyte carbonic anhydrases from various species*

Values for guinea-pig isoenzymes were measured by isoelectric focusing (see the Materials and Methods section); they are mean values ±S.E.M. (number of observations).

Species	HACA	LACA	References
Guinea-pig	7.3 ± 0.3 (4)	5.2 ± 0.2 (4)	See the text
Man	7.4	5.7	Ricki *et al.* (1964)
Horse	>10.0	6	Furth (1968)
Ox	5.2	—	Shimizu & Matsuura (1962)
Dolphin	5.2	—	Shimizu & Matsuura (1962)
Tuna	5.0	—	Shimizu & Matsuura (1962)
Rat	7.3, 7.7	8.1	McIntosh (1969)

kinetic type in other species (Table 9). Thus the 'high activity' and 'low activity' carbonic anhydrases of horse erythrocytes possess isoelectric pH values of >10 and 6 respectively (Furth, 1968). The bovine, tuna and dolphin erythrocyte enzymes (only one major isoenzyme has been reported in these species) have isoelectric pH values of about 5 (Shimizu & Matsuura, 1962), although these enzymes are akin to the 'high activity' carbonic anhydrases described above in carbon dioxide hydratase activity and amino acid composition. In contrast with the human, horse and guinea-pig blood isoenzymes, the isoelectric pH value of the

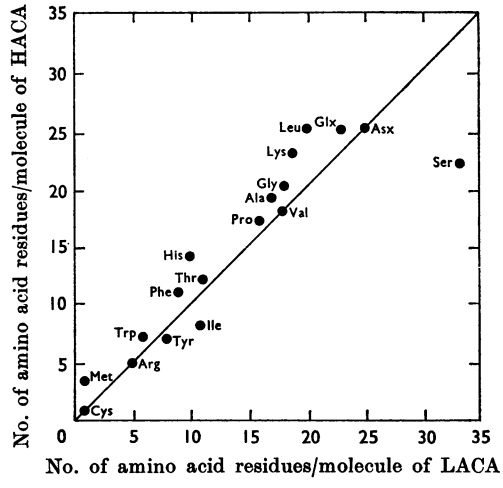

Fig. 7. Comparison of the amino acid compositions of guinea-pig blood 'high activity' and 'low activity' carbonic anhydrases (method of Nyman & Lindskog, 1964). Data are from Table 5.

rat blood 'low-activity' isoenzyme is greater than those of the two rat blood 'high-activity' isoenzymes (McIntosh, 1969; Table 9).

The minor form of carbonic anhydrase (secondary 'high activity' carbonic anhydrase, HACA-2) had two-thirds the enzymic activity of the major 'high activity' enzyme, both major and minor forms

being indistinguishable in their amino acid compositions. However the two enzymes showed marked differences in chromatographic and electrophoretic behaviour, and the differences could be due to a smaller proportion of amide groups in the 'secondary high activity' isoenzyme (they may also be examples of 'conformational isoenzymes'; see Epstein & Schechter, 1968). It is therefore possible that the 'secondary high activity' isoenzyme arose from 'high activity' carbonic anhydrase, deamidinated during preparative handling of the enzymes (see Funakoshi & Deutsch, 1969). In the guinea-pig tissues studied, the minor form was always present in the same proportion to the major 'high activity' enzyme (about 10%), regardless of the concentration of 'low activity' carbonic anhydrase in a tissue.

These results have a special importance in regard to the functional significance of the carbonic anhydrases. The differences in the tissue distributions of the two isoenzymes imply that the isoenzymes are functionally independent enzymes, for each has a selective and peculiar distribution. The occurrence of the 'high activity' carbonic anhydrase in blood, stomach, proximal colonic mucosa and caecal mucosa, and its virtual absence from the small-intestinal mucosa and distal colonic mucosa, do not permit us to associate it with a particular physiological event. In particular we cannot, as a result of these findings, associate it with the transport of bicarbonate–carbon dioxide, since the ileum, proximal colon and distal colon resemble one another rather closely in their handling of bicarbonate–carbon dioxide (Parsons, 1956; Swallow & Code, 1967; Edmonds, 1967). It is possible, though we consider it unlikely, that the origin of the secreted bicarbonate in the proximal colon differs from its origin in the ileum and distal colon. The rather restricted distribution of the 'low activity' carbonic anhydrase, apart from endowing this enzyme with an hitherto unrecognized importance, suggests some connexion with the micro-organisms in the large intestine. 'Low activity' carbonic anhydrase may therefore be related in some fashion to the absorption and subsequent metabolism of a substance, or a group of substances, whose formation is dependent upon the micro-organisms inhabiting the large intestine, such as ammonium ions or the volatile fatty acids. If this is so, interesting questions are raised in regard to the physiological role of this enzyme in the erythrocytes.

M. J. C. is a Medical Research Council Scholar. We are further indebted to the Medical Research Council for a grant for the purchase of equipment.

REFERENCES

Armstrong, J. McD., Myers, D. V., Verpoorte, J. A. & Edsall, J. T. (1966). *J. biol. Chem.* **241**, 5137.

Bencze, W. L. & Schmid, K. (1957). *Analyt. Chem.* **29**, 1193.

Byvoet, P. & Gotti, A. (1967). *Molec. Pharmac.* **3**, 142.

Carter, M. J. & Parsons, D. S. (1968*a*). *J. Physiol., Lond.*, **198**, 13*P*.

Carter, M. J. & Parsons, D. S. (1968*b*). *Nature, Lond.*, **219**, 176.

Carter, M. J. & Parsons, D. S. (1970). *Biochim. biophys. Acta*, **206**, 190.

Cohn, E. J. & Edsall, J. T. (1943). *Proteins, Amino Acids and Peptides*, chapter 16. New York: Reinhold Publishing Corp.

Edmonds, C. J. (1967). *J. Physiol., Lond.*, **193**, 589.

Edsall, J. T. (1968). *Ann. N.Y. Acad. Sci.* **151**, 41.

Epstein, C. & Schechter, A. N. (1968). *Ann. N.Y. Acad. Sci.* **151**, 85.

Funakoshi, S. & Deutsch, H. F. (1969). *J. biol. Chem.* **244**, 3438.

Furth, A. J. (1968). *J. biol. Chem.* **243**, 4832.

Goodwin, T. W. & Morton, R. A. (1946). *Biochem. J.* **40**, 628.

Hirs, C. H. W. (1956). *J. biol. Chem.* **219**, 611.

Hsiao, S.-h. & Putnam, F. W. (1961). *J. biol. Chem.* **236**, 122.

Huehns, E. R. & Shooter, E. M. (1961). *J. molec. Biol.* **3**, 257.

Keilin, D. & Mann, T. (1940). *Biochem. J.* **34**, 1163.

Lamm, O. (1929). *Z. phys. Chem.* A, **143**, 177.

Lindskog, S. (1960). *Biochim. biophys. Acta*, **39**, 218.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

McIntosh, J. E. A. (1969). *Biochem. J.* **114**, 463.

Marriq, C., Luccioni, F. & Laurent, G. (1965). *Biochim. biophys. Acta*, **105**, 606.

Meldrum, N. U. & Roughton, F. J. W. (1933). *J. Physiol., Lond.*, **80**, 113.

Moore, S. & Stein, W. H. (1963). In *Methods in Enzymology*, vol. 6, p. 819. Ed. by Colowick, S. P. & Kaplan, N. O. New York and London: Academic Press.

Nyman, P. O. (1961). *Biochim. biophys. Acta*, **52**, 1.

Nyman, P. O. & Lindskog, S. (1964). *Biochim. biophys. Acta*, **85**, 141.

Parsons, D. S. (1956). *Q. Jl exp. Physiol.* **41**, 410.

Philpot, J. St L. & Stanier, J. E. (1956). *Biochem. J.* **63**, 214.

Rickli, E. E., Ghazanfar, S. A. S., Gibbons, B. H. & Edsall, J. T. (1964). *J. biol. Chem.* **239**, 1065.

Sen, M., Drance, S. M. & Woodford, V. R. (1963). *Can. J. Biochem.* **41**, 1235.

Shimizu, C. & Matsuura, F. (1962). *Bull. Jap. Soc. scient. Fish.* **28**, 924.

Squire, P. G., Starman, B. & Li, C. H. (1963). *J. biol. Chem.* **238**, 1389.

Svensson, H. (1962). *Archs Biochem. Biophys.* Suppl. no. 1, p. 132.

Swallow, J. H. & Code, C. F. (1967). *Am. J. Physiol.* **212**, 717.

Tappan, D. V., Jacey, M. J. & Boyden, H. M. (1964). *Ann. N.Y. Acad. Sci.* **121**, 589.

van Goor, H. (1948). *Enzymologia*, **13**, 73.

Van Holde, K. E. & Baldwin, R. L. (1958). *J. phys. Chem., Ithaca*, **62**, 734.

Wetlaufer, D. B. (1962). *Adv. Protein Chem.* **17**, 303.