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THE ISOENZYMES OF CARBONIC ANHYDRASE: TISSUE, SUBCELLULAR DISTRIBUTION AND FUNCTIONAL SIGNIFICANCE, WITH PARTICULAR REFERENCE TO THE INTESTINAL TRACT

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SUMMARY

1. The total carbonic anhydrase activity in some guinea-pig tissues has been measured using a pH-stat procedure. Stomach, gall bladder, proximal colon and caecum all possess more carbonic anhydrase activity per unit amount of protein than does whole blood.

2. The carbonic anhydrase activity of the small intestine is low. Reasons are given for supposing that activity found there is not entirely due to contamination by whole blood, and it is suggested that in this tissue the enzyme may be localized in some cell type other than the columnar absorbing cells.

3. Evidence is presented which indicates that heavy metals interfere with the activity of the enzyme as measured in tissue homogenates.

4. The distribution and concentration of the two major isoenzymes of carbonic anhydrase have been measured in different tissues. Blood and proximal colon contain both isoenzymes in comparable concentrations, the ratio of the concentration of the 'low activity' isoenzyme to that of the 'high activity' being about 2. The gastric mucosa contains much 'high activity' carbonic anhydrase, but only a negligible amount of the 'low activity' isoenzyme. In the caecal mucosa, the 'low activity' isoenzyme is predominant, the ratio of its concentration to that of the 'high activity' isoenzyme being about 9. It is also found that more than 1·5% of the protein in the caecal mucosa is accounted for as carbonic anhydrase enzymes.

5. It is found that some 45% of the total carbonic anhydrase activity of sucrose homogenates of the guinea-pig colon is bound to particles. The activity is located mainly in the nuclear and microvillous fraction and in the 'high-speed supernatant' fraction. The form of enzyme bound is

largely of the 'high activity' variety. When the tissue is homogenized in potassium chloride solutions less than 4% of the total activity is recovered in particulate fractions. The amount of activity which is bound to particulate fractions increases as the ionic strength or pH of the homogenate is lowered.

6. The findings are discussed in relation to the possible physiological roles of the isoenzymes in tissues other than blood. Possible relationships between the presence of the enzymes and the metabolism and transport of ammonium and fatty acids are considered.

INTRODUCTION

Meldrum & Roughton (1933) reported the isolation from ox blood of the enzyme carbonic anhydrase (EC 4.2.1.1), which catalyses the reactions between carbon dioxide, water and bicarbonate. The presence of such an enzyme in mammalian blood had been predicted on the ground that the spontaneous rate of dehydration of bicarbonate was too slow to account for the observed rate of excretion of carbon dioxide through the lungs (Meldrum & Roughton, 1933). More recently, the erythrocyte carbonic anhydrases of several species have been isolated in the form of two distinct isoenzymes (Edsall, 1968; Furth, 1968; McIntosh, 1969; Carter & Parsons, 1970*a*).

The two major types of carbon anhydrase isoenzyme are of peculiar interest, because one type ('high activity' carbonic anhydrase) is many times more potent a catalyst of the reactions between carbon dioxide, water and bicarbonate than the other type of enzyme ('low activity' carbonic anhydrase). The two major sorts of enzyme both have molecular weights of about 30,000 and each comprises a single polypeptide chain attached to a zinc atom. However, they show marked dissimilarities in amino acid composition and in some physicochemical properties (Edsall, 1968).

We took up the question of the physiological role of carbonic anhydrase in the gastrointestinal epithelial tissues noted for the transport of inorganic ions. The luminal contents are acidic in the stomach and upper small intestine, but alkaline in the more aboral segments (Parsons, 1956); these acid–base changes apparently involve transport of hydrogen ions and bicarbonate ions. Chloride ions are known to be transported actively in the stomach (Hogben, 1965) and in at least some parts of the intestine (see, e.g. Kinney & Code, 1964). A study was therefore made of the distribution of carbonic anhydrase in an attempt to clarify its relation to these transport processes. The existence of two types of carbonic anhydrase isoenzyme, with quite different properties, added further interest.

Only a few attempts have been made to isolate carbonic anhydrases from tissues other than blood (Keilin & Mann, 1940; Sen, Drance & Woodford, 1963; Byvoet & Gotti, 1967). Recently McIntosh (1969) has given a kinetic description of two enzymically (both 'high activity') active fractions from rat dorsolateral prostate gland. We have reported elsewhere the isolation and characterization of both 'high activity' and 'low activity' carbonic anhydrases from guinea-pig stomach, colonic mucosa, and caecal mucosa (Carter & Parsons, 1970*a*, *b*). Here we present quantitative data on the distribution of the major carbonic anhydrase isoenzymes in some guinea-pig tissues, and discuss the possible physiological significance of the isoenzymes.

METHODS

Measurement of carbonic anhydrase activity: the pH-stat method

When carbon dioxide is added to water it undergoes spontaneous hydration to form carbonic acid, and the carbonic acid then spontaneously dissociates into bicarbonate ions and hydrogen ions. In comparison with the concentrations of carbon dioxide the concentration of carbonic acid is usually very small, and for this reason carbonic acid can be neglected in writing the equation for the over-all reaction:

$$CO_2 + H_2O \underset{k_{HHCO_3}}{\overset{k_{CO_2}}{\rightleftharpoons}} H^+ + HCO_3^-,$$

where k_{CO_2} is the rate constant for the spontaneous hydration of carbon dioxide (dimension: sec^{-1}), and k_{HHCO_3} is the rate constant for the spontaneous dehydration of bicarbonate ions (dimensions: M^{-1}, sec^{-1}). The progress of the hydration reaction can be described by the equation:

$$-\frac{d[CO_2]}{dt} = k_{CO_2}.[CO_2] - k_{HHCO_3}.[HCO_3^-].[H^+],$$

and the progress of the dehydration reaction by:

$$-\frac{d[HCO_3^-]}{dt} = k_{HHCO_3}.[HCO_3^-].[H^+] - k_{CO_2}.[CO_2],$$

under standard conditions.

Another reaction contributes to the measured rates of hydration and dehydration, viz. an hydroxylation reaction:

$$CO_2 + OH^- \underset{k_{HCO_3^-}}{\overset{k_{OH^-}}{\rightleftharpoons}} HCO_3^-.$$

But k_{OH^-} is reported to be approximately 1000 M^{-1}, sec^{-1} at 0° C (Roughton & Booth, 1946) and accordingly the contribution of this reaction to the total rate of hydration is less than 2% at pH 7·4.

In the pH-stat method the rate of hydration of carbon dioxide is measured as the rate at which a standard solution of sodium hydroxide (0·1–0·5 N) must be added to 5·0 ml. of a reaction mixture, in order to keep the solution at the desired pH (see

Carter & Parsons, 1968*b*, for details of the apparatus). The substrate for the hydration reaction is supplied by continuously bubbling a gas mixture of 5% carbon dioxide–95% air (v/v) through the reaction mixture. The rate of flow of gas is monitored with a flowmeter; the rate of flow of gas necessary to keep the solution saturated for the duration of a measurement has been found empirically (Table 1). The substrate concentration during each measurement thus remains constant and can be calculated accurately using Henry's law.

In order that the method should have a high sensitivity, two features of its design were specially considered. First, the reaction volume was made as small as possible, viz. 5 ml. Secondly, attention was paid to the buffer strength of the mixture. The buffer concentration was low, so that an ample error signal for the pH-stat system was produced by the acid formed during the hydration reaction. On the other hand, the buffer concentration was not so low that the pH of the reaction mixture oscillated with an unacceptably large amplitude about the set pH.

TABLE 1. The effect of the rate of gas flow upon the rate of hydration of carbon dioxide. The reaction mixture contained iminazole sulphate 2 mM. 0° C; pH 7·4

Gas flow-rate (ml./min)	Rate of hydration of CO_2 (μ-mole/min)	
	− Enzyme	+ Enzyme
25	2·3	10·5
60	2·4	10·9
80	2·4	10·7
100	2·4	10·8

To measure the rate of hydration of carbon dioxide, 4·8 ml. distilled water was transferred to the reaction vessel, allowed to cool to 0° C and saturated with the carbon dioxide–air gas mixture. 0·2 ml. of a solution containing iminazole sulphate buffer was injected. This addition caused the pH of the solution in the reaction vessel to rise rapidly to the set pH, and at this point a small (10–50 μl.) volume of a test solution was injected and the titration assembly was switched on. The rate of the subsequent addition of sodium hydroxide solution was recorded graphically and a measurement of the rate of hydration of carbon dioxide could be obtained for the initial 30–60 sec of the reaction.

To measure the rate of dehydration of bicarbonate ions, the procedure was modified as follows: The carbon dioxide–air gas mixture was replaced by 100% nitrogen, which was bubbled through a caustic soda solution before entering the reaction vessel. A suitable volume of a sodium phosphate solution was transferred to the reaction vessel, the supply of nitrogen was turned on, and the buffer solution was allowed to cool to 0° C. The titrant was 0·1–1·0 N sulphuric acid. The pH of the buffer solution was adjusted with the acid titrant to a value just less than the set pH. The titration assembly was switched on and 0·05–0·5 ml. 0·5 M sodium bicarbonate solution was then injected. The bicarbonate solution was prepared just before a series of measurements and was kept at 0–1° C. The rate of hydration of bicarbonate was calculated from the rate of addition of the standard acid titrant.

Spontaneous rate constants for the hydration and the dehydration reactions were measured with the pH-stat method (Table 2). It was thus possible to compare the rate constants obtained using the pH-stat method with the values obtained by other different methods (summarized by Edsall & Wyman, 1958). That the rate constants obtained with the pH-stat method agree well with those described in the literature was taken as sufficient evidence for the accuracy of our method.

A unit of enzyme activity, measured by the pH-stat method, was defined as that amount of enzyme activity that increases the rate of hydration of carbon dioxide by 1 μ-mole/min in 5 ml. reaction mixture at 0° C. Fig. 1 shows total rate of hydration of carbon dioxide (μ-mole/min) plotted against the amount of an homogenate of guinea-pig colon added to a reaction mixture. The intercepts give a measure of the spontaneous contribution to the total rate; the enzymically catalysed rate is equal to the total rate less the spontaneous contribution. The linear relationship between rate of reaction and amount of enzyme preparation used, obtained from a variety of different tissues, suggested that the method was suitable for assaying carbonic anhydrase activity over the range of reaction rates studied.

TABLE 2. Equilibrium and kinetic data on the spontaneous reactions between carbon dioxide and water. The reaction mixture contained iminazole sulphate, 2 mM. 0° C

	Dimensions	Present work	Other data	Author
(*a*) $10^{-4}\,k_{CO_2}$	sec^{-1}	$20{\cdot}2 \pm 0{\cdot}2$	21, 20·5	(i) (ii)
(*b*) k_{HHCO_3}	$M^{-1}\,sec^{-1}$	7536 ± 106	$8000 = k_{H_2CO_3}/K_{H_2CO_3}$	(i)
(*c*) pK_1	—	6·576	6·578	(iii)
(*d*) $k_{H_2CO_3}$	sec^{-1}	1·875	2	(i)
(*e*) $K_{H_2CO_3}$	—	926	952	(i)

(*a*) and (*b*) are defined in Methods.

$$K'_{H_2CO_3} = \frac{[H^+].[HCO_3^-]}{[H_2CO_3]}.$$

(*c*) is the negative logarithm of the over-all dissociation constant, i.e.

$$-\log(k_{CO_2}/k_{HHCO_3}).$$

In calculating (*d*) and (*e*) it was assumed that, at equilibrium $K'_{H_2CO_3} = 2{\cdot}5 \times 10^{-4}$ M (Roughton, 1941).

(*d*) is the rate constant for the reaction: $H_2CO_3 \rightarrow CO_2 + H_2O$.

(*e*) is the equilibrium constant,

$$K_{H_2CO_3} = \frac{[CO_2].[H_2O]}{[H_2CO_3]}.$$

(i) 'Thermal method', Roughton (1941).

(ii) 'Isotope exchange', Mills & Urey (1940).

(iii) E.m.f. measurements, Harned *et al.*, cited by Edsall & Wyman (1958).

Preparation of tissues

Guinea-pigs, 300–500 g, were decapitated, bled, and the abdomens opened. Carotid blood was collected in a vessel containing heparin and stored at 0–2° C. Segments of small intestine and colon were removed and washed through with ice-cold 0·9 % sodium chloride. The stomach and caecum were removed, opened, and the contents washed away by agitating the tissue in a large volume of 0·9 % sodium chloride. The gall bladder was pulled from the liver, adhering parenchymal tissue being removed with forceps. Intestinal segments were laid on absorbent paper, opened longitudinally and scrapings of mucosal tissue were taken with a glass slide. Mucosal tissue was plucked from the stomach wall with forceps. All tissues were homogenized in water, using a tight fitting Teflon plunger in a glass tube (Jencons).

About fifty strokes were sufficient to break all but a small proportion of the cells, as judged by phase-contrast microscopy.

Tissue fractionation. The proximal colon was removed from a single guinea-pig and washed through with 0·3 M sucrose. The tissue was homogenized in 8 ml. ice-cold 0·3 M sucrose, using a Viridia (Chance Bros., Ltd.) glass tube (12 × 2·5 cm). A stainless-steel plunger (minimum clearance, 0·1 mm) of the Philpot & Stanier (1956) type was used. Fifty return strokes were usually sufficient to break all but a very small proportion of the cells (homogenates were inspected with a phase contrast microscope). More strokes were used when necessary. Only a very small fraction (< 0·5 %) of guinea-pig erythrocytes are broken by this procedure, and this fact influenced the choice of animal for the more detailed study of carbonic anhydrase distribution.

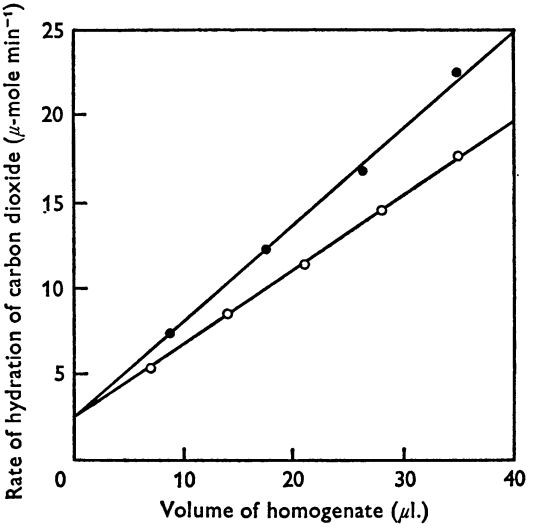

Fig. 1. Plots of rates of carbon dioxide hydration against the volume of a homogenate added to the reaction mixture, pH 7·4, 0° C. (○) Reaction mixture contained 5 mM iminazole sulphate, 3·8 mM carbon dioxide, and the homogenate contained 4·9 mg protein/ml. (●). Reaction mixture was supplemented with EDTA (sodium salt) (1 mM), and the homogenate contained 1 mg protein/ml. For details of the method for measuring the carbon hydration rate, see text.

The homogenized tissue was centrifuged at 1500 *g* for 10 min (Mistral 6L; Measuring and Scientific Instruments, Ltd.). The supernatant (Sp_1) was decanted, and the sediment (S_1) was washed three times by resuspension and recentrifugation in three successive aliquots of fresh medium (4 ml. each). The supernatant (Sp_1's) were pooled and a measured volume (*ca.* 15 ml.) was centrifuged at 8875 *g* for 20 min. (Spinco Model L, SW 39 rotor; Beckman Instruments Co.). This supernatant (Sp_2) was decanted and the sediment (S_2) was washed twice with 5 ml. aliquots of fresh medium. The Sp_2's were pooled and a measured volume (*ca.* 15 ml.) of the pooled supernatants was centrifuged at 134,000 *g* for 60 min. Then this supernatant (Sp_3) was decanted and the sediment (S_3) was washed once with fresh medium; the two Sp_3's were pooled. Sediments were resuspended and stored in 0·3 M sucrose (2–3 ml.).

Preparation of crude carbonic anhydrase from guinea-pig tissues

The purification of carbonic anhydrase from guinea-pig tissues has been described in detail elsewhere (Carter & Parsons, 1970*b*). Crude erythrocyte carbonic anhydrase was obtained from an haemolysate of washed guinea-pig erythrocytes by (*a*) the addition of ammonium sulphate to 45% saturation, followed by centrifugation to remove the precipitated haemoglobin and stroma, (*b*) gel filtration using 'Sephadex G-75', and (*c*) by adsorption of the haemoglobin on DEAE-Sephadex (Armstrong, Myers, Verpoorte & Edsall, 1966). All three methods gave similar results. The crude enzyme was dialysed against 10 mM sodium phosphate, pH 7·0, 0° C, and its volume reduced to < 0·5 ml.

Homogenates of whole stomach and of mucosal tissue from the small intestine, caecum, proximal colon and distal colon prepared as described by Carter & Parsons (1970*b*) were centrifuged at 134,000 *g* for 60 min, 0–2° C, and the supernatants so obtained fractionated by the addition of ammonium sulphate to 55% saturation followed by centrifugation at 50,000*g* for 20 min. The supernatants obtained from the ammonium sulphate step were further fractionated by gel filtration using 'Sephadex G-75', and the single peaks of enzymically active material obtained from each tissue were dialysed against 10 mM sodium phosphate, pH 7·0, 0° C and then reduced in volume to 0·1–0·3 ml.

2-(p-indophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium reductase (INT-reductase) activity.

This was measured by the method of Pennington (1961). The activity of this enzyme in a particular subcellular fraction is related to the succinic dehydrogenase activity and was therefore taken to be a measure of the mitochondrial content of that fraction.

Protein concentration

This was measured by the method of Lowry, Rosebrough, Farr & Randall (1951). Bovine plasma albumen formed the reference protein.

Disk electrophoresis

Acrylamide gel electrophoresis was performed as described by Davis (1964) as modified by Carter & Parsons (1970*b*).

RESULTS

a. Occurrence of carbonic anhydrase isoenzymes in guinea-pig tissues

In a preliminary survey of blood and some gastrointestinal tissues of various animals, it was found that the gastric mucosa, proximal colonic mucosa and caecal mucosa contained carbonic anhydrase activity in amounts comparable with that of the blood (Carter & Parsons, 1968*a*). Isoenzymes of carbonic anhydrase were isolated by these workers from these tissues in a highly purified form. The isoenzymes are of two sorts, one type of isoenzyme ('high activity' carbonic anhydrase, HACA) having forty times the catalytic activity of the other type ('low activity' carbonic anhydrase, LACA), as determined by the pH-stat assay. 'High activity'

carbonic anhydrases proved indistinguishable by a number of criteria, whatever the tissue source; 'low activity' carbonic anhydrases from different tissues were also very similar. However, 'high activity' enzymes differed strikingly from 'low activity' enzymes in their amino acid compositions, and in a number of their physical and kinetic properties (Carter & Parsons, 1970*a*, *b*).

In the present work crude carbonic anhydrase from guinea-pig blood and other tissues was analysed by acrylamide gel electrophoresis (Fig. 2). Marked variations were observed in the tissue distributions of the two

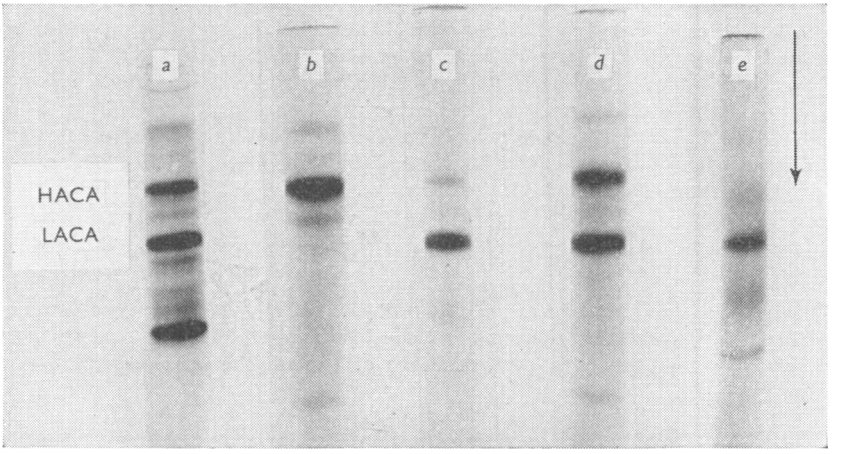

Fig. 2. Acrylamide gel electrophoretic analysis of crude carbonic anhydrase preparations from (*a*) blood, (*b*) stomach, (*c*) caecal mucosa, (*d*) proximal colonic mucosa, (*e*) distal colonic mucosa.

Voltage gradient, 40 V/cm. Gels were run for 1½–2 hr. The current through each gel was 2 mA. Gels were stained in 1% naphthalene black in 5% acetic acid for 2 hr.

major isoenzymes. Blood and proximal colonic mucosal tissue contained both isoenzymes in comparable concentrations (LACA/HACA = 2, Carter & Parsons, 1970*b*). The gastric mucosa contained much 'high activity' carbonic anhydrase, but only a negligible amount of 'low activity' carbonic anhydrase. In the caecal mucosa, however, the 'low activity' isoenzyme was the preponderant form of carbonic anhydrase (LACA/HACA = 9, Carter & Parsons, 1970*b*). The very small quantity of either isoenzyme in the small-intestinal mucosa precluded the possibility that they might be isolated in reasonable quantity from that tissue. However, it was established that significantly more 'high activity' than 'low activity' carbonic anhydrase was present (Fig. 3). In the proportions of the two major isoenzymes that it contained, the distal colonic mucosa

resembled the caecal mucosa rather than the proximal colonic mucosa (Fig. 2).

b. Total carbonic anhydrase activity in guinea-pig tissues

In preliminary experiments on the subcellular distribution of carbonic anhydrase activity in guinea-pig colonic mucosal cells, it was found that the total enzyme activity recovered in the subcellular fractions exceeded the total activity of the starting homogenate. It was decided to supplement the standard reaction mixture (5 mM iminazole sulphate) with

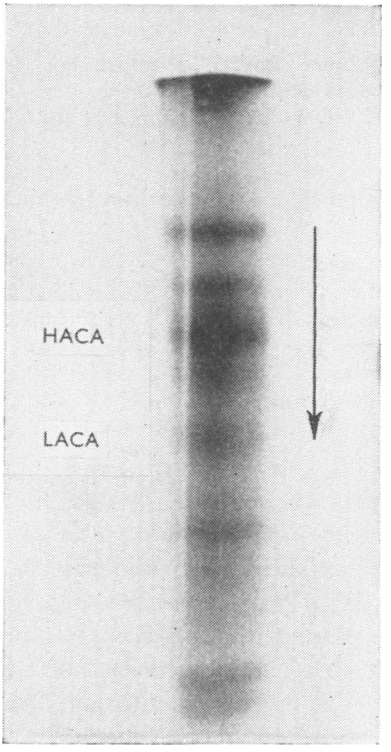

Fig. 3. Acrylamide gel electrophoretic analysis of crude carbonic anhydrase from the small-intestinal mucosa. See also caption to Fig. 2.

bovine serum albumin at a concentration of 25 mg/l., in order that the stability of the enzyme during a measurement be assured. The bovine serum albumin produced a marked increase in the specific activities of the homogenates and 1 mM-EDTA produced an even greater increase in activity.

Some of the assays were repeated using 'Tris' sulphate or sodium phosphate buffers instead of iminazole sulphate. The 'Tris' or phosphate

buffered reaction mixture gave specific activities very similar to those obtained with iminazole sulphate. But when either phosphate buffered reaction mixture or Tris-buffered reaction mixture was repeatedly extracted with a solution of dithizone in carbon tetrachloride (Malmström, 1953) a homogenate possessed an enzymic activity that was almost as high as that measured in the presence of 1 mM-EDTA. These findings are in accord with the view that the 'activation' of carbonic anhydrase is best attributed to the binding of inhibitory metals. The carbonic anhydrase activities of some tissues obtained from bled guinea-pigs were measured in the presence of EDTA and albumin (Table 3). The relative carbonic

TABLE 3. Carbonic anhydrase activity of guinea-pig tissues (pH-stat method). Reaction mixture contained iminazole sulphate, 5 mM; EDTA, 1 mM; carbon dioxide, 3·8 mM. 0° C, pH 7·4. Values are of mean ± S.E. of mean (number of observations)

Tissue	Specific activity (μ-mole min^{-1} mg protein^{-1})
Whole blood	239·1 ± 7·8 (6)
Gastric mucosa	833·7 ± 36·7 (6)
Gall bladder mucosa	341·6 ± 44·3 (5)
Jejunal mucosa	22·8 ± 3·4 (6)
Ileal mucosa	32·9 ± 3·1 (6)
Caecal mucosa	282·5 ± 24·2 (6)
Proximal colonic mucosa	555·7 ± 25·4 (6)

anhydrase activities of the tissues studied were much the same as when they were measured in the absence of EDTA and albumin, but the activity of the gastric mucosa was increased by a factor of 13 while the activities of most other tissues were increased by a factor of about 5. The difference in the size of the 'activation' may be related to the differences in the kinds of carbonic anhydrase present in the tissues.

Table 3 shows that no less than four tissues of the gastrointestinal tract of the guinea-pig possess more carbonic anhydrase activity per unit amount of protein than does the whole blood. The gastric mucosa indeed possesses three to four times as much activity as whole blood. The markedly selective manner in which the enzyme activity is distributed in the gastrointestinal tract is noteworthy, for it has an important bearing on the physiological significance of the enzyme (see Discussion).

The measured activity of the tissue whole homogenates is the sum of the contributions from the isoenzymes therein. From the proportions of the 'high activity' and 'low activity' carbonic anhydrases in the tissues studied (Carter & Parsons, 1970*b*, and see section *a supra*) it is estimated that the contribution of the 'low activity' isoenzyme to the total carbonic anhydrase activity of whole blood and of proximal colonic mucosal tissue

is less than 5% under our conditions of assay. The contribution of the 'low activity' isoenzyme to the stomach tissue is negligible, but to the caecal mucosal tissue that contribution amounts to 15–20%. With less exactitude, we can say that the 'low activity' enzyme also makes a substantial contribution to the total carbonic anhydrase activity of the distal colonic mucosal tissue.

The data of Table 3 do not prove that carbonic anhydrase is present within the cells of the small-intestinal mucosa. However, there are three reasons for believing that the activity detected was not due to contamination of the tissue by blood. Firstly, at least one tenth of the protein of the mucosal tissue would have to be of blood origin in order to account for the measured activity; yet estimates of this contamination based on the absorbance of haemoglobin at 410 nm put the percentage of blood at about 3%. Secondly, in a medium containing sucrose, 0·3 M, sodium phosphate, 30 mM, pH 7·0, some 75% of the activity was found in the particle-free supernatant obtained from an homogenate, even though a negligible proportion ($< 0{\cdot}5\%$) of erythrocytes were broken using these conditions. Thirdly, the observed pattern of carbonic anhydrase isoenzymes in the mucosal extracts was distinctly different from that found in erythrocytes (Figs. 2 and 3). The exact location of the carbonic anhydrase activity and its relation to transport processes in the small intestine are unknown. The small amount of enzyme activity found suggests that carbonic anhydrase might occur in but a few of the cell types found in the small-intestinal mucosa, although there is some histochemical evidence that it is rather generally distributed in the absorbing epithelial cells (Korhonen, Korhonen & Hyyppä, 1966).

c. The tissue concentrations of the 'high activity' and 'low activity' carbonic anhydrases

Using the pH-stat method for assaying carbonic anhydrase activity, the specific activities of the 'high activity' isoenzymes are 130×10^3 μ-mole min^{-1} mg $protein^{-1}$ and those of the 'low activity' isoenzymes are 3×10^3 μ-mole min^{-1} mg $protein^{-1}$ (Carter & Parsons, 1970*b*). From a knowledge of the specific activities of the purified isoenzymes, the proportions of the isoenzymes found in the tissues studied (section *a* above) and the specific activities of the whole tissue homogenates (using the pH-stat method of assay; section *b* above), the concentrations of the two major isoenzymes in guinea-pig tissues have been estimated (Fig. 4).

The relatively small enzymic activity of the distal colonic mucosal tissue tends to obscure the presence of an appreciable amount of 'low activity' carbonic anhydrase in that tissue. About 0·5 mg of 'low activity' carbonic anhydrase was obtained from the distal colonic mucosae of

twelve guinea-pigs (cf. Carter & Parsons, 1970*a*, Table 1). The quantity of 'high activity' enzyme was too small to enable us to estimate the exact proportion of the two major enzymes in this tissue.

d. The subcellular distribution of carbonic anhydrase activity

Homogenates of proximal colonic mucosal tissue were prepared in 0·3 M sucrose (pH 6·9 ± 0·1) and fractionated as described in the Methods section. Three particulate cell fractions (S_1, S_2, S_3) with a high-speed

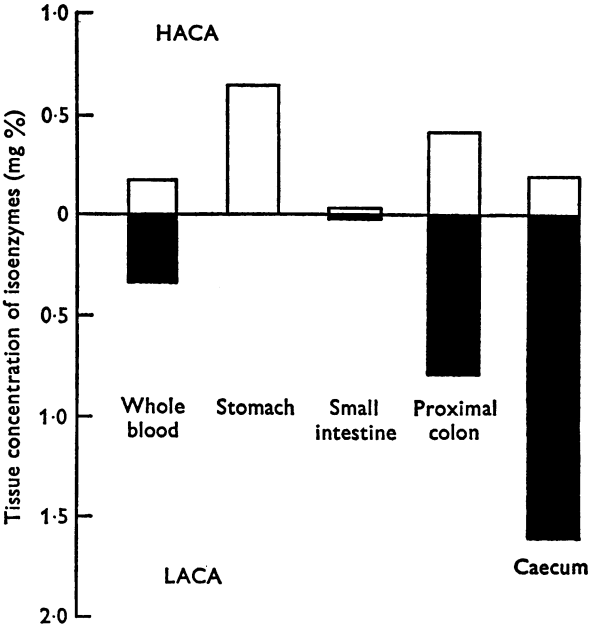

Fig. 4. Estimated concentrations, mg enzyme/100 mg tissue protein, of the 'high activity' (HACA) and 'low activity' (LACA) carbonic anhydrases in the blood and in some gastrointestinal mucosal tissues of the guinea-pig. Note that these estimates are based upon estimates of the protein content of purified enzymes deduced from a knowledge of the specific extinction coefficients at 280 nm, while the protein content of whole homogenates were measured by comparison with albumin, using the method of Lowry *et al.* (1951).

supernatant (Sp_3) were obtained. The three sediments and the high-speed supernatants were examined with a phase-contrast microscope and their contents are described in Table 4. The distribution of INT-reductase activity showed that contamination of the 'nuclei and microvillous sheet' fraction and the high-speed sediment with mitochondria was tolerably low.

Tables 5 and 6 show that some 45% of the total carbonic anhydrase

activity of the guinea-pig colonic mucosa was particle bound. It is also found that the particle-bound carbonic anhydrase activity was located mainly in the 'nuclei and microvillous sheet' fraction (18·5% of the total activity) and the 'high-speed sediment' (20%). The 'mitochondrial' fraction contained a much smaller proportion of the total activity (7%). These data also show that none of the fractions had either a very high or a very low specific activity. The 'particle-free supernatant', the 'high-speed

TABLE 4. Composition of subcellular fractions of homogenates of proximal colonic mucosa. Homogenates prepared and fractionated in 0·3 M sucrose. For definition of fractions see Methods. See also Results, section *d*

Fraction	Appearance in phase contrast	Name of fraction
S_1	Microvillous sheets, nuclei, a few granules, a small number of erythrocytes and intact cells	'Nuclei and microvillous sheet' fraction
S_2	Granules	'Mitochondrial fraction'
S_3	Clear	'High speed sediment'
Sp_3	Clear	'Particle-free supernatant'

TABLE 5. Analysis of subcellular fractions of mucosal cells from the proximal colon of the guinea-pig. Values are of means of seven experiments ± S.E. of mean. Protein was measured by the method of Lowry *et al.* (1951). INT-reductase activity was measured by the method of Pennington (1961). The pH-stat method was used for carbonic anhydrase activity (see Methods section). For details of the reaction mixture see legend to Table 3

		Carbonic anhydrase activity		INT-reductase activity	
Fraction	Total protein (mg)	Total activity (10^{-3} × units)	Specific activity (μ-mole min^{-1} mg protein^{-1})	Total activity (arbitrary units)	Specific activity (μ-mole min^{-1} mg protein^{-1})
Homogenate	34·3 ± 2·3	19·45 ± 1·44	567 ± 25	11·5 ± 0·8	0·34 ± 0·03
S_1	7·5 ± 0·9	3·31 ± 0·43	441 ± 34	1·1 ± 0·3	0·14 ± 0·02
S_2	5·0 ± 0·3	1·18 ± 0·13	236 ± 17	9·9 ± 0·8	1·98 ± 0·09
S_3	5·0 ± 0·3	3·58 ± 0·51	716 ± 79	0·6 ± 0·2	0·12 ± 0·03
Sp_3	16·4 ± 0·1	9·86 ± 0·64	601 ± 34	0·1 ± 0·04	0·01 ± 0·004
Recovered	33·9 ± 2·1	17·93 ± 1·16	—	11·7 ± 0·8	—

sediment' and the 'nuclei and microvillous sheet' fractions all had comparable specific activities and they were all similar to that of the whole homogenate. Only the 'mitochondrial' fraction was markedly different in that respect, its specific activity being less than one-half that of the whole homogenate.

A homogenate of proximal colonic mucosal cells was centrifuged at 134,000 *g* for 60 min. The 'total particulate' fraction obtained was resuspended in a medium containing 0·3 M sucrose, 30 mM sodium phosphate,

pH 7·0, and then centrifuged as above. The supernatant so obtained contained more than 95% of the enzyme activity that previously was insoluble. The supernatant was made 55% saturated in ammonium sulphate, pH 7·0, and centrifuged at 50,000 *g* for 20 min, and the supernatant obtained from that step was dialysed against 10 mM sodium phosphate, pH 7·0, and reduced in volume. The partially purified preparation of 'particle-bound' carbonic anhydrase activity was analysed by acrylamide

TABLE 6. Subcellular distribution of carbonic anhydrase activity, protein and INT-reductase activity, expressed as percentages of the quantities of each item recovered from guinea-pig colon. Values are of means ± S.E. of mean. Recalculated from data in Table 5

Fraction	Protein	Carbonic anhydrase	INT-reductase
Homogenate	102·8 ± 1·3	108·5 ± 3·1	98·2 ± 1·9
Recovered	100·0	100·0	100·0
S_1	21·9 ± 1·9	18·5 ± 2·5	9·7 ± 2·3
S_2	14·7 ± 0·5	6·6 ± 0·5	84·0 ± 2·1
S_3	15·1 ± 1·0	20·0 ± 2·1	5·1 ± 1·2
Sp_3	48·3 ± 1·2	55·0 ± 2·1	1·3 ± 0·3
Total particulate	51·7 ± 1·2	45·0 ± 2·1	98·8 ± 0·3

TABLE 7. Percentage particle-bound carbonic anhydrase activity in various tissues. Activity measured in the absence of EDTA and albumin

Tissue	(*a*)	(*b*)	(*c*)	(*d*)
Liver (rat)	15	14	—	—
Kidney (rat)	20	24	—	—
Submaxillary gland (rat)	25	—	—	—
Cerebral cortex (rat)	25	—	—	—
Shell gland	—	—	20	—
Colonic mucosa (guinea-pig)	—	—	—	22

(*a*) Karler & Woodbury (1960).
(*b*) Datta & Shepard (1959).
(*c*) Bernstein *et al.* (1968).
(*d*) Present study; 5 ml. reaction mixture contained 3·8 mM-CO_2, 5 mM iminazole sulphate, pH 7·4 (see Methods section).

gel electrophoresis, which showed that it comprised 'high activity' carbonic anhydrase and only a negligible proportion of 'low activity' isoenzyme.

The proportion of particle-bound carbonic anhydrase activity reported here in Table 6 (viz. 45%) is a good deal higher than has been reported for other tissues. It could be a feature peculiar to the colonic mucosal cells, but another explanation is suggested by the data collected in Table 7. These show the distribution of carbonic anhydrase activity between 'particulate' and 'soluble' fractions when the enzyme assay mixture

contained no EDTA and no albumin. The specific activities of the proximal colonic mucosal homogenates were much lower (55μ-mole min^{-1} mg $protein^{-1}$ and the presence of a chelatable inhibitor may be assumed. The proportion of particle-bound activity in the proximal colon was less (22%) when measured under these conditions than when EDTA and albumin were present in the assay mixture (45%). Table 7 shows that the proportions of particle bound carbonic anhydrase activity reported for other tissues are similar to the values found by us in the absence of a chelating agent. It is almost certainly true that the low proportion of particle-bound carbonic anhydrase activity

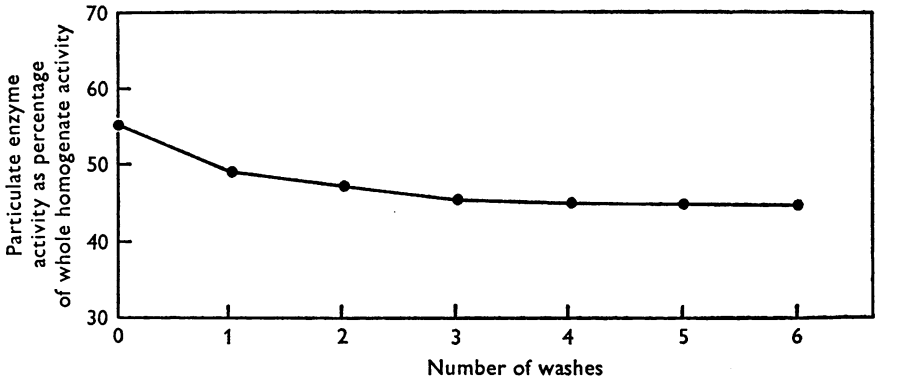

Fig. 5. Particulate carbonic anhydrase activity (expressed as a percentage of the total activity of the whole homogenate) plotted against the number of washes with 0·3 M sucrose (2–4° C).

in proximal colonic mucosal homogenates was due to the presence of an inhibitory agent. If the same was true for the other data collected in Table 7 those data serve to emphasize the value of the precautions recommended here (above) and elsewhere (Magid, 1967) that a chelating agent should be an ingredient in the assay mixture for carbonic anhydrase activity.

That the high proportion of particulate activity in proximal colonic mucosal tissue (Table 6) was bound rather firmly under these conditions is evident from the results obtained by repeatedly washing a total particulate fraction with aliquots of fresh medium (Fig. 5). A homogenate was prepared in the way already described and 5 ml. of it were centrifuged at 134,000 *g* for 40 min. The sediment was resuspended and recentrifuged with successive 5 ml. portions of fresh 0·3 M sucrose.

The distribution of carbonic anhydrase activity was strongly affected by the conditions used for homogenizing. First, when proximal colonic mucosal cells were homogenized in isotonic potassium chloride, only a small proportion ($< 4\%$) of the total enzymic activity was recovered with

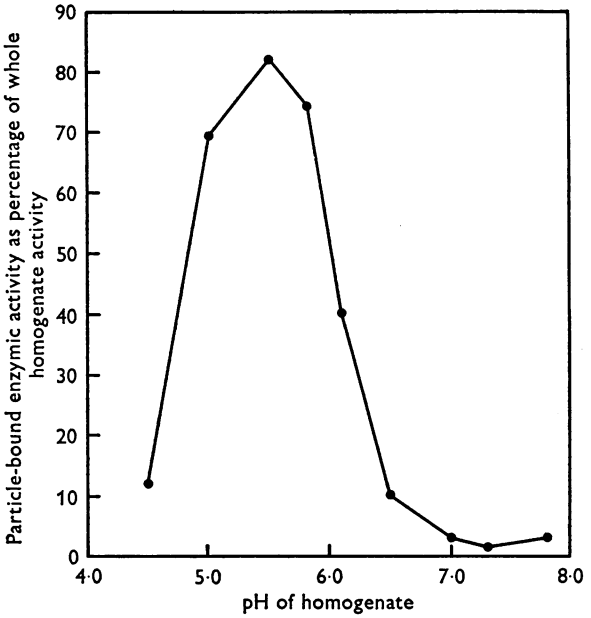

Fig. 6. Particulate carbonic anhydrase activity (expressed as a percentage of the total activity of the homogenate) plotted against the pH of the homogenate (2–4° C). 0·3 M sucrose medium was supplemented with either sodium acetate (10 mM) or sodium phosphate (10 mM).

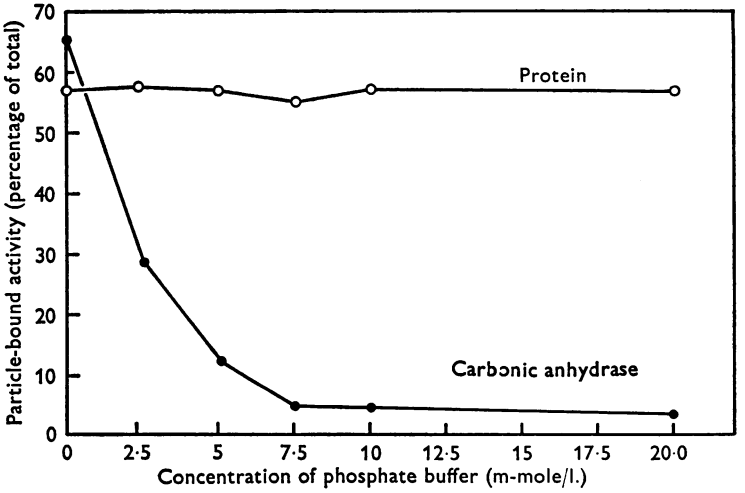

Fig. 7. Particulate carbonic anhydrase activity (●) and particulate protein (○) (expressed as percentages of whole homogenate carbonic anhydrase activity and protein respectively) plotted against the concentrations of sodium phosphate buffer, pH 7·0, in successive washing media. The homogenate was prepared in 0·3 M sucrose.

the particulate matter. Secondly, it was observed that a very high proportion of the total enzymic activity was particle-bound when an homogenate was adjusted to pH 5·5 before centrifugation (Fig. 6). However, it was also noted that whereas at pH 7·0 50% of the whole homogenate protein was particulate, at pH 5·5 over 80% of the whole homogenate protein was particulate. Thirdly, the particle-bound enzyme activity (using 0·3 M sucrose) was eluted by washing the particulate fraction with dilute solutions of sodium phosphate in 0·3 M sucrose (Fig. 7).

DISCUSSION

'Activators' of carbonic anhydrase

The present work has led to a revision of our earlier estimates of the carbonic anhydrase activities of blood and gastrointestinal tissues (Carter & Parsons, 1968*a*). This revision follows our observation that plasma albumin and EDTA increase the measured rates of hydration catalysed by tissue extracts. Carbonic anhydrase 'activation' has received much attention in the past and was thoroughly reviewed by van Goor (1948) The 'activating' substances include many proteins, peptides, amino acids and other substances. A property that these substances have in common is the ability to bind or to chelate metals. The apparent activation of carbonic anhydrase found in the present experiments is therefore believed to result from the binding of inhibitory metals by the 'activator'. This explanation is consistent with the observation that EDTA at very low concentrations produced a sixfold stimulation of carbonic anhydrase activity (Ho & Sturtevant, 1960). However, these authors claimed to have evidence that the activation was not due to the binding of inhibitory metals by EDTA, and suggested that the activation was due to a conformational alteration in the enzyme induced by the binding of EDTA to the zinc atom of the enzyme.

Buffer salts may not be the sole source of inhibitory agents. As van Goor (1948) has pointed out, activation may be found even when crude homogenates are used, and it might be expected that the enzyme would be adequately shielded from inhibitory metals by the large excess of protein in the homogenates. A plot of the catalysed rate of hydration of carbon dioxide against the amount of an homogenate added to the reaction vessel was always linear (Fig. 1). If the enzyme were partially inhibited this linearity would suggest that the homogenate itself contained an inhibitory agent. We note that Lindskog (1960) found appreciable quantities of Cu in a highly purified preparation of bovine carbonic anhydrase.

Subsequent to these findings, the reaction mixture was routinely supplemented with EDTA (1 mM) and with bovine plasma albumin (25 mg/l.). Chelating agents have not been used when measuring carbonic anhydrase

activity in the past, and it is probable that much previous work was bedevilled by the presence of inhibitory metals. The not insubstantial literature dealing with the 'activation' of carbonic anhydrase is proof of that (van Goor, 1948). More recently, unexplained variations in activity in the course of subcellular distribution studies (Karler & Woodbury, 1960; Bernstein *et al.* 1968), during purification of the enzyme (Sen *et al.* 1963) and in serological investigations (Wistrand & Rao, 1968) may well have been due to the presence of a chelatable inhibitor. When precautions are taken to exclude inhibitory metals from the assay system, the somewhat surprising fact emerges that no less than four tissues of the gastrointestinal tract possess more carbonic anhydrase activity per unit amount of protein than does the whole blood (Table 3).

Tissue distribution of carbonic anhydrase isoenzymes

The guinea-pig tissues studied contained either one or both of two carbonic anhydrase isoenzymes. The two isoenzymes are distinguished most strikingly by the difference in their enzymic activities in respect of the reactions between carbon dioxide, bicarbonate and water. We wish to consider here the physiological significance of the isoenzymes, using also the information obtained from the tissue distributions of the enzymes.

Both the 'high activity' and the 'low activity' enzymes coexist within the same erythrocytes (Hansson, 1965), but clearly the occurrence of a much less active isoenzyme cannot be construed as a physiological advantage if its function is solely that of catalysing the reactions between carbon dioxide and water. It has been implied that in erythrocytes *in vivo* the human 'high activity' and 'low activity' carbonic anhydrases would both contribute substantially to the total catalysed rates of hydration and of dehydration (Edsall, 1968), but other kinetic measurements suggest that it is not true for the guinea-pig (Carter & Parsons, 1970*a*) and rat (McIntosh, 1969) erythrocyte carbonic anhydrases (however, no measurements have yet been made at 37° C). In the mucosa of the large intestine, which also contains both 'high activity' and 'low activity' isoenzymes, the precise cell locations of the isoenzymes are unknown. If the two isoenzymes are sited in different types of cells, it is possible that the low capacity of the 'low activity' isoenzyme means that the catalysed reaction is a rate-limiting step.

Studies with other vertebrate tissues have given the impression that carbonic anhydrase is essentially a soluble cell constituent (Datta & Shepard, 1959; Karler & Woodbury, 1960; Shepard, 1962; Mitchell, Mitchell & Hanahan, 1965; Bernstein *et al.* 1968), though a membrane-associated role in ion-transporting epithelia has been proposed on the basis of physiological (Rector, Carter & Seldin, 1965) and histochemical

(Hansson, 1968) observations. A carbonic anhydrase in the mid-gut epithelium of lepidoptera larvae is entirely associated with particulate cell material when the tissue is homogenized under conditions similar to those used here (Turbeck & Foder, 1970). Using a conventional homogenizing medium, 45% of the 'high activity' carbonic anhydrase of the proximal colonic mucosa was located in particulate matter, and reasons have been given for believing that this may be the more usual situation in regard to the carbonic anhydrase activity of mammalian tissues. The carbonic anhydrase in the colonic mucosa was associated with the light membrane fractions and with the nuclei and microvillous fraction.

In the present work some carbonic anhydrases were isolated in a highly purified form, not only from erythrocytes but also from the stomach, colonic mucosa and caecal mucosa of the guinea-pig, and the enzymes obtained from these tissues were compared. Thus, according to some of their physical and kinetic properties, the 'high activity' enzymes were indistinguishable from one another whatever the tissue source, and the same was also true for the 'low activity' enzymes (Carter & Parsons, 1970*a*, *b*). While both forms were found in the erythrocytes, the stomach contained almost exclusively the 'high activity' isoenzyme and the caecal mucosa contained predominantly the 'low activity' isoenzyme. The colonic mucosa contained both forms in approximately the same proportions as they occurred in the erythrocytes, while the small-intestinal mucosa contained both isoenzymes in only small amounts. There seems to be a selective tissue distribution that is peculiar to each of the two major isoenzymes, for the two enzymes are not invariably associated with one another. The findings imply a functional difference between the two kinds of carbonic anhydrase, and despite its relatively poor catalytic ability the 'low activity' carbonic anhydrase gains a special importance from these findings that hitherto it has lacked. In contrast, the data of McIntosh (1969) suggest that the 'high activity' carbonic anhydrase present in rat prostate gland is different in important respects from the erythrocyte 'high activity' carbonic anhydrase of that species.

Possible physiological role of isoenzymes of carbonic anhydrase

The idea that carbonic anhydrase in erythrocytes is concerned simply with the catalysis of the reactions between carbon dioxide and water has so far stood the test of time (Roughton, 1964). With respect to carbonic anhydrase activity, the 'high activity' enzyme is presumably the more important of the two enzymes, and it can be considered to be an unregulated enzyme catalysing an event which leads to the uptake of carbon dioxide by blood and excretion of carbon dioxide through the lungs. We have found 'high activity' carbonic anhydrase in high concentrations in

the stomach and the proximal colonic mucosa, but only at low concentrations in the small-intestinal mucosa and distal colonic mucosa. In the past, the carbonic anhydrase activity of tissues other than blood has intuitively been connected with the transport of hydrogen ions, carbon dioxide and bicarbonate ions. There is evidence which suggests that the relation between carbonic anhydrase and these transport processes is tenuous (Carter, 1970). For example, the ileum, proximal colon and distal colon, while morphologically discrete, handle saline solutions containing carbon dioxide-bicarbonate in very similar fashions, and each of these segments of the gut maintains a quantitatively similar hydrogen ion gradient between artificial saline solutions and plasma (Parsons, 1956; Swallow & Code, 1967; Edmonds, 1967). Thus the distribution of 'high activity' carbonic anhydrase does not suggest a close link between that enzyme and the transport of hydrogen ions and carbon dioxide-bicarbonate ions. We cannot at present relate the sharply restricted tissue distribution of 'high activity' carbonic anhydrase in the gastrointestinal tract to any physiological event peculiar to the tissues in which it occurs.

The classical theory concerning blood carbonic anhydrase must remain incomplete while the functions of the isoenzymes in the stomach and in the large-intestinal mucosa remain unknown. If the presence of carbonic anhydrase in tissues other than blood is not related to certain ion-transporting functions, there remains the dubious notion that the enzyme is concerned simply with 'pH regulation' (see Bonting, Caravaggio, Canady & Hawkins, 1964). It is possible that carbonic anhydrase is present in some cells in order to facilitate the passage of carbon dioxide from tissues. The direct product of the reactions catalysed by urease and yeast carboxylase is carbon dioxide (Krebs & Roughton, 1948), and carbonic anhydrase would be of no value where these enzymes are concerned. However there is a certain amount of evidence that bicarbonate ions or carbonic acid can be products of oxidative metabolism in mammalian brain tissue (see for example, Severinghaus, Hamilton & Cotev, 1969). The mitochondria-associated carbonic anhydrase activity (Tables 5 and 6) may therefore be connected with carbon dioxide excretion at the subcellular level.

The 'low activity' carbonic anhydrase is most abundantly present in the caecal mucosa. The sharpness with which the distribution of 'low activity' carbonic anhydrase dissociates the mucosa of the large intestine from that of the small intestine suggests that the presence of the 'low activity' isoenzyme may be connected with the presence of micro-organisms. A possible connexion between carbonic anhydrase and the absorption of volatile fatty acids from the rumen has already been suggested (Aafjes, 1967). The suggestion deserves attention in relation to the occurrence of carbonic anhydrase in the guinea-pig large intestinal mucosa.

Another product of microbial action is ammonia, and in non-ruminant animals by far the largest and most constant source of ammonia is the large intestine, especially the caecum (Folin & Denis, 1912; Parnas & Klisiecki 1926; Silen, Harper, Mawdsley & Weirich, 1955). This ammonia may be of nutritive value (see, for example, Richards, Metcalfe-Gibson, Ward, Wrong & Houghton, 1967; Snyderman, 1967). It is formed by the break-down of proteins and urea, and there is evidence that a special carbon dioxide/bicarbonate-dependent mechanism exists for its absorption (Mossberg, 1967). The possible importance of carbonic anhydrase in connexion with ammonia was considered by Meldrum & Roughton (1933) in their report of the purification and properties of ox blood carbonic anhydrase.

Evidence for a close relationship between carbonic anhydrase and ammonia is to be found in several tissues. The kidney has already received a good deal of attention in that respect (Pitts, 1963). That carnivorous animals excrete more acid urine than herbivorous animals has been known for more than a century (Bernard, 1856) and renal carbonic anhydrase is said to be involved in the secretion of hydrogen ions (Pitts, 1963). But the titratable acidity of the urine of carnivorous animals is increased by the simultaneous excretion of ammonia (Walter, 1877), and it is therefore possible that carbonic anhydrase is also concerned with that process. Thus, not only do the kidneys of herbivorous animals secrete less acid urine than do carnivores, they also secrete much less ammonia (Beccari, 1917) and contain much less carbonic anhydrase activity (van Goor, 1948). The guinea-pig kidney contains very small quantities of both 'high activity' and 'low activity' isoenzymes (unpublished observations). A search for 'low activity' carbonic anhydrase in the kidneys of omnivorous animals would be of value. Carbonic anhydrase has also been implicated in the excretion of ammonium ions by fish gills (Maetz & Garciá Romeu, 1964).

In the reproductive systems of both birds and mammals the movements of ammonia seem related to the presence of carbonic anhydrase. Thus in the shell gland of birds carbonic anhydrase and ammonia are seen as playing closely interrelated parts in shell formation (Campbell & Speeg, 1969). In rabbits a rise in the carbonic anhydrase activity of the endometrium during pregnancy (Lutwak-Mann, 1955) parallels the appearance of much ammonia in the venous effluent from that organ (Parnas & Klisiecki, 1926).

A search for the counterparts of guinea-pig 'low activity' carbonic anhydrase in some tissues might establish a broader correlation between that sort of carbonic anhydrase and ammonium transport and metabolism, leading to a more concrete link than can be given here. Such a search has recently revealed the presence, in ox rumen epithelium, of 'low

activity' carbonic anhydrase. In its quantity, and in its properties, the rumen enzyme is comparable with the caecal mucosal 'low activity' isoenzyme (Carter, 1971).

We conclude that a considerable amount remains to be discovered about carbonic anhydrase, for there are two isoenzymes, differing very greatly in their kinetic and chemical details, and differing in their tissue distribution, for which no clear role is apparent. Whatever are the functions of the carbonic anhydrase isoenzymes, these may involve the catalysis of physiological reactions other than those between carbon dioxide, bicarbonate and water.

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