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STUDIES ON A CARBONIC ANHYDRASE FROM THE MIDGUT EPITHELIUM OF LARVAE OF LEPIDOPTERA

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SUMMARY

1. Carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) is demonstrated in the epithelium of the larval midgut of six species of lepidoptera.

2. With 0.25 M sucrose as the homogenizing medium, all activity was found in the particulate fractions.

3. The distribution of carbonic anhydrase among the particulate fractions was not parallel to that of succinate cytochrome *c* reductase, which was used as a mitochondrial marker enzyme; the lighter fractions contained relatively more carbonic anhydrase activity.

4. The effect of various solubilization procedures was studied. Repeated freezing and thawing had no effect. Treatment with deoxycholate, Tween-80 or sonication released some of the activity in an active soluble form, but the total activity of the homogenate was not increased.

5. The enzyme is sensitive to anions. In 10 mM phosphate as basic medium, thiocyanate inhibits at all concentrations ranging from 0–100 mM. Sulphate, chloride, nitrate, bromide, and iodide all stimulate the activity with a maximum at 10–25 mM.

6. The enzyme was demonstrated histochemically in the goblet cell of the epithelium; the columnar cell was devoid of reaction. The precipitate was grainy and localized in the luminal part of the cell.

7. pH and total CO_2 of larval haemolymph and gut contents were determined and the physiological role of the midgut carbonic anhydrase is discussed in relation to these data.

INTRODUCTION

Carbonic anhydrase is present in the epithelium of the intestinal tract of mammals, amphibia and other classes[1]. In the gastric mucosa the enzyme is believed to participate in the sequence of reactions leading to secretion of H^+ (ref. 2), whereas its role in other parts of the digestive tract is obscure. In larvae of lepidoptera feeding on a natural diet the pH of the haemolymph is in the region of 6.5 and the pH of the

intestinal contents about 10 (ref. 3). The mechanism responsible for the maintenance of this hydrogen ion concentration gradient is not known.

The isolated midgut of the larva of *Hyalophora cecropia* transports potassium actively from the haemolymph to the lumen. Potassium carries about 90% of the short-circuit current[4]. In the presence of 10^{-5} M ethoxzolamide, a carbonic anhydrase inhibitor, the short-circuit current was inhibited by 36%. This finding was correlated with the pH gradient across the epithelium and a potassium–hydrogen ion linked transport was suggested[5]. The presence of carbonic anhydrase in lepidoptera midgut epithelium does not seem to have been demonstrated earlier. In the midgut of *Philosamia cynthia*, a species closely related to *H. cecropia*, no carbonic anhydrase was found[2]. The enzyme is present in tissues but not in blood of pupae of *H. cecropia* and *Agapema galbina*[6]. In orthoptera, carbonic anhydrase has been demonstrated in preparations of gut from adult insects[7].

The present study was designed to determine whether carbonic anhydrase is present in the larval midgut epithelium of lepidoptera and to explore the properties and subcellular distribution of the enzyme.

MATERIALS

The larvae of the species *Hyalophora cecropia*, *Aglais urticae*, *Pieris brassicae* and *Vanessa io* were all reared indoors and fed on a natural diet. Larvae of *Phalera bucephala* and *Cosmotriche potatoria* were collected in nature. The later instars of the larvae were used. The larvae were killed by decapitation and the midgut dissected out and freed from Malpighian tubules. The gut was opened and the perithrophic membrane was removed together with the intestinal contents.

The epithelium was separated from the muscular tissue by gentle treatment in a Dounce homogenizer (Kontes Glass Co., Vineland, N.J.) in 0.25 M sucrose at 0° followed by filtration through a nylon cloth. The filtrate was homogenized in a Teflon-glass homogenizer for 1 min. The homogenate was centrifuged at $600 \times g$ for 5 min and the sediment containing the unbroken cells and the nuclei was discarded. The supernatant is referred to as 'total homogenate'.

METHODS

Carbonic anhydrase assay

The pH-stat method was chosen because of its advantage over the various spectrophotometric and electrometric pH-changing methods, especially when enzyme preparations with high protein content are assayed. In the pH-stat method a low buffer concentration can be used, thus avoiding specific ion effects. The technique has been described earlier[8], but owing to the small amounts of tissue available, the volume of the reaction medium was reduced from 10 to 1 ml. The reaction chamber was correspondingly smaller in size and the sintered glass disc in the bottom of the chamber was replaced by a canula in a rubber stopper. The reaction medium contained 10 mM orthophosphate, 0.5 mM EDTA and 50 mM $NaHCO_3$ (initial concentration) in a volume of 1 ml. The dehydration reaction was followed at 0.1° ± 0.1° and pH 7.30 ± 0.02 by automatic titration with 0.2 M H_2SO_4 delivered from a microsyringe. The quanta of acid added were registered every minute. A stream of fine bubbles of nitrogen

saturated with water vapour at the experimental temperature was maintained through the reaction chamber. The nitrogen flow was 19 ml/min measured at 20°. The spontaneous dehydration reaction was run for 10 min, at the 10th minute the enzyme was added and the reaction followed for 5 min. The rate of the spontaneous reaction v_{sp} was calculated by linear regression from the amounts of acid added from the 6th to the 10th minute, and the rate of the enzyme-catalyzed reaction v_e was calculated from the values of the 11th to the 15th minute. The enzyme activity A in terms of units was calculated from

$$A = \frac{v_e}{v_{sp} \cdot 0.883} - 1$$

and expressed in units per mg protein. The constant 0.883 was determined from 10 assays in which water was added at the 10th minute instead of enzyme; the standard deviation was 0.071. The constant corresponds to an observable first-order constant of the spontaneous dehydration reaction at pH 7.30 and 0° of $3.9 \cdot 10^{-4}$ sec^{-1}, a value which agrees with those given in the literature[9]. Between A and the enzyme concentration there is a linear correlation in the range $0 < A < 1$. At higher values of A a calibration curve was used. When $A > 2$ the activity was redetermined with a lower enzyme concentration. All assays were in duplicate. In 10 assays with $A_{mean} = 1.22$, the standard deviation was 0.106. When solubilizing reagents such as Tween-80, deoxycholate and digitonin had been used in the preparation of the enzyme, 10 μl silicone anti-foam emulsion (Wacker Chemie) was added prior to the addition of enzyme. Control experiments showed that neither the spontaneous nor the enzyme-catalyzed reaction was influenced by the presence of the anti-foam emulsion.

Succinate cytochrome c reductase

The technique of DE DUVE *et al.*[10] was employed using a Beckman DB spectrophotometer with a 10-inch recorder. The temperature of the cell compartment was maintained at 25° by means of a thermostat. The final incubation medium contained 33 mM potassium phosphate buffer, 27 mM nicotinamide, 0.2 mM sodium cyanide, 0.04 mM oxidized cytochrome *c*, and 33 mM sodium succinate. The final volume was 3.0 ml. The change in absorbance was followed at 550 mμ in a cell with a light path of 1 cm. The enzyme preparation was diluted with the homogenizing medium and 0.2 ml of this mixture was preincubated in the cell in the presence of all components except succinate. The latter was added in a volume of 0.3 ml to start the reaction. The preincubation was run for 3 min to allow the endogenous activity to be estimated. The incubation with succinate was run for 5 min and was approximately linear during this period. The unit used was 1 μmole of substrate converted per minute under the conditions described.

Protein

The method of LOWRY *et al.*[11] was used.

pH

The pH of haemolymph and intestinal contents was measured by a Radiometer pH meter 26 equipped with a microelectrode type G 297 or a glass electrode type G 202 C and a calomel electrode type K 401. The standard temperature 0° for the measurement of pH in both haemolymph and intestinal contents was chosen because the coagulation rate of the haemolymph is low at this temperature.

Total carbon dioxide

This was determined by the method described by CONWAY[12].

Histochemical demonstration of carbonic anhydrase

Since thin frozen sections of the midgut epithelium disintegrate when floated and thawed on the surface of the incubation medium, the following procedure was developed. The midgut was treated in an all-glass Dounce homogenizer (Kontes Glass Co, Vineland, N.J.) with a loosely fitting pestle, in a cold fixative consisting of hydroxyadipaldehyde 10% in 0.1 M sodium cacodylate buffer (pH 7.3). By careful treatment the epithelium could be separated from the muscle layer as single cells and clusters of cells. The suspension was fixed for approximately 16 hours at 4°. The histochemical method used was that described by HANSSON[13]. The supernatant was decanted from the sedimented cells and one volume of the sediment was mixed with three volumes of freshly prepared incubation medium containing 1.17 mM phosphate. Drops of this suspension were placed on a Millipore filter (type SMWPO-190 R) which was immediately floated on the incubation medium for 5 min. To the medium of the controls $6 \cdot 10^{-6}$ M acetazolamide was added. The filters were counterstained with hematoxyline and eosine, dehydrated and mounted.

Electron microscopy of homogenate fractions

A sample of the rehomogenized sediment was mixed with 1% osmium tetroxide in 5,5-diethylbarbiturate and acetate buffer (pH 7.4) and fixed for 1 hour. After centrifugation at 75 000 × *g* for 30 min, the pellet was imbedded in vestopal, sectioned and examined in a Hitachi HU 11E electron microscope.

RESULTS

Carbonic anhydrase content of lepidoptera larval midgut

Table I shows that the midgut epithelium of the six examined species of lepidoptera contains carbonic anhydrase. *H. cecropia* is an American species, while the remainder are Danish species. The enzymic activity was in the range 0.2 to 2.1 units per mg protein. Haemolymph from *H. cecropia* was found to be without carbonic anhydrase activity; in insect species where the haemolymph has no respiratory function, it contains no carbonic anhydrase[6].

TABLE I

CARBONIC ANHYDRASE ACTIVITY IN LARVAL MIDGUT OF SOME SPECIES OF LEPIDOPTERA

The enzyme preparation assayed was the total homogenate. The results are expressed in units per mg protein and are mean values of *n* experiments ± S.E.

Species	*n*	*Carbonic anhydrase activity*
Hyalophora cecropia	6	2.1 ± 0.19
Pieris brassicae	2	0.7 ± 0.1
Phalera bucephala	2	0.4 ± 0.1
Vanessa io	2	0.5 ± 0.3
Aglais urticae	1	0.2
Cosmotriche potatoria	1	0.5

TABLE II

INTRACELLULAR DISTRIBUTION OF CARBONIC ANHYDRASE AND SUCCINATE CYTOCHROME *c* REDUCTASE IN LARVAL MIDGUT EPITHELIUM OF *H. cecropia*

The total homogenate was centrifuged at 2000 × *g* for 10 min. The sediment was suspended by gentle homogenization and assayed. The supernatant was centrifuged at 5000 × *g* for 10 min and the resulting sediment was resuspended and assayed. The procedure was repeated according to the data shown in the left column of the table. Results are expressed in units per mg protein as the arithmetic mean of 4 experiments ± S.E.

Fraction	*Carbonic anhydrase*	*Succinate cytochrome c reductase*
2000 × *g* · 10 min sed.	1.3 ± 0.45	1.5 ± 0.31
5000 × *g* · 10 min sed.	3.7 ± 0.84	1.7 ± 0.81
10 000 × *g* · 10 min sed.	4.4 ± 1.9	1.5 ± 0.11
40 000 × *g* · 10 min sed.	3.4 ± 0.6	0.2 ± 0.04
90 000 × *g*. 60 min sed.	0	0
90 000 × *g*. 60 min supernat.	0	0

Subcellular distribution of carbonic anhydrase

Following a differential-centrifugation procedure described in the legend of Table II, the fractions were assayed for carbonic anhydrase and succinate cytochrome *c* reductase, an enzyme which is believed to be truly mitochondrial. It is seen that both enzymic activities are confined to the particulate matter. However, the ratio between carbonic anhydrase and succinate cytochrome *c* reductase activity is not constant, but is higher in the lighter than in the heavier fractions.

Effect of solubilization procedures

The results obtained on exposing the total homogenate to various physical and chemical treatments are shown in Table III. Repeated freezing and thawing affected neither the total activity nor the distribution of the activity between the particulate

TABLE III

EFFECT OF SOLUBILIZATION PROCEDURES ON CARBONIC ANHYDRASE FROM *H. cecropia* MIDGUT

Samples of the total homogenate were treated in different ways, all procedures performed at 0°. Freezing and thawing indicates that a sample of the homogenate was frozen and thawed three times. Sonication was performed in three periods of 10 sec each, the homogenate and the probe of the ultrasonic power unit (MSE series 30216) being allowed to cool in the intervals. Tween-80 or sodium deoxycholate was added at the concentration indicated, the sample rehomogenized for 30 sec and incubated for 60 min. After any of these treatments, the homogenate was centrifuged at 100 000 × *g* for 30 min, the supernatant decanted and the sediment rehomogenized in 0.25 M sucrose. The volumes of the two fractions were determined, samples were assayed and the total enzymic activity was calculated.

Treatment	*Total carbonic anhydrase activity*		
	Sediment	*Supernatant*	*Total*
No treatment	110	0	110
Freezing and thawing	110	0	110
Sonication	75	36	111
Tween-80 5%	28	12	40
Deoxycholate 1%	93	20	113

TABLE IV

pH AND TOTAL CO_2 OF FIFTH INSTAR *H. cecropia* HAEMOLYMPH AND INTESTINAL CONTENTS

Results are expressed as the arithmetic mean ± S.E. Values in parentheses refer to number of experiments.

	pH (at o°)	*Total CO_2 (mM)*
Haemolymph	7.0 ± 0.042 (9)	4.7 ± 0.70 (6)
Intestinal contents	10.6 ± 0.17 (8)	51.3 ± 6.4 (7)

and soluble fraction. Sonication lowered the activity of the sediment, while there was a corresponding increase in the supernatant giving an unaltered total activity. A similar result was obtained with deoxycholate. Treatment with Tween-80 lowered the total activity, although some activity appeared in the supernatant.

Inhibition by acetazolamide

A total homogenate of *H. cecropia* was centrifuged 7000 × *g* for 10 min and the rehomogenized sediment was assayed for carbonic anhydrase by the standard technique. Varying concentrations of acetazolamide were added to the medium and the I_{50} (50% inhibition value) was calculated as $3 \cdot 10^{-8}$ M.

Effect of anions

The midgut carbonic anhydrase is sensitive to anions at concentrations ranging from 0–100 mM (Fig. 1). Thiocyanate inhibits at all concentrations, whereas the other anions examined stimulate the activity with a maximum at 10–25 mM. Bromide and iodide have less effect than sulphate, chloride and nitrate.

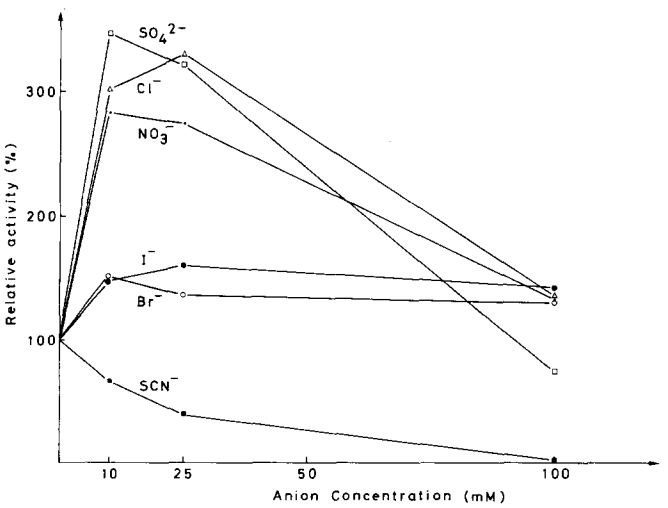

Fig. 1. Influence of anions on *H. cecropia* midgut carbonic anhydrase. The total homogenate was centrifuged at 100 000 × *g* for 30 min and the sediment rehomogenized in 0.25M sucrose. The potassium salts of the anions mentioned were added to the standard assay medium containing 10 mM phosphate and 0.5 mM EDTA.

pH and total CO_2 of H. cecropia haemolymph and intestinal contents

H. cecropia was chosen because the transepithelial potential and other relevant biophysical data are known for this species, as it has been the object of several investigations[4,5,14].

Histochemical demonstration of carbonic anhydrase

The single-layered epithelium of the larval midgut of lepidoptera consists of three types of cells, columnar cells, goblet cells and regenerative cells; these last ones, placed basally, are supposed to replace degenerating epithelial cells at each ecdysis and their number and appearance varies through larval life[16]. The fine structure of the midgut epithelium of *H. cecropia* has been studied by electron microscopy[17]. No regenerative cells were seen, but it should be noted that only mature fifth instar larvae were used. We can establish that apart from the presence of regenerative cells, the morphology of the midgut epithelium of *A. urticae* closely resembles that of *H. cecropia*. Luminally the columnar cell has a regular array of microvilli and in the basal region numerous deep infoldings with mitochondria oriented parallel to these. The goblet cell has been given this name because of its similarity to the vertebrate goblet cell, but in contrast to the latter it does not secrete mucus, and the internal structure is quite different. It bears a certain resemblance to the mammalian parietal cell of the gastric mucosa; the goblet cell chamber may be compared with the intracellular canaliculi of the parietal cell. The goblet cell chamber is lined with numerous cytoplasmic projections, each of which contains an elongated mitochondrion. In the parietal cell the projections are devoid of mitochondria, which are, however, extraordinarily abundant in the cell itself. Both the goblet cell and the parietal cell have scanty reticulum. In the luminal part of the goblet cell some of the projections are subdivided into villus-like units, forming a canal through which the cell chamber is confluent with the lumen of the gut. These villi are devoid of mitochondria, but scattered around the apical pole of the cell there are mitochrondria, various dense bodies and multivesicular bodies. This is the region in which the carbonic anhydrase is demonstrated histochemically, see Fig. 2.

The left part of the figure shows two goblet cells with their basally located nuclei

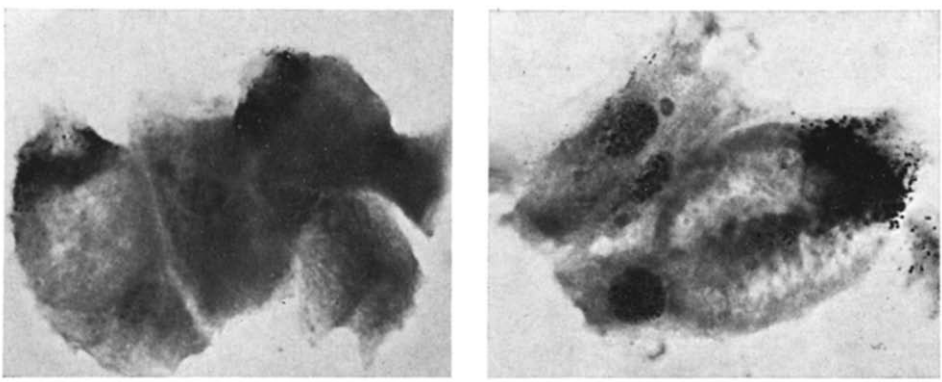

Fig. 2. Epithelial cells from the larval midgut of *A. urticae*. Histochemical demonstration of carbonic anhydrase. Magnification × 730.

and between them two columnar cells with centrally located nuclei. On the right part of the figure a swollen goblet cell is seen together with two columnar cells. A positive reaction for carbonic anhydrase is seen in the luminal poles of the goblet cells, whereas the columnar cells are devoid of reaction. No regenerative cells could be identified. In control experiments in which 10^{-6} M acetazolamide was added to the medium, no precipitate appeared. When clusters of cells were seen from their luminal pole, an annular distribution of the precipitate in the goblet cell was evident; thers is a suggestion of this in the cell to the extreme left of Fig. 2. The precipitate varied somewhat in amount, but it was always grainy and always located luminally.

Electron microscopy of homogenate fractions

Fig. 3 shows that a sediment with carbonic anhydrase activity contains, in addition to more or less intact mitochondria, electron-dense bodies, microsomal and vesicular particles.

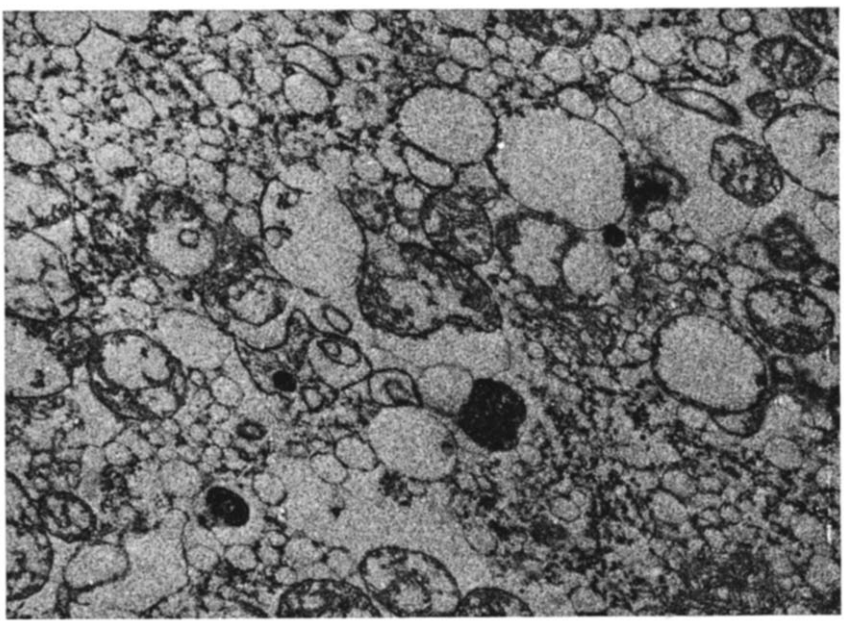

Fig. 3. Electron micrograph of material with carbonic anhydrase activity sedimenting at 5000 × *g* for 10 min following the procedure described in the legend of Table II. Magnification × 29 400.

DISCUSSION

The most striking property of the carbonic anhydrase from the larval midgut of lepidoptera is the fact that it is confined to the particulate fractions of the homogenate. Following a conventional homogenization technique with 0.25 M sucrose as medium, no activity is found in the soluble fraction.

Although carbonic anhydrase has been demonstrated in numerous tissues, there have been only a few studies of its subcellular distribution. The mammalian red

cell carbonic anhydrase, most thoroughly investigated, is a soluble constituent of the cell sap. In the avian shell gland the enzyme is found in the soluble fraction, while mitochondrial and microsomal fractions had very low activities[18]. In homogenates of mammalian kidney and cerebral cortex prepared in 0.25 M sucrose, the greater part of the carbonic anhydrase activity was found in the soluble fraction, the mitochondrial fraction contained 10–20% and the nuclear and microsomal fractions each about 5% of the total activity[19]. However, the identity of the subcellular particle containing the carbonic anhydrase was not established. In human lens about four-fifths of the activity was found in the particulate fraction, but when Tween-80 was added to the saline used as homogenizing medium, nearly all of the activity was found in the supernatant and the specific activity of the homogenate was increased about 70 times, suggesting a solubilizing effect of Tween[20].

Most or all of the carbonic anhydrase activity of the larval midgut is found in the fractions containing mitochondrial material, but the distribution of the activity between the fractions does not follow that of the applied mitochondrial tracer enzyme, succinate cytochrome *c* reductase. This does not rule out the possibility that the midgut carbonic anhydrase is mitochondrial, as it may be associated with the lighter mitochondrial fraction or with mitochondria having a weaker succinate cytochrome *c* reductase activity than the remainder of the mitochondrial population. The possibility that the enzyme is located in another organelle with sedimentation properties close to those of mitochondria should be considered. Electron micrographs of homogenate fractions showing carbonic anhydrase activity revealed their heterogeneity, and in the luminal part of the goblet cell where the enzyme is demonstrated histochemically both mitochondria, dense bodies and multivesicular bodies are found. The identity of the organelle containing the enzyme cannot be established at the light microscopical level. However, the distinctly granular appearance of the reaction product is in concordance with the results obtained by differential centrifugation. The author of the histochemical method used by us has recently investigated the carbonic anhydrase in a number of mammalian epithelia involved in active transport of electrolytes[21]. In distal renal tubules he found striated precipitates, which perhaps corresponded to mitochondria, but in none of the epithelia examined was there a staining pattern like that of larval goblet cells.

None of the solubilization procedures applied increased the total carbonic anhydrase activity of the homogenate. Thus, the enzyme does not seem to occur in a latent form, in a manner similar to that of many mitochondrial and lysosomal enzymes[22]. Freezing and thawing had no effect on the midgut carbonic anhydrase. An identical result was found with the carbonic anhydrase activity of the mitochondrial fraction from rat brain. The results obtained by treatment of the midgut homogenate with Tween-80 differ greatly from those obtained with lens homogenate; this may probably be explained by a different kind of binding of the enzyme to the subcellular particles of the two tissues. No reports of treatment of particulate carbonic anhydrases with sonication or with deoxycholate have been found, but membrane-bound enzymes are known to be released by these means.

Like carbonic anhydrase from red cells, that of the larval midgut is sensitive to anions, but there are differences in the pattern. Thiocyanate inhibits both enzymes in all concentrations examined, but complete inhibition occurs at 5 mM in the case of red cells[23] and at 100 mM with midgut enzyme. The stimulation of the midgut enzyme

in the range 10–25 mM by sulphate, chloride, nitrate, bromide and iodide is not seen with red cell enzyme and is possibly related to its particulate properties. The red cell enzyme is increasingly inhibited by these anions in the range 0–100 mM[23]. The order of effectiveness with which different anions inhibit the red cell enzyme corresponds to the lyotrophic series of Hofmeister, but this sequence is not applicable to the midgut enzyme.

The concentration of acetazolamide required to obtain 50% inhibition of the midgut carbonic anhydrase is $3 \cdot 10^{-8}$ M; this is in the range of carbonic anhydrases from mammalian and avian blood and from dog kidney[20].

In *H. cecropia* the total CO_2 of the haemolymph is about 5 mM and that of the intestinal contents about 50 mM. The pH values are 7.0 and 10.6, respectively. Since the pK_a values of carbonic acid are 6.3 and 10.3, it is possible to calculate the concentrations of HCO_3^- and CO_3^{2-}. The HCO_3^- concentration of the haemolymph is 4.2 mM and that of the gut contents 17 mM. The concentrations of CO_3^{2-} are 0.002 and 33 mM, respectively. The potential difference across the *H. cecropia* midgut epithelium measured *in vitro* is about 100 mV with the lumen side positive[4]. According to the Nernst equation this potential corresponds to a ratio of 50 between the concentrations of a monovalent ion and 2000 when the ion is divalent. The observed potential is sufficient to explain only the HCO_3^- concentration gradient across the epithelium. H^+ and CO_3^{2-} must be involved in an active transport mechanism and possibly carbonic anhydrase plays a role in this as it does in the gastric mucosa, where it is believed to facilitate the formation of HCO^-_3 from CO_2 and OH^- formed within the cell because of the secretion of H^+ (see ref. 2). In addition to the resemblance between the fine structure of the midgut goblet cell and that of the gastric parietal cell, the respective tissues have another point in common. An ATPase with alkaline pH optimum, which is stimulated by bicarbonate, has been demonstrated in both *H. cecropia* midgut[24] and in frog gastric mucosa[25], where it is believed to participate in the acid secretion.

The active potassium transport of the midgut is electrogenic and it has been shown that at least eight potassium ions are transported for every bicarbonate ion produced metabolically, even if all the oxygen is used for the potassium transport[14]. If ethoxzolamide is added to the bathing media of the biophysical experiment, the potassium transport is inhibited about one-third, indicating that it may be connected with a carbonic anhydrase-catalyzed reaction[5]. In this connection it should be noted that the goblet cell is thought to be the principal unit of the midgut potassium transport[17].

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