Histochemical localization of carbonic anhydrase in Malpighian tubules of *Culex pipiens*

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Summary. Carbonic anhydrase (CA) activity has been localized histochemically by Hansson's method in Malpighian tubules of *Culex pipiens.* The enzyme has been observed on membranes of the cytoplasmic inclusions of Malpighian cells; no CA activity has been found in other cytoplasmic structures. The possible meaning of the localization of the enzyme is discussed.

Carbonic anhydrase (CA) is an enzyme having paramount importance in kidney function as it is involved both in urine acidification and in bicarbonate tubular reabsorption[1-3] acting as a catalyst in the hydration of CO_2 and in the dehydration of the bicarbonate ions following the reactions:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3$$

The first studies on the CA activity of kidney were performed on mammals, using biochemical methods, by Davenport and Wilhelmi[4]; later Pollak et al.[5], using the same methods, showed the presence of the enzyme in distal and proximal tubules.

The enzyme is present in the kidneys of many vertebrate species, and a relationship between its activity, the excretion of more or less urine and concentration of ammonia is generally accepted. In this connection Van Goor[1] reports that CA activity is lower in herbivorous mammals than in carnivorous mammals, whose urine is more acid and contains more ammonia.

Many studies have been made, starting in 1958, on the histochemical localization of CA in various segments of nephrons, both in mammals and in other vertebrates. But, as far as we are aware no papers have been published describing studies at the histochemical level, on the Malpighian tubules of insects, even though the problem of CA presence in these excretory organs has been, and still is, the object of many studies. For example, Gooding[6] has shown that acetazolamide, a specific inhibitor of CA activity, influences urine production in *Glossina morsitans,* while other results[7,8] indicate that *Calliphora* and *Rhodnius* Malpighian tubules are insensitive to acetazolamide. In addition, Edwards and Patton[9] have reported the absence of this enzyme from the Malpighian tubules of *Acheta domestica* and recent research by Szibbo and Scudder[10] shows that HCO_3^- excretion by the Malpighian tubules of *Cenocorixa bifida* may play an important role in the regulation of haemolymph pH. In our laboratory the CA activity has been studied both in the stomach of some vertebrates[11-14] and in fowl kidney[15]. The consideration that insects are mostly uricotelic led us to extend our research to the Malpighian tubules of *Culex pipiens.*

These observations have been carried out using Hansson's method[16] which is specific but does not give sufficient resolution of the structures for sections obtained with a cryostat, and which is difficult to apply to observations at the electron microscope level. However, this problem may be avoided using the same method on semi-thin sections obtained after embedding the samples in hydrophilic plastic. This procedure does not interfere with the histochemical reaction and allows a better resolution of the different structures[14,17].

Materials and methods. Our observations were made on female imagos of *Culex pipiens,* obtained from laboratory colonies and fed on 0.5% sucrose solution. The Malpighian tubules, drawn under a Wild binocular, were collected in 0.7% NaCl solution and then fixed for 15 min at room temperature (22-24 °C) in 0.1 M pH 7.4 Millonig buffer containing 0.5% glutaraldehyde and 4% formaldehyde. This fixative was chosen since, in our earlier experiments[11,13-15], it gave the best results for preservation both of the structures and the enzymatic activity. The use of formaldehyde and glutaraldehyde as fixatives for CA histochemistry has

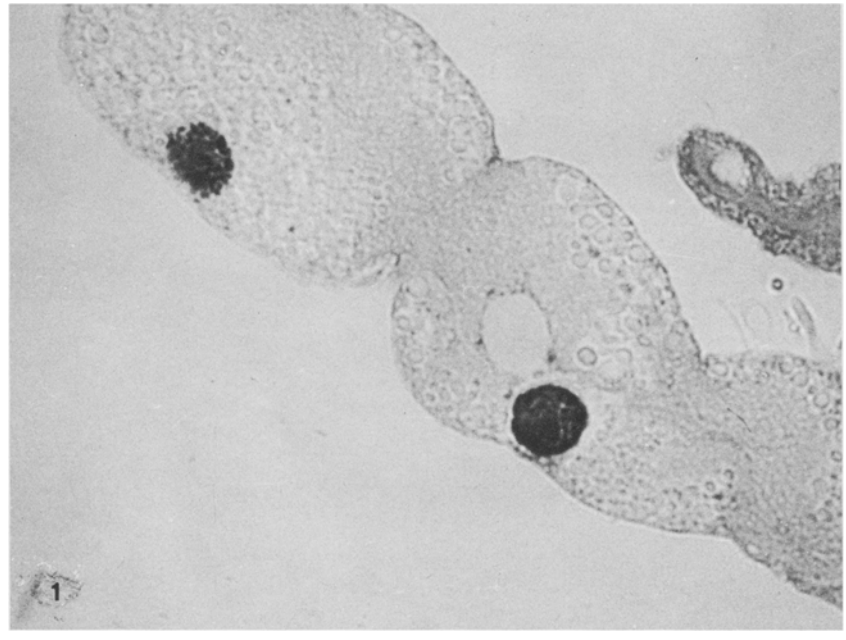

Fig. 1. Malpighian tubules of *Culex pipiens.* Embedded in type JB-4 (Polysciences) plastic; section, 2 μm thick, stained with Hematoxylin/Eosin, vesicular structure of the typical cytoplasmic inclusions is evident. × 360.

been suggested by various authors, among whom are Lonnerholm[18] and, recently, Sugai and Ito[19].

For morphological observations the tubules, after rinsing with Millonig buffer, were placed on slides and stained with Hematoxylin/Eosin.

Other samples were embedded in type JB-4(Polysciences) plastic; semi-thin sections of 1–2 µm were stained with Hematoxylin/Eosin by floating, and others were transferred, using Millipore filters, into the incubation medium where they stayed floating for 2–3 min at room temperature (22–24 °C).

After rinsing with distilled water, the samples were treated with $(NH_4)_2S$ (1%) for 3 min, rinsed again and then dried and placed on slides by means of a Technicon mounting medium.

The incubation medium[16] contains cobalt ions which precipitate as cobalt-phosphate complex and are displayed by blackening as cobalt-sulphide.

The controls were performed incubating the samples in the presence of 10^{-5} M acetazolamide, which acts as a specific inhibitor of CA.

The validity of this technique, and more generally of histochemical methods for CA demonstration, has been questioned by some authors who do not accept its specificity[20,21]. Other authors, such as Musser and Rosen[22] and Lightfoot and Cassidy[23] have a different opinion. The subject has already been treated by one of us[11,12] and an extensive critical study on the histochemistry of CA was made by Lonnerholm[18] in support of the specificity of Hansson's method.

Results and discussion. Figure 1 shows Malpighian tubules of *Culex pipiens* obtained using sections 1–2 µm thick. In particular, the vesicular structure of typical cytoplasmic inclusions is evident. Figure 2 shows the results of histochemical reaction. It is evident that the reaction is positive only at level of the membranes of the cytoplasmic inclusions. No positive reaction is present on the cellular membrane, on the nucleus or on other cytoplasmic structures. In samples incubated in Hansson's medium containing acetazolamide, the reaction is not present, except for some cytoplasmic vesicles, indicated by arrows (figure 3). These

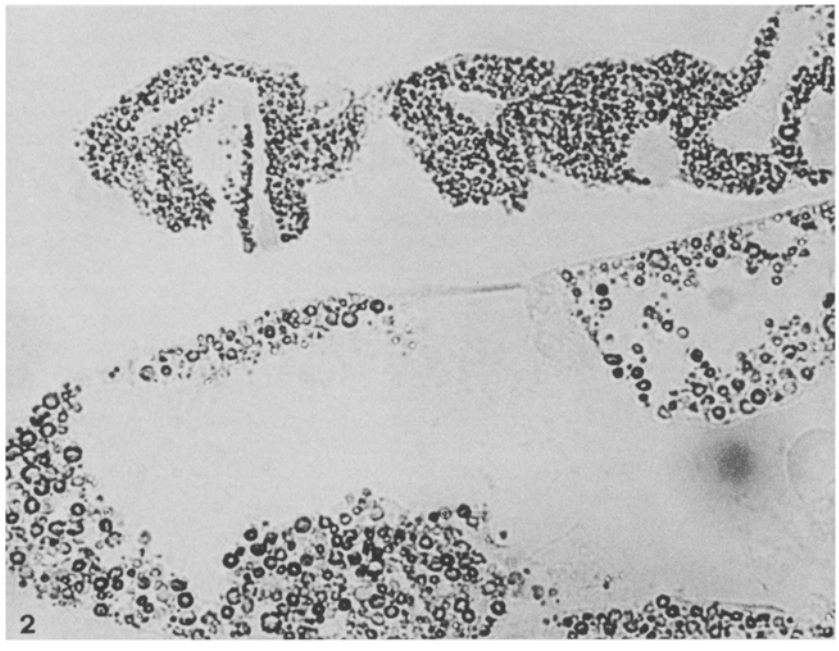

Fig. 2. Malpighian tubules of *Culex pipiens*. Histochemical reaction for carbonic anhydrase (CA) by Hansson's method. The CA activity is localized only on the membranes of the cytoplasmic inclusions. × 860.

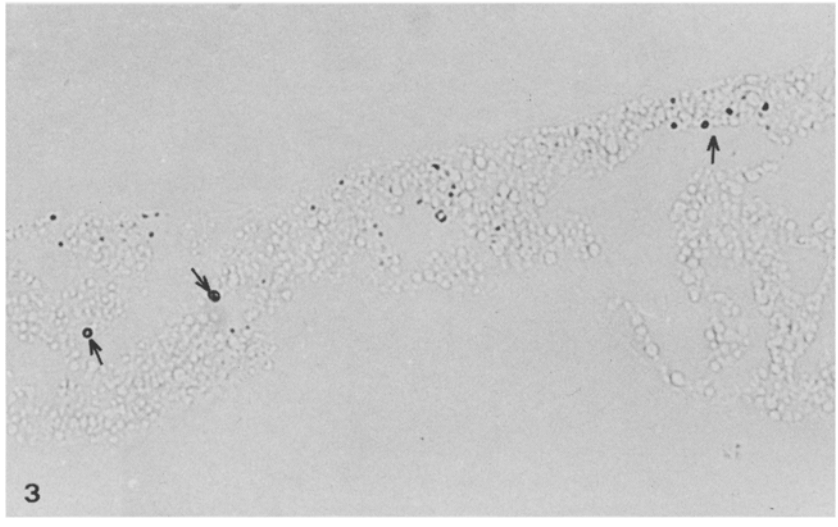

Fig. 3. Malpighian tubules of *Culex pipiens*. Histochemical reaction for carbonic anhydrase (CA) by Hansson's method, with addition to the medium of 10^{-5} M acetazolamide. The staining is present only in very few cytoplasmic vesicles (arrows). Explanation in the text. × 290.

dark stained vesicles, which also appear after inhibition, might be due to the presence, in some cytoplasmic inclusions, of phosphates[24] which, as observed by Lonnerholm[18], precipitate independently of CA activity, as well as carbonates[24] which might have analogous behaviour. Alternatively, they might also be due to an extraordinarily high enzyme concentration. The results obtained show that CA is present in Malpighian tubules of *Culex pipiens*. In particular, our observations show that CA activity is present on the membranes of the cytoplasmic inclusions (figure 2). The localization of this enzyme on cellular surfaces, on the other hand, has been observed already, at ultrastructural level, on the microvilli of the parietal cells in the gastric mucosa of rat and mouse[12,19,25]. No evidence of CA presence has been found at the level of the cellular membranes.

These results agree with what is known both about the enzyme function and about the characteristics of cytoplasmic inclusions, described by Berkaloff[26] and Smith[27]. In fact CA has been localized in the shell glands of birds' eggs and in the glands which produce the shell in molluscs[2], which means that this enzyme is involved in the mechanism of calcium deposition. Calcium is present in many granular formations of the cytoplasmic inclusions in Malpighian tubules[26].

Moreover the vesicles of the Malpighian tubule cells are responsible for the transfer, from the haemolymph to the tubular lumen, of the catabolites and particularly of the uric acid (urate granules[26]).

As already shown in mesonephros and metanephros of the fowl[15] and in practically all excretory organs of vertebrates, both histochemically and biochemically[1,2,28,29], the CA plays a determining role in the excretion process and in urine acidification in uricotelic, ureotelic and ammoniotelic animals. Therefore, it can be assumed that the presence of the enzyme in the Malpighian tubules is a 'physiological necessity' and that its localization on surfaces is necessary for ionic transport and for preservation of the cellular pH[2].

1 H. Van Goor, Enzymologia *13*, 73 (1948).
2 J.M. Carter, Biol. Rev. *47*, 465 (1972).
3 R.P. Steinmetz, Physiol. Rev. *54*, 890 (1974).
4 W.H. Davenport and E.A. Wilhelmi, Proc. Soc. exp. Biol. Med. *48*, 53 (1941).
5 E.V. Pollak, H. Mattenheimer, H. De Bruin and K. Weinman, J. clin. Invest. *44*, 169 (1965).
6 H.R. Gooding, Acta trop. *32*, 96 (1975).
7 M.J. Berridge, J. exp. Biol. *48*, 159 (1968).
8 S.H.P. Maddrell, J. exp. Biol. *51*, 79 (1969).
9 L.J. Edwards and R.L. Patton, J. Insect Physiol. *13*, 1333 (1967).
10 C.M. Szibbo and G.G.E. Scudder, J. Insect Physiol. *25*, 931 (1979).
11 P. Palatroni, Rc. Accad. naz. Lincei *56*, 249 (1974).
12 P. Palatroni, Rc. Accad. naz. Lincei *58*, 797 (1975).
13 P. Palatroni and G. Menghi, Riv. Ist. Norm. Pat. *21*, 7 (1977).
14 P. Palatroni, G. Gabrielli and B. Scattolini, Experientia *36*, 678 (1980).
15 S. Russo-Caia and P. Palatroni, Riv. Ist. Norm. Pat., *21*, 15 (1977).
16 J.H.P. Hansson, Acta physiol. scand. *73*, 427 (1968).
17 Y. Ridderstrale, Acta physiol. scand. *98*, 465 (1976).
18 G. Lonnerholm, Acta physiol. scand., suppl. 418 (1974).
19 N. Sugai and S. Ito, J. Histochem. Cytochem. *28*, 511 (1980).
20 F.T. Muther, J. Histochem. Cytochem. *20*, 319 (1972).
21 A. Churg, Histochemie *36*, 293 (1973).
22 L.G. Musser and S. Rosen, J. Histochem. Cytochem. *20*, 951 (1972).
23 G.F. Lightfoot and M.M. Cassidy, J. Histochem. Cytochem. *21*, 402 (1973).
24 P. Razet, Traité de Zoologie, Tome VIII, fasc. IV, 517 (1976).
25 S.A.M. Cross, Histochemie *22*, 219 (1970).
26 A. Berkaloff, Thesis Sci. nat., Paris 1961.
27 S.D. Smith, in: Insect cells. Their structure and function. Oliver and Boyd, Edinburgh 1968.
28 S. Rosen, Histochem. J. *4*, 35 (1975).
29 G. Lonnerholm, Acta physiol. scand. *81*, 433 (1973).

Immunocytochemical localization of cyclic AMP in *Tetrahymena*[1]

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Summary. Intracellular localization of adenosine 3',5'-cyclic monophosphate (cyclic AMP) in *Tetrahymena* was demonstrated immunocytochemically and changes in the staining pattern during culture growth and cell division were examined using an indirect immunofluorescent technique.

The presence of cyclic AMP, adenylate cyclase and cyclic AMP phosphodiesterase in *Tetrahymena* has been demonstrated biochemically, and changes in the levels of cyclic AMP were noted during culture growth and the cell cycle[3-6]. These studies suggested that cyclic AMP may possibly be a regulator in cell function. Intracellular localization of cyclic AMP in this organism has heretofore not been demonstrated, and we report here the immunocytochemical localization of cyclic AMP, and its changes during the culture growth and cell division in *Tetrahymena*. *T. pyriformis* NT-1 and *T. pyriformis* GL were grown at 39.5 and 28 °C, respectively, in enriched proteose-peptone medium, according to the method described by Thompson[7]. NT-1 cells were collected from the medium during the exponential and stationary phases and GL cells were harvested during the heat-synchronized cell division[6] by centrifugation at 500 × g. Pellets containing living cells were frozen immediately in an aluminium foil boat filled with OCT compound (Lab-Tek) by immersion in acetone and dry ice. Cryostat sections (10 μm) of unfixed samples were prepared and then airdried on glass slides. Localization of cyclic AMP was determined by an indirect immunofluorescent technique, as described by Steiner et al.[8]. Cyclic AMP-specific antiserum, prepared by the method of Steiner et al., was carefully screened for cross-reactivity with various other nucleotides and nucleosides using radioimmunoassay[9,10]. The cyclic AMP content in the cells was determined by the radioimmune method of Honma et al.[11].

Cyclic AMP fluorescence was localized in the cell membrane, cytoplasm, nuclear membrane, nucleus, cilia and