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SOLUBILIZATION OF ANION ATPase FROM NECTURUS OXYNTIC CELLS

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SUMMARY

An SCN^--inhibited, HCO_3^--stimulated ATPase (ATP phosphohydrolase, EC 3.1.6.3) has been shown to be localized in the oxyntic cells of Necturus gastric mucosa. Carbonic anhydrase is also associated with this cell type. High activity of the ATPase was found in a pure smooth microsomal fraction of a homogenate of these oxyntic cells. A method is described for solubilization of the enzyme resulting in a marked increase in the sensitivity to HCO_3^- and SCN^-. High activity of the enzyme is found in tissues of Necturus which transport H^+ and/or Cl^- and which have a high carbonic anhydrase content.

INTRODUCTION

Amphibian gastric mucosa has been shown to have an ATPase (ATP phosphohydrolase, EC 3.1.6.3) which is inhibited by SCN^- and stimulated by HCO_3^- (refs. 1, 2). This ATPase is found in high concentration in the microsomal fraction of the gastric homogenate[2,3] and its appearance in the stomach during ontogenetic development coincides with the development of acid secretory potential[4].

On the basis of these observations, it was postulated that this gastric ATPase was involved in ion transport across the luminal membrane of the oxyntic cell[1]. More specifically, it was suggested that the enzyme catalyzed a HCO_3^-–Cl^- exchange mechanism in the luminal membrane[5].

As, however, enzymes with similar properties were found to be present in other tissues[2] and since the methods used for gastric mucosa did not allow localization of the enzyme in a given cell type, the possibility remains that this ATPase is not involved in acid secretion.

In the present paper, suspensions of isolated oxyntic cells were prepared, and shown to contain in their smooth microsomal fraction a particulate ATPase of high specific activity and sensitivity to SCN^- and HCO_3. The enzyme was solubilized with significant increase in specific activity, HCO_3^- activation and SCN^- inhibition. In addition the distribution of the enzyme in different tissues of Necturus was examined.

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid.

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METHODS

Isolated oxyntic cells were prepared from Necturus gastric mucosa as previously described[6]. Five cell fractions were successively collected, with a progressive increase in oxyntic cell content. Oxyntic cells in the cell suspension were identified using the nitro BT stain for succinate dehydrogenase[7] as outlined in the previous paper[6]. Cell suspensions were homogenized in 0.25 M sucrose with 20 mM Tris–HCl (pH 7.4) using a teflon pestle homogenizer at 3000 rev. / min for 20 strokes.

The cell homogenate produced by the above methods was centrifuged to produce a crude nuclear ($1 \cdot 10^4 \times g \cdot$min), heavy mitochondrial ($1 \cdot 10^5 \times g \cdot$min), light mitochondrial ($7 \cdot 10^5 \times g \cdot$min), microsomal ($6 \cdot 10^6 \times g \cdot$min), and supernatant fraction.

In some experiments the $7 \cdot 10^5 \times g \cdot$min and $6 \cdot 10^6 \times g \cdot$min fractions were layered on a linear sucrose gradient (20–50 %). Bands were collected following 16 h centrifugation in an SW25 rotor, and the sucrose density was measured by an Abbe refractometer. Enzyme assay was carried out on each fraction.

An aliquot of each band was fixed in 1 % OsO_4, centrifuged and the pellet embedded in epon. Thin sections were then examined by light and electron microscopy.

Another aliquot of the band containing the highest ATPase specific activity was analyzed for RNA content by measurement of absorption at 260 nm and for phospholipid composition. For the latter, 1 ml of sample was incubated overnight with 25 ml of chloroform–methanol (1:1, by vol.), filtered and 3 ml saline added to the filtrate with mixing. The top layer was removed following centrifugation. The filter paper was washed with chloroform–methanol and this was again mixed with saline, centrifuged and the top layer removed. The bottom layers were combined, washed three times with saline–chloroform (1:1, by vol.) and then solid Na_2SO_4 was added to bottom layer of final washing. This was filtered, and the filtrate dried under N_2. The dried material was dissolved in chloroform and an aliquot taken for phosphate determination[8] and thin-layer chromatography[9].

Solubilization of the enzyme was carried out using Triton X-100. Other detergents, such as lubrol and sodium dodecyl sulfate were relatively ineffective. In preliminary studies, varying ratios of Triton: protein (as measured by the Lowry *et al.*

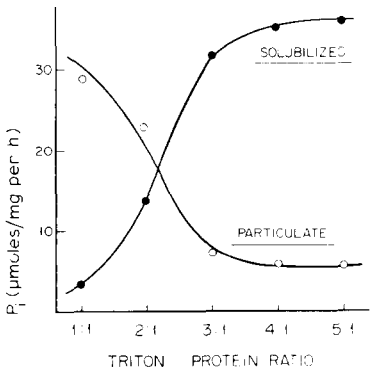

Fig. 1. The effect of varying Triton: protein ratio on solubilization of the HCO_3^--activated ATPase showing that at about a 3:1 ratio the maximal solubilization is obtained.

method) were used. Since it was shown that a Triton: protein ratio of 3:1 resulted in a high specific activity of the supernatant (Fig. 1) and gave the most stable enzyme preparations and the most reproducible results, this ratio was used in all subsequent experiments. After treatment with triton, the suspension was centrifuged at 0° for 1 h at $1 \cdot 10^5 \times g$. The supernatant was used as the soluble enzyme, and stored in the refrigerator.

For measuring activity in other organs, whole homogenate was assayed for ATPase activity, HCO_3^- stimulation and SCN^- inhibition following Triton X-100 solubilization and compared with whole gastric homogenate.

The enzyme was assayed in a medium containing 2 mM ATP or other nucleotide, 2 mM $MgCl_2$ 20 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES) buffer (pH 7.4) with or without 20 mM $NaHCO_3$ or 10 mM NaSCN. The phosphate liberated was measured by the method of FISKE AND SUBBAROW[10] or YODA AND HOKIN[11] following a 20-min incubation. Results are expressed in μmoles phosphate liberated per mg protein per h. Protein was measured by the LOWRY *et al.*[12] method.

Carbonic anhydrase was measured according to PHILPOT AND PHILPOT[13].

RESULTS

Fig. 2 shows the relationship between the specific activity of the ATPase, carbonic anhydrase and the number of oxyntic cells present in the preparation as measured by succinate dehydrogenase staining. The activity of both enzymes is related to the number of oxyntic cells. The first two cell fractions which have little HCO_3^--activated ATPase are also relatively devoid of oxyntic cells.

Table I shows the distribution of the ATPase activity in different subcellular fractions before solubilization with Triton. The highest activity appears to be in the mitochondrial and microsomal fractions. In electron microscopy, these fractions are

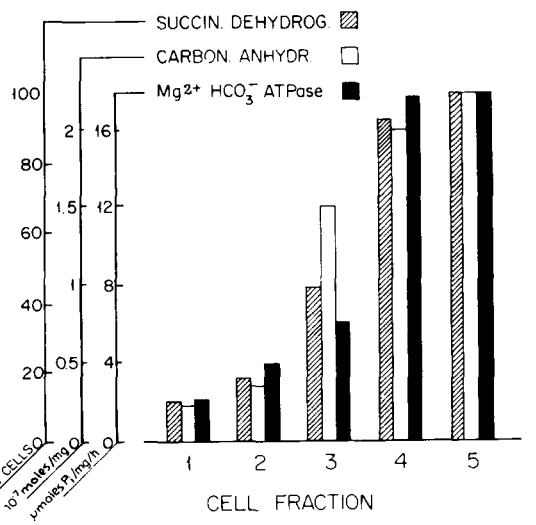

Fig. 2. The relationship between HCO_3^--ATPase and carbonic anhydrase activity and percent of succinate dehydrogenase cells obtained by sequential isolation from pronase treated Necturus gastric mucosa.

TABLE I

ATPase ACTIVITY MEASURED IN THE WHOLE AND FRACTIONATED HOMOGENATE OF A SUSPENSION OF OXYNTIC CELLS (> 80 %) OF NECTURUS GASTRIC MUCOSA

Fraction	*ATPase activity* (*μmoles P_i per mg per h*)	
	Mg^{2+}	Mg^{2+}, HCO_3^-
Whole homogenate	11.8	15.7
$1 \cdot 10^4 \times g \cdot$min (nuclear)	10.2	13.5
$1 \cdot 10^5 \times g \cdot$min (mitochondrial)	13.5	18.9
$7 \cdot 10^5 \times g \cdot$min (light mitochondrial)	14.4	20.4
$6 \cdot 10^6 \times g \cdot$min (microsomal)	20.7	27.3
Supernatant	<0.1	<0.1

TABLE II

SPECIFIC AND TOTAL ACTIVITY OF BANDS COLLECTED FROM A DENSITY GRADIENT FRACTIONATION (16 h, SW25, 25000 rev./min, 20–50 % BUFFERED SUCROSE, 5°) OF THE $6 \cdot 10^6 \times g \cdot$min FRACTION OF TABLE I

Sucrose density	*ATPase activity* (*μmoles P_i per mg per h*)		*Total activity* (*μmoles P_i per h*)	
	Mg^{2+}	Mg^{2+}, HCO_3^-	Mg^{2+}	Mg^{2+}, HCO_3^-
1.05	209	289	8.36	11.56
1.09	102	159	12.24	19.08
1.12	45	47	2.7	2.82
1.14	8	7	0.58	0.51
1.20	<1	<1	<0.04	<0.04

mixtures containing both mitochondria and microsomal aggregates. The supernatant does not contain ATPase activity.

Linear gradient centrifugation of the microsomal fraction shows great concentration of activity in the threshold band and in a band suspending at a density of 1.09 (Table II). Electron micrographs show this latter band to be a relatively homogeneous suspension of smooth vesicles (Figs. 3 and 4).

The whole oxyntic cell of this species contained 32.8 *μg* phospholipid phosphorus per mg protein consisting of 47 % phosphatidyl choline, 32 % phosphatidyl ethanolamine, 11 % sphingomyelin and 7.6 % cardiolipin. The high activity vesicular fraction contained 27.5 *μg* phospholipid per mg protein consisting of 35 % phosphatidyl choline, 60 % phosphatidyl ethanolamine and 5 % sphingomyelin. There was also a high content of neutral lipids, accounting for 50 % of total lipid. Pronase treatment of the mucosa does not significantly affect the properties of the isolated oxyntic cells used as the starting material for this preparation[6], and whether the isolated oxyntic cell is used, or scrapings of the whole mucosa are used, the high activity fraction bands at the same density.

Table III compares some of the properties of the particulate and solubilized enzyme. The degree of HCO_3^- activation markedly increases with solubilization, as does inhibition by SCN^-. In some preparations, a dependence on HCO_3^- for ATPase

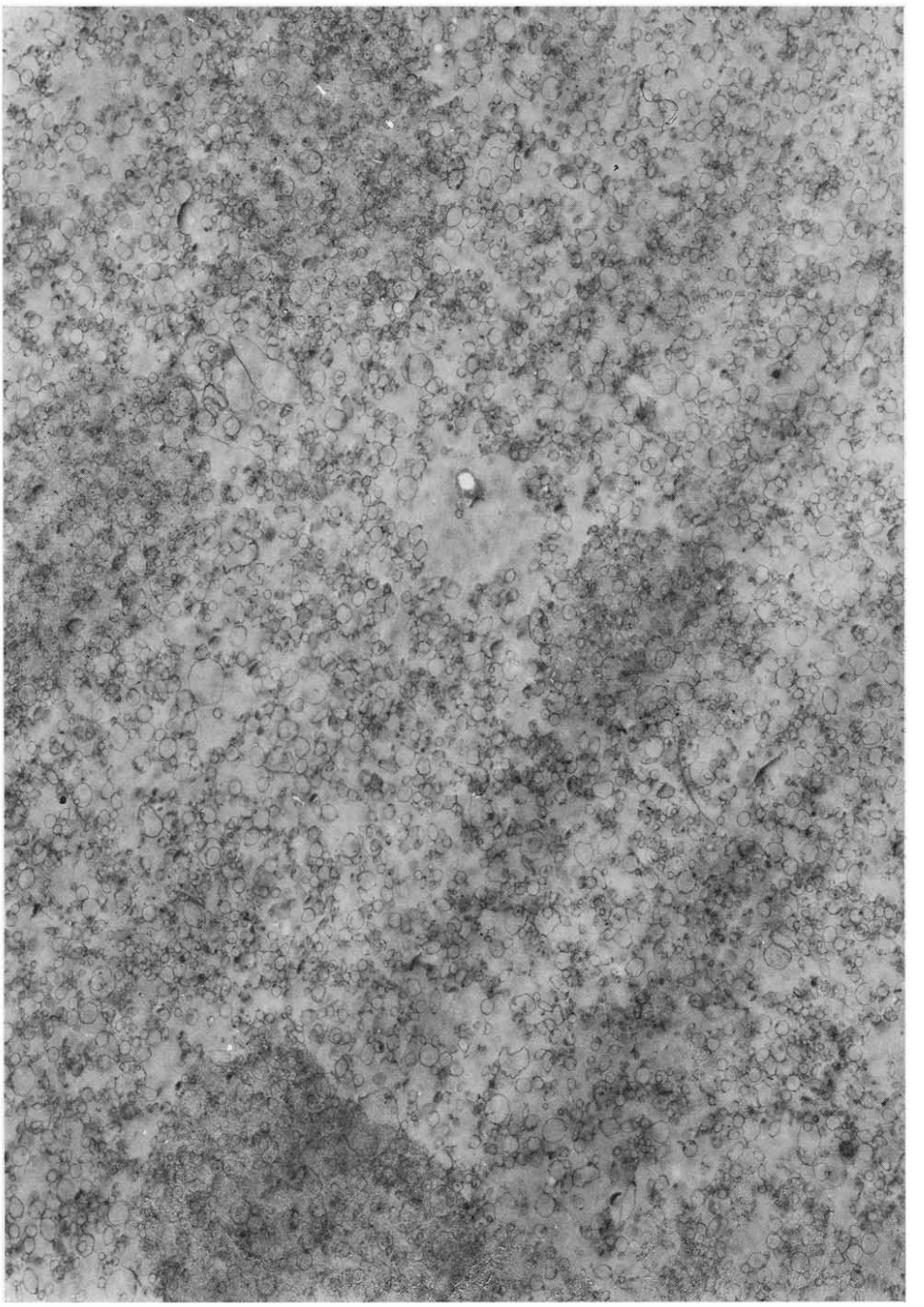

Fig. 3. Electron micrograph of part of the band collected at a sucrose density of 1.09, showing a fairly homogeneous composition. × 6000.

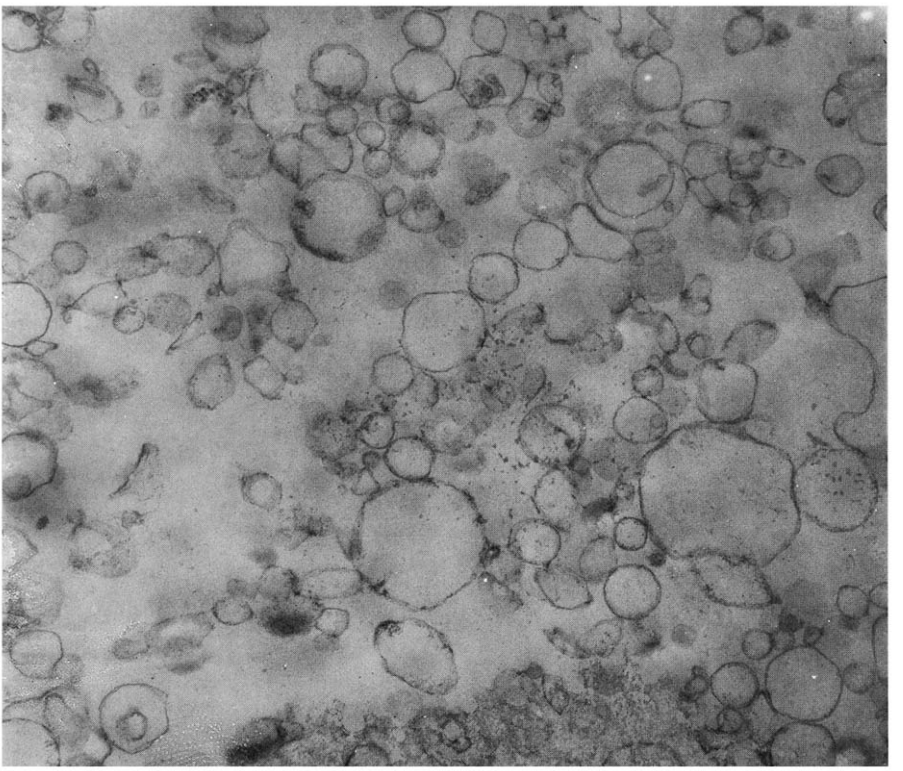

Fig. 4. Detail of band collected at a density of 1.09. Smooth-surfaced microsomes dominate the picture. × 30000.

TABLE III

ATPase ACTIVITY OF THE PARTICULATE AND SOLUBILIZED ENZYME PREPARATION SHOWING THE STIMULATION AND INHIBITION OBTAINED WITH 20 mM HCO_3^- AND 10 mM SCN^-, RESPECTIVELY

Enzyme	*ATPase activity (μmoles P_i per mg per h)*			
	Mg^{2+}	HCO_3^-	SCN^-	$HCO_3^- + SCN^-$
Particulate	21.4	31.03	16.7	15.23
Soluble	32.6	101.06	14.2	12.98

activity occurred, but as a general rule the HCO_3^- stimulation of ATPase activity of the soluble preparation ranged from 3 to 7 times in Necturus.

Fig. 5 shows the distribution of enzyme in different tissues of the Necturus. The highest activity was obtained in the gills of this animal. ATPase of the antrum had a low specific activity and was not sensitive to HCO_3^- and SCN^-.

DISCUSSION

The present work shows that in Necturus gastric mucosa, a SCN^-–HCO_3^--sensitive ATPase is localized in the oxyntic cell. This enlarges on the observation of FORTE

et al.[4] that in developing tissue the appearance of the enzyme coincides with the functional differentiation of the oxyntic cell.

Electron microscopic studies have also shown that the HCO_3^--sensitive ATPase is localized in the apical microvilli of the toad oxyntic cell[14], and carbonic anhydrase has apparently a similar localization[15]. The high activity of the ATPase in the smooth vesicular elements of the oxyntic cell homogenates substantiates these electron microscopic observations in the intact tissue. These vesicles may be derived from the

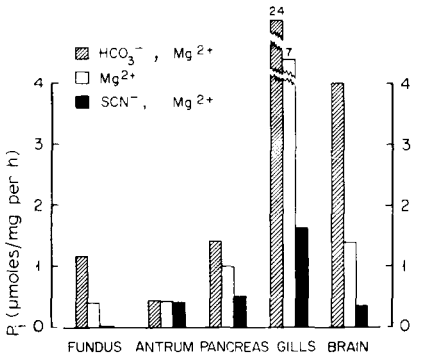

Fig. 5. The occurrence of the HCO_3^--activated SCN^--inhibited ATPase (solubilized) in Necturus gills, gastric fundus, antrum, kidney and brain.

apical microvilli of the oxyntic cell, the rest of the plasma membrane of the cell, or the smooth-surfaced endoplasmic reticulum. The larger proportion is presumably derived from the apical microvilli since these comprise the largest amount of smooth membrane elements in the oxyntic cell. Although these vesicles had a high ATPase activity, there was virtually no nucleoside mono- or diphosphatase activity.

Solubilization of the ATPase was achieved using a non-ionic detergent, Triton X-100. The method produced a highly active preparation, and has been applied to a variety of tissues from various species (G. SACHS, unpublished observations).

As a consequence of solubilization there was a marked increase in the sensitivity of ATP hydrolysis to HCO_3^- stimulation and SCN^- inhibition. The solubilized enzyme showed a 10–15 % residual activity in the presence of SCN^-, and a better than 3-fold stimulation by HCO_3^-.

The activation of the basal Mg^{2+}-ATPase activity by HCO_3^-, or conversely the inhibition of this basal Mg^{2+}-ATPase activity by SCN^-, may be considered analogous to the activation of the $(Na^+ + K^+)$-ATPase by the presence of $Na^+ + K^+$ or the inhibition of this enzyme by ouabain.

Thus, in the case of the Triton-solubilized enzyme, there would appear to be a selective extraction of the HCO_3^--activated enzyme. The activity in the absence of added HCO_3^- could be due to the presence of a non-specific ATPase, or also to activation of the "HCO_3^--ATPase" by, for example, OH^-. Hence there will be SCN^- inhibition in the absence of added base, as was found to be the case. The SCN^--insensitive activity may then be regarded as being due to a non-specific Mg^{2+}-ATPase.

It has been suggested that acid secretion is a consequence of inward HCO_3^- transport across the oxyntic cell membrane[16] and DURBIN AND KASBEKAR[5] have

suggested that this ATPase is involved in HCO_3^-–Cl^- exchange. The marked increase of HCO_3^- activation obtained with the partial purification occurring as a consequence of solubilization could, therefore, be regarded as circumstantial evidence that this enzyme could indeed be involved in HCO_3^- transport.

In view of this, when the activity in different tissues is compared in Necturus, the highest activity was found in the gills. Certain gills absorb Na^+ and secrete H^+, perhaps by an Na^+–H^+ exchange mechanism[17]. This tissue has a high carbonic anhydrase content[18]. Na^+ reabsorption is inhibited by diamox[19] as is the H^+ secretion[17]. In the case of the gastric mucosa diamox inhibits Cl^- transport[20] and acid secretion[21]. The effect, therefore, of a carbonic anhydrase inhibitor on transport in these tissues, and the presence of an enzyme stimulated by HCO_3^-, a product of the carbonic anhydrase reaction, would also argue for a role of the ATPase in transport. The ATPase itself is not sensitive to diamox, hence since diamox inhibits transport in both gills and gastric mucosa, it is likely that carbonic anhydrase is functionally linked to the ATPase.

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