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# IS THERE A PLASMA MEMBRANE-LOCATED ANION-SENSITIVE ATPase?

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## Summary

A study of the intracellular localization of $HCO_3^-$-stimulated, $SCN^-$-inhibited magnesium-dependent ATPase was performed in gill tissue of the rainbow trout (*Salmo irideus*), rabbit kidney and rabbit gastric mucosa. Tissue homogenates were subjected to centrifugal fractionation, and the microsomal (60 min 100 000 × *g*) and light mitochondrial (20 min 20 000 × *g*) fractions were further fractionated by density gradient centrifugation. Subfractions were characterized by marker enzyme assays and electron microscopic observation.

In trout gill indications for an exclusively mitochondrial localization were found. In kidney no definite conclusions could be drawn. In rabbit gastric mucosa initially an apparently non-mitochondrial $HCO_3^-$-stimulated ATPase, in addition to a mitochondrial one, was found and its characteristics were studied. Further studies showed that this ATPase also appears to be of mitochondrial origin and probably represents mitochondrial inner membranes.

Possible explanations for earlier conflicting reports concerning the localization of this enzyme in gastric mucosa and other tissues are discussed.

## Introduction

Several tissues possess a $Mg^{2+}$-ATPase activity, which is insensitive towards cations but which can be stimulated by various anions. Since bicarbonate is prominent among the stimulating anions, the enzyme activity has usually been called $HCO_3^-$-ATPase, but in view of the lack of specificity for this anion we prefer to use the term "anion-sensitive ATPase".

In analogy to the cation-sensitive ATPases, which act as cation pumps, a role of anion-sensitive ATPase in anion transport and indirectly in $H^+$ transport has been suggested [1—3]. However, no definitive proof for either of these roles has been supplied so far.

In an earlier paper on the anion-sensitive ATPase activity in lizard gastric

Abbreviation: DCCD, dicyclohexylcarbodiimide.

mucosa we have shown that its properties are quite different from those of ($Na^+$ + $K^+$) -ATPase and that this pleads against a pump function for the enzyme [4]. In view of the persisting uncertainty about the role of the enzyme, further studies are warranted. A clear requirement for a cellular transport system is its localization in the plasma membrane. Such localization has been alleged for anion-sensitive ATPase in the case of dog gastric mucosa [2,5]. On the other hand, there is evidence that mitochondria contain an anion-sensitive ATPase activity [6,7]. Hence, some authors have suggested that the anion-sensitive ATPase activity in microsomes is derived from mitochondrial contamination [8—11].

In order to settle this problem we have made a thorough study of the intracellular localization of the anion-sensitive ATPase activity in three different tissues, where active anion transport is thought to occur: rainbow trout gill, rabbit kidney and rabbit gastric mucosa. The results of this study, reported in this paper, do not support the presence of an anion-sensitive ATPase of plasma membrane origin in trout gill and rabbit gastric mucosa and doubt is thrown on its presence in the plasma membrane of rabbit kidney.

## Methods

*Tissue preparation.* Male and female New Zealand white rabbits, weighing 2—3 kg, are fasted 24 h before the start of each experiment. The animals are killed by a blow on the neck, immediately followed by carotic exsanguination. The stomach and kidneys are quickly removed and cooled on ice. The stomach is opened and washed out with water. The mucosa is separated from the underlying muscular layer and suspended in a buffer solution containing 0.25 M sucrose, 25 mM Tris/maleate (pH 7.6).

Kidneys and mucosa are minced with scissors and homogenized in nine volumes (v/w) of this buffer. Homogenization is carried out in a teflon/glass homogenizer (Potter/Elvehjem) with 4—5 strokes at 850 rev./min. All operations are performed at 0—4° C.

Rainbow trouts (*Salmo irideus*), 25—30 cm long, obtained from a local trout hatchery, are decapitated and cooled on ice. The gill filaments are cut from the gill arches, and are rinsed and homogenized in five volumes of the same buffer with a Polytron homogenizer (type PT 10-20-3500, Kinematica GmbH, Luzern, Switzerland) at setting 3—4 for 45 s at 0—4° C.

*Centrifugal fractionation.* The homogenates are filtered through four layers of surgical gauze and fractionated by centrifugation in a nuclear (10 min 1000 × $g$), heavy mitochondrial (10 min 10 000 × $g$), light mitochondrial (20 min 20 000 × $g$) and microsomal (60 min 100 000 × $g$) pellet and a remaining supernatant. The nuclear and heavy mitochondrial fractions are washed once with homogenization buffer, recentrifuged and the remaining supernatants are combined with the initial ones.

*Density gradient centrifugation.* Light mitochondrial and microsomal pellets are further fractionated by resuspending them in homogenization buffer and layering them on top of a linear gradient of 30—55% (w/v) sucrose in 25 mM Tris/maleate (pH 7.6). Gradients are routinely centrifuged for 16—17 h at 24 000 rev./min (64 000 × $g_{av}$) in the SW 25.1 rotor of the Spinco Beckman

type $L_2$ ultracentrifuge or in the SB 110 rotor of the IEC type B 60 ultracentrifuge. Approx. 25 fractions are collected with a density gradient removing apparatus (Auto-Densi-flow $II_c$, Buchler Instruments, Searle Analytic Inc., Fort Lee N.J., U.S.A.) connected with a fraction collector (LKB, Bromma, Sweden). Sucrose concentrations are measured with an Abbe refractometer at 20°C.

For further studies of the ATPase activities after centrifugation in the gradients, appropriate subfractions are pooled, diluted 1 : 1 with 25 mM Tris/maleate buffer (pH 7.6), centrifuged 60 min 100 000 × *g*, and resuspended in homogenization buffer.

*Protein determination.* Protein concentrations are estimated by the method of Lowry et al. [12] with bovine serum albumin, dissolved in homogenization buffer, serving as standard. In gradient fractions protein concentrations are determined by measuring 280 nm absorbance ($A_{280nm}$) after dilution with two volumes of 2% (w/v) sodium dodecyl sulphate to eliminate scattering in the cuvet. Corrections are made for the absorbance of sucrose.

*Assay of marker enzymes.* Cytochrome *c* oxidase is estimated after Cooperstein and Lazarow [13] at room tempeature (22°C) in the presence of 0.6% Tween-80 and expressed as the rate of the initial decrease in 550 nm absorbance with an initial extinction of 0.5 (equivalent to a cytochrome *c* concentration of approx. 17 μM). A unit of enzyme activity is defined as a decrease of one extinction unit per min.

5'-Nucleotidase is measured according to Heppel and Hilmoe [14] with small changes in the conditions.

$(Na^+\text{-}K^+)$-ATPase is measured as described by Bonting (ref. 15, p. 261) and modified by Schuurmans Stekhoven et al. [16].

*Assay of anion-sensitive ATPase.* Different media are used, each of which contains one major anion: $HCO_3^-$, $Cl^-$, $SCN^-$, formiate, acetate, $I^-$, azide, $NO_3^-$, $ClO_4^-$, $SO_3^{2-}$, $SO_4^{2-}$, oxalate or citrate. The incubation medium, after addition of 20 μl enzyme preparation to 300 μl medium, has the following final composition: 100 mM Tris, 2 mM $MgCl_2$, 2 mM $Na_2ATP$, $10^{-4}$ M ouabain and 50 mM sodium salt of one of the monovalent anions or 27.5 mM $Na_2SO_3$, $Na_2SO_4$ or disodium oxalate or 16.7 mM trisodium citrate. The pH of the medium is adjusted to 8.4 with the corresponding acid, except in the case of $SCN^-$, $I^-$ or azide, where acetic acid is used. After incubation for 30 min at 37°C the reaction is stopped by addition of 1 ml ice-cold 8.6% trichloroacetic acid. Blanks are run at 0°C. Then 1 ml of 9.2% $FeSO_4 \cdot H_2O$ in 0.66 M $H_2SO_4$, 1.15% ammonium-molybdate is added, and after 20 min at room temperature the tubes are centrifuged for 10 min at 3000 × *g*. The 700 nm absorbance of the supernatants is read and compared with that of a phosphate standard treated in the same way.

When inhibitors in ethanolic solution are applied, controls are run containing the same amount of ethanol (<3%, v/v). Preincubation for 15 min at 0°C (DCCD, 60 min, 0°C; sodium dodecyl sulphate, 30 min, 0°C) and 5 min at 37°C in the absence of ATP precedes the incubation in this case.

*Lipid analysis.* Lipid extraction is carried out according to Folch et al. [17], except that a chloroform/methanol/water mixture (60 : 30 : 35, by vol.) is used to replace the homogenization buffer, which is removed through centrifugation at 60 min 100 000 × *g*. The total lipid extract is washed with 0.2

volume 0.1 M KCl and concentrated by evaporation. The concentrated extract is dissolved in benzene/ethanol (4 : 1, by vol.). This solution is used for phospholipid analysis by two-dimensional thin-layer chromatography according to the method of Broekhuyse [18].

*Electron microscopy.* Samples from the density gradient fractions are fixed by addition of 50% glutaraldehyde to a final concentration of 1%, filtered through Millipore filters (pore-diameter, 0.01 μm) coated with a layer of rabbit erythrocytes, and further processed according to Baudhuin et al. [19].

## Materials

Cytochrome *c* (horse heart), $Na_2ATP$, $Na_3CTP$, $Na_3GTP$, $Na_3ITP$, $Na_3UTP$, $Na_2AMP$ and ADP are obtained from Boehringer (Mannheim, Germany), Tween-80 from Atlas Goldschmidt GmbH (Germany), NaSCN from UCB (Belgium).

Sodium dodecyl sulphate, oligomycin and quercetin are purchased from Sigma (St. Louis, Mo., U.S.A.), sucrose from the British Drug Houses Ltd. (England). Dicyclohexylcarbodiimide (DCCD), from Nutritional Biochemical Corp., is vacuum distilled before use. Aurovertin D (isolated by Dr. R. Bertina and obtained as a generous gift from the B.C.P. Jansen Institute, University of Amsterdam) is dissolved in abs. ethanol and the concentration is determined spectrophotometrically at 367.5 nm using an absorbance coefficient of 42.7 $mM^{-1} \cdot cm^{-1}$ [20].

All other reagents are from E. Merck (Darmstadt, Germany) and are of analytical grade.

## Results

*ATPase activities in main fractions*

Table I presents the specific ATPase activities in homogenates of trout gill, rabbit kidney and rabbit gastric mucosa. In all three tissues the activity is stimulated by bicarbonate and inhibited by thiocyanate, relative to the activity in chloride medium. After fractionation the largest anion effects are seen in the heavy mitochondrial (10 min 10 000 × *g*) fraction, but the microsomal fraction (60 min 100 000 × *g*) is also anion sensitive in all three cases (Figs. 1a–1c). Since most authors, who claim to have found a non-mitochondrial anion-sensitive ATPase, were using a microsomal fraction, we decided to study the distribution

TABLE I

SPECIFIC ACTIVITY OF ANION-SENSITIVE ATPase IN HOMOGENATES OF DIFFERENT TISSUES

Results are expressed in μmol ATP per h per mg protein with the standard error.

| Tissue | Medium | | | *n* |
|---|---|---|---|---|
| | $HCO_3^-$ | $Cl^-$ | $SCN^-$ | |
| Gastric mucosa | 17.1 ± 3.6 | 12.4 ± 1.7 | 6.1 ± 1.2 | 5 |
| Kidney | 23.7 ± 4.1 | 17.7 ± 2.9 | 7.5 ± 1.2 | 4 |
| Gill | 11.7 ± 2.2 | 5.1 ± 0.9 | 2.1 ± 0.4 | 5 |

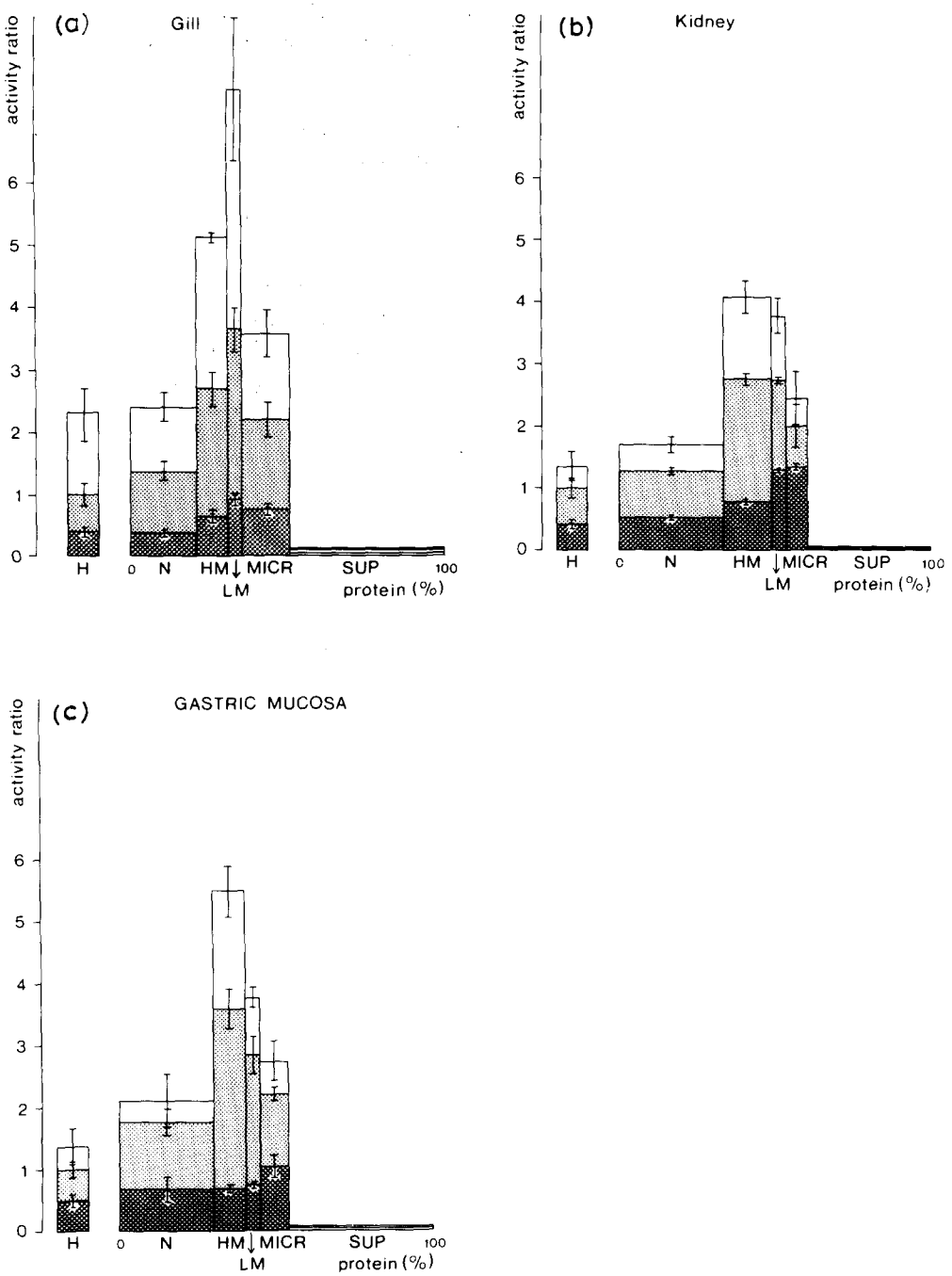


Fig. 1. Distribution of anion-sensitive ATPase activities in main fractions of gill (a), kidney (b) and gastric mucosa (c). Ratios of the specific activity in $HCO_3^-$ medium (total column), $Cl^-$ medium (darker part of column) and $SCN^-$ medium (darkest part of column) to the specific activity of the homogenate in $Cl^-$ medium are plotted against the protein distribution in percent. Abbreviations: H is the homogenate; N, HM, LM and MICR are the fractions sedimenting at 10 min 1000 × *g*, 10 min 10 000 × *g*, 20 min 20 000 × *g* and 60 min 100 000 × *g*, respectively, SUP is the remaining supernatant. Means of five (a), three (b) and five (c) experiments with standard errors are given.

patterns in the light mitochondrial (20 min 20 000 × *g*) and the microsomal (60 min 100 000 × *g*) fractions after density gradient centrifugation.

*ATPase activities in fractions of gill*

The distribution patterns for the light mitochondrial and microsomal fractions from gill tissue, rabbit kidney and rabbit gastric mucosa have been determined, and are presented in Figs. 2–4.

In gill tissue (Figs. 2a and 2b) the $HCO_3^-$-stimulated ATPase activity distribution virtually coincides with the cytochrome *c* oxydase pattern. Although 5′-nucleotidase, used as a marker for plasma membranes, show peak activities at two or more places in the gradients, it is clear that there can hardly be a relationship between 5′-nucleotidase and anion-sensitive ATPase activity. This is particularly evident, when one observes that the high activity of 5′-nucleotidase at the low sucrose concentrations coincides with an anion-insensitive $Mg^{2+}$-

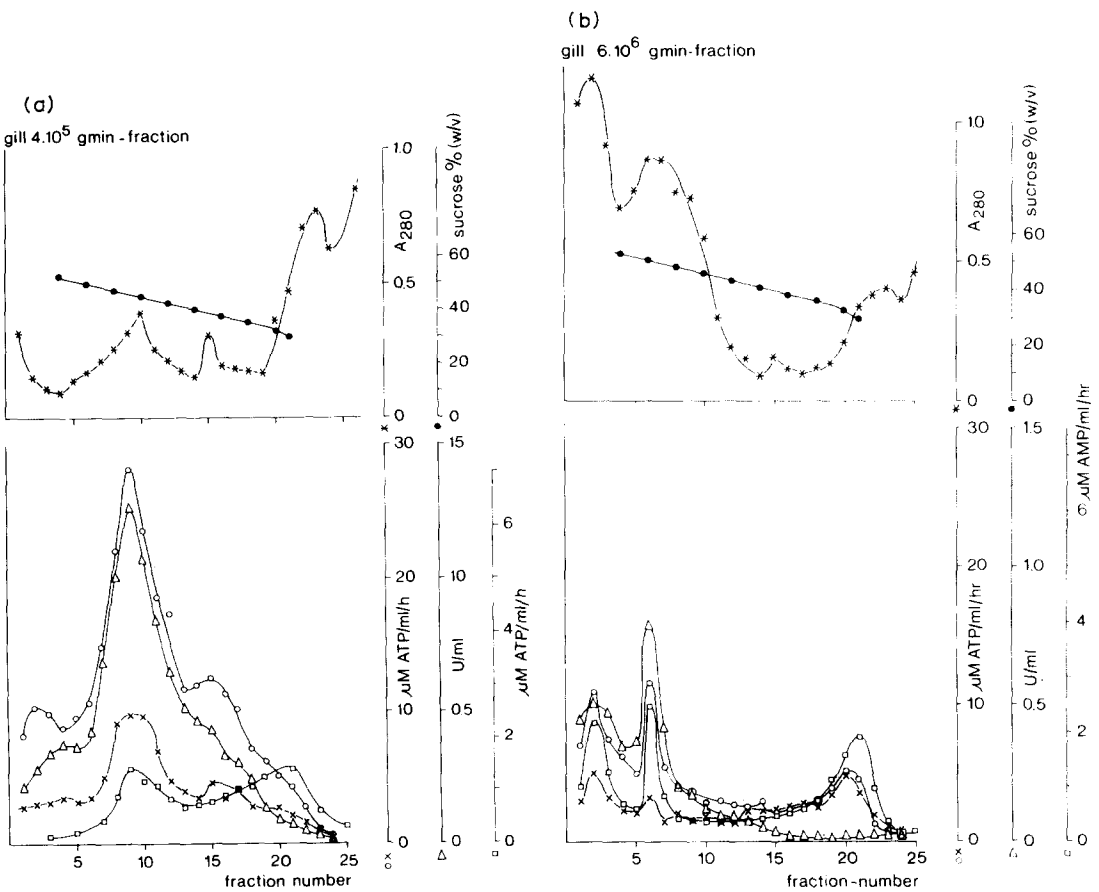


Fig. 2. Enzyme distribution pattern after density gradient centrifugation (16 h 64 000 × $g_{av}$) of (a) light mitochondrial (20 min 20 000 × *g*) and (b) microsomal (60 min 100 000 × *g*) fractions of trout gill (typical experiment representative for seven (a) and three (b) experiments, respectively). Symbols: ●———●, distribution of sucrose; ★———★, 280 nm absorbance, after dilution and correction; ○———○, ATPase activity in $HCO_3^-$ medium; χ———χ, ATPase activity in $Cl^-$ medium; ●———●, $(Na^+ + K^+)$-ATPase activity; △———△, cytochrome *c* oxidase activity, □———□, 5′-nucleotidase activity.

ATPase activity (Fig. 2b), and that the second (and third in Fig. 2b) 5′-nucleotidase peak activity is found at high densities (>45% sucrose, w/v). Therefore, it is very unlikely that the latter two 5′-nucleotidase activities are localized in plasma membranes, but more likely that they represent a non-specific phosphatase activity. Thus both the coincidence of anion-sensitive ATPase with cytochrome *c* oxydase and the presence of an anion-insensitive ATPase coinciding with 5′-nucleotidase at the lower sucrose concentrations indicates nearly complete absence of a non-mitochondrial anion-sensitive ATPase activity in this tissue.

*ATPase activities in fractions of kidney*

The distribution pattern for rabbit kidney (Figs. 3a and 3b) shows that both in the light mitochondrial and in the microsomal fraction a strong overlap of cytochrome *c* oxydase, 5′-nucleotidase and ($Na^+ + K^+$)-ATPase activities occurs. The $HCO_3^-$-stimulated ATPase is distributed over a large part of the density gradient (34—48% or 33—45% sucrose (w/v) in Figs. 3a and 3b, respectively),

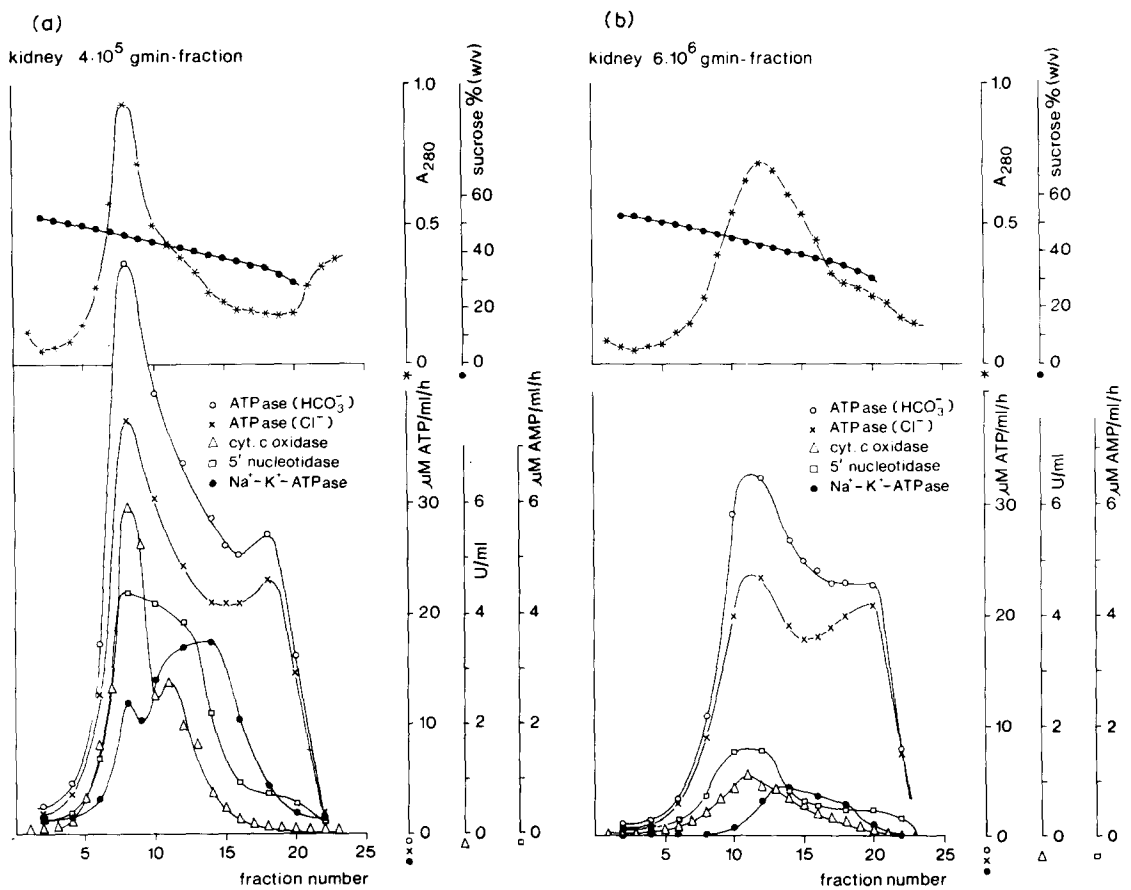


**Fig. 3. Enzyme distribution pattern after density gradient centrifugation (16 h 64 000 × $g_{av}$) of (a) light mitochondrial (20 min 20 000 × *g*) and (b) microsomal (60 min 100 000 × *g*) fractions of rabbit kidney. Typical experiment representative for three (a) and five (b) experiments, respectively. Symbols: see legend Fig. 2.**

although especially in the microsomal fraction at the lower sucrose concentrations a $Mg^{2+}$-ATPase activity is observed, which is less stimulated by $HCO_3^-$. Thus the gradient subfractionation of both fractions from rabbit kidney does not permit a definitive conclusion. If a non-mitochondrial $HCO_3^-$-ATPase activity is present, it will be extremely difficult to separate it from contaminating mitochondria in this tissue for further studies.

*ATPase activities in fractions of gastric mucosa*

In gastric mucosa three $Mg^{2+}$-ATPase peaks are obtained upon subfractionation of both the light mitochondrial and microsomal fractions (Fig. 4). One sediments at low sucrose concentrations, coincides with 5'-nucleotidase, and has no anion sensitivity. This peak is most prominent in the microsomal fraction, is called peak I, and possibly represents a plasma membrane fraction. This fraction is relatively insensitive to inhibition by $SCN^-$ (not shown). Two $HCO_3^-$-

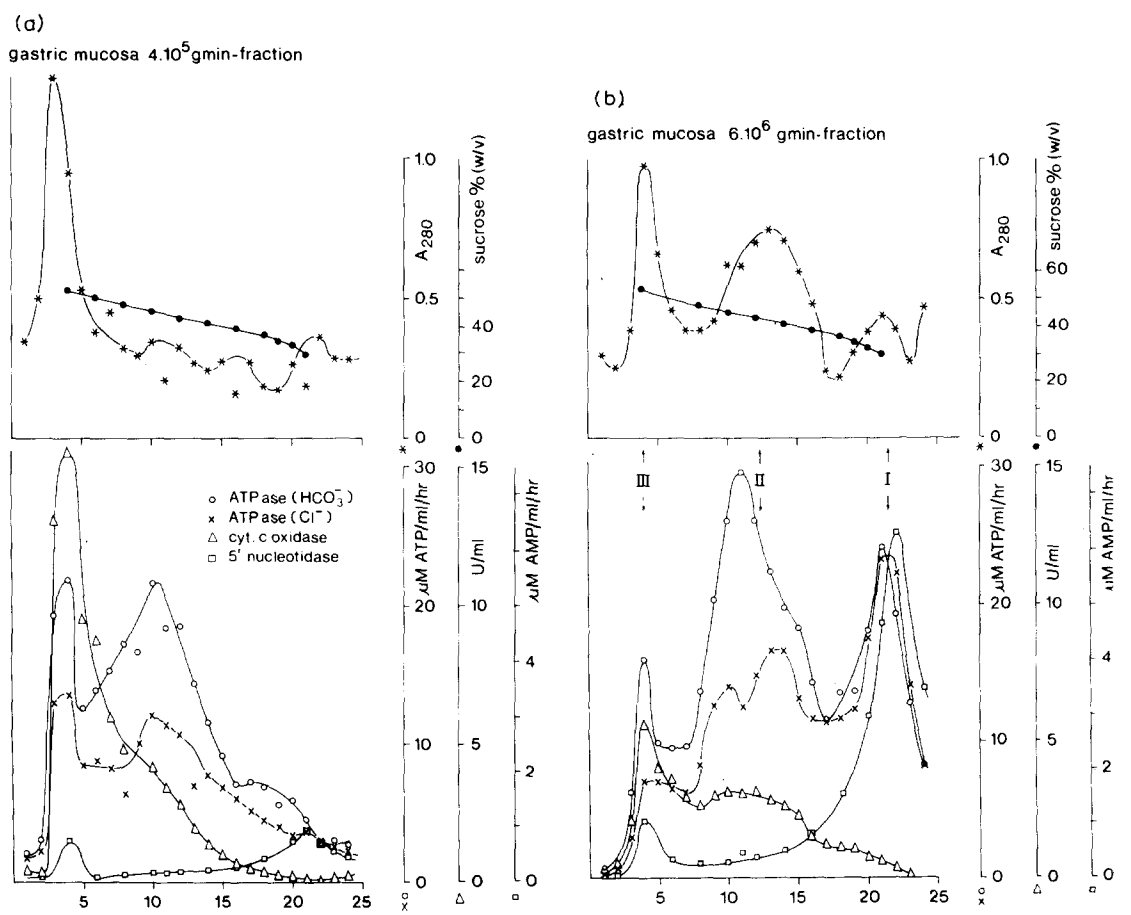


Fig. 4. Enzyme distribution pattern after density gradient centrifugation (16 h 64 000 × $g_{av}$) of (a) light mitochondrial (20 min 20 000 × $g$) and (b) microsomal (60 min 100 000 × $g$) fractions of rabbit gastric mucosa. Typical experiment representative for six (a) and 28 (b) experiments, respectively. Symbols: see legend Fig. 2.

stimulated ATPase peaks are seen in both the light mitochondrial and microsomal fractions, one sedimenting at 40—44% (w/v) sucrose (peak II) and one sedimenting at 48—52% (w/v) sucrose (peak III). Peak III coincides with the highest cytochrome *c* oxydase level. The low cytochrome *c* oxydase level in peak II could suggest a non-mitochondrial origin for peak II. However, other intracellular markers like glucose-6-phosphatase, RNA (endoplasmic reticulum) or $(Na^+ + K^+)$-ATPase (plasma membrane) do not coincide with $HCO_3^-$-stimulated ATPase activity in peak II (not shown).

Separation of a crude microsomal fraction from bullfrog oxyntic cells in a discontinuous density gradient also gives a discrepancy between cytochrome *c* oxydase and $Mg^{2+}$-ATPase activity in the fractions sedimenting at the 37—43% and the 43—55% (w/v) sucrose interface, although the authors do not comment on this finding [21].

*Properties of anion-sensitive ATPase*

At this point the characteristics of the (peak II) $HCO_3^-$-stimulated ATPase from rabbit gastric mucosal microsomes have been studied in order to determine the optimal assay conditions for all further experiments.

Table II shows the dependence of the activity on the presence of various divalent cations. The $SO_4^{2-}$ medium is used in order to avoid precipitates formed with cations other than magnesium in the $HCO_3^-$ medium. $Zn^{2+}$ also produces a precipitate in the $SO_4^{2-}$ medium. In the absence of any divalent cation the activity is practically zero. Manganese can replace magnesium to a large extent, calcium and zinc only very partially. The slight activity observed in the inhibitory $SCN^-$ medium is least influenced by the nature of the divalent cation. Similar results were found for dog gastric mucosa, where $Mn^{2+}$ effectively substituted for $Mg^{2+}$, but $Ca^{2+}$ and $Zn^{2+}$ did not [2].

In Table III the effects of different anions are presented, the activity in $Cl^-$ medium being set at 1.00. Azide appears to be even more inhibitory than thiocyanate, which is also found for gastric mucosa of frog [22] and dog [29]. Oxalate and $SO_3^{2-}$ are even more stimulatory than $HCO_3^-$, which is also the case for dog gastric mucosa [2], rabbit submandibular gland [24], cat pancreas [25] and rat liver mitochondrial ATPase [7].

TABLE II

EFFECT OF DIVALENT CATIONS ON "PEAK II" ANION-SENSITIVE $Mg^{2+}$-ATPase

Relative activities in the presence of 2 mmol/l of the cation are presented. Averages with standard error for three experiments are given. Peak II represents the 40—44% sucrose (w/v) subfraction of the microsomal fraction of rabbit gastric mucosa. n.d., not determined.

| Cation | Medium | | |
|---|---|---|---|
| | $SO_4^{2-}$ | $Cl^-$ | $SCN^-$ |
| $Mg^{2+}$ | ≡1.00 | ≡1.00 | ≡1.00 |
| $Mn^{2+}$ | 0.94 ± 0.03 | 0.82 ± 0.05 | 0.89 ± 0.08 |
| $Ca^{2+}$ | 0.37 ± 0.05 | 0.39 ± 0.05 | 0.88 ± 0.06 |
| $Zn^{2+}$ | n.d. | 0.35 ± 0.06 | 0.22 ± 0.05 |
| None | 0.01 ± 0.01 | 0.02 ± 0.01 | 0.04 ± 0.04 |

TABLE III

EFFECTS OF DIFFERENT ANIONS ON "PEAK II" ANION-SENSITIVE $Mg^{2+}$-ATPase

Relative activities (activity in $Cl^-$ medium set at 1.00) are presented with S.E. for four experiments. Peak II represents the 40—44% sucrose (w/v) subfraction of the microsomal fraction of rabbit gastric mucosa.

| Major anion | Relative activity | Major anion | Relative activity |
|---|---|---|---|
| Azide | 0.10 ± 0.01 | citrate | 1.18 ± 0.04 |
| $SCN^-$ | 0.28 ± 0.02 | $SO_4^{2-}$ | 1.29 ± 0.03 |
| $ClO_4^-$ | 0.41 ± 0.01 | acetate | 1.38 ± 0.06 |
| $NO_3^-$ | 0.65 ± 0.03 | $HCO_3^-$ | 1.54 ± 0.04 |
| $Cl^-$ | ≡1.00 | oxalate | 1.63 ± 0.05 |
| $I^-$ | 1.05 ± 0.05 | $SO_3^{2-}$ | 2.59 ± 0.16 |
| Formiate | 1.13 ± 0.10 | | |

Table IV shows the substrate specificity of the ATPase activity, indicating that only GTP and ITP can replace ATP to a considerable extent. Activity with ADP, AMP, CTP and UTP is negligible or low. Similar results were obtained for dog gastric mucosa [2] and cat pancreas [25].

In Fig. 5 the pH dependence of the ATPase activity in the $Cl^-$ medium and the strongly stimulating $SO_3^{2-}$ medium is presented. The curves are broad and flat with an optimum around pH 8.4. The activities in dog and frog gastric mucosa and cat pancreas showed an optimum at pH 8.3 [2,22,25]. In the presence of $NaHCO_3$ values of pH 7.4 and 7.6 were obtained for dog gastric mucosa and cat pancreas [2,25], which lower values may be due to the increasing $CO_2/HCO_3^-$ ratio towards the $pK_1 = 6.52$ value for $H_2CO_3$.

The effect of varying the magnesium concentration, in the presence of 2 mM ATP is shown in Fig. 6. Optimal activity in both the $HCO_3^-$ medium and the $Cl^-$ medium is around 0.6 mM $Mg^{2+}$ ($Mg^{2+}$/ATP ratio = 0.3). This means that all activities, measured in our standard media containing 2 mM $MgCl_2$, are underestimated by approx. 25%. For frog gastric mucosa an optimal $Mg^{2+}$/ATP ratio of about 0.5 has been reported [22].

Fig. 7 shows that there is some effect of ionic strength on both peak II and peak III $HCO_3^-$-ATPase activities, leading to decreased activities above 50 and

TABLE IV

SUBSTRATE SPECIFICITY OF "PEAK II" ANION-SENSITIVE $Mg^{2+}$-ATPase

Relative activities (activity for ATP set at 1.00) are presented with S.E. for three experiments. Peak II represents the 40—44% sucrose (w/v) subfraction of the microsomal fraction of rabbit gastric mucosa.

| Substrate | Medium | | |
|---|---|---|---|
| | $HCO_3^-$ | $Cl^-$ | $SCN^-$ |
| ATP | ≡1.00 | ≡1.00 | ≡1.00 |
| AMP | 0.02 ± 0.00 | 0.01 ± 0.00 | 0.08 ± 0.04 |
| ADP | 0.06 ± 0.02 | 0.08 ± 0.03 | 0.28 ± 0.09 |
| GTP | 0.52 ± 0.04 | 0.50 ± 0.07 | 0.60 ± 0.05 |
| CTP | 0.07 ± 0.01 | 0.10 ± 0.01 | 0.30 ± 0.03 |
| UTP | 0.11 ± 0.01 | 0.14 ± 0.02 | 0.34 ± 0.02 |
| ITP | 0.60 ± 0.03 | 0.63 ± 0.05 | 0.67 ± 0.01 |

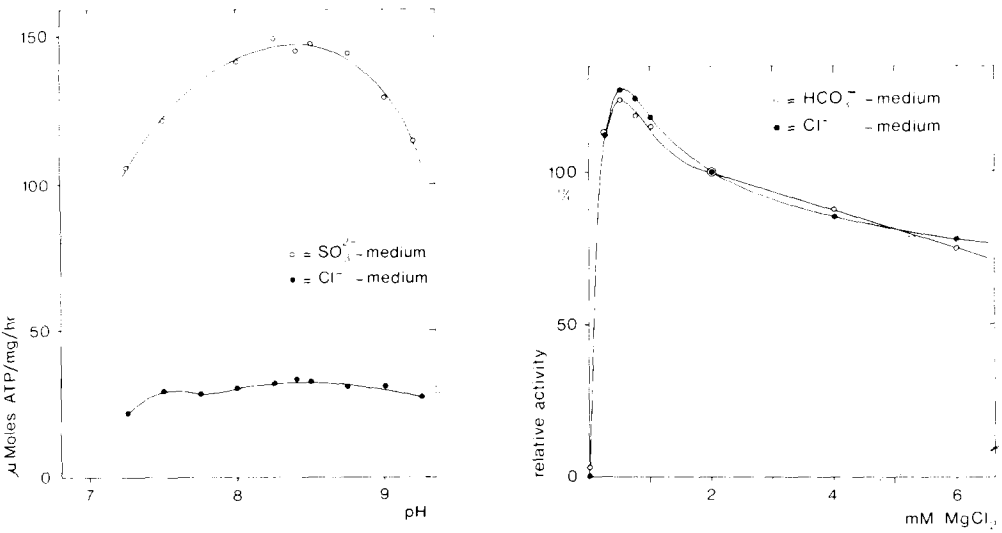


Fig. 5. Activity of (peak II) anion-sensitive ATPase of rabbit gastric mucosal microsomes as a function of pH in $SO_3^{2-}$ medium (○) and $Cl^-$ medium (●). The pH values of the media are adjusted with the related acid (mean of two experiments).

Fig. 6. Effect of $Mg^{2+}$ concentration on (peak II) anion-sensitive ATPase activity. The activity in the presence of 2 mM $MgCl_2$ is set at 100%. Activities are measured in the presence of 2 mM $Na_2ATP$ in $HCO_3^-$ medium (○) and $Cl^-$ medium (●) (mean of two experiments).

TABLE V

EFFECTS OF VARIOUS AGENTS ON ANION-SENSITIVE $Mg^{2+}$-ATPase ACTIVITIES IN TWO SUB-FRACTIONS FROM GASTRIC MUCOSAL MICROSOMES

$pI_{50}$ is the negative logarithm of molar inhibitor concentration at half-maximal inhibition. Rest activity is percent ATPase activity remaining at maximal inhibition. $pI_{max}$ is the negative logarithm of molar inhibitor concentration required for obtaining 99% of the maximal inhibition. $HCO_3^-$ medium was used in all experiments.

| Agent | Peak II ATPase | | | Peak III ATPase | | | n |
|---|---|---|---|---|---|---|---|
| | $pI_{50}$ | Rest activity (%) | $pI_{max}$ | $pI_{50}$ | Rest activity (%) | $pI_{max}$ | |
| Quercetin | 4.2 | 6 | 3.7 | 4.1 | 8 | 3.7 | 2 |
| DCCD | 6.6 | 16 | 5.8 | 6.7 | 16 | 6.0 | 2 |
| Sodium dodecyl sulphate | 3.9 | 3 | 3.5 | 3.8 | 2 | 3.5 | 2 |
| Oligomycin | 7.2 | 15 | 5.0 | 7.1 | 20 | 4.9 | 2 |
| Aurovertin D | 6.1 | 46 | 4.9 | 6.2 | 50 | 5.1 | 2 |
| Oligomycin + aurovertin D * | — | 9 | — | — | 17 | — | 2 |
| Acetazolamide | — | 93 (10 mM) | — | — | 93 (10 mM) | — | 1 |

* Oligomycin ($2 \cdot 10^{-5}$ M) and aurovertin D ($3.5 \cdot 10^{-5}$ M) have also been tested in combination, which gives a further increased inhibition.

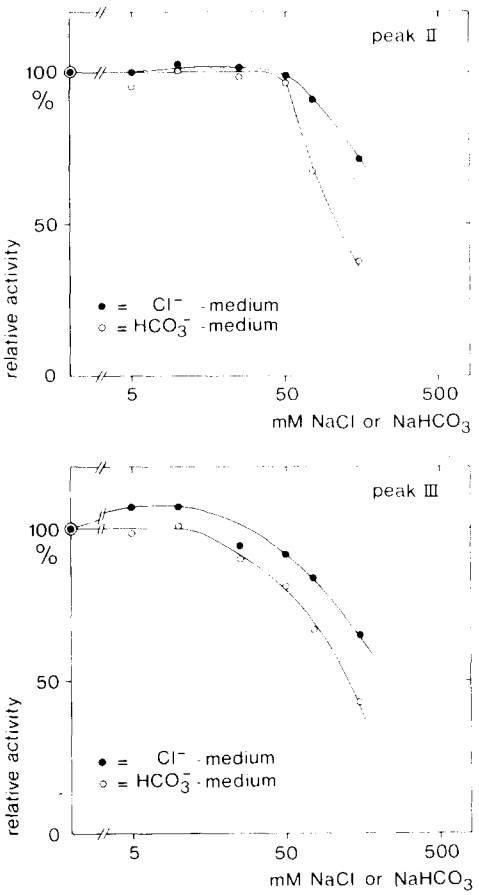


**Fig. 7. Effect of ionic strength on anion-sensitive ATPase activity of subfractions II and III of gastric mucosal microsomes. $HCO_3^-$ and $Cl^-$ media are prepared by using increasing concentrations of $NaHCO_3$ or NaCl, respectively, and brought to pH 8.4 with $CO_2$ or HCl, respectively. The media with 50 mM $NaHCO_3$ (○) and 50 mM NaCl (•) represent the media routinely used. Mean ratios over the activity at 0 mM added salt (=100%) are shown for two experiments.**

25 mM $NaHCO_3$, respectively. The routinely used concentration of 50 mM in our assay medium is optimal for peak II activity and about 20% suboptimal for peak III activity. The behaviour of peak II activity is rather similar to that previously found by us for lizard gastric mucosa [4]. In frog gastric mucosa an optimal value of 20 mM added $NaHCO_3$ was obtained at a lower buffer concentration [22].

The effects of various inhibitory substances on peak II and peak III $HCO_3^-$-stimulated ATPase activities are presented in Table V. Both half maximal inhibitor concentrations (expressed as $pI_{50}$) and percent activity remaining at maximal inhibition are very similar for the two peaks. The inhibition by aurovertin and oligomycin of the two activities and particularly the nearly complete inhibition by a combination of these two substances, strongly suggests a mitochondrial origin. The plasma membrane localized ($Na^+ + K^+$)-ATPase is 300 times less sensitive to inhibition by oligomycin [26].

TABLE VI

PHOSPHOLIPID COMPOSITION OF THREE SUBFRACTIONS OF RABBIT GASTRIC MUCOSA MICROSOMES

Microsomal fraction of rabbit gastric mucosa subjected to density gradient centrifugation. Values are percentages of total phosphorus with standard error for four experiments.

| Phospholipid | I | II | III |
|---|---|---|---|
| Phosphatidylethanolamine | 28.0 ± 1.0 | 28.6 ± 0.5 | 24.4 ± 0.7 |
| Phosphatidylcholine | 27.2 ± 0.7 | 43.5 ± 1.5 | 49.6 ± 0.7 |
| Phosphatidylinositol | 4.7 ± 0.3 | 6.0 ± 0.4 | 6.6 ± 0.3 |
| Phosphatidylserine | 13.1 ± 1.0 | 5.3 ± 0.4 | 4.2 ± 0.2 |
| Sphingomyelin | 18.8 ± 1.5 | 6.7 ± 1.0 | 6.2 ± 0.7 |
| Lysophosphatidylcholine | 2.5 ± 1.6 | — | 0.9 ± 0.4 |
| Diphosphatidylglycerol (cardiolipin) | 1.2 ± 0.4 | 7.0 ± 1.1 | 3.1 ± 0.5 |
| Unidentified | 4.7 ± 0.6 | 2.7 ± 0.8 | 4.2 ± 1.0 |

*Electron microscopic characterization of subfractions*

Peak I consists mainly of vesicular structures (Fig. 8a) and could originate from the plasma membrane or from microvilli located on the apical cell surface of the parietal cell. The ATPase activity in this subfraction is not anion sensitive (cf. Fig. 4b), in contrast to the morphologically similar vesicular fraction obtained at slightly higher densities by Blum et al. [2]. Peak III contains rough endoplasmic reticulum and a sizable number of small-sized mitochondria (Fig. 8c). Peak II has fewer mitochondria, most of them lacking an outer membrane, and contains less endoplasmic reticulum, while the large black dots may represent lysosomes (Fig. 8b).

*Phospholipid composition of subfractions*

As a further means of characterizing the subfractions we have determined the phospholipid composition of peaks I, II and III (Table VI). There is a close resemblance between the phospholipid patterns of peak II and III, while peak I shows lower levels of phosphatidylcholine and phosphatidylinositol and higher levels of phosphatidylserine and sphingomyelin, resembling more a plasma membrane fraction. The high level of diphosphatidylglycerol (cardiolipin) in peak II may indicate an enrichment of mitochondrial inner membrane in this fraction [27,28].

*Further experiments on the origin of peak II*

The results reported in the previous three sections raise the possibility that peak II, which contains the major anion-sensitive ATPase activity in the microsomal fraction, may be of mitochondrial origin like peak III despite the difference in density and the ratios between the cytochrome *c* oxydase and anion-sensitive ATPase activities. In order to obtain more information on this point, some further experiments have been carried out.

Recentrifugation of peaks II and III, pooled after an initial separation in density gradients by the normal procedure, for 16 h in density gradients, results in the return of both peaks to the same places in the gradient which they occupied after the initial density gradient centrifugation. When the fractions

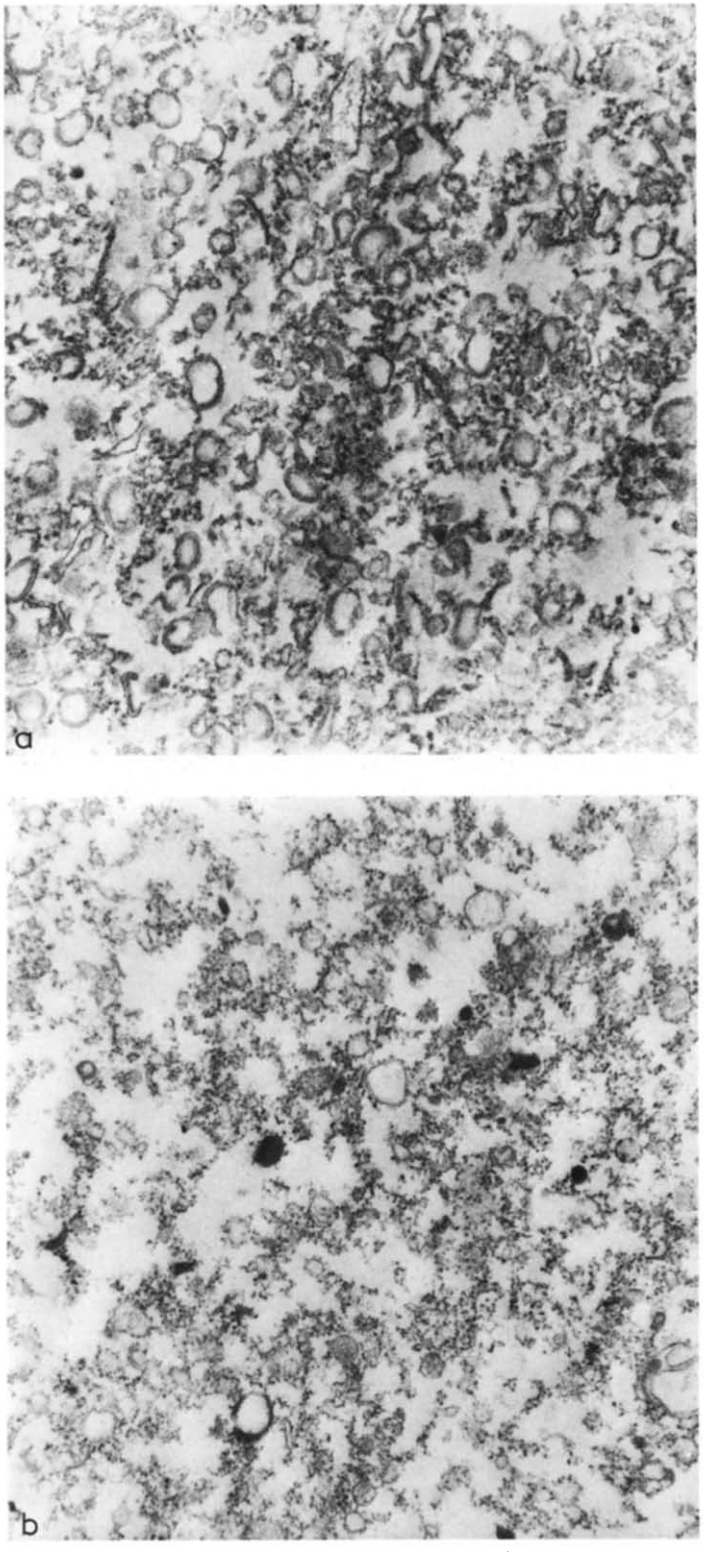


Fig. 8. For legend see opposite page.

(microsomes, 60 min 100 000 × *g* and light mitochondria, 20 min 20 000 × *g*), resuspended in 55% (w/v) sucrose, are placed on top of a layer of 64.5% (w/v) sucrose and subjected to centrifugation by flotation in a gradient of 55 to 30% (w/v) sucrose, again the normal pattern is obtained. Prolonged centrifugation of microsomal and light mitochondrial fractions on a normal sucrose gradient for 65 h instead of the usual 16 h yields about the same enzyme distribution pattern, indicating that after 16 h equilibrium has been reached.

However, when centrifugation is stopped after 8 h, a different pattern is obtained (Fig. 9). At that time peak fraction III has already reached its normal position, indicating that it consists of rather large particles. Peak fraction II is still near the top of the gradient, indicating that the particles in this fraction, which contains the (peak II) $HCO_3^-$-ATPase activity, must be of small size. They could originate from disruption of mitochondria, which are abundant in the parietal cells of the gastric mucosa [29]. This would also agree with the more evenly distributed cytochrome *c* oxydase activity. Very drastic homogenisation of the gastric mucosa could disrupt the mitochondria to such an extent that after 16 h of centrifugation peak II has not yet reached equilibrium.

The following experiment supports this suggestion. When the light mitochondrial fraction (20 min 20 000 × *g*) is sonicated 4 × 20 s with a Branson sonifier at 0°C at maximal output and is then subjected to density

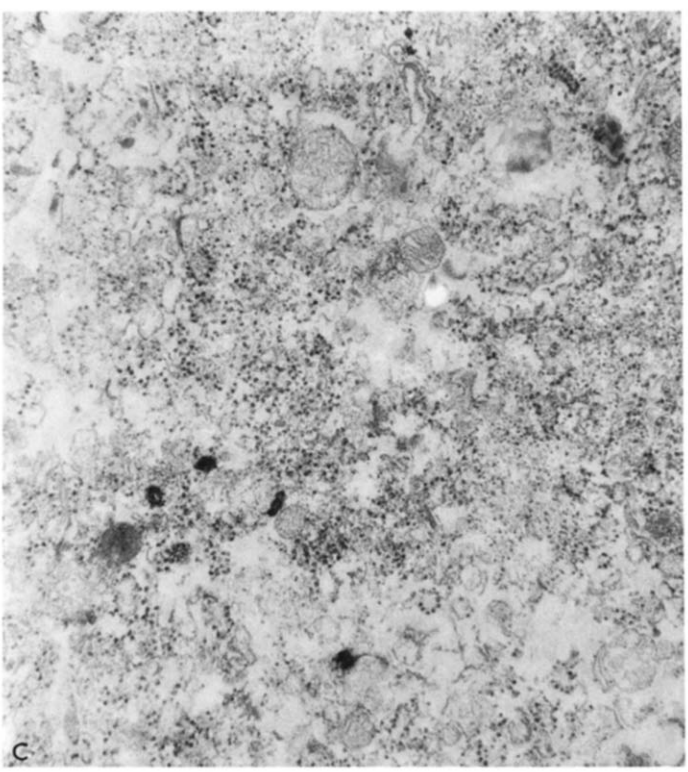

Fig. 8. Electron micrographs of subfractions I, II and III, obtained by density gradient centrifugation of rabbit gastric mucosal microsomes (34 200 ×).

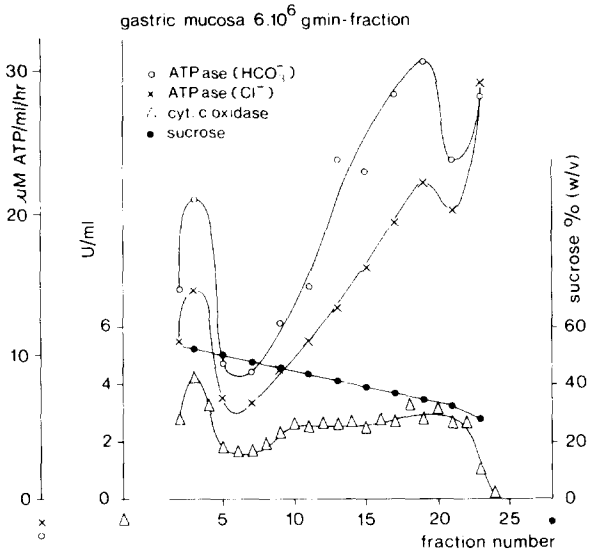


**Fig. 9. Enzyme distribution pattern of gastric mucosal microsomes on the normal density gradient after a shorter (8 h instead of the usual 16—17 h) centrifugation at 64000 × $g_{av}$. Activities of ATPase in $HCO_3^-$ medium (○) and $Cl^-$ medium (χ) together with cytochrome *c* oxidase (△) and sucrose concentration (●) are shown (typical experiment representative for two experiments).**

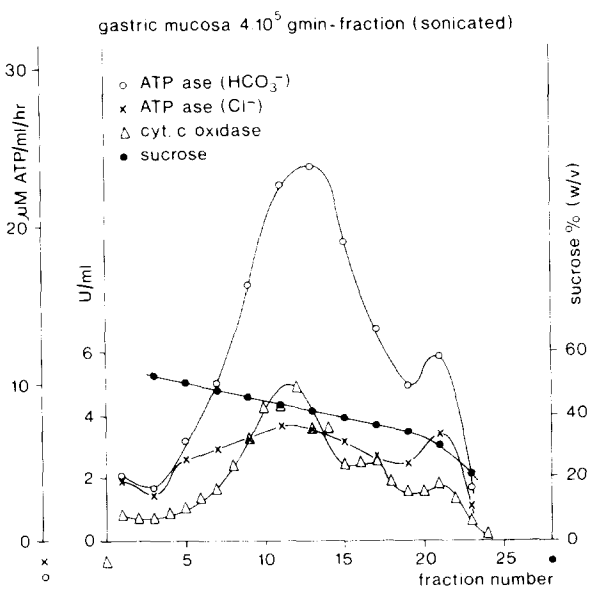


**Fig. 10. Enzyme distribution pattern of light mitochondrial (20 min 20 000 × *g*) fraction of gastric mucosa after sonication with a Branson sonifier 4 × 20 s at 0°C and centrifugation on the normal density gradient for 16 h at 64 000 × $g_{av}$. Activities of ATPase in $HCO_3^-$ medium (○) and $Cl^-$ medium (χ) together with cytochrome *c* oxidase (△) and sucrose concentration (●) are shown (typical experiment representative for two experiments).**

gradient centrifugation, a marked change in enzyme distribution pattern is obtained (Fig. 10). The $HCO_3^-$-ATPase activity has disappeared from peak III, and is increased in peaks I and II (cf. Fig. 4a). At the same time, cytochrome *c* oxydase activity has shifted partly from peak III to the top of the gradient, indicating a strong relationship between cytochrome *c* oxidase and $HCO_3^-$-ATPase activity.

## Discussion

Anion-sensitive ATPase activity has been investigated in a number of tissues. In most studies the difference between the ATPase activity in a medium with and without 20—25 mM $NaHCO_3$ has been called $HCO_3^-$-ATPase. This appears to involve the tacit assumption that the activity without bicarbonate and the additional activity in the presence of bicarbonate would represent two distinct enzymes. This assumption has certainly not been proven, and our own previous [4] and present results as well as work on mitochondrial ATPase [7] give considerable evidence to the contrary. We, therefore, prefer to use the ratio of enzyme activity with various anions to characterize the anion-sensitive ATPase activity. In the present study the activities with either bicarbonate, chloride or thiocyanate as major anion have been measured.

The question to be answered by this study was: is there an anion-sensitive ATPase located in plasma membranes? The fractionation studies in the three tissues gill, kidney and gastric mucosa, chosen for their possession of active anion transport, indicate that the major activity occurs in the mitochondrial fraction.

Although for rainbow trout gill a discrepancy between succinate dehydrogenase and $HCO_3^-$-ATPase distribution has been found [30], the enzyme distribution pattern in our density gradients shows that virtually all $HCO_3^-$-stimulated ATPase activity in the trout gill must be of mitochondrial origin. The 5′-nucleotidase activity on top of the gradients does not coincide with either cytochrome *c* oxidase or $HCO_3^-$-stimulated ATPase, which would seem to be sufficient proof for the absence of a non-mitochondrial $HCO_3^-$-stimulated ATPase in this tissue.

The density gradient pattern of the rabbit kidney fractions does not permit a definitive conclusion to be drawn. A better separation seems to have been achieved by free-flow electrophoresis [31], but these authors did not determine a mitochondrial marker enzyme in their final separation pattern, assuming that a lowering of succinate dehydrogenase activity during purification of the microvilli was sufficient proof for the presence of a non-mitochondrial anion-sensitive ATPase. This need not be the case, however, as is shown for the (peak II) $HCO_3^-$-stimulated ATPase of gastric mucosal microsomes in this paper. An increasing ratio of $HCO_3^-$-ATPase to succinate dehydrogenase activity could reflect activation through loss of an ATPase inhibitor protein [32] or increase in substrate accessibility of the ATPase during purification. Further experiments, in which inhibitor effects on various subfractions of kidney are compared (van Amelsvoort et al., to be published) lend further support to a wholly or largely mitochondrial origin of the activity in the kidney.

Density gradient fractionation of gastric mucosal microsomes yields three

subfractions, two of which (peak II and III) contain anion-sensitive ATPase activity, whereas the third peak (peak I) is apparently a plasma membrane fraction lacking anion sensitivity, as was also the case for gastric mucosal microsomes of pig [11] and rat [8]. Peak III is clearly a mitochondrial fraction. Hence our attention has been directed to peak II, which has a low cytochrome *c* oxidase level.

The properties of (peak II) $HCO_3^-$-ATPase are very similar to those of the $HCO_3^-$-ATPase activities studied by other investigators: sulfite stimulates maximally, optimal pH is 8.4, manganese but not calcium or zinc can replace magnesium, only GTP and ITP can replace ATP to some extent, optimal $Mg^{2+}$/ATP ratio is 0.3, effects of ionic strength.

However, there is a striking difference in the morphological appearance of the subfraction with that described by other authors for their anion-sensitive ATPase-containing fraction. In necturus oxyntic cells and dog gastric mucosa the anion-sensitive ATPase activity was localized in a vesicular fraction sedimenting at density 1.09—1.12 [33,2]. In our results only subfraction I, which is anion insensitive, appears to contain vesicular structures, but not subfraction II. Furthermore, the nearly identical effects of various inhibitors on (peak II) and (peak III) $HCO_3^-$-ATPase and the phospholipid composition strongly suggest that (peak II) $HCO_3^-$-ATPase is of mitochondrial origin, and may in view of the high cardiolipin content largely consist of mitochondrial inner membranes.

The experiments involving recentrifugation, centrifugation by flotation and prolonged (65 h) centrifugation indicate that in our case equilibrium has been reached after 16 h, and also that (peak II) $HCO_3^-$-ATPase does not originate from peak III by osmotic lysis of (peak III) mitochondria during centrifugation of the microsomal fraction. The very different distribution pattern obtained after 8 h centrifugation, compared to that after 16 h, indicates that equilibrium has barely been reached after 16 h. This suggests that, when more drastic homogenization has yielded smaller submitochondrial particles, equilibrium may not even be reached after 16 h. Our results after sonication appear to bear out this suggestion. This may explain why Sachs et al. [5] appear to find an anion-sensitive ATPase activity of gastric mucosa in an apparent plasma membrane fraction.

Summarizing we can say that previous reports of a non-mitochondrial $HCO_3^-$-stimulated ATPase activity in gastric mucosa and other tissues are in considerable doubt. The only hard evidence for a non-mitochondrial $HCO_3^-$-stimulated ATPase so far has been obtained for rabbit erythrocytes, which have no mitochondria [34]; however, this $HCO_3^-$-ATPase activity differs from all others by being insensitive to $SCN^-$. This means that further doubt has been thrown on a role of the anion-sensitive ATPase as an anion pump except for rabbit erythrocyte where such a role cannot be excluded.

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