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GASTRIC HCO_3^--STIMULATED ATPase: EVIDENCE AGAINST ITS MICROSOMAL LOCALIZATION IN RAT FUNDUS MUCOSA

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SUMMARY

Rat gastric mucosa was shown to contain a Mg^{2+}-dependent ATPase which is stimulated by HCO_3^- at pH 8–9.

Triton X-100 solubilizes this HCO_3^--stimulated, Mg^{2+}-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3).

The gastric mucosa was resolved into five subcellular fractions by differential centrifugation. A large granule fraction (Fraction M), 28 000 $g \cdot$ min, was characterized by cytochrome *c* oxidase (marker enzyme for mitochondria). A microsomal fraction (Fraction P), 2 760 000 g · min, was characterized by 5′-nucleotidase(5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) (plasma membrane).

The Mg^{2+}-dependent ATPase was demonstrated to have a bimodal mitochondrial membranous localization: 24% of its activity is associated with cytochrome *c* oxidase, and 75% with 5′-nucleotidase(5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) at pH 8.

The HCO_3^- addition resulted in two opposite effects: (1) a strong stimulation (84%) in Fraction M; (2) a slight inhibition (12%) in Fraction P.

Fraction M was subfractionated by equilibration on a sucrose gradient. It gave rise to a homogeneous mitochondrial (*d*, 1.17–1.21) Mg^{2+}-dependent ATPase, closely associated with cytochrome *c* oxidase. This ATPase is strongly stimulated ($\times 2$) by HCO_3^-. The subfractionation of Fraction P gave rise to two distinct ATPases: (1) the major one is associated with membranous (*d*, 1.10–1.15) material marked by 5′-nucleotidase and is slightly inhibited by HCO_3^-; (2) the other is associated with denser (*d*, 1.17–1.21) material and is stimulated by HCO_3^-.

The bicarbonate-stimulated fraction of the Mg^{2+}-dependent ATPase activity found in the gastric microsomal fraction is assumed to arise from mitochondrial cross-contamination. Further support comes from the optimal HCO_3^- concentration. In addition, SCN^- is shown to specifically inhibit the ATPase of Fraction M.

From these results it appears that the implication of HCO_3^--stimulated ATPase in the gastric secretion of H^+ is not as clear as had been suggested. However,

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in the view of an ATPase-supported model for H^+ secretion, attention can be directed towards the Mg^{2+}-dependent ATPase found to be associated with microsomes.

INTRODUCTION

From a theoretical point of view, a membrane-bound anisotropic ATPase [1–3] may be a useful model to elucidate the H^+ secretory mechanism in the stomach. A HCO_3^--stimulated, Mg^{2+}-dependent ATPase found in the frog gastric microsomes [4] aroused great interest. A further implication of the HCO_3^- was of particular interest with respect to the carbonic anhydrase requirement in the gastric acid secretory process [5].

This ATPase has been studied extensively in the gastric mucosa of various species [4, 6–10]. Evidence has been given for its relationship with the ontogenic development of the acid-secreting cell [9] and its association with oxyntic cell-enriched fractions [7]. SCN^- inhibition or HCO_3^- stimulation were found to be interchangeable properties of this enzyme [7]. It was readily soluble in Triton X-100 [7, 8] and its buoyant density was given to be 1.09–1.12 in a sucrose gradient [11, 12]. Unlike the mitochondrial ATPase [8, 11, 12], it was claimed to be associated with microsomal structures probably derived from the luminal membrane of the acid-secreting cell [8, 13].

On the other hand, the presence of a HCO_3^--stimulated Mg^{2+}-dependent ATPase was confirmed in microsomes from other tissues involving HCO_3^- transcellular transport, i.e. the pancreas [14], gills [7], brain [7] and submandibular gland [15].

In the light of these data, HCO_3^--stimulated, Mg^{2+}-dependent ATPase was implicated in tentative models as linking H^+ and HCO_3^- in a directional membrane translocation mechanism [11]. These models, although fascinating, could be challenged in so far as the membranous localization of the HCO_3^--stimulated, Mg^{2+}-dependent ATPase has not been clearly demonstrated. It is well known that mitochondrial ATPase can be activated by various anions [16] including HCO_3^- [17]. In this respect, the consideration of the possible cross-contamination of microsomes by mitochondria is of particular importance. In fact, recent reports by Katz et al. on rat kidney [18] and Izutsu et al. on dog submandibulary gland [19] argued for a mitochondrial origin of the HCO_3^--stimulated ATPase found in their microsomal fractions.

RESULTS

In crude fundus extracts, the rate of the Mg^{2+}-dependent hydrolysis of ATP exhibited two optimum pH values, pH 7 and pH 8–9 (Fig. 1). The rate of hydrolysis was accelerated by bicarbonate at pH 8–9. The surfactant action of Triton X-100 destroyed the enzymatic activity at pH 7 but preserved the pH 8–9 peak of activity and allowed it to be completely recovered in a 100 000 g · 1 h supernatant.

The subcellular structure associated with this enzymatic activity has been investigated in fractions obtained by differential centrifugation (Fig. 2).

The pH 8, Mg^{2+}-dependent ATPase was found to be widely distributed from

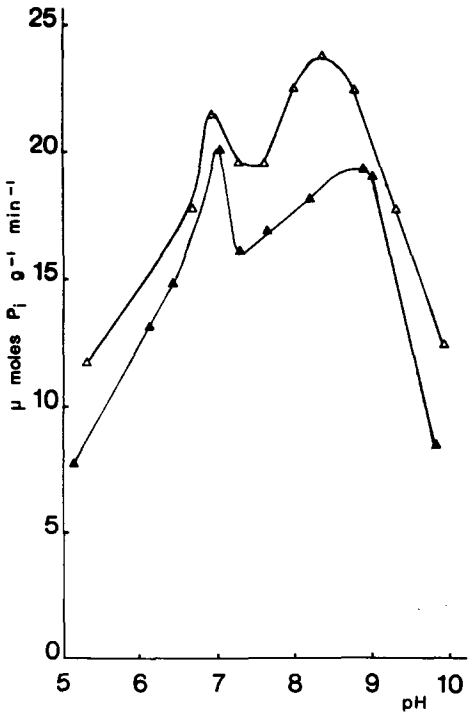

Fig. 1. pH dependence of ATPase activity in crude rat fundus extract. ATPase was tested in the presence of 3 mM ATP(disodium salt), 3 mM $MgCl_2$ with (△) or without (▲) 20 mM $NaHCO_3$ 20 min at 37 °C. Activity is expressed per g of fresh tissue and per minute. The pH range was obtained with 20 mM imidazole–HCl buffer; the pH was measured continuously during the incubation. Each assay was made in triplicate.

large granules, Fraction M, 9500 $g \cdot 3$ min, to microsomes, Fraction P, 92000 $g \cdot 30$ min, but was mainly associated with the 5'-nucleotidase membrane marker. The addition of HCO_3^- (20 mM) results in a significant enhancement ($\times 1.84$) of the activity in the M fraction and a slight decrease ($\times 0.88$) in the P fraction. No consistent activity was recovered in the supernatant (Table I).

If we consider the small granule Fraction L, 37000g · 6.7 min, as an intermediate fraction between Fractions P and M in the size-partitioning procedure, we note that a linear relationship exists between 5'-nucleotidase (plasma membrane) and cytochrome *c* oxidase (mitochondrial inner membrane) data (Fig. 3). Thus, a linear model was tested for the Mg^{2+}-dependent ATPase and for HCO_3^--stimulated, Mg^{2+}-dependent ATPase assuming that (a) a certain amount of their activities follows the cytochrome *c* oxidase subcellular pattern while (b) another part follows the 5'-nucleotidase pattern (Fig. 4). In the case of Mg^{2+}-dependent ATPase, computation from Fractions M, L and P gives (a) 24 % ± 2 % and (b) 75 % ± 3 % (mean ± S.E. of 3 fractionations). The sum (a)+(b) gives 99%, which accounts for a consistent recovery of the total activity of the extract. The same computation for HCO_3^--stimulated, Mg^{2+}-dependent ATPase led to (a) 62% ± 7%, (b) 24% ± 13% and (a)+(b) = 86%. On the basis of these results a dual localizationcan be postulated for the Mg^{2+}-

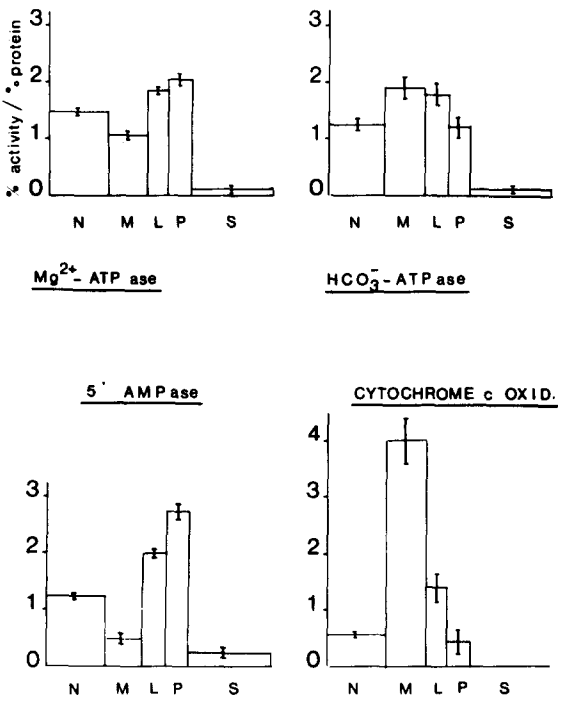

Fig. 2. Differential fractionation of rat gastric mucosa. The subcellular patterns (mean of three experiments ± S.E.) are standardized. ATPase was estimated in the presence of 2 mM ATP, 2 mM $MgCl_2$, with or without 20 mM $NaHCO_3$ (pH 8) (100 mM imidazole–HCl). The 5′-nucleotidase and cytochrome *c* oxidase recovery were, respectively, 102 and 95%. Fraction N, 1200 *g* × min, nuclear and cell debris fraction; Fraction M, 28 200 *g* × min, large granules; Fraction L, 252 000 *g* × min, small granules; Fraction P, 2 760 000 *g* × min, microsomes; Fraction S, supernatant.

TABLE I

ATPase ACTIVITY IN THE SUBCELLULAR FRACTIONS OF RAT GASTRIC MUCOSA

ATPase was estimated in the presence of 2 mM ATP (disodium salt), 100 mM imidazole (pH 8.0), 2 mM $MgCl_2$, with or without 20 mM $NaHCO_3$. Incubation: 20 min at 37 °C. Activities are expressed in μmoles P_i/mg protein per h (mean of three fractionations ± S.E.).

Activator	Mg^{2+}	$Mg^{2+}+HCO_3^-$	$\frac{Mg^{2+}+HCO_3^- - Mg^{2+}}{Mg^{2+}}$ (%)	Protein (mg/g tissue)
Crude homogenate	11.73 ± 1.95	13.46 ± 1.49	14	154.7
N 1200 *g* · min	19.30 ± 1.02	20.46 ± 1.51	6	40.6
Cytoplasmic extract	9.38 ± 0.39	11.84 ± 0.46	26	113.3
M 28 800 *g* · min	13.66 ± 1.99	25.08 ± 0.52	84	25.3
L 252 000 *g* · min	24.03 ± 0.88	28.70 ± 2.26	19	14.8
P 2 760 000 *g* · min	26.26 ± 2.66	22.90 ± 2.19	−12	13.7
S	1.62 ± 0.15	1.80 ± 0.12	11	54.5
Recovery (%)	103	105		97

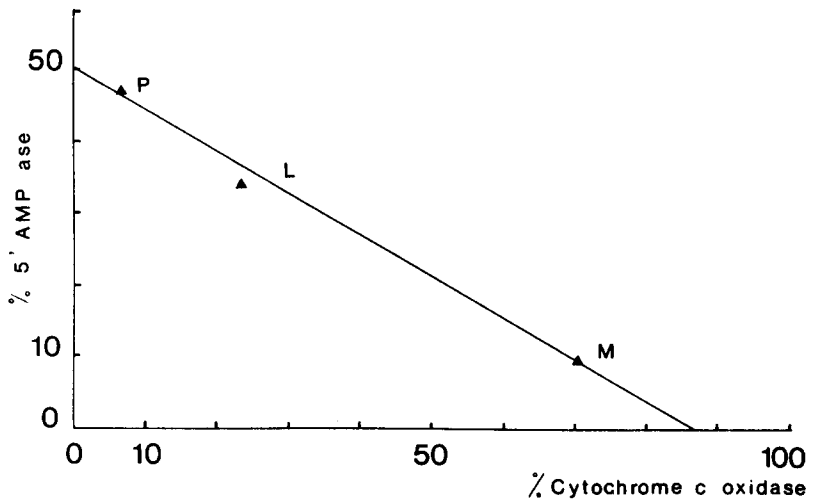

Fig. 3. 5'-Nucleotidase distribution versus cytochrome *c* oxidase distribution along a fractionation. The percentage of the markers in the respective fractions are collected. 100 % is taken as the extract (Fractions M+L+P+S) activity.

dependent ATPase recovered in the rat fundus extracts: (1) the major part, 75%, is associated with (plasma) membranes while (2) the remaining part, 24%, is associated with mitochondria. The addition of HCO_3^- causes a dramatic decrease (from 75% to 24%) in membranes in relation to a net inhibition of the associated enzyme. On the other hand, it produces an increase (from 24% to 62%) of the mitochondrial contribution, resulting from a marked stimulation of the associated enzymatic activity.

The HCO_3^--stimulated, Mg^{2+}-dependent ATPase of the rat fundus extract

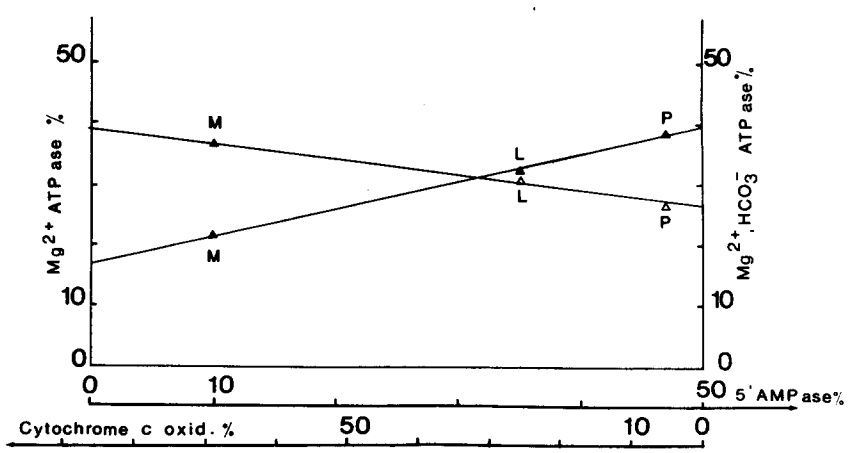

Fig. 4. Mg^{2+}-dependent (Mg^{2+}-ATPase, ▲) and HCO_3^--stimulated, Mg^{2+}-dependent (Mg^{2+}, HCO_3^--ATPase, △) ATPase. The percentage of activity in the fractions are referred to 5'-nucleotidase and cytochrome *c* oxidase in the same fractions using a bimodal model assuming that the ATPase activity is coupled to the membrane and to the mitochondria. 100 % activity is taken as the extract (Fractions M+L+P+S).

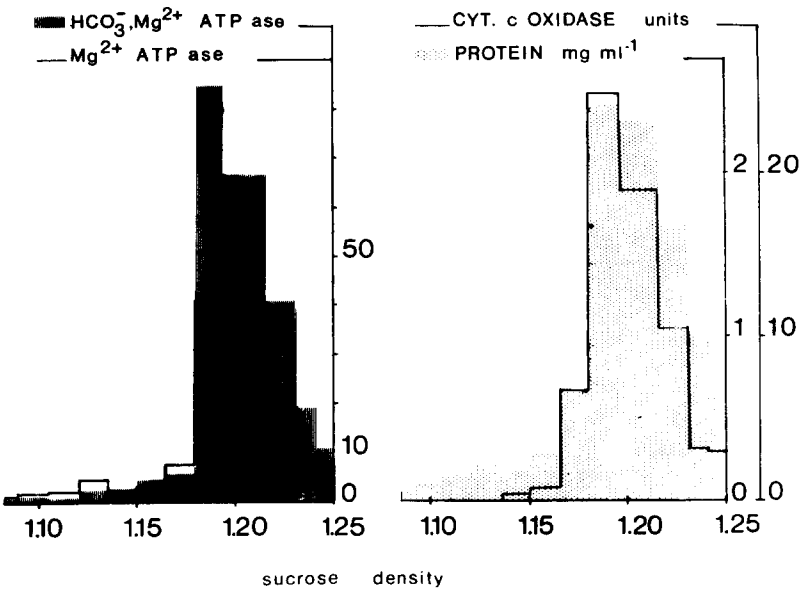

Fig. 5. Fraction M equilibration on a sucrose density gradient varying from 1.09–1.25. Fraction M, 28 000 $g \times$ min was homogeneously distributed on a linear gradient and centrifuged for 20 h at 20 000 rev./min at 0–4 °C. ATPase was estimated in the presence of 2 mM ATP, 2 mM $MgCl_2$, at 37 °C (pH 8.0) (imidazole, 100 mM), with or without $NaHCO_3$ (20 mM). The recovery obtained for Mg^{2+}-dependent ATPase was 97 % and for HCO_3^--stimulated, Mg^{2+}-dependent ATPase, 81 %. Protein equilibration of the fraction is given in the second part of the figure with cytochrome *c* oxidase as a marker. Cytochrome *c* oxidase units are expressed in Δ log A/h per ml of fraction $\times 10^{-2}$. The recovery for protein was 97 % and for cytochrome *c* oxidase 90 %.

is, therefore, assumed to be solely localized in mitochondria (or in subcellular organelles similar in size).

The M and P fractions were then subjected to isopycnic equilibration on a linear sucrose gradient.

The M fraction (Fig. 5) showed a single peak of activity for the Mg^{2+}-dependent ATPase at a density of 1.17–1.21, closely associated with the cytochrome *c* oxidase profile and strongly stimulated ($\times 2$) by 20 mM HCO_3^-. Furthermore, with respect to the protein recovery, the material of this fraction is assumed to be consistently associated with the mitochondria.

On the other hand, the P fraction (Fig. 6) exhibits a substantial amount of dense (*d*, 1.17–1.21) proteic material separate from the light (*d*, 1.10–1.15) material marked by 5′-nucleotidase. It gives a single peak of activity for Mg^{2+}-dependent ATPase at a density 1.10–1.15 of which is markedly ($\times 0.75$) inhibited by 20 mM HCO_3^-. However, as expected, a slight activation ($\times 1.25$) is observed at the level of the 1.17–1.21 density material (mitochondria).

Kinetic studies were performed with both P and M fractions (Fig. 7). Unlike the Fraction M, in which a definite stimulation was obtained for incubation times up to 30 min, HCO_3^- addition had no marked effect on the P fraction. However, a close examination of the kinetic data for Fraction P denotes; (1) a slight inhibition (over 15 min), (2) a poor stimulation (under 15 min). This dual phenomenon was

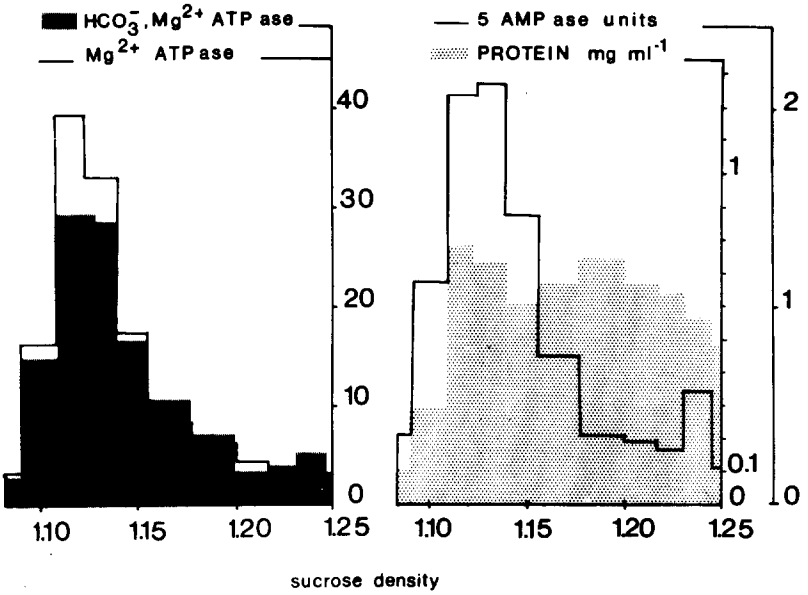

Fig. 6. Fraction P equilibration on a sucrose gradient varying from 1.09–1.25. Fraction P (2 760 000 $g \times$ min) was homogeneously distributed on a linear sucrose gradient and centrifuged for 20 h at 20 000 rev./min, 0–4 °C. ATPase was estimated as in Fig. 5 with a 20-min incubation. The recovery of HCO_3^--stimulated, Mg^{2+}-dependent ATPase was 68 % and that of Mg^{2+}-dependent ATPase was 64 %. Protein equilibration of the fraction is given with a 5′-nucleotidase marker profile. 5′-Nucleotidase units are expressed in moles of PO_4^-/h per ml of fraction. The recovery was 94% protein and for 91 % for 5′-nucleotidase.

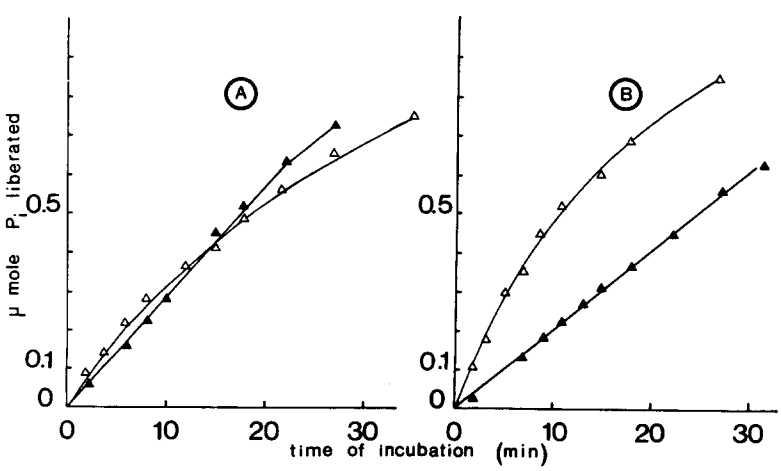

Fig. 7. Fractions P and M kinetics. The incubation medium contained 2 mM ATP 100 mM imidazole (pH 8.0), 2 mM $MgCl_2$ with (△) or without (▲) 20 mM $NaHCO_3$ at 37 °C. A, Fraction P; B, Fraction M.

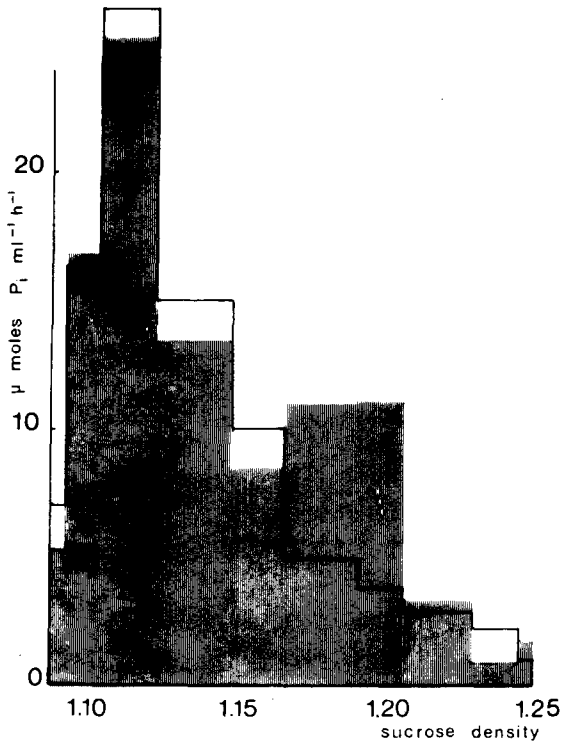

Fig. 8. Fraction P equilibration on a sucrose gradient. Equilibration was performed as in Fig. 6 and ATPase was estimated by a 5-min incubation. The recovery of HCO_3^--stimulated, Mg^{2+}-dependent ATPase was 80 % and that of Mg^{2+}-dependent ATPase was 78 %.

repetitively obtained. Particular attention was paid to the possibility of the net activation of the fraction obtained with less than 15 min of incubation: the Fraction P subfractionation on a sucrose gradient was tested with a 5-min incubation time for the ATPase assay (Fig. 8). Under those conditions, it was clearly shown that the stimulation is due to the mitochondrial contribution whose importance increases with decreasing incubation time (Fig. 8 compared to Fig. 6).

The HCO_3^--stimulated, Mg^{2+}-dependent ATPase was then studied with respect to the $MgCl_2$ and $NaHCO_3$ concentrations. The optimal conditions were found to be 2 mM Mg^{2+} (ATP: Mg^{2+} ratio 1 : 1) for the P and M fractions, and 60–80 mM HCO_3^- for Fraction M. These concentrations provide a maximally 4-fold HCO_3^- stimulation of Mg^{2+}-dependent ATPase in Fraction M. In addition, mitochondrial ATPase was shown to be inhibited by SCN^-; 50 mM NH_4SCN inhibited 90% of the ATPase activity (Mg^{2+} activity as well as HCO_3^--stimulated Mg^{2+}-dependent activity). In the same conditions less than a 10% inhibition is observed in Fraction P.

DISCUSSION

From the data presented it would appear that the rat gastric mucosa exhibits a consistent HCO_3^--stimulated, Mg^{2+}-dependent ATPase activity closely similar,

with respect to pH optimum [4, 8], Triton solubilization [7] and SCN^- inhibition [4, 8] to that described in gastric microsomes from amphibian (frog [4, 9], toad [10], *Necturus* [7]) and mammalian species (dog [8]). However, in our hands no extramitochondrial activity was recovered in the subcellular fractions, therefore, a conflict arises concerning the subcellular structure associated with this enzyme.

Admittedly, our conflicting results can be due to our choice of the rat as an experimental animal, although the discrepancy between rat and dog [12] in gastric enzyme behavior was not expected.

It is more likely that these differences are due to the subcellular fractionation procedures used, as well as to their analytical interpretation.

The gastric mucosa is extremely difficult to homogenize because of the presence of connective tissue. Without the use of a proteolytic enzyme [20] a great deal of force is required for its mechanical disruption. In addition, the mammalian parietal cell (likewise the amphibian oxyntopeptic cell) is very rich in mitochondria. Therefore, when fractionating, cross-contamination of microsomes by mitochondria (and/or mitochondrial membranes) is a matter of fact, even in "pure" preparations.

In this respect, as recently reviewed [21], homogenization by drastic use of a high speed motor-driven pestle [7, 8], nitrogen cavitation [12], and by ultraturax [10], which were used in previous and similar studies, are probably contributing factors to this cross-contamination.

The situation appears to be circumvented in a more reliable way by using marker enzymes [21] rather than by an electron microscopic examination of the subcellular fractions [4, 7, 8, 12].

5′-Nucleotidase and cytochrome *c* oxidase have been well documented in rat gastric mucosa (unpublished results). They provide, in this material, a reliable characterization of plasma membranes and mitochondria, respectively. In spite of the cellular heterogeneity in gastric mucosa, each of these organelles is found to be related to a single subcellular population.

The classical resolution [22] of the homogenate in a five-fraction scheme of differential centrifugation delivers a typical microsomal fraction which accounts for about 6% of the total cytochrome *c* oxidase activity. This leads us to speculate that the mitochondria represent about 13% of the microsomal proteins. Consequently, these mitochondria, whether intact or damaged, represent a material whose contribution in the ATP hydrolysis is not negligible. It must be mentioned that this contribution can pass unnoticed because mitochondrial marker enzymes can be underestimated in aged preparations: for cytochrome *c* oxidase, we have observed a 45% activity decrease in 24 h.

The isopycnic equilibration in sucrose gradients gives, for the gastric mucosa, a reliable pattern in which as expected [21], the plasma membrane equilibrates at a density of 1.10–1.15 while the mitochondria equilibrate at 1.17–1.21. The 5′-nucleotidase is enriched 2.8 fold on the membrane density (recovery 91%). The cytochrome *c* oxidase is enriched 5.9 fold on the mitochondrial density (recovery 90%). Usually, no more than 10% of the total 5′-nucleotidase is recovered on the 1.17–1.21-mitochondrial zone.

We used the SW_{25} rotor for isopycnic equilibration. No more than 16–22 mg protein (3–6 rats) were subjected to equilibration and the more reliable results were obtained by distributing the material homogeneously along the gradient. The local

material concentration in the gradient was found to be low enough to prevent aspecific aggregation and droplet sedimentation. Under these conditions 20 h are required to achieve the equilibration of all types of organelles. However, relatively enriched preparations are required to obtain a satisfactory separation. The equilibration of the total homogenate is obscured by various artifacts resulting from hazardous associations due to mucus and, to a lesser extent, pepsinogen and from adsorption due to the relative concentration of the material in the gradient.

On the other hand, the stimulation by HCO_3^- of mitochondrial ATPase, from liver [17] is a well-known phenomenon. This mitochondrial ATPase does not appear to be basically different from the HCO_3^--stimulated, Mg^{2+}-dependent ATPase of gastric mucosa with regard to optimum pH, HCO_3^- concentration and the Mg^{2+}: ATP ratio. These two enzymes are readily solubilized by Triton X-100 under the same experimental conditions. In an attempt to distinguish, in the gastric mucosa, between the two enzymes from a biochemical point of view, only two major differences were reported [11], (1) dinitrophenol was shown to be inactive on the "microsomal" enzyme while it stimulated the mitochondrial enzyme; (2) BO_3^- were reported to inhibit mitochondrial ATPase while they stimulated the microsomal enzyme. These effectors were not involved in the present study. However, in a similar study on dog submandibular gland [19], the HCO_3^- stimulation was found to be correlated in each subcellular fraction with the succinate dehydrogenase activity and with dinitrophenol stimulation.

These results, following a previous report from Katz et al. [18] on rat kidney, support the hypothesis that HCO_3^--stimulated activity found in gastric microsomes results from mitochondrial cross-contamination.

Such an assumption does not definitively exclude the possible role of HCO_3^- stimulated, Mg^{2+}-dependent ATPase in H^+ transport by the gastric mucosa although this implicating role is not as obvious as it has been. The fact that this enzyme originates from mitochondria obviously renders self-fulfilling the studies that relate its activity to isolated acid-secreting cell fractions in which enrichment is followed by mitochondrial marker enrichment [7]. In addition, it has been shown recently that, in rat gastric mucosa, the HCO_3^--stimulated rate of ATP hydrolysis was not specifically concerned with various stimulants or inhibitors of gastric acid secretion with regard to the marked effect of these agents on Mg^{2+}-dependent ATP hydrolysis and on carbonic anhydrase activity [23].

However, a membrane-bound ATPase still remains an attractive model for the gastric acid secretory mechanism. In this context, attention can be directed towards the Mg^{2+}-dependent ATPase found to be associated with 5′-nucleotidase-marked membranes in rat gastric mucosa. This enzyme appears to be distinct from (Na^+–K^+)-ATPase (unpublished results). The Mg^{2+}-dependent ATPase is inhibited by HCO_3^-. However, this latter result may be due to a damaging action of $NaHCO_3$ on the associated membrane (unpublished results). Further studies are required to better define its enzymatic characteristics and its functional role in the gastric mucosa.

METHODS

Stomachs of fed male Wistar rats weighing between 250 and 300 g were used in this study.

Tissue homogenization. Stomachs were washed with a solution containing 0.25 M sucrose, 30 mM imidazole (pH 7.4). Fundic mucosa was scraped and homogenized in an approximate 1/10 (w/v) solution of 0.25 M sucrose, 3 mM imidazole (pH 7.4). The homogenization was performed with scissors and a teflon homogenizer (Potter–Elvehjem), 4–5 strokes at 2000 rev./min. All the operations were performed between 0–4 °C.

Differential centrifugation. The homogenate was fractionated in a 0.25 M homogeneous sucrose medium using the PR_2 International centrifuge, rotor No. 269, and the L_2 Spinco Beckman centrifuge, rotor No. 50 Ti. Five fractions were obtained. The fractions are defined by the specific enrichment of the following markers [22]: (1) the DNA "N fraction", $200 \times g$ during 6 min, represented by nucleus and cellular debris; (2) the cytochrome *c* oxidase "M fraction", $9500 \times g$ during 3 min and 2 s; representing the large granules; (3) the acid phosphatase "L fraction", $37\,000 \times g$ during 6 min and 42 s, for the small granules; (4) 5'-nucleotidase and RNA "P fraction", $92\,000 \times g$ during 30 min, for the microsomes. The supernatant of the fraction P was called S. The extract used in the preliminary studies is the supernatant of the Fraction N.

Isopycnic centrifugation. The M and P fractions were distributed homogeneously along a 23–55% linear sucrose gradient (density 1.09–1.25) and centrifuged for 20 h at 20 000 rev./min in the SW_{25} rotor of the L_2 Spinco Beckman centrifuge. The fractions were collected with a syringe by perforating the tube at the various sucrose levels. The density was determined by equilibrating drops of the fractions in an organic gradient (petroleum benzin–1,2-dichlorobenzene) standardized with KBr solutions [24].

Assays. Substrates used for the biochemical assays were obtained from Sigma Chemical Co. The protein concentrations were determined by the method of Lowry et al. [25]. Cytochrome *c* oxidase was estimated after Cooperstein and Lazarow [26]. 5'-Nucleotidase was measured in the presence of 2 mM 5'-AMP. 100 mM Tris buffer (pH 7.5), with 0.6 mM $MnCl_2$ as activator. DNA and RNA were determined following the method of Schneider [27]. Acid phosphatase was estimated by measuring the *p*-nitrophenol liberated from *p*-nitrophenyl phosphate (pH 5.0) using a Gilford single beam spectrophotometer ($\lambda = 410$ nm).

ATPase activity was assayed in a final volume of 1 ml which contained: 2 mM ATP (disodium salt), 2 mM $MgCl_2$, 100 mM imidazole (pH 8.0) with or without 20 mM $NaHCO_3$. The reaction was run at 37 °C during 5–20 min according to the protein concentration. The reaction was stopped by 2 ml of 8% trichloroacetic acid and the tubes were kept in ice. P_i was measured by the method of Fiske and SubbaRow [28] ($\varepsilon : 3.3 \cdot 10^3\ l \cdot mole^{-1} \cdot cm^{-1}$).

Solubilization was carried out using Triton X-100 as reported by Blum et al. [8].

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