BBA 75711

PROPERTIES OF SOLUBLE ATPase OF GASTRIC MUCOSA

II EFFECT OF HCO_3^-

A. L. BLUM, G. SHAH, T. ST. PIERRE, H. F. HELANDER, C. P. SUNG, V. D. WIEBELHAUS AND G. SACHS*

Division of Gastroenterology and Department of Chemistry, University of Alabama, Birmingham, Ala, and Veterans Administration Hospital, Birmingham, Ala. and Smith Kline and French Laboratories, Philadelphia, Pa. (U.S.A.)

(Received April 23rd, 1971)

SUMMARY

An ATPase has been solubilized from dog gastric mucosa which was localized to the smooth vesicular fraction of the tissue homogenate. With solubilization, there was a marked increase in the HCO_3^- activation of the enzyme, and several other oxyanions were found to stimulate ATPase activity, such as borate, selenite, arsenite, arsenate and sulfite. The stimulation was a function of the concentration and *pK* of the base. Additionally, methane sulfonyl chloride inhibited the enzyme, particularly the base activated component. Based on these observations a tentative model for the HCO_3^- activation mechanism is suggested.

INTRODUCTION

An SCN^--inhibited, HCO_3^--stimulated ATPase (EC 3.1.6.3) has been isolated from the gastric mucosa and other tissues of a variety of species[1,2], and has been shown to be localized in the smooth membrane fraction derived from isolated oxyntic cells and to be absent from the surface epithelial cell of the gastric mucosa[3]. Since SCN^- inhibits acid secretion *in vivo*[4] or *in vitro*[5] the ATPase inhibition by SCN^- suggests a role for this enzyme in H^+ transport by the gastric mucosa. Moreover, since carbonic anhydrase inhibitors, such as diamox, inhibit acid secretion *in vivo* by 90 %[6] and Cl^- or H^+ secretion *in vitro*[7,8] and the uncatalyzed rate of HCO_3^- production from CO_2 and H_2O is 10 % of the catalyzed rate, the HCO_3^- stimulation of this ATPase may have considerable functional significance. The wide distribution of this enzyme[2,3] also indicates a more general importance than a role in gastric acid secretion.

Accordingly a method was developed for the preparation of a stable solubilized ATPase from dog gastric mucosa, which confirmed the localization of this enzyme to the smooth vesicular membrane fraction. Using this preparation, it was shown that activation occurred with a variety of oxyanions as a function of the concentration and

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanosulfonic acid; DFP, diisopropyl fluorophosphate.

* To whom correspondence should be addressed.

basicity of the anion. In addition electrophilic reagents, such as methane sulfonyl chloride inhibited the activation by more than 60 %, suggesting the involvement of a serine-OH in the reaction.

METHODS

The method for the preparation of a soluble, HCO_3^--activated ATPase from purified suspensions of amphibian (*Rona catesbiana* and *Necturus maculosus*) oxyntic cells has been previously described[3]. For this study, however, large quantities of enzyme were necessary, and hence fresh dog gastric mucosa was chosen as a source of enzyme.

The mucosa was scraped off the stomach into approximately 10 vol. of ice-cold 0.25 M sucrose containing 20 mM Tris–HCl buffer, pH 7.4. The suspension was homogenized using a teflon homogenizer (30 strokes, 2000 rev./min) and fractionated by differential centrifugation into crude nuclear, heavy mitochondrial, light mitochondrial, microsomal, and supernatant fractions. All operations were carried out at 0°.

The highest specific activity of the HCO_3^--activated ATPase was found in the crude microsomal fraction. Electron microscopy showed that this was a heterogeneous mixture of cellular elements. Accordingly the microsomal pellet was resuspended in 0.25 M sucrose, the suspension layered on a 20–50 % linear sucrose gradient and centrifuged at 25000 rev./min for 16 h in a SW25 rotor in the Beckman L2 ultracentrifuge at 4°. Five bands were collected and assayed for ATPase activity, as well as sucrose concentration by an Abbé refractrometer, and studied morphologically. For the latter, an aliquot of the bands was fixed in 1 % OsO_4 solution buffered with veronal acetate at pH 7.4 (ref. 9) and embedded in Epon.

The soluble enzyme was prepared from the crude microsomal pellet. The pellet was stirred for 1 h at 0° with 20 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid), pH 7.4, 60 mM NaCl, and Triton X-100 at a 3:1 triton to protein ratio which had been shown to be optimal for enzyme extraction[3]. The mixture was centrifuged for 1 h at 100000 × *g* and the supernatant was used without further purification.

Assay of ATPase activity was carried out in a solution containing 3 mM ATP, 3 mM $MgCl_2$, 20 mM HEPES buffer at appropriate pH, about 0.1–0.2 mg enzyme protein in 1 ml, and with additions as detailed in the text. The phosphate released was measured in duplicate by the method of YODA[10] following a 20 min incubation at 37°.

For studies with diisopropyl fluorophosphate (DFP) or methane sulfonyl chloride, preincubation was necessary for inactivation of the enzyme, as was true for the (Na^+, K^+)-ATPase[11]. Thus the enzyme was preincubated with varying concentrations of these inhibitors at 37° for 1 h, and subsequently assayed for Mg^{2+} and Mg^{2+} + HCO_3^--ATPase activity.

For comparison of enzyme activity in ^2H_2O as compared to water, the microsomal pellet was extracted in the usual way, and an aliquot dialyzed against ^2H_2O and another aliquot dialyzed against water for 24 h. The assay medium contained ^2H_2O instead of water for all the reagents.

Proteins were measured by the LOWRY method[12] corrected for interference by triton or sucrose.

RESULTS

Localization

Table I shows the localization of the ATPase in different subcellular fractions of the dog gastric mucosa. It can be seen that the highest specific activity of the ATPase, and the highest percentage stimulation by HCO_3^- was obtained in the crude microsomal fraction, although in terms of total activity a considerable amount is present in the crude mitochondrial fraction. The enzyme appeared to be absent from the soluble supernatant.

TABLE I

ATPase ACTIVITY OF GASTRIC SUBCELLULAR FRACTIONS

ATPase specific and total activity of subcellular fractions of dog gastric mucosa. The enzyme was incubated for 20 min at 37° in a medium containing about 0.1 mg enzyme, 3 mM Na_2ATP, 3 mM $MgCl_2$, 20 mM HEPES buffer, pH 7.4, with and without 20 mM $NaHCO_3$ in a final volume of 1 ml.

Fraction	*Specific activity* ($\mu moles\ P_i \cdot mg^{-1} \cdot h^{-1}$)		*Total activity of* HCO_3^- *ATPase* ($\mu moles\ P_i \cdot h^{-1}$)
	Mg^{2+}	$Mg^{2+} + HCO_3^-$	$[Mg^{2+} + HCO_3^-] - [Mg^{2+}]$
Total homogenate	6.73	7.20	574
Nuclear ($10^4 \times g$)	6.35	7.49	69.4
Mitochondrial ($10^5 \times g$)	5.92	7.22	246.0
Light mitochondrial ($4 \cdot 10^5 \times g$)	11.35	15.09	58.3
Microsomal ($6 \cdot 10^6 \times g$)	17.18	25.42	172.2
Supernatant	1.75	1.75	—

TABLE II

ATPase ACTIVITY OF BANDS

Specific and total activity of bands collected following density gradient centrifugation (20–50% sucrose, 16 h, SW25 rotor, 25000 rev./min, 5°) of the $6 \cdot 10^6 \times g$ "microsomal" fraction. Incubation conditions as in Table I.

Sucrose density	*Specific activity* ($\mu moles\ P_i \cdot mg^{-1} \cdot h^{-1}$)		*Total activity of* HCO_3^- *ATPase* ($\mu moles\ P_i \cdot h^{-1}$)
	Mg^{2+}	$Mg^{2+} + HCO_3^-$	$[Mg^{2+} + HCO_3^-] - [Mg^{2+}]$
1.06	15.00	21.1	0.3
1.08	22.5	32.4	2.1
1.12	18.5	29.9	11.8
1.15	11.1	13.7	1.5
1.22	3.7	4.5	0.6

Table II shows the activation obtained in the 5 bands following density gradient fractionation of the microsomal pellet obtained in Table I. The highest specific activity was found in the second and third bands corresponding to sucrose densities

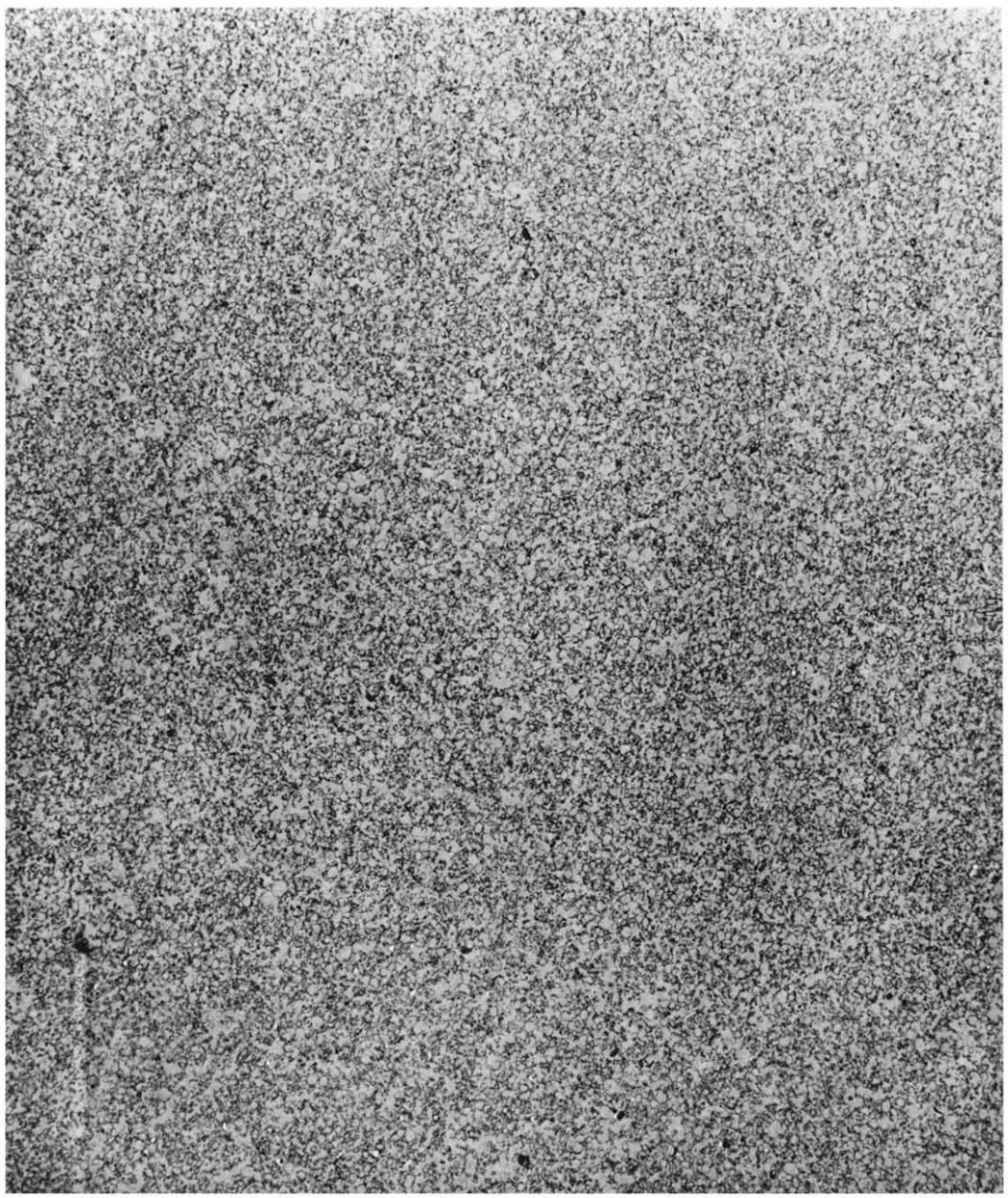


Fig. 1. Electron micrograph of band recovered from the density gradient at a sucrose density of 1.08. This survey shows the relatively homogeneous composition of this band. × 3400.

of 1.08 and 1.12. These consisted of largely smooth microsomes (Figs. 1–4). The bands at 1.06, 1.16 and 1.22 density contained a mixture of rough and smooth microsomes and occasional mitochondria.

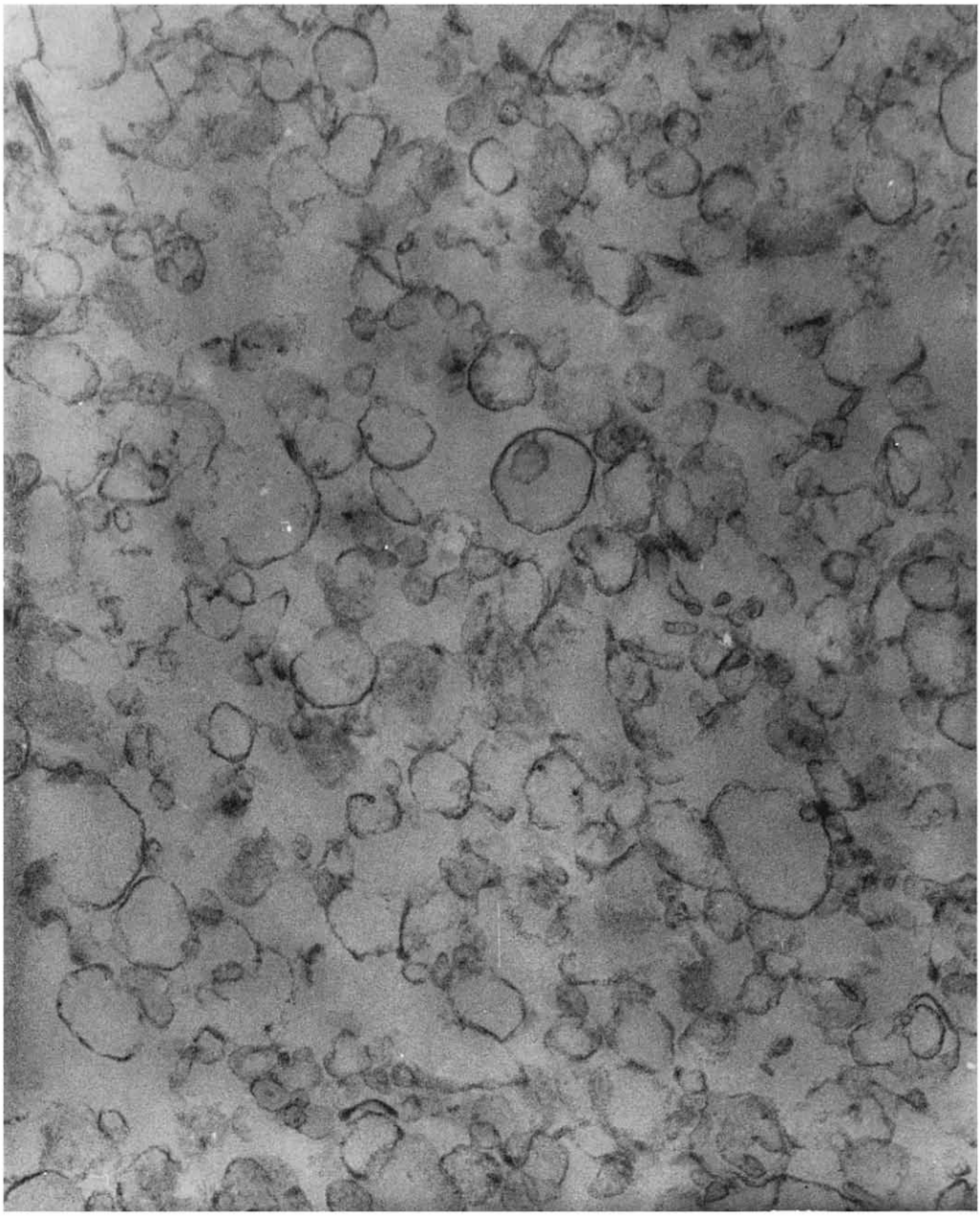


Fig. 2. High magnification of the material displayed in Fig. 1. Smooth membranes dominate the picture. × 56000.

Solubilization

Various detergents were tried in an attempt to solubilize the enzyme, such as lubrol, sodium dodecyl sulfate, sodium deoxycholate and Triton X-100. Of these only

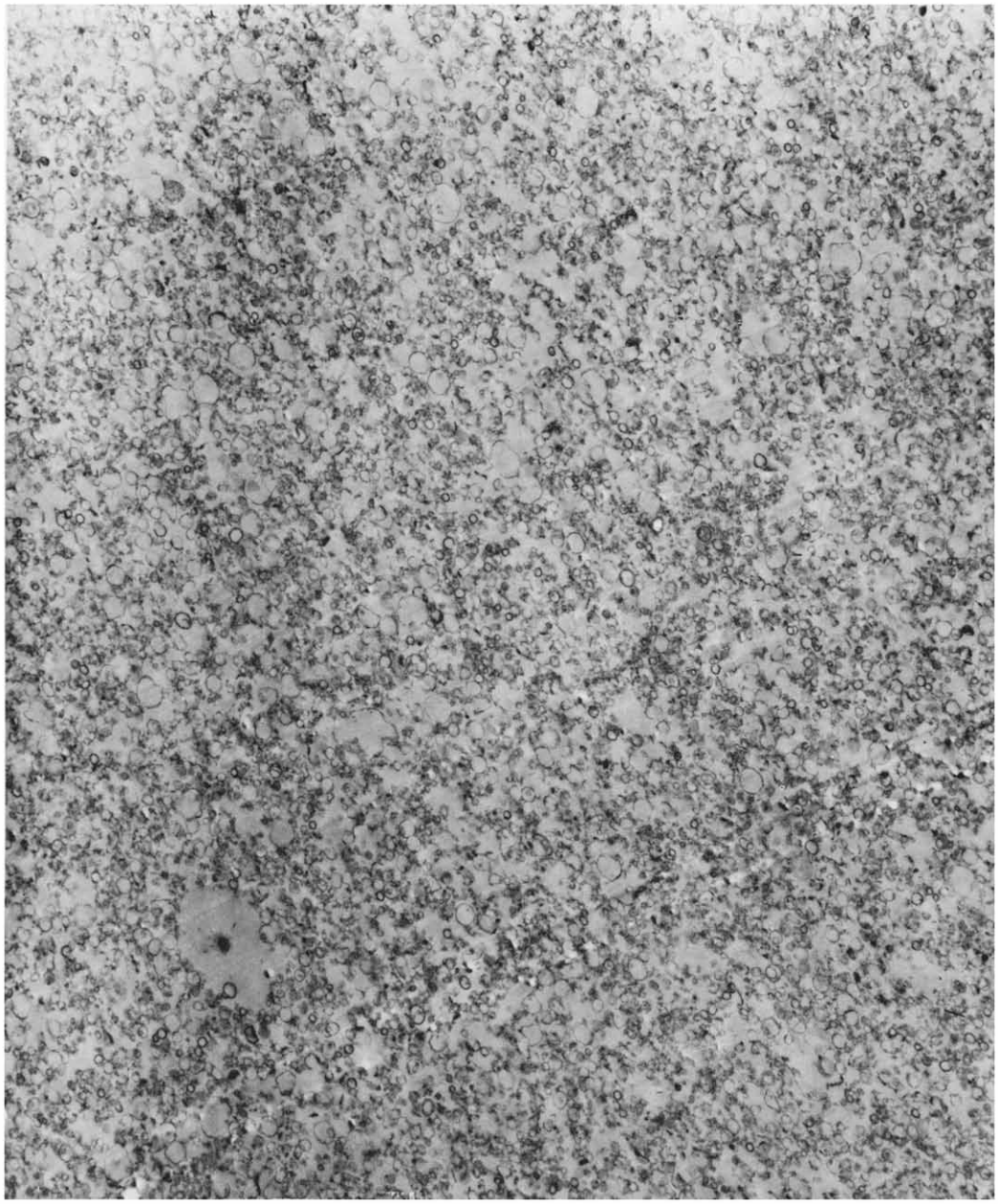


Fig. 3. Survey of section from band collected at a density of 1.12. This band is also fairly homogeneous in composition. × 6500.

the latter was successful, and, as in the case of Necturus[3] a Triton:protein ratio of 3:1 gave optimal results. Concomitant with solubilization there was a decrease in the specific activity of the Mg^{2+} enzyme, but a marked increase in the degree of HCO_3^-

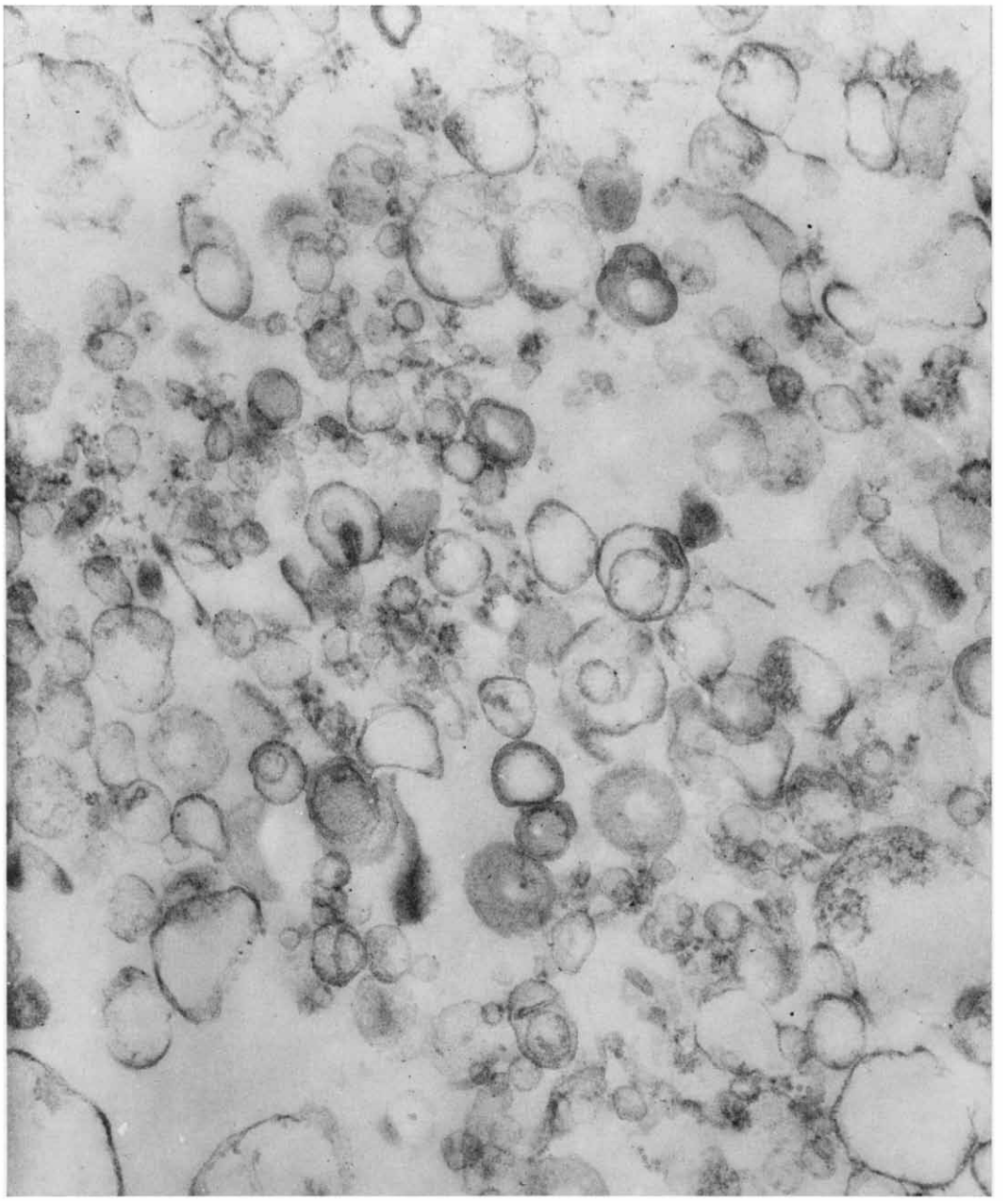


Fig. 4. Detail of the material from the 1.12 band. The great majority of particles are smooth-surfaced membranes. × 56000.

activation, resulting in a higher specific activity of the enzyme in the presence of HCO_3^-. Both the light mitochondrial and microsomal fraction gave active preparations with similar properties.

The preparation did not lose activity after storage for 1 week at refrigerator temperature, but one cycle of freezing and thawing resulted in more than 50 % loss of enzyme activity. Fresh preparations of the enzyme gradually increased in activity over the first 24–48 h of storage, similar to the lubrol solubilized (Na^+, K^+)-ATPase[13]. Heating to 60° for 5 min abolished the HCO_3^- activation. These results are summarized in Table III.

TABLE III

ATPase ACTIVITY

Assay conditions as in Table I. The soluble enzyme was prepared from a mixture of the light mitochondrial and microsomal fractions. Data in μmoles $P_i \cdot mg^{-1} \cdot h^{-1}$.

Fraction	Mg^{2+}	$Mg^{2+} + HCO_3^-$
Total homogenate	7.3	8.39
Light mitochondrial	12.1	16.7
Microsomal	19.4	27.3
Soluble enzyme	13.2	44.6
Soluble enzyme heat treated	6.8	7.1

TABLE IV

SPECIFICITY OF ATPase

Assay as in Table I, except other nucleotides substituted for ATP. Data in μmoles $P_i \cdot mg^{-1} \cdot h^{-1}$.

Substrate	Mg^{2+}	$Mg^{2+} + HCO_3^-$
ATP	7.2	23.3
GTP	4.4	5.3
CTP	<0.01	<0.01
UTP	0.7	0.65
ADP	<0.02	<0.02
AMP	<0.01	<0.01

Properties of solubilized enzyme

The solubilized enzyme from dog mucosa hydrolyzed GTP at about 50 % of the ATP rate, and UTP at about 10 %. There was no significant hydrolysis of CTP, ADP or AMP (Table IV).

Mn^{2+} substituted effectively for Mg^{2+} with this enzyme, but Ca^{2+} and Zn^{2+} did not, when added at equal concentrations. In fact Ca^{2+}, when added in the presence of Mg^{2+} inhibited the enzyme, especially the HCO_3^- activation.

The pH optimum of the enzyme is illustrated in Fig. 5. A fairly broad pH–activity profile was obtained with an optimum at 8.3. In the presence of HCO_3^-, the peak activation occurred at pH 7.4.

Effect of activators

A. Specificity. The stimulation of the enzyme by HCO_3^- was not confined to this base. Various other bases such as selenite, borate, arsenite, arsenate and sulfite were also effective in increasing the activity of the enzyme. These data are summarized

in Table V. The effect of some of these bases on the soluble enzyme from Necturus is inserted for comparison. Based on the effective concentration of the bases at the pH of the experiment this activation suggested that there might be a relationship between the pK of the base, and its activating effect. The following method was used to calculate the activation constant k', assuming the simplest possible kinetic scheme:

Let Mg^{2+} rate $= V_1 = k_1[ES]$;

Mg^{2+} rate + base catalyzed rate $= V_2 = k_1[ES] + k_2[B]\,[ES]$

where $[B]$ is concentration of basic species at given pH, and $[ES]$ is enzyme substrate complex. Then

$$\frac{V_2 - V_1}{[B]V_1} = \frac{k_2}{k_1} = k'$$

and ES does not have to be known, and k' can be taken as equivalent to the k_{cat} of organic reactions.

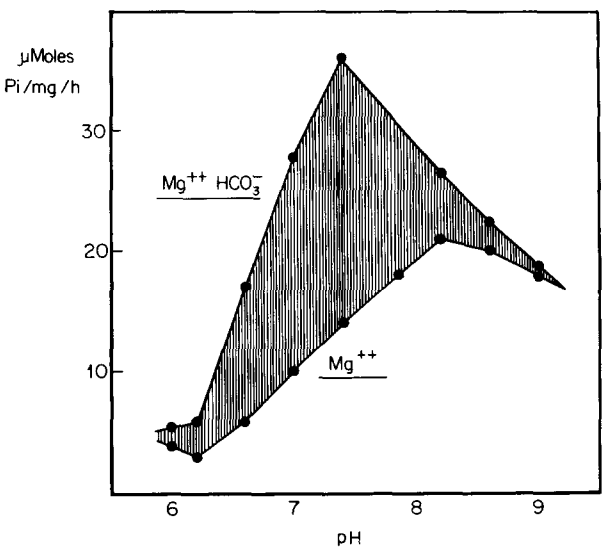


Fig. 5. A plot of activity *vs.* pH for dog enzyme with and without 20 mM HCO_3^-.

TABLE V

RELATIVE RATES OF ATPase WITH BASES

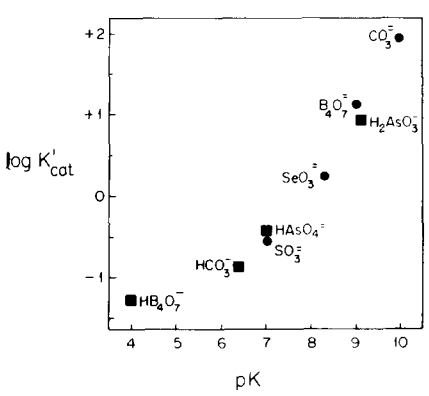
The concentration of base added is 20 mM, the pH of the final mixture was 7.4. The activity for Mg^{2+} alone is taken as 1 to allow direct comparison (Mean ± S.E., $n \geqslant 5$). Data in µmoles $P_i \cdot mg^{-1} \cdot h^{-1}$.

Base	*Necturus*	*Dog*
HCO_3^-	3.5 ± 0.49	3.39 ± 0.11
SeO_3^{2-}	5.6 ± 0.15	3.00 ± 0.38
$H_2AsO_3^-$	4.3 ± 0.8	3.60 ± 0.44
$HB_4O_7^-$	1.8 ± 0.18	1.52 ± 0.20
$HAsO_4^{2-}$	—	5.08 ± 0.56
SO_3^{2-}	—	4.81 ± 0.68

Accordingly, if a proton transfer step is involved in the activation mechanism, a plot of log k' (*i.e.* log $V_2-V_1/[B]V_1$) *vs.* pK of the base should be a straight line[14] *i.e.*

$$\log k' = \alpha + \beta(\mathrm{p}K_a)$$

Fig. 6 shows this plot for the enzyme for dog mucosa. The fact that this is a curve shows that the actual situation is more complex than assumed here, but nevertheless, the Brønsted relationship is evident. A similar plot may be obtained for the enzyme obtained from Necturus and Fig. 7 shows a plot of log k' for Necturus against log k' for dog, giving a slope of approximately 1.



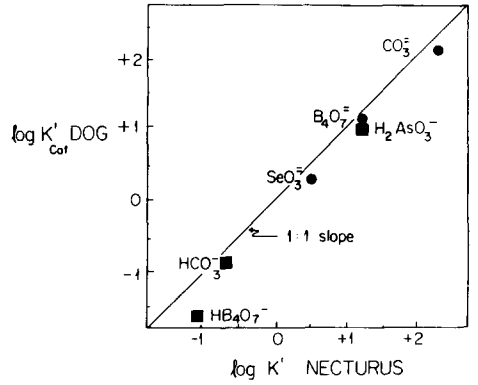


Fig. 6. Acid base catalysis of dog ATPase. A plot of log k' *vs.* pK for various bases for the soluble ATPase from dog gastric mucosa.

Fig. 7. Acid base catalysis of ATPase. A plot of log k' for the dog *vs.* log k' for Necturus, showing 1:1 relationship.

B. Effect on kinetic constants. Bicarbonate increased both the K_m and the v_{max} of the ATPase. The K_m was increased from 0.132 ± 0.039 to 0.265 ± 0.044 mM (7 experiments ± S.D.) and the v_{max} from 19.2 ± 3.8 to 46.47 ± 7.7 μmoles $P_i \cdot mg^{-1} \cdot h^{-1}$.

The kinetics approximately fit an equation of the form:

$$\frac{v_{max}}{V} = \frac{K_m}{[S]} + \frac{K_B}{[B]} + 1$$

where v_{max} is maximal velocity, V is measured velocity, K_m is dissociation constant for ATP, K_B is constant fr HCO_3^-, and $[S]$ and $[B]$ are concentrations of ATP and bicarbonate, respectively.

A plot of log $V/v_{max}^- V$ *vs.* log [ATP] gave a straight line with n (the Hill coefficient) = 1. Similar results were obtained for a plot against log $[HCO_3^-]$.

C. Effect of irreversible inhibitors. Both DFP and methane sulfonyl chloride inhibited the ATPase activity. In the case of methane sulfonyl chloride the base (HCO_3^-) activation was considerably more sensitive to the inhibitor, except at the highest concentration. Table VI shows the effect of preincubation with varying concentrations of methane sulfonyl chloride.

D. Effect of ^2H_2O. Dialysis against $^2HO_2$, followed by assay in a ^2H_2O medium reduced the HCO_3^- stimulation of the ATPase activity from 2.5 fold to 0.1, *i.e.*

TABLE VI

EFFECT OF PREINCUBATION WITH METHANE SULFONYL CHLORIDE

Concn. (*M*)	*ATPase activity as % of control*	
	Mg^{2+}	$Mg^{2+} + HCO_3^-$
Control	100	100
10^{-5}	100	90
10^{-4}	100	72
10^{-3}	100	54
10^{-2}	50	36

the HCO_3^- stimulation was virtually abolished. This may be used as additional evidence for the requirement of a proton transfer step in the HCO_3^- activation step. However, the data could be explained in other ways, such as alteration in protein conformation. Similar treatment of the (Na^+, K^+)-ATPase has only a slight effect on the $Na^+ + K^+$-activity, however (unpublished observation).

DISCUSSION

Several ATPases have been shown to occur in mammalian tissues. Thus, in most cells the cell membrane contains a Mg^{2+} ATPase and a ($Mg^{2+} + Na^+, K^+$)-ATPase. The latter enzyme, which is involved in Na^+ transport, has recently been solubilized and extensively purified[15]. Mitochondria also contain an ATPase(F_1)which has also been purified, and localized in the inner membrane of mitochondria[16,17]. The enzyme discussed here is distinct from both of these. In contrast to the F_1 ATPase, this enzyme is cold stable and heat labile, is not stimulated by dinitrophenol (unpublished observations), and is localized in a smooth vesicle fraction derived from the gastric mucosa. However, the F_1 ATPase is also partially sensitive to HCO_3^-[18].

The localization of the HCO_3^- activated enzyme in the smooth vesicular fraction of the dog gastric mucosa and in a similar fraction derived from purified amphibian oxyntic cells[3] might suggest some involvement in the acid secretory process. Moreover, the increase observed in HCO_3^- activation upon solubilization of the enzyme implies that this activation may have considerable functional significance, particularly since diamox inhibits acid secretion[6].

Since it was then found that various oxyanions stimulated this ATPase, and that the degree of stimulation was a function of the concentration of the anion and its pK, it may be concluded that a proton transfer step is involved in the activation by HCO_3^-, the only one of these bases of importance biologically. This finding appears to be unique in enzyme catalysis, that externally added base accelerates the reaction by a general acid-base or nucleophilic mechanism. This could be due to a variety of mechanisms, such as true general base catalysis, general acid-specific base catalysis or nucleophilic catalysis. Kinetic information does not distinguish between these possibilities[19]. The lack of effect of Br^-, a strong nucleophile, might imply that nucleophilic catalysis is not involved. The similarity of response of dog and Necturus shows that the enzymes are similar in both species. At this stage our observations of base catalysis are limited to the structurally similar oxybases, and

do not predict the effects of structurally unrelated bases. However, they relate directly to the mechanism whereby HCO_3^-, the presumed physiological activator, accelerates the enzyme reaction.

Additional insight into the enzyme mechanism may be obtained from the effect of DFP[2] or methane sulfonyl chloride. The latter compound was studied in more detail since its effects cannot be due to F^- release[21]. Since both compounds inhibit the ATPase, a serine-OH group may be involved in the reaction. Additional evidence which is consistent with this possibility is the broad pH optimum curve, and also the decreasing effect of HCO_3^- towards pH 9, the pK of the serine-OH.

Based on these two lines of evidence, a tentative hypothesis for the mechanism of HCO_3^- activation of this enzyme would be as follows: The active center of the enzyme contains a serine-OH group, and a binding site for the base. The base would then act to remove the proton from serine-OH, increasing the nucleophilic reactivity of this group towards the γ-PO_4 of ATP. Work is in progress to purify the enzyme so that more detailed kinetics can be obtained.

In relation to the possibility that this enzyme is involved in acid secretion, the activation by HCO_3^- may relate to HCO_3^- transport by this enzyme. In this case the secretory membrane of the acid secreting cell would contain the ATPase[22], and a source of HCO_3^- such as the CO_2 carbonic anhydrase system[23]. Acid secretion would then result from HCO_3^- transport into the cell, and diffusion of H^+, accompanied by anion such as Cl^- into the lumen. This mechanism is formally equivalent to the chemi-osmotic theory of oxidative phosphorylation[24], when $CO^2{}_3^-$ is substituted for O^{2-}. Moreover, depending on whether the enzyme is oriented as in the gastric cell, or in the opposite direction, as in the pancreas, H^+ secretion or HCO_3^- secretion could result.

ACKNOWLEDGMENT

This investigation was supported by National Institutes of Health grants AM08541, AM09260, TIAM05286; National Science Foundation grants GB8351, GB25364 and Swedish Medical Research Council grant B-71-12R-3279.

REFERENCES

1 D. K. KASBEKAR AND R. P. DURBIN, *Biochim. Biophys. Acta*, 105 (1965) 472.
2 G. SACHS, W. E. MITCH AND B. I. HIRSCHOWITZ, *Proc. Soc. Exp. Biol. Med.*, 119 (1965) 1023.
3 V. D. WIEBELHAUS, C. P. SUNG, H. F. HELANDER, G. SHAH, A. L. BLUM AND G. SACHS, *Biochim. Biophys. Acta*, 241 (1971) 49.
4 F. G. MOODY, *Am. J. Phys.*, 220 (1971) 467.
5 H. W. DAVENPORT, *Am. J. Phys.*, 129 (1940) 505.
6 S. M. CAIN, W. N. STANISKY AND A. B. OTIS, *Fed. Proc.*, 18 (1959) 22.
7 C. A. M. HOGBEN, in A. M. SHANES, *Electrolytes in Biological Systems*, Am. Physiol. Soc., 1955.
8 G. SACHS, L. C. CLARK AND G. M. MAKHLOUF, *Proc. Soc. Exp. Biol. Med.*, 134 (1969) 694.
9 H. F. HELANDER, *J. Ultrastruct. Res.*, Suppl. 4:1, 1962.
10 A. YODA AND L. E. HOKIN, *Biochem. Biophys. Res. Commun.*, 40 (1970) 880.
11 G. SACHS, E. Z. FINLEY, T. TSUJI AND B. I. HIRSCHOWITZ, *Arch. Biochem. Biophys.*, 134 (1969) 497.
12 O. H. LOWRY, N. I. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
13 A. KAHLENBERG, N. DULAK, J. F. DIXON, P. GALSWORTHY AND L. E. HOKIN, *Arch. Biochem. Biophys.*, 131 (1969) 253.
14 J. N. BRØNSTED, *Z. Phys. Chem.*, 102 (1922) 169.

15 S. UESUGI, N. C. DULAK, J. F. DIXON, T. D. HEAUM, J. L. DAHL, J. F. PERDUE AND L. E. HOKIN, *J. Biol. Chem.*, 246 (1971) 531.
16 H. S. PENEFSKY, M. E. PULLMAN, A. DATH AND E. RACKER, *J. Biol. Chem.*, 235 (1960) 3322.
17 Y. KAGAWA AND E. RACKER, *J. Biol. Chem.*, 241 (1916) 2475.
18 E. RACKER, *Fed. Proc.*, 21 (1962) 54A.
19 W. P. JENCKS, *Catalysis in Chemistry and Enzymology*, McGraw Hill, 1969, p. 221.
20 G. SACHS AND B. I. HIRSCHOWITZ, *Proc. Soc. Exp. Biol. Med.*, 120 (1965) 702.
21 H. YOSHIDA, K. NAGAI, M. KAMLI AND Y. NAGAKAWA, *Biochim. Biophys. Acta*, 150 (1968) 162.
22 C. S. KOENIG AND J. D. C. VIAL, *J. Histochem. Cytochem.*, 18 (1970) 340.
23 S. C. CROSS, *Histochem.*, 22 (1970) 219.
24 P. MITCHELL, *Biol. Rev.*, 41 (1966) 448.