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# $H^+/K^+$-ATPase contents of human, rabbit, hog and rat gastric mucosa

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**A monoclonal antibody (mAb 95–111) was used to titrate the amounts of $H^+/K^+$-ATPase in subcellular fractions of the fundus of rats, pigs, rabbits and humans. All four had similar amounts of $H^+/K^+$-ATPase: $2.1 \pm 0.5$ (human), $1.9 \pm 0.4$ (rabbit), $4.4 \pm 0.5$ (rat) and $4.2 \pm 0.8$ (hog) mg ATPase/g wet tissue. The antigen concentrations and $H^+/K^+$-ATPase enzymatic activities of subcellular fractions were linearly correlated in all species but rat suggesting that human, rabbit and hog $H^+/K^+$-ATPases have similar rates of catalysis (223 $\mu$mol $P_i$/h per mg ATPase). The non-correlation of rat data probably reflects the known lability of the rat enzyme. Hence, immuno-titration promises to be a more reliable method of estimating rat ATPase than the currently used enzymatic assay. The $H^+/K^+$-ATPase content of human biopsies was 20-fold higher than previously-published (Smolka, A. and Weinstein, W.M. (1986) Gastroenterology 90, 532–539) suggesting that the previous immuno-titration underestimated the $H^+/K^+$-ATPase content of the human fundus.**

## Introduction

Immunological titrations are sensitive techniques for measuring the concentrations of interesting proteins [1] and the use of monoclonal antibodies makes them extremely specific. We have prepared a monoclonal antibody, mAb 95–111, specific for the catalytic $\alpha$ subunit of $H^+/K^+$-ATPase [2]. This antibody inhibits $H^+/K^+$-ATPase activity but does not react with $Na^+/K^+$-ATPase [2]. This mAb has been used to measure the $H^+/K^+$-ATPase contents of the fundic mucosa in the developing rat [3]. In this study, we have measured the amounts of immunoreactive $H^+/K^+$-ATPase in the fundic mucosa of four mammalian species, rat, hog, rabbit and human and measured the $H^+/K^+$-ATPase enzymatic activity of these tissus. A comparison of the two sets of data indicates that the immuno-titration values correlate well with the enzymatic values for all species but rat. The reasons for this exception are discussed. The human immunotitrations and enzymatic values are in good agreement and the human titer is similar to those for the other three species examined but it is 20-fold higher than previously published by others [4].

## Materials and Methods

### *Materials*

Phosphoenolpyruvate (monocyclohexylammonium salt), ATP ($Mg^{2+}$ or $Na^+$ salts), Ponceau S and diaminobenzidine were purchased from Sigma (U.S.A.). bovine serum albumin, Hepes, dithiothreitol, were from Boerhinger-France. Sucrose, sodium carbonate, sodium hydrogen carbonate, sodium chloride, potassium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, Tris, HCl, 1,2-di(2-aminoethoxy)ethane-*N,N,N′,N′*-tetraacetic acid (EGTA), and, sodium dodecyl sulfate (SDS) were purchased from Prolabo (France). $^{125}I$ (sodium salt) was from Amersham. Chloramine T, hydrogen peroxide and sodium metabisulfite were purchased from Merck (F.R.G.). Rabbit antimouse IgG(H + L) antibody and peroxidase labeled sheep antimouse IgG(H + L) was from Biosys (France). Trisacryl 2000 was purchased from IBF (France). Nitrocellulose sheets (0.22 $\mu$m) were from Pharmacia-LKB.

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*Methods*

*1. Hog gastric fractions.* Fresh hog stomachs were obtained from the slaughterhouse (Domont Abattoirs, Pontoise). The fundic mucosa was scraped off and homogenized first with a mincer then with a blender in 50 mM Hepes/0.25 M sucrose/2 mM dithiothreitol adjusted to pH 7.2 with solid Tris. Homogenate was centrifuged at 4°C for 10 min at $800 \times g$, the resulting supernatant (hog extract) was centrifuged at $40000 \times g$ for 7 min (Sorvall RC5C) at 4°C to obtain the mitochondrial pellet (P2 fraction). The mitochondrial supernatant was centrifuged at $100000 \times g$ for 30 min at 4°C (Beckman L5 65) to pellet the microsomes (P3 fraction). The standard fraction having a $K^+$-stimulated activity of 80 μmol $P_i$/h per mg protein was prepared by isopycnic equilibration of P3 on a 35% sucrose cushion.

*2. Rat gastric fractions.* The subcellular fractions were prepared by the method of Im and Blakeman [5]. Adult male Wistar rats, weighing 150–200 g, were killed by cervical dislocation. The rumen and the antrum were excised and the fundic epithelium was scraped off, homogenized first with scissors, then by 20 passes through a Potter-Elvehejm homogenizer (pistons A and B) in 5 mM Tris-HCl, 1 mM 1,2-di(2-aminoethoxy)ethane-*N,N,N′,N′*-tetraacetic acid, 0.25 M sucrose (pH 7.5). The homogenate was centrifuged to prepare the P2 and P3 fractions.

*3. Rabbit gastric fractions.* New Zealand rabbits were killed by an intravenous injection of sodium pentobarbital. The fundic mucosa was scraped off, homogenized first with scissors, then by 20 passes through a Potter-Elvehejm homogenizer (pistons A and B) in the same buffer as for hog. The P2 and P3 subcellular fractions of were obtained as described above for hog.

*4. Human gastric fractions.* All subjects gave their informed consent: 15 gastric biopsies were obtained from five fasted human subjects prepared for endoscopy under standard conditions at Hospital Bichat (Paris) and immediately placed in liquid nitrogen. The biopsies were thawed immediately before assay, weighed, homogenized by 20 passes through a Potter-Elvehejm homogenizer (pistons A and B) in 1.5 ml of 40 mM Hepes (pH 7.2) and centrifuged at $15000 \times g$ for 30 min at 4°C to obtain the S1 (supernatant) and the P1 (pellet) fractions.

*5. Proteins.*

Proteins were measured by the method of Bradford, using bovine serum albumin as standard [6].

*6. Enzymatic $H^+/K^+$-ATPase activity.* The ($H^+/K^+$)ATPase activity was measured as previously described [7] in 40 mM Hepes (pH 7.0), in the presence of 2 mM ATP-Mg, 1 μM nigericin, 4 mM phosphoenolpyruvate, 1 unit/ml pyruvate kinase, with or without 20 mM KCl.

*7. Iodination of antimouse IgG(H + L).* Iodination was performed essentially as described by Mc Conahey [8]. Aliquots of rabbit antimouse IgG(H + L) (50 μl, 1 mg/ml) were diluted in 50 μl of 0.1 M phosphate buffer (pH 7.5) and 1 mCi $^{125}I^-$ (10 μl) was added followed by Chloramine T (1 μg in 5 μl phosphate buffer). After 10 min, the reaction was stopped by adding freshly prepared sodium metabisulfite (5 μg in 10 μl phosphate buffer). Free radioiodine was separated from $^{125}$I-IgG by chromatography on Trisacryl 2000, ($20 \times 1$ cm column equilibrated with 0.1 M phosphate buffer plus 0.1% bovine serum albumin. The first radioactive peak eluted, corresponding to the IgG, had a specific activity of 16 mCi/mg protein.

*8. Radioactive-linked immunosorbent assay (RaLISA).* Assay samples were diluted to 1.25 to 40 μg protein/ml in 15 mM carbonate/35 mM bicarbonate, as described by Douillard and Hoffman [9] and 100-μl aliquots were incubated overnight at 0–4°C in the wells of polyvinyl chloride microplates (Flow laboratory). The solutions were decanted and the wells rinsed ($4 \times 5$ min) with 200 μl of phosphate-buffered saline plus 0.1% bovine serum albumin. The last rinse was left for 1 h to saturate the sites on the plastic wells. Monoclonal antibody (100 μl, 0.1 μg/ml) was added to each well and incubated for 1 h at room temperature. The plates were rinsed four times with phosphate-buffered saline plus 0.1% bovine serum albumin and $^{125}$I-labelled antimouse IgG(H + L) was added for 1 h (200000 cpm ± 2% per well). Unbound radioactivity was decanted and the wells were washed three times with phosphate-buffered saline plus 0.1% bovine serum albumin, cut out and their radioactivity counted (1275 minigamma LKB).

*9. Monoclonal antibody (mAb 95–111).* Monoclonal antibody was prepared from mice immunized with $H^+/K^+$-ATPase-enriched membrane fractions as previously described [2,3]. The mAb 95–111 was purified by DEAE Affi-Gel blue chromatography [10]. Either ascites fluid or purified mAb was used in the assays.

*10. Western blots.* Membrane proteins were separated by SDS-polyacrylamide gel electrophoresis on a 5–20% polyacrylamide separating gel, transferred to a nitrocellulose sheet [11] and stained with Ponceau S [12]. The nitrocellulose sheet was saturated for 30 min in 50 mM Hepes, 1% bovine serum albumin, 0.5% Tween 20 (pH 7) and incubated for 1 h in the same buffer containing 0.4 μg/ml ascites fluid protein ($8 \cdot 10^{-10}$ M, 95–111 mAB). The sheet was rinsed four times with Hepes-bovine serum albumin-Tween and incubated with peroxidase-labeled sheep antimouse IgG(H + L) (0.5 μg/ml) for 1 h, rinsed ($4 \times 5$ min) with Hepes-bovine serum albumin-Tween and stained with diaminobenzidine plus hydrogen peroxide.

*11. Data analysis.* Results are expressed as the means ± S.E. of three experiments. Regression lines were calculated by the least-squares method. As no pure $H^+/K^+$-ATPase is available, a hog membrane prepara-

tion was used as standard. This standard fraction contained 364 μg 95 kDa polypeptides per mg protein by spectrophotometric scanning of the Coomassie blue-stained SDS-polyacrylamide gel electrophoresis and had a $K^+$-ATPase specific activity of 80 μmol $P_i$/h per mg protein [7,13].

## Results and Discussion

*1. Immuno-titration assay*

*a. Assay and calculation.* Subcellular fractions were used to coat polyvinyl chloride microplates, and plastic-bound antigens were assayed with a saturating concentration of mAb 95–111 (0.1 μg mAb/ml, $6 \cdot 10^{-10}$ M). The immuno-titrations of subcellular fractions of hog, rabbit, rat and human fundic mucosa all gave hyperbolic plots which all plateaued between 10–40 μg coating proteins/ml (Fig. 1).

Such plots suggest that the microplate sites were saturated with the antigenic solutions. Similar saturations have been obtained using other binding surfaces or coating protocols [1,4,14,15]. Regardless the support or protocol used, the important point is to be able to compare all the samples to be assayed to the same standard, which requires that all fractions have the same binding affinity as the standard. Hence, we shall be able to assume that the maximal values of bound antigen reflect the relative concentrations of $H^+/K^+$-ATPase in the unknown and standard preparations.

The titration curves were assumed to be simple hyperbolas and plotted according to the following Michaelis Menten-derived equation:

$$\frac{1}{\text{cpm}} = \frac{K_a}{\text{cpm}_{max}} \cdot \frac{1}{c} + \frac{1}{\text{cpm}_{max}} \quad (1)$$

where cpm and $c$ are experimental parameters; cpm is the measured plastic-bound radioactivity and $c$ is the concentration of sample proteins used to coat the microplates. $K_a$ is the coating affinity, i.e., the concentration of sample required to saturate 50% of the polyvinyl chloride microplate sites. $\text{cpm}_{max}$ is the amount of antigen-antibody complexes measured at saturation of the polyvinyl chloride microplate sites.

A set of straight lines was obtained when 1/cpm was plotted against $1/c$. This is in good agreement with the previous analysis by Smoka and Weinstein [4], even if

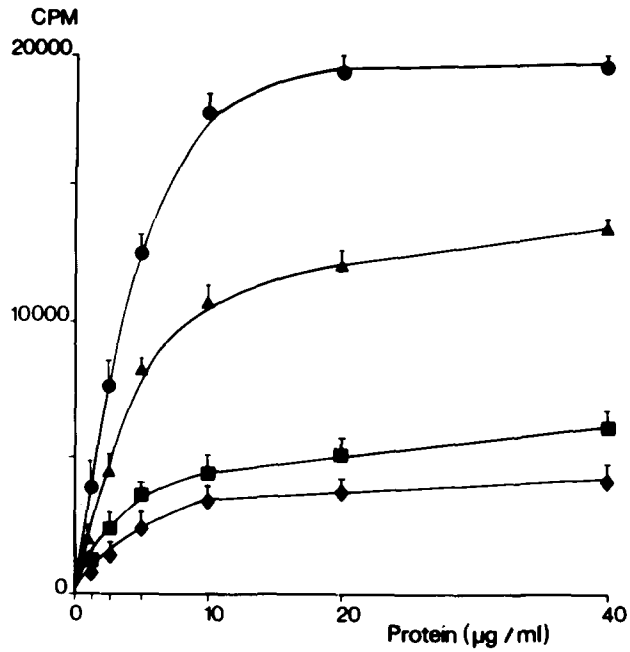


Fig. 1. Titration of four hog gastric fractions with 95–111 monoclonal antibody. Sucrose gradient-purified membranes (●———●), P3 fraction (▲———▲), P2 (fraction (■———■), and extract (◆———◆) were prepared as described in Methods. Varying concentrations of hog subcellular fractions (0–40 μg protein/ml) were incubated overnight in microplate wells and decanted. Microplate-bound antigens were titrated as described in Methods. Each value is the mean of three determinations.

TABLE I

*Immunological titration of hog, rat, rabbit and human gastric $H^+/K^+$-ATPase*

Hog, rat, rabbit and human fundic fractions were prepared as described in Methods. polyvinyl chloride microplate wells were coated with increasing concentrations of (sucrose gradient-purified) hog fundic mucosa membranes (standard), or hog, rat, rabbit, human subcellular fractions, as in Fig. 1. Microplate-bound antigens were titrated using 0.3 μg/ml of ascites fluid or 0.1 μg/ml of purified mAb 95–111 and 200000 cpm of $^{125}$I-labelled rabbit anti mouse IgG. $\text{cpm}_{max}$ and $K_a$ were extrapolated from the linear plots of the data according to Eqn. 1. A hog membrane fraction containing 364 μg 95 kDa/mg protein was used as standard to calculate the antigen concentration (column 3). The immuno-titers (column 4) were calculated knowing that the hog extract contained 60 mg protein/g wet mucosa, hog P2: 12 mg/g, hog P3: 9.6 mg/g, rat P2+P3: 30.3 mg/g, rabbit P2+P3: 17 mg/g and human biopsies P1+S1: 76 mg/g.

| Fractions | $K_a$ (μg prot/ml) | $\text{cpm}_{max}$ | Antigen concn. (μg ATPase/mg prot) | Immuno-titer (mg ATPase/g muc) |
|---|---|---|---|---|
| Standard | 3.8±0.5 | 22405±150 | 364 | |
| Hog extract | 4.1±0.4 | 4300±450 | 70± 7 | 4.2±0.8 |
| Hog P2 | 3.5±0.3 | 5925±315 | 100±17 | 1.2±0.3 |
| Hog P3 | 3.7±0.5 | 13820±355 | 218±11 | 2.1±0.1 |
| Rat P2+P3 | 3.8±0.4 | 8960±430 | 145±10 | 4.4±0.5 |
| Rabbit P2+P3 | 3.9±0.5 | 6720±580 | 112±12 | 1.9±0.4 |
| Human S1+P1 | 3.8±0.4 | 1820±140 | 28± 3 | 2.1±0.5 |
| Human (from Ref. 4) | | | | 0.1±0.02 |

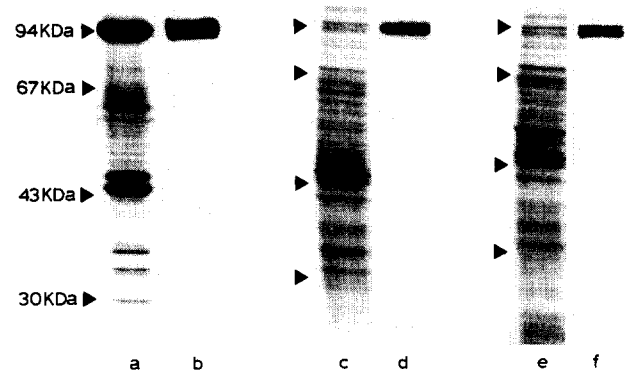


Fig. 2. Western blots of hog and rat gastric fractions using 95–111 mAb. Hog membranes, 4 μg (lanes a and b); rat P3 fraction, 15 μg (lanes c and d) and rat P2 fraction, 15 μg (lanes e and f) were analyzed on SDS-polyacrylamide gel electrophoresis (5–20%) and transferred to nitrocellulose. Lanes a, c and e were stained with Ponceau S. Lanes b, d and f were incubated with 0.4 μg/ml of mAb 95–111 ascites fluid and immune complexes were revealed as described in Methods.

these authors prefered a 'three parameter logistic' to the two parameter analysis. $K_a$ and $cpm_{max}$ were obtained by extrapolation (Table I). The subcellular fractions of hog, rabbit, rat and the human gastric fractions had similar $K_a$ values, demonstrating that their proteins had the same binding affinity for microplates. It was therefore assumed that the $cpm_{max}$ values reflected the relative concentrations of $H^+/K^+$-ATPase in the unknown and standard preparations (Table I). The titers, expressed as mg $H^+/K^+$-ATPase per g wet mucosa were then calculated by multiplying the immuno-concentration of $H^+/K^+$-ATPase by the amounts of proteins coming, in each fraction, from the homogenization of 1 g wet mucosa (Table I).

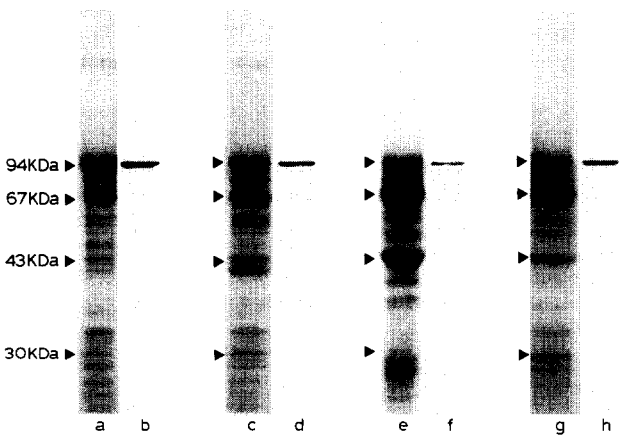


Fig. 3. Western blots of rabbit and human gastric fractions using 95–111 mAb. Rabbit P3 fraction, 10 μg (lanes a and b); rabbit P2 fraction, 15 μg (lanes c and d); human S1 fraction, 20 μg (lanes e and f) and human P1 fraction, 20 μg (lanes g and h) were analyzed on SDS-polyacrylamide gel electrophoresis (5–20%) and transferred to nitrocellulose. Lanes a, c, e and g were stained with Ponceau S. Lanes b, d, f and h show the immuno-reactive $H^+/K^+$-ATPase detected as in Fig. 2.

*b. Monoclonal antibody specificity.* Although mAb 95–111 was raised against hog $H^+/K^+$-ATPase [2], it also reacted with the human, rabbit and rat gastric subcellular fractions. Western blots demonstrated that the epitope was carried by the 95 kDa band of the α $H^+/K^+$-ATPase subunit in all the species tested Figs. 2 and 3). mAb 95–111 is therefore suitable for titrating $H^+/K^+$-ATPase in these species.

*2. Immunotitration of hog, human, rat and rabbit gastric $H^+/K^+$-ATPase*

The hog extract, which is the fundic epithelium homogenate minus the nuclear fraction, contained 4.2 mg ATPase/g wet mucosa (Table I). This is in good agreement with the titer of the P2 plus P3 fractions (3.3 mg ATPase/g wet mucosa) because P2 and P3 account for most of the extract $H^+/K^+$-ATPase. The human S1 plus P1 fractions represent the entire biopsies and contained 2.1 mg ATPase/g wet biopsy (Table I). The rat and the rabbit P2 + P3 subcellular fractions contained 4.4 and 1.9 mg ATPase/g wet mucosa, respectively (Table I). If it is well established that P2 and P3 fractions are enriched in $H^+/K^+$-ATPase, this does not mean that P2 and P3 fractions account for the total $H^+/K^+$-ATPase activity of the homogenate. In the present conditions, the rat P2 + P3 fractions accounted for 84% of the homogenate $H^+/K^+$-ATPase enzymatic activity. Hence, we may suggest that the immunological titer of the entire rat gastric mucosa is 5.2 mg ATPase/g wet mucosa. The rabbit P2 + P3 fractions accounted for 47% of the rabbit homogenate $H^+/K^+$-ATPase enzymatic activity suggesting that the entire rabbit gastric mucosa contains 4 mg ATPase/g wet mucosa.

To our knowledge, nobody ever compared the $H^+/K^+$-ATPase contents of these four species. The data show that the concentrations of $H^+/K^+$-ATPase in the gastric mucosa of hog (4.2 mg/g), rabbit (4 mg/g), rat (5.2 mg/g) and human (2.1 mg/g) are similar.

*3. Comparison of the $H^+/K^+$-ATPase immuno-titers and enzymatic activities*

Even though immunological titration of $H^+/K^+$-ATPase is seldom used, there have been many reports of $H^+/K^+$-ATPase enzymatic activity (Refs. 2, 3, 5, 7, 13, 16, for example) and the specific activity values have been used as references to discuss preparation purity. The comparison of the enzyme activity and of the antigen concentration (Table I) of gastric subcellular fractions is therefore of interest. Indeed, on the one hand, antigen concentration is the mass of proteins

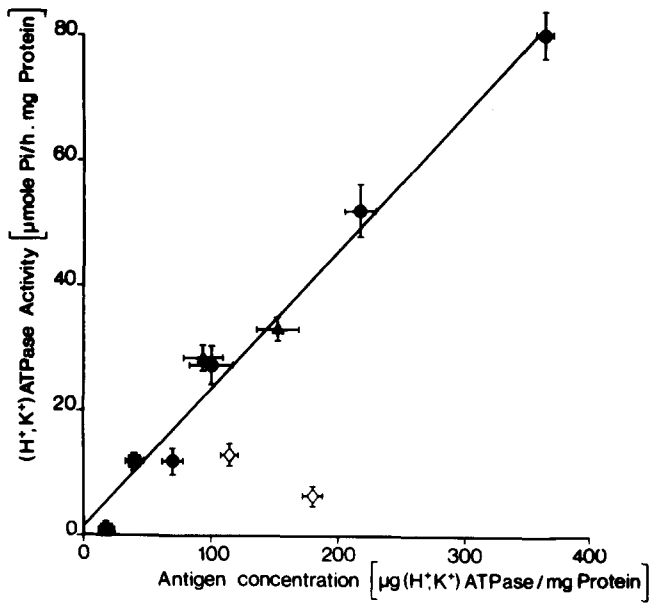


Fig. 4. Correlation between $H^+/K^+$-ATPase titer and activity in gastric subcellular fractions of different species. The specific activity of $K^+$-stimulated ATPase was measured as described in Methods. Results were plotted as a function of the antigen concentration ($\mu$g ATPase/mg protein as defined in Table I). A linear regression $y = 0.222\ x + 1$ was calculated from all values but the rat's ($r = 0.986$). ●, Hog samples; ▲, rabbit samples; ■, human samples; ◇, rat samples.

which are $H^+/K^+$-ATPases in 1 mg of total proteins. On the other hand, the enzyme activity is the activity of those $H^+/K^+$-ATPases in 1 mg of the same total proteins. Plots of antigen concentrations as a function of specific $K^+$-stimulated ATPase activities demonstrate a linear correlation in all species but rat ($\approx 0.222\ x + 1$ ($r = 0.986$)) (Fig. 4). The existence of this linear correlation suggests that human, rabbit and hog $H^+/K^+$-ATPases have the same catalytic rates that can be extrapolated to 223 $\mu$mol $P_i$/h mg ATPase (the activity of a fraction containing 1 mg ATPase/mg protein). In agreement with this, an analysis of our own and published data [13], indicates that highly enriched $H^+/K^+$-ATPase preparations have a specific activity of about 160–220 $\mu$mol $P_i$/h per mg.

Rat values do not fit the correlation. Several hypothesis can be made to explain this discrepancy: either the presence of EGTA during homogenization modifies the antigen epitope, or, the rat ATPase has a slower catalytic rate than the human, rabbit and hog ATPases. As the antibody titrates more ATPase than the enzyme activity suggests, a third hypothesis is that rat $H^+/K^+$-ATPase is partially inactivated and that mAb 95–111 titrates both the active and the inactive enzyme. This is very likely because, the rat $H^+/K^+$-ATPase is well known to be rapidly inactivated in vitro [5]. Indeed, rat $H^+/K^+$-ATPase remained undetected until Im and Blakeman [5] demonstrated that its in vitro isolation requires special care. They used 1,2-di(2-aminoethoxy)ethane-*N,N,N′,N′*-tetraacetic acid to stabilize the $H^+/K^+$-ATPase activity. In our hands, although 1,2-di(2-aminoethoxy)ethane-*N,N,N′,N′*-tetraacetic acid improved the enzyme stability and allowed to measure the activity in fresh in vitro preparations, it did not prevent a complete inactivation after 48–72 hours at 0–4°C. Therefore, the difference in rat values may be due to the ATPase inactivation. The mechanism of rat $H^+/K^+$-ATPase inactivation in vitro is unclear: phospholipases have been implicated [17]. The present results indicate that ATPase proteolysis is not implicated, since the mAb 95–111 epitope is found only at 95 kDa in the rat fundic extracts as in those of the other species.

*4. Analysis of the human $H^+/K^+$-ATPase immuno-titers*

Smolka and Weinstein [4] previously reported that the human fundic mucosa contains 0.10 mg ATPase/g wet biopsy (as compared to 2.1 mg/g in the present data) (Table I). This apparent 20-fold difference in ATPase content cannot readily be explained.

Assuming, as it is likely, that their biopsies contained the same amount of protein/g wet biopsy as ours, their immunological titer suggests 1.3 $\mu$g of ATPase/mg protein. Therefore, to account for the $H^+/K^+$-ATPase enzymatic activity of human biopsies (5.5 + 0.6 $\mu$mol $P_i$/h per mg protein), the specific activity of human ATPase should be 4230 $\mu$mol $P_i$/h per mg ATPase (5.5 : 0.0013) which is highly unlikely.

This indicates that their immuno-titer of human biopsies was underestimated. There may have been an arithmetic error, but as the titer of the standard fraction is not given, this cannot be verified. Alternatively, the tested biopsies might have been poor in parietal cells. Indeed, Helander [18] demonstrated that parietal cells are not uniformely distributed throughout the fundic mucosa. This hypothesis cannot be checked because the $H^+/K^+$-ATPase activity of the biopsies was not measured.

*5. Conclusion*

The present results demonstrate that human, rabbit, rat and hog fundic mucosa contain similar amounts of $H^+/K^+$-ATPase per g of mucosa and suggest that human, rabbit and hog $H^+/K^+$-ATPases have similar catalytic rates, indicating that, if $H^+/K^+$-ATPase alone is responsible for $H^+$ secretion human, rabbit, rat and hog fundic mucosas all have similar $H^+$-secretion capacities per g of tissue.

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