# THE ATP-DEPENDENT PROTON PUMP IN LYSOSOME MEMBRANES

## Still a valid hypothesis

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## 1. Introduction

There is now considerable evidence for a proton pump or some energy-dependent system in lysosome membranes that functions to maintain intralysosomal acidity. Although most of this evidence is indirect, direct pH measurements by fluorescence in lysosomes of living macrophages have been made [1]. Inhibitors of ATP formation and certain bases known to accumulate in lysosomes were shown to reversibly raise intralysosomal pH. These results must be interpreted in terms of an energy-dependent proton or acid pump. The inhibitory effects of ionophores on the stimulation of intralysosomal proteolysis by ATP in cell-free suspensions must also be interpreted in terms of such a pump [2]. Evidence for a proton pump in rat liver lysosome membranes has also been obtained [3,4].

Attempts to detect an ATP-induced lowering of pH in rat liver tritosomes (TritonWR-1339-filled lysosomes) in alkaline media by methylamine distribution have not been successful [5–7]. The reason for this is not clear, but a number of possible explanations exist; these include short incubation periods at low temperatures, possible interference of Triton WR-1339 with the proton pump, and a relative impermeability of liver tritosomes to alkaline buffers.

Experimental data interpreted as evidence against a proton pump in rat liver tritosomes has been presented [5]. Here I point out that the evidence against the existence of a proton pump in [5,6] is based on inadequate and improperly interpreted data. Additional evidence supporting the proton pump hypothesis is also presented.

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## 2. Materials and methods

The preparation of $^{125}$I-labelled, formaldehyde-treated bovine serum albumin and cell-free mouse liver and kidney suspensions containing labelled albumin-filled phagolysosomes has been described in [8,9]. In all experiments 2–4 mg ($9–12 \times 10^6$ cpm) labelled protein were injected intravenously into adult mice, and the animals were sacrificed 30 min later. The organs were homogenized in ice-cold 0.25 M sucrose containing 1 mM EDTA (pH 7.0). Radioactive particles (labelled protein-filled phagolysosomes) obtained by centrifugation of these homogenates contained $\geqslant 3 \times 10^5$ cpm. Unwashed pellets containing these phagolysosomes were suspended in incubation mixtures (preincubated at 35°C) containing 2.5 mM ATP (disodium salt), 5 mM $MgCl_2$ or 2 mM $CaCl_2$, 50 mM $\beta$-mercaptoethanol, and buffers as indicated in the text, in 12 ml 0.25 M sucrose. This concentration of ATP was $\geqslant$10-times the quantity required for 50% maximum stimulation of intralysosomal proteolysis. Intralysosomal proteolysis was measured during incubation of phagolysosome suspensions at 35°C by precipitation of undegraded protein with 5% trichloroacetic acid as in [8–10]. Phosphate was assayed according to Fiske and Subbarow [11]. Radioactivity measurements were made in a Beckman 1000 $\gamma$ spectrometer with automatic sample changer. The efficiency of this system was 80% for $^{125}$I.

All chemicals were reagent grade. ATP (disodium salt) was obtained from Sigma Chemical Co, St Louis, MO.

### 3. Results and discussion

In order to demonstrate a stimulation of intralysosomal proteolysis by additions of ATP, the activity must first be inhibited by pH 7–8 buffer; i.e., buffer must penetrate the lysosome and raise the intralysosomal pH. This inhibition is not always easily accomplished in liver phagolysosomes unless relatively high buffer concentrations are used [8], or unless phagolysosomes are first preincubated on ice in the presence of alkaline buffer. Mouse liver phagolysosomes appear to be relatively more impermeable to many substances than are kidney phagolysosomes [12]. Figure 1 shows an experiment in which there was little significant stimulatory effect of ATP because 0.05 M borate buffer (pH 8) did not inhibit intralysosomal proteolysis. A higher concentration of buffer (0.1 M) inhibited proteolysis by ~10%, compared with the pH 5 control, and this inhibition was reversed by ATP. It is important to note that the stimulating effect of ATP was not evident until after ⩾20 min incubation. It must also be emphasized that the effects of buffers on intralysosomal proteolysis in liver phagolysosomes is variable, and in some experiments significant inhibitions may be obtained with low concentrations of Tris or borate buffers [10]. The reason for this variability is not known. Relatively low concentrations of ammonium chloride (pH 8) will consistently inhibit intralysosomal proteolysis by ~40–60% and this inhibition is reversed by MgATP or CaATP (fig. 2).

In the attempts [5] to demonstrate that ATP had no effect on intralysosomal pH, the pH in tritosomes incubated at pH 6.5 and 7.5, and in the presence of bicarbonate, with and without ATP, was measured.

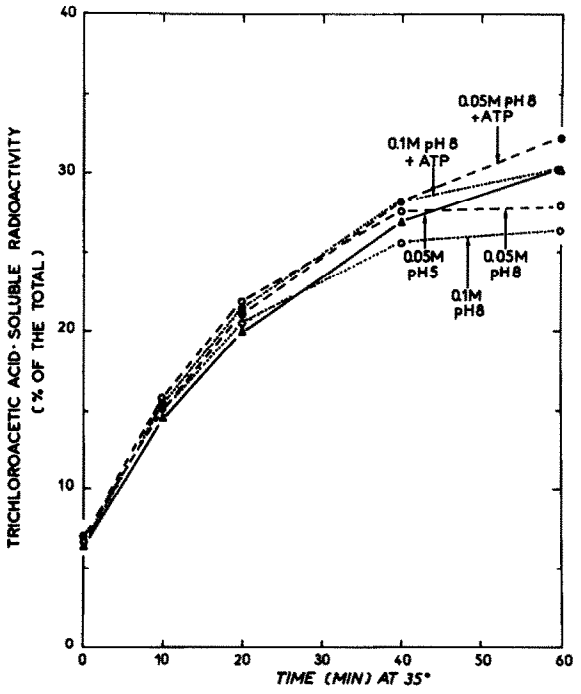


Fig. 1. Lack of a stimulatory effect of ATP on intralysosomal proteolysis in mouse liver phagolysosomes unless proteolysis is inhibited by pH 8 buffer. All samples contained 0.25 M sucrose, 0.05 M β-mercaptoethanol and buffer in 12 ml. Buffer concentrations are indicated: pH 5, Tris acetate; pH 8, sodium borate. ATP (neutralized to pH 8 with NaOH) was 2.5 mM.

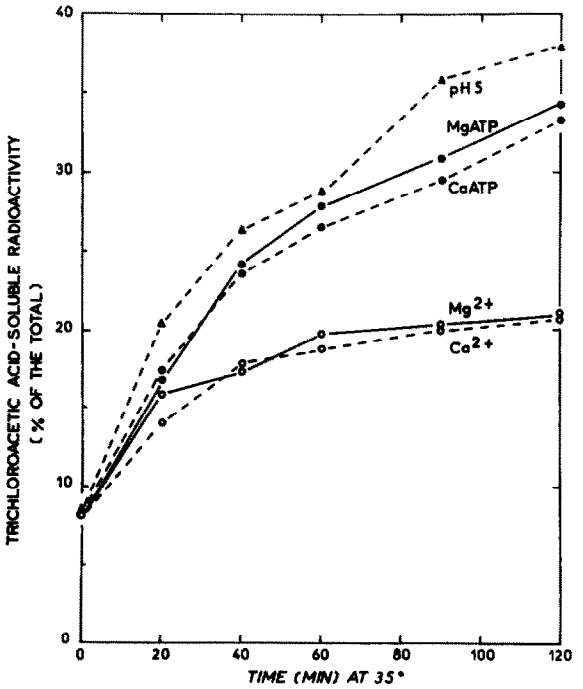


Fig. 2. Inhibition of intralysosomal proteolysis by 25 mM ammonium chloride buffer (pH 8) and reversal by ATP in mouse liver phagolysosomes. Incubation conditions and concentrations of ATP, $Mg^{2+}$, $Ca^{2+}$, are described in section 2.

However, their experiments do not show that pH 7.5 buffer or bicarbonate raised intralysosomal pH above controls without buffer or ATP. One measurement was made in a control in KCl medium at pH 6.5. Raising the pH to 7.5 in KCl medium raised the intralysosomal pH by ~6.5%, and ATP decreased this to ~3.8% above the control. In view of the short incubation periods (5 min), and the extremely small pH differences between control and experimental conditions in [5] it is not surprising that ATP had such a small effect.

The stimulatory effect of ATP on intralysosomal proteolysis was suggested [5,6] to be due to the liberation of protons into the medium during ATP hydrolysis. It was asserted [5] that a decrease in the medium pH from 7.5–7.1 due to ATP hydrolysis is sufficient to cause the observed drop in intralysosomal pH of 0.2 units. Figure 3 shows an experiment in

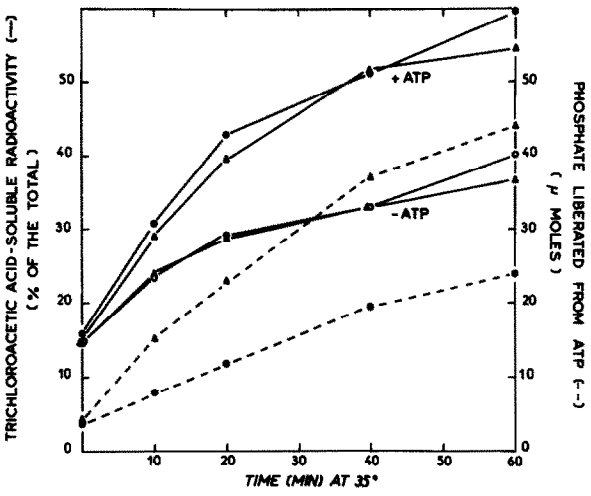


Fig. 3. ATP hydrolysis and intralysosomal proteolysis in mouse kidney phagolysosome suspensions incubated at 35°C. A kidney homogenate from a mouse injected intravenously with 2 mg $^{125}$I-labelled bovine serum albumin was divided into two parts: 75% and 25% of the total. Each of these was then equally divided and centrifuged 5 min at 500 × *g* followed by 10 min at 30 000 × *g* to sediment protein-filled phagolysosomes. Each pellet was then suspended in 12 ml incubation media containing 0.25 M sucrose, 0.05 M β-mercaptoethanol, 5 mM $MgCl_2$ without (○,△) and with (●,▲) 2.5 mM ATP. 25% homogenate is indicated by circles and 75% homogenate by triangles. Dashed lines: phosphate liberated from ATP.

Table 1
Effect of buffer concentration on the stimulation of intralysosomal proteolysis by ATP

| Tris acetate at pH 8 (mM) | Trichloroacetic acid-soluble radioactivity produced in 60 min/2 ml (cpm) | | × Stimulation |
|---|---|---|---|
| | −ATP | +ATP | |
| 25 | 1088 | 2442 | 2.24 |
| 50 | 770 | 1762 | 2.29 |
| 100 | 423 | 1103 | 2.61 |
| 200 | 210 | 552 | 2.63 |

Trichloroacetic acid-soluble radioactivity was determined in mouse kidney phagolysosome suspensions at zero time and after 60 min incubation at 35°C. Radioactivities at zero time were subtracted from those obtained after 60 min. Total radioactivity/2 ml sample was 5138–5799 cpm

which intralysosomal proteolysis was stimulated exactly the same if twice the quantity of ATP was hydrolyzed. Furthermore, additions of $KH_2PO_4$ to mouse kidney phagolysosome suspensions, in quantities 2-times the molar concentration of ATP that produces a 2-fold stimulation of intralysosomal proteolysis, have no significant stimulatory effect (J. L. M., unpublished). Raising buffer concentrations to levels such that no pH changes due to ATP hydrolysis are possible results in the same degree of stimulation of intralysosomal proteolysis by ATP as that observed at lower concentrations. Table 1 shows that higher buffer concentrations resulted in greater inhibitions of intralysosomal proteolysis due to increased penetration of buffer into the phagolysosomes. However, the degree of stimulation of proteolysis was the same at all buffer concentrations, ~2.2–2.6-fold. These results are consistent with a proton pump operating at maximum capacity and delivering the same quantity of protons at all buffer concentrations. Thus, the stimulatory effects of ATP cannot be due to acidification of the medium as proposed [5,6], but must be due to a transfer of the protons released by ATP hydrolysis into the phagolysosome. Therefore the work in [5] should be interpreted as direct evidence for a proton pump, rather than as evidence against it, since their data show that ATP lowered the pH in tritosomes by 0.2 pH units upon prolonged incubation at pH 7.1–7.5.

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