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CHANGES IN MITOCHONDRIAL ULTRASTRUCTURE INDUCED BY GRAMICIDIN *PLUS* PROTAMINE

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SUMMARY

The changes in isolated rat liver mitochondria induced by protamine or gramicidin and by protamine *plus* gramicidin have been investigated electron microscopically. The results are as follows:

1. Inhibition of succinate (*plus* rotenone) oxidation induced by protamine caused mitochondrial shrinkage with collapse of outer and inner lamina of mitochondrial membrane and of cristae.

2. Both inhibition and structural changes can be partially reversed by citrate.

3. After protamine *plus* gramicidin significant damage of mitochondria with almost complete destruction of matrix, cristae and outer lamina was noted.

4. A protective influence of NH_4^+ on the mitochondrial structure in the presence of gramicidin or gramicidin *plus* protamine was observed.

The presented results suggest that: (1) Changes in mitochondrial structure may be ascribed to a difference in the proton gradient on both sides of the mitochondrial membrane. (2) A large pH gradient may cause damage of mitochondrial structure. (3) The transport of anions by A^-/H^+ symport may be the cause of structural changes of isolated rat liver mitochondria.

INTRODUCTION

HACKENBROCK[1] has shown that isolated rat liver mitochondria undergo reversible structural transformations as their metabolic state is changed. The conformational oxidative phosphorylation theory[2] was based on electron microscopical observations of changes in mitochondrial structure dependent on the state of energy. A close relationship between mitochondrial structure and state of energy seems to be confirmed at present[3]. The mechanism of structural changes of mitochondria is still the main problem of discussion[4–8].

MITCHELL[9,10] suggested that differences in the proton gradient on both sides of the mitochondrial membrane could be the reason of mitochondrial structural

Abbreviations (Figs. 2–6): mit, mitochondria; cit, citrate; succ, succinate; prot, protein; gra, gramicidin.

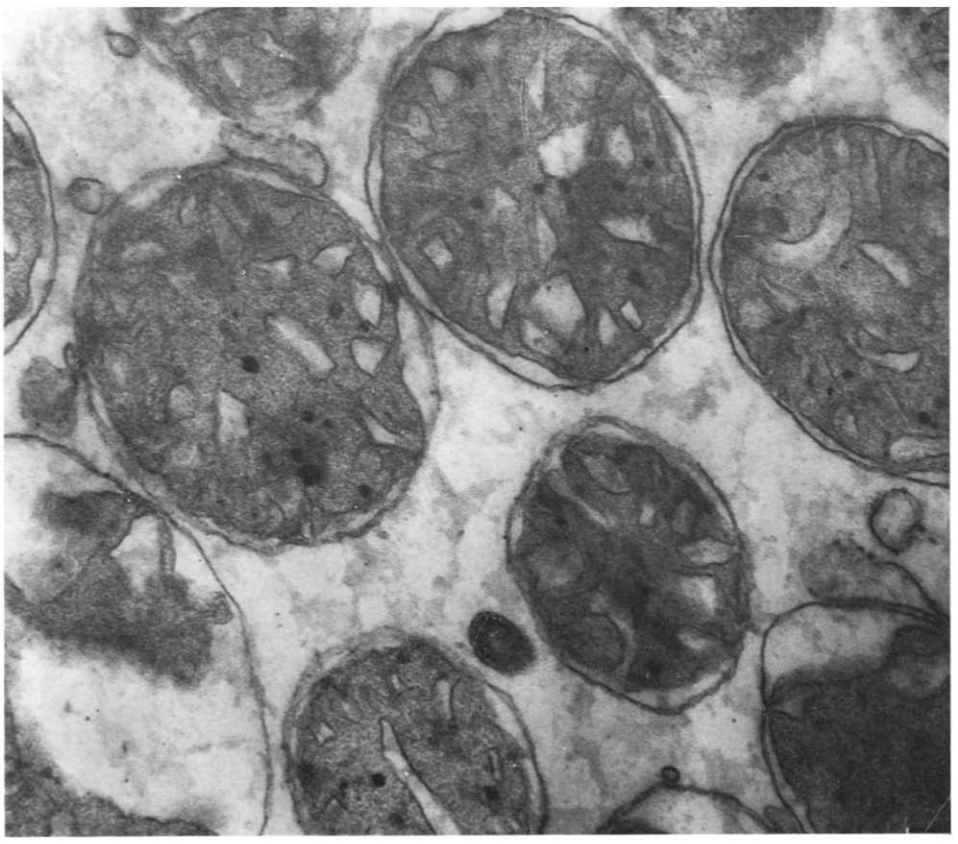
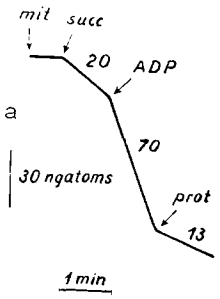


Fig. 1. Freshly isolated in 0.25 M sucrose mitochondria. The mitochondria are round in shape, with prominent outer and inner lamina of mitochondrial membrane. The lumen of mitochondrial cristae is wide, angular in shape. × 23000.

changes. On the other hand, in previous papers from our laboratory[11–14] we proposed that basic protein – protamine, affects mitochondrial metabolic processes as an inhibitor of energy transfer. This polycation probably reacts with the mitochondrial membrane and inhibits protons back-diffusion into the mitochondrion[13]. This is followed by inhibition of respiration of isolated mitochondria. These suggestions are based on the observation that protamine does not inhibit succinate oxidation in sonicated submitochondrial particles but inhibits succinate oxidation in intact mitochondria[13]. This inhibition may be partially reversed by addition of dinitrophenol, dicoumarol, menadione, citrate or isocitrate, reagents which probably conduct protons into mitochondria by A^-/H^+ symport[13]. Another uncoupling agent, gramicidin[15], increases permeability of the mitochondrial membrane not only for protons but also for univalent cations. Gramicidin added after protamine was without effect on the inhibition of succinate (*plus* rotenone) oxidation by protamine. Inhibition of respiration of mitochondria treated with protamine *plus* gramicidin can not be reversed by either agent conducting protons into mitochondria[13]. The

present results suggest that protamine acts by inducing a large proton gradient. Therefore, it seemed interesting to investigate structural changes of mitochondria by electron microscopy.



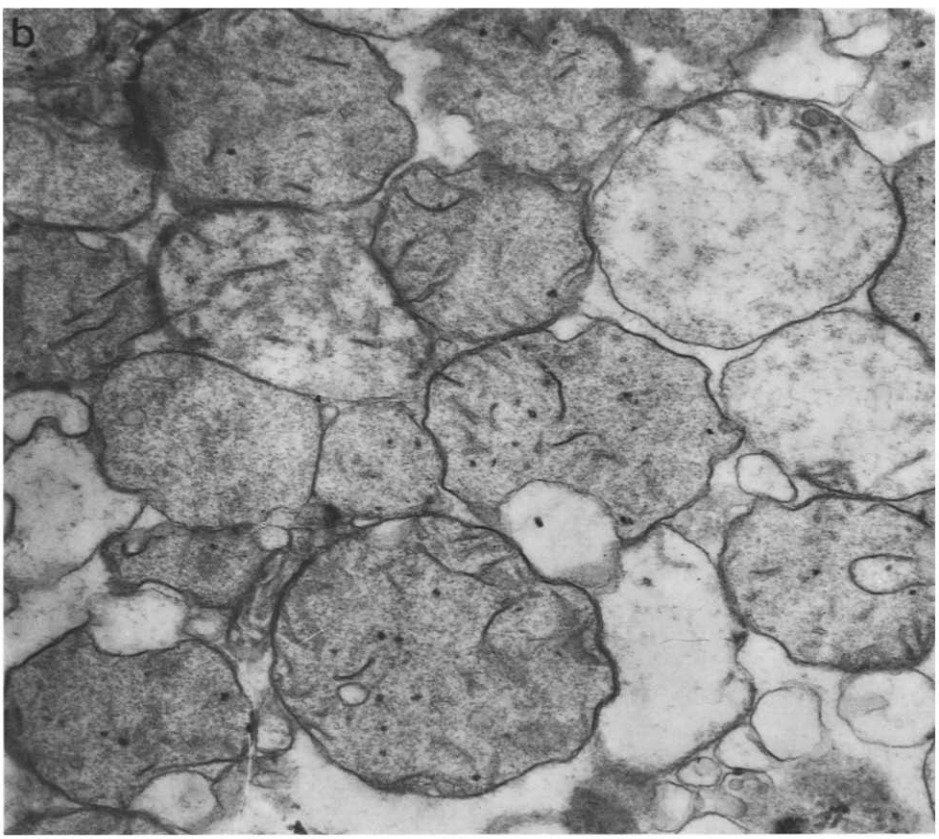
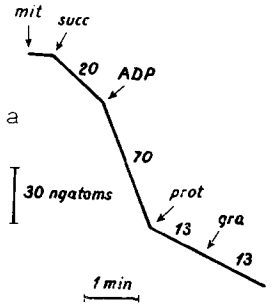


Fig. 2. Mitochondria after incubation with protamine. (a) Effect of protamine on succinate (*plus* rotenone) oxidation. For experimental conditions, see MATERIAL AND METHODS. (b) The outer and inner lamina of mitochondrial membrane are contracted and the mitochondria are of different size. The outer compartment is visible only in a few places as small vesicles between outer and inner lamina. Mitochondrial cristae are shrinked and tortuous. The mitochondrial matrix is insignificantly granular and electron-optically dense. × 25000.

MATERIALS AND METHODS

Rat liver mitochondria were prepared and suspended in 0.25 M sucrose with 3 mM Tris–HCl (pH 7.3)[16]. Respiration was measured using a Clark oxygen electrode at 25° in 3.5 ml of medium (pH 7.3) containing: 15 mM KCl, 50 mM Tris–HCl, 5 mM potassium phosphate, 5 mM $MgSO_4$ and 4 μg rotenone. Other additions: 10 mM



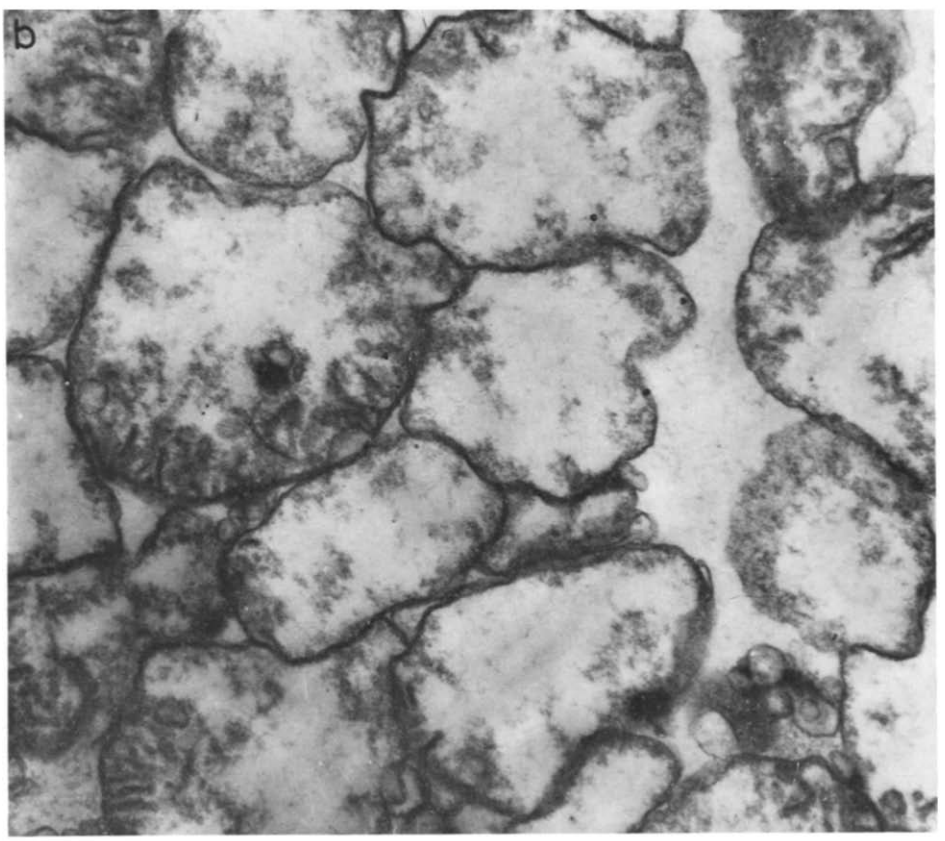
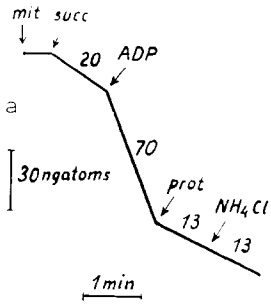


Fig. 3. Mitochondria after incubation with protamine followed by gramicidin. (a) Effect of gramicidin on protamine-inhibited respiration. For experimental conditions, see MATERIALS AND METHODS. (b)The mitochondria are swollen. Their membrane is formed in most mitochondria only by the inner lamina; the outer lamina is present only in a few places. The mitochondria matrix is visible in very small concentration around numerous mitochondrial cristae. × 21 600.

succinate, 1 mM ADP, 300 μg protamine, 3 μg gramicidin, 10 mM NH_4Cl, 7 mM citrate were added as indicated in the figures. Oxygen consumption was expressed as ngatoms O/min per mg protein. The reaction was started by adding 0.1 ml of suspension of freshly prepared rat liver mitochondria in the amount corresponding to 8 mg of mitochondrial protein. After the measurement of respiration had been accom-



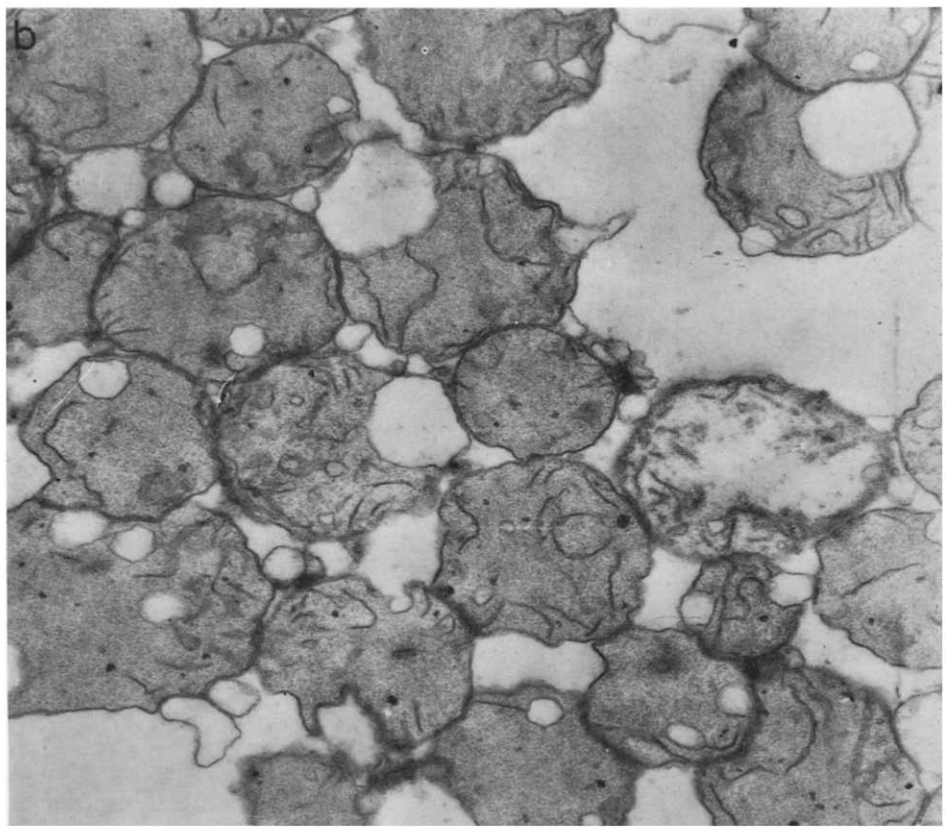


Fig. 4. Mitochondria after incubation with protamine followed by ammonium. (a) Effect of NH_4Cl on protamine-inhibited respiration. Experimental conditions, see MATERIALS AND METHODS. (b) Mitochondria are contracted with numerous small electron-optically empty vesicles between inner and outer lamina. The outer compartment as well as cristae are collapsed and tortuous. The mitochondrial matrix is electron-optically dense. × 22000.

plished, samples (3 ml) were removed from the oxygen electrode chamber for electron microscopy. They were centrifuged for 40 sec at 10000 × *g*, and the pellets obtained were fixed in 2 % osmium tetroxide for 1 h. The fixative was brought to pH 7.4 with 0.21 M sodium phosphate containing 50 mM sucrose. After fixation the pellets were stained for 4 min with 1 % uranyl acetate in 25 % ethanol at 0°, dehydrated with ethanol and embedded in Epon 812. Sections were cut with a glass knife, poststained with uranyl acetate and lead citrate. Specimens were examined in a JEM 7A electron microscope at 80 kV. The protein content of the suspension was estimated by the biuret method[17]. Protamine sulphate *ex* herring, was used after adjusting to pH 7.3 with Tris.

Reagents: Succinate was purchased from Sigma Chemicals Co. ADP, gramicidin, protamine were Koch-Light products. Citrate and NH_4Cl were from P.P.H. Polskie Odczynniki Chem. Gliwice. Rotenone was a generous gift from Professor L. Wojtczak.

RESULTS

Experiments performed are presented in the figures. A part of these results was presented in June 1970 in Szczecin at the Meeting of Polish Biochemical Society, and published in *FEBS Lett.*[13,14]. There was a contraction of membrane and cristae of the rat liver mitochondria after incubation with protamine (Fig. 2). The two laminae of mitochondrial membrane were so close to each other that it was very difficult to visualize the outer compartment as well as the space of the cristae. The tortuous shape of the mitochondrial membrane and cristae is a morphological demonstration of mitochondrial contraction. Although the small vesicles between outer and inner lamina may be caused by protamine, it is also possible that they are the result of preparative damage. Oxygen consumption by these mitochondria was 13 ngatoms/min per mg protein. The addition of NH_4^+ to mitochondria incubated with protamine did not significantly change their structure nor the oxygen consumption (Fig. 4). The structure of mitochondria and their respiration changed when protamine was followed by citrate (Fig. 6). The regular shape of mitochondria was again visible (Fig. 5) after gramicidin had been added to contracted mitochondria incubated previously with protamine and NH_4^+. Oxygen consumption was 42 ngatoms/min per mg protein. At the same time small vesicles were formed in the outer compartment. All these changes after addition of gramicidin may indicate that contraction of mitochondria due to protamine and NH_4^+ is reversible, and that a different pressure inside the mitochondria after incubation with gramicidin is the cause of their round shape. Gramicidin destroyed mitochondria incubated with protamine and was without affect on mitochondrial respiration (Fig. 3). There was damage of matrix, cristae and often the outer lamina of the mitochondrial membrane. The shrinkage of mitochondria caused by protamine was diminished. The appearance of all these changes may be caused by the lack of a protective effect of NH_4^+. The mitochondria incubated with gramicidin were round shaped with well-visible outer and inner lamina of membrane, a small amount of cristae and often damaged outer lamina. The mitochondria were swollen; they seemed to be in the early state of disruption.

The conclusions based on the presented results are as follows:

(1) Protamine causes significant contraction of mitochondria and their membranes; this effect is reversible by citrate.

(2) Gramicidin causes a swelling of mitochondria.
(3) The actions of NH_4^+ and protamine on mitochondrial shrinkage are additive.

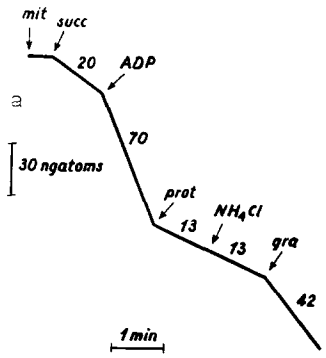
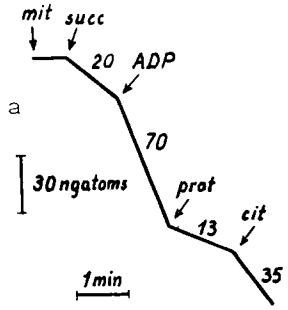


Fig. 5. Mitochondria after incubation with protamine followed by NH_4^+ and gramicidin. (a) Reversed effect of gramicidin on protamine-inhibited respiration in the presence of NH_4Cl. For experimental conditions, see MATERIALS AND METHODS. (b) The mitochondria are swollen. The three layers of mitochondrial membrane are well visible as well as the structure of cristae. There are small, electron-optically empty vesicles between inner and outer lamina in many mitochondria. The mitochondrial matrix in the whole inner compartment is osmophilic and microgranular. × 21 000.

(4) NH_4^+ protects mitochondria against damage caused by gramicidin. Gramicidin in the presence of NH_4^+ produces only structural swelling of mitochondria.

(5) Gramicidin destroys mitochondrial matrix, cristae and outer lamina of membrane of mitochondria incubated with protamine.



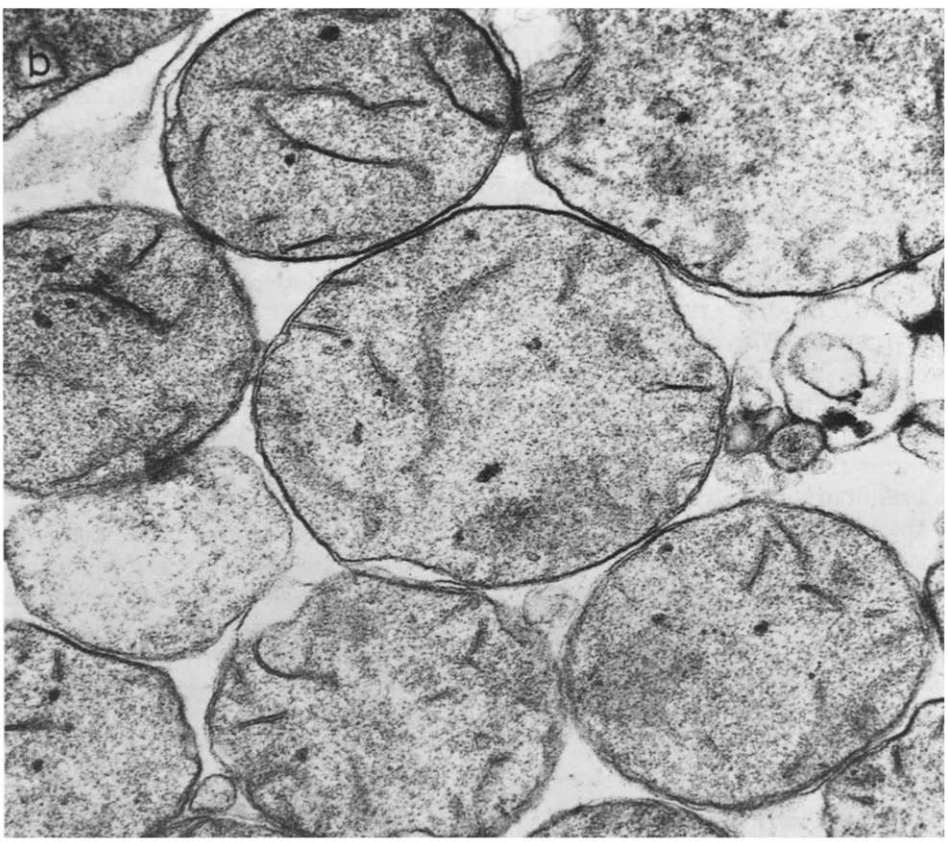


Fig. 6. Mitochondria after incubation with protamine followed by citrate. (a) Effect of citrate on protamine-inhibited respiration. For experimental conditions, see MATERIALS AND METHODS. (b) The mitochondria are round, not significantly contracted. The outer compartment is narrow but visible. Mitochondrial ciristae are mostly elongated and narrow; part of them is wide, angular in shape. The mitochondrial matrix has a fine granular structure and is of low electron density. × 32 000.

DISCUSSION

It seems that under the influence of protamine a significant shrinkage of mitochondrial membrane and cristae occurs (Fig. 2). This reaction of the mitochondrial membrane is followed by inhibition of back-diffusion of protons into the mitochondrion. Respiration of isolated mitochondria inhibited by protamine cannot be reversed by uncouplers or weak acids in the absence of Mg^{2+} in the medium. If Mg^{2+} was added to the medium before protamine, the systems conducting protons into mitochondria reversed this inhibition. It would appear that systems which conduct protons into mitochondria are penetrating the mitochondrial membrane as unionized-form A^-/H^+ symport. Probably these processes appear in those places of the mitochondrial membrane which are bound with previously added Mg^{2+}. This view is supported by the finding that Mg^{2+} added to isolated mitochondria after protamine was without effect on the irreversibility of inhibition. Gramicidin which increases the permeability of the mitochondrial membrane to protons and univalent cations did not reverse this inhibition. On the other hand, citrate, which is supposed to penetrate mitochondria during A^-/H^+ symport, reversed protamine-induced changes. These findings suggest also that H^+ is conducted into mitochondria during A^-/H^+ symport. The irreversibility of inhibition of respiration in the presence of protamine *plus* gramicidin seems to be connected with the damage of mitochondrial structure (Fig. 3). This is probably a result of a two-step action of gramicidin. Extrusion of H^- is the first step of this action, the second step being the accumulation of these protons and univalent cations. It seems that the extrusion of H^+ from mitochondria by gramicidin, when protamine inhibits back-diffusion of protons, causes a significant increase of OH^- concentration inside the mitochondrion followed by destruction of mitochondrial structure (Fig. 3). SLATER[18] has calculated that the pH of the cristae space according to MITCHELL's[9,10] hypothesis would be 13.5. It seems that in our conditions when protamine *plus* gramicidin were added, the pH is very strongly alkaline inside the matrix space; this may be the cause of the changes which are visible on the electron micrographs. The protective effect of NH_4^+ on mitochondrial matrix structure and on respiration (Fig. 4) confirms also the view that the damage of mitochondria may be a result of strong alkalinization of matrix. Presented suggestions are also supported by the results of CHAPPELL AND CROFTS[15] who observed acidification inside the mitochondrion when NH_4^+ was added in the presence of gramicidin. In our experiments NH_4^+ was added first and prevented the destruction of mitochondrial structure caused by protamine *plus* gramicidin (Fig. 5). It is interesting that ammonia-forming enzymes in mitochondria are activated by extremly alkaline conditions[19,20].

The present results are in reasonable agreement with the chemiosmotic as well as with the conformational theory of phosphorylation.

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