SWELLING OF RAT LIVER MITOCHONDRIA WITH *N*-(*N*-ACETYL-4-SULPHAMOYLPHENYL)-MALEIMIDE

G.ZIMMER and K.KRIEGER

Institut für Vegetative Physiologie, Universität Frankfurt (Main), Germany

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1. Introduction

Sulphydryl groups play an important role through lipid peroxidation when swelling in mitochondria is initiated by heavy metals, cysteine, and alkylating reagents such as maleimides. At rising concentrations of deoxycholate [1], and thiophens [2], the ATPase of mitochondrial preparations after an activation and subsequent inhibition phase revealed a second increase of activity. Contrary to myosin ATPase, the ATPase in mitochondrial membranes cannot be inhibited completely by blocking the SH-groups within the concentration range equivalent to the titratable sulphydryl groups [3]. A complete understanding of the mechanism responsible for this failure to achieve complete inhibition has not yet been attained.

In our investigation with *N*-(*N*-acetyl-4-sulfamoylphenyl)-maleimide (ASPM), synthesized in 1965 by Merz, Pfleiderer and Wieland [4], we found a sequence of alternating activations and inhibitions of mitochondrial swelling with increasing μmolar concentrations of the reagent. This was interpreted as the consecutive blocking of enzyme-activating and enzyme-inhibiting sulphydryl groups which are gradually set free in the course of swelling when the inner mitochondrial membrane is unfolded [5]. When ASPM is added at 2 μM concentration, the ATPase in the membranes is activated and inhibited alternatively in the course of swelling. The change in conformation of the membrane proteins, which causes this process and which may also occur in a mere Tris-KCl-buffer without SH-reagent [6] is discussed.

2. Materials and methods

Wistar rats of a local strain, weighing 150–200 grams were used. Preparation of the mitochondria was performed in a conventional manner as described elsewhere [7] with the exception that the final stock suspension was about 8–10 ml. The swelling curves were observed photometrically in KCl 0.125 M, Tris 0.02 M, adjusted to pH 7.5 with HCl, by an Eppendorf photometer at 546 nm, temperature 21°C. The absorbance was read every minute. For the ATPase determinations see legend to fig. 4. Protein concentrations were determined by the method of Lowry [8].

3. Results

The figures show that very small concentrations of ASPM or NEM (about 0.1 μM) have an accelerating effect on swelling. Higher concentrations (0.5 – 5 μM), cause, depending on the concentration of mitochondria, either an inhibition of swelling speed (fig. 1) or a more pronounced acceleration. Further increased concentrations of 5 (–10) μM cause an acceleration, whereas concentrations of 10–20 μM were shown to have a swelling-inhibiting effect (fig. 2). The increase of the concentration to 50 and 100 μM again further activates swelling (fig. 2). Analogous tests with NEM at rising concentrations did not always reveal such clear results regarding activation or inhibition of swelling, namely at higher concentration range (fig. 3). The course of membrane ATPase activity at the same ASPM concentration (2 μM) shows the

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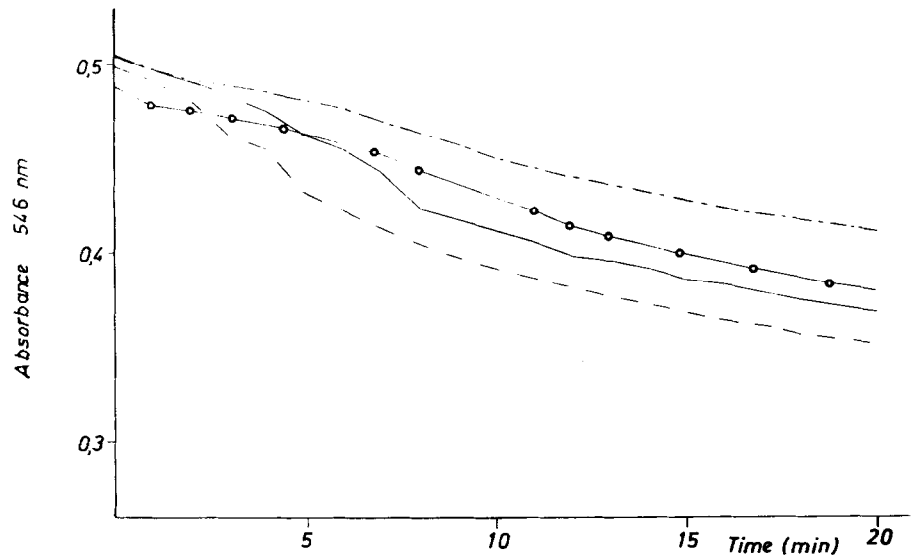

Fig. 1. Swelling curves as obtained with *N*-(*N*-acetyl-sulphamoylphenyl)-maleimide (ASPM). Medium: Tris 0.02 M, KCl 0.125 M, pH 7.5 with HCl. Temperature 21°C. Protein concentration: about 0.11 mg/ml Tris-KCl. ASPM concentrations:
– – – – 0.1 μM – – – – 1 μM –o–o– 5 μM ——— control.

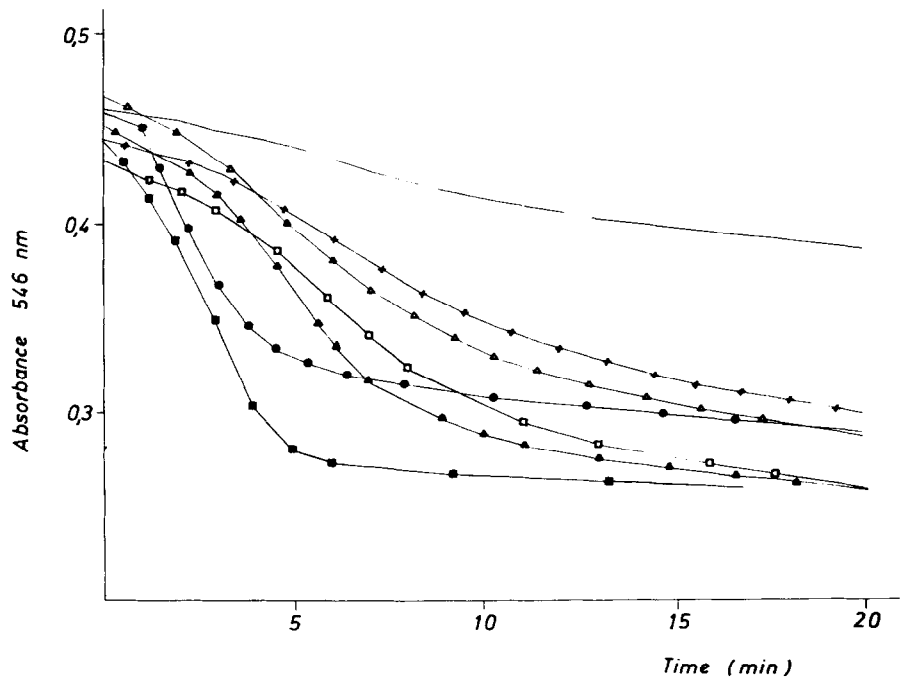

Fig. 2. Swelling curves as obtained with *N*-(*N*-acetyl-sulphamoylphenyl)-maleimide. Conditions see fig. 1.
ASPM concentrations:
–●–●–● 10 μM
–★–★–★– 20 μM –△–△– 50 μM –□–□– 100 μM
–▲–▲– 200 μM –■–■– 500 μM ——— control

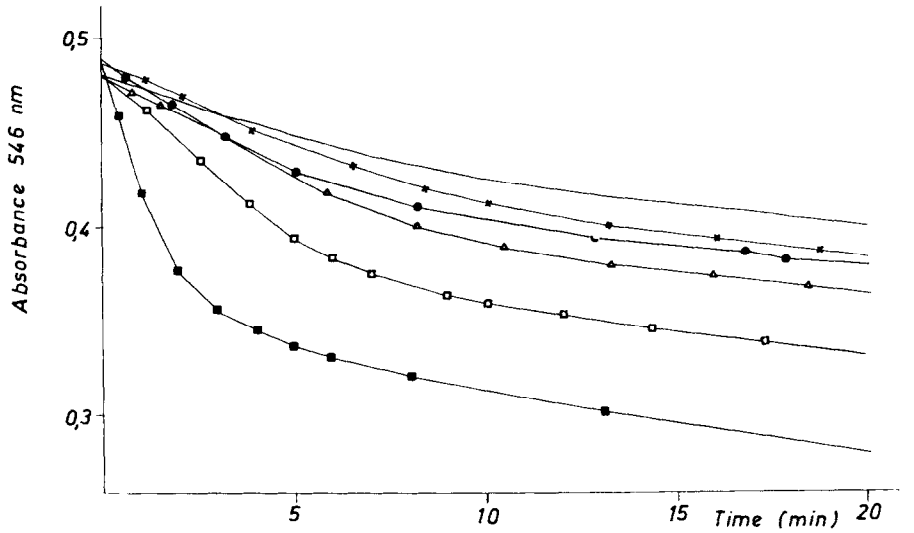

Fig. 3. Swelling curves as obtained with *N*-ethyl-maleimide (NEM). Conditions: see fig. 1. NEM concentrations:
—●—●—●— 10 μM —★—★—★— 20 μM —△—△— 50 μM
—□—□— 100 μM —■—■— 500 μM ——— control.

timely sequence of activation and inhibition phases (fig. 4).

4. Discussion

As NEM shows a similar behaviour in activating and inhibiting the swelling as ASPM, but ASPM reveals the activating and inhibiting phases (oscillations) more clearly, the difference between these substances must be related to the different substitution. The relatively large substitution of ASPM apparently prevents a great number of molecules from reacting simultaneously at narrow space. Owing to this fact activation and inhibition phases are sharper contrasted by ASPM. Since further SH-groups are gradually set free in the course of swelling [9], the process of alkylation can take place continuously as has been shown by autoradiography [7]. Activation is followed by inhibition and vice versa.

Penniston et al. [10] have demonstrated that the subunits of the membranes take part in conformative changes. It may be that it is just the same change of conformation, which can be initiated by a potassium buffer alone that is accelerated when SH-reagents are present. The course of the swelling curves will then be accordingly slower, corresponding to the values of the controls shown in the figures. Whatever similarity or non-similarity between these processes exists, the oscillations between activation and inhibition periods may serve to elucidate the missing complete inhibition of mitochondrial membrane ATPase by SH-reagents. If the activating SH-groups are alkylated, an excess of reagent will block the inhibiting sulphydryl groups and then again the newly introduced activating groups and so on, so that if these processes interfere with one another to a small degree, a total inhibition of the ATPase in the mitochondrial membranes will not occur. Obviously this is connected with the law of the unfolding of the inner mitochondrial membranes.

Contrary thereto the mitochondrial ATPase activity in the soluble mitochondrial proteins sometimes was found to fall to zero or to almost zero in the course of swelling (Zimmer, unpublished observations). This might be considered as another indication leading to the assumption that the membrane ATPase can be only partially inhibited because it represents a structurally bound enzyme system of the liver mitochondria.

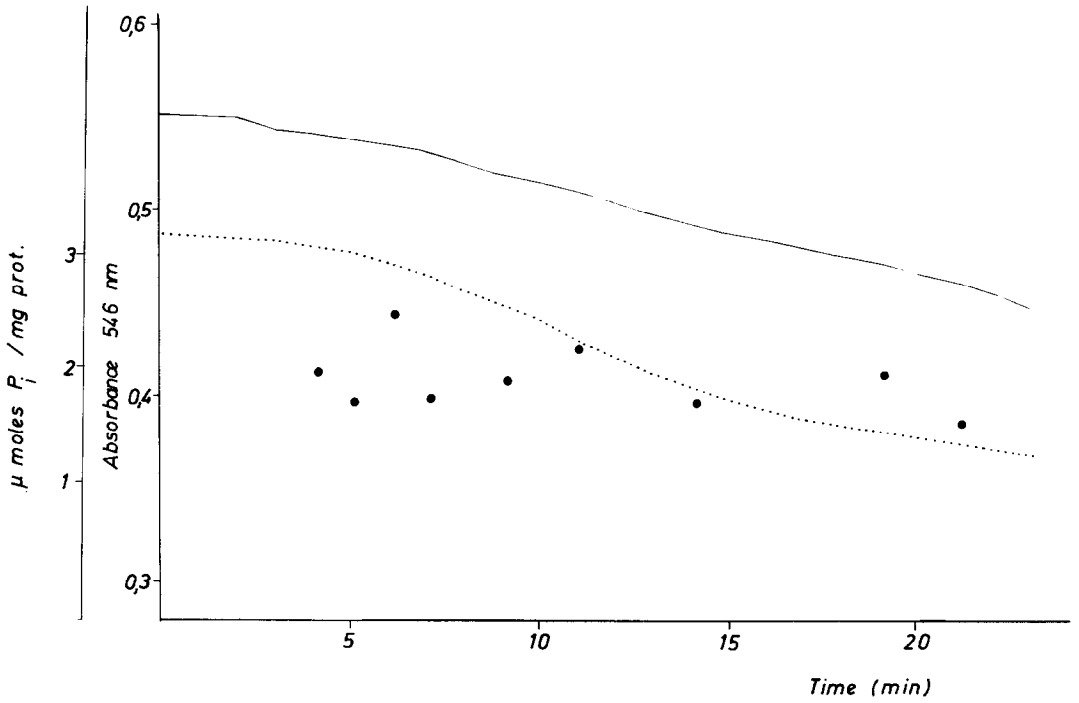

Fig. 4. Swelling curves and ATPase activities in mitochondrial membranes. Conditions of incubation: see fig. 1. • membrane ATPase activity, ... swelling curve with 2 μM ASPM, ——— swelling curve control.

ATPase determinations: Each point represents the mean of 2 determinations. Incubation medium was the same as swelling medium. At the chosen time, an aliquot of the stock suspension (about 0.11 mg protein/ml Tris-KCl) was centrifuged at 20000 g for 3 min in 200 ml Tris-KCl. The supernatant was decanted off and the mitochondrial pellets were superficially washed with 0.25 M sucrose to remove the salt solution. The total time required for the incubation of the mitochondria in the Tris-KCl buffer until the termination of the washing of the pellets was noted. Then the mitochondria sediment was suspended in ice-cold 0.25 M sucrose, and diluted to 45 ml for sonification, which was carried out in a Branson S 75 sonifier with a current of 2.5 – 3 A for 20 min. Afterwards, the protein content was determined in 0.5 ml of the sonicated mitochondria. 36 ml of the clear solution were then centrifuged for 20 min at 13500 × *g* in the ultracentrifuge to remove unbroken mitochondria. Then the supernatant was centrifuged for 60 min at 100000 × *g*. Each membrane pellet resulting from this last centrifugation was suspended in 0.4 ml 0.003 M potassium phosphate buffer pH 8.7, according to the proposal of [11].

After 30 min standing the ATPase activity was determined with the following additions: 0.7 ml 0.1 M KCl, 0.8 ml 0.125 M H istidine, adjusted to pH 7.5, 0.2 ml 0.05 M $MgCl_2$, 0.2 ml Protein and 0.1 ml 0.1 M ATP (neutralized) = total of 2.0 ml. Incubation 5 min at 28°C, terminated by addition of 1.5 ml 8% perchloric acid 2 ml used to determine phospnate by the method of ref. [12].

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References

[1] P.Siekevitz, H.Löw, L.Ernster and O.Lindberg, Biochim. Biophys. Acta 29 (1958) 378.

[2] T.J.Franklin, C.W.Jones and E.R.Redfearn, Biochim. Biophys. Acta 131 (1967) 240.

[3] W.W.Kielley, in: Proc. 5th Intern. Congr. Biochemistry, Moscow, Vol. V (Pergamon Press, Oxford, London, New York, Paris, 1961) p. 378.

[4] H.Merz, G.Pfleiderer and Th.Wieland, Biochem. Z. 342 (1965) 66.
[5] G.A.Blondin and D.E.Green, Proc. Natl. Acad. Sci. U.S. 58 (1967) 612.
[6] G.Zimmer, H.Ette and P.Geck, submitted for publication.
[7] G.Zimmer, Hoppe-Seyler's Z. Physiol. Chem. 345 (1966) 187.
[8] O.H.Lowry, N.J.Rosebrough, A.L.Farr and R.J.Randall, J. Biol. Chem. 193 (1951) 265.
[9] J.D.Robinson, Arch. Biochem. Biophys. 113 (1966) 526.
[10] J.T.Penniston, R.A.Harris, J.Asai and D.E.Green, Proc. Natl. Acad. Sci. U.S. 59 (1968) 624.
[11] W.W.Kielley and R.K.Kielley, J. Biol. Chem. 200 (1953) 213.
[12] C.H.Fiiske and Y.Subbarow, J. Biol. Chem. 66 (1925) 375.