ADP-DEPENDENT INHIBITION OF SARCOSOMAL ADENINE NUCLEOTIDE TRANSLOCASE BY *N*-ETHYLMALEIMIDE

Pierre LEBLANC* and Hubert CLAUSER
Institut de Biochimie, Université de Paris-Sud, Centre d'Orsay, 91-Orsay, France

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1. Introduction

It seems presently established that ADP** induces structural modifications in mitochondria, independently of its action on the oxidophosphorylating system, hence independently of its influence on the mitochondrial energy level, which in turn is known to modify the morphology of these organelles [1, 2]. Energy-independent, ADP dependent structural modifications are revealed by electron microscopy [3]. They are also revealed by slight, but very rapid modifications of the turbidity of mitochondrial suspensions [4]. On the other hand, Zimmer [5] has shown that ADP induces an increase of the total -SH groups which may be alkylated in mitochondria, under non-phosphorylating conditions, due to the presence of oligomycin. In the course of investigations on the accumulation of large amounts of calcium phosphate in the presence of ADP by hog heart sarcosomes [6] the turbidimetric technique of Stoner and Sirak [4] has been used and the ADP dependent jump in optical density, first reported by these authors, has been systematically studied; it is established in the present work that this turbidimetric jump is modified by the alkylating agent NEM, which amplifies the phenomenon and inhibits its reversion by atractyloside. It is further demonstrated that the alkylation kinetics of reactive groups by NEM and the inhibition kinetics of the atractyloside induced reversion of the optical jump are similar. In addition, alkylation of these groups by NEM parallels an inhibition of adenine nucleotide translocation, mediated by the adenine nucleotide-translocase exchange reaction [7–10]; the possibility of correlating these various phenomena is discussed.

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** Abbreviations: AN: Adenine nucleotides; ADP: adenosine diphosphate; NEM: *N*-ethylmaleimide.

2. Materials and methods

The previously described method for isolating sarcosomes [6] has been modified as follows: the tissue was homogenized in 270 mM sucrose, 60 mM KCl, 20 mM Tris-HCl pH 7.6. The mitochondrial pellets obtained after the first washing procedure were suspended in 10 ml of 270 mM sucrose, 60 mM KCl, 10 mM Tris-HCl pH 7.4; 5 ml of this suspension were carefully layered over 30 ml of 1 M sucrose, centrifuged at 20,000 *g* (20 min). After recentrifugation to eliminate sucrose in excess, the pellets were resuspended in a 150 mM KCl, 10 mM Tris-HCl pH 7.2 solution, adjusted to 30 mg protein/ml, which constitutes the stock mitochondrial suspension, and may be used for 3 days when kept at 0°, without obvious modifications of the sarcosomes responsiveness.

The incubation medium for all experiments was as follows: 70 mM sucrose (BDH Chemicals Ltd), 70 mM Tris-HCl (Calbiochem), 12 mM Na-succinate (Calbiochem), 1.5 mM EDTA (Prolabo), 2 μg/ml rotenone and 3 μg/ml oligomycin (Sigma Chem. Lab.).

The optical jumps were recorded on a Gilford 240 recording spectrophotometer at 540 nm (expansion scale 150 units optical density).

Alkylation by ^{14}C-NEM (CEA Saclay, France) was

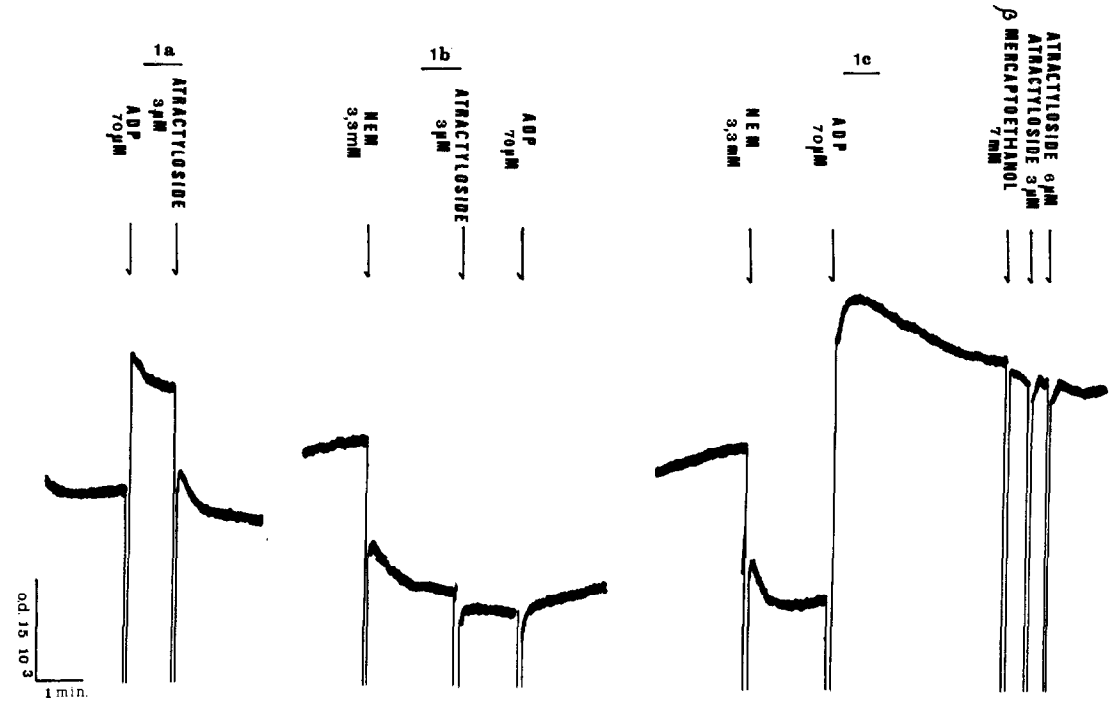

Fig. 1. Influence of NEM on the ADP induced shrinkage of hog heart sarcosomes. Protein concentration 1 mg/ml. Initial absorbance: 1.8 cm^{-1}; 20°, pH 6.8. Final volume 3 ml. The sarcosomes are preincubated in the absence of NEM (Sigma Chem.) for 2 min (for details, see Materials and methods).

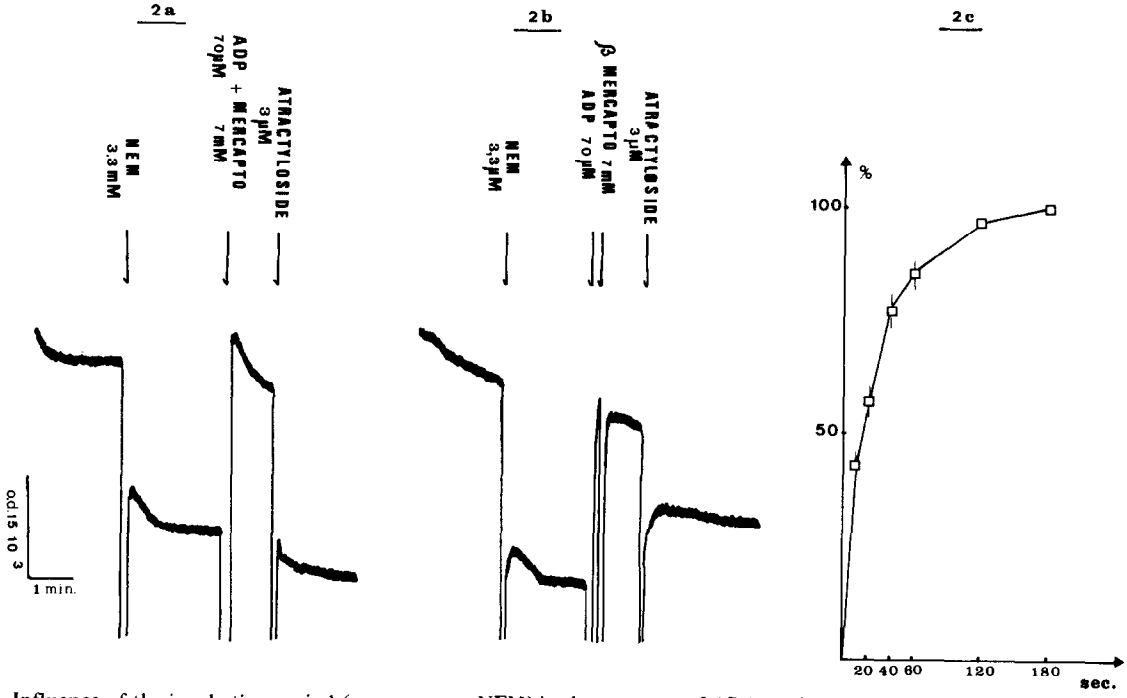

Fig. 2. Influence of the incubation period (sarcosomes + NEM) in the presence of ADP on the reversibility of the ADP induced optical jump. On fig. 2a, β-mercaptoethanol and ADP (P.L. Biochemicals Inc.) are added simultaneously; on fig. 2b, β-mercaptoethanol is added 10 sec after the addition of ADP; experimental conditions as in fig. 1. Fig. 2c, percentage of inhibition of the atractyloside dependent reversion of the optical jump as a function of the incubation period in the presence of NEM plus ADP.

measured as follows: 500 μl of the reaction medium were pipetted with an Eppendorf-micropipette and quickly added to 0.7 ml of a solution containing 10% TCA and 10 mM β-mercaptoethanol in a refrigerated (0°) plastic centrifuging tube (Eppendorf 3200 microcentrifuge).

After 1 min centrifugation at maximal speed, the supernatant was discarded, the tube and the pellet were rinsed with a small volume of 5% TCA, the pellet was resuspended in 1 ml of 5% TCA, recentrifuged (30 sec), the supernatant discarded again and the pellet rinsed with distilled H_2O. The pellet was dissolved in 500 μl of "soluene 100" (Packard) and the solution counted with a Packard 3375 scintillation counter after addition of 10 ml of the usual scintillation medium (toluene, PPO, POPOP).

^{14}C-ADP (Radiochemical Centre, Amersham) exchange was estimated by separating the sarcosomes from an aliquot of the medium by filtration under negative pressure through a 0.6 μ Schleicher–Schüll membrane [9, 10] washed once with 10 ml of a 100 mM sucrose, 100 mM Tris-HCl pH 7.4 solution and dried; the filter was dipped into 10 ml of the scintillation medium and the total radioactivity counted with a Packard 3375 scintillation counter.

Proteins were measured according to Jacobs et al. [11], cytochrome *a* was assayed following the method of Griffiths and Wharton [12].

3. Results

3.1. *The action of NEM on the ADP induced optical jump [4]*

Comparing fig. 1c (NEM present) and 1a (NEM absent), it appears that the alkylating agent increases the optical jump observed with ADP and abolishes its reversion by atractyloside. Fig. 2a shows that by adding ADP simultaneously with β-mercaptoethanol, the action of NEM is abolished. When (fig. 2b) β-mercaptoethanol is added 10 sec. after ADP, partial reversion of the optical jump occurs on addition of atractyloside. Fig. 2c shows the percentage inhibition of reversion of the phenomenon with atractyloside, as a function of the incubation time in the presence of NEM and ADP. Fig. 1b shows that atractyloside, when added before ADP, prevents the optical jump, even in the presence of NEM.

3.2. *Alkylation kinetics of sarcosomal reactive groups by ^{14}C-NEM*

Fig. 3a shows that, under the influence of ADP, additional reactive groups which may be titrated by NEM are revealed. Fig. 4 shows alkylation kinetics of these groups, at 20° and at 2° the maximum amount titrated at both temperatures is identical, which further confirms their specificity. Fig. 3b demonstrates that, as for the optical jump (fig. 1b), atractyloside inhibits the unmasking of additional alkylable groups by ADP.

3.3. *Action of NEM on nucleotide-exchange in sarcosomes*

Fig. 5a shows, in agreement with current views [9, 10], that NEM does not inhibit adenyl-nucleotide exchange when the sarcosomes are incubated in the absence of ADP. Conversely, it appears that in the presence of this nucleotide, NEM inhibits the exchange reaction; the extent of this inhibition parallels the incubation period with NEM. The percentage inhibition of the exchange reaction, measured after 15 min incubation, plotted versus incubation time in the presence of NEM and ADP is indicated on fig. 5b. It has been controlled that no nucleotide escape from the sarcosomes occurred both at 2° and 20° in the presence of NEM. Such an escape, if proportional to the incubation period in the presence of NEM, would be an alternative explanation for the inhibition of the exchange reaction, without implying an alteration of translocase activity. In addition, control experiments also demonstrated that the percentage inhibition of translocase activity in the presence of ADP depends on the incubation period with NEM and does not increase any more, once the alkylating agent has been eliminated by adding β-mercaptoethanol.

On fig. 6, the kinetics of the various parameters studied are compared. Fig. 6a shows the alkylation kinetics by NEM of reactive groups unmasked by ADP and the inhibition kinetics by NEM of the atractyloside induced reversion of the optical jump, both at 20°. Fig. 6b shows the alkylation kinetics at 2° and the inhibition kinetics by NEM of the

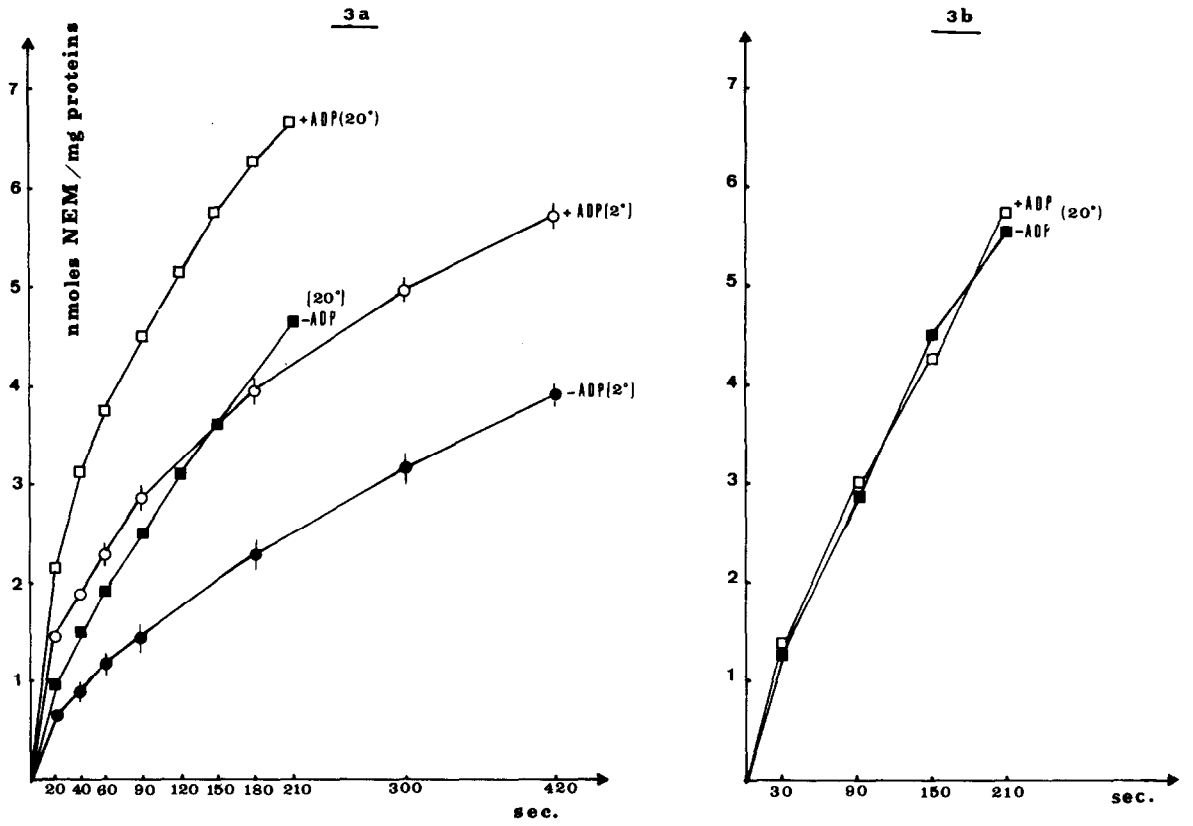

Fig. 3. Titration by ^{14}C-NEM of additional alkylable groups under the influence of ADP and inhibition of this titration by atractyloside. a) Experiments performed at 20°. Incubation of the sarcosomes started at 0 min; at 2 min "cold" NEM is added to a final conc. of 3.3 mM; at 4 min 0.5 μmoles ^{14}C-NEM (3.8×10^6 cpm) are added with or without ADP (200 nmoles). Final vol 3.5 ml. Proteins 1 mg/ml; pH 6.8. Experiments performed at 2°: at 3 min "cold" NEM is added to a final conc. of 3.3 mM; at 7 min 1.5 μmoles ^{14}C-NEM (6×10^6 cpm) are added with or without ADP (1200 nmoles). Final vol 5.5 ml. Proteins 1 mg/ml; pH 6.8. b) Experiments performed at 20° in the presence of atractyloside; same conditions as above. Atractyloside (5 μM, final conc.) is added at 3 min 50 sec.

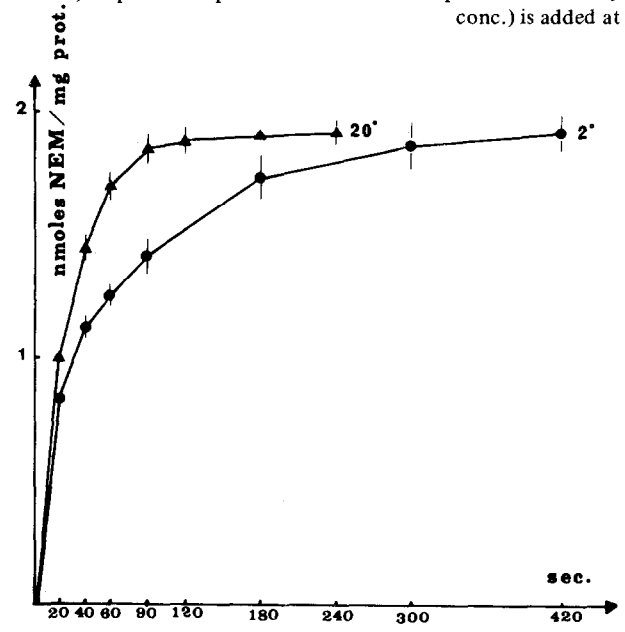

Fig. 4. Alkylation kinetics of the reactive groups "unmasked" by ADP. The kinetics shows the difference between the alkylation kinetics in the presence and in the absence of ADP as shown in fig. 3a. (▲—▲—▲): 20° (average of 4 exp.), (●—●—●): 2° (average of 3 exp.).

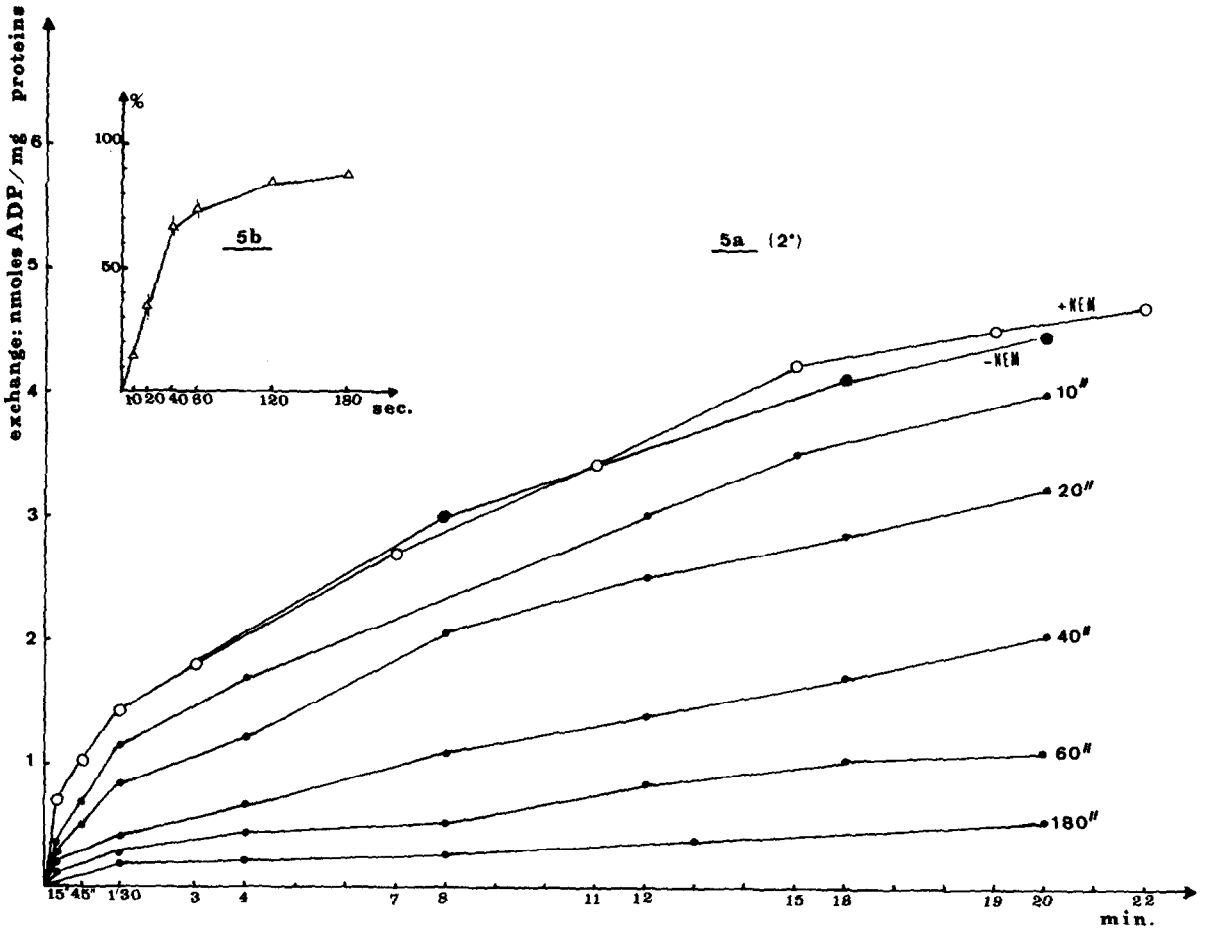

Fig. 5. Inhibition of adenine nucleotide exchange in sarcosomes by NEM preincubated in the presence of ADP. Exchange kinetics for various preincubations periods. a) All experiments performed at 2°, final vol 5.5 ml; proteins 1 mg/ml, pH 6.8. Incubation of sarcosomes *started at 0 min.* (•—•—•): Control without NEM; *additions: 6 min 45 sec: β-mercaptoethanol* 5.4 mM (final conc.); 7 min: ADP 220 μM (final conc.); 9 *min:* ^{14}C-ADP (600 nmoles, 58 × 10^4 cpm). (o—o—o): Control with NEM; additions: 3 min: NEM 3.3 mM (final conc.); subsequent additions as above. (•—•—•): Increasing pre*incubation* periods of NEM with ADP; additions: 3 min: NEM 3.3 mM; 7 *min*: ADP 220 μM; β-mercaptoethanol 5.4 mM is added 10, 20 and 40 sec., 1 min and 3 min after ADP. ^{14}C-ADP (600 nmoles, 58 × 10^4 cpm) added 30 sec after β-mercaptoethanol. b) Percentage inhibition of adenine nucleotide exchange (determined at 15 min incubation) as a function of the preincubation period with NEM and ADP.

ADP exchange reaction at this temperature. Inhibition of ADP exchange obtains at both temperatures but the rate of the exchange reaction at 20° is fast enough to prevent any precise estimation of the percentage inhibition at this temperature. Hence this parameter had to be measured at 2°.

4. Discussion

The main conclusions which may be drawn from the results obtained so far concerning ADP induced changes in the mitochondrial membrane are as follows:

1) the reactive groups unmasked by ADP seem to be rather specific with respect to the thiol groups mentioned by Sabadie-Pialoux and Gautheron [15] and by Foucher and Gaudemer [16] which are titrated by different reagents in the absence of oligomycin, and with respect to the -SH groups of the phosphate translocator which are not unmasked by ADP, and the titration of which ought to be completed within

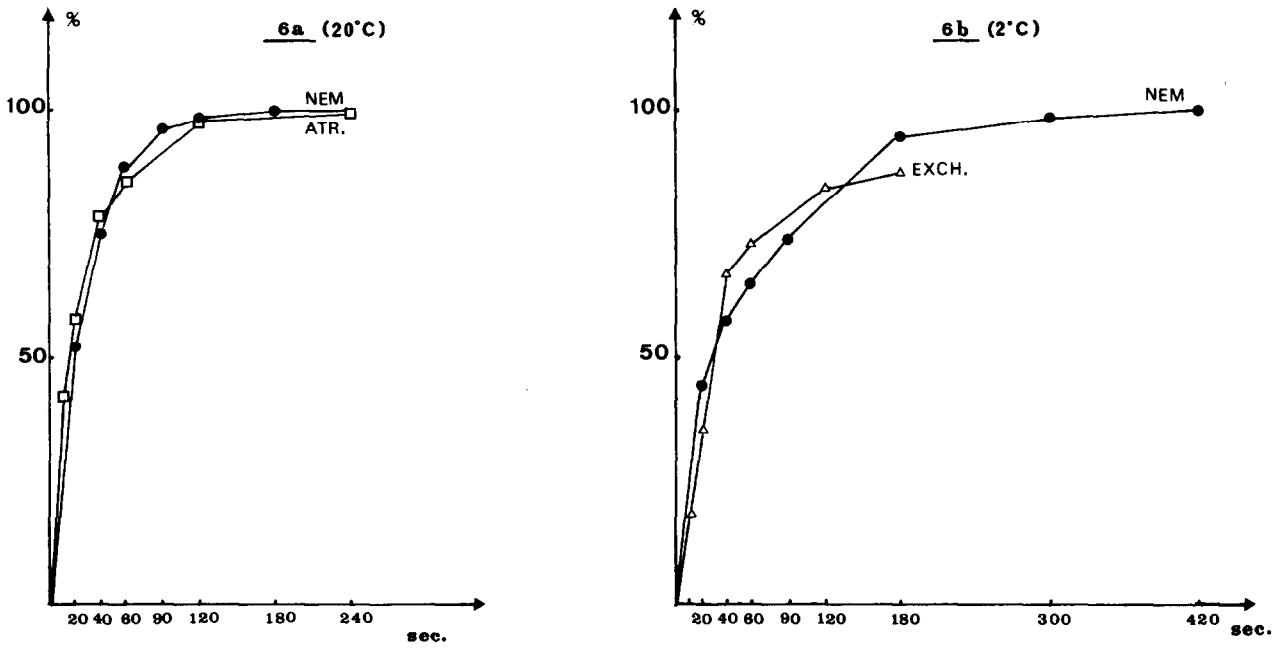

Fig. 6. Compared kinetics of ADP dependent alkylation by NEM, of ADP and NEM dependent inhibition of the atractyloside induced reversal of the "optical jump" and ADP and NEM dependent adenine nucleotide-translocase inhibition. a) Alkylation kinetics of reactive groups revealed by ADP (●—●—●) and inhibition kinetics of atractyloside induced reversion of the ADP dependent optical jump (□—□—□) in the presence of NEM; compared plots, expressed as percentages of the maximum effect. Experiments performed at 20°. b) Alkylation kinetics of reactive groups revealed by ADP (●—●—●) and inhibition kinetics of adenine nucleotide translocation (△—△—△) in the presence of NEM; compared plots, expressed as percentages of the maximum effect. Experiments performed at 2°.

the first 2 min of our preincubation period [17, 20] at 2°.

2) NEM inhibits the atractyloside dependent reversion (fig. 1c) of the ADP induced optical jump. The kinetics of this inhibition (fig. 6a) closely parallel the alkylation kinetics of the reactive groups unmasked by ADP. The parallelism extends to the atractyloside sensitivity of both parameters, suggesting their close correlation.

3) Fig. 5a shows that, as opposed to current views [9, 10], AN-translocase activity is sensitive to alkylating agents such as NEM, provided that the alkylation takes place in the presence of ADP. This inhibition stringently depends on the incubation time of the sarcosomal material in the presence of both NEM and ADP (fig. 5b), and its kinetics at 2° closely parallels alkylation kinetics, indicating that these parameters too may be correlated (fig. 6b).

4) It must be emphasized that the maximal amount of reactive groups unmasked by ADP (measured at 20° and at 2°) is about 1.3 nmoles/nmole of cytochrome *a* content(which we found to be 1.35 nmoles/mg of protein in fresh sarcosomes). This amount is stoichiometrically close to the amount of ADP and atractyloside and gummiferin binding sites in beef heart and rat liver mitochondria [21, 24]. According to Vignais et al. [24] adenine nucleotide translocase is an allosteric enzyme, ADP acting on this system both as a substrate and as an activator. Comparing these results with our own data it may be postulated that the groups titrated in the presence of ADP are closely linked to the translocase system and that the conformational change induced by ADP on this system is responsible for their unmasking. Hence all parameters studied in the present work may be related to a conformational change of AN-

translocase since all of them seem to be sensitive to the same activator (ADP), to the same specific inhibitor (atractyloside) [13, 14] and to the alkylating agent solely in the presence of the activator.
5) The nature of the reactive groups which are unmasked by ADP is presently unknown. Alkylation kinetics at the concentrations of NEM used point to the unmasking of additional -SH groups. However, preliminary experiments, which are not reported in the present work indicate that the groups alkylated by NEM exhibit comparatively low reactivities with other thiol reagents (mercurials, iodoacetamide); hence an unusually reactive -NH_2 group [25, 26] especially if located in the vicinity of an histidyl residue might also be alkylated. Additional experiments will be necessary to settle this point.
6) It also remains unexplained why endogenous adenine nucleotides, which are abundant in fresh sarcosomes, seem unable to induce the actions reported above and how the sole conformational change of the translocase system could provoke the overall effects observed macroscopically on the sarcosomal suspension by electron microscopy [3] and turbidimetry [4].

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References

[1] C.R. Hackenbrock, J. Cell. Biol. 30 (1966) 269.
[2] D.E. Green, J. Asai, R.A. Harris and J.T. Penniston, Arch. Biochem. Biophys. 125 (1968) 684.
[3] N.E. Weber and P.V. Blair, Biochem. Biophys. Res. Commun. 41 (1970) 821.
[4] C.D. Stoner and H.D. Sirak, Federation Proc. 29 (1970) 734.
[5] G. Zimmer, FEBS Letters 9 (1970) 274.
[6] P. LeBlanc, M. Bourdain and H. Clauser, Biochem. Biophys. Res. Commun. 40 (1970) 754.
[7] E. Pfaff, M. Klingenberg and H.W. Heldt, Biochim. Biophys. Acta 104 (1965) 312.
[8] P.V. Vignais and E.D. Duee, Bull. Soc. Chim. Biol. 48 (1966) 1169.
[9] H.H. Winkler, F.L. Bygrave and A.L. Lehninger, J. Biol. Chem. 243 (1968) 20.
[10] G. Brierley and R.L. O'Brien, J. Biol. Chem. 240 (1965) 4532.
[11] E.E. Jacobs, M. Jacobs, D.R. Sanadi and L.B. Bradley, J. Biol. Chem. 223 (1956) 147.
[12] D.E. Griffiths and D.C. Wharton, J. Biol. Chem. 236 (1961) 1850.
[13] P.V. Vignais, E.D. Duee, M. Colomb, A. Reboul, A. Cheruy, O. Barzu and P.M. Vignais, Bull. Soc. Chim. 52 (1969) 471.
[14] M. Klingenberg, FEBS Letters 6 (1970) 145.
[15] N. Sabadie-Pialoux and D. Gautheron, Biochem. Biophys. Acta 234 (1971) 9.
[16] B. Foucher and Y. Gaudemer, FEBS Letters 13 (1971) 95.
[17] A. Fonyo, Biochem. Biophys. Res. Commun. 32 (1968) 624.
[18] D.D. Tyler, Biochem. J. 111 (1969) 665.
[19] N. Haugaard, N.H. Lee, R. Kostrzewa, R.S. Horn and E.S. Haugaard, Biochem. Biophys. Acta 172 (1969) 198.
[20] B. Guérin, M. Guérin and M. Klingenberg, FEBS Letters 10 (1970) 265.
[21] M.J. Weidemann, M. Erdel and M. Klingenberg, European J. Biochem. 16 (1970) 313.
[22] P.V. Vignais, P.M. Vignais and M.G. Colomb, FEBS Letters 8 (1970) 328.
[23] M. Klingenberg, G. Falkner, H. Erdelt and K. Grebe, FEBS Letters 16 (1971) 296.
[24] P.V. Vignais, P.M. Vignais and G. Defaye, FEBS Letters 17 (1971) 281.
[25] D.G. Smyth, Atsuo Nagamatsu and J.S. Fruton, J. Amer. Chem. Soc. 82 (1960) 4600.
[26] D.G. Smyth, D.O. Blumenfeld and W. Konigsberg, Biochem. J. 91 (1964) 589.