NUCLEOPHILIC SITES IN ENERGIZED MITOCHONDRIAL MEMBRANES

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1. Introduction

pH indicators have been used in studies with mitochondria and submitochondrial particles with increasing frequency in recent years [1–3]. The absorbancy changes observed have been so far interpreted mostly as due to acid-base transitions [1–3]. The acid-base transition however has also been suggested to occur because of migration of the indicator between the inner and the outer mitochondrial aqueous phase [3]. The hypothesis that some of the responses of the indicator might reflect conformational changes of the membrane has not yet been confirmed [4].

Fluorescent probes have also been used to follow the metabolic transitions of mitochondrial membranes [5]. The fluorescence changes during energization have been interpreted as due either to conformational changes of the membrane [5,6] or to migration of the probes between phases of different hydrophobicity [7].

In the present paper we report our studies with another pH indicator, neutral red. It will be shown that at acidic pH the indicator responses to energization of submitochondrial particles cannot be interpreted as acid-base transitions but rather as due to an attractive interaction between dye molecules leading to phenomena of the stacking type.

Abbreviations:
EGTA: ethylene glycol bis(β-aminoethyl ether)-*N*, *N*-tetracetic acid;
FCCP: carbonyl cyanide-*p*-trifluoromethoxy phenylhydrazone;
HEPES: *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid.

2. Experimental

Rat liver mitochondria were prepared as described previously. Submitochondrial particles were kindly given to us by Dr. Azzi.

Absorbancy changes were measured in a duel wavelength spectrophotometer. Spectral studies were carried out on the Hitachi Perkin-Elmer split-beam spectrophotometer Mod. 124. With highly concentrated solutions of neutral red cuvettes with a shorter light path were used. Analysis of the binding of the dye to mitochondria and submitochondrial particles was carried out as follows. The membrane preparation was incubated in the presence of neutral red under the specified conditions and then centrifuged. The supernatant was brought to pH 4 with the addition of HCl and recentrifuged if necessary. The absorbancy of the supernatant was read at 530 nm and the concentration of neutral red calculated from a calibration curve.

Neutral red was a product of Merck. Agar was a product of Difco.

3. Results

3.1. *The binding of neutral red to submitochondrial particles*

Neutral red is bound to a large extent by submitocondrial particles. Addition of ATP plus Mg^{2+} to the particles resulted in a large increase in the amount of neutral red bound (table 1). The ATP induced binding was completely abolished by FCCP. A similar, although smaller, increase of binding was observed when the

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Table 1

Passive and active binding of neutral red to submitochondrial particles.

Additions	pH	Dye bound	
		With FCCP	Without FCCP
		(μmoles/g protein)	
ATP + Mg	6.5	41.0	69.5
ATP + Mg	7.5	40.0	76.0
Succinate + oligomycin	6.8	24.0	33.3
Succinate + oligomycin	7.5	24.0	35.9

The medium contained 0.1 M KCl and 10 mM HEPES at the pH indicated. 50 μM neutral red and 0.375 mg protein/ml in the presence of ATP, 20 μM neutral red and 0.64 mg protein/ml in the presence of succinate. 2 mM ATP, 2.5 mM $MgCl_2$, 1 mM succinate, 2 μg oligomycin and 10^{-6} M FCCP.

energization was carried out through the addition of succinate plus oligomycin. The binding of neutral red due to addition of ATP was not affected by changes of the pH of the medium in the range between 6.5 and 8.0.

The large binding of neutral red to the deenergized submitochondrial particles was not accompanied by variations of the extinction coefficient or of the absorption spectra. This was obtained by following the interaction of neutral red with the particles in the dual wavelength and in the split-beam spectrophotometers at variable dye and particles concentrations.

3.2. *Absorbancy changes due to energization*

Addition of ATP plus Mg^2 to submitochondrial particles incubated in a KCl medium at pH 7.8 induced a slight increase of absorbancy at 530 nm which is the maximum of extinction of the cationic form of the dye (fig. 1). On the other hand when ATP was added to particles incubated at pH 6.5 a very large decrease of absorbancy at 530 nm was observed (note the difference in the scale on the ordinate between the two experiments). Fig. 1 also shows that the dye did not show acid-base transitions when pulses of HCl and of NaOH were added at the end of the phase of decreased absorbancy at pH 6.5. However after FCCP had restored the initial absorbancy the pulse of HCl did cause a base-acid transition. At pH 7.9 there was a similar response of the dye to acid-base transitions before and after addition of FCCP.

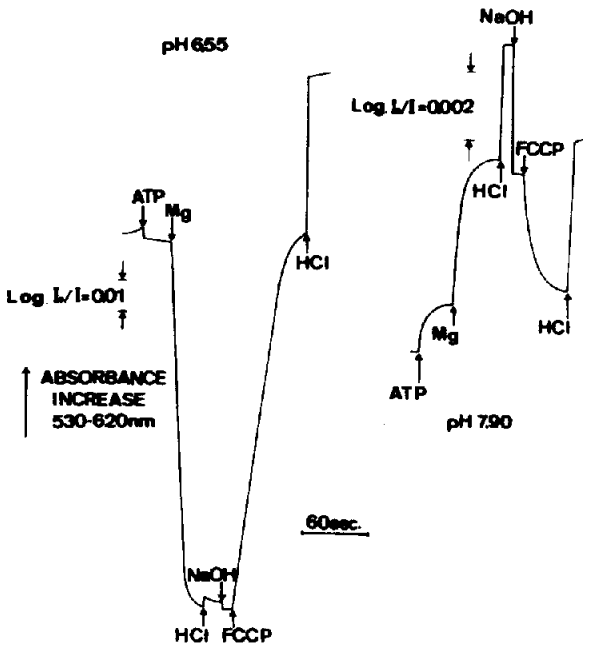
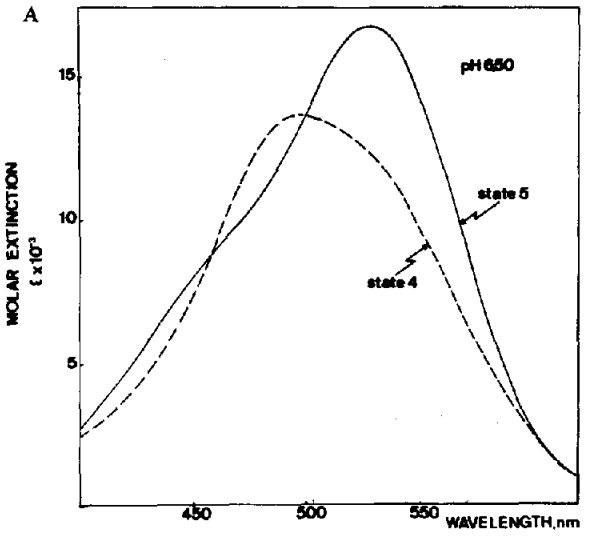


Fig. 1. Absorbancy changes of neutral red during energization. The medium contained in 2 ml: 0.82 mg protein/ml of submitochondrial particles, HEPES-Cl 25 mM, KCl 0.1 M, neutral red 25 μM, 1 mM HCl or NaOH, 2.5 mM $MgCl_2$; 100 μM ATP.

A possible interpretation of the change of absorbancy of fig. 1 is that it corresponds to an acid-base transition. However this is not supported by the analysis of the spectra. Fig. 2 shows that the decrease of absorbancy observed at pH 6.5 is accompanied by a spectral shift of the dye. The new form has a maximum at about 490 nm. The spectral shift did not occur at pH 7.8, where a small increase of absorbancy at 530 nm was observed. When the spectral shift was followed at increasing concentrations of particles, three isosbestic points were observed at 610, 500 and 457 nm respectively. The spectral changes shown in fig. 2 also eliminate the possibility that the decreased absorbancy of the dye of fig. 1 might be due to a redox change [8]. This however was very unlikely because of lack of reducing power in these particles.



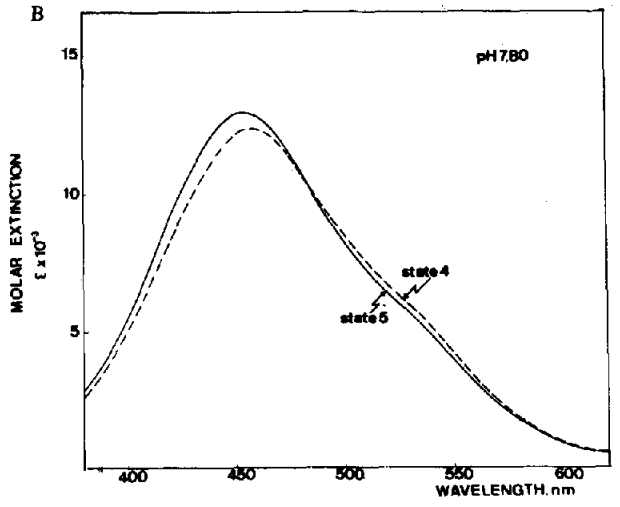


Fig. 2 A and B. Absorption spectra of neutral red in "State 5" and "State 4" at pH 6.5 and 7.8. The medium contained in 2.5 ml: 0.35 mg protein/ml of submitochondrial particles, 10 mM HEPES-Cl, neutral red 25 μM, KCl 0.1 M, $MgCl_2$ 2.5 mM, and in "State 4", 140 μM ATP.

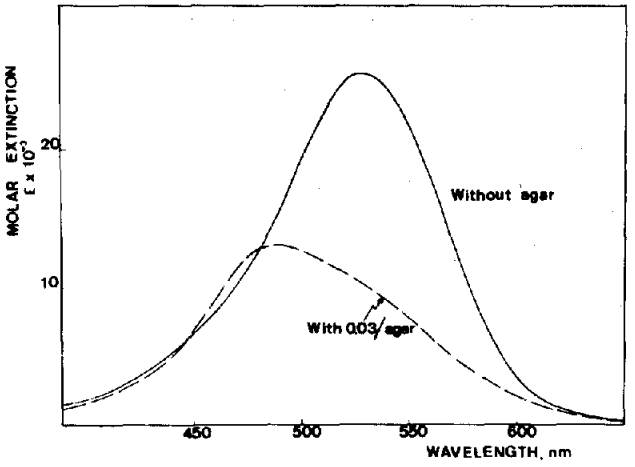


Fig. 3. Absorption spectra of neutral red in the absence and presence of agar. The medium contained in 2.5 ml: sodium acetate 10 mM, pH 4.3, neutral red 25 μM, agar 0.03%.

In order to interpret the spectral shift, the metachromatic behaviour of neutral red was investigated. In confirmation of the data of Bartels [9], decrease of the extinction coefficient and shift of the maximum to shorter wavelengths were observed by measuring the spectra of the cationic, neutral red at increasing concentrations of the dye. Increasing the salt concentration also caused a similar phenomenon. Spetral shift were not observed by increasing the dye concentration at alkaline pH.

Spectral shifts were also obtained during interaction of the dye with various chromotropic substrates [10]. Addition of increasing amounts of ribonucleic acid to neutral red in high concentrations resulted in an inverse metachromatic effect, i.e. shift of the maximum to longer wavelength. On the other hand addition of agar to 25 μM neutral red at pH 4.0 gave a spectral shift similar to that observed during energization of the submitochondrial particles (fig. 3). When the spectral shift of fig. 3 was obtained by sequential addition of increasing amount of agar three isosbestic points were obtained at 620, 480 and 450 nm. Addition of agar to neutral red at pH 6.5 also resulted in a spectral shift, whereas no shift was observed at pH 7.5.

4. Discussion

The shift of the maximum of neutral red to lower wavelength by increasing the dye concentration in solution has been attributed by Bartels [9] to a monomer-dimer transition. Above a certain dye concentration the formation of higher aggregates takes place. In the case of other cationic dyes [11–13] the forma-

tion of dimers or of higher aggregates occurs both in solution and after addition of polyelectrolites such as polystyrene sulphonic acid or polymetacrilic acid. The function of the polyelectrolite would be that of binding electrostatically the positive charged form of the dye thereby facilitating the stacking phenomena of the dye molecules by London-Van der Waals forces.

The capacity of agar to cause a metachromatic change of neutral red similar to that observed with the energized submitochondrial particles suggests that the structures of the two systems are somewhat related [10]. Since agar is a sulphuric ester of a high polymeric carbohydrate, the parallelism suggests the presence of strongly electronegative sites in the energized membrane. It is important to note that although neutral red is bound to a large extent by the deenergized particles no spectral shifts are observed. It would therefore appear that the formation of highly nucleophilic sites responsible for the shift is a consequence of the structural changes of the membrane occurring during energization. It should be noted that the proton pump model for ion transport does indeed imply the formation of highly nucleophilic sites during energization [14].

In order to identify the sites responsible for the binding of neutral red to the energized and deenergized membranes a quantitative analysis of the binding parameters has also been carried out both with mitochondria and submitochondrial particles (Colonna et al., in preparation). From these data it appears that energization results in an increase not of the number of the binding sites but only of the affinity of the membrane for neutral red. For a fraction of the sites, the increase of affinity is about two orders of magnitude in respect to the deenergized membrane. Furthermore it appears that only the high affinity sites are involved in the mechanism of the spectral shift.

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