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# 10-N NONYL-ACRIDINE ORANGE: A FLUORESCENT PROBE WHICH STAINS MITOCHONDRIA INDEPENDENTLY OF THEIR ENERGETIC STATE

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The specificity of binding of 10-N Nonyl Acridine Orange to mitochondria, and more precisely to inner membranes, is demonstrated by subcellular fractionation of hepatocytes. Unlike Rhodamine 123, which is a preferential marker of the transmembrane potential, Nonyl Acridine Orange binding is essentially independent of the mitochondria energization state although a low uptake of this dye, in response to the potential, may be measured. So 10-N Nonyl acridine orange is an appropriate marker of the mitochondrial membrane surface per unit of cell mass. 

Mitochondria biogenesis involves two distinct processes: formation of mitochondrial membranes and the compartments they define, and differentiation of the organelles for oxidative phosphorylation. Because mitochondria generated a high membrane potential ($\Delta\Psi$) (1), their energization state is easily measurable today by methods based on the use of fluorescent lipophilic cations (2-6). However, these probes, which are mainly distributed electrophoretically in the mitochondrial matrix in response to $\Delta\Psi$ (7-10), can only be used to study potential changes and it is nearly always impossible to objectively determine changes in the total mitochondrial surface per unit of cell mass during different biological processes (11, 12). A such evaluation of mitochondrial membranes growth would probably indicate the changing metabolic needs of cells in differentiating cell systems and in other non-steady-state situations.

New derivatives synthetized from acridine orange by grafting an aliphatic chain of variable length to the quaternary ammonia (13, 14) have different binding properties compared to acridine orange, known to intercalate between bases of double-stranded nucleic acids (15). Hence Septinus et al. (16) and Ratinaud et al. (17), using fluorescence microscopy, have observed that nonyl-acridine orange (NAO) would preferentially incorporated in the mitochondria of Hela cells and mouse splenocytes.

Abbreviations: CCCP, Carbonyl Cyanide m-chlorophenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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In this study, direct proofs are given on the specific incorporation of NAO in inner mitochondrial membranes by a mechanism which didn't involve potential. Accordingly, NAO could be used as a possible marker of the mitochondrial membrane surface per unit of cell mass even when the cells are fixed.

## Materials and Methods

*Subcellular fractionation.* After liver homogenization, aliquots of homogenate (10 ml at about 100 mg/ml of protein) were incubated at 4°C for 20 min with acridine orange or one of its derivatives (Fig. 1). Nuclear fraction, mitochondrial fraction, microsomal fraction and cell sap were separated by differential centrifugations in 0.25 M sucrose, 2 mM Tris-HCl buffer (pH: 7,4) according to Gonzalez-Cadavid and Campbell (18).

*Preparation of mitochondria.* Rat-liver mitochondria were isolated in 0.25 M sucrose, 2 mM Tris-HCl buffer (pH: 7,4) (19). Protein was determined by a biuret procedure using crystalline bovine serum albumin as standard (20).

*Mitochondrial fractionation.* Purified mitochondria were incubated for 15 min with different concentrations of NAO then washed twice in 0.25 M sucrose, 2 mM Tris-HCl (pH 7.4). Outer membranes, inner membranes and matrix were separated by osmotic pressure technique (21).

*Preparation of hepatocytes.* Isolated rat-liver cells were preparated by enzymatic perfusion (22). Incubations were carried out at 20°C in a standard Krebs-Ringer bicarbonate buffer, containing 120 mM NaCl, 24 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 2 mM $CaCl_2$, 2 mM KCl, 2 mM $KH_2PO_4$,1.5% bovine serum albumin and 10 mM Hepes (pH: 7.4). High $K^+$ medium contained 120 mM KCl, 24 mM $KHCO_3$ instead of the corresponding sodium salts and 2 mM NaCl. Cell fixation was made in cold ethanol (3:7 v/v) for at least 30 min, cell suspension was centrifuged and rinsed twice.

*Spectrofluorimetric and cytofluorimetric analysis.* Fluorescence measurements were made with a Jobin & Yvon JY3 spectrofluorimeter. Cytofluorimetric analysis was performed using a ORTHO 50H (Ortho Diagnostic Instruments), equipped with helium-neon (Spectra-Physics) and argon (Innova 90-4, Coherent) lasers. Green fluorescence, emitted by rhodamine 123 or NAO, was collected between 500 and 540 nm. Data were analysed with a computer (MCA 3000, ODAM) connected to the cytometer.

*Cytochrome c oxidase activity.* Cytochrome c oxidase activity was measured spectrophotometrically by following ferrocytochrome c oxidation at 550 nm (23). The oxydation rate was calculated using a molar absorption coefficient of 19600 and expressed as µmoles of cytochrome c oxidized/min/mg of protein.

*Chemicals.* Pentyl-acridine orange, octyl-acridine orange and nonyl-acridine orange were a gift of H. W. Zimmerman ( Institüt für Physikalische Chemie der Universität FREIBURG FRG). Acridine orange was obtained from Polysciences, dodecyl-acridine orange from Molecular Probes and Rhodamine 123 (laser grade) from Eastman Kodak Co. Oligomycin, valinomycin and CCCP were purchased from Boehringer and nigericin from Sigma. All other reagents were of the highest purity commercially available.

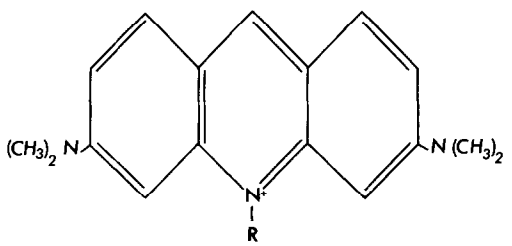


Fig. 1. Chemical structure of acridine orange [3,6-bis (dimethyl-amino) acridine] (R = H) and its N-alkylated derivatives ( R = $C_nH_{2n+1}$).

## Results

*Distribution of acridine orange and its derivatives in the subcellular fractions*

Whereas acridine orange is intercalated into nucleic acids of nuclei and microsomes (table I), its N-alkyl derivatives were mainly incorporated by mitochondria (table II). The derivative with 9 carbones (NAO) presented the highest specificity to mitochondria as there was near 65 % of the dye added to the initial homogenate in these organelles (table I). This result is supported by the fluorescence quantum yield of NAO, either free or bound, which is close to 0,990. Taking into account this fact and the distribution of cytochrome c oxidase (enzymatic marker of mitochondria) present in nuclei and microsomes fractions (result of contamination), the NAO incorporation in mitochondria would be more than 80 %.

NAO-stained mitochondria didn't release the dye even when they are washed twice and their fractionation showed a preferential incorporation in inner membranes (table III).

*NAO uptake by energized and de-energized isolated mitochondria*

Comparatively to the control probe Rhodamine 123 (Rh 123), which is closely incorporated by mitochondria according to energization state (the ratio between the dye uptake in the de-energized and energized states varied between 0.2 and 0.4), NAO uptake (Fig. 2) was found to be mainly independent of energization (ratio between 0.85 and 0.95).

In the presence of valinomycin ($K^+$ ionophore) or CCCP (protonophore) which both collapse the transmembrane potential, the uptake (Table IV) decreased by only 12 % for NAO and 90 % for the potential probe Rh 123. With nigericin, which collapses the $\Delta pH$ and hyperpolarizes the membrane, there is a slight increase in the uptake of the two probes. Hence, only the $\Delta\Psi$ component is involved in the uptake differences measured in presence of ionophores or uncoupler. The slight NAO incorporation increase observed with energized mitochondria may be due to the positive charge of the dye attracted by the negative potential inside mitochondria.

Table I. ANALYSIS OF DYES UPTAKE BY SUBCELLULAR FRACTIONS

| Fractions | | Nuclear | Mitochondrial | Microsomal | Cell sap |
|---|---|---|---|---|---|
| Cyt. c oxidase activity (%) | | 18.2 | 74.0 | 7.8 | 0 |
| 0.1 μM | AO | 16.4 | 38.8 | 36.9 | 7.9 |
| | NAO | 7.3 | 56.1 | 36.6 | 0 |
| 1 μM | AO | 14.2 | 20.9 | 49.5 | 15.4 |
| | NAO | 9.9 | **64.5** | 25.6 | 0 |
| 10 μM | AO | **19.5** | 8.3 | **61.2** | 11.0 |
| | NAO | 9.8 | 61.8 | 24.5 | 3.9 |

In all the subcellular fractions fluorescence quantum yield is 0.990±0.009 for NAO and 0.991±0.006 for AO.
The results are expressed for the individual fractions, as percentage distribution of the total AO or NAO fluorescences and cytochrome c oxidase activity recovered in all fractions.

Table II. ACRIDINE ORANGE DERIVATIVES UPTAKE BY RAT-LIVER MITOCHONDRIAL FRACTION

| Dye (μM) | % of fluorescence in mitochondrial fraction | | | | |
|---|---|---|---|---|---|
| | AO | PAO | OAO | NAO | DAO |
| 0.1 | 38.8 | 43.6 | 40.3 | **56.1** | 35.0 |
| 0.5 | 25.0 | 41.9 | 49.4 | **62.2** | 36.2 |
| 1.0 | 21.0 | 49.5 | 56.0 | **64.5** | 40.3 |

Fluorescence quantum yield of all dyes is 0.990±0.010.
The results are expressed as in Table I. Acidine Orange (AO) derivatives are: Pentyl (PAO), Octyl (OAO), Nonyl (NAO) and Dodecyl (DAO) Acridine Orange.

*Incorporation of NAO in isolated hepatocytes*

The contribution of plasma membrane potential was evaluated by comparing dyes uptakes by cells incubated in either low and high $K^+$ media. The replacement of low $K^+$ medium by high $K^+$ medium, to depolarise the plasma membrane, which leads to a slight decrease in Rh 123 incorporation, didn't change NAO uptake (table V). Modification of the mitochondrial transmembrane potential by the use of the ionophores valinomycin, CCCP and nigericin leads to an expected changements of the Rh 123 incorporation while no significant change in NAO uptake was observed. Moreover it appears that fixed cells incorporated the dye as much as unfixed cells. This result confirms that NAO is preferentially a mitochondrial surface marker.

## Discussion

Fluorescent lipophilic cations (cyanins, safranin, rhodamines etc...) are used today for the in situ study of mitochondrial respiratory activity (24, 25). They are essentially incorporated in response to the transmembrane potential (9, 26) and cannot provide any data about the quantitative information concerning the mitochondrial mass. Our comparative study of acridine orange derivatives binding in hepatocyte subcellular fractions proves that nonyl- acridine orange is the more specific for mitochondria and confirms the microscopic observations of Septinus et al (16) and Ratinaud et al (17). Moreover, the dye accumulation was shown to be particularly specific for inner mitochondrial membranes.

Table III. NAO DISTRIBUTION IN SUBMITOCHONDRIAL FRACTIONS

| Fractions | Cyt. c Oxidase (%) | NAO concentration | | |
|---|---|---|---|---|
| | | 0.1 μM | 1 μM | 10 μM |
| | | % of fluorescence collected in different fractions | | |
| Matrix | 0.3 | 2.8 | 0.6 | 2.9 |
| Inner membrane | 91.8 | 78.4 | 86.4 | 96.2 |
| Outer membrane | 7.9 | 18.8 | 13.0 | 0.9 |

Fluorescence quantum yield of NAO bound to each fraction is 0.991±0.001.
The results are expressed for the individual fractions as percentage distribution of the total recovered in all fractions as in Table I.

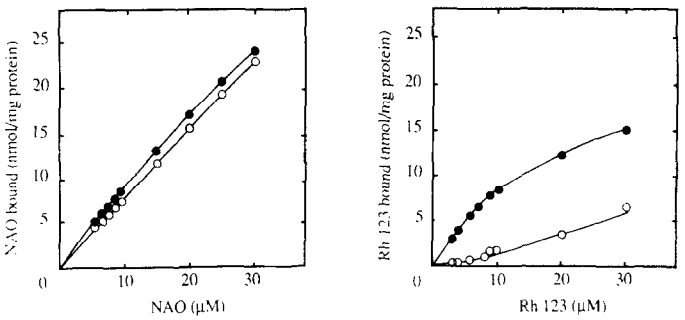


Fig. 2. Uptake of NAO and Rh 123 by rat-liver mitochondria.
Mitochondria (1mg/ml of protein) were incubated at 20°C for 1.5 min with 5 to 30 μM NAO or Rh 123 in basic reaction medium containing 150 mM sucrose/ 5 mM $MgCl_2$/ 10 mM disodium succinate/ 2.5 μM rotenone/ 5 mM KPi buffer/ 20 mM potassium-Hepes buffer (pH 7.4). After centrifugation for 2 min at 10 000 x g, free dye in the supernatant was determined by absorbance at 496 nm for NAO and at 500 nm for Rh 123. Bound dye was taken as total minus free. Full symbols are with no other additions (energizeed). Open symbols are with addition of 1 mM $NaN_3$, 2 μM CCCP and 2 μg/ml oligomycin (de-energized).

At the energized state, the amount of NAO uptake by mitochondria was slightly greater than at the de-energized state. Therefore energized state involves two types of binding, the first, which is responsable for about 85 % of the uptake, may be due to the hydrophobic interactions of the molecule with mitochondrial constituents of the inner membrane and explains the specificity of the probe to the organelle; the second leads to an additional uptake of only 15 % and is dependent on the mitochondrial energization state and more specifically on $\Delta\Psi$. The $\Delta\Psi$-dependent uptake corresponds to an electrophoretic movement of a small quantity of the fluorochrome across the internal membrane due to its quaternary ammonia positive charge.

NAO uptake by hepatocytes, evaluated by flow cytometry, still almost unchanged even when potential changed or when cells were fixed. This observation agree with the results obtained with isolated mitochondria.

All these results indicate that NAO can be considered as an excellent mitochondrial mass marker. It will be useful for several studies such as the analysis of mitochondrial surface changes in situ during the cell cycle, during cellular ageing and when studying drugs whose act on mitochondria biogenesis.

Table IV. NAO AND RHODAMINE 123 UPTAKE BY ENERGIZED AND DE-ENERGIZED MITOCHONDRIA

| Additions | Dye bound (nmol/mg protein) | |
|---|---|---|
| | NAO | Rh123 |
| None | 4.90 ± 0.03 | 4.45 ± 0.10 |
| CCCP | 4.30 ± 0.11 | 0.35 ± 0.01 |
| Valinomycin | 4.30 ± 0.05 | 0.50 ± 0.01 |
| Nigericin | 4.95 ± 0.04 | 4.60 ± 0.08 |

Dyes (5 μM) were incubated for 1.5 min with isolated mitochondria (1 mg/ml of protein) at 20°C and the amount of NAO or Rh 123 bound was determined as in Fig. 2. Additions of uncoupler or ionophores were 2 μM CCCP, 70 ng/ml nigericin and 20 ng/ml valinomycin.

Table V. NAO AND RHODAMINE 123 UPTAKE BY HEPATOCYTES TREATED WITH IONOPHORES

| Conditions | Relative Fluorescence Intensity | |
|---|---|---|
| | NAO | Rh 123 |
| Control low $K^+$ | 102.5 ± 2.4 | 101.0 ± 2.8 |
| Control high $K^+$ | 100.8 ± 3.0 | 91.0 ± 2.7 |
| + Valinomycin | 108.3 ± 4.8 | 44.7 ± 3.9 |
| + CCCP | 101.0 ± 5.2 | 42.7 ± 2.6 |
| + Nigericin | 103.6 ± 4.4 | 122.5 ± 8.5 |
| Fixed cells | 91.2 ± 5.1 | 21.1 ± 3.6 |

Isolated hepatocytes ($10^6$/ml) were preincubated for 15 min at 20°C. in low $K^+$ medium alone or in high $K^+$ medium containing either 10 µM CCCP or 10 µg/ml valinomycin or 10 µM nigericin. Incubations were continued for 15 min after addition of 5 µM of NAO or Rhodamine 123 and the relative green fluorescences were collected with a flow cytometer.

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