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# Structural and Functional Aspects of the Respiratory Chain of Synaptic and Nonsynaptic Mitochondria Derived from Selected Brain Regions

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## Abstract

Studies on brain mitochondria are complicated by the regional, cellular, and subcellular heterogeneity of the central nervous system. This study was performed using synaptic and nonsynaptic mitochondria obtained from cortex, hippocampus, and striatum of male Sprague–Dawley rats (3 months old). Ubiquinone content, detected by HPLC analysis, was about 1.5 nmol/mg protein with an approximate $CoQ_9/CoQ_{10}$ molecular ratio of 2:1. The activities of several respiratory chain complexes were also studied (succinate–cyt. *c* reductase, NADH–cyt. *c* reductase, succinate–DCIP, ubiquinol$_2$–cyt. *c* reductase, and cytochrome oxidase), and generally found to be higher in mitochondria from cortex than from other regions. Study of the activities of some of these enzymes vs. $1/T$ (Arrhenius plots) showed a straight line with an activation energy between 7 and 10 kcal/mol in all the three areas considered. Only $CoQ_2H_2$–cyt. *c* reductase activity revealed a biphasic temperature dependence. Also anisotropy (as fluorescence polarization) of the hydrophobic probe DPH showed a deviation from linearity; the break points for both enzymatic activity and anisotropy were found at about 23–24°C.

**Key Words:** Rat brain mitochondria; ubiquinone levels; Arrhenius plots; fluorescence anisotropy; brain heterogeneity.

## Introduction

Evidence for the heterogeneity of cerebral mitochondria has been increasing in many morphological, histochemical, and biochemical studies (Hansford,

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1983); as regards cerebral tissue, in noting the uneven distribution of certain mitochondrial enzymes along a sucrose gradient, Van den Berg and coworkers (Reijnierse *et al.*, 1975) emphasized the existence of many mitochondrial subpopulations.

In several studies, the relative activity of some mitochondria varied depending on the extent to which these mitochondrial preparations were enriched with synaptosomes (Salganicoff and Koeppe, 1968; Dienel *et al.*, 1977). Moreover, the centrifugation techniques used caused the sedimentation of highly heterogeneous fractions (Clark and Nicklas, 1970). These problems can and ought to be circumvented using preparations of intrasynaptic mitochondria free of synaptosomes.

In 1977, a method was developed whereby three distinct populations of relatively pure mitochondria, heterogeneous with respect to their biochemical characteristics, were prepared from rat forebrains (Lai *et al.*, 1977): two mitochondrial populations were derived from synaptosomes and a third consisted of "free" nonsynaptic mitochondria.

Some biochemical studies have searched for enzymes distinguishing between two major (or perhaps three) populations of mitochondria, perikaryal and intrasynaptic, either *in situ* (Hajos and Kerpel-Fronius, 1969) or *in vivo* (Hajos and Kerpel-Fronius, 1971; Deshmukh *et al.*, 1980), or in isolated subcellular fractions *in vitro* (Salganicoff and Koeppe, 1968; Wilson, 1972).

However, the brain is made up of heterogeneous tissue variously distributed in different zones with distinct metabolic activities responsible for diverse functions.

Some discrepancies discussed in the literature (Hansford, 1983) on the distribution of several enzymes in these fractions are probably related to the above differences. For example, in intrasynaptic mitochondria remarkable differences arise for the ubiquinone content and activities of related enzymes (Battino *et al.*, 1988, 1990); the specific activities of superoxide dismutase and cytochrome oxidase in "light" and "heavy" intrasynaptic mitochondria from rat brain cerebral cortex at different ages from 4 to 24 months (4-month intervals) varied in a very different way (Vanella *et al.*, 1988, 1989), and the different enzyme activities evolved independently within only 2 weeks (Benzi, 1979; Benzi *et al.*, 1980) or in 8-week intervals (Villa *et al.*, 1989b).

In this context, the aim of this study was to obtain further information on the properties of the brain respiratory chain by evaluating the structural and functional features of both synaptic and nonsynaptic mitochondria obtained from three specific areas of rat brain, i.e., cerebral cortex, hippocampus, and striatum. In this case, these areas possess specific attributes (memory, learning, motor control, integrative processes) and are of considerable functional, pharmacological, and neurochemical importance.

The structural studies concern the detection of ubiquinone and respiratory cytochrome levels in the inner mitochondrial membrane, whereas the functional aspects involve studies on both the specific activities of individual respiratory complexes and the integrated activities of part of the respiratory chain. In view of the reported peculiar characteristics of brain mitochondria and of current ideas concerning the role of lipid alterations in several pathological processes and in aging (Lenaz, 1988), we have also investigated the temperature dependence and the effects of the lipid environment on the enzymatic activities of the same mitochondria.

## Materials and Methods

### *Chemicals*

Different ubiquinone homologs were a kind gift from Eisai Co., Tokyo; they were stored as solutions in absolute ethanol at −20°C at concentrations ranging between 1 and 10 mM as determined spectrophotometrically at 275 nm using extinction coefficients typical of each homolog (Degli Esposti *et al.*, 1981; Lenaz and Degli Esposti, 1995) and according to Mayer and Isler (1971). Ubiquinone-2 was reduced using the method of Rieske (1967), and kept at −20°C in absolute ethanol under slight acidic conditions at concentrations ranging between 1 and 5 mM. Ubiquinol-2 solutions were stable for at least a week under the conditions used.

All chemicals used were purchased from Sigma Chemical Co. Ltd., St. Louis, Missouri 63178, USA.

### *Solvents*

All solvents were pure reagents of Merck and Carlo Erba.

### *Animals*

The experiments were performed on male Sprague-Dawley rats (Cobs-Charles River) aged 14 weeks. The animals were selected according to randomized experimental procedures and kept from birth under standard cycling and caging conditions (temperature 22 ± 1°C, relative humidity 60 ± 5%, lighting cycle 12 h light and 12 h darkness, low noise disturbances), fed with a standard certified pellet diet (Charles River) and with water *ad libitum*, and housed two per cage. The animal used for each experiment was sacrified at 9:00 a.m. to avoid any circadian changes in enzyme activity.

### *Preparation of Free and Intrasynaptic Mitochondria*

The different types of mitochondria were prepared from cerebral cortex (right side), hippocampus, and striatum of a single animal according to the

original method of Lai *et al.*, (1977) as subsequently adapted for selected areas by Leong *et al.* (1984) and previously described in detail (Villa *et al.*, 1989a,b). The male rats, aged 14 weeks, were killed by decapitation and the subsequent procedure was performed at 0–4°C. Brain areas were isolated ($\leqslant 20$ sec) in a refrigerated box at 0–4°C and immediately placed in an isolation medium (0.32 M sucrose, 1.0 mM $K^+$-EDTA, 10 mM Tris-HCl; pH 7.4). Briefly, the homogenate in the isolation medium was obtained by a Teflon-glass homogenizer (Braun S homogenizer) and centrifuged three times at $3.6 \times 10^3 g \cdot$ min in a Beckman J2-21 supercentrifuge, rotor JA-17; supernatants were combined and centrifuged at $288 \times 10^3 g \cdot$ min to obtain the "crude" mitochondrial pellet containing synaptosomes. Isolation of free mitochondria as such from synaptosomes was performed on a discontinuous Ficoll–sucrose gradient consisting of 12% w/w Ficoll and 7.5% Ficoll soluted in a basic solution; the gradient was centrifuged at $175.2 \times 10^4 g \cdot$ min in an OTD65B Sorvall ultracentrifuge equipped with an AH-650 type rotor; manual soft control of acceleration was set at position number 4. The myelin fraction was sucked off, the synaptosomal band at the 7.5–12% (w/w) Ficoll interphase was collected by aspiration, and the purified mitochondrial pellet was resuspended in a small volume of 0.32 M sucrose, 25 mM K-phosphate, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) 0.5 mM), and phenylmethylsulfonyl fluoride (PMSF) (1 mM), pH 7.6. The latter two chemicals were used as proteolysis inhibitors. The isolated synaptosomal fraction was pelleted and lysed by resuspension in 5 ml of 6 mM Tris-HCl, pH 8.1. After resuspension the pellet obtained from lysed synaptosomes was layered on a discontinuous Ficoll gradient consisting of two layers of 4.5% (w/w) and of 6% (w/w) Ficoll in a basic solution. This gradient was centrifuged at $280.2 \times 10^3 g \cdot$ min; at the end of centrifugation, the upper phase or "light mitochondrial fraction" was clearly separated from the pellet, i.e., the "heavy mitochondrial fraction." After isolation, the washed pellets were finally resuspended in a small volume of 0.32 M sucrose, 25 mM K-phospate, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) (0.5 mM), and phenylmethylsulfonyl fluoride (PMSF) (1 mM), pH 7.6.

Mitochondria immediately frozen in liquid nitrogen were stored at $-80$°C for 2–3 days. The suspension was frozen and thawed at least 3 times before enzymatic assays to allow complete accessibility of substrates to the enzymes of the inner membrane.

### *Analytical Determinations*

Protein concentration was determined according to Lowry *et al.* (1951).

The mitochondria were assayed for ubiquinone content by reversed-phase HPLC analysis (Takada *et al.*, 1982; Tsai *et al.*, 1985) after extraction

with methanol and light petroleum using the method of Kröger (1978). Separations were performed using a Spherisorb S5 ODS I 25 × 0.46 cm column with a guard column containing the same material as the main column; the mobile phase was prepared by dissolving 7.0 g $NaClO_4 \cdot H_2O$ in 1000 ml ethanol/$H_2O$/70% $HClO_4$ (969 : 30 : 1). The flow rate was 1 ml/min. Measurements were performed at 25°C. The HPLC system was a Waters Data Module M730-Model 721 programmable system controller equipped with a Lambda-Max Model 481 LC spectrophotometer. The cytochrome content was evaluated by the differential spectra (dithionite reduced minus ferricyanide oxidized in the presence of 1% deoxycholate) in a Jasco (Uvidec-610) double-beam spectrophotometer according to Vanneste (1966) and Nicholls (1976).

### *Enzyme Assays*

The mitochondria were assayed for respiratory chain activities at 25°C in a 25 mM K-phospate buffer, pH 7.5, by standard spectrophotometric methods under quasi-saturating substrate concentrations. NADH–cytochrome *c* reductase, succinate–cytochrome *c* reductase, and ubiquinol-2–cytochrome *c* reductase activities were measured, in the above buffer with the addition of 1 mM KCN, by monitoring the absorbance increase of cytochrome *c* (Sigma horse heart type III) upon reduction at 550–540 nm in a Sigma ZWS dual-wavelength spectrophotometer equipped with a rapid-mixing apparatus designed in this laboratory (mixing time 200 ms) using an extinction coefficient of 19.1 $mM^{-1} \cdot cm^{-1}$ for cytochrome *c* (Degli Esposti and Lenaz, 1982a,b). In the determination of ubiquinol-2–cytochrome *c* reductase activity the final ethanol concentration never exceeded 0.2% and the $CoQ_2H_2$ concentration never exceeded 15 $\mu$M according to Battino *et al.* (1986a). NADH–cytochrome *c* reductase activity was measured only with some mitochondrial preparations lacking in protease inhibitors in the resuspending medium because the activity was rotenone-sensitive only in this case. Cytochrome oxidase activity was assayed using reduced cytochrome *c* [reduced by dithionite and purified on a Sephadex G-25 column (Degli Esposti and Lenaz, 1982a; Battino *et al.*, 1986b; Smith *et al.*, 1974)] as substrate by monitoring the absorbance decrease of cytochrome *c* upon oxidation at 417–409 nm; the extinction coefficient used for cytochrome *c* in this case was 40.7 $mM^{-1} \cdot cm^{-1}$ (Battino *et al.*, 1986b). Succinate–ubiquinone reductase activity was measured indirectly by following the ubiquinone-2 dependent reduction of 2,6-Dichloroindophenol (DCIP) (Hatefi and Stiggall, 1978) as the absorbance decrease at 600 nm utilizing a Perkin-Elmer 559 UV-VIS spectrophotometer and using an extinction coefficient (reduced minus oxidized) of 21 $mM^{-1} \cdot cm^{-1}$ for DCIP at 600 nm.

*Inhibitors*

NADH–cytochrome *c* reductase activity was about 88% rotenone-sensitive (0.1 μg/ml), succinate–cytochrome *c* reductase and succinate–DCIP reductase activities about 95% malonate-sensitive (10 mM), ubiquinol-2–cytochrome *c* reductase activity over 95% antimycin A-sensitive (0.25 μg/ml), and cytochrome oxidase activity 100% KCN-sensitive (1 μM).

*Fluorescence Polarization Studies*

Mitochondrial membrane suspensions were treated with the lipid-soluble fluorophore 1,6-diphenyl-1,3,5-hexatriene (DPH) (obtained from Molecular Probes, Junction City, Oregon), and steady-state fluorescence polarization measurements were accomplished either in a Jasco FP 550 spectrofluorometer or in a Perkin-Elmer MPF-4 fluorescence spectrophotometer. The polarization of fluorescence was expressed in terms of the fluorescence anisotropy *r*. The fluorescence anisotropy of DPH in membranes is determined mainly by the maximal hindered anisotropy and provides an estimate of the static component of "fluidity," i.e., of lipid order (Heyn, 1979; Jähnig, 1979; Van Blitterswijk *et al.*, 1981; Brasitus *et al.*, 1984). The anisotropy parameter varies directly with the rotational relaxation time of the probe and hence is inversely related to the lipid fluidity (Shinitzky and Inbar, 1976; Shinitzky and Barenholz, 1978; Brasitus and Schachter, 1980a). For temperature scans, temperature was varied with a B. Braun Frigomix 1497-Thermomix 1480 circulating water bath. The temperature dependence of the fluorescence anisotropy was studied over the range 8–38°C, and Arrhenius plots were made to detect thermotropic transitions (Shinitzky and Barenholz, 1978; Brasitus *et al.*, 1980). Samples were heated to 40°C and then cooled to 6°C followed by a heating scan to 40°C. The operation was repeated three times.

## Results

The HPLC method detected, in addition to ubiquinone-9, the known major benzoquinone component in rat tissues, and also a consistent amount of ubiquinone-10 at a $CoQ_9/CoQ_{10}$ molecular ratio of 2:1, with retention times of 7.26 and 8.53 min, respectively (Fig. 1). This feature was underlined not only for nonsynaptic mitochondria, as previously reported (Battino *et al.*, 1988, 1989a,b), but also for the two populations of synaptic mitochondria. The results are shown in Table I. The total amount of ubiquinone was higher in nonsynaptic mitochondria and lower in synaptic mitochondria; nevertheless the $CoQ_9/CoQ_{10}$ ratio was nearly the same in each population. The

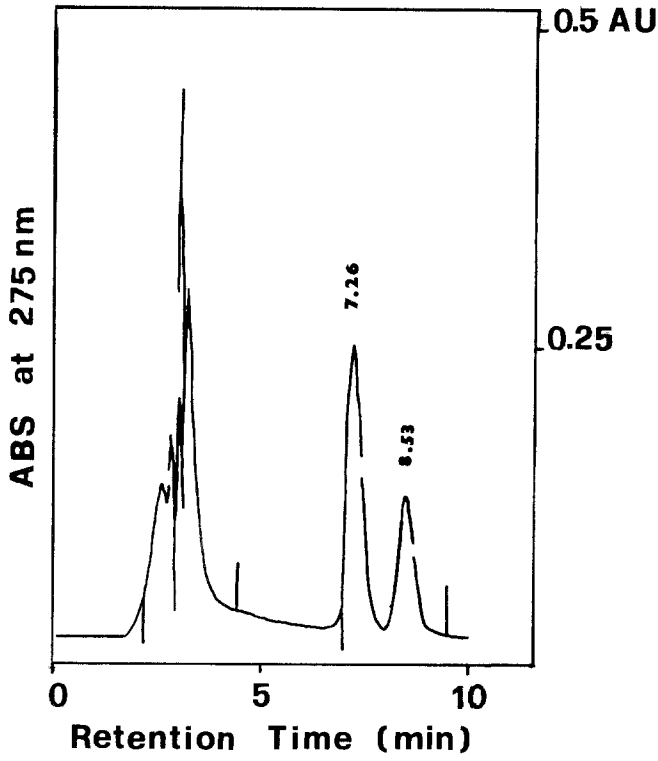


**Fig. 1.** Reversed-phase HPLC profile of $CoQ_9$ (Rt = 7.26 min) and $CoQ_{10}$ (Rt = 8.53 min) after extraction from rat brain mitochondria (see text).

cytochrome concentrations were investigated by their differential spectra only in nonsynaptic mitochondria because the synaptic mitochondrial fractions were too small for cytochrome determinations (Fig. 2).

No important differences arose between the different regions: In fact (Table II), the content of *b* and $a/a_3$ cytochromes was roughly similar in the three areas, and the only exception was for cytochromes $c + c_1$ that were significantly higher in hippocampus and striatum (about 50%) than in cerebral cortex. Respiratory chain activities revealed (Table III) intricate relationships with regard to the different areas and distinct mitochondrial populations as previously described for hippocampus only (Villa *et al.*, 1986, 1989a,b). A constant feature was the presence of higher specific activities (for each enzyme examined) in cerebral cortex in comparison with hippocampus and striatum both of synaptic and nonsynaptic mitochondria, while the behavior of different mitochondrial fractions in the same area was more complex; in fact, higher maximal velocities were detected either in the nonsynaptic

**Table I.** $CoQ_9$ and $CoQ_{10}$ Levels in Nonsynaptic (FM) and in "Light" or "Heavy" (LM and HM) Synaptic Mitochondria from Rat Brain Regions as Detected by HPLC Analysis[a]

| | Cortex | | Hippocampus | | Striatum | |
|---|---|---|---|---|---|---|
| | $CoQ_9$ | $CoQ_{10}$ | $CoQ_9$ | $CoQ_{10}$ | $CoQ_9$ | $CoQ_{10}$ |
| FM | 884.86 ± 73.91 | 456.14 ± 49.23 | 756.43 ± 96.6 | 429.43 ± 54.50 | 952.86 ± 72.91 | 409.29 ± 47.53 |
| LM | 845.86 ± 145.09 | 427.29 ± 54.29 | — | — | 588.00 ± 79.70 | 290.00 ± 56.15 |
| HM | 478.50 ± 93.10 | 247.93 ± 51.08 | — | — | — | — |

[a]CoQ concentrations are expressed in pmol/mg mitochondrial protein. Results are mean ± S.D. for number of rats ⩾ 7.

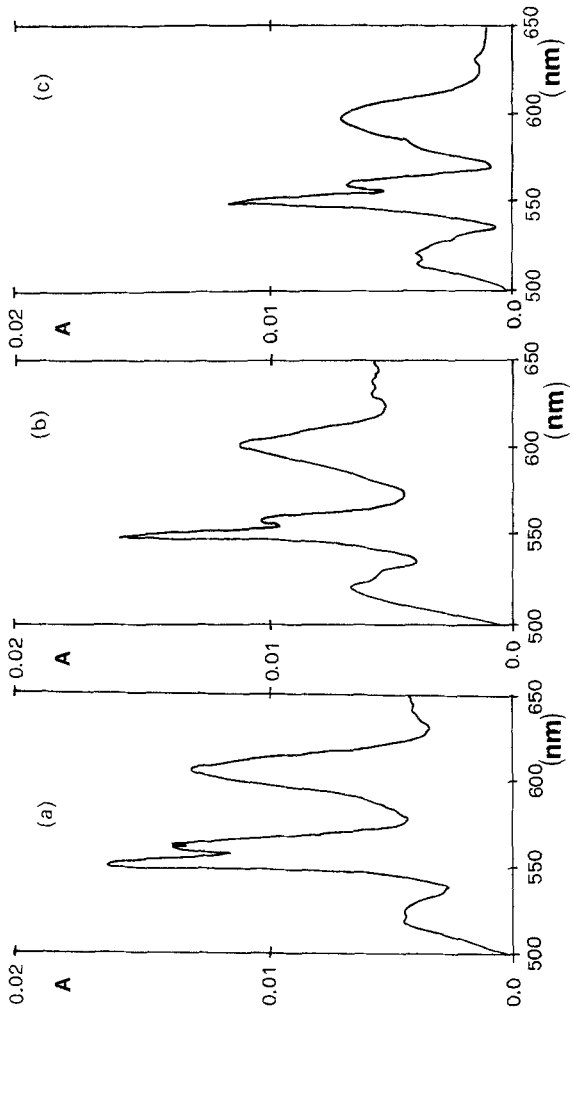


**Fig. 2.** Differential spectra of cytochrome $c + c_1$, $b$, and $a + a_3$ (dithionite reduced minus ferricyanide oxidized) obtained from the nonsynaptic fraction. (a) cortex; (b) hippocampus; (c) striatum. Mitochondria were suspended in 0.1 M phosphate buffer, pH 7.4, at a final protein concentration ranging from 0.9 up to 3.0 mg/ml depending on the sample availability; the final volume was 1 ml.

**Table II.** Cytochrome $c + c_1$, $b$, and $a + a_3$ Concentrations Evaluated by the Differential Spectra (See Text for Explanations) in Nonsynaptic Mitochondria of Rat Brain Regions[a]

| | $c + c_1$ | $b$ | $a + a_3$ |
|---|---|---|---|
| Cortex | 0.195 ± 0.015 | 0.120 ± 0.010 | 0.140 ± 0.018 |
| Hippocampus | 0.290 ± 0.023 | 0.090 ± 0.011 | 0.120 ± 0.016 |
| Striatum | 0.320 ± 0.021 | 0.100 ± 0.009 | 0.115 ± 0.014 |

[a]Cytochrome concentrations are expressed in nmol/mg mitochondrial protein. Results are means ± S.D. for number of rats ⩾ 10.

**Table III.** Respiratory Chain Activities of Nonsynaptic and Synaptic Mitochondria from Rat Brain Regions

| | Cortex | Hippocampus | Striatum |
|---|---|---|---|
| | | Succ–$c$ | |
| FM | 0.196 ± 0.076 | 0.159 ± 0.048 | 0.134 ± 0.039 |
| LM | 0.171 ± 0.050 | 0.154 ± 0.051 | 0.159 ± 0.045 |
| HM | 0.075 ± 0.019 | 0.062 ± 0.011 | 0.063 ± 0.019 |
| | | Succ–DCIP | |
| FM | 0.082 ± 0.048 | 0.053 ± 0.016 | 0.059 ± 0.038 |
| LM | 0.051 ± 0.019 | 0.049 ± 0.023 | 0.052 ± 0.037 |
| HM | 0.032 ± 0.017 | 0.017 ± 0.008 | 0.018 ± 0.013 |
| | | $CoQ_2H_2$–$c$ | |
| FM | 2.397 ± 0.445 | 1.513 ± 0.175 | 1.776 ± 0.666 |
| LM | 2.647 ± 1.097 | 2.153 ± 0.995 | 2.028 ± 0.823 |
| HM | 1.137 ± 0.471 | 0.505 ± 0.129 | 0.766 ± 0.415 |
| | | Cytochrome oxidase | |
| FM | 2.217 ± 0.342 | 1.677 ± 0.259 | 2.021 ± 0.380 |
| LM | 2.328 ± 0.498 | 1.870 ± 0.427 | 1.819 ± 0.688 |
| HM | 1.125 ± 0.307 | 0.698 ± 0.203 | 0.762 ± 0.219 |

[a]Enzymatic activities are expressed in μmol/min/mg mitochondrial protein. Results are means ± S.D. for number of rats ⩾ 10.

mitochondrial fraction or in the light intrasynaptic one, depending on the enzymic activity and the brain area considered. Nevertheless, the heavy mitochondrial fraction disclosed the lowest activities without any exception for areas or enzymes.

The turnover numbers could be calculated for both ubiquinol–cytochrome $c$ reductase and cytochrome oxidase in nonsynaptic mitochondria from the cyt. $b$ and $a + a_3$ content: Values around $600\,s^{-1}$ for ubiquinol–cyt. $c$ reductase and around $500\,s^{-1}$ for cytochrome oxidase were found in all three areas.

The temperature dependence of respiratory activities exhibited different behaviors for various enzymes. Under our experimental conditions, cytochrome oxidase and NADH–cytochrome $c$ reductase activities of nonsynaptic

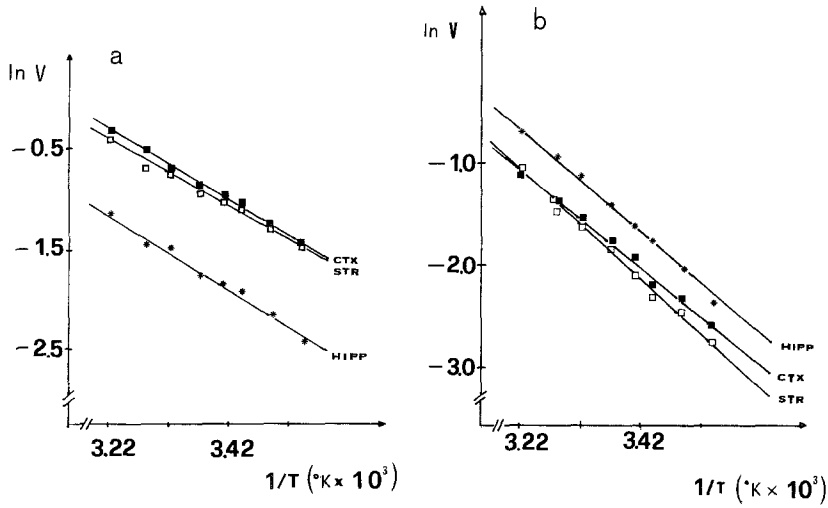


**Fig. 3.** The Arrhenius plots of cytochrome oxidase (a) and NADH–cytochrome *c* reductase (b) in nonsynaptic mitochondria showed a straight line of the activity vs. $1/T$ in all the three brain areas considered. ■, cortex; □, striatum; ∗, hippocampus.

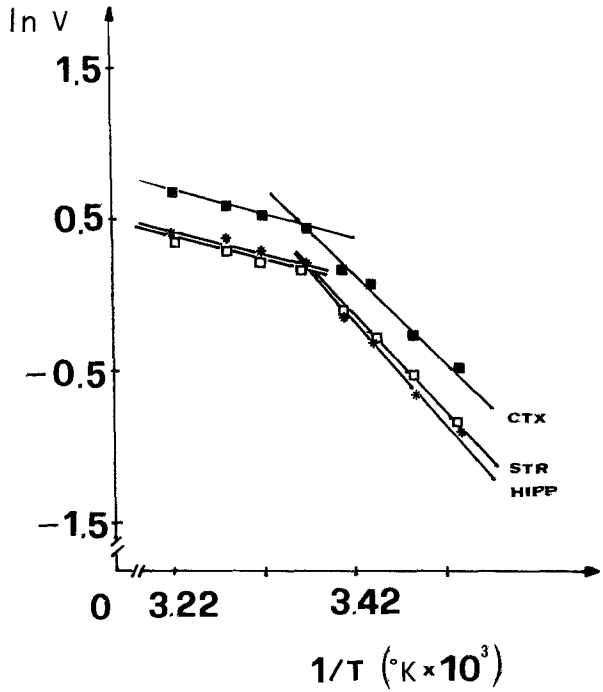


**Fig. 4.** The Arrhenius plots of ubiquinol-2-cytochrome *c* reductase activity ($bc_1$ complex) of nonsynaptic mitochondria revealed a biphasic temperature dependence with breaks at 24°C. ■, cortex; □, striatum; ∗, hippocampus.

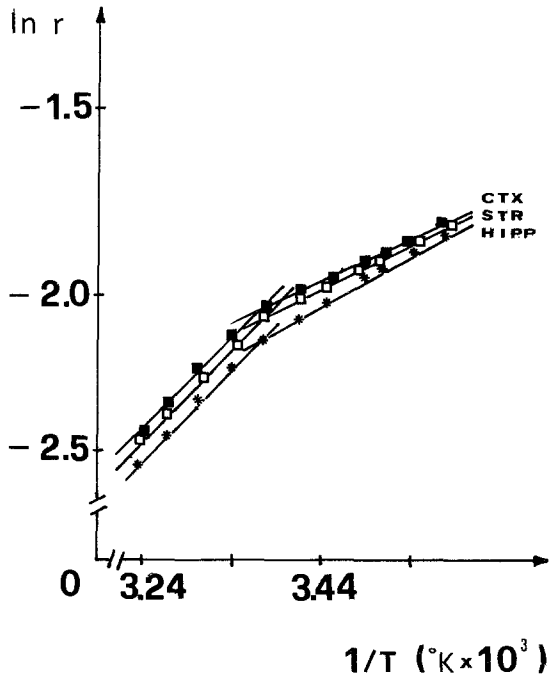


**Fig. 5.** Arrhenius plots of anisotropy of nonsynaptic mitochondria by fluorescence polarization of DPH showed also broken lines with the discontinuity points localized at 23°C. ■, cortex; □, striatum; ∗, hippocampus.

**Table IV.** Activation Energy Values Calculated from Arrhenius Plots of Figs. 3 and 4[a]

| | NADH–cyt. *c* reductase | $CoQ_2H_2$–cyt. *c* reductase | | Citochrome oxidase |
|---|---|---|---|---|
| | | Below break | Above break | |
| Cortex | 9.63 | 11.69 | 3.44 | 7.08 |
| Hippocampus | 10.03 | 13.38 | 3.17 | 7.53 |
| Striatum | 10.95 | 12.04 | 3.17 | 6.88 |

[a]Activation energies are expressed in kcal/mol.

mitochondria displayed monophasic Arrhenius plots (Battino *et al.*, 1986b) (Fig. 3), whereas Ubiquinol-2–cytochrome *c* reductase ($bc_1$ complex) exhibited a clear discontinuity in the Arrhenius plots at approximately 24°C in all three areas (Fig. 4), with large differences in activation energies above and below the break (Table IV); Arrhenius plots of anisotropy of nonsynaptic mitochondria from the three areas also displayed a deviation from linearity with a break at about 23°C (Fig. 5). Only heating scans are provided, but cooling scans also showed break points in Arrhenius plots at the same temperature.

## Discussion and Conclusions

The data obtained support the results of other studies (Salganicoff and Koeppe, 1968; Blokhuis and Veldstra, 1970; Dennis *et al.*, 1977; Owen *et al.*, 1977; Lai and Clark, 1979; Leong *et al.*, 1984; Villa *et al.*, 1986, 1989b) on the concept of heterogeneity of brain mitochondria. Most of the enzymatic studies in brain mitochondria have been carried out using whole brain hemispheres or cortex (Clark and Nicklas, 1970; Dienel *et al.*, 1977; Lai *et al.*, 1977; Benzi, 1979, 1981; Benzi *et al.*, 1980; Hansford, 1983). Particular significance needs to be given to these considerations when subcellular investigations are made (Villa *et al.*, 1978, 1989a,b; Benzi *et al.*, 1980; Villa, 1981). Different enzymes have been analyzed, and different separation techniques have been used in various investigations (Lai and Clark, 1976; Dienel *et al.*, 1977; Booth and Clark, 1978; Deshmukh *et al.*, 1980; Leong *et al.*, 1984); these results are not therefore directly comparable with one another. In addition, synaptosomes are readily fractionated into soluble and particulate fractions but less readily separated (Villa *et al.*, 1989a) into their components: mitochondria, synaptic vesicles, and plasma membrane that overlap considerably in buoyant density on gradients.

It seems clear that differences between perikaryal and synaptic mitochondria do in fact exist, although the nature and significance of these differences are not clear. The overall evidence for the biochemical diversity of these populations of mitochondria from rat brain areas (Deshmukh *et al.*, 1980; Leong *et al.*, 1984) is quite convincing. When careful examination is made of the possible changes induced, for example, by aging and drugs on subcellular fractions, striking differences appear for an extended pattern of enzyme activities of free mitochondrial fractions of different areas (Benzi *et al.*, 1988a,b, 1989a,b) in normal individuals or during ischemia (Villa, 1981; Villa *et al.*, 1981, 1982a); therefore, when specific brain metabolic pathways are considered in aging (Dienel *et al.*, 1977; Benzi *et al.*, 1980, 1988a; Deshmukh *et al.*, 1980; Leong *et al.*, 1984; Vanella *et al.*, 1988, 1989), the microheterogeneity of intrasynaptic mitochondria must be considered for correctly evaluating the effects of aging. This type of research, coming from single animals and from specific brain areas, is particularly appropriate when attempting to follow the maturation processes of the subcellular compartment site that appears to be selectively affected by drugs (Villa *et al.*, 1982b, 1986, 1989c).

In this work this heterogeneity involves both the three areas considered and the three populations of mitochondria isolated from each area. In particular two aspects seem to be clear:

(a) With respect to the three areas, the highest levels of respiratory chain components and activities are exhibited by cortex:

(b) As for the mitochondrial fractions, the lowest levels and activities are present in the heavy fraction of synaptic mitochondria; it is reasonable to assume that the levels of respiratory components and activities are correlated to the energy potentiality of the respective mitochondria.

The major analytical points displayed by this study are the following:

(i) Presence of both $CoQ_9$ and $CoQ_{10}$ in roughly 2 : 1 ratio in all populations. The major CoQ homolog described in rat mitochondria has been $CoQ_9$. It is not clear whether the high $CoQ_{10}$ content is the result of the improved analytical procedure (HPLC) or is a special feature of the rat population employed and of the dietary contribution. The total CoQ content of brain mitochondria is in line with the result found by other investigators using spectrophotometric techniques (Parsons and Basford, 1967; Munn, 1974).

(ii) The cytochrome content of the different mitochondrial populations were generally similar, except for cytochromes $c + c_1$ that were significantly higher in hippocampus and striatum than in cerebral cortex. However, this difference could presumably be due to cytochrome $c$ since the $b : c_1$ ratio in the mitochondrial $bc_1$ complex is always 2 : 1; it cannot be established whether the higher level of cyt. $c$ is a real feature of these areas or is due to decreased loss during the isolation procedure, owing to stronger association to the membrane or to lower membrane damage. Also, in this case, data were in good agreement with literature indications (Sacktor and Packer, 1962; Munn, 1974).

NADH dehydrogenase and succinate dehydrogenase attracted attention because of their peculiar behavior. (A) In the first case, it was found that activity was completely rotenone-insensitive. The only way to obtain a good sensitivity of Complex I to its specific inhibitor was not to treat mitochondria with protease inhibitors during preparation; in this case rotenone inhibited the reaction with about 88% of efficiency. The remaining activity could be ascribed to outer membrane activity which involves an outer membrane dehydrogenase and a $b_5$ cytochrome; the amount of activity that was completely rotenone-insensitive found in mitochondria treated with TLCK and PMSF was one-tenth the activity detected in the absence of these substances, i.e., of the same order of magnitude as that part of NADH oxidation ascribed to outer membrane activity. It might be suggested that both TLCK and PMSF separately or in combination (as we found) affect irreversibly the first mitochondrial complex of these electron chains, probably binding to its protein moiety and damaging even the primary structure or the relationships

with cofactors. (B) The activities which involved the second respiratory complex, i.e., succinate–cytochrome *c* reductase and succinate–CoQ (by DCIP) reductase, turned out to be much lower than the NADH dehydrogenase activity while in beef heart mitochondria (Gutman, 1985), for example, the two activities do not differ appreciably, being both on the order of 1.0 μmol/min/mg protein. A possible explanation for this result may be a lower number of succinate dehydrogenase complexes in these rat brain mitochondria.

Breaks in Arrhenius plots of enzymatic activities, with large differences in activation energies above and below the break, were formerly detected in our laboratory with bovine heart mitochondria (Bertoli *et al.*, 1973; Sechi *et al.*, 1973; Curatola *et al.*, 1983; Parenti-Castelli *et al.*, 1983, 1987; Baracca *et al.*, 1984, 1986a,b; Lenaz *et al.*, 1984, 1986, 1987a,b; Solaini *et al.*, 1984; Calanni-Rindina *et al.*, 1986; Battino *et al.*, 1986c).

The reason for the biphasic temperature dependence of membrane enzyme activities is controversial and ascribed to several possible causes (Lenaz and Parenti-Castelli, 1985): (I) changes in the rate-limiting step in the kinetic pathway of the enzymic reaction (Dixon and Webb, 1964); (II) temperature-dependent conformational changes of the enzyme: in this case the Arrhenius plot of an enzyme having two forms with different catalytic activities could display a discontinuity at the temperature where the amount of the two forms is equal (Biosca *et al.*, 1983); (III) solvent properties could induce alterations in the linearity of Arrhenius plot, and this phenomenon could be referred to transitions in some particular "vicinal" water molecules (Drost-Hansen, 1969); (IV) the temperatures at which Arrhenius plot discontinuities occur could be strongly influenced by the physical state of lipids (Raison, 1973), even if there is no coincidence with the calorimetric transition temperature, and sometimes very large difference (Lenaz, 1979), and these discontinuities occur independently of the lipid composition of the membrane (Dean and Tanford, 1978; Kaizu *et al.*, 1980).

Other reasons might also be offered to explain this phenomenon, such as the possibility that lipids tightly bound to membrane proteins could force rearrangements in some protein microdomains influenced by medium (i.e., lipid) viscosity that could induce variations in the transition between conformational states involved in the catalytic process (Beece *et al.*, 1980; McMurchie *et al.*, 1983).

Some evidence (Parenti-Castelli *et al.*, 1979; Curatola *et al.*, 1983) suggests ascribing the origin of breaks to conformational changes mediated by temperature-dependent properties of the lipids (Lenaz and Parenti-Castelli, 1985; Battino *et al.*, 1986c; Lenaz, 1987; Lenaz *et al.*, 1987b); this hypothesis is partially confirmed in this study by the temperature-dependent studies of anisotropy (as fluorescence polarization) of the hydrophobic probe DPH that

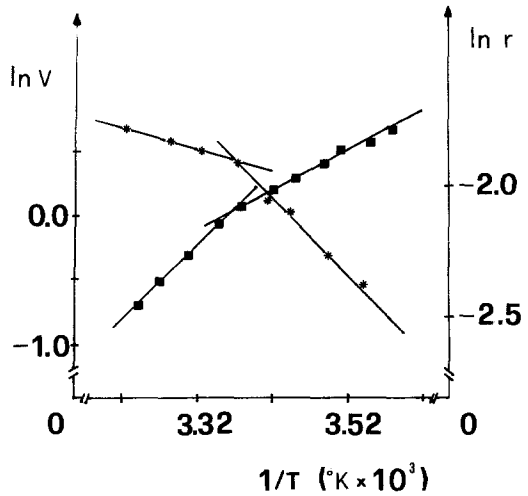


**Fig. 6.** Comparison between break points in the Arrhenius plots of $CoQ_2H_2$–cytochrome *c* reductase activity and in the anisotropy of the fluorescence probe DPH in cortex nonsynaptic mitochondria. Similar findings are present in hippocampus and striatum nonsynaptic mitochondria. ■, anisotropy of DPH vs. $1/T$; ∗, $bc_1$ complex activity vs. $1/T$.

also showed a deviation from linearity with a break at 23°C, in the same temperature range as for $bc_1$ complex activity (Fig. 6).

The reason for the lack of breaks in the Arrhenius plots of some of the activities investigated may be found in the nature of the rate-limiting step at the different temperatures; clearly a break will be found only if a shift of the rate-limiting step occurs at a given temperature.

It must be borne in mind that lipid fluidity of these mitochondrial membranes was assessed by the steady-state fluorescence polarization of a hydrophobic probe (as DPH): It is widely recognized that steady-state fluorescence polarization can be a useful empirical parameter of lipid fluidity for comparison purposes, even if the absolute significance may bear some ambiguity (Brasitus and Schachter, 1980b).

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