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THE OCCURRENCE OF CYTOCHROMES IN NUCLEI ISOLATED FROM RAT THYMUS

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SUMMARY

1. Rat-thymus nuclear preparations were purified with the detergent Triton X-100. No cytoplasmic contamination could be detected by electron microscopy. The RNA/DNA ratio for these nuclei was about 0.12, the protein/DNA ratio about 1.54.

2. By low-temperature spectrophotometry the presence of cytochromes *a*, a_3, *b* and *c* could be demonstrated in the detergent-treated nuclei, after reduction with $Na_2S_2O_4$, or with NADH in the presence of CN^-. Cytochrome b_5 or hemoglobin were not detected.

3. A quantitative comparison of the spectra obtained with nuclei and mitochondria proved that the cytochromes must be components of the nuclei. About 40% of the cellular cytochrome aa_3 is present in the nuclei.

4. The possible localization of a nuclear respiratory chain in the inner nuclear membrane is discussed.

INTRODUCTION

It is generally believed that mitochondria are the only sites of respiratory-chain-linked oxidative phosphorylation in all cells that contain these organelles. However, there is now convincing evidence that thymus nuclei are capable of oxidative phosphorylation[1–5]. The responses of nuclear phosphorylation and oxygen uptake to inhibitors of the mitochondrial respiratory chain strongly suggested that nuclear oxidative phosphorylation is coupled to a similar respiratory chain. This would imply that thymus nuclei contain cytochromes.

The presence of cytochromes in rat-thymus nuclei has now been demonstrated by low-temperature spectrophotometry.

METHODS AND MATERIALS

Isolation and purification of nuclei

Thymus nuclei were isolated in 0.25 M sucrose which contained 3 mM $CaCl_2$ as previously described[2]. These nuclei contained small amounts of cytoplasm and a small number of intact cells. The nuclear fraction was purified further by treatment with the detergent Triton X-100 (ref. 6). A 4% (v/v) solution of Triton X-100 in

0.25 M sucrose–3 mM $CaCl_2$ was added slowly and under continuous shaking to a diluted (3–6 mg protein per ml) nuclear suspension to a final concentration of 0.25 % (v/v). The suspension was shaken 5–10 min at room temperature and several times vigorously expelled from a transfer pipette. Subsequently, the suspension was centrifuged for 7 min at 900 × *g*, or the Triton-containing suspension was layered over 2 M sucrose which contained 3 mM $CaCl_2$ and 0.25 % Triton X-100 and centrifuged for 15 min at 15000 × *g* (Spinco rotor SW 25). The pellets were suspended in 0.25 M sucrose–3 mM $CaCl_2$. Identical spectra were obtained with both procedures.

Nuclei treated with Triton X-100 appeared to be free of cytoplasmic and whole cell contamination as judged by light microscopy as well as by electron microscopy.

The RNA/DNA ratio for the preparations was 0.12 and the protein/DNA ratio 1.54 (mean of 10 experiments). Before Triton treatment these ratios were respectively 0.182 and 2.25 (ref. 5). Triton-treated nuclei did not contain detectable amounts of nucleotides, succinate: cytochrome *c* reductase or cytochrome *c* oxidase (EC 1.9.3.1).

Analytical methods

DNA, RNA and protein were determined as described previously[7].

Low-temperature difference spectra (reduced *minus* oxidized) were obtained manually with a dual-wavelength spectrophotometer (American Instruments Co., Silverspring, Md., U.S.A.) fitted with a low-temperature attachment of our own design (Fig. 1). The distance from the centre of the cuvette to the photomultiplier surface was 40 mm. The depth of the cuvettes was 4 mm. In the lower position of the cuvettes the Dewar vessel was filled with liquid air to about 1 cm below the photomultiplier window. The lower cuvette always contained the reduced sample.

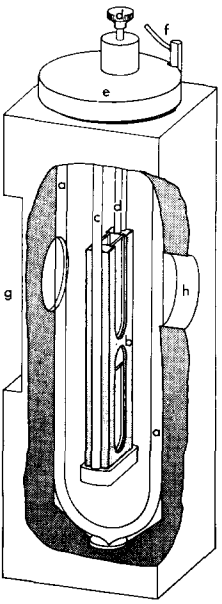

Fig. 1. Cell and cell holder for low-temperature spectrophotometry; a, unsilvered Dewar vessel; b, cells, aluminium with plexiglass windows; c, cell alignment and sliding rods; d, positioning rod, to bring one of the two cells in the light path. The sliding rods are attached to the cover (e). The assembly is hold in the right position by a pawl (f). g, space for shutter and attachment of photomultiplier. h, light-entrance hole. The holder is made of aluminium.

Difference spectra were measured on devitrified samples containing 50 % (v/v) glycerol[8] by the dual-wavelength technique[9]. In this procedure the difference between the transmittances of reduced and oxidized samples at a certain wavelength is measured with respect to a reference wavelength. The reference wavelengths used were 470 mμ in the range of 410–470 mμ; 540 mμ from 540 to 570 mμ and 610 mμ from 590 to 610 mμ. The slit-width was 0.2 mm corresponding to a spectral half band-width of about 1.2 mμ. It was assumed that the amplification obtained by freezing was constant between 540 and 610 mμ.

RESULTS

Fig. 2 shows the difference spectrum (reduced with $Na_2S_2O_4$ *minus* oxidized) of Triton-treated nuclei after layering over 2 M sucrose. The spectrum shows both α bands of cytochrome *c* (547 and 549 mμ) and the α bands of cytochrome *b* (shoulder at 560–563 mμ) and of cytochromes aa_3 (603 mμ). The Soret region showed the γ band of cytochromes aa_3 (445 mμ) and of cytochrome *b* (430 mμ). The (reduced + CO) *minus* reduced spectrum in the Soret region showed the typical a_3–CO spectrum with a peak at 429 mμ and a minimum at 447 mμ.

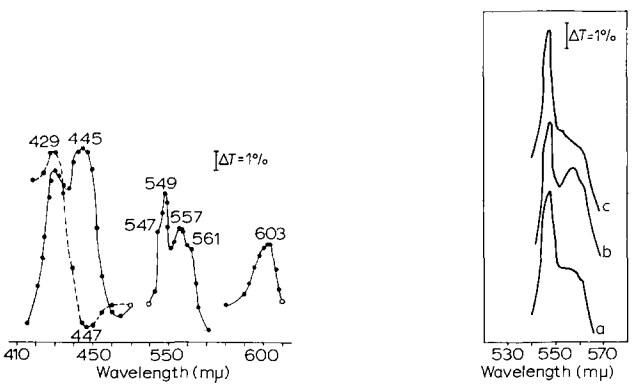

Fig. 2. Low-temperature spectrum of thymus nuclei. Thymus nuclei were treated with Triton X-100 and layered over 2.0 M sucrose as described under METHODS. The cells contained 0.125 M sucrose, 1.5 mM $CaCl_2$, 50 % (v/v) glycerol, 3.1 mg nuclear protein and 2.0 mg DNA per ml. Reduction with $S_2O_4^{2-}$. ●—●, reduced *minus* oxidized spectrum; ●- - -●, (reduced + CO) *minus* reduced spectrum; ○, reference wavelengths.

Fig. 3. Denatured hemoprotein in thymus nuclei. The cells contained 0.125 M sucrose, 1.5 mM $CaCl_2$, 50 % (v/v) glycerol and nuclei corresponding with 1.8 mg protein and 1.4 mg DNA per ml. (a) Nuclear suspension treated for 4 min with 0.25 % Triton X-100, sedimented and resuspended in 0.25 M sucrose–3 mM $CaCl_2$. Reduced ($Na_2S_2O_4$) *minus* oxidized spectrum at liquid-air temperature. (b) Treated for 15 min with Triton X-100, otherwise as under (a). (c) Treated for 15 min with Triton X-100. (Reduced + CO) *minus* oxidized spectrum at liquid-air temperature.

The broad maximum between 555 and 560 mμ did not originate from contamination with microsomal cytochrome b_5, but must be ascribed to a denatured hemoprotein. Fig. 3 shows that this band was not present in the (reduced + CO) *minus* oxidized spectrum[10] and that the appearance of this band was dependent on the duration of the Triton X-100 treatment of the nuclei.

The possibility was considered that the cytochrome spectrum of these nuclear

preparations was caused by small mitochondrial fragments that were strongly adsorbed by the nuclei. It has been demonstrated by DOUNCE[11] that in liver nuclei this source of contamination could be eliminated by treatment of the nuclei with arabic gum. The spectrum of a nuclear preparation that has been treated with Triton X-100 and arabic gum is shown in Fig. 4. Treatment with arabic gum removed some of the cytochrome *c* and increased the band of denatured hemoprotein, but the bands of cytochrome aa_3 were not affected. This suggested, but did not prove, that the spectrum of Triton-treated nuclei was not due to contamination with mitochondrial fragments that had escaped detection in the electron micrographs.

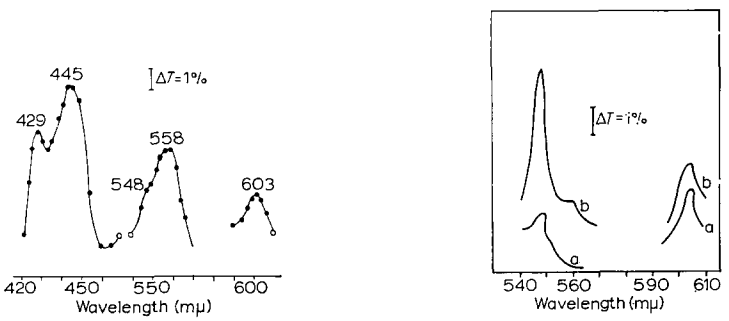

Fig. 4. Low-temperature spectrum of thymus nuclei treated with arabic gum. Thymus nuclei were suspended in a medium containing 0.25 M sucrose, 3 mM $CaCl_2$ and 1 % arabic gum and treated with 0.25 % Triton X-100 for 4 min. The suspension was layered over a medium containing 2.0 M sucrose, 3 mM $CaCl_2$, 0.25 % Triton X-100 and 1 % arabic gum was centrifuged for 15 min at 15000 × *g*. The pellet was resuspended in 0.25 M sucrose–3 mM $CaCl_2$. The cells contained 0.125 M sucrose, 1.5 mM $CaCl_2$, 50 % (v/v) glycerol and nuclei corresponding to 3.1 mg protein and 2.0 mg DNA per ml. ●—●, reduced ($Na_2S_2O_4$) *minus* oxidized spectrum; ○, reference wavelength.

Fig. 5. Reduction by substrate of cytochromes in thymus nuclei. Reduced *minus* oxidized spectra at liquid-air temperature. The cells contained 0.125 M sucrose, 1.5 mM $CaCl_2$, 50 % (v/v) glycerol and nuclei corresponding to 2.5 mg protein per ml. (a) Triton-treated nuclei incubated with 5 mM KCN for 10 min at room temperature before addition of glycerol. (b) As in (a), but incubated with 5 mM KCN + 5 mM NADH.

The cytochrome spectrum of the nuclear preparations could also be obtained by incubation of the nuclear preparation with KCN or NADH + KCN (Fig. 5). After 10 min incubation with 5 mM KCN the cytochrome aa_3 band was fully developed, but the cytochrome *c* band was weak. Incubation with 5 mM NADH + 5 mM KCN gave a spectrum similar to that obtained after reduction with $S_2O_4^{2-}$. The band around 555 mμ was completely absent. This observation is in agreement with conclusion that this band in the $S_2O_4^{2-}$-reduced spectra is due to a denatured hemoprotein.

In order to demonstrate quantitatively that the spectrum observed with the nuclear preparations could not be ascribed to contamination with mitochondria or mitochondrial fragments it had to be shown that spectra obtained with mitochondria and nuclei could be compared quantitatively. It was shown that: (a) The transmittance difference $\Delta T_{548-540\ m\mu}$ was proportional to the protein concentration between 0 and 10 % transmittance for thymus mitochondria (Fig. 6) and nuclei (Fig. 7). (b) The amplification of the cytochrome *c* band in thymus mitochondria at the temperature of liquid air was about 30 times with respect to the intensity of the band at 25°.

With thymus nuclei the amplification could not be measured very accurately, since very dense nuclear suspensions had to be used at 25°, but it could be established that amplification of the cytochrome *c* band was between 20- and 30-fold. In any case it could be concluded that the amplification obtained with nuclei was not greater, but rather somewhat smaller, than that obtained with mitochondria. (c) The low-temperature spectra of mitochondria and nuclei were additive (Table I).

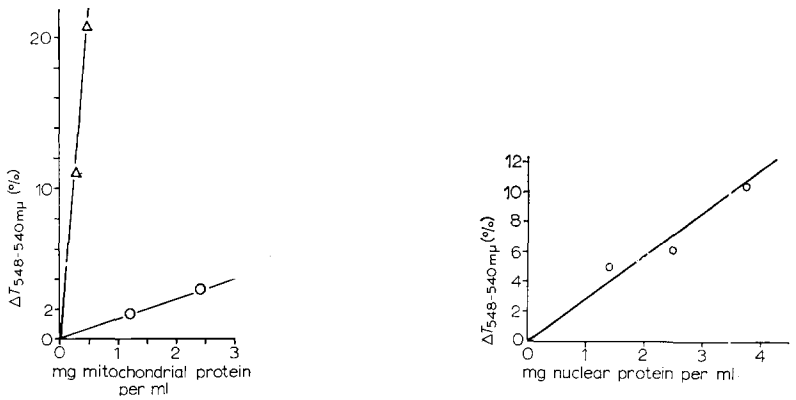

Fig. 6. Relation between $\Delta T_{548-540\,m\mu}$ and mitochondrial protein concentration at 25° (○—○) and at liquid-air temperature (△—△). The cells contained 0.125 M sucrose, 50% (v/v) glycerol and the indicated amount of mitochondrial protein. The transmittance difference at liquid-air temperature was measured in a 4-mm cell, but recalculated to a light-path of 10 mm. Reduction with $Na_2S_2O_4$.

Fig. 7. Relation between $\Delta T_{548-540\,m\mu}$ and nuclear protein concentration at the temperature of liquid air. The cells contained 0.125 M sucrose, 1.5 mM $CaCl_2$, 50% (v/v) glycerol and the indicated amount of nuclear protein per ml. Light-path, 4 mm. Reduction with $Na_2S_2O_4$.

TABLE I

ADDITIVITY OF LOW-TEMPERATURE SPECTRA OF NUCLEI AND MITOCHONDRIA

Thymus mitochondria were isolated as described in ref. 2. Thymus nuclei were treated with Triton X-100 as described under METHODS without layering. The mitochondrial suspension contained 1.1 mg protein per ml and the nuclear suspension 2.7 mg protein per ml. Nuclei + mitochondria: 0.55 mg mitochondrial protein + 1.35 mg nuclear protein per ml. The transmittance differences were determined from the reduced ($Na_2S_2O_4$) *minus* oxidized spectra.

Preparation	ΔT (%) (548–540 mμ)	ΔT (%) (610–603 mμ)
a. Mitochondria	11.5	5.8
b. Nuclei	9.9	2.5
c. Nuclei + mitochondria	9.8	3.8
d. Calculated: $1/2 \times \Delta T$(a + b)	10.2	4.2

It may be concluded that spectra obtained with nuclei and mitochondria could be compared, at least in a semi-quantitative way.

It may then be calculated that the cytochrome aa_3 band at 604 mμ in the nuclear spectra cannot be explained by mitochondrial contamination:

(1) The $\Delta T_{610-604\,m\mu}$ of Triton-treated nuclei was 0.8% per mg protein (mean of 6 experiments). The $\Delta T_{610-604\,m\mu}$ of thymus mitochondria was 4% per mg

protein (mean of 4 experiments). If the band obtained with the nuclear preparation were due to contamination, it would have to contain $(0.8/4) \times 100 = 20\%$ mitochondrial protein.

(2) From the fractionation experiments with thymus[5,7] it could be calculated that a mixture of the complete mitochondrial and nuclear fractions from thymus tissue would contain 14 % mitochondrial protein and 86 % nuclear protein. In the course of the Triton treatment about 30 % of the protein is lost from the nuclear fraction (this can be calculated from the protein/DNA ratios of the nuclei before and after Triton treatment). Therefore a mixture of the Triton-treated nuclear fraction and the mitochondrial fraction would contain about 80 % nuclear protein and 20 % mitochondrial protein. It follows from this calculation that, in order to explain the cytochrome aa_3 band in the spectrum of Triton-treated nuclei by mitochondrial contamination it would have to be assumed that these nuclei contained the total mitochondrial fraction. According to the fractionation data[5,7] the nuclear fraction not treated with Triton X-100 contained only 5–10 % of the mitochondria and there is no doubt that the detergent-treated nuclei contained less mitochondrial contamination.

Nuclei isolated in sucrose–$CaCl_2$ contained about 2 % mitochondrial protein[5,7], which would contribute about 0.07 % per mg protein to the $\Delta T_{610-604\,m\mu}$, *i.e.* less than one-tenth of the transmittance difference observed.

It must be concluded that cytochrome oxidase in nuclear preparations cannot be explained by contamination with mitochondria, but must be a constituent of the nuclei. Comparable results were obtained from similar calculations for cytochromes *b* and *c*. It could not be excluded, however, that some of the cytochrome *c* might be due to absorption of mitochondrial cytochrome *c* that was solubilized by the detergent treatment.

DISCUSSION

The results presented allow an approximate calculation of the distribution of cytochrome aa_3 (as measured spectrophotometrically) over nuclei and mitochondria. Triton-treated thymus nuclei give a $\Delta T_{610-604\,m\mu}$ of 0.8 % per mg protein. The nuclear fraction has lost about 30 % protein by Triton treatment. If it is assumed that this protein loss concerns only nuclear protein, pure nuclei isolated in sucrose–$CaCl_2$ would have yielded a $\Delta T_{610-614\,m\mu}$ of 0.5 % per mg protein. From the protein distribution in the mixture of nuclei *plus* mitochondria in a homogenate of thymus tissue, the percentage of total cytochrome aa_3 in the nuclei may be calculated. This equals 100 (% nuclear protein $\times$ ΔT per mg nuclear protein)/(% nuclear protein $\times$ ΔT per mg nuclear protein + % mitochondrial protein $\times$ ΔT per mg mitochondrial protein) = $100\ (86 \times 0.5)/(86 \times 0.5 + 14 \times 4) = 43$. Thus about 40 % of the total cytochrome aa_3 was present in the nuclei and 60 % in the mitochondria. The concentration in the mitochondria (per mg protein) is about 8 times greater than that in the nuclei.

These results are in good agreement with the observation that calf-thymus nuclei contain 20–30 % of the succinate dehydrogenase activity (EC 1.3.99.1) and of the peptide-bound flavin in calf thymus[12].

There seems to be a discrepancy between the enzymatic and spectral determi-

nation of the distribution of succinate:cytochrome *c* reductase and cytochrome oxidase. Presumably this discrepancy has to be explained by the inability of the nuclei to react with exogenous succinate and cytochrome *c*.

The localization of the respiratory chain in the thymus nuclei is not known. The respiratory chain in mitochondria as well as in bacteria is strongly associated with a membrane structure. Therefore it seems likely that in the nuclei the cytochromes will also be membrane-bound. Since the inner nuclear membrane is the only membrane structure that is present in detergent-treated nuclei[6,12,13], it seems quite likely that the cytochromes are present in this membrane. This would agree with the localization of the mitochondrial respiratory chain in the inner membrane of mitochondria[14] and of bacterial cytochromes in the plasma membrane[14].

It seems clear from this and the foregoing paper[5], as well as from the work of McEWEN *et al.*[1,2], that the role of the cell nucleus in energy metabolism and particularly in respiratory chain-linked oxidative phosphorylation needs reevaluation. It has been proved by CONOVER AND SIEBERT[12] that cytochromes are not present in liver nuclei, so that respiratory chain-linked oxidative phosphorylation is evidently not present in the nucleus of the liver cell. We agree with SIEBERT[15], that it might be premature to generalize from the findings with either liver or thymus nuclei. There are good indications, however, that nuclear oxidative phosphorylation might be a general property of lymphatic tissues[16,17].

ADDENDUM

After completion of the manuscript, a paper by YAMAGATA, UEDA AND SATO[18] came to our attention in which the presence of cytochromes *b* and *c* in calf-thymus nuclei was demonstrated. These authors, however, found no convincing evidence for the presence of cytochrome aa_3.

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