THE OCCURRENCE OF CYTOCHROMES IN THE MEMBRANOUS STRUCTURES OF CALF THYMUS NUCLEI

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Nuclei isolated from calf and rat thymus have been shown to catalyze an aerobic synthesis of ATP. In previous papers, we have reported the occurrence of diaphorases (Nonomura and Ueda, 1963) and cytochromes (Yamagata et al., 1966) in calf thymus nuclei, and presented evidence that their occurrence was not due to contamination by cytoplasmic constituents.

Recently, Conover (1967) and Betel (1967) have observed cytochrome components, resembling those of mitochondria, in thymus nuclei by low-temperature spectrophotometry. Since the aerobic phosphorylation in thymus nuclei differs from its mitochondrial counterpart in several respects (McEwen et al., 1963), it may be expected that the nuclear electron transport system differs from that of mitochondria. Yamagata and Sato (1968) have recently reported that the thymocyte contains the same species of cytochrome c in both the nucleus and mitochondria.

In the present work, we made an attempt to compare the cytochrome components of the nuclear membrane fraction from calf thymus with

those of mitochondria and microsomes, and came to the conclusion that cytochrome *a* + a_3 and a *b*-type cytochrome or cytochromes are consistently present in the nuclear membranous structures.

METHODS

Nuclear fraction, which was prepared from calf thymus in 0.25 M sucrose-3 mM $CaCl_2$ (Sucrose-Ca) by the method of Allfrey *et al.* (1957), was mixed with 2 volumes of 2.3 M sucrose-3 mM $CaCl_2$ and layered over 1.95 M sucrose-3 mM $CaCl_2$, and centrifuged at 7,500 x *g* for 30 min. The pellet formed at the bottom was collected and called "naked nuclei". For more complete removal of cytoplasmic constituents, the naked nuclei were resuspended in Sucrose-Ca containing 0.25% Triton X-100, and recentrifuged immediately. The final pellet of purified nuclei was suspended in Sucrose-Ca and called "Triton nuclei".

Nuclear membranous fractions were prepared as follows: The Triton nuclei were digested with DNase (15 μg/mg protein) in Tris-HCl buffer (0.05 M, pH 7.4) containing $MgSO_4$ (5 mM) at 0° for 16 hours. The digest was mixed with an equal volume of 2 M NaCl and centrifuged at 7,500 x *g* for 10 min. The pellet thus obtained was resuspended in 0.05 M Tris-HCl buffer, pH 7.4, and called "heavy nuclear membranous fraction". The supernatant was centrifuged again at 78,000 x *g* for 90 min, and the resulting pellet was suspended in Tris-HCl buffer (0.05 M, pH 7.4) and called "light nuclear membranous fraction".

Low-temperature (liquid nitrogen) spectra were recorded in a Shimadzu MPS spectrophotometer fitted with a low-temperature attachment of our own design (Kawai, 1967 and 1968), using cuvettes of 2.0 mm optical path.

RESULTS AND DISCUSSION

As can be seen in Curve B of Fig. 1, the NADH-reduced minus air-oxidized low-temperature difference spectrum of Triton nuclei showed absorption peaks in the α region at 604 mμ, 549 mμ and a shoulder at 557 mμ, indicating the presence of cytochromes $\underline{a}$ + $\underline{a}_3$, $\underline{c}$ and a $\underline{b}$-type cytochrome, respectively. On the other hand, the spectra of mitochondria and microsomes from the thymus were markedly different from that of nuclei (Curves A and C of Fig. 1). These results are in good agreement with previous observations (Yamagata *et al.* 1966).

Since the inner nuclear membrane appeared to be the only membranous structure that is present in the Triton nuclei, it was likely that the nuclear cytochromes are localized in this structure. In Fig. 2, the NADH-reduced minus oxidized difference spectra of nuclear membranous fractions are compared to those of the mitochondrial fragments that have been treated with the Triton and DNase in the same way. The spectrum of the mitochondrial fragments showed peaks in the α-band region at 604, 559 and 553 mμ, indicating the presence of cytochromes $\underline{a}$ + $\underline{a}_3$, $\underline{b}$ and $\underline{c}_1$, respectively. The most striking finding from Fig. 2 was that the difference spectra of nuclear membranous fractions showed the α-band of cytochrome $\underline{a}$ + $\underline{a}_3$ and unknown α-bands at 553 and 557 mμ, whereas no α-band of cytochrome $\underline{b}$ could be detected, contrary to the reports of Conover (1967) and Betel (1967). The unknown α-bands also differed from that of cytochrome $\underline{b}_5$, but considerable amounts of protoheme and cytochrome $\underline{a}$ heme were extracted with HCl-acetone from the nuclear membranous fractions which had contained very little hemoglobin. Therefore, it seems that the nuclear membranous fractions obtained from calf thymus cells contain cytochrome $\underline{a}$ + $\underline{a}_3$ and a $\underline{b}$ type cytochrome or cytochromes having α-bands at 553 and 557 mμ.

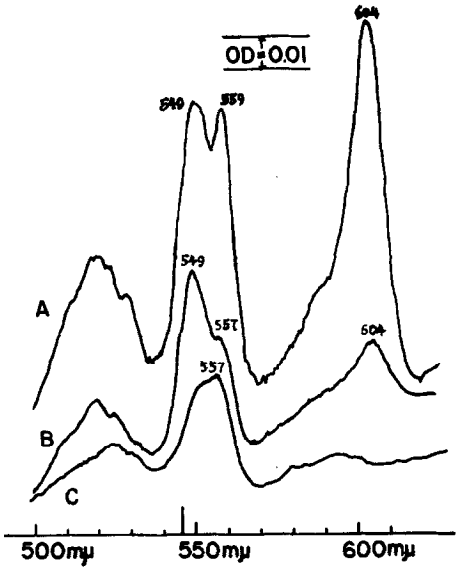

Fig. 1

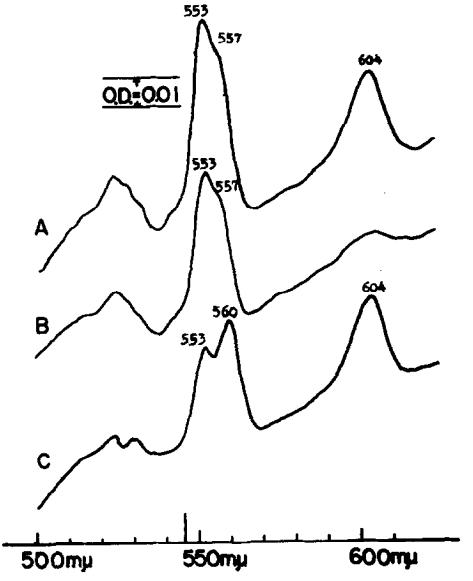

Fig. 2

Fig. 1. NADH-reduced minus air-oxidized low-temperature difference spectra of calf thymus fractions.
(A) Mitochondria in the sample cuvette were reduced with a few mg of solid NADH in the presence of KCN (2 mM). Both the sample and the reference cuvettes contained 46.5 mg of mitochondrial protein per ml of 50 mM Tris-HCl buffer (pH 7.4).
(B) Triton nuclei were reduced with NADH as in (A). Both sample and reference cuvettes contained 44.7 mg of nuclear protein per ml of 50 mM Tris-HCl buffer (pH 7.4).
(C) Microsomes (40.0 mg protein per ml) were reduced as in (A).

Fig. 2. Low-temperature difference spectra of heavy nuclear membranous fraction and mitochondrial fragments.
(A) Heavy nuclear membranous fraction in the sample cuvette was reduced with a few mg of solid NADH in the presence of KCN (2 mM) prior to the addition of glycerol. Both the sample and the reference cuvettes contained 51 μg of nuclear phospholipids per ml of 50% (v/v) glycerol containing 50 mM Tris-HCl buffer (pH 7.4).
(B) The same sample as (A), but reduced with NADH in the presence of Antimycin A (300 μg/ml).
(C) Mitochondria, treated with Triton X-100 and DNase as described in Methods, were reduced with NADH as in (A) of Fig. 1. Both the sample and the reference cuvettes contained 304 μg of mitochondrial phospholipids.

The following experiments also supported the above conclusion: Incubation of the nuclear preparation with ascorbate gave a spectrum

similar to those obtained with NADH, whereas incubation with succinate produced no detectable cytochrome spectra. In the presence of Antimycin A, the broad peak between 553 mμ and 557 mμ fully developed with NADH, while the *a* peak of cytochrome $\underline{a} + \underline{a}_3$ decreased markedly (Curve B of Fig. 2).

Table 1. RNA, Phospholipids and Cytochrome Contents of Calf Thymus Fractions

	Nuclear membranous fr.		Mt.	Ms.
	heavy	light		
RNA (μg/mg protein)	35±2.5(4)	28±4.6(4)	70±18(4)	171±32(4)
PL (μg/mg protein)	149±25 (5)	213±24 (4)	155±35(4)	244±40(4)
Cytochromes (μμmoles/μg PL)				
$\underline{c} + \underline{c}_1$			0.55-0.67	
$\underline{b}$			0.49-0.55	
$\underline{b}$-type	0.29-0.50	0.45-1.34		
$\underline{b}_5$				0.21-0.22
$\underline{a} + \underline{a}_3$	0.25-0.50	0.36-0.80	0.86-1.13	

Values in parentheses represent the number of experiments. The standard deviations are also shown. The upper- and lower-limits are shown, when less than 3 experiments were made.
Mt: mitochondria; Ms: microsomes; PL: phospholipids.
It was assumed that the cytochromes detected in nuclear preparation had the same extinction coefficients and low-temperature enhancement ratios as those of corresponding mitochondrial cytochromes.

The contents of cytochromes in the nuclear preparations are almost comparable to those in mitochondria, as compared on the basis of the phospholipid content (Table 1). This value can not be explained simply as due to the extent of mitochondrial contamination (less than 3%) or of microsomal contamination (less than 8%) in the final nuclear preparations.

It may be concluded from this and the previous papers that the cytochromes associated with isolated nuclei are actually of nuclear origin and reside in the membranous structures, perhaps in the inner nuclear membrane, at concentrations comparable to those of mitochondrial pigments, and that the nuclear cytochrome system is composed at least of cytochromes c, a + a_3 and a b type cytochrome. No definite statement can be made as yet concerning the occurrence of cytochrome c_1 in the nuclear membranous structures. Further work is in progress to elucidate the question.

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