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Isolation and Characterization of Nuclear Membranes from Calf and Rat Thymus

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Summary: A method is described for isolating nuclear membranes from rat and calf thymus glands which involves fragmentation of isolated nuclei, extraction with high salt concentrations and purification of the membrane material in a continuous sucrose gradient. The procedure yields a single band of nuclear membranes at a density of about 1.19 g/cm^3 with a 50% recovery of the total phospholipids of the nuclei. The structural preservation and purity of the nuclear and nuclear membrane fractions has been examined in the electron microscope and quantified by morphometry.

Chemical composition of the nuclear membranes (protein, RNA, DNA, the various phospholipids, and cholesterol) and enzyme activities (ATPases, glucose-6-phosphatase, NADH- and NADPH-cytochrome *c* oxidoreductases, and cytochrome *c* oxidase) are compared with the corresponding data from total nuclei, condensed chromatin, and other subcellular fractions (light and heavy microsomes, mitochondria). The biochemical data (e. g. the relatively high contents of RNA, protein, phosphatidylcholine, phosphatidylethanolamine, $Mg^{2\oplus}$-ATPase, and NADH-cytochrome *c* oxidoreductase, coincident with low contents of cholesterol, sphingomyelin, cardiolipin, $(Na^{\oplus}+K^{\ominus})$-ATPase, and cytochrome *c* oxidase) emphasize the relationship of the nuclear envelope with the membranes of the endoplasmic reticulum. Findings of low amounts of cardiolipin and cytochrome oxidase activity are not interpreted as support for the concept of the existence of these components in thymus nuclei and nuclear membranes but rather are attributed to the mitochondrial contamination in these fractions and can, indeed, be quantitatively accounted for by the mitochondrial contamination as determined by morphometry.

Isolierung und Charakterisierung von Zellkernmembranen aus Kälber- und Rattenthymusdrüsen

Zusammenfassung: Eine Methode zur Isolierung von Kernmembranen aus Thymusdrüsen von Ratte und Kalb wird beschrieben. Die Kernmembranen wurden durch mechanische Zerkleinerung der isolierten Kerne mit anschließender Extraktion in Medien hoher Ionenstärke gewonnen und über einen kontinuierlichen Rohrzuckergradienten, in dem sie eine einheitliche Bande der

* Some results of the present article are contained in the thesis of E. D. Jarasch and the „Diplomarbeit" of C. E. Reilly (Faculty of Biology, University of Freiburg, Germany).

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Abbreviations: ER: endoplasmic reticulum; TMPD: *N,N,N′,N′*-tetramethylphenylenediamine, Wurster's blue.

Enzymes:

ATPase, ATP phosphohydrolase (EC 3.6.1.3.)

Glucose-6-phosphatase, D-glucose-6-phosphate phosphohydrolase (EC 3.1.3.9)

NADH dehydrogenase, NADH:cytochrome *c* oxidoreductase (EC 1.6.99.3)

NADPH dehydrogenase, NADPH:cytochrome *c* oxidoreductase (EC 1.6.99.1)

Cytochrome *c* oxidase, cytochrome *c*:oxygen oxidoreductase (EC 1.9.3.1).

Dichte 1,19 g/cm³ bildeten, gereinigt. Die Ausbeute an Kernmembranphospholipiden betrug 50% des Gesamt-Phospholipidgehaltes der Kerne. Die Strukturerhaltung und Reinheit der Kern- und Kernmembranfraktionen wurde elektronenoptisch und morphometrisch untersucht.

Die chemische Zusammensetzung (Protein-, RNA-, DNA-, Phospholipid- und Cholesteringehalt) und Enzymaktivitäten (ATPasen, Glucose-6-phosphatase, NADH- und NADPH-Cytochrom-*c*-Oxidoreduktasen, sowie Cytochrom-*c*-Oxidase) in den Kernmembranen wurden mit den entsprechenden Werten in den Kernen, im Heterochromatin und in anderen Zellfraktionen (leichte und schwere Mikrosomen sowie Mitochondrien) verglichen. Der relativ hohe Gehalt der Kernmembranen an RNA, Protein, Lecithin, Phosphatidyläthanolamin, an $Mg^{2\oplus}$-ATPase- und NADH-Cytochrom-*c*-Oxidoreduktase-Aktivität, in Verbindung mit den niedrigen Werten an Cholesterin, Sphingomyelin, Cardiolipin und an $(Na^{\oplus}+K^{\oplus})$-ATPase- und Cytochrom-Oxidase-Aktivität unterstreicht ihre nahe Verwandtschaft mit Membranen des Endoplasmatischen Retikulums. Der gemessene, niedrige Gehalt an Cardiolipin und an Cytochrom-Oxidase-Aktivität wird nicht als Bestandteil der Thymuskerne und -kernmembranen gedeutet, sondern der, auch morphometrisch bestimmten, geringen Verunreinigung solcher Fraktionen mit Mitochondrien zugeschrieben.

Many studies have been made, over the last decades, of nuclei isolated from mammalian thymus tissue (e. g.[1–4], see also[5–14]). The isolated thymus nucleus has thus become one of the "classic" reference fractions in current biochemistry. Some of these studies have suggested the presence in thymus nuclei of activities not normally considered to be localized in the nuclei of other cell types, particularly striking examples of this being respiration and oxidative phosphorylation[5–11], which are usually thought to be confined to the inner mitochondrial membranes. Subfractionation studies of the thymus nucleus have so far focused onto the preparation of various chromatin fractions and "nucleoli"[12–14]. In order to further localize and characterize the putative membrane-bound activities, Ueda and his associates have recently developed procedures for the isolation of membrane-enriched fractions from thymus nuclei[15–17].

Following from our earlier work[18] we describe in this paper procedures, which do not employ detergents, for the isolation of calf and rat thymus nuclei and nuclear membranes, and the basic characterization of these preparations chemically, biochemically and morphologically. This provides a basis for further examinations of the proposed localization of the activities in question in thymus nuclear membranes.

Materials and Methods

Reagents

ATP, glucose 6-phosphate, cytochrome *c*, NADH, NADPH, and isocitrate dehydrogenase were bought

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from Boehringer Mannheim GmbH; bovine serum albumin, oligomycin, ouabain, and isocitrate from Serva GmbH & Co., Heidelberg; and TMPD from Schuchardt GmbH & Co., München (this chemical was post-purified by recrystallization from acidic ethanol). Rotenone was obtained from Sigma Chemical Co., St. Louis, Mo. (this chemical was post-purified by recrystallization from trichloroethylene) and [γ-^{32}P]ATP, specific radioactivity about 1 Ci/mmol from the Radiochemical Centre, Amersham, England.
All other chemicals were analytical grade reagents obtained from E. Merck, Darmstadt.

Isolation media

Medium A: 0.4M sucrose, 10mM $CaCl_2$,70mM KCl.

Medium B: 1.8M sucrose, 3mM $CaCl_2$, 70mM KCl.

Medium C: 2.0M sucrose, 3mM $CaCl_2$, 70mM KCl.

High salt concentration medium: 0.4M sucrose, 1.5M KCl.

All media were buffered with 10mM Tris/HCl buffer, pH 7.2.

Animal material

Thymus glands from calves were obtained at the local slaughter house immediately (i. e. within 5 min at the best) after death by electric shock. The glands were immersed in a large volume of ice-cold medium A, cut into thin slices and the connective tissue was largely removed before homogenization. Pieces of the gland were processed in parallel for electron microscopy (for methods see below) in order to follow possible cytological *post mortem* changes.
Rat thymi were removed immediately after decapitation of the animals, cut into pieces, incubated in ice-cold medium A and immediately homogenized. About 20 glands of three weeks old rats of both sexes (strain BR 46 – Wistar II, purchased from F. Brünger, 4801 Bokel, Westf., Germany) were pooled for one experiment, yielding about one g wet weight of tissue.

Isolation procedures

Nuclei and nuclear membranes were initially isolated as described by Reilly[18]. In later experiments we used the following more advanced procedures which are essentially modifications of those described by Franke *et al.*[19] and Kartenbeck *et al.*[20] for mammalian liver tissue.

Nuclei: The tissue from which nuclei were isolated was homogenized in at least a tenfold volume of medium A using a loosely fitting glass-Teflon Potter-Elvehjem homogenizer, filtered through a single layer of nylon cloth (mean mesh lumen 120 μm) and centrifuged at $375 \times g$ for 10 min. The supernatant was decanted and collected for preparation of the mitochondrial and microsomal fractions. The pellet was resuspended in two volumes of medium B using the Potter-Elvehjem homogenizer, then layered on top of an equal volume of medium B, and centrifuged at $20000 \times g$ for 1 h. This supernatant was combined with that of the previous centrifugation step. The nuclear pellet was resuspended in medium A and centrifuged at $500 \times g$ for 10 min. The resulting pellet was resuspended in medium C to give a final sucrose concentration of 1.8M and then layered over a cushion of pure medium C. After centrifugation ($80000 \times g$ for 30 min) in a swinging bucket rotor the pellet consisting of purified nuclei was gently resuspended in medium A and finally pelleted at $500 \times g$ for 10 min.

Nuclear membranes: The purified nuclear pellet was disrupted by resuspension in 0.4M sucrose using a rotating blades homogenizer (Fa. E. Bühler, 74 Tübingen, Germany), followed by sonication (15 pulses for 2 sec at 8 Amp with a Branson Sonifier S125, Branson Instruments, Stamford, Conn., U.S.A.). A solution of 2.5M KCl in 10mM Tris/HCl buffer, pH 7.2, was added to give a final KCl concentration of 1.5M. This suspension was extracted for 3 h with moderate stirring at 4 °C. After centrifugation ($1000 \times g$ for 10 min) the resulting pellet was sonified again in about 20 vol. of the high salt concentration medium and stirred for another hour in the cold. Large nuclear and heterochromatic fragments were then removed by a low speed centrifugation ($1000 \times g$ for 10 min). The supernatants of both extraction steps were combined, and the membrane material was sedimented at $110000 \times g$ for 1 h. After decanting, the pellet was resuspended in ca. 5 *ml* of the high salt concentration medium and layered on top of a linear sucrose gradient (density from 1.12 to 1.26 g/cm^3) made up in 70mM KCl, 3mM $CaCl_2$ and 10mM Tris/HCl, pH 7.2. After centrifugation at $80000 \times g$ for 3 h in swinging buckets, the nuclear membranes banded at a density of about 1.19 g/cm^3. The banded material was collected, diluted with medium A and finally pelleted at $110000 \times g$ for 1 h.
For comparison we prepared in parallel the condensed chromatin fraction ("heterochromatin", "inactive chromatin" of the literature, e. g.[13,21]).

Condensed chromatin: This was obtained from the purified nuclear fraction by a modification of the method of Frenster *et al.*[12]. The nuclei were washed twice in plain Tris/HCl buffer (10mM, pH 7.2) and then disintegrated by combination of both the rotating blades homogenizer and sonication, exactly as described above for the preparation of the nuclear membranes. This suspension was centrifuged at $1000 \times g$ for 5 min. The

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pellet was resuspended in medium C and sedimented through a cushion of medium C by centrifugation at 80000 × *g* for 30 min in a swinging bucket rotor. The pellet was designated as the "condensed chromatin" fraction.

Mitochondria: A mitochondria-enriched fraction was prepared from the combined supernatants of the first and second centrifugation steps of the nuclear isolation. The suspension was diluted with Tris/HCl buffer, pH 7.2, in order to achieve a final sucrose concentration of 0.75M, and centrifuged at 10000 × *g* for 5 min. The pellet containing the mitochondria was resuspended in 0.25M sucrose and sedimented again at 10000 × *g* for 5 min.

Microsomes were obtained from the combined supernatants of these two centrifugation steps by centrifugation at 110000 × *g* for 90 min. The sedimented material was sonicated, extracted with high salt concentrations, and purified through a linear sucrose gradient in the same way as described for the nuclear membranes. A major microsomal band was consistently observed at a mean peak density of 1.17 g/cm³. In addition, a small lighter band (1.14 g/cm³) sometimes appeared which, presumably, was enriched in plasma membranes.

Chemical analyses

Protein was routinely determined by the procedure of Lowry *et al.*[22] with bovine serum albumin as standard. The gross composition of the cellular fractions was analyzed as described elsewhere[19,20]. Phospholipids were separated by two-dimensional thin-layer chromatography using two different separation systems[23,24]. Cholesterol was determined by the method of Clark *et al.*[25].

Enzyme assays

The incubation mixture of the glucose-6-phosphatase assay was prepared according to Nordlie and Arion[26], that for the ATPases was described by Franke *et al.*[19]. The enzyme activities were measured by the release of inorganic phosphate as described by Martin and Doty [27]. Rat thymus ATPases were also determined with [γ-^{32}P]ATP using the isotope distribution method of Lindberg and Ernster[28]. NADH-cytochrome *c* oxidoreductase was assayed according to Mahler[29] in the presence of 1mM KCN, with and without 3μM rotenone. NADPH-cytochrome *c* oxidoreductase was measured as described by Masters *et al.*[30] in the presence of 1mM isocitrate and about 200 mU of isocitrate dehydrogenase from pig heart as the NADPH regenerating system. Cytochrome *c* oxidase was assayed polarographically with a Beckman O_2 macroelectrode equipped with a Beckman Physiological Gas Analyzer (Model 160) and adapted for a 1.8 m*l* thermostatted cuvette of the Gilson Oxygraph, Model K. The oxygen consumption was measured according to Chuang *et al.* [31] after addition of ascorbate, TMPD, and cytochrome *c*.

Specific enzyme activities are expressed as mU/m*g* protein. 1 mU corresponds to the turnover of 1 nmol of substrate/min.

Electron microscopy

Samples (tissue pieces and pelleted fractions) were prepared as described elsewhere, usually with inclusion of 3mM $CaCl_2$ or $MgCl_2$ in the fixative (e. g.[32]). Fixation with glutaraldehyde and osmium tetroxide was carried out by simultaneous[33] or sequential use (4% glutaraldehyde in 50mM sodium cacodylate buffer, pH 7.0, 30min; cold buffer wash; 2% osmium tetroxide, 2 h; cold water wash; 1% uranyl acetate, 8 h). For methods of dehydration, embedding, sectioning, and electron microscopy see the references given above. Micrographs were taken with a Siemens Elmiskop IA.

Results

Morphology of fractions

The fractions were routinely examined by light and electron microscopy. Under the conditions used, the isolated thymocyte nuclei (Fig. 1) exhibited a very condensed state of their chromatin, with the interchromatinic regions showing loosely packed aggregates of granulo-fibrillar masses. The nuclear envelope was well preserved in some nuclei but was partially lacking in others. Nuclear surface

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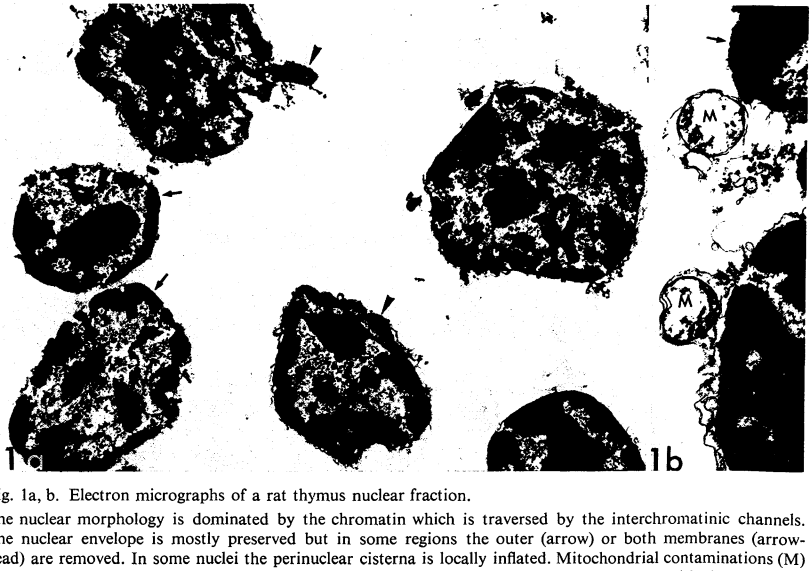

Fig. 1a, b. Electron micrographs of a rat thymus nuclear fraction.
The nuclear morphology is dominated by the chromatin which is traversed by the interchromatinic channels. The nuclear envelope is mostly preserved but in some regions the outer (arrow) or both membranes (arrowhead) are removed. In some nuclei the perinuclear cisterna is locally inflated. Mitochondrial contaminations (M) which could be found in the nuclear fraction are often in a conspicuously close association with the surface of the nuclei, demonstrating a membrane-to-membrane adhesion. Fig. 1a: × 9000; Fig. 1b: × 15000

regions were noted in which only the outer nuclear membrane was absent, besides others in which both membrane leaflets were lacking. Not infrequently, parts of the perinuclear cisterna were inflated, resulting in bizarre shaped sac formations (Fig. 1a).

However, some contaminations of the nuclear fractions were noted and appear in our opinion, as well as those of others[6,7,34,35] to be to some extent inevitable with this tissue. In morphometric determinations from the electron micrographs we traced, e. g., the relative lengths of membraneous components recognized in nuclear fractions. We found that 72% of the total membrane profile length was clearly identified as nuclear membrane whereas 9.5% was of mitochondrial origin. The remaining 18.5% included endoplasmic reticulum and plasma membranes (but probably also some vesiculated nuclear and mitochondrial membranes). In our nuclear fractions the contamination with whole cells was negligibly low (one cell per 300 nuclei), a figure which is much below that reported in the fractions of other authors[6,34,35].

A special remark should be added concerning the mitochondrial contamination (Fig. 1b). Associations of the nuclear membrane with outer mitochondrial membrane are not uncommon *in situ* and seem to be stabilized by electron-opaque cross-linking threads (compare[36]). From our routine fixations of thymus tissue we got the impression that the number of such nucleo-mitochondrial associations is especially enhanced by *post mortem* changes (compare also[37]). Consequently, rat thymus is a superior material for preparing

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Table 1. Gross composition of the homogenate and cellular fractions from calf thymus.

Fraction	% dry weight*				
	Protein	Phospholipids	Cholesterol	RNA	DNA
Homogenate	68.9	8.3	2.1	3.4	17.3
Nuclei	59.0	4.1	0.6	2.8	33.5
Nuclear membranes	70.0	16.1	2.2	4.2	7.5
Microsomes	72.0	17.7	4.2	5.7	0.4

* Values were corrected to yield 100.0%. Carbohydrates and nonpolar lipids other than cholesterol were not determined.

nuclei compared to calf thymus tissue since the *post mortem* time interval until use can be kept much shorter by the experimenter.

The membrane fragment fractions (Fig. 2) isolated from calf and rat thymus nuclei consisted of vesicular and occasional short cisternal membrane formations. The size of the vesicles varied, with diameters ranging from 30 to 300 nm. As a consequence of both the low pore frequency and the small envelope fragment size, pores were relatively infrequent. It might be noteworthy that, even after the harsh high salt extraction procedure, occasionally pore complex substructures including the central granule were still identifiable (Fig. 2a).

The buoyant densities of the nuclear and inner mitochondrial membranes are almost identical. Therefore, mitochondrial contaminants of the nuclear fraction tend to be enriched by aggregation to and co-banding with the nuclear membranes (Fig. 2b).

The intimate association of the inner nuclear membrane with the peripheral condensed chromatin (for review see[38]) is often maintained and results in finely fibrillar strands adhering to the nuclear membrane vesicles which presumably represent the sheared-off DNA fragments (see also[18]).

The heterochromatin preparation and the other membraneous fractions (light and heavy microsomes and mitochondria) showed the normal appearance, with a higher frequency of obvious plasma membrane formations occurring in the light microsomes.

Chemical composition of fractions

Table 1 shows the gross composition of calf thymus cellular fractions. Data from rat thymus were similar (compare also Table 2). The relatively high DNA content of thymus tissue is explained by the fact that about half of the cellular mass in the thymocyte is occupied by the nucleus. The DNA/protein ratio of the isolated nuclei is markedly higher than in mammalian liver nuclei (w/w ratios of 0.57 compared to 0.33 determined previously in our laboratory[19]), but resembles the values observed in other nuclei with most of their chromatin present in the condensed form (e. g. avian erythrocyte nuclei[39]). RNA/protein ratios determined in both homogenate and nuclei are about half those in the corresponding liver fractions and are somewhat in contrast to the data reported by Betel and Klouwen[7], the difference possibly reflecting differences of age, physiological conditions or relative contents of thymocytes compared to other cell types in the glands. The RNA/DNA ratios of the homogenate and of the isolated nuclei are low when compared to data reported for mammalian liver and are close to the RNA/DNA ratios observed in cells not actively transcribing such as the nucleated erythrocytes[39,40].

In the calf thymus nuclear membrane fraction we observed a relatively high DNA content, but this was somewhat variable in different experiments. The DNA content was generally lower in the rat thymus nuclear membranes (2.2 to 4.5% of dry weight).

Thymus tissue contains a considerable amount of cholesterol, most of which was recovered with the "microsomal" fraction. This fraction did, however, also contain plasma membrane material, and it is probable that the high cholesterol content is a reflection of this.

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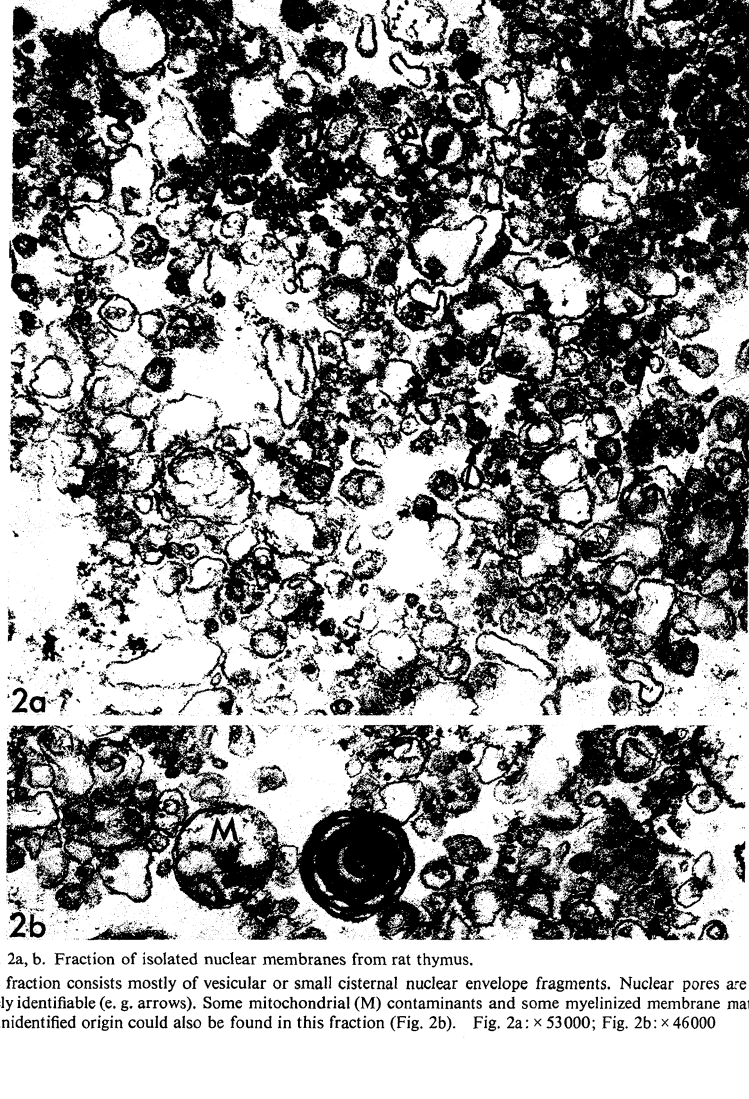

Fig. 2a, b. Fraction of isolated nuclear membranes from rat thymus.

The fraction consists mostly of vesicular or small cisternal nuclear envelope fragments. Nuclear pores are only rarely identifiable (e. g. arrows). Some mitochondrial (M) contaminants and some myelinized membrane material of unidentified origin could also be found in this fraction (Fig. 2b). Fig. 2a: × 53000; Fig. 2b: × 46000

Table 2. Weight per weight ratios of phospholipids and protein in calf and rat thymus fractions.
PL = phospholipid; PC = phosphatidylcholine (lecithin); DPG = diphosphatidylglycerol (cardiolipin).

Fraction	Calf thymus			Rat thymus		
	PL/Protein	PC/PL	DPG/PL	PL/Protein	PC/PL	DPG/PL
Homogenate	0.12	0.59	0.021	0.12	0.49	0.018
Nuclei	0.07	0.65	0.012	0.07	0.53	0.008
Condensed chromatin	0.09	0.62	0.007	0.08	0.54	0.002
Nuclear membranes	0.23	0.68	0.017	0.19	0.52	0.008
Crude mitochondria	0.19	0.54	0.046	0.16	0.46	0.062
Microsomes	0.26	0.56	0.009	0.37	0.53	0.012

Table 2 gives some selected ratios (w/w) for the chemical components present in the cellular fractions from calf and rat thymus. The phospholipid/protein ratios as well as the ratios of the major phospholipid component, phosphatidylcholine, to the total phospholipid content are similar in both tissues, although the phosphatidylcholine content was always lower in the rat thymus fractions than in the corresponding fractions from calf.

Diphosphatidylglycerol (cardiolipin) contents are especially interesting since this phospholipid is generally considered to be confined to the inner mitochondrial membrane and to provide a mitochondrial marker. It can be seen from the table that the cardiolipin/phospholipid ratio of calf thymus nuclei was about half that of the total homogenate, and that in the nuclear membranes there was even an enrichment relative to the nuclei. In rat thymus nuclei cardiolipin constituted only about 8% of that present in the homogenate whereas ca. 77% was recovered in the mitochondrial plus microsomal fractions. In this case the relative amount of cardiolipin in the nuclear fractions was lower than in calf thymus and no enrichment was observed in the nuclear membranes relative to the nuclei. This suggests a variable degree of mitochondrial contamination in our nuclear membrane preparation. It is difficult to calculate exactly the mitochondrial contamination on the basis of the cardiolipin content, because even in our best mitochondrial preparations (i. e. those with the highest cytochrome oxidase activity) only at most 50% of the total membrane material could be unequivocally identified under the electron microscope as being of mitochondrial origin. Assuming that cardiolipin is solely localized in the inner mitochondrial membranes (estimated to represent roughly 16% of the total phospholipid) one could estimate a contamination by these membranes totalling about 5% of the membrane phospholipid material of the isolated rat thymus nuclei and nuclear membranes. The corresponding values of the calf thymus fractions were 9 and 13% for the nuclei and nuclear membranes, respectively. These data are in agreement with the findings of the cytomorphometrical investigations (see above).

In both calf and rat thymus the condensed chromatin preparations contained much less cardiolipin on a w/w basis than the nuclei or nuclear membranes, and again this content was smaller in the rat. Procedures for isolating heterochromatin also select for the chromatin-associated membranes (i. e. nuclear membranes) relative to all other membranes not tightly attached to chromatin. Consequently, as expected, the condensed chromatin contained the least amount of contaminating membrane material, including the mitochondrial ones, though even here occasional mitochondria-derived membrane profiles could be identified in the electron microscope. Therefore, the decrease in the ratio of cardiolipin to total phospholipids, in going from isolated nuclei to the heterochromatin fraction, militates against the possibility that cardiolipin is an endogenous constituent of the nuclear membrane in thymus.

The phospholipid pattern of calf thymus fractions is given in Table 3. On the whole, the distribution of the various phospholipid components is quite similar to that observed in other tissues from a variety of vertebrate species (for review see[41];

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Table 3. Phospholipid composition of cellular fractions from calf thymus.

Phospholipid	% weight of total lipid phosphorus				
	Nuclei	Nuclear membranes	Heavy microsomes	Light microsomes enriched in plasma membranes	Crude mitochondria
Phosphatidylcholine	65.0	68.0	56.4	44.5	54.2
Lysophosphatidylcholine	1.4	1.7	1.8	0.9	3.2
Phosphatidylethanolamine	17.9	15.0	21.8	31.0	14.3
Lysophosphatidyl-ethanolamine	4.5	4.8	2.7	0.9	8.0
Phosphatidylinositol +phosphatidylserine	8.0	6.1	5.0	7.7	4.4
Sphingomyelin	1.1	1.0	8.7	13.2	9.2
Diphosphatidylglycerol	1.2	1.7	0.9	0.2	4.6
Unidentified phospholipids	0.9	1.7	2.7	1.6	2.1

see also[24,42]). Quantitatively, phosphatidylcholine and phosphatidylethanolamine are the most important phospholipids in all fractions investigated. (The relatively high content of lysophosphatidylcholine and lysophosphatidylethanolamine in some of the fractions possibly reflects artefactual saponification in the course of the preparation). Phosphatidylcholine and phosphatidylethanolamine, together with their lyso derivatives, amount to about 80 to 90% of the total phospholipid in the thymocyte membranes. Sphingomyelin was present in the nuclear membranes only in minor amounts, but was significantly enriched in the light microsomal fraction, suggesting its higher content in plasma membranes.

Enzyme activities

The specific activities of certain membrane-bound enzymes are summarized in Table 4, both on a protein and on a phospholipid basis.

$Mg^{2\oplus}$-dependent ATPase activity was found to be highest in the mitochondria, somewhat lower in the microsomes, and lowest in the nuclei and the condensed chromatin. The high activity of the mitochondria is especially striking when expressed on the basis of the phospholipid content. However, not all ATPase activity in mitochondria is reported to be membrane bound (e. g.[43,44]), therefore this value may be an overestimation of the true membraneous ATPases. About 30% of the mitochondrial activity could be inhibited by 50μM oligomycin. Stimulation by 0.5mM 2,4-dinitrophenol was not observed, indicating that the mitochondria in our preparations were in an already uncoupled state. Neither oligomycin nor dinitrophenol had any effect on the other fractions studied. The specific activities per phospholipid of the ATPase were nearly identical in the nuclei and nuclear membranes, supporting the membraneous localization of this activity. In the condensed chromatin, however, the $Mg^{2\oplus}$-ATPase activity expressed on phospholipid basis was considerably reduced when compared to the nuclei or nuclear membranes; an unexpected finding in relation to the concept that nuclear $Mg^{2\oplus}$-ATPase is mostly bound to the membrane[20,45,46].

$(Na^{\oplus}+K^{\oplus})$-stimulated, ouabain-inhibited ATPase was present in thymus microsomes and, to a lesser degree, in the mitochondrial fractions. These activities were almost completely inhibited in the presence of 50μM oligomycin. Practically no $(Na^{\oplus}+K^{\oplus})$-ATPase activity was observed in the nuclei, in the condensed chromatin, and in the

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Table 4. Enzyme activities of rat thymus homogenate and cellular fractions.

Enzyme	Homogenate	Nuclei	Condensed chromatin	Nuclear membranes	Microsomes	Crude mitochondria
$Mg^{2\oplus}$-ATPase						
[mU/mg protein]	73.4	17.5	11.7	52.0	208	428
[mU/mg phospholipid]	612	250	146	274	547	2680
$(Na^{\oplus}+K^{\ominus})$ stimulated $Mg^{2\oplus}$ – ATPase						
[mU/mg protein]	20.0	2.0	1.3	2.2	180	60
[mU/mg phospholipid]	167	29	16	12	474	375
Glucose-6-phosphatase						
[mU/mg protein]	10.0	1.5	trace	1.0	2.2	2.4
[mU/mg phospholipid]	83	21	–	5	6	15
Rotenone insensitive NADH – cytochrome *c* oxidoreductase						
[mU/mg protein]	6.0	4.6	5.1	17.3	31.2	8.2
[mU/mg phospholipid]	50	66	64	93	82	51
NADPH – cytochrome *c* oxidoreductase						
[mU/mg protein]	2.5	trace	0	0	trace	0
[mU/mg phospholipid]	21	–	–	–	–	–
Cytochrome *c* oxidase						
[mU/mg protein]	94	40	16	75	182	330
[mU/mg phospholipid]	784	570	200	398	478	2060

nuclear membranes. The addition of 10mM $CaCl_2$ to the enzyme assays inhibited both ATPase activities by about 50%. Activity of both ATPases in the presence of 10mM $CaCl_2$ (instead of $MgCl_2$) was in all the fractions only about 15% of that with $MgCl_2$.

Glucose-6-phosphatase activity seems to be absent from both rat and calf thymus; the very low activity found in rat thymus homogenate was almost exclusively (90%) recovered with the non-sedimentable protein fraction and can possibly be attributed to unspecific phosphohydrolases. Similarly, no NADPH-cytochrome *c* oxidoreductase activity was detected in thymus membrane fractions. The low activity which we observed in the homogenate was extracted into the supernatant during the fractionation procedure. Since NADPH-cytochrome *c* oxidoreductases from other tissue (liver, adrenal cortex) are tightly bound to the membrane and become solubilized only after harsh treatments such as lipase digestion or prolonged sonication[47,48], the cellular nature of this activity in thymus remains unclear.

Oxidized cytochrome *c* was reduced by rat thymus fractions with NADH as electron donor. 3μM rotenone inhibited the activity in the total homogenate by 40%. The highest specific activity of the rotenone-insensitive NADH-cytochrome *c* oxidoreductase was found in the microsomal fraction, followed by the nuclear membranes with about half of this activity. When calculated on the basis of the phospholipid content, however, the activities in the nuclei and nuclear subfractions were comparable to those observed in the microsomes. While the mitochondria-enriched fraction exhibited a relatively lower rotenone-insensitive activity, the rotenone-sensitive NADH-cytochrome *c* oxido-

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reductase was confined to this fraction. However, only 30% of this latter activity originally present in the homogenate could be recovered in the various fractions, indicating that the integrity of the mitochondrial respiratory chain, which is necessary for the rotenone-inhibited electron transfer from NADH to cytochrome *c*, was largely destroyed during the course of the isolation.

Cytochrome *c* oxidase (Table 4) was highest in the mitochondria but rather high activities were also observed in the microsomal preparation. These two fractions contained about 76% of the cytochrome *c* oxidase activity of the homogenate while they contributed only 24% of the total protein content. The remaining activity was recovered with the nuclei (the figure is much below that reported by Betel[8]). This activity is of special interest in the view of the many reports on "nuclear respiration" in thymus. The preparation of nuclear membranes from isolated nuclei resulted in an enrichment in cytochrome *c* oxidase activity by a factor of 2 with less than 30% recovery of the total nuclear activity. However, when expressed on a phospholipid basis, cytochrome *c* oxidase was considerably decreased in nuclear membranes compared to the activity in the corresponding nuclei. In the condensed chromatin cytochrome *c* oxidase activity was much reduced. This fraction contained 50% of the nuclear protein, but only 20% of the oxidase activity. Another 27% of protein associated with 48% of the nuclear activity was recovered in a particulate fraction sedimentable at $110000 \times g$ for 1 h from the supernatant of the heterochromatin sedimentation (at $1000 \times g$ for 1 min; see Methods). In all fractions the cytochrome *c* oxidase activity was competely abolished in the presence of 1mM KCN or 1mM Na_2S. An inhibition by 80 to 85% was observed with 1mM NaN_3.

Discussion

The method for isolating thymus nuclear membranes developed in our laboratory (compare also[18,19]) differs from that described by Ueda *et al.*[15] since it does not involve the use of strong membranolytic detergents but rather maintains the membrane architecture. It shows some preparative parallels to the procedure most recently reported from Ueda's group[17] but does not use deoxyribonuclease for separation of the chromatin.

Our procedure yields thymus nuclear membrane fractions which are comparable in membrane integrity, relative biochemical activities, and purity to the fractions obtained from mammalian liver material with various methods[19,20,45,49–53]. Our gross composition data of the isolated thymus nuclear membranes from both animals agree fairly well with the calf thymus data from Ueda's laboratory with respect to relative contents of protein, RNA, total phospholipid, phosphatidylcholine, and phosphatidylethanolamine[15–17]. In contrast to their fractions, our preparations contain significant amounts of DNA associated with the membranes (for data on DNA attached to nuclear membranes in other tissues see[19,20,39,45,50–53]) and contain less sphingomyelin and cholesterol. Since the latter lipid components are concentrated in plasma membranes in various cell systems[39,54–59] the enrichment in nuclear membranes or endoplasmic reticulum fractions must be considered as possibly indicating contamination with plasma membranes or whole cells.

An occurrence of cardiolipin has so far been only reported in rat thymus homogenate[60] but not in other studies of isolated thymus nuclei[61] and nuclear membranes[16]. Our figure of 0.008 cardiolipin/total phospholipids (w/w) correlates well with

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our morphometric calculations for mitochondrial contamination. Therefore, we suggest that this amount of cardiolipin (for bovine liver see also [42,62]) should not be considered as being a true nuclear membrane constituent.

The nuclear envelope fragments made by this procedure are relatively small compared to the nuclear envelope "ghost" preparations obtained after more gentle disruption of the nuclei from liver, hepatoma, *Tetrahymena*, and plant cells[51,52,63–65]. This might be explained by the intense sonication which is necessary for sufficient recovery of membrane materials on the sucrose gradients (recoveries of membrane phospholipids from nuclei were between 43 and 51% in the course of the present study). Similar small-sized nuclear membrane vesicles have been reported for preparations from avian erythrocytes[39] and mammalian liver[19,20,45,46,50,53,66,67]. Small-sized vesicles are also contained in the calf thymus nuclear membrane fractions described by Matsuura and Ueda[17], particularly in their light subfraction.

In general, the enzyme activity pattern of the thymus nuclear membrane apparently reflects its close relationship with the endoplasmic reticulum which has also been enzymologically demonstrated for liver nuclear membranes[19,20,46,49–51,68]. This is illustrated by the presence of $Mg^{2\oplus}$-ATPase and the near absence of cation-stimulated ATPase, as well as by the presence of the rotenone-insensitive NADH-cytochrome *c* oxidoreductase. Our finding that the latter enzyme activity is constitutive to the nuclear and endoplasmic reticulum membranes disagrees with the early statement of Stern and Timonen[69] on their calf thymus nuclear preparations but corresponds to the spectrophotometric observation of cytochrome b_5 (the characteristic pigment of the NADH-dependent microsomal electron transfer system; for review see[70]) in the calf thymus nuclear membrane fractions of Matsuura and Ueda[17]. On the other hand, the thymocyte nuclear membranes exhibit some tissue specificities in membrane-bound enzymes, e. g. the lack of glucose-6-phosphatase and NADPH-cytochrome *c* oxidoreductase which are characteristic for nuclear and endoplasmic reticulum membranes from liver[20,51,68,71].

Our measurements of cytochrome *c* oxidase and cardiolipin lead us to be sceptical of results suggesting a unique nuclear membrane locus for cytochrome aa_3-dependent oxygen consumption and of oxidative phosphorylation. These suggestions have not only come from studies on thymus nuclei and nuclear membranes but also from various other types of nuclei such as mammalian liver[45,72–74] and even plants[75]. The data presented in this article, however, are compatible with the interpretation that the cytochrome oxidase activity found in thymus nuclei and nuclear membranes is due to the presence of mitochondrial contaminants. This is especially suggested by the correlation of cardiolipin present with the cytochrome oxidase activity as well as by the decreases in cytochrome oxidase activity per membrane phospholipid when one preparatively selects for nuclear membranes *versus* other membranes by preparing the condensed chromatin (Table 4). We have found, as have others[6,7,34,35], that thymus nuclei are hard to purify from a certain amount of mitochondrial fragments. It is likely that any minor contamination of non-nuclear membrane material in a nuclear fraction becomes enriched

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and almost totally recovered in nuclear membranes prepared from it by means of sucrose gradient techniques. (So, for instance, contamination of nuclei with 1% mitochondrial membranes, on a protein basis, results in ca. 7 to 10% of this contamination in the corresponding nuclear membranes.) Since none of the thymus nuclear fractions reported in the literature has been convincingly shown to be free of considerable contamination (i. e. roughly 5% on membrane protein or phospholipid basis) with other cellular membranes (in particular mitochondrial and plasma membranes) we tend to conclude that at the moment there is no justification for considering cytochrome oxidase to be a true nuclear membrane constituent.

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