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OXIDATIVE PHOSPHORYLATION IN NUCLEI ISOLATED FROM RAT THYMUS

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SUMMARY

1. Nuclei were isolated from rat thymus in 0.25 M sucrose–3 mM $CaCl_2$. Fractionation of thymus tissue showed that the nuclear fraction could be contaminated with 10% intact cells at the most.

2. The nuclear fraction contained about 50% of the cellular ATP. Under anaerobic conditions nuclear ATP is degraded. When, after an anaerobic incubation, the nuclei are incubated under aerobic conditions endogenous nuclear ATP is resynthesized. The amount of ATP found cannot be accounted for by mitochondria or intact cells. It could also be shown that the ATP synthesis is not due to such contamination.

3. Nuclear ATP synthesis and respiration were inhibited by amytal, rotenone, and carbon monoxide. The latter inhibition was reversed by light. 2,4-Dinitrophenol abolished ATP synthesis and accelerated respiration. Oligomycin inhibited respiration and phosphorylation. The inhibition of respiration by oligomycin could be released by dinitrophenol. ATP synthesis was also inhibited by lewisite. This inhibition could be reversed by BAL. No inhibition, however, was found with arsenite or arsenite + BAL.

4. A P:O ratio between 0.6 and 1.0 was obtained for nuclear phosphorylation. This must be considered as a minimal value.

5. It is concluded that thymus nuclei are capable of respiratory-chain-linked oxidative phosphorylation.

INTRODUCTION

Aerobic ATP synthesis in calf-thymus nuclei has been demonstrated by OSAWA, ALLFREY AND MIRSKY[1] and MCEWEN, ALLFREY AND MIRSKY[2–4], and in rat thymus by CREASEY AND STOCKEN[5], ORD AND STOCKEN[6] and ourselves[7,8]. The mechanism of this ATP synthesis is essentially unknown, although it shows striking similarities with oxidative phosphorylation[2–4,9].

The present paper describes a study on the occurrence and the mechanism of aerobic ATP synthesis in nuclei isolated from rat thymus. The results indicate

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that the thymus nucleus is capable of the synthesis of ATP by a process that must be considered as respiratory-chain-linked oxidative phosphorylation.

Part of the results have been published in preliminary form[8–11].

MATERIALS AND METHODS

Fractionation of thymus tissue

The fractionation of thymus and the isolation of the nuclear fraction were performed essentially according to the method developed by KLOUWEN AND BETEL[7], with some modifications as outlined below.

Thymus glands were obtained from male and female albino rats, 5–7 weeks old and weighing 130–160 g. Rats under light ether narcosis were decapitated with scissors and the thymus glands removed. 3–8 thymus glands were pooled. They were finely minced with scissors and 1 vol. (w/v) 0.5 M sucrose and 8 vol. 0.25 M sucrose–3.3 mM $CaCl_2$ were added. The mince was homogenized in a Potter–Elvehjem homogenizer. After homogenization the suspension was filtered through 6 layers of nylon gauze in a stainless-steel Dounce homogenizer (clearance 0.08 mm). After seven strokes in this homogenizer, the tissue suspension was filtered through 12 layers of nylon gauze and centrifuged for 7 min at 900 $\times$ *g*. The supernatant was discarded and the pellet suspended in 0.25 M sucrose–3 mM $CaCl_2$ and centrifuged again. The pellet from this centrifugation consisted mainly of nuclei and was suspended in 0.25 M sucrose–3 mM $CaCl_2$. In general 1.5–3 ml sucrose–$CaCl_2$ were added finally per original thymus. The final suspension contained about 12 mg protein and 0.4 mg DNA P per ml. The isolation procedure was performed at 0–4°. Thymus mitochondria were isolated from the supernatant obtained from both centrifugations by centrifugation for 15 min at 13600 $\times$ *g*. The mitochondrial sediment was suspended in 0.25 M sucrose–3 mM $CaCl_2$, or in 0.25 M sucrose. The supernatant of the mitochondrial fraction was not fractionated.

Isolation of thymus cells

Thymus glands were finely minced in a modified Krebs–Ringer medium (0.13 M NaCl, 4.4 mM KCl, 3 mM $CaCl_2$, 1.1 mM $MgSO_4$, 9 mM Tris; pH 7.4) or in 0.25 M sucrose–3 mM $CaCl_2$. The tissue mince was gently squeezed through 4 layers of nylon gauze with the aid of a glass rod and the cells were washed through the gauze with the medium used. The cell suspension was centrifuged for 7 min at 900 $\times$ *g* and the pellet was suspended in the isolation medium.

Analytical methods

Unless otherwise indicated, the acid-soluble extract was prepared from the nuclear and cellular pellet after centrifugation of the suspension as previously described[7]. In these extracts ATP, ADP and AMP were determined by enzymatic methods[12]. Electrophoresis of acid-soluble extracts was performed according to KLOUWEN[13]. The determination of DNA, RNA and protein has been described[7]. Oxygen uptake was measured polarographically (Oxygraph Model K, Gilson Medical Electronics, Middleton, Wisc. U.S.A.). The medium contained 0.25 M sucrose, 15 mM NaCl, 50 mM Tris and 3 mM $CaCl_2$; the pH was 7.4. The oxygen concentration in the air-saturated medium was 0.241 mM.

Succinate–cytochrome *c* reductase activity was determined according to RABINOWITZ AND DE BERNARD[14] and cytochrome oxidase according to COOPERSTEIN AND LAZAROW[15]. The unit of succinate–cytochrome *c* reductase is the amount that reduces 1 μmole cytochrome *c* per min at 25°. One unit of cytochrome oxidase is the amount that initially oxidizes 1 μmole reduced cytochrome *c* per min. The initial velocity was calculated from the first-order rate constant by multiplication with the initial concentration of reduced cytochrome *c* (12.7 μM). Both activities were proportional to enzyme concentration for all tissue fractions tested. Both determinations also measured the activities present in intact cells as can be seen from the good agreement between both activities in thymus homogenate and thymus cells (Table I).

TABLE I

SUCCINATE–CYTOCHROME *c* REDUCTASE AND CYTOCHROME OXIDASE ACTIVITY IN THYMUS HOMOGENATE AND THYMOCYTES

Thymus glands were finely minced in 0.25 M sucrose–3 mM $CaCl_2$. From one part of the mince the homogenate was prepared, from a second part thymocytes were isolated in sucrose–$CaCl_2$.

Expt.		*munits per mg protein*	
		Succinate–cytochrome c reductase	*Cytochrome oxidase*
1	Homogenate	7.5	13.0
	Thymocytes	6.9	10.7
2	Homogenate	4.5	14.0
	Thymocytes	5.4	13.6

Deoxyribonuclease I (1 × crystallized) was obtained from Worthington Biochemical Corporation, Freehold, N.J. All other enzymes were obtained from C. F. Boehringer and Soehne, Mannheim, Germany. Oligomycin and antimycin A were purchased from Wisconsin Alumni Research Foundation, Madison, Wisc. Rotenone was a generous gift from Dr. D. E. GRIFFITH, University of Oxford, England, and atractyloside from Dr. T. AIELLO, University of Palermo, Italy. Lewisite and 2,3-dimercaptopropanol were gifts from Drs. P. J. CHRISTEN, Medical Biological Laboratory-TNO, Rijswijk, The Netherlands. All other reagents were obtained from E. Merck, Darmstadt, Germany or British Drug House Ltd., London, England.

RESULTS

Fractionation of thymus tissue

The results of a typical fractionation are shown in Table II. The nuclear fraction contained respectively 5 and 7 % of the succinate–cytochrome *c* reductase and cytochrome oxidase activities of the homogenate. In four other fractionation experiments 5–10 % of both activities were recovered in the nuclear fraction. Thus 5–10 % of the mitochondria present in the homogenate are recovered in the nuclear fraction (*cf.* ref. 7). If it is assumed that these mitochondria are exclusively present in intact cells,

TABLE II

FRACTIONATION OF RAT THYMUS

Data were obtained from an experiment in which three thymus glands were pooled.

Fraction	*Protein*		*DNA*		*RNA*		*Succinate–cytochrome reductase*		*Cytochrome oxidase*	
	mg	%	*mg*	%	*mg*	%	*munits*	%	*munits*	%
Homogenate	173	100	40.8	100	17.2	100	1020	100	2810	100
Nuclei	86	51	38.5	94	7.2	42	47	5	196	7
Mitochondria	17	10	0.5	1	3.4	18	908	89	2020	73
Supernatant	65	37	0.1	—	5.3	31	57	6	—	—
Yield	168	98	39.1	95	15.9	91	1012	100	2216	80

we may conclude that the maximal contamination of the nuclear fraction could exist of 10 % intact cells.

The RNA/DNA ratio of the nuclear fraction was 0.182 ± 0.027 (mean of 20 determinations ± S.D.). This value is higher than obtained by isolation methods using non-aqueous methods[16] but lower than reported by FAURÈS AND ERRERA for rat-thymus nuclei isolated in sucrose–$CaCl_2$ (ref. 17).

Adenine nucleotides in thymus nuclei

The presence of nucleotides in calf-thymus nuclei has been demonstrated by OSAWA, ALLFREY AND MIRSKY[1]. Rat-thymus nuclei also proved to contain a considerable amount of nucleotides. The main nucleotide in these nuclei is ATP. The nuclea· preparations contained 0.50 ± 0.07 μmole ATP per mg DNA P (mean of 45 determinations ± S.D.). In addition to ATP the nuclear fraction also contained GTP, UTP and CTP. By high-voltage electrophoresis of acid-soluble extracts from the nuclei it could be demonstrated that ATP accounted for 70–80 % of the nucleoside triphosphates (*cf.* ORD AND STOCKEN[6]). The nuclei also contained relatively small amounts of AMP and ADP (Table III).

TABLE III

NUCLEOTIDE COMPOSITION OF NUCLEAR ACID-SOLUBLE EXTRACT AS DETERMINED BY HIGH-VOLTAGE ELECTROPHORESIS

Values obtained in a single experiment.

	$A_{260\,m\mu}$ (% *of total*)
Bases + nucleosides	6
AMP	10
ADP	7
ATP	54
CTP	5
GTP + UTP	13
Unidentified fractions	4

The following evidence demonstrates that the amount of ATP found cannot be explained by contamination of the nuclei with cytoplasm or absorption of cytoplasmic ATP during the isolation:

a. Isolated thymocytes contain 0.88 μmole ATP per mg DNA P (mean of 8 determinations, range 0.7–1.0 μmole). If it is assumed that the nuclear fraction contains 10% intact cells, this contamination can explain the presence of maximally 0.1 μmole ATP per mg DNA P.

TABLE IV

ADENINE NUCLEOTIDES IN THYMOCYTES, THYMUS HOMOGENATE AND NUCLEI

Thymocytes were prepared in modified Krebs–Ringer solution. Homogenate and nuclei were prepared in 0.25 M sucrose–3 mM $CaCl_2$. In this experiment the fractions were not centrifuged before preparing the acid-soluble extract. $HClO_4$ was added to the suspensions.

Fraction	*μmoles per mg DNA P*			
	ATP	*ADP*	*AMP*	*Sum*
Thymocytes	0.90	0.15	0.10	1.15
Homogenate	0.58	0.15	0.57	1.30
Nuclei isolated from this homogenate	0.57	0.09	0.07	0.73

TABLE V

DEGRADATION OF ADDED ATP BY THE NUCLEAR SUSPENSION

1.50 μmoles ATP were added to 2 ml of the nuclear suspension (3.75 mg DNA per ml) and incubated at 25° and pH 7.2. At the time indicated 1 ml 10% $HClO_4$ was added. After centrifugation ATP was determined in the neutralized supernatant.

Addition	*Incubation time (min)*	*μmoles ATP*
None	0	0.20
1.50 μmoles ATP	0	1.70
1.50 μmoles ATP	5	0.75
1.50 μmoles ATP	15	0.20
None	15	0.20

b. Table IV shows that practically all ATP in the tissue homogenate is recovered in the nuclear fraction. Cytoplasmic ATP is completely degraded into AMP during homogenization. Since added ATP is not absorbed by the nuclei, but rapidly degraded into AMP (Table V; *cf.* McEWEN, ALLFREY AND MIRSKY[2]), it seems unlikely that part of the cytoplasmic ATP is absorbed by the nuclei during isolation. It must be concluded that nearly all ATP found in the nuclear fraction must be present inside the nuclei.

ATP synthesis

The isolated nuclei are unable to synthesize ATP from added AMP and ADP, even under conditions favorable for mitochondrial oxidative phosphorylation (*cf.*

McEWEN, ALLFREY AND MIRSKY[2]). Only endogenous AMP and ADP can be converted into endogenous ATP.

In order to measure nuclear ATP synthesis it is essential first to degrade endogenous ATP into AMP and ADP. In the experiments of McEWEN, ALLFREY AND MIRSKY[2] and ORD AND STOCKEN[6] this was accomplished by a time lag between the killing of the animal and the excision of the thymus gland. Due to the anaerobic conditions in the tissue, the ATP level decreased rapidly and AMP accumulated. Nuclei isolated rapidly from these glands contained a large amount of AMP. When these nuclei were incubated under aerobic conditions in a medium containing only 0.25 M sucrose and 3 mM $CaCl_2$, a rapid conversion of AMP into ATP occurred.

In our hands, this method of measuring ATP synthesis yielded a variable and small synthesis[7]. This was undoubtedly due to the fact that this synthesis from intranuclear AMP resumed even during the isolation of the nuclei.

Since it was likely that the disappearance of ATP was caused by the anaerobic conditions in the thymus tissue, it was decided to incubate freshly isolated nuclei *in vitro* in a N_2 atmosphere. As a result of this anaerobic incubation the ATP content of the nuclei was decreased. When these "*in vitro* aged" nuclei were incubated subsequently under aerobic conditions, a rapid resynthesis of ATP occurred. The mean values of ATP content of "0 min" nuclei, "*in vitro* aged" nuclei and aerobically ncubated nuclei are summarized in Table VI.

TABLE VI

ATP CONTENT AND ATP SYNTHESIS IN THYMUS NUCLEI

In these experiments the nuclear suspension was divided in three parts. From one part the acid-soluble extract was prepared immediately after isolation. The second part was incubated for 15 min at 30° under N_2: aged *in vitro*. The third part was incubated under N_2 and subsequently aerobically incubated for 45 min at room temperature. Isolation and incubation of the nuclei in 0.25 M sucrose–3 mM $CaCl_2$. The values represent the mean ± S.D. (number of determination).

ATP (μmole per mg DNA P)		
After isolation	*Aged in vitro*	*After aerobic incubation*
0.50 ± 0.07 (45)	0.11 ± 0.08 (71)	0.35 ± 0.07 (54)

A number of observations argue against the participation of mitochondria in the observed ATP synthesis.

a. ATP synthesis was not enhanced by addition of mitochondrial substrates, such as succinate, α-oxoglutarate, glutamate or pyruvate; nor by a medium favorable for mitochondrial oxidative phosphorylation. Moreover, addition of cytoplasm or thymus mitochondria had no effect (Table VII). Furthermore, thymus mitochondria isolated in sucrose–$CaCl_2$ or $MgCl_2$ proved to be completely uncoupled. No esterification of phosphate could be measured with succinate or α-oxoglutarate as substrates.

From these observations it seems extremely unlikely that free mitochondria contribute to the ATP synthesis.

b. The possibility was considered that the ATP synthesis occurs in mitochondria absorbed by the nucleus or which are present in small adherent tags of cytoplasm. In this case a transport of ADP from the nuclei to these mitochondria and a

TABLE VII

ABSENCE OF INFLUENCE OF CYTOPLASM AND MITOCHONDRIA ON NUCLEAR ATP SYNTHESIS

In Expt. 1 part of the nuclei was suspended in the cytoplasm fraction, *i.e.*, the supernatant obtained after the first centrifugation of the homogenate. In Expt. 2 nuclei and mitochondria were isolated in 0.25 M sucrose–3 mM $MgCl_2$. Mitochondria (10 mg protein) obtained from 3 thymus glands were added to nuclei obtained from 2 thymus glands. *ΔATP*: ATP after aerobic incubation for 45 min *minus* ATP after anaerobic incubation.

Expt.		*ΔATP (μmole per mg DNA P)*
1	Nuclei in sucrose–$CaCl_2$	0.29
	Nuclei in cytoplasm	0.27
2	Nuclei in sucrose–$MgCl_2$	0.22
	Nuclei + mitochondria	0.22
	Nuclei + mitochondria + 10 mM ADP + 1 mM EDTA + 3 mM $MgCl_2$ + 20 mM succinate + 15 mM phosphate buffer (pH 7.4)	0.21

TABLE VIII

EFFECT OF ATRACTYLOSIDE ON NUCLEAR ATP SYNTHESIS

The nuclei were suspended in 0.25 M sucrose–3 mM $CaCl_2$ to a final concentration of 0.8 mg DNA P per ml.

Atractyloside (μM)	*ΔATP (μmole per mg DNA P)*
0	0.21
50	0.24
100	0.21

reverse transport of the ATP synthesized has to occur. This transport in and out of the mitochondria could be expected to be inhibited by atractyloside[18,19]. However, atractyloside had no effect on nuclear ATP synthesis (Table VIII). The concentrations used completely inhibited oxidative phosphorylation with added ADP in thymus and liver mitochondria.

The possibility that the ATP synthesis is entirely due to contaminating intact cells has also been excluded. As has been pointed out, contamination with maximally 10% intact cells could explain at most 0.07–0.10 μmole ATP per mg DNA P. If it is assumed that only intact cells are responsible for the ATP synthesis, this could never exceed 0.10 μmole ATP per mg DNA P. The synthesis is in fact 2–3 times this value. Since the actual contamination does not exist of 10% intact cells, the contribution of cells to ATP synthesis will be minor.

It has been demonstrated by ALLFREY AND MIRSKY[20] that treatment of thymus nuclei with deoxyribonuclease (EC 3.1.4.5) removes part of the DNA and is accompanied by a strong inhibition of nuclear phosphorylation. The effect of deoxyribonuclease on phosphorylation could be counteracted by the presence of polyanions

TABLE IX

EFFECT OF DEOXYRIBONUCLEASE ON ATP CONTENT AND ATP SYNTHESIS IN THYMUS NUCLEI AND THYMUS CELLS

Nuclei were isolated in 0.25 M sucrose–3 mM $CaCl_2$, cells in Krebs–Ringer medium. Suspensions of nuclei and cells were divided in 3 equal parts. One part was incubated aerobically with 0.5 mg/ml deoxyribonuclease for 30 min at 30°, the second part with deoxyribonuclease + 10 mg/ml heparin, and the third without additions. After this treatment, the ATP synthesis in the three parts was determined. "0 min": μmole ATP/2 thymus glands after incubation for 30 min at 30°. ΔATP: μmole ATP/2 thymus glands synthesized during aerobic incubation (45 min) after preceding anaerobic incubation for 15 min at 30°. The nuclear suspensions in Expts. 1 and 2 contained, before deoxyribonuclease treatment, 0.75 and 0.84 mg DNA P per ml, respectively. The cell suspensions in Expts. 3 and 4 contained, before deoxyribonuclease treatment, 0.50 and 0.83 mg DNA P per ml, respectively.

Expt.		*–Deoxyribonuclease*		*+ Deoxyribonuclease*		*+ Deoxyribonuclease + heparin*	
		"0 min"	*ΔATP*	*"0 min"*	*ΔATP*	*"0 min"*	*ΔATP*
1	Nuclei	0.55	0.26	0.09	0.06	0.55	0.25
2	Nuclei	0.78	0.38	0.10	0.02	0.70	0.38
3	Cells	0.78	0.74	0.71	0.62	0.69	0.67
4	Cells	0.86	0.66	0.72	0.52	0.78	0.64

during incubation with this enzyme. These findings were confirmed (Table IX). Furthermore, it could be demonstrated that deoxyribonuclease had only a minor effect on resynthesis of ATP in thymocyte preparations. This minor effect is probably due to damaged cells in these preparations. As has been demonstrated by MCEWEN, ALLFREY AND MIRSKEY[2], deoxyribonuclease has no effect on mitochondrial oxidative phosphorylation. Also from these experiments it seems extremely unlikely that intact cells make an important contribution to nuclear ATP synthesis.

From the experiments presented in this section it is concluded that thymus nuclei are capable of an independent aerobic ATP synthesis.

Oxygen uptake by nuclear suspensions

Nuclear preparations isolated in sucrose–$CaCl_2$ showed a significant endogenous respiration, amounting to 3–5 μmoles O_2/h per mg DNA P (3.5–7 μl/h per mg protein). Addition of mitochondrial substrates such as succinate, β-hydroxybutyrate, α-oxoglutarate, pyruvate or glutamate, stimulated oxygen uptake only slightly (0–10%). The respiration of thymus mitochondria, on the other hand, was strongly stimulated by substrates. Therefore it is unlikely that the endogenous respiration of the nuclei can be explained by contamination with free mitochondria[9].

The endogenous respiration of isolated thymus cells was 2–3 times as high as the respiration of nuclear preparations (7–15 μmoles O_2/h per mg DNA P). If the nuclear preparations were contaminated with 10% intact cells, this could explain only 20–30% of the observed respiration. Furthermore, the respiration of nuclear preparations was strongly inhibited by treatment with deoxyribonuclease, which had no effect on the oxygen uptake of intact cells (Table X). In agreement with MCEWEN, ALLFREY AND MIRSKY[2,4] it must be concluded that thymus nuclei exhibit an independent endogenous respiration.

TABLE X

EFFECT OF DEOXYRIBONUCLEASE TREATMENT ON THE ENDOGENOUS RESPIRATION OF NUCLEI AND THYMOCYTES

Nuclei were isolated in sucrose–$CaCl_2$; cells in Krebs–Ringer medium. Nuclear and cellular suspensions were incubated for 15 min at 30° with and without 0.5 mg deoxyribonuclease/ml. After incubation the suspensions were centrifuged and resuspended. Oxygen uptake was measured polarographically in the medium described in Fig. 1. The respiration was calculated on the DNA P content before deoxyribonuclease treatment.

Expt.	*Preparation*	*Oxygen uptake (μmole/h per mg DNA P)*	
		Untreated	*Treated with deoxyribonuclease*
1	Nuclei (0.06 mg DNA P/ml)	3.8	1.6
	Cells (0.04 mg DNA P/ml)	5.4	5.4
2	Nuclei (0.07 mg DNA P/ml)	3.0	1.2
	Cells (0.05 mg DNA P/ml)	6.1	6.1

Inhibition of nuclear ATP synthesis and respiration

The effect of inhibitors of the mitochondrial respiratory chain on ATP synthesis in calf-thymus nuclei has been studied by OSAWA, ALLFREY AND MIRSKY[1], and MCEWEN, ALLFREY AND MIRSKY[2,4], who found complete or partial inhibition of nuclear ATP synthesis by 1 mM cyanide, 1 μg/ml antimycin, 1 mM amytal and 1 mM azide. No inhibition could be demonstrated by a gas mixture containing 95 % CO–5 % O_2. The effect of these inhibitors on nuclear respiration was not studied. Fig. 1 shows the effect of rotenone, antimycin and amytal on the respiration of rat-thymus nuclei. The concentrations giving maximal inhibition of respiration inhibited ATP synthesis completely. These results confirm the observations of OSAWA, ALLFREY AND MIRSKY[1] and MCEWEN, ALLFREY AND MIRSKY[2,4]. They strongly suggest the presence of a respiratory chain in thymus nuclei.

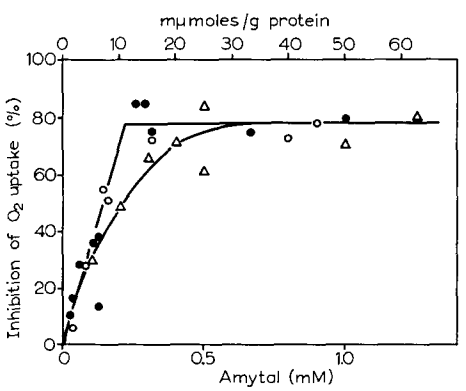


Fig. 1. Inhibition of nuclear endogenous respiration. ○, antimycin; ●, rotenone; △, amytal. The upper scale refers to antimycin and rotenone, the lower to amytal. The assay medium contained 0.25 M sucrose, 15 mM NaCl, 3 mM $CaCl_2$, 50 mM Tris; the pH was 7.4.

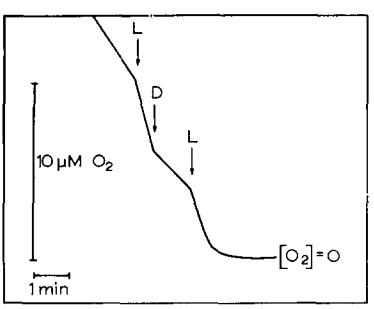
TABLE XI

LIGHT-REVERSIBLE INHIBITION OF ATP SYNTHESIS BY CARBON MONOXIDE

Four 100-ml flasks containing 3–5 ml of a nuclear suspension (containing about 0.6 mg DNA P/ml) were flushed with CO for 5 min, closed with rubber caps and incubated for 15 min at 30° in the dark. To one flask (A) $HClO_4$ was added immediately after this incubation. The second flask (B) was flushed with air and aerobically incubated for 45 min at 25°. To the third and fourth flasks a known volume of oxygen was added with a gas-tight syringe. One of these flasks (C) was incubated in dark for 45 min, the other flask (D) was illuminated during incubation with a Radium type R 16420 "cold light" lamp. After incubation the acid-soluble extracts were prepared from the nuclear suspensions in B, C and D. In these extracts ATP was determined. ΔATP light = ATP in D *minus* ATP in A; ΔATP dark = ATP in C *minus* ATP in A; ΔATP control = ATP in B *minus* ATP in A.

Gas mixture	*ΔATP (μmole per mg DNA P)*		
	Light	*Dark*	*Control (air)*
1% O_2–99% CO	0.13	0.05	—
1% O_2–99% CO	0.20	0.06	0.32
2% O_2–98% CO	0.17	0.04	0.20
4% O_2–96% CO	0.17	0.07	0.18
4% O_2–96% CO	0.22	0.07	0.34
6% O_2–94% CO	0.21	0.18	0.21
6% O_2–94% CO	0.35	0.21	0.30

In contrast with the results of the aforementioned authors we were able to demonstrate light-reversible inhibition of respiration and phosphorylation by carbon monoxide. Reproducible effects on ATP synthesis could only be obtained with oxygen concentrations below 4% (Table XI). From polarograph tracings as depicted in Fig. 2, the double-reciprocal plot (Fig. 3) was constructed. From this graph it was



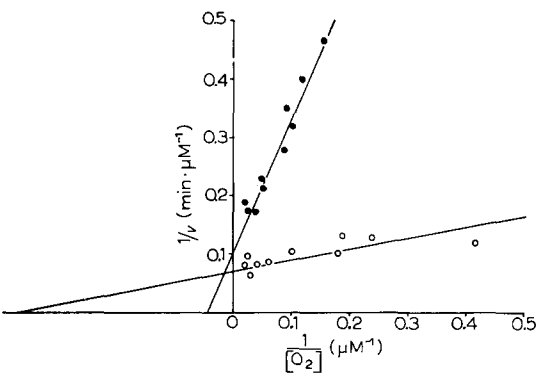


Fig. 2. Light-reversible inhibition of nuclear respiration by carbon monoxide. The oxygen concentration was measured polarographically. The polarograph vessel contained: 0.25 M sucrose, 3 mM $CaCl_2$, 15 mM NaCl, 50 mM Tris buffer (pH 7.4), and nuclei corresponding to 0.15 mg DNA P/ml. CO was passed into the medium. Assuming that the medium was saturated with CO, the concentration of the latter was about 950 μM. Temperature, 25°; L, illuminated; D, dark.

Fig. 3. Lineweaver–Burk plot of the light-reversible inhibition by carbon monoxide of nuclear respiration. Oxygen concentrations and respiratory rates were measured in experiments as described in Fig. 2. The points were obtained in two experiments in which the polarograph vessel contained, respectively, 0.30 and 0.22 mg DNA P/ml. Oxygen uptake was calculated as μmoles per min per 0.3 mg DNA P. ●—●, dark; ○—○, illuminated.

calculated that the apparent K_m (O_2) of the enzyme was 2.8 μM. Furthermore, it can be seen that the respiration is completely sensitive to carbon monoxide and not only that part that could be brought about by contamination. These experiments are good evidence for the presence of cytochrome oxidase (EC 1.9.3.1) in the nuclei.

One difference between mitochondrial and nuclear phosphorylation with respect to the effects of inhibitors has so far been found. It has been demonstrated by SANADI and co-workers[21–23] that arsenite in the presence of BAL is a potent uncoupler of mitochondrial phosphorylation. No effect, however, was found on nuclear phosphorylation (Table XII). Lewisite (dichloro(2-chlorovinyl)arsine), on the other hand, inhibited nuclear phosphorylation completely and this inhibition could be reversed by an equivalent amount of BAL. In the presence of lewisite an appreciable inhibition of the respiration was found (Table XII). This difference between the action of arsenite–BAL on mitochondria and nuclei could be due to a different permeability or affinity for the arsenical. Whether the inhibition by lewisite is to be ascribed to uncoupling or to inhibition of a sulphydryl group-dependent reaction (*e.g.* the oxidation of pyruvate) or both, cannot be decided from these experiments.

TABLE XII

EFFECT OF ARSENITE, LEWISITE AND BAL ON NUCLEAR ATP SYNTHESIS

The nuclear suspension in 0.25 M sucrose–3 mM $CaCl_2$ contained 0.32 mg DNA P per ml. Lewisite, arsenite and arsenite + BAL were added before anaerobic incubation. In Expt. 2, BAL was added just before aerobic incubation. Respiration was measured in a separate experiment in the medium given in Table I at 25°. ΔATP = ATP after aerobic incubation for 45 min at room temperature *minus* ATP after anaerobic incubation.

Expt.	*Additions*	*ΔATP (μmole per mg DNA P)*	*Respiration (μmoles/l per min)*
1	None	0.29	10.5
	0.1 mM arsenite	0.29	9.5
	0.1 mM arsenite + 0.1 mM BAL	0.28	—
2	None	0.19	10.5
	0.1 mM lewisite	0.0	3.2
	0.1 mM lewisite + 0.1 mM BAL	0.20	—

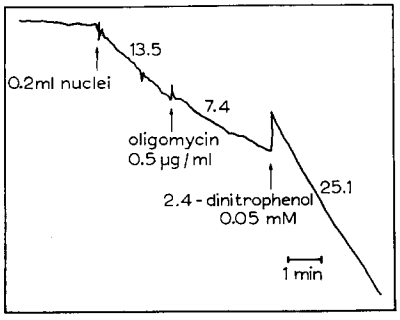


Fig. 4. Effect of oligomycin and 2,4-dinitrophenol on nuclear respiration. Medium as in Fig. 2. Nuclei containing 0.16 mg DNA P/ml. The values above the tracing are respiratory rates in μM/min. Final reaction volume, 2.0 ml.

We also studied the effects of oligomycin and 2,4-dinitrophenol on respiration and phosphorylation in thymus nuclei. Fig. 4 shows the effects of both compounds on nuclear respiration. Oligomycin inhibited respiration maximally to about 50%, the inhibition being released by 0.5 mM dinitrophenol. Dinitrophenol enhanced oxygen uptake in the absence of oligomycin. The stimulation of respiration was between 1.5–2 times in 12 nuclear preparations. Both compounds inhibited ATP synthesis practically completely (Table XIII).

TABLE XIII

INHIBITION OF ATP SYNTHESIS BY OLIGOMYCIN AND 2,4-DINITROPHENOL

Experimental conditions: see Table VI. Additions were made just before aerobic incubation. The nuclear suspension contained 0.46 mg DNA P per ml.

Additions	*μmole ATP per mg DNA P*	
	After anaerobic incubation	*After aerobic incubation*
None	0.04	0.28
Oligomycin (0.5 μg/ml)	0.04	0.06
None	0.07	0.25
2,4-Dinitrophenol (50 μM)	0.07	0.05

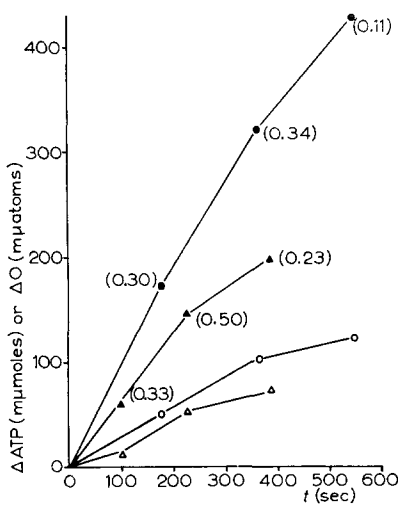


Fig. 5. ΔATP:ΔO ratio in thymus nuclei. Nuclei were incubated under N_2 for 15 min. 0.5 ml of the anaerobic suspension was added to the polarograph vessel containing 1.7 ml buffer. At the times indicated, 0.2-ml samples were withdrawn and acidified with 0.2 ml 10% $HClO_4$. In these acid-soluble extracts ATP was determined. Oxygen uptake and concentration were calculated from the polarograph tracing. Oxygen uptake during the first 30 sec after addition of the nuclear suspension was calculated by extrapolation to the time of addition. Two experiments are depicted. Expt. 1: ●—●, ΔO; ○—○, ΔATP. Expt. 2: △—△, ΔATP; ▲—▲, ΔO. The calculated ΔATP:ΔO ratios are given in brackets. For this calculation the ΔATP and ΔO with respect to preceding point was taken. The oxygraph vessel contained in both experiments 1.8 mg protein/ml and 0.07 mg DNA P/ml.

The effects of inhibitors of the respiratory chain together with the effects of dinitrophenol and oligomycin on nuclear ATP synthesis and respiration justify the conclusion that ATP synthesis in thymus nuclei must be considered as oxidative phosphorylation coupled to a respiratory chain. The results also suggest that the mechanism of nuclear phosphorylation, at least qualitatively, does not differ essentially from the mechanism of oxidative phosphorylation in mitochondria.

Efficiency of nuclear phosphorylation

The efficiency of oxidative phosphorylation is generally expressed as the P:O ratio. Since nuclei do not phosphorylate added ADP, and the ATP formed cannot be trapped, the standard methods for the estimation of P:O ratios could not be used. An attempt was therefore made to measure the initial rates of ATP synthesis and respiration after aging *in vitro* under N_2. The results of two experiments are shown in Fig. 5. The ΔATP:ΔO ratios obtained were between 0.3 and 0.5. Since, in the nuclei, ATP synthesis starts from AMP rather than from ADP, two phosphate molecules are esterified per molecule ATP synthesized. The P:O ratio is therefore twice the ΔATP:ΔO ratio and will be between 0.6 and 1.0. This phosphorylation efficiency is low compared with the efficiency that can be obtained with mitochondria.

DISCUSSION

Judging from the small variations in the RNA/DNA ratios of the nuclear fraction and the constancy of the distribution of the marker enzymes over the fractions, the fractionation of thymus tissue and the isolation of nuclei according to the method described yielded reproducible results.

ALLFREY, LITTAU AND MIRSKY[24] found a rather large variation in the number of intact cells in nuclear preparations from calf thymus. A possible explanation of our different experience in this respect could be that the use of coaxial homogenizers yields more reproducible results than blendor-type homogenizers. It may be mentioned in passing that the methods described by ALLFREY, LITTAU AND MIRSKY[24] for the removal of intact cells and tagged nuclei from nuclear preparations from calf thymus were completely unsuccessful with rat thymus. In contrast with calf thymocytes, rat thymocytes did not flotate during centrifugation in dense sucrose or ficoll media, but sedimented along with the nuclei.

From the fractionation data it is concluded that the nuclear fraction is contaminated with 5–10% of the mitochondria present in the tissue homogenate. Intact cells formed the most serious source of contamination, since cells can perform complex functions which could be erroneously ascribed to the nuclei. Therefore it was assumed for the purpose of calculation that the mitochondrial contamination was present entirely in the form of intact cells, and that this amounted to 10%.

The experimental results showed clearly that neither the ATP content nor ATP synthesis in the nuclear preparations could be explained quantitatively by contamination with 10% intact cells, and also could not be ascribed to contamination with free mitochondria. Therefore, ATP must be present in nuclei which are capable of aerobic resynthesis of endogenous ATP.

The respiration of the nuclear preparations is comparable to that observed

by McEWEN, ALLFREY AND MIRSKY[2,4] for calf-thymus nuclei. The effects of inhibitors of the mitochondrial respiratory chain on nuclear ATP synthesis and respiration suggest that in the nuclei also, respiration and phosphorylation are closely linked with each other. The light-reversible inhibition of phosphorylation and respiration by carbon monoxide at low oxygen concentrations strongly suggest the presence and function of cytochrome oxidase in the nuclei.

The coupling between respiration and phosphorylation could be demonstrated by the effects of 2,4-dinitrophenol and oligomycin on both processes. The stimulating effect of 50 μM 2,4-dinitrophenol on nuclear respiration is not in contradiction with the inhibitory effect of 2 mM 2,4-dinitrophenol on the respiration of calf-thymus nuclei reported by ALLFREY AND MIRSKY[20]. It is known that in mitochondria too, high concentrations of this uncoupler inhibit respiration[25].

From the experiments with inhibitors of the mitochondrial respiratory chain and with dinitrophenol and oligomycin, the conclusion is justified that thymus nuclei are capable of oxidative phosphorylation coupled to a respiratory chain.

The efficiency of nuclear oxidative phosphorylation was low when determined from the rates of ATP synthesis and oxygen uptake. It is possible that the rate of ATP synthesis is underestimated, due to ATP turnover during resynthesis[26]. On the other hand, the possibility must be considered that the low efficiency is an intrinsic property of nuclear phosphorylation. It is also possible that nuclei make an efficient use of high-energy intermediates[27].

It has been argued that nuclear phosphorylation must be considered as a minor process and does not make a major contribution to the energy pool of the cell[28,29]. However, the thymus nucleus contains about 50 % of the cellular ATP and maintains this ATP level under aerobic conditions for about 2 h in a medium containing only sucrose and $CaCl_2$. That ATP is turned over during this time must be concluded from the rapid decrease in the ATP level in the presence of inhibitors of ATP synthesis. If it is assumed that the thymus nucleus *in situ* also maintains 50 % of the cellular ATP, this must be considered as a substantial contribution to the total energy pool.

SIEBERT AND HUMPHREY[29] have argued that the rate of ATP synthesis in the nuclei is extremely low when compared with the rate that can be obtained with mitochondria from the same tissue. In our view the comparison between isolated nuclei, which phosphorylate only endogenous nucleotides at the expense of endogenous substrate, and maximally stimulated mitochondria cannot be justified. Preliminary experiments with thymocytes have shown that the rate of ATP synthesis in these cells is only 2–4 times the rate observed in nuclei, when synthesis is determined under identical conditions. It is clear then that the net rates of ATP synthesis in isolated nuclei and in mitochondria in the cell do not differ to a large extent. An exact evaluation of the relative contribution of cytoplasm and nucleus to the total ATP production in the thymocytes cannot be given at present. Studies on the rate of ATP production and consumption in cells and nuclei and on the possible compartmentation of ATP in thymocytes could clarify this point.

The occurrence of oxidative phosphorylation in these nuclei implies that they probably contain a respiratory chain. Spectrophotometric studies have revealed that thymus nuclei do contain cytochromes[30]. The results of these studies will be described in a separate paper.

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