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Studies of Energy-yielding Reactions in Thymus Nuclei*

I. COMPARISON OF NUCLEAR AND MITOCHONDRIAL PHOSPHORYLATION

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It has been shown previously that nuclei isolated from calf thymus tissue are capable of many biosynthetic reactions, including the synthesis of proteins (1–3) and ribonucleic acids (2, 4, 5). These reactions require energy in the form of adenosine triphosphate. Studies of the composition of nuclei isolated from thymus, liver, and other tissues have made it clear that nuclei *do* contain adenosine triphosphate and other nucleotides (6–8) in concentrations comparable to those seen in the cytoplasm, provided the isolation is in media that do not extract adenosine triphosphate, *i.e.* in *nonaqueous* media. In 1957, Osawa *et al.* (7) showed that thymus nuclei isolated in 0.25 M sucrose-0.003 M $CaCl_2$ can cause a net synthesis of adenosine triphosphate by an aerobic process that differs from oxidative phosphorylation in mitochondria in its insensitivity to calcium ions and methylene blue. Subsequent studies showed that nuclear adenosine triphosphate synthesis comes to a halt when nuclei are treated with deoxyribonuclease, but resumes when polyanions, such as deoxyribonucleic acid, ribonucleic acid, heparin, polyethylene sulfonate, or polyacrylic acid, are added to the deoxyribonuclease-treated nuclei (9, 10).

The purpose of this study is to show differences between ATP synthesis by calf thymus nuclei and oxidative phosphorylation by calf thymus mitochondria and to provide evidence for the localization of the ATP-synthetic process within the nucleus. It is shown that ATP synthesis in thymus nuclei is insensitive to carbon monoxide, calcium ions, and methylene blue, whereas oxidative phosphorylation by thymus mitochondria is strongly inhibited by these agents, even in the presence of nuclei. Both nuclei and mitochondria are inhibited by cyanide, azide, 2,4-dinitrophenol, Dicumarol, antimycin A, and Amytal. On the other hand, respiration and ATP synthesis in the nucleus are both inhibited by treatment of the nuclear suspension with deoxyribonuclease; under the same conditions, mitochondrial phosphorylation is not affected. Mitochondrial ATP synthesis can be inhibited by histones derived from the thymus nucleus, whereas nuclear ATP synthesis seems more resistant to added histone. Evidence is also presented that nuclear ATP is derived from an endogenous nucleotide pool and that added AMP, ADP, and ATP do not penetrate readily into the thymus nucleus.

EXPERIMENTAL PROCEDURE

Isolation of Calf Thymus Nuclei—All operations were performed at 2–4°. Fresh calf thymus tissue was minced with scissors and transferred to a blender containing 10 volumes of 0.25 M sucrose-0.003 M $CaCl_2$ (or, in some cases, 0.003 M $MgCl_2$). The suspension was homogenized at 1000 r.p.m. for 4 minutes, and the nuclei were isolated as described previously (2). Nuclear fractions thus isolated from calf thymus tissue have been shown to satisfy a number of morphological and chemical criteria of purity, but a critical examination in the electron microscope shows that they contain a small proportion of intact thymocytes (3, 11–13). Good preparations have fewer than 10% cells; poor preparations may contain 17 to 25% whole cell contamination. The latter can be removed by density gradient centrifugation. A simple procedure we have employed uses the synthetic polysaccharide, Ficoll (Pharmacia, Ltd., Vanlose, Copenhagen, Denmark), to increase the density of isotonic sucrose. Nuclear suspension, 100 ml in 0.25 M sucrose-0.003 M $CaCl_2$, was carefully layered over 75 ml of Ficoll solution (230 mg of Ficoll per ml of 0.25 M sucrose-0.003 M $CaCl_2$) and centrifuged in a horizontal rotor at 800 × *g* for 10 minutes. The interphase layer, which contained many small intact thymocytes, nuclei, and a small proportion of red cells, was removed with a syringe. This "cell fraction" was washed twice with sucrose-$CaCl_2$ before resuspending it for incubation experiments. Electron microscopy of a number of "cell fractions" has given cell counts ranging from 55 to 80%. The free nuclei, because of their higher density, pass the Ficoll density barrier and gather as a white sediment at the bottom of the centrifuge tube. This sediment was washed twice to remove the Ficoll and resuspended in 0.25 M sucrose-0.003 M $CaCl_2$. Electron microscopy of the nuclear sediment shows fewer than 10% cells. If further purification is required, the layering over Ficoll can be repeated. Two such fractionations give a nuclear preparation that is better than 95% pure. Unfortunately, Ficoll is somewhat toxic to nuclei, and their metabolic activity is decreased by prolonged exposure to this substance.

Isolation of Thymus Mitochondria—Fresh calf thymus tissue was finely minced with scissors. Mince in 10-g portions was added to approximately 50 ml of 0.25 M sucrose in a heavy walled glass tube, and homogenized with a motor-driven Teflon pestle. The suspension was centrifuged at 1000 × *g* for 7 minutes to remove nuclei and whole cells. The supernatant was then centrifuged again at 1000 × *g* for 7 minutes, discarding the sediment. The supernatant fraction was centrifuged at 6000 × *g* for 15 minutes. The resulting sediment, which contained the mitochondria, was resuspended, with 5 ml of the isolation medium for every 10 g of fresh tissue originally used. In isolations in which sucrose alone was used as the isolation medium,

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the yields were low, because in the absence of Ca and Mg ions, the nuclei form a heavy gel that traps many of the mitochondria. Electron microscopy showed that thymus mitochondria isolated in this way were largely intact and similar in form to mitochondria isolated from liver tissue (14).

Incubation Procedure for Nuclei—Incubations were at 37° after the addition of 1.0 ml of nuclear suspension (containing approximately 40 mg of nuclei (dry weight) per ml), to 0.5 ml of buffer (0.1 M Tris-Cl in 0.25 M sucrose, pH 7.4), and 0.4 ml of a "substrate + salts" solution. When glucose was used as substrate, the latter solution contained 100 μmoles of glucose, 25 μmoles of $MgCl_2$, and 8.0 mg of NaCl per ml. When succinate was used as substrate, the "substrate-salts" solution contained 100 μmoles of sodium succinate, pH 7.4, and 25 μmoles of $MgCl_2$ per ml. When no substrate was added to the nuclei, this solution contained 100 μmoles of sucrose, 25 μmoles of $MgCl_2$, and 8.0 mg of NaCl per ml. Nuclear respiration was measured by the conventional Warburg methods. ATP levels in the nuclei were determined as described below.

Incubation Procedure for Mitochondria—Mitochondrial suspensions were incubated for 15 minutes at 37° in a medium containing 30 μmoles of substrate, 50 μmoles of potassium phosphate buffer, pH 7.4, 0.03 μmole of cytochrome *c*, 20 μmoles of $MgSO_4$, 5 μmoles of EDTA, 4 μmoles of ATP, 5 to 8 mg (dry weight) of mitochondria, and a phosphate acceptor system consisting of 50 units of hexokinase (Sigma, type II) and 90 μmoles of glucose. The final volume was 2.0 ml. Respiration was measured by the conventional Warburg method. The mitochondria were preincubated for 5 minutes before the acceptor system was added from one side arm of the flask. The reaction was stopped by adding 0.1 ml of 20% trichloroacetic acid from the other side arm. Phosphate was measured in the deproteinized extract by Allen's method (15). In experiments in which nuclei were mixed with mitochondria, the trichloroacetic acid extract was neutralized to the phenolphthalein end point and centrifuged to remove nuclear histone, which otherwise interferes with the phosphate determination.

Measurement of ATP Levels in Nuclei—After incubation, the nuclear suspensions were placed in an ice bath, and cold perchloric acid was added to a final concentration of 2%. The suspensions were centrifuged, and the supernatant fraction was collected. These extracts were neutralized to pH 6 to 7 with KOH, chilled, and centrifuged to remove the potassium perchlorate formed. ATP was separated from the other nucleotides in the extract by chromatography on Dowex 1-formate (200 to 400 mesh) following the scheme of Potter and Siekevitz (16). The samples (approximately 20 ml in volume) were washed on to the columns with 20 ml of water. The fraction containing AMP and ADP was eluted with 70 ml of 4 N formic acid. ATP was then eluted with 70 ml of 4 N formic acid-0.4 M ammonium formate. The optical density of each fraction was read at 260 mμ in a Beckman model DU spectrophotometer against a "blank" consisting of the appropriate eluent which had been run through a parallel blank column.

RESULTS

Oxygen Dependence of Nuclear ATP Synthesis—The ATP levels in duplicate nuclear suspensions after 30 minutes of incubation in air and in nitrogen are compared in Table I. Results are shown for nuclei without added substrates and for nuclei given a glucose supplement. It is clear that optimal ATP levels are maintained only under aerobic conditions, although anaerobic glycolysis will support some ATP synthesis. The Q_{O_2} for the usual nuclear preparations is 1.5 to 2.5, without added glucose. A slight inhibition of this respiration, reminiscent of the Crabtree effect (17), is obtained when glucose is added to the incubation medium.

TABLE I

Oxygen dependence of ATP synthesis in thymus nuclear suspensions

Nuclei were incubated at 37° for 30 minutes under the conditions described in "Experimental Procedure."

Conditions of experiment	Oxygen uptake	ATP level
	μl O_2/30 min/ml	*mμmoles/ml*
Aerobic, no substrate added	79.5	260
Aerobic + 2×10^{-2} M glucose	71.8	310
Nitrogen atmosphere, no substrate added		68
Nitrogen + 2×10^{-2} M glucose		180

Time Course of Nuclear ATP Synthesis and Disappearance—The synthesis of ATP in nuclear suspensions at 37° is a very rapid reaction. The time course of net ATP production is plotted in Fig. 1, in which it can be seen that the initial increase in ATP is matched by a corresponding decrease in AMP + ADP. After reaching a maximum at 10 minutes, the ATP level falls off slightly, while levels of nucleoside, AMP, and ADP increase. At 10 minutes, 80% of the total nucleotide is in the form of ATP.

Under anaerobic conditions, however, nuclear ATP levels fall rapidly; two-thirds of the ATP disappears within 5 minutes (see Fig. 2). Although some of this loss undoubtedly represents utilization, one does not always observe a corresponding increase in the levels of AMP and ADP, and nucleoside concentrations increase only slightly. Thus, the findings suggest that, under anaerobic conditions, ATP is either utilized or stored in a form that is not extractable in dilute acid. Thymus nuclei contain an RNA polymerase that can form a polynucleotide rich in polyadenylic acid (18). In either case, it is evident that free ATP levels are not maintained in the absence of oxygen. Earlier experiments on the effects of inhibitors (other than anaerobiosis) showed that an inhibitor which blocks nuclear ATP synthesis and not its utilization causes the free ATP level to run down (7).

Inability of Nuclei to Phosphorylate Added AMP and ADP Directly—Added nucleotides do not penetrate thymus nuclei readily; instead, they are largely hydrolyzed to nucleosides and free bases. Thus, when nuclei are incubated at 37° with AMP-8-C^{14} or ADP-8-C^{14}, most of the isotope appears in the nucleoside fraction and only a small part appears as nuclear ATP (Table IIA). The adenosine-8-C^{14} produced as a result of this extensive hydrolysis of the added nucleotides is transported into the nucleus (19). Subsequent utilization of the nucleoside for both ATP and RNA synthesis occurs (4). That this is the sequence of events is shown by experiments in which an excess of unlabeled adenosine is added to dilute the C^{14}-adenosine derived from the C^{14}-AMP or ADP. The addition of an excess of C^{12}-adenosine together with the C^{14}-AMP or C^{14}-ADP reduces the uptake of C^{14} into nuclear ATP and RNA by over 60%.

Also, the appearance of radioactivity in ATP when nuclei are

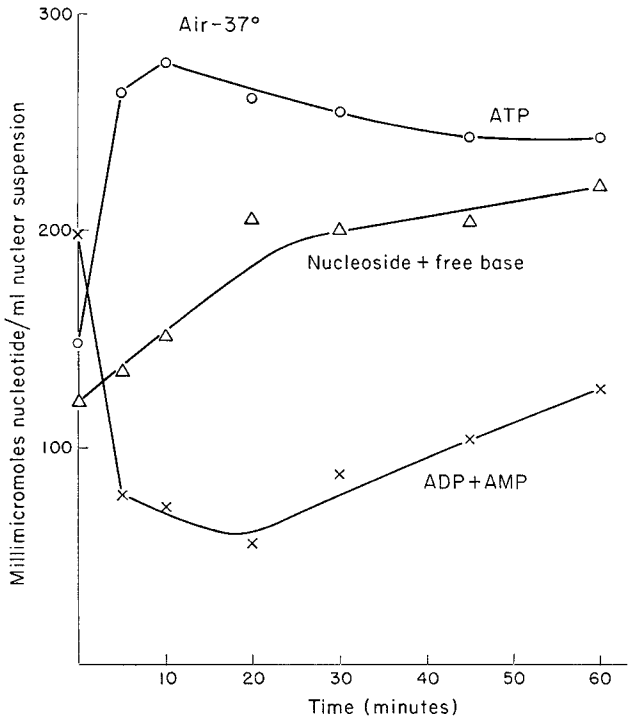

FIG. 1. The time course of ATP synthesis in suspensions of isolated calf thymus nuclei. The nuclear ATP level is plotted as a function of the time of incubation at 37°. The incubation conditions and analytical methods are described in "Experimental Procedure."

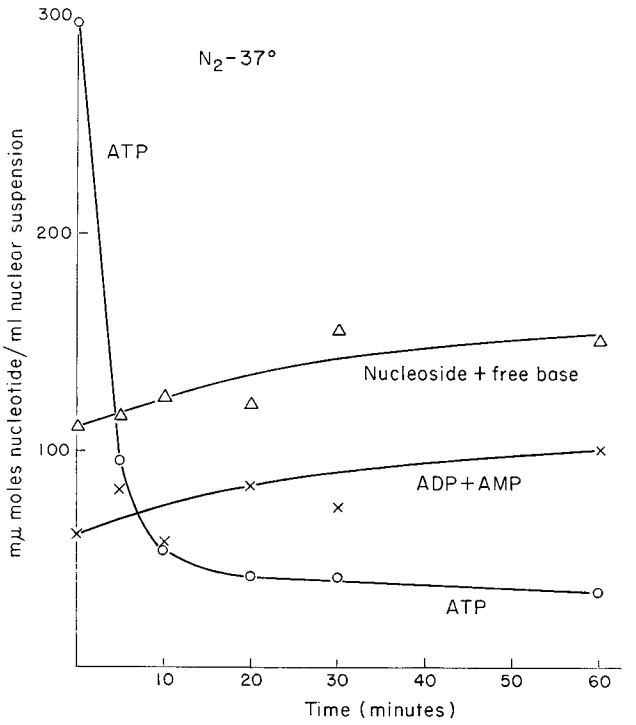

FIG. 2. The time course of ATP disappearance in nuclear suspensions under anaerobic conditions. The nuclear ATP level is plotted as a function of the time of incubation under nitrogen at 37°. The conditions of the experiment are described in "Experimental Procedure."

exposed to C^{14}-AMP or ADP is not due to the action of adenylate kinase, because the uptake takes place only in the presence of oxygen. (Adenylate kinase has been shown to be present in calf thymus nuclei by Miller and Goldfeder (20).) The data in Table IIB show that the appearance of C^{14} in nuclear ATP is much higher in air than it is in a nitrogen atmosphere.

Nuclear Impermeability to ATP in Medium—The rapid and extensive hydrolysis of added AMP or ADP in thymus nuclear suspensions contrasts with the stability of these compounds in the intranuclear "pool" (see Figs. 1 and 2). Intranuclear ATP is also quite stable under aerobic conditions. This is not the case for ATP added to the medium. The instability of the latter can be shown by tracer experiments with C^{14}-labeled ATP. Within 5 minutes, 78% of the added C^{14}-ATP was hydrolyzed to C^{14}-AMP and ADP, and, in 30 minutes, 40% of the added counts were present as C^{14}-adenosine and adenine. The latter compounds can penetrate isolated thymus nuclei and are used as precursors for the synthesis of nuclear ATP and nucleic acids. In this way, some of the counts originally added as C^{14}-ATP make their way into nuclear ATP and RNA. However, when C^{14}-ATP is added to nuclei suspended in an excess of unlabeled adenosine and adenine, the penetration of C^{14} into the nucleus is minimized. As shown in Fig. 3, only 3% of the added counts are taken up in 30 minutes at 37°. In contrast, the penetration of C^{14}-adenosine and C^{14}-adenine (measured in parallel experiments) is very rapid. It is clear from these experiments that ATP, as such, does not readily enter the nuclear "pool." Like added AMP or ADP, it is first hydrolyzed to the nucleoside and free base. The findings make it very unlikely that the increase in nuclear ATP content observed when thymus nuclear suspensions are incubated in air represents a transfer of ATP into the nucleus from some extranuclear phosphorylating system.

Criteria of Nuclear Purity—There is very good evidence that the aerobic synthesis of ATP observed in thymus nuclear suspensions is indeed a nuclear process and is not due to contamination by mitochondria and whole cells. The point is important because thymus nuclear fractions as usually prepared in isotonic sucrose solutions are not 100% pure, but do contain small amounts of cytoplasmic contamination and a small proportion of intact cells. The cells most frequently found are very small thymocytes, consisting of a nucleus surrounded by a thin rim of cytoplasm. The cytoplasm is usually too small to be evident in the light microscope, although differential staining reactions are helpful and have been used (21). With the electron microscope, however, more reliable cell counts can be made. Good nuclear preparations have fewer than 10 small thymocytes per 100 nuclei; poor preparations may contain 17 to 25 whole cells per 100 nuclei. Most of the cells can be removed by centrifugation through a Ficoll density gradient, as described in "Experimental Procedure." Alternatively, the cells can be broken by a brief blending in hypotonic sucrose solution.[1] By this means, the over-all level of cytoplasmic contamination can be reduced to less than 5% by weight.

Highly purified thymus nuclear fractions prepared by centrifugation through Ficoll density gradients are capable of aerobic phosphorylation. They respire and show a net synthesis of ATP. A comparison of Ficoll-purified nuclei with a "cell fraction" containing approximately 65% of intact, small lymphocytes gave Q_{O_2} of 0.8 for the nuclei and 2.4 for the cells.

[1] V. G. Allfrey, unpublished observations.

TABLE II

Hydrolysis and utilization of added C^{14}-AMP and ADP by thymus nuclear suspensions

Incubations were in the medium described in "Experimental Procedure," containing no added substrate. AMP-8-C^{14} and ADP-8-C^{14} were added to different flasks at levels of 1.4×10^4 c.p.m. per ml of incubation mixture. In Experiment A, the total volume was 8.0 ml, and the nucleotides were separated on small Dowex 1-formate columns as described in "Experimental Procedure." In Experiment B, the total volume was 32 ml, and the nucleotides were separated on large Dowex 1 columns with the use of the gradient elution scheme described ealier (7). The eluate fractions were taken to dryness in stainless steel cup-planchets and counted in a thin window gas flow counter.

Precursor added	Conditions of experiment	Distribution of radioactivity in							Total counts recovered in acid-soluble fraction
		Nucleoside + base		AMP + ADP		ATP		RNA	
		c.p.m.	*% total*	*c.p.m.*	*% total*	*c.p.m.*	*% total*	*c.p.m./mg*	
A. AMP-8-C^{14}	Control	41,700	59	23,000	33	5,600	8	570	84,600
AMP-8-C^{14}	+ 50 μmoles of C^{12}-adenosine	48,400	65	24,000	32	2,200	2	159	91,000
ADP-8-C^{14}	Control	51,100	67	20,000	26	5,500	7	559	91,200
ADP-8-C^{14}	+ 50 μmoles of C^{12}-adenosine	54,700	67	24,000	30	2,700	3	100	108,800
B. AMP-8-C^{14}	Aerobic	146,700	73	22,400	11	33,000	16	1,735	202,000
AMP-8-C^{14}	Anaerobic	176,800	88	24,100	11	1100	1	512	202,000
ADP-8-C^{14}	Aerobic	201,200	81	9,500	3	38,700	16	2,981	249,400
ADP-8-C^{14}	Anaerobic	251,400	94	15,800	5.5	1,200	0.5	711	268,400

The nuclei contained 4.4 mμmoles of ATP per mg, whereas the cell fraction contained 9.9 mμmoles of ATP per mg. Evidently, the intact cells respire more actively and synthesize more ATP than do the isolated nuclei, but the lower rates of respiration and ATP synthesis in Ficoll-treated nuclei are due in part to the toxicity of this synthetic polysaccharide. Also, high ATP levels have been detected in nuclei purified in nonaqueous media (7), and in these preparations, contamination by whole cells was not a problem.

Electron microscopy indicates the presence of some swollen but structurally intact mitochondria adhering to some nuclei. One of the consequences of this mitochondrial contamination is that nuclear suspensions will oxidize added succinate, even though nuclei freed of mitochondrial contamination will not. The reason for this is that succinate and similar dicarboxylic acids do not enter the intranuclear "pool" of metabolites.[2, 3] The contribution of mitochondria to the respiration of nuclear suspensions can be estimated in several ways, among them by the stimulation of respiration when succinate is added to the suspension. Nuclear suspensions receiving a succinate supplement show a Q_{O_2} increment of 5. Thymus mitochondria in the same medium have a Q_{O_2} of 125. When necessary, the mitochondrial contribution to nuclear respiration can be selectively inhibited by CO (see the following), or the mitochondria can be selectively destroyed by high speed blending. (The latter is a less effective procedure and damages the nuclei to some extent.)

Effect of Inhibitors of Mitochondrial Oxidative Phosphorylation on Nuclear ATP Synthesis—In previous work, it was shown that a number of well known inhibitors of mitochondrial oxidative phosphorylation inhibit nuclear ATP synthesis (7, 9). Table III summarizes the effects of these inhibitors on the nuclear system. Azide, cyanide, 2,4-dinitrophenol, antimycin A, Dicumarol, and Amytal inhibit nuclear ATP synthesis and ATP-dependent C^{14}-alanine incorporation into protein. However, three other well known inhibitors of mitochondrial oxidative phosphorylation, calcium ions, methylene blue, and carbon monoxide, have little or no effect on nuclear ATP levels and protein synthesis (Table III). In subsequent sections, use is made of these last inhibitors to show that it is extremely unlikely that mitochondria in the nuclear suspensions provide ATP to nuclei.

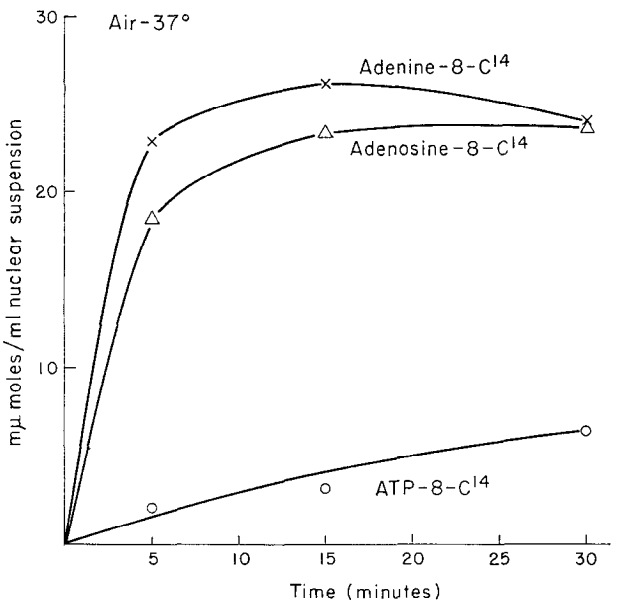

FIG. 3. The time course of penetration of C^{14}-labeled adenine, adenosine, and ATP into the "acid-soluble" fraction of isolated calf thymus nuclei. The experimental conditions are similar to those described in studies of amino acid transport into nuclear "pools" (19).

Properties of Isolated Thymus Mitochondria—Mitochondria isolated from the supernatant fraction after isolation of nuclei

[2] B. S. McEwen, V. G. Allfrey, and A. E. Mirsky, manuscript in preparation.

[3] M. Izawa and A. E. Mirsky, manuscript in preparation.

TABLE III

*Effects of inhibitors of mitochondrial oxidative phosphorylation on nuclear ATP synthesis**

Inhibitor added	Concentration	Change in ATP level	Change in alanine-1-C^{14} uptake into protein
		%	%
NaCN	10^{-3} M	−100	−76
2,4-Dinitrophenol	2×10^{-4} M	−100	−84
NaN_3	1×10^{-3} M	−100	−91
Antimycin A	1 μg per ml	−61	−89
Dicumarol	3×10^{-5} M	−47	−96
Amytal	1×10^{-3} M		−86
Methylene blue	2×10^{-5} M	+31	+3
Methylene blue	2.5×10^{-4} M	+21	−18
Ca ions	2×10^{-3} M	0	0
Ca ions	3×10^{-3} M	0	0
Ca ions	4×10^{-3} M	0	0
CO	95% CO-5% O_2	0	0

* Nuclei were incubated for 30 minutes at 37° under the conditions described in "Experimental Procedure." Alanine-1-C^{14} incorporation into nuclear proteins was determined as described by Allfrey *et al.* (2).

TABLE IV

*Effects of inhibitors on respiration and phosphorylation by isolated thymus mitochondria**

Conditions of experiment	Oxygen uptake	Phosphate uptake	P:O ratio	Uncoupling
	μl O_2/15 min	*μmoles P/15 min*		%
A. Control, air	149	24.4	2.08	
95% CO-5% O_2	43	−3.3	<0	100
B. Control	158	24.4	1.95	
+ thymus histone, 0.5 mg per ml	52	−4.2	<0	100
C. Control	216	32.4	1.88	
+ DNase, 0.5 mg per ml	220	31.8	1.80	0

* Thymus mitochondria were isolated and incubated as described in "Experimental Procedure."

in 0.25 M sucrose-0.003 M $CaCl_2$ are incapable of ATP synthesis. This is due both to the inhibitory effects of calcium ions (see the following) and to the disruption of mitochondrial structure during the blending of the tissue suspension. It is in accord with the previous observation by Osawa *et al.* (7) that the entire supernatant fraction of a thymus homogenate cannot synthesize ATP once the nuclei have been removed.

It has been noted that thymus nuclear preparations contain swollen but structurally intact mitochondria adhering to some of the nuclei. To study what the properties of these mitochondria might be, structurally intact mitochondria have been isolated from calf thymus tissue in 0.25 M sucrose without added salts and also in sucrose containing calcium or magnesium ions. All preparations actively respire, but phosphorylation by mitochondria prepared in calcium containing media is extensively uncoupled from respiration. Mitochondria isolated in 0.25 M sucrose respire and phosphorylate most actively with succinate, showing P:O ratios between 1.5 and 2.0. The P:O ratios with β-hydroxybutyrate and with α-ketoglutarate (plus malonate) are 1.05 and 1.08, respectively, and the respiration is very low with these substrates.

Comparative Effects of Carbon Monoxide on Respiration and ATP Synthesis in Nuclei and Mitochondria—Respiration and phosphorylation by calf thymus mitochondria are inhibited by carbon monoxide. The effect of 95% CO-5% O_2 on mitochondrial suspensions containing succinate is shown in Table IVA.

It was shown previously that nuclear ATP synthesis, unlike that in mitochondria, is not sensitive to carbon monoxide (10). The effects of carbon monoxide on nuclear respiration and ATP levels are summarized in Table V. In nuclear suspensions without added substrate in an atmosphere of 95% CO-5% O_2, ATP synthesis is normal, and respiration is inhibited only slightly. When succinate is added, carbon monoxide effectively prevents the increment in respiration that is due to mitochondrial contamination; as before, there is no change in nuclear ATP levels.

It should be emphasized that the failure of carbon monoxide to inhibit nuclear ATP synthesis and endogenous respiration is not due to a failure of carbon monoxide to reach mitochondria in the presence of nuclei. CO (95%) inhibits mitochondrial respiration in the presence of nuclei (the succinate-stimulated respiration of the nuclear preparation) to the *same extent* that it inhibits respiration of isolated mitochondria. (Compare Table IVA with Table V.) At this level of inhibition, mitochondrial phosphorylation is completely uncoupled (Table IVA).

Comparative Effects of Calcium and Magnesium Ions on Respiration and Phosphorylation in Thymus Nuclei and Mitochondria—The synthesis of ATP in suspensions of thymus mitochondria is sensitive to calcium ions, since calcium uncouples phosphorylation from respiration (22). The uncoupling effect of calcium is especially apparent when mitochondria are isolated in the presence of 0.003 M $CaCl_2$ (Table VI). By contrast, the presence of another divalent cation, magnesium, in the isolation medium does not result in the uncoupling of mitochondrial oxidative phosphorylation (Table VI).

When mitochondria isolated in the absence of calcium or magnesium ions are mixed with thymus nuclei which have been isolated in the presence of one of these ions, the selective uncoupling action by calcium ions is still apparent. Table VI

TABLE V

Effect of carbon monoxide on respiration and ATP content of isolated thymus nuclei

Incubations were carried out in the medium described in "Experimental Procedure" containing no added substrate. The flasks were pregassed for 10 minutes with 95% CO-5% O_2 before 30 minutes of incubation in the dark at 37°.

Conditions of experiment	Endogenous respiration		Succinate-stimulated respiration		ATP content
	μl O_2/60 min	*% inhibition*	*μl O_2/60 min*	*% inhibition*	*mμmoles/ml suspension*
Aerobic	125				
95% CO-5% O_2	103	18			
Aerobic	60				224
95% CO-5% O_2	54	10			207
Aerobic			205		
95% CO-5% O_2			66	68	
Aerobic			122		189
95% CO-5% O_2			35	71	231

shows that mitochondria in the presence of magnesium nuclei have a P:O ratio approaching 1.2, whereas mitochondria in the presence of calcium nuclei are completely uncoupled.

It follows from this information that if the mitochondria which are always present in the nuclear suspension are responsible for oxygen-dependent nuclear ATP synthesis, one would expect to find a substantial difference between ATP levels in nuclei isolated in calcium-containing sucrose and nuclei isolated in magnesium-containing sucrose. In fact, this is not found to be the case. Nuclei prepared in 0.25 M sucrose-0.003 M $MgCl_2$ had a Q_{O_2} of 1.41 and an ATP level of 1.62 mμmoles per mg (dry weight) after 30 minutes of incubation at 37°, whereas a comparable nuclear suspension, prepared from the same thymus gland in 0.25 M sucrose-0.003 M $CaCl_2$, had a Q_{O_2} of 1.67 and an ATP level of 1.50 mμmoles per mg.

Comparative Effects of Methylene Blue on Respiration and ATP Synthesis in Thymus Nuclei and Thymus Mitochondria—Methylene blue appears to have no effect on nuclear ATP synthesis. In fact, ATP levels are often increased by the presence of methylene blue in the medium (Table III). In contrast, methylene blue is known to uncouple mitochondrial oxidative phosphorylation (22). Table VII shows that 5×10^{-5} M methylene blue uncouples oxidative phosphorylation by thymus mitochondria in the presence of nuclei isolated in 0.25 M sucrose-0.003 M $MgCl_2$. Thus, if nuclei do sequester methylene blue, they do not bind enough to prevent its action as a mitochondrial inhibitor.

Effects of Thymus Nuclear Histones on Respiration and ATP Synthesis in Nuclei and Mitochondria—A mixture of thymus nuclear histones can be prepared by extracting the isolated nuclei with 0.2 N HCl and subsequently lyophilizing the dialyzed extract. Histone mixtures prepared in this way act as potent inhibitors of oxidative phosphorylation in isolated mitochondria. Some effects are shown in Table IVB. The histone fraction inhibits mitochondrial respiration (with succinate as substrate) by 67%, and it uncouples phosphorylation completely. In contrast, the isolated thymus nuclei are relatively insensitive to low levels of histone. Under these conditions, nuclear respiration is inhibited only slightly, and nuclear ATP levels are virtually unaffected. At higher histone concentrations, nuclear phosphorylation is also inhibited, as shown in Fig. 4, which compares respiration and phosphorylation in nuclei and mitochondria under conditions of increasing histone concentration. Because the inhibition observed is a function of the number of nuclei or mitochondria present, as well as histone concentration, comparisons were made of respiration and ATP synthesis in suspensions containing 0.1 mg of histone per mg (dry weight) of nuclei or mitochondria. Mitochondrial respiration was inhibited 76% and phosphorylation was completely uncoupled, whereas nuclear respiration was down 7 to 13%, and nuclear ATP

TABLE VI

Effect of calcium ions in uncoupling mitochondrial oxidative phosphorylation in presence of isolated nuclei

Mitochondria prepared as described in "Experimental Procedure" were resuspended in 0.25 M sucrose containing either 0.003 M $MgCl_2$ or 0.003 M $CaCl_2$ and added to suspensions of thymus nuclei prepared in sucrose-$CaCl_2$ or sucrose-$MgCl_2$. The conditions of incubation are described in "Experimental Procedure."

Conditions of experiment	Ca concentration of medium	Oxygen used (−ΔO)	Phosphate bound (−ΔP)	P:O ratio
	M	*μatoms*	*μmoles*	
Mitochondria isolated in 0.25 M sucrose	0	16.5	30.3	1.83
Mitochondria isolated in 0.25 M sucrose-0.003 M $CaCl_2$	0.003	6.8	3.5	0.52
Mitochondria isolated in 0.25 M sucrose-0.003 M $MgCl_2$	0	16.1	29.0	1.80
Mitochondria isolated in 0.25 M sucrose mixed with nuclei prepared in sucrose-calcium	0.003	6.8	−4.1	<0
Mitochondria isolated in 0.25 M sucrose mixed with nuclei prepared in sucrose-magnesium	0	7.9	9.4	1.19

TABLE VII

Effect of methylene blue in uncoupling mitochondrial oxidative phosphorylation in presence of isolated nuclei

Mitochondria prepared as described in "Experimental Procedure" were resuspended in a suspension of thymus nuclei isolated in 0.25 M sucrose-0.003 M $MgCl_2$. The conditions of incubation are described in "Experimental Procedure."

Conditions of experiment	Oxygen used (−ΔO)	Phosphate bound (−ΔP)	P:O ratio
	μatoms	*μmoles*	
Mitochondria isolated in 0.25 M sucrose + nuclei prepared in sucrose-magnesium	7.9	9.4	1.19
Above + 5×10^{-5} M methylene blue	8.9	3.2	0.36

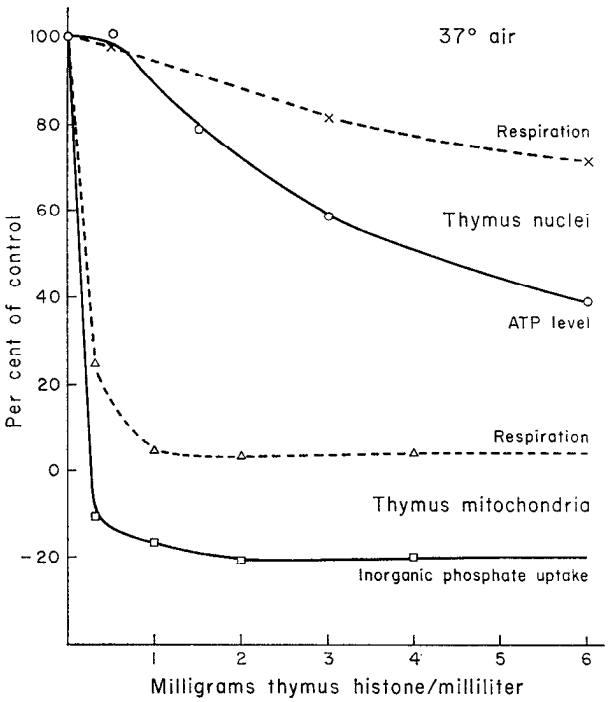

FIG. 4. The effects of added thymus histones on respiration and phosphorylation in suspensions of thymus nuclei (*upper curves*) and isolated thymus mitochondria (*lower curves*). The conditions of incubation are described in detail in "Experimental Procedure."

TABLE VIII

Effects of deoxyribonuclease on respiration and ATP levels in isolated thymus nuclei

The nuclei were preincubated at 37° for 20 minutes in an incubation medium containing 0.5 mg per ml of crystalline pancreatic DNase (Worthington). The DNA-depleted nuclei were collected by centrifugation and resuspended in 0.25 M sucrose-0.003 M $CaCl_2$. The suspension was added to the incubation medium described in "Experimental Procedure." In the "restoration" experiments, polyethylene sulfonate (molecular weight, 12,900) was added to the DNase-treated nuclei to give a final concentration of 1.5 mg per ml. In all cases, the added substrate was glucose. Respiration was measured over a 2-hour interval at 37°.

Conditions of experiment	Oxygen uptake				ATP level*	
	$\mu l\ O_2$ (0–60 min)	% control	$\mu l\ O_2$ (60–120 min)	% control	mμmoles ATP/ml	% control
Control	41	100	31	100	282	100
Nuclei + DNase, 1 mg	31	76	16	52	56	20
DNase-treated nuclei + polyethylene sulfonate	37	90	26	84	321	114

* Data from a parallel experiment under the same experimental conditions.

levels were 20 to 22% lower than observed in a histone-free medium.

Comparative Effects of Deoxyribonuclease Treatment on Respiration and Phosphorylation in Nuclei and Mitochondria—In earlier work on nuclear phosphorylation, it was observed that treatment of thymus nuclear fractions with pancreatic deoxyribonuclease results in a loss of capacity to maintain nuclear ATP levels (9, 10). As a result, nuclear protein synthesis and RNA synthesis come to a halt. It has now been found that DNase treatment impairs respiration and glycolysis as well. Some effects are shown in Table VIII, which compares oxygen consumption and ATP content in "control" nuclei with those of nuclei pretreated with pancreatic deoxyribonuclease. In these experiments, the nuclei were exposed to DNase for 30 minutes at 37°, centrifuged down, and resuspended in the incubation medium as described previously (9). It can be seen that removal of the DNA results in a lowered rate of nuclear respiration, and that nuclear ATP levels are much reduced after DNase treatment.

In accord with earlier observations that ATP synthesis can be restored to DNase-treated nuclei by the addition of polyanions (10), it has been found that a supplement of polyethylene sulfonate also restores the respiration of DNA-depleted nuclei (Table VIII).

On the other hand, isolated mitochondria are not affected by DNase treatment under these conditions. The data in Table IVC show that both respiration and phosphorylation are practically unchanged after enzyme treatment.

DISCUSSION

Occurrence of ATP and ATP-dependent Reactions in Cell Nuclei from Various Tissues—The occurrence of a nucleotide pool in the nucleus *in vivo* is now well established by the findings of several laboratories that nuclei from a variety of cell types (derived from mammals, birds, and fish) contain ATP and other phosphorylated nucleosides. Among the nuclei tested were preparations from rabbit appendix, intestinal mucosa, and liver (6, 8), rat liver (23), calf liver (7), chicken erythrocytes, and trout sperm (7). It should be stressed that all these nuclear isolations were carried out in nonaqueous media, under conditions that preclude a loss or exchange of nucleotides between nucleus and cytoplasm.

In certain cases, it has been possible to demonstrate a net ATP synthesis in isolated nuclei, or to show the occurrence of nuclear biosynthetic processes which are ATP-dependent. For example, ATP-dependent amino acid incorporation into protein can be observed in nuclei from calf thymus, mouse AKR lymphoma cells (1), and Novikoff hepatoma (24). Similarly, an uptake of P^{32}-labeled orthophosphate into RNA, DNA, and organic phosphates has been observed in nuclei from appendix tissue (25) and calf thymus (26). However, not all preparations of isolated nuclei retain their capacity to synthesize ATP. In certain cases, such as the nuclei prepared from pea seedlings, it may be possible to add ATP or an ATP-generating system to stimulate biosynthetic processes (27), but in rat liver nuclei, ATP supplements do not lead to appreciable protein-synthetic activity (24). Similarly, we have observed that calf liver nuclei isolated in sucrose solutions do not retain their nucleotides,[4] and it is not surprising that such nuclei are unable to activate amino acids for protein synthesis.

Failure of Whole Cells to Account for Nuclear ATP Synthesis—The question arises as to whether the few whole cells present in the nuclear fractions are responsible for the phosphorylations observed, and the evidence indicates that the free nuclei are themselves capable of performing the synthesis of ATP. Although some intact cells are found in thymus nuclear suspensions, they are a small minority; yet over 80% of the total mononucleotides in the suspension can be phosphorylated to ATP in a 10-minute incubation. Since the nucleotide levels in thymus nuclei isolated in sucrose solutions are comparable to those observed in thymus nuclei isolated in nonaqueous media (7), and the concentration of nucleotides in thymus cytoplasm does not exceed that of the nonaqueous nuclei, it follows that the few cells which occur in the thymus nuclear suspensions examined here could not account for more than a small part of the total nucleotide present, nor could they account for the observed ATP synthesis. Furthermore, it should be pointed out that treatment of thymus nuclear suspensions with deoxyribonuclease inhibits ATP synthesis up to 80%, yet the enzyme does not attack intact cells in the suspension. Other indirect evidence for the localization of ATP in the isolated nuclei themselves comes from autoradiography experiments in which it was proven that nuclei free of cytoplasmic contamination can actively incorporate amino acids into protein, but only in an atmosphere containing oxygen (2, 21). As in cytoplasmic systems, the amino acid activation reactions in nuclear protein synthesis require ATP (28).

Intranuclear Localization of ATP Synthesis—The isolated calf thymus nucleus can retain its nucleotides during an isolation in isotonic sucrose media, and, as shown above, such nuclei do not seem to be permeable to external ATP. The failure of thymus nuclei to phosphorylate added AMP (observed previously by Osawa *et al.* (7)) and their failure to phosphorylate ADP added to the medium can now be explained in terms of nuclear "pool" permeability, since nucleotides, as such, do not readily

[4] A. E. Mirsky, unpublished observations.

penetrate the intranuclear pool. When an incorporation of C^{14}-labeled AMP or ADP into nuclear ATP is observed, most of it is due to prior hydrolysis to the corresponding nucleoside or free base, either of which can enter the nucleus. (The occurrence of phosphatases and of nucleoside phosphorylase in thymus nuclear suspensions has already been demonstrated by Stern *et al.* (29).)

It is difficult to imagine how the few mitochondria present in the nuclear suspension could effect ATP synthesis for the nucleus. Not only does the ATP synthesis in the nuclear suspension involve the phosphorylation of intranuclear AMP and ADP, but all nucleotides outside of the nuclear pool are rapidly hydrolyzed. It is conceivable, but hardly likely, that some mitochondria attached to the nucleus might communicate with the inside of the nucleus in such a manner that nucleotides passing between nuclei and mitochondria would not be "outside" of the nuclear pool, but, under these circumstances, one would expect ATP synthesis in the nuclear suspension to be sensitive to carbon monoxide, methylene blue, and calcium ions, inhibitors which have already been shown to block mitochondrial oxidative phosphorylation and not to inhibit nuclear ATP synthesis. It has been adequately shown, by mixing mitochondria and nuclei, that carbon monoxide, calcium ions, and methylene blue can selectively inhibit mitochondrial phosphorylation despite the presence of nuclei, which might tend to bind these substances.

Inhibitors such as azide, cyanide, 2,4-dinitrophenol, antimycin A, Amytal, and Dicumarol block both nuclear and mitochondrial ATP synthesis. Several reports in the literature suggest that these inhibitors of oxidative phosphorylation by mitochondria are not entirely specific for mitochondria. Thus, it has been reported that antimycin A may block 3,4-dihydroxyphenylalanine oxidase, a copper-containing enzyme (30). Furthermore, rabbit liver aldehyde oxidase is inhibited by Amytal, antimycin A, and cyanide (31). It remains to be seen which nuclear enzymes are inhibited by these agents.

It is not yet known that ATP synthesis in the nucleus involves a direct coupling of phosphorylation to oxidation, as it does in mitochondria. Until that time, it is premature to call the observed nuclear ATP synthesis "oxidative phosphorylalation." What has been demonstrated is an oxygen-dependent ATP synthesis that occurs within the nucleus and supplies ATP for nuclear functions. In a subsequent paper, the nature of the metabolic pathways that supply the free energy for ATP synthesis will be discussed, and the possibility that "substrate-linked" phosphorylations may contribute to nuclear ATP levels will be examined.

Effect of Thymus Histones and DNase on Nuclear Respiration and ATP Synthesis—The sensitivity of nuclear respiration and ATP synthesis to treatment with deoxyribonuclease at first suggested that DNA plays a role in nuclear phosphorylation, and some evidence relating DNA and magnesium ions to ATP-binding sites in the nucleus has already been presented (32). It seems unlikely, however, that DNA plays a specific intermediary role in phosphate transfer to AMP or ADP, because a large part of the nuclear DNA can be replaced by polyethylene sulfonate, polyacrylic acid, or other polyanions, with no loss of ATP-synthetic activity (10). It may be suggested that at least part of the effect of removing DNA from the nucleus may be indirect and result from a release of histones which were previously bound to the DNA in the chromosomes. It has already been observed that the addition of histones and other basic polypeptides to nuclei inhibits a number of ATP-dependent synthetic reactions (33).

Nuclei are much less susceptible to histone inhibition than are mitochondria from the same tissue. Although it is possible that histones do not penetrate nuclei as well as they do mitochondria, the difference could be due to the "trapping" of added histone by nucleic acids in the nucleus, and the absence of such complex formation in mitochondria, which are not as basophilic. The effect of histones on mitochondrial oxidative phosphorylation is probably due to their combination with cytochrome *c* oxidase, since Person and Fine (34) and Conrad *et al.* (35, 36) have reported that histones and other basic proteins strongly inhibit this enzyme. It is unlikely, however, that this is the mechanism of histone inhibition of nuclear phosphorylation reactions, since cytochrome *c* oxidase (an enzyme inhibited by carbon monoxide) cannot be detected in thymus nuclei (37), nor can carbon monoxide inhibition of nuclear respiration be demonstrated.

Other experiments have indicated some clear differences between the effects of added histones and deoxyribonuclease treatment of isolated nuclei. Metabolic pathways of glucose utilization are affected differently by these procedures, and different histone fractions have different effects. These findings will be reported in a subsequent paper.

SUMMARY

A comparison has been made between oxidative phosphorylation by isolated calf thymus mitochondria and aerobic adenosine triphosphate (ATP) synthesis in suspensions of calf thymus nuclei.

1. The nuclear system is insensitive to carbon monoxide, calcium ions, and methylene blue, whereas thymus mitochondria are strongly inhibited by these agents even in the presence of thymus nuclei. Use is made of these three inhibitors to show that ATP synthesis by thymus nuclei is not due to mitochondria in the nuclear suspension.

2. Both the nuclear and mitochondrial ATP synthesis are inhibited by cyanide, azide, 2,4-dinitrophenol, antimycin A, Dicumarol, and Amytal. The possibility is considered that the nucleus contains oxidative enzymes, other than those found in mitochondria, which are inhibited by these six compounds.

3. Evidence is presented that nuclear ATP is derived from an endogenous nucleotide pool and that added adenosine phosphate, adenosine diphosphate, or adenosine triphosphate do not penetrate the nuclear pool.

4. Nuclear respiration and phosphorylation are inhibited by treatment with deoxyribonuclease, whereas mitochondria are not affected.

5. Mitochondrial ATP synthesis can be inhibited by low concentrations of histones extracted from the thymus nucleus. Nuclear ATP synthesis can also be inhibited by histones, but much higher concentrations are required.

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Studies of Energy-yielding Reactions in Thymus Nuclei: I. COMPARISON OF NUCLEAR AND MITOCHONDRIAL PHOSPHORYLATION

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