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Studies on Energy-yielding Reactions in Thymus Nuclei

III. PARTICIPATION OF GLYCOLYSIS AND THE CITRIC ACID CYCLE IN NUCLEAR ADENOSINE TRIPHOSPHATE SYNTHESIS*

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It was shown in the preceding paper that calf thymus nuclei contain enzymes of glycolysis, the citric acid cycle, and the hexose monophosphate shunt (1). Siebert *et al.* (2, 3) have found enzymes of these pathways in nuclei from pig kidney, rat liver, and beef brain and glycolytic enzymes have been found in other nuclei by Stern and Mirsky (4) and Dounce and Beyer (5). In addition, nuclei from many tissues contain adenosine triphosphate and other nucleotides (2, 6–8). The intranuclear localization of these enzymes and nucleotides is unequivocal, because the nuclei were isolated in "nonaqueous" media under conditions which prevent loss or exchange of enzymes and other water-soluble molecules during isolation (9).

In view of the well demonstrated role of glycolysis and the citric acid cycle in cellular energy production, it seemed of interest to study the role of these pathways in ATP production by cell nuclei. However, most cell nuclei (such as those of liver) do not retain acid-soluble nucleotides[1] and lose certain water-soluble enzymes (10) during isolation in aqueous solutions. Calf thymus nuclei, however, do retain both acid-soluble nucleotides (7) and soluble enzymes (10) after isolation in 0.25 M sucrose-0.003 M CaCl₂, and they carry out energy-dependent processes such as protein synthesis (11, 12) and RNA synthesis (13–15). Energy for these processes is supplied by an oxygen-dependent ATP synthesis which was shown in the first paper of this series to be independent of mitochondrial oxidative phosphorylation (16).

In the experiments described in the present paper, well known metabolic inhibitors and the enzyme DNase were employed to prove that both glycolysis and the citric acid cycle take part in aerobic nuclear ATP synthesis. Inhibitors of glycolysis, such as iodoacetic acid, and of the citric acid cycle, such as fluoroacetic acid, inhibit both respiration and ATP synthesis by isolated thymus nuclei. Furthermore, preincubation of the nuclei with pancreatic DNase, a procedure which inhibits nuclear ATP synthesis (17, 18), inhibits glycolysis and the citric acid cycle without affecting the first two enzymes of the hexose monophosphate shunt or the catabolism of ascorbic acid-1-C¹⁴ to C¹⁴O₂. Evidence is also presented that di- and tricarboxylic acids such as succinic, malonic, and citric acids do not penetrate rapidly, if at all, into the "acid-soluble" pool of isolated nuclei. Finally, thymus nuclei appear to have an endogenous substrate, and

some experiments are presented which suggest that this substrate is probably not glycogen or glucose.

EXPERIMENTAL PROCEDURE

Isolation of Nuclei from Calf Thymus Tissue—Nuclei were isolated in isotonic sucrose solutions (0.25 M sucrose-0.003 M CaCl₂) as described previously (13). Nuclei prepared in this way were shown to satisfy a number of morphological and chemical criteria of purity, but examination with the electron microscope showed that they contain some intact thymocytes (9, 10, 12, 19). Good preparations have fewer than 10% cells; poor preparations may contain 17 to 25% whole cells. These whole cells can be removed by centrifugation through sucrose solutions containing the synthetic polysaccharide Ficoll (Pharmacia, Ltd., Vanlose, Copenhagen, Denmark), as described previously (16).

Unfortunately, Ficoll is somewhat toxic to nuclear metabolism. Therefore, a slightly modified procedure was developed for isolating nuclei. This procedure, which was described in the preceding paper (1), involves blending thymus tissue in slightly hypotonic sucrose solutions (0.20 M sucrose-0.0033 M CaCl₂) and then raising the sucrose concentration to 0.25 M. Nuclei prepared in this way were found by observation with the electron microscope to regularly contain less than 6% whole cells (usually approximately 3 to 4%).[2]

For the experiments with mannitol, nuclei were isolated in 0.25 M mannitol with 0.003 M CaCl₂ by the same procedure that is used for isolating nuclei in isotonic sucrose (13). All solutions contained only mannitol and no sucrose whatever.

Incubation Conditions for Thymus Nuclei—The usual incubation mixture contained nuclear suspension, buffer, and substrate in the following proportions: for every milliliter of nuclear suspension (approximately 40 mg, dry weight) was added 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 MgCl₂, and 8.0 mg of NaCl per ml. When acetate, lactate, or pyruvate were used as substrates, the "substrate + salts" solution contained 100 μmoles of the acid (neutralized to pH 7.4 with sodium hydroxide), 25 μmoles of MgCl₂, and 3 mg of NaCl per ml. When no substrate was added to the mixture, the "substrate + salts" solution contained 100 μmoles of sucrose, 25 μmoles of MgCl₂, and 8 mg of NaCl per ml. Incubations of nuclei isolated in mannose or mannitol were carried out in a

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

[2] V. C. Littau, unpublished observations.

medium containing mannose or mannitol substituted for sucrose but identical in all other respects to the solutions described.

Incubation of Nuclei with Deoxyribonuclease—Thymus nuclei were incubated for 20 minutes at 37° in the nuclear incubation medium described above without added substrate. A control flask contained no DNase; the other flask contained 0.5 mg per ml or 1.0 mg per ml of pancreatic DNase (Worthington Biochemical Corporation). After incubation, the nuclei were re-isolated from the incubation mixture by centrifugation and resuspended in a volume of 0.25 M sucrose-0.003 M CaCl₂ equal to the original volume of nuclear suspension.

Labeling of Nuclei with Radioisotope—Nuclei were incubated for 15 minutes at 37° in the nuclear incubation medium described above. No substrate was added except for the isotope (1.5 μc of acetate-2-C^{14} per ml of nuclear suspension, or 0.5 μc of pyruvate-3-C^{14} per ml of nuclear suspension). After incubation, the nuclei were reisolated from the incubation medium by adding 4 ml of incubation mixture to 8 ml of cold 0.25 M sucrose-0.003 M CaCl₂ and centrifuging. The supernatant fluid, which contains isotope that had not penetrated into nuclei, was discarded. The nuclear sediment was then resuspended in 5 ml of 0.25 M sucrose-0.003 M CaCl₂ for use in a subsequent incubation.

Incubation of Nuclei for Transport Experiments—Thymus nuclei were incubated at 37° in the usual nuclear incubation medium as described above. No substrate was added except for the metabolite being tested. Samples (1 ml) of the incubation mixture were removed at 0, 15, and 30 minutes, mixed with 11 ml of cold 0.25 M sucrose-0.003 M CaCl₂, and centrifuged. The supernatant solution was discarded and the walls of the tube were washed with 10 ml of 0.25 M sucrose-0.003 M CaCl₂, taking care not to disturb the nuclear sediment. Metabolites present in the nuclear "pool" were extracted from the sediment with 3 ml of 2% perchloric acid. The perchloric acid was precipitated as potassium perchlorate by adding KOH, and the extract was plated in cup planchets and counted in a gas flow counter as described previously (20). The counts per minute were expressed as millimicromoles of metabolite.

Measurement of C$^{14}O_2$ Production from Labeled Metabolites—Thymus nuclei were incubated at 37° for 60 minutes in the incubation medium described above. No substrate was added except for 1 μc of the metabolite being tested: glucose-1-C^{14} (2.0 μc/μmole), glucose-6-C^{14} (2.1 μc/μmole), uniformly labeled sucrose-C^{14} (4.14 μc/μmole), pyruvate-3-C^{14} (3.6 μc/μmole), acetate-2-C^{14} (1.97 μc/μmole), succinate-1-C^{14} (6.2 μc/μmole), citrate-1,5-C^{14} (1.1 μc/μmole), or ascorbic acid-1-C^{14} (1.35 μc/μmole). Incubations were carried out in small Warburg flasks containing 2 ml of incubation mixture. At the end of the incubation 0.1 ml of 50% trichloroacetic acid was added from the side arm to liberate the C$^{14}O_2$ from the medium. The C$^{14}O_2$ was collected in sodium hydroxide in the center well of the flask, plated as BaCO₃, and counted in a gas flow counter. Counts were corrected for self-absorption of BaCO₃ (21) and expressed as counts per minute produced by 1 ml of nuclear suspension.

Measurement of Respiration and ATP Levels of Thymus Nuclear Suspensions—Respiration of nuclear suspensions was measured by conventional Warburg manometry. ATP was extracted from the incubation mixture and separated from other nucleotides by chromatography on small Dowex 1-formate columns, as described previously (16).

Estimation of Glycogen in Thymus Nuclei and Thymus Tissue—Fresh thymus tissue was minced into small pieces with scissors

and was then dehydrated with three changes (10 ml per change) of 88% ethanol, one change of 95% ethanol, and two changes of diethyl ether. After driving off the ether, the sample was weighed and heated for 2 hours at 100° in 3 ml of 1 N HCl to hydrolyze any glycogen present. The hydolysate, after removing the residue by centrifugation, was neutralized with KOH and used for the estimation of glucose described below. Thymus nuclei, isolated in hypotonic sucrose as described, were dehydrated and hydrolyzed by the same procedure used for whole tissue. It was estimated that the dehydration steps removed virtually all of the sucrose from the nuclei. Whole tissue and nuclei prepared in nonaqueous solvents were hydrolyzed directly in 1 N HCl at 100° for 2 hours.

Glucose was estimated in the neutralized hydrolysate by the procedure of Keston (22). This involved a reaction with glucose oxidase and peroxidase which produces a colored product that is estimated spectrophotometrically. The standard curve for glucose was measured in the presence of the hydrolysate in order to correct for a slight inhibition of the reaction by the hydrolysate. (Enzymes and other reagents for the estimation of glucose by this method are available in kit form from Worthington Biochemical Corporation.)

RESULTS

Effects of Metabolic Inhibitors on Nuclear Metabolism and ATP Levels

The endogenous ATP levels in suspensions of calf thymus nuclei isolated in sucrose solutions reflect the balance between ATP synthesis and utilization, and inhibitors which block ATP synthesis and not utilization result in lower ATP levels (16).

Glycolysis—Several key enzymes and metabolites of glycolysis were shown to be normal constituents of calf thymus nuclei (1). Two inhibitors have revealed the important role which glycolysis plays in nuclear ATP synthesis. Iodoacetic acid, an inhibitor of the glycolytic enzyme, triosephosphate dehydrogenase (23), decreased nuclear respiration and nuclear ATP levels (see Table I). At a concentration of 10^{-3} M, this inhibitor also blocked the conversion of C^{14}-labeled glucose to C$^{14}O_2$ by 75% (Table I). Sodium fluoride, an inhibitor of the glycolytic enzyme, enolase (23), blocked nuclear respiration and ATP synthesis to a considerable extent (see Table I).

Citric Acid Cycle—Several key enzymes of this pathway, including succinic dehydrogenase, appear to be normal constituents of calf thymus nuclei (1). Two inhibitors of the citric acid cycle have revealed that this pathway, as well as glycolysis, contributes to nuclear ATP synthesis. Fluoroacetic acid, which is converted to fluorocitrate, an inhibitor of the citric acid cycle enzyme, aconitase (24), inhibited nuclear respiration and ATP synthesis (Table I). This inhibitor was also found to block the conversion of C^{14}-labeled acetate and pyruvate to C$^{14}O_2$ by 84% and 93%, respectively. Dehydroacetic acid, an inhibitor of the nuclear succinic dehydrogenase described in the preceding paper (1), inhibited nuclear respiration and ATP synthesis (Table I). This inhibitor blocked the conversion of C^{14}-labeled glucose to C$^{14}O_2$ by 87% (see Table I). In contrast to dehydroacetic acid, the more common succinic dehydrogenase inhibitor, malonate (25), had no effect on nuclear respiration and ATP synthesis and inhibited the conversion of C^{14}-labeled glucose to C$^{14}O_2$ by only 16% (see Table I). Malonic acid, however, is a

TABLE I

Effects of metabolic inhibitors on nuclear metabolism and ATP synthesis

Nuclei for Experiments A to C were isolated in isotonic sucrose solutions, whereas nuclei for Experiment D were isolated in isotonic sucrose after homogenizing the tissue briefly in hypotonic sucrose. Incubations were carried out for 60 minutes at 37° in the usual nuclear incubation medium containing the indicated inhibitors without added substrate. ATP levels are expressed as millimicromoles per ml of nuclear suspension, and respiration as microliters per hour per ml of nuclear suspension. In parallel experiments for $C^{14}O_2$ production, 1 μc of glucose-6-C^{14} (specific activity, 2.1 μc per μmole) was added to 2 ml of incubation mixture and $C^{14}O_2$ production was measured as described in "Experimental Procedure." $C^{14}O_2$ produced is expressed as counts per minute per 60 minutes per ml of nuclear suspension.

Conditions of experiment	Concentration	$C^{14}O_2$ from glucose-6-C^{14}		Oxygen uptake		ATP level	
		Amount	Inhibition	Amount	Inhibition	Amount	Inhibition
	M	c.p.m./60 min/ml	%	$\mu l/O_2/60$ min/ml	%	$m\mu moles/ml$	%
A. Control		11,630		103		159	
Iodoacetic acid	10^{-4}			83	20	120	25
Iodoacetic acid	10^{-3}	2,955	75	70	32	17	89
NaF	10^{-2}			72	30	38	76
B. Control		11,630		125		160	
D-2-Deoxyglucose	2×10^{-2}	185	98	114	9	154	4
C. Control		5,890		91		155	
Fluoroacetic acid	10^{-2}			54	40	89	43
Malonic acid	10^{-2}	4,980	16	92	0	154	0
D. Control		2,950		35		60	
Dehydroacetic acid	5×10^{-2}	371	87	30	14	44	27

very effective inhibitor of the succinic dehydrogenase found in disrupted nuclei, as described in the previous paper (1). The explanation for these seemingly contradictory observations is that malonic acid does not penetrate very rapidly, if at all, into intact thymus nuclei. (The permeability properties of isolated thymus nuclei will be taken up again in a later section.) It should also be noted that fluoride can inhibit succinic dehydrogenase (26). Thus the effects of sodium fluoride, ascribed to the inhibition of enolase, may also be due in part to the inhibition of nuclear succinic dehydrogenase (see Table I).

Production of $C^{14}O_3$ from C^{14}-labeled Metabolites by Isolated Thymus Nuclei

The ability of inhibitors of both glycolysis and the citric acid cycle to inhibit nuclear respiration and ATP synthesis indicates the important contributions which these pathways make to nuclear metabolism. It was also noted in the previous section that suspensions of thymus nuclei convert glucose-6-C^{14} to $C^{14}O_2$ and that inhibitors of glycolysis and the citric acid cycle, which penetrate into the nucleus, block the conversion to a considerable extent (75 to 90%). Thus the main pathway for the conversion of glucose-6-C^{14} to $C^{14}O_2$ involves glycolysis and the citric acid cycle. (Another pathway which seems to be present in thymus nuclei, the hexose monophosphate shunt, does not oxidize the 6-position of glucose.) Thymus nuclei also utilize products of glycolysis, such as pyruvate and acetate, coverting both acetate-2-C^{14} and pyruvate-3-C^{14} to $C^{14}O_2$ (Table II). This catabolism is undoubtedly due to the citric acid cycle, since $C^{14}O_2$-production from these two metabolites was blocked 84% and 93%, respectively, by 10^{-2} M fluoroacetic acid.

It seems very likely for several reasons that the complete glycolytic pathway and citric acid cycle are present in thymus nuclei. First, the conversion of glucose-6-C^{14} to $C^{14}O_2$ requires both pathways as was noted above. Second, several turns of a complete citric acid cycle are required to convert pyruvate labeled

TABLE II

$C^{14}O_2$ production from C^{14}-labeled metabolites by isolated thymus nuclei and inhibition by DNase

Nuclei were isolated in isotonic sucrose solutions and were incubated in the usual nuclear incubation medium for 60 minutes at 37° without adding substrate except for the labeled metabolite being tested. To test the effect of DNase, nuclei were incubated with the enzyme for 20 minutes (see "Experimental Procedure"), reisolated, and then incubated with 1 μc of the isotope in 2 ml of incubation mixture. The control flasks contained nuclei incubated without DNase, reisolated, and then incubated with the isotope.

Experiment	Metabolite added	$C^{14}O_2$ produced		Inhibition by DNase
		Control	+ DNase	
		c.p.m./60 min/ml		%
A	Glucose-6-C^{14}	7,900	3,150	49
B	Glucose-6-C^{14}	7,130	1,830	74
A	Pyruvic acid-3-C^{14}	15,800	2,700	83
	Acetic acid-2-C^{14}	12,800	3,200	75
	Citrate-1,5-C^{14}*	370	1,540	Stimulation
	Succinate-1-C^{14}*	2,500	5,500	Stimulation
A	Glucose-1-C^{14}	40,800	44,200	None
B	Glucose-1-C^{14}	46,800	45,900	None
A	Ascorbate-1-C^{14}	6,250	6,550	None

* Does not penetrate into intact nuclei (see Fig. 1).

in the 3-position or acetate labeled in the 2-position to $C^{14}O_2$. However, it is not possible to test directly for CO_2-production from added citric acid cycle intermediates. Succinate-1-C^{14} and citrate-1,5-C^{14} are not extensively converted to $C^{14}O_2$ by intact isolated thymus nuclei (Table II), because these acids do not readily penetrate into isolated thymus nuclei (see the next sec-
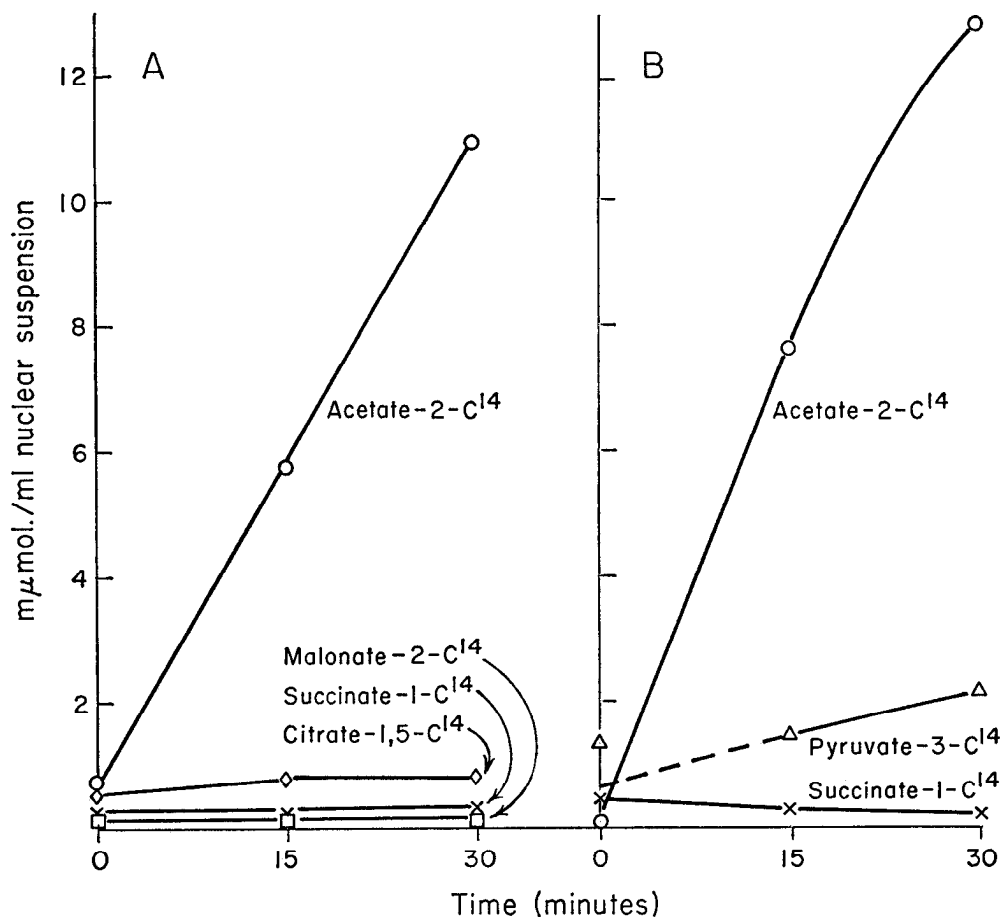
Penetration into Thymus Nuclei - 37°

Fig. 1. The time course of penetration of C^{14}-labeled metabolites into the acid-soluble pool of calf thymus nuclei isolated in isotonic sucrose after brief blending of the tissue in hypotonic sucrose. The incubation conditions and techniques for measuring penetration are described in "Experimental Procedure." *A* and *B* show two separate experiments done under identical conditions.

tion). However, one can demonstrate the formation of C^{14}-citrate in nuclei from acetate-2-C^{14} added to the medium.[3]

The first two enzymes of the hexose monophosphate shunt, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, are normal constituents of calf thymus nuclei (1). These enzymes are evidently very active in thymus nuclei isolated in sucrose solutions. Table II shows that nuclei convert 4 times more glucose-1-C^{14} than glucose-6-C^{14} to $C^{14}O_2$. This is usually interpreted to mean that the shunt is operating in addition to glycolysis and the citric acid cycle (27). Whether the observed ratio of 4 means that glucose metabolism via the shunt is actually 4 times greater than by way of glycolysis and the citric acid cycle is doubtful, because it is not certain how much of the isotope is diluted by endogenous metabolites of the respective pathways (27).

Isolated thymus nuclei also break down ascorbic acid-1-C^{14} to $C^{14}O_2$ (Table II). Whether some part of the glucuronic acid pathway is present in thymus nuclei is open to question. However, thymus nuclei are known to contain appreciable quantities of ascorbic acid (28).

Failure of Di- and Tricarboxylic Acids to Penetrate into Thymus Nuclei

Isolated thymus nuclei transport amino acids into an intranuclear pool by a process which seems to depend on energy from nuclear ATP synthesis (20). When it was found that malonate failed to inhibit nuclear metabolism and that C^{14}-succinate and citrate were not extensively metabolized by thymus nuclear suspensions, it was decided to examine the permeability of the nucleus to these substances. Fig. 1A shows that whereas acetate-2-C^{14} penetrated rapidly into the nuclear pool, citrate-1,5-C^{14}, succinate-1-C^{14}, and malonate-2-C^{14} did not appear to enter at all. Other dicarboxylic acids, such as glutamic acid, also failed to penetrate into the nuclear pool.[4] Pyruvate-3-C^{14} penetrated more slowly than acetate-2-C^{14} (Fig. 1B). Glucose has been observed to penetrate so rapidly that the time course cannot be measured under these conditions.[4]

The selective exclusion of di- and tricarboxylic acids from the acid-soluble pool of isolated thymus nuclei may explain the selectivity of the "acetate effect" described by Osawa, Allfrey, and Mirsky (7). In this effect, monocarboxylic acids (such as acetic,

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[3] B. McEwen, unpublished observations.

[4] M. Izawa, unpublished observations.

formic, propionic, and monochloroacetic) were observed to extract acid-soluble nucleotides and potassium ions from isolated thymus nuclei at pH values below 5. Di- and tricarboxylic acids, particularly succinic and citric, did not extract these substances even at pH 4.2 to 4.4. It now seems likely that these two acids had no effect because they did not penetrate into the nuclei.

Inhibition of Glycosis and Citric Acid Cycle by Treatment of Nuclei with DNase

In previous work it was shown that treatment of thymus nuclei with deoxyribonuclease inhibits ATP synthesis and respiration (16). Table II shows that DNase treatment also inhibited $C^{14}O_2$ production from glucose-6-C^{14}, acetate-2-C^{14}, and pyruvate-3-C^{14} by 70% or more. The inhibition by DNase was selective, in that $C^{14}O_2$ production from ascorbic acid-1-C^{14} and via the shunt, i.e. from glucose-1-C^{14} was not affected. $C^{14}O_2$ production from labeled succinate and citrate (neither of which penetrate rapidly into thymus nuclei) was increased very slightly by DNase treatment, due possibly to a greater rate of penetration of these metabolites into the damaged nuclei.

As a further demonstration of the action of DNase on energy metabolism via glycolysis, it was found that treatment with this enzyme also blocked lactic acid and pyruvic acid production from endogenous substrate and from glucose added to the incubation medium (see Table III). Demonstration of lower levels of citric acid cycle intermediates in DNase-treated nuclei could not be as unequivocally interpreted as evidence for direct inhibition of citric acid cycle activity, however, because inhibition of pyruvate production would result in lower levels of citric acid cycle intermediates.

Therefore, an attempt was made to show that $C^{14}O_2$ production via the citric acid cycle is inhibited by DNase under conditions in which other effects of DNase could be ruled out. First, it was necessary to avoid the possibility that the inhibition of $C^{14}O_2$ production by DNase could be due to an inhibition of "transport" or penetration of the metabolite into the nucleus. Accordingly, nuclei were incubated with acetate-2-C^{14} or with pyruvate-3-C^{14}, centrifuged, and washed to remove substrate which had not penetrated (see "Experimental Procedure"), and then treated with DNase. The subsequent $C^{14}O_2$ production from these previously labeled nuclei incubated with DNase was compared with the $C^{14}O_2$ production by nuclei which were treated with DNase before exposure to the isotope. It can be seen from Table IV that $C^{14}O_2$ production from acetate-2-C^{14} was inhibited by DNase whether or not penetration of the isotope occurred before DNase treatment. Also, it is necessary to rule out the possibility that the DNase effect on acetate catabolism is due to the depletion by DNase treatment of ATP, which is required for the activation of acetate to acetyl coenzyme A (29). Table IV shows that $C^{14}O_2$ production from nuclei prelabeled with pyruvate-3-C^{14}, which does not require ATP for entry into the citric acid cycle, is also sensitive to DNase.

Previous studies of the effect of DNase on thymus nuclei showed that nuclear ATP synthesis, which is inhibited when nuclei are treated with DNase, is not inhibited by DNase treatment when polyanions such as DNA, RNA, heparin, polyethylene sulfonate, or polyacrylic acid are present during the treatment with DNase (18). When nearly all of the DNA is removed with DNase in the presence of such polyanions, ATP synthesis and

Table III

Inhibition of lactic and pyruvic acid production by treatment of thymus nuclei with DNase

Nuclei were isolated in isotonic sucrose solutions and incubated with or without DNase as described in "Experimental Procedure." These nuclei were then incubated in the usual nuclear incubation medium without added substrate or with added glucose for 60 minutes at 37° and then acidified by adding an equal volume of 5% trichloroacetic acid in an ice bath. The supernatant solution, after centrifuging down the acid-precipitated nuclei, was used for the estimation of pyruvic and lactic acid, as described previously (1). Results are expressed as millimicromoles of acid per mg, dry weight.

Conditions of experiment	Lactic acid		Pyruvic acid	
	Amount	Inhibition	Amount	Inhibition
	mμmoles/mg	%	*mμmoles/mg*	%
No substrate added				
Control	46.5		2.2	
Previously treated with 0.5 mg/ml DNase	33.1	19	1.2	45
Glucose, 2×10^{-2} M				
Control	138		5.6	
Previously treated with 0.5 mg/ml DNase	50.2	62	3.0	46

amino acid incorporation into nuclear proteins are not noticeably affected. A similar "restorative" effect of the polyanion, polyethylene sulfonate, has now been shown for $C^{14}O_2$ production from acetate-2-C^{14} via the citric acid cycle. Table IV shows that nearly complete restoration of nuclear respiration and complete restoration of $C^{14}O_2$ production were achieved when polyethylene sulfonate was present during DNase treatment.

A partial explanation for the effects of DNase treatment and the "restorative" effects of polyanions was offered in a previous paper (16) and bears repeating in the present report. Removing DNA with DNase unmasks histones, including the strongly basic arginine-rich histones, and these free histones are known to inhibit nuclear metabolism. (Histones added to suspensions of thymus nuclei have been observed to inhibit nuclear ATP synthesis and respiration (16), amino acid incorporation into nuclear proteins (30), and nuclear RNA synthesis (31).) Polyanions added to nuclear suspensions would probably combine with the histones and thereby make them ineffective as inhibitors.

ATP Synthesis Associated with Citric Acid Cycle Metabolism

The inhibition of ATP synthesis in thymus nuclear suspensions by inhibitors of the citric acid cycle provides indirect evidence that the citric acid cycle is involved in nuclear ATP production. More direct evidence was obtained by showing that substrates which are metabolized exclusively by way of the citric acid cycle support nuclear ATP synthesis when the glycolytic contribution to nuclear ATP synthesis is partially inhibited by iodoacetic acid. Pyruvate, lactate, and acetate were chosen as substrates because they penetrate into nuclei; moreover, they appear to be catabolized exclusively by the citric acid cycle, since $C^{14}O_2$ production from acetate-2-C^{14} and from pyruvate-3-C^{14} was inhibited more than 80% by 10^{-2} M fluoroacetic acid and 5×10^{-2} M dehydro-

TABLE IV

Inhibition of citric acid cycle in thymus nuclei by treatment with DNase and restoration of activity by polyethylene sulfonate

Nuclei for Experiments A to C were isolated in isotonic sucrose solutions after blending the tissue briefly in hypotonic sucrose. Nuclei for Experiments A and C were previously labeled with acetate-2-C^{14} or pyruvate-3-C^{14} as described in "Experimental Procedure" and then incubated with or without 1 mg per ml of DNase. Nuclei for Experiment B were incubated with DNase as described in "Experimental Procedure" and then incubated with 1 μc per 2 ml of acetate-2-C^{14}. For Experiment D, nuclei were isolated in isotonic sucrose solutions and incubated with 0.5 mg per ml of DNase with or without 2 mg per ml of polyethylene sulfonate (molecular weight, 12,900). Respiration is expressed as microliters of oxygen consumed per ml of nuclear suspension and $C^{14}O_2$ production as counts per minute produced per ml of nuclear suspension.

Conditions of initial incubation	Conditions of final incubation	DNA removed	Respiration		$C^{14}O_2$ production	
			0–30 min	30–60 min	0–30 min	30–60 min
		%	$\mu l/ml$		c.p.m./ml	
A. Nuclei labeled with acetate-2-C^{14}	Control		14.5	11.1	3680	2220
	1 mg/ml DNase	85	10.6	4.4	2280	220
B. Nuclei incubated						
Without DNase	1 μc Acetate-2-C^{14}		14.5	11.1	2860	1580
With 1 mg/ml DNase	1 μc Acetate-2-C^{14}	85	9.5	1.5	285	270
C. Nuclei labeled with pyruvate-3-C^{14}	Control		13.6	8.6	504	361
	1 mg/ml DNase	94	10.3	6.9	420	84
D. Nuclei incubated						
Without DNase	1 μc Acetate-2-C^{14}		62		6350	
With 0.5 mg/ml DNase	1 μc Acetate-2-C^{14}	68	49		3060	
With 0.5 mg/ml DNase + 2 mg/ml PES*	1 μc Acetate-2-C^{14}	66	59		6720	

* PES, polyethylene sulfonate.

TABLE V

Maintenance of nuclear ATP levels and respiration by citric acid cycle metabolites when glycolysis is inhibited

Nuclei were isolated in isotonic sucrose solutions after homogenizing the tissue briefly in hypotonic sucrose. They were incubated in large Warburg flasks for 30 minutes at 37° in the usual nuclear incubation medium: 5 ml of nuclear suspension, 2.5 ml of buffer, and 2.0 ml of "substrate + salts" solution containing sucrose when no substrate was added or acetate, pyruvate, or lactate as indicated. Iodoacetic acid (10^{-3} M) was present, where indicated, from the start of the incubation. Anaerobic conditions were produced by flushing with nitrogen during the incubation.

Conditions of experiment	Substrate	Respiration	ATP level	
			$m\mu moles/ml$	Control
		$\mu l/30 \, min/ml$		%
A. Control, aerobic	Endogenous	35.0	70.0	100
+ Iodoacetate, aerobic	Endogenous	23.8	44.8	64
	Acetate, 2 × 10^{-2} M	26.6	47.3	68
	Pyruvate, 2 × 10^{-2} M	28.0	87.0	124
	Lactate, 2 × 10^{-2} M	33.0	64.4	92
B. Control, aerobic	Endogenous		72.8	100
+ Iodoacetate, aerobic	Endogenous		53.2	73
	Pyruvate, 2 × 10^{-2} M		71.5	98
Control, anaerobic	Endogenous		54.6	100
+ Iodoacetate, anaerobic	Endogenous		49.0	90
	Pyruvate, 2 × 10^{-2} M		33.1	68

acetic acid, inhibitors of aconitase and succinic dehydrogenase, respectively.

Table V shows the results of adding pyruvate, lactate, and acetate, as substrates, to nuclear suspensions to which 10^{-3} M iodoacetic acid was also added as an inhibitor of glycolysis. ATP levels, otherwise depleted by the effect of iodoacetic acid, were maintained by pyruvate, and to a lesser extent by lactate. Respiration was also partially maintained by these substrates. The maintenance of ATP levels by pyruvate, furthermore, required an aerobic atmosphere (see Table V, *B*). Acetate, how-ever, failed to support nuclear ATP levels in the presence of iodo-acetic acid, although it seems to have maintained respiration to some extent (Table V, *A*). (This is possibly explained by the increased requirement for ATP to produce acetyl coenzyme A, which enters the citric acid cycle.)

Search for an Endogenous Substrate for Nuclear Metabolism

Suspensions of calf thymus nuclei isolated in isotonic sucrose solutions carry on respiration, ATP synthesis, and amino acid incorporation into protein for nearly an hour in the absence of

TABLE VI

Glycogen in thymus tissue and thymus nuclei

Glycogen was estimated as glucose after hydrolysis (see "Experimental Procedure"), and is expressed as millimicromoles of glucose per mg, dry weight.

Sample analyzed	Glycogen level (as glucose)
	$m\mu moles/mg$
Fresh tissue	5.5
Aqueous nuclei	3.4
Nonaqueous tissue	4.4
Nonaqueous nuclei	2.0

TABLE VII

Nuclear metabolism in absence of added metabolizable sugar

Nuclei were isolated in isotonic mannitol as described in "Experimental Procedure." All incubations were carried out in the usual nuclear incubation medium in which sucrose had been replaced by mannitol. When no substrate was added, the "substrate + salts" solution contained 0.1 M mannitol in place of sucrose. Respiration is expressed as the Q_{O_2} (microliters of O_2 consumed per hour per mg, dry weight). Alanine incorporation into protein was measured over a 60-minute period (12). Lactic acid was estimated after a 30-minute incubation under the same experimental conditions. The nuclear suspensions were extracted with 5% trichloroacetic acid and lactic acid was measured enzymatically as described previously (1).

Conditions of experiment	Alanine incorporation	Q_{O_2}	Lactic acid
	$c.p.m./mg$		$m\mu moles/mg$
Nuclei			
Zero time			10.7
No substrate added	184	2.60	27.2
Sucrose, 2×10^{-2} M	234	2.27	27.6
Glucose, 5×10^{-3} M	229	2.15	
Glucose, 2×10^{-2} M	226	2.18	

any added substrate.[5] It will now be shown that nuclear suspensions contain endogenous substrates which are probably not glycogen or free glucose.

Both thymus nuclei and whole thymus tissue apparently contain negligible quantities of glycogen (see Table VI). The analyses in Table VI show that 1 ml of a nuclear suspension, containing 40 mg (dry weight) contained only 0.1 μmole of glucose as glycogen. Even complete oxidation of this amount of glucose could not account for the observed oxygen consumption in the nuclear suspension (1 to 3 μmoles of O_2 per hour). The virtual absence of glycogen from nuclei is in agreement with the findings of Siebert for nuclei from rat liver (2) and is consistent with the findings of Ord and Stocken for rat thymus nuclei (32). Although this suggests that mammalian nuclei do not contain glycogen, it should be noted that Himes and Pollister have found glycogen deposits in frog liver nuclei (33).

The possibility was next considered that sucrose in the isolation and incubation medium might be metabolized. On adding uniformly labeled sucrose-C^{14} to the incubation medium, it was observed that small amounts of sucrose were converted to CO_2. However, sucrose, as such, does not seem to be the main "en-

[5] B. McEwen, unpublished observations.

dogenous" substrate for the nuclear suspension. $C^{14}O_2$ production from uniformly labeled sucrose-C^{14} was inhibited by 84% in the presence of D-2-deoxyglucose, a glucose analogue which interferes with glycolysis (34). The same concentration of 2-deoxyglucose had no effect on nuclear ATP levels and only a slight effect on endogenous respiration (see Table I). A similar experiment showed that stored, free glucose is not an endogenous substrate, since 2-deoxyglucose inhibited $C^{14}O_2$ production from glucose-6-C^{14} by 98% although it had no effect on nuclear ATP synthesis (see Table I).

Further evidence for the utilization of endogenous substrate by nuclear suspensions is the observation that nuclei isolated in mannitol, i.e. in the absence of metabolizable sugar, maintained respiration for 60 minutes, incorporated amino acids into protein, and produced lactic acid (Table VII). The nature of the endogenous substrate or substrates remains to be discovered.

DISCUSSION

The presence of a number of enzymes of glycolysis and the citric acid cycle in cell nuclei is now well established, since these enzymes are readily detected in nuclei isolated from a variety of tissues with nonaqueous isolation procedures (1–5). No attempt has been made to demonstrate all of the enzymes of the citric acid cycle in "nonaqueous" nuclei, but enzymes or metabolites representing four of the eight steps in the citric acid cycle have been found (1). That thymus nuclei do contain a complete citric acid cycle is indicated by the ability of thymus nuclei isolated in isotonic sucrose to convert pyruvate-3-C^{14} and acetate-2-C^{14} to $C^{14}O_2$, as was shown in the present paper. The nearly complete inhibition of this $C^{14}O_2$ production by two inhibitors of the citric acid cycle, fluoroacetic acid and dehydroacetic acid, indicates that these acids are metabolized exclusively by way of the citric acid cycle. Aqueous thymus nuclei are also capable of net ATP synthesis in vitro (7, 16). The effects of specific inhibitors presented in this paper indicate that ATP production depends on the citric acid cycle as well as glycolysis, and this is in accord with the earlier observations on the oxygen-dependence of nuclear ATP synthesis (7, 16).

Since the citric acid cycle is usually associated with electron transport to molecular oxygen, this raises the question whether ATP synthesis in nuclei involves an "oxidative phosphorylation" as well as substrate-linked phosphorylations. Some evidence which suggests an "oxidative phosphorylation" in thymus nuclei was presented in the first paper of this series (18). It was also shown at that time that the ATP synthesis observed in thymus nuclear suspensions could not be due to mitochondrial contamination since it was not inhibited by carbon monoxide, calcium ions, or methylene blue (16).

Although small numbers of intact cells are found in suspensions of thymus nuclei, these cells cannot account for all of the ATP synthesis, and their actual contribution to over-all ATP levels is small (16). The effect of DNase is an important means of distinguishing nuclear from cellular metabolism and ATP synthesis, since cells are not attacked by DNase under experimental conditions employed, whereas nuclei are readily attacked by this enzyme. DNase treatment reduces ATP levels in nuclear suspensions by 80% or more (16) and was shown in the present report to inhibit $C^{14}O_2$ production from labeled metabolites by 75% or more. These results indicate that the contribution of intact cells to energy metabolism in thymus nuclear suspensions is minor.

SUMMARY

Aerobic adenosine triphosphate (ATP) synthesis occurring in calf thymus nuclei was shown to depend upon both glycolysis and the citric acid cycle.

1. Inhibitors of glycolysis, such as iodoacetic acid, and of the citric acid cycle, such as dehydroacetic acid and fluoroacetic acid, blocked nuclear respiration and reduced nuclear ATP levels.

2. It was possible to demonstrate ATP synthesis associated with the citric acid cycle under conditions in which glycolysis was inhibited with iodoacetic acid.

3. Treatment of nuclear suspensions with DNase, a procedure which inhibits nuclear respiration and ATP synthesis, selectively inhibited glycolysis and the citric acid cycle without affecting $C^{14}O_2$ production from glucose-1-C^{14} via the hexose monophosphate shunt or the breakdown of ascorbic acid-1-C^{14} to $C^{14}O_2$.

4. Di- and tricarboxylic acids, such as succinate, citrate and the succinic dehydrogenase inhibitor, malonate, did not penetrate rapidly, if at all, into pools of isolated thymus nuclei. Monocarboxylic acids, such as acetate, penetrated very rapidly.

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Studies on Energy-yielding Reactions in Thymus Nuclei: III.
PARTICIPATION OF GLYCOLYSIS AND THE CITRIC ACID CYCLE IN
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Bruce S. McEwen, V. G. Allfrey and A. E. Mirsky

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