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# Studies on Energy-yielding Reactions in Thymus Nuclei

## II. PATHWAYS OF AEROBIC CARBOHYDRATE CATABOLISM\*

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The means by which cell nuclei obtain free energy for RNA, DNA, and protein synthesis and other processes is a question of considerable importance to our understanding of cellular physiology and function. Mitochondria are known to have a highly organized, membrane-bound system for the oxidative phosphorylation of adenosine diphosphate to adenosine triphosphate. Our observations point to the conclusion that nuclei from at least one mammalian tissue, namely, the thymus gland, also have a system for the oxygen-dependent synthesis of adenosine triphosphate (1, 2). Evidence for an independent nuclear phosphorylation system was presented in the first paper of this series (2) in which a direct comparison of nuclei and mitochondria from the thymus gland showed that mitochondria cannot account for the ATP synthesis which occurs in suspensions of thymus nuclei. The nuclear system is sensitive to many of the same inhibitors which block mitochondrial oxidative phosphorylation but differs from this process by being insensitive to carbon monoxide, calcium ions, and methylene blue and sensitive to treatment of the nuclei with DNase (2).

The purpose of this paper is to present evidence that three major pathways of carbohydrate catabolism, the hexose monophosphate shunt, glycolysis, and the citric acid cycle, are all present in thymus nuclei. The operation of the citric acid cycle involves succinic dehydrogenase, and evidence is presented that this enzyme is found in thymus nuclei as well as in thymus mitochondria. In much of the work to be described we used nuclei isolated in nonaqueous solvents, a procedure which has been shown to provide the most reliable information about the localization of water-soluble substances within nuclei (3). Evidence from other laboratories, which will be discussed, indicates that nuclei from a wide variety of other tissues also contain enzymes of these three pathways. The third paper of this series, which follows this one, presents evidence that glycolysis and the citric acid cycle participate in the aerobic ATP synthesis occurring in suspensions of isolated intact thymus nuclei.

### EXPERIMENTAL PROCEDURE

*Isolation of Nuclei from Calf Thymus in Nonaqueous Media*—Fresh calf thymus tissue was frozen in liquid nitrogen and lyophilized. The dry tissue was ground in petroleum ether and fractionated in cyclohexane-carbon tetrachloride mixtures of gradually increasing specific gravity as described previously (4). The nuclear fraction, which sediments at a specific gravity of 1.38

to 1.39, leaving behind lighter cytoplasmic debris, has been shown to meet a number of criteria of purity (3): (a) absence of cytoplasmic debris as shown by staining; (b) a DNA content of 25.8% of the dry weight as compared with 15.7% for whole tissue; (c) absence of contaminating serum proteins as shown by immunochemistry; (d) absence of certain enzymes from nuclei, such as alkaline (phenolphthalein) phosphatase. Moreover, the possibility was excluded that contaminants are ground into nuclei during isolation (3).

*Use of Nuclei and Tissue Prepared in Nonaqueous Media for Estimation of Enzyme Distribution between Nucleus and Cytoplasm*—To measure intracellular enzyme distributions, nuclear specific activities were compared with specific activities in whole tissue. To insure that enzymes in whole tissue were treated in the same way as in nuclei, lyophilized, unfractionated tissue was ground and extracted with the same solvents used for the nuclear isolation.

For enzyme determinations, tissue and nuclei prepared in nonaqueous media and stored as a dry powder at  $-20^{\circ}$  were suspended in ice-cold 0.06 M phosphate buffer, pH 7.0 with the use of a Sonifier (Branson Instruments, Inc., Stamford, Connecticut) which operates at 20 kc. Aliquots (0.1 ml) of the resulting suspensions, containing 0.1 to 4 mg of nuclei or tissue, dry weight, were used in the procedures described below. Knowing the specific activity of an enzyme per mg (dry weight) in nuclei and in whole tissue, one can calculate the specific activity of the enzyme in the cytoplasm, since the nucleus is known to comprise 60% of the dry weight of thymus tissue on the average (4).

*Methods for Measuring Enzyme Activities in Thymus Nuclei and in Whole Tissue*—The following measurements were carried out at  $30^{\circ}$  in a Beckman DU spectrophotometer. Pyruvate kinase was measured at 340  $m\mu$  by the coupled reaction in which the product, pyruvate, is reduced to lactate in the presence of DPNH and lactic dehydrogenase (5). Isocitric dehydrogenase (6), glucose 6-phosphate dehydrogenase (7), and 6-phosphogluconate dehydrogenase (8) were all measured by following the reduction of TPN at 340  $m\mu$ . Aldolase was measured by a method which involved the conversion of all the product to dihydroxyacetone phosphate with triose phosphate isomerase and reduction of this intermediate by DPNH with  $\alpha$ -glycerophosphate dehydrogenase (9). Lactic dehydrogenase was measured as the reduction of pyruvate to lactate by DPNH (10). Malic dehydrogenase was measured as the reduction of oxaloacetate to malate by DPNH (11). Reagents for the last three determinations are available in kit form from C. F. Boehringer und Soehne, Mannheim, Germany (distributed in the United States

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by California Corporation for Biochemical Research). Alkaline phenolphthalein phosphatase was measured by following the formation of free phenolphthalein from sodium phenolphthalein phosphate at 553  $m\mu$  (7).

Succinic dehydrogenase was measured by following the reduction of 2,6-dichlorophenolindophenol at 600  $m\mu$  with phenazine methosulfate as an electron carrier between the enzyme and the dye by a method similar to that described by Ells (12). The concentrations of indophenol and phenazine methosulfate were  $3$  to  $6 \times 10^{-5}$  M and 0.05 mg per ml, respectively. The rate of reaction was expressed as the difference between the rate of reaction in a cuvette containing succinate and the rate in a cuvette without added succinate and with the succinic dehydrogenase inhibitor, malonate (13). After an initial burst of reduction, lasting a minute or so, during which a nonenzymatic reduction of indophenol took place, the reaction was nearly linear for about 5 minutes. Over the range of activities normally employed, the rate was proportional to enzyme concentration. When the nonenzymatic reducing activity (possibly ascorbic acid) was too large, 80% of it could be removed by dialysis for 6 hours against 0.06 M sodium phosphate buffer with 0.001 M sodium succinate, pH 7.0.

Succinic oxidase was measured by conventional Warburg manometry with  $2 \times 10^{-2}$  M sodium succinate in the nuclear incubation medium described previously (2). The rate was nearly linear over the 30-minute period of incubation at 37°.

*Estimation of Metabolite Concentrations in Thymus Tissue and Thymus Nuclear Extracts*—For certain analyses, nuclei and tissue prepared in nonaqueous media were extracted three times with 5% trichloroacetic acid. The combined extract was neutralized with potassium hydroxide and small aliquots were used directly for the estimation. Malic acid was determined with purified malic dehydrogenase (Worthington Biochemical Corporation) by the method of Hohorst, Kreutz, and Bücher (14).  $\alpha$ -Ketoglutaric acid was measured by reductive amination with DPNH and purified glutamic dehydrogenase (15). Lactic acid was measured with DPN and purified lactic dehydrogenase at pH 9.0 in the presence of hydrazine to trap the product (16). Pyruvic acid was measured with DPNH and purified lactic dehydrogenase (17). Reagents and enzymes for the last three determinations are contained in kits available from C. F. Boehringer und Soehne.

For the succinate estimation, nuclei and tissue prepared in nonaqueous media were extracted three times with 1 N sulfuric acid. The combined extract (approximately 10 ml) was adsorbed to 20 g of Celite No. 535 (Johns-Manville) which had been washed with diethyl ether and dried in an oven at 110°. The citric acid cycle acids were extracted from the Celite with 500 ml of diethyl ether. Nearly complete recovery of malic acid indicated that extraction was very efficient. After evaporating the ether, the dry residue was redissolved in 0.06 M sodium phosphate buffer, pH 7.0, for the estimation. Succinate was determined enzymatically by the method of Rodgers (18) utilizing as a source of succinic dehydrogenase activity a washed mitochondrial preparation from calf thymus. The method involves the reduction of 2,6-dichlorophenolindophenol, which is followed spectrophotometrically at 600  $m\mu$ . Results were corrected for other substances capable of reducing the indophenol by running controls in the presence of the specific succinic dehydrogenase inhibitor, malonic acid (13).

*Determination of Peptide-bonded Flavin*—Peptide-bonded flavin

in nuclei and in whole tissue was measured according to the procedure described by Singer, Hauber, and Kearney (19). Two successive digestions (5 to 7 hours each) with trypsin and chymotrypsin were found necessary to remove the peptide-bound flavin from nuclei and from whole tissue. (Several drops of toluene were added to each tube to discourage growth of microorganisms during the digestions.)

Fluorometric determinations were made with a Turner model 110 fluorometer in 4-ml tubes. The filters used were those recommended for the determination of riboflavin. All measurements were made at the pH of maximal fluorescence (pH 3.3) in a 0.03 M glycine buffer. Readings were corrected for nonflavin fluorescence by adding sodium hydrosulfite (20) and were corrected for internal quenching with internal riboflavin standards. The flavin content was calculated with respect to the fluorescence of riboflavin at pH 7.0.

*Isolation of Thymus Nuclei in Isotonic Sucrose Solutions*—Method 1. In this procedure, cells are broken under slightly hypotonic conditions since this increases their fragility. All operations were performed at 2–4°. Fresh calf thymus tissue (50 g) was chopped with scissors and homogenized in a Waring Blender in 450 ml of 0.20 M sucrose-0.0033 M  $\text{CaCl}_2$  for 3 minutes at 1000 r.p.m. The homogenate was filtered through gauze and flannelette to remove pieces of tissue and then blended for 2 minutes at 1000 r.p.m. After filtering through flannelette, a sufficient quantity of 0.5 M sucrose-0.0033 M  $\text{CaCl}_2$  was added (usually 75 ml) to raise the sucrose concentration to 0.25 M, and the blending was continued for another 2 minutes at 1000 r.p.m. The homogenate was filtered once again through flannelette and centrifuged at  $800 \times g$  for 7 minutes. The nuclear pellet was washed once by resuspending it in 0.25 M sucrose-0.0030 M  $\text{CaCl}_2$ , and recentrifuged at  $800 \times g$  for 7 minutes. Finally, the pellet was resuspended in 40 ml (final volume) of 0.25 M sucrose-0.0030 M  $\text{CaCl}_2$ , giving a nuclear suspension which contained between 30 and 50 mg per ml, dry weight. Electron microscopy revealed that these nuclear preparations regularly contained 4 to 6% whole cells. When compared with earlier preparations isolated in isotonic sucrose (which contained 7 to 9% intact cells (21)), the "hypotonic" preparations showed lower levels of acid-soluble nucleotide, including ATP, and lower metabolic activity, including respiration and amino acid incorporation. This difference is due in part to damage induced by hypotonic shock.

Method 2. Nuclei were also prepared in 0.25 M sucrose-0.0030 M  $\text{CaCl}_2$  as described previously (21). Such preparations currently have variable cell counts, ranging from 7 to 25% cells, the contamination consisting of very small intact thymocytes. Other laboratories have reported that this method results in cell counts of 3 to 8% (22, 23).

*Purification of Thymus Nuclei Isolated in Sucrose Solutions and Treatment for Measurement of Succinic Dehydrogenase*—Portions (5 ml) of a suspension of nuclei isolated in hypotonic or isotonic sucrose as described were layered over 25 ml of concentrated sucrose (2.0, 2.1, or 2.2 M) containing 0.003 M  $\text{CaCl}_2$  and centrifuged in the swinging bucket rotor (SW 25) of a Spinco model L preparative ultracentrifuge at 22,000 r.p.m. for 40 minutes. The floating debris and sucrose were removed by suction and the pellet was resuspended in 0.25 M sucrose-0.003 M  $\text{CaCl}_2$ . For the measurements of succinic dehydrogenase activity the original nuclear suspension or the purified nuclear suspension was treated for 30 seconds in the Sonifier to break most of the nuclei and then incubated for 5 minutes at 37° in a medium

TABLE I  
Distribution of enzymes between nucleus and cytoplasm of thymus tissue

Enzyme activities were estimated by the methods described in "Experimental Procedure." Data are expressed as the optical density change at the prescribed wave length per minute per mg, dry weight, at 30°.

Pathway	Enzyme	Specific activity			Relative specific activity
		Nuclei	Tissue	Cytoplasm*	
		$\Delta O.D./min/mg$			$N/T \times 100$
A. Shunt	Glucose-6-P dehydrogenase	0.00504	0.0148	0.0295	34.0
	6-PG† dehydrogenase	0.00184	0.00288	0.00445	63.8
B. Glycolysis	Aldolase	0.0116	0.0328	0.0648	35.4
	Pyruvate kinase	0.132	0.348	0.672	37.9
	Lactic dehydrogenase	0.226	0.503	0.918	44.9
C. Citric acid cycle	Isocitric dehydrogenase	0.0081	0.0418	0.0924	19.4
	Succinic dehydrogenase, pH 6.6	0.0011	0.0025	0.0046	44.0
	Succinic dehydrogenase, pH 7.6	0.0012	0.0036	0.0072	33.3
	Malic dehydrogenase	0.371	0.514	0.728	72.7
D. Other	Alkaline phosphatase	0.00027	0.00492	0.0119	5.6

\* Calculated (see "Experimental Procedure").

† 6-PG, 6-phosphogluconate.

containing 0.5 mg per ml of DNase, 5 ml of a sonicated nuclear suspension in 0.25 M sucrose, 0.003 M  $CaCl_2$ , 0.1 ml of 1 M  $MgCl_2$ , and 2 ml of 0.06 M sodium phosphate buffer, pH 7.0. By sonicating and depolymerizing the DNA, the formation of a gel, which occurs at pH values exceeding 7.2, was avoided and the intranuclear succinic dehydrogenase was made accessible to the medium.

#### RESULTS

*Use of Nuclei Isolated in Nonaqueous Media in Studying Distribution of Water-soluble Substances Within the Cell*—In studying the distribution of a water-soluble substance among the cell organelles after cell fractionation, it is necessary to make sure that the particular organelle has neither lost nor gained the substance during fractionation. Isolation of cell nuclei in isotonic sucrose solutions by the conventional techniques frequently results in the loss of small molecules, such as acid-soluble nucleotides,<sup>1</sup> and enzyme proteins, such as adenosine deaminase and nucleoside phosphorylase (7).

The most reliable information on whether an enzyme or small ionized molecule is normally present within the nucleus is provided by studies on nuclei which have been isolated from quickly frozen, lyophilized tissue in the complete absence of water, with organic solvents for density gradient centrifugation (4). Such solvents prevent the exchange of water-soluble substances between the nucleus and cytoplasm during fractionation. A résumé of the methods and criteria of purity for the isolation of nuclei in nonaqueous media is found in "Experimental Procedure."

The extent of cytoplasmic contamination in the nuclear fraction isolated in nonaqueous media can be estimated in several ways (3). Enzyme distributions usually reveal characteristic differences in composition between the nucleus and the cytoplasm, so that an assay for characteristic cytoplasmic enzymes is a measure of cytoplasmic contamination. Thymus nuclei, for

example, do not contain appreciable amounts of cytochrome *c* oxidase (24), and very little alkaline phosphatase (7). Judged by their content of alkaline (phenolphthalein) phosphatase, the nuclei used in the present experiments are approximately 95% pure (Table I). The ratio of nuclear enzyme activity to that of the whole tissue ( $N/T \times 100$ ) is called the "relative specific activity." From this figure and the fact that nuclei comprise 60% of the dry weight of thymus tissue, one can calculate the enzyme level in the cytoplasm. When the  $N/T \times 100$  is low, *i.e.* 5 to 10, the presence of the enzyme in the nucleus is doubtful and the activity observed probably represents cytoplasmic contamination. Relative specific activities in excess of 5 to 10 are considered to indicate nuclear localization of the enzyme.

The problem arises that some enzymes may be selectively inactivated in the nucleus or cytoplasm during the course of isolation. Thus, the  $N/T \times 100$  ratios observed might not reflect the actual enzyme distribution in the fresh tissue. As a precaution against selective inactivation, the homogenized tissue is treated with the same solvents used for the nuclear isolation. It should be pointed out, however, that this precautionary measure would not preclude selective damage to organized enzyme systems, such as those of the mitochondrion, which are inactivated by organic solvents. (In the present work we have not measured the activity of such multienzyme systems.) For other enzymes which survive lyophilization and treatment with organic solvents, the  $N/T \times 100$  ratios are probably representative of the natural enzyme distributions in the cells. In the special case of succinic dehydrogenase the activity distribution can be supported by direct chemical means, *i.e.* by the determination of peptide-bound flavin.

*Enzymes of Hexose Monophosphate Shunt Pathway in Thymus Nuclei*—It was shown previously that calf thymus nuclei have the first enzyme of the hexose monophosphate shunt, glucose 6-phosphate dehydrogenase (7). This has been confirmed (see Table I). In addition, the second enzyme of this pathway, 6-phosphogluconate dehydrogenase, was also found in thymus

<sup>1</sup> A. E. Mirsky, unpublished observations.



TABLE II

Distribution of metabolites between the nucleus and cytoplasm of thymus tissue

Metabolite concentrations were estimated as described in "Experimental Procedure." Data are expressed as millimicromoles of metabolite per mg, dry weight.

Pathway	Metabolite	Concentration			Relative concentration
		Nuclei	Tissue	Cytoplasm*	
		$\mu\text{moles/mg}$			$N/T \times 100$
Glycolysis	Pyruvic acid	0.54	0.47	0.35	115
	Lactic acid	62.9	71.8	85.0	87.7
Citric acid cycle	$\alpha$ -Ketoglutaric acid	0.24	0.22	0.19	109
	Succinic acid	2.9	2.3	1.4	126
	Malic acid	0.42	0.40	0.38	105

\* Calculated (see "Experimental Procedure").

nuclei (Table I). Subsequent reactions of the shunt lead to the formation of ribose 5-phosphate, needed for ribonucleic acid synthesis. In view of the ability of thymus nuclei to incorporate free purines into nuclear RNA (25), it appears that the nucleus does have the enzymes required for ribose phosphate formation.

**Enzymes and Metabolites of Glycolysis in Thymus Nuclei**—Calf thymus nuclei isolated in nonaqueous media contain several glycolytic enzymes and metabolites. Table I shows that the glycolytic enzymes, aldolase, pyruvate kinase, and lactic dehydrogenase, were all found to have an  $N/T \times 100$  well in excess of 30. Furthermore, a large proportion of the lactic and pyruvic acid of the thymus cell was found in the nucleus (Table II). The large excess of lactic acid in both nucleus and cytoplasm may reflect postmortem changes.

**Enzymes and Metabolites of Citric Acid Cycle in Thymus Nuclei**—Since we have observed that thymus nuclei have an oxygen-dependent means of making ATP for energy-dependent reactions (1, 2), it was important to establish whether nuclei have enzymes, such as those of the citric acid cycle, for the oxidative breakdown of glycolytic products. Accordingly, the specific activities of three citric acid cycle enzymes (isocitric, succinic, and malic dehydrogenases) were determined in nuclei isolated in nonaqueous media. The relative specific activities ( $N/T \times 100$ ) for all three dehydrogenases indicate nuclear localization (Table I). In addition, three metabolites of the citric acid cycle,  $\alpha$ -ketoglutaric acid, succinic acid, and malic acid, were found to be present in nearly the same concentrations (millimicromoles per mg, dry weight) in thymus nuclei and tissue prepared in nonaqueous media (Table II).

Isocitric and malic dehydrogenases are soluble enzymes which can occur in the soluble space of the cytoplasm as well as inside mitochondria (26, 27). The present evidence shows that they also occur in the nucleus. The occurrence of succinic dehydrogenase in nuclei is more surprising since it is not a soluble enzyme and since the experimental data on the intracellular distribution of succinic dehydrogenase indicates that most of this enzyme is found in the mitochondrial fraction of many tissues (26). As a result, it is widely believed that succinic dehydrogenase is exclusively localized in the mitochondrion. The tests which will now be described show that, although most of the succinic dehydrogenase in thymus tissue does occur in mitochondria, a

significant amount is present in the nuclei and is not due to mitochondrial contamination.

Because succinic dehydrogenase is inactivated by the organic solvents used in the isolation of nuclei in nonaqueous media, the actual levels of activity reported in Table I are low. This low recovery of activity raised doubts whether the relative specific activities of succinic dehydrogenase reported in Table I accurately reflect the distribution of that enzyme between the nucleus and cytoplasm. As a result, two independent approaches were followed. First, succinic dehydrogenase activity was measured in thymus nuclei isolated in sucrose solutions and purified as much as possible from contaminating mitochondria. Second, succinic dehydrogenase was measured in nonaqueous nuclei by a method which does not involve an enzyme assay but instead employs a fluorometric determination of the peptide-bound flavin prosthetic group of the enzyme.

**Succinic Dehydrogenase Activity in Thymus Nuclei Purified in Sucrose Gradients**—Electron micrographs show that thymus nuclei, as ordinarily isolated in isotonic sucrose, contain some mitochondria attached to the nuclear surface (2). Nuclei may be separated from such mitochondria by layering nuclear suspensions over 2.0 to 2.2 M sucrose and centrifuging as described in "Experimental Procedure." Free mitochondria and nuclei with attached cytoplasm remain in the interface between the heavy and light sucrose solutions, and only nuclei with little or no attached cytoplasm are found in the sediment. During centrifugation through hypertonic sucrose, nuclei lose many soluble enzymes. However, succinic dehydrogenase is normally tightly bound to both mitochondria and nuclei, and is not lost during centrifugation.

To check the nuclear preparation for mitochondrial contamination, succinic oxidase activity was measured, and it was hoped that the nuclei could be freed of succinic oxidase activity although succinic dehydrogenase activity remained. In this connection, it should be noted that the succinic oxidase activity of the usual thymus nuclear suspensions is due mostly to mitochondrial contamination, since 95% CO-5% O<sub>2</sub> inhibits succinic oxidase activity of the nuclear preparation to the same extent (70%) that it inhibits the succinic oxidase activity of isolated mitochondria (2). In contrast, endogenous respiration and oxygen-dependent ATP synthesis of thymus nuclear suspensions are virtually unaffected by 95% CO-5% O<sub>2</sub> (2).

Table III shows that after nuclei were centrifuged through 2.2 M sucrose, succinic oxidase activity was reduced to a very low level so that the relative specific activity with respect to an homogenate of thymus tissue ( $N/T \times 100$ ) was only 9.9. In contrast, succinic dehydrogenase activity remained associated with the nuclei, with  $N/T \times 100$  values of 40.9 and 31.5 at pH 6.6 and pH 7.6, respectively (see Table III). It was reassuring to note that the  $N/T \times 100$  in purified aqueous nuclei agreed very well with the  $N/T \times 100$  found in the nonaqueous nuclei (compare Table I with Table III). The retention of succinic dehydrogenase by nuclei after mitochondria were removed strongly suggests that the primary dehydrogenase is normally present in thymus nuclei. (Since the mitochondrial enzyme is tightly bound it is unlikely that a transfer of succinic dehydrogenase from mitochondria to nuclei could have occurred during the purification of the nuclei.)

**Peptide-bonded Flavin of Thymus Nuclei and Thymus Tissue**—In addition to demonstrating succinic dehydrogenase activity associated with highly purified thymus nuclei, it was possible to

demonstrate the presence of the flavin coenzyme typical of this enzyme in thymus nuclei. The method which we followed was described recently by Singer *et al.* and involves proteolytic digestion of the enzyme after interfering substances and acid-soluble flavin coenzymes have been extracted (19). Thymus nuclei isolated in nonaqueous media were found to have appreciable amounts of peptide-bonded flavin (Table IV). In fact, the relative concentration ( $N/T \times 100$ ) was somewhat higher than the relative specific activity of succinic dehydrogenase reported in Table III. Peptide-bonded flavin has been detected in nuclei from a variety of other tissues and these results will be reported elsewhere.

**Some Properties of Thymus Succinic Dehydrogenase**—Under the assay conditions employed, two peaks of succinic dehydrogenase activity were observed, one at pH 6.6 and the other at pH 7.6 (Fig. 1). The ratio of activity at pH 6.6 to activity at pH 7.6 often differed among the subcellular fractions from the thymus. The possible significance of these two pH optima will be considered in the "Discussion."

Another property of thymus succinic dehydrogenase concerns its ability to react directly with 2,6-dichlorophenolindophenol in the absence of phenazine methosulfate to mediate electron transfer. Table V shows that succinic dehydrogenase in whole tissue, in nuclei, and in mitochondria reacted with 2,6-dichlorophenolindophenol at both pH 6.6 and pH 7.6. Added phenazine methosulfate stimulated the pH 7.6 activity about 2-fold and stimulated pH 6.6 activity to a lesser extent. According to

TABLE III

*Measurement of succinic dehydrogenase activity in nuclear suspensions in relation to mitochondrial contamination*

Whole homogenate from calf thymus was prepared by homogenizing fresh tissue in a Waring Blendor in 0.25 M sucrose-0.003 M  $\text{CaCl}_2$  for 4 minutes at 1000 r.p.m. and filtering through gauze and flannelette. Nuclei were isolated in isotonic sucrose by Method 1 and purified further by centrifugation through 2.0, 2.1, and 2.2 M sucrose, as described in "Experimental Procedure." Aliquots (1 ml) were used without further treatment for the succinic oxidase estimation. Another portion of each fraction was sonicated and treated with DNase for the succinic dehydrogenase determination. Details of these procedures are given in "Experimental Procedure."

Description of fraction	Succinic oxidase $\mu\text{l O}_2/30 \text{ min/mg}^*$	Relative specific activity $N/T \times 100$	Succinic dehydrogenase			
			pH 6.6	$N/T \times 100$	pH 7.6	$N/T \times 100$
			$\Delta\text{O.D.}/\text{min}/\text{mg} \times 10^3$			
Whole thymus homogenate	4.48		18.6		26.6	
Nuclei						
Isolated in isotonic sucrose	2.01	44.9	11.8	63.5	13.6	51.2
Centrifuged, 2.0 M sucrose	0.51	11.3	8.8	47.3	9.3	35.0
Centrifuged, 2.1 M sucrose	0.49	10.9	7.4	39.7	7.8	29.3
Centrifuged, 2.2 M sucrose	0.43	9.9	7.6	40.9	8.4	31.5
Cytoplasm†	10.5		35.0		54.0	

\* Dry weight.

† Calculated (see "Experimental Procedure").

TABLE IV

*Peptide-bonded flavin of thymus nuclei and thymus tissue*

Analyses were performed on thymus nuclei and thymus tissue prepared in nonaqueous media, following the procedures described in "Experimental Procedure." Flavin content is expressed in terms of micromicromoles of riboflavin per mg, dry weight.

Tissue fraction	Flavin content	Relative concentration
	$\mu\text{moles/mg}^*$	$N/T \times 100$
Nuclei.....	5.6	51
Tissue.....	11.6	
Cytoplasm†.....	19.3	
Nuclei.....	6.9	66
Tissue.....	10.4	
Cytoplasm†.....	15.6	
Nuclei.....	6.6	
Nuclei.....	9.1	

\* Dry weight.

† Calculated (see "Experimental Procedure").

Singer *et al.*, the solubilized, purified succinic dehydrogenase from beef heart mitochondria does not react with 2,6-dichlorophenolindophenol but does react with phenazine methosulfate (28). Green, Mii, and Kohout, on the other hand, isolated a particulate succinic dehydrogenase from beef heart mitochondria which did react directly with 2,6-dichlorophenolindophenol (29). The difference between the two enzymes, aside from the solubility of the former, is that the former contains flavin and non-heme iron only whereas the latter contains, in addition, some heme iron and possibly some other electron carriers. The implication of the ability of thymus nuclear succinic dehydrogenase to react with 2,6-dichlorophenolindophenol is that these nuclei may contain heme or other electron carriers capable of transferring electrons from the primary dehydrogenase to the indophenol. No direct evidence to support this idea is available yet, although nuclei isolated in nonaqueous media have an iron content of 0.0066% (4).

Thymus nuclear succinic dehydrogenase was inhibited at pH 6.6 and pH 7.6 by the well known inhibitor, malonic acid (13) (Fig. 2). In addition, another less known inhibitor of succinic dehydrogenase, dehydroacetic acid (30), also inhibited the nuclear enzyme at both pH values (Fig. 2). Intact thymus nuclei isolated in isotonic sucrose are impermeable to malonic acid but are permeable to dehydroacetic acid (31). Use will be made of this permeability in the next paper of this series to show the effect of inhibiting the nuclear succinic dehydrogenase upon nuclear ATP synthesis (31).

## DISCUSSION

### *Presence of Enzymes of Carbohydrate Catabolism in Nuclei from Many Tissues*

Studies of nuclei from a variety of other tissues suggest that the three major pathways of carbohydrate catabolism present in calf thymus nuclei may be present in other nuclear types.

**Hexose Monophosphate Shunt**—Stern and Mirsky (7) demonstrated the presence of glucose 6-phosphate dehydrogenase in nuclei from calf liver as well as calf thymus. Siebert *et al.* (32, 33) found glucose 6-phosphate dehydrogenase and 6-phospho-

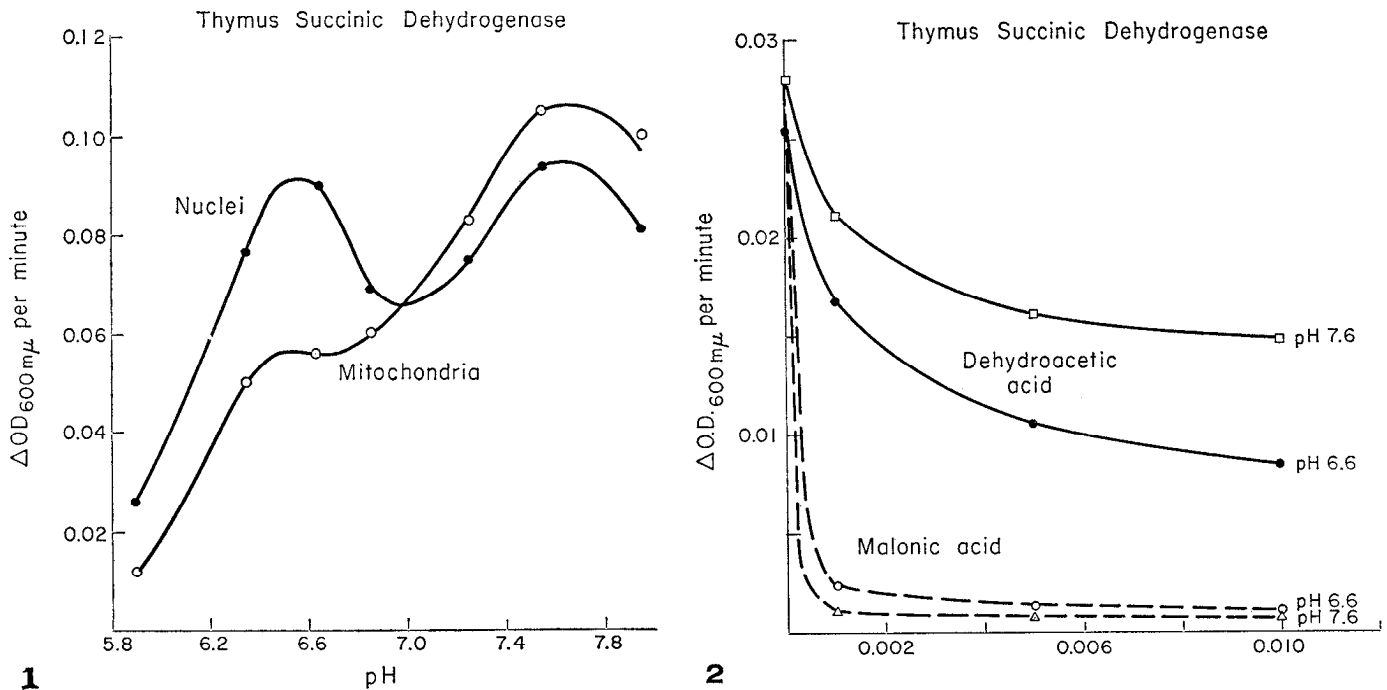


FIG. 1 (left). The pH dependence of succinic dehydrogenase activity in thymus nuclei and thymus mitochondria. Nuclei were isolated by Method 1 (see "Experimental Procedure"); mitochondria were isolated in isotonic sucrose as described previously (2). Nuclei were treated by sonication and DNase as described in "Experimental Procedure" to make nuclear succinic dehydrogenase accessible to the medium.

FIG. 2 (right). The effect of increasing concentrations (moles per liter) of malonic acid and dehydroacetic acid on thymus nuclear succinic dehydrogenase activity at pH 6.6 and pH 7.6. Nuclei were isolated by Method 1 and were treated by sonication and DNase as described in "Experimental Procedure" before measuring enzyme activity.

TABLE V  
Effect of electron acceptors on thymus succinic dehydrogenase activity

Thymus mitochondria were isolated in 0.25 M sucrose as described previously (2). Nuclei for Fraction B were isolated by Method 1 and were treated by sonication and DNase as described in "Experimental Procedure." Nuclei for Fraction C were isolated by Method 1 and purified by centrifugation through 2.1 M sucrose (see "Experimental Procedure") before being treated by sonication and DNase.

Subcellular fraction	pH	Succinic dehydrogenase activity		
		2,6-Dichlorophenol-indophenol	+0.05 mg/ml Phenazine methosulfate	+0.33 mg/ml Phenazine methosulfate
		$\Delta O.D._{600}/min/mg \times 10^3$		
A. Thymus mitochondria	6.6	512	587	605
	7.6	432	885	1080
B. Thymus nuclei	6.6	12.5	12.7	
	7.6	8.1	14.0	
C. Thymus nuclei	6.6	6.3		9.0
	7.6	4.2		9.3

gluconate dehydrogenase in nuclei isolated in nonaqueous media from rat liver, pig kidney, and beef brain. Evidence from tracer experiments that the shunt pathway is functional in thymus nuclei will be presented in a subsequent paper (31).

**Glycolysis**—Dounce and Beyer (34) found the enzyme aldolase

in nuclei from rat liver and dog kidney. Stern and Mirsky (35) found that nuclei from wheat germ contain the glycolytic enzymes aldolase, phosphoglycerate dehydrogenase, enolase, and pyruvate kinase. Siebert *et al.* (32, 33) found 10 glycolytic enzymes and a number of glycolytic metabolites in nuclei isolated in nonaqueous media from rat liver, pig kidney, and beef brain. The presence of metabolites of glycolysis in these nuclei implies that the pathway is functional. This has been shown directly; intact thymus nuclei isolated in sucrose solutions produce lactate and pyruvate from added glucose and convert glucose-6- $C^{14}$  to  $C^{14}O_2$  (31).

**Citric Acid Cycle**—Siebert *et al.* (32, 33) have found the enzymes isocitric dehydrogenase and malic dehydrogenase and the metabolite malic acid in nuclei isolated in nonaqueous media from rat liver, pig kidney, and beef brain. The presence of metabolites in these nuclei and in thymus nuclei again implies that the citric acid cycle is functioning. This has been shown directly; intact thymus nuclei isolated in sucrose solutions convert labeled acetate and pyruvate to  $C^{14}O_2$  (31). Moreover, glycolysis and the citric acid cycle in thymus nuclei are functional to the extent that they provide the free energy for nuclear ATP synthesis (31).

Succinic Dehydrogenase in Cell Nuclei

Two independent approaches to the localization of succinic dehydrogenase have supported our initial observations on nonaqueous preparations that significant amounts of the enzyme activity are present in thymus nuclei. First, succinic dehydrogenase activity remains associated with thymus nuclei isolated in isotonic sucrose solutions and purified from mitochondria by



density gradient centrifugation. Second, very pure thymus nuclei isolated in nonaqueous media contain significant quantities of peptide-bound flavin, the coenzyme which is characteristic of succinic dehydrogenase. From the flavin analysis and from the measurement of enzyme activity, it can be estimated that between 20% and 30% of the succinic dehydrogenase of the thymus tissue is present in nuclei.

As to the possible significance of the two pH optima in thymus nuclei and thymus tissue, any speculation, such as the possibility that two isomeric enzymes are involved, should be tempered with caution until more detailed studies are made. Arrighoni and Singer (36) have outlined more rigorous kinetic tests which increase the diagnostic significance of the phenazine methosulfate-dichlorophenolindophenol assay method; their methods, combined with purification steps, might resolve the origin of the two pH activity maxima.

Two instances are known of dual pH optima for fumarate reductase (the reverse reaction of succinic dehydrogenase). Warringa and Giuditta (37) reported that fumarate reduction to succinate, catalyzed by an enzyme from *Micrococcus lactilyticus*, showed a peak of activity at pH 5.5 and another peak at pH 7.5 to 7.6. These authors considered the possibility that two enzymes were responsible for the two pH optima, but seemed to favor the alternative that only one enzyme species was involved. Another case of two pH optima for fumarate reduction was reported by Lara (38) for an enzyme from *Propionibacterium pentosaceum*.

In addition to the evidence presented in this paper that thymus nuclei contain succinic dehydrogenase, there are suggestions that nuclei from other tissues may contain this enzyme. Defendi and Pearson (39) reported that nuclei in the intact, nucleated chicken erythrocyte react with tetrazolium dyes in the presence of succinate. These authors said that the formazan deposits are found just inside the nuclear membrane and are not formed when the succinic dehydrogenase inhibitors malonate or fluoride are present. Quite recently, De and Chatterjee (40-42) have reported that nuclei in intact rat liver cells and in human cancer cells similarly react with tetrazolium salts. They said that the formazan deposits are located around the nucleoli. Sedar and Rosa (43) also found by electron microscopy that formazan deposits are occasionally seen in nuclei in thin sections of rat liver cells which have been incubated with succinate.

#### Relation between Nuclear and Cytoplasmic Enzymes

Recently, Vesell and Bearn (44) have reported that nuclei of young cells in the erythrocyte series contain a lactic dehydrogenase isozyme which does not seem to be present in the cytoplasm. This finding suggests the possibility that differences may exist between some nuclear enzymes and their cytoplasmic counterparts. Malic dehydrogenase is known to exist in two forms in the cytoplasm of many types of cells, one form in the mitochondria and the other in the soluble cytoplasmic space (27, 45); and one can imagine that differences might exist between the nuclear and cytoplasmic malic dehydrogenases, as well.

Another aspect of the relation between nuclear and cytoplasmic enzymes is the degree of communication between the nucleus and cytoplasm. It does not seem likely that the nucleoplasm is simply an extension of the cytoplasmic space, as Siebert has suggested (32). If this were the case, certain soluble enzymes

such as alkaline phenolphthalein phosphatase would not be virtually absent from nuclei. Nor would the distributions of other enzymes vary as much as they do. For thymus nuclei, relative specific activities ( $N/T \times 100$ ) were observed to range from 19.4 to 72.7 (Table I). In the data of Siebert *et al.* (32, 33), relative specific activities for different enzymes vary from approximately 50 to as high as 300. Furthermore, at least in some cell types the nuclear membrane acts as a transport barrier to low molecular weight substances (46) and can be shown to sustain a definite membrane potential (47). This is even stronger evidence against open communication between the nucleus and cytoplasm.

#### SUMMARY

Because thymus nuclei produce adenosine triphosphate by an oxygen-dependent process, the presence of pathways of oxidative carbohydrate catabolism was examined. Great care was taken to exclude cytoplasmic contamination.

1. Enzymes of (a) the hexose monophosphate shunt, (b) glycolysis, and (c) the citric acid cycle were found in thymus nuclei.

2. Metabolites of glycolysis and the citric acid cycle were found in thymus nuclei.

3. The presence of succinic dehydrogenase in thymus nuclei was confirmed by two independent methods. First, thymus nuclei isolated in isotonic sucrose were freed of mitochondrial contamination by gradient centrifugation and succinic dehydrogenase was found to remain associated with nuclei. Second, the peptide-bound flavin coenzyme characteristic of succinic dehydrogenase was detected by fluorescence measurement in highly purified nuclei isolated in nonaqueous media.

4. Some properties of thymus nuclear succinic dehydrogenase were described. These include pH dependence, effect of known inhibitors, and the ability of the enzyme to react with electron acceptors.

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**Studies on Energy-yielding Reactions in Thymus Nuclei: II. PATHWAYS OF  
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