Studies on Energy-yielding Reactions in Thymus Nuclei

II. PATHWAYS OF AEROBIC CARBOHYDRATE CATABOLISM*

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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 immunology 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 such 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.

* This research was supported in part by a grant (RG4919) from the United States Public Health Service.
† Graduate Fellow of The Rockefeller Institute.
by California Corporation for Biochemical Research). Alkaline
phenolphtalein phosphatase was measured by following the
formation of free phenolphtalein from sodium phenolphtalein
phosphate at 535 mμ (7).

Succinic dehydrogenase was measured by following the re-
duction of 2,6-dichlorophenolindophenol at 600 mμ with pheno-
zine methosulfate as an electron carrier between the enzyme
and the dye by a method similar to that described by Elia (12).
The concentrations of indophenol and phenazine methosulfate
were 3 to 6 × 10⁻⁴ 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 de-
hydrogenase 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 nor-
mally employed, the rate was proportional to enzyme concentra-
tion. 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 × 10⁻⁴ 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). α-Keto-
glutaric acid was measured by reductive amination with DPNH
and purified glutamic dehydrogenase (15). Lactic acid was
measured with DPN and purified lactic dehydrogenase (15). Pyru-
vic acid was measured with DPNH and purified lactic dehy-
drogenase (17). Reagents and enzymes for the last three de-
terminations 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 ad-
sorbed 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 evap-
orating 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 in-
volves the reduction of 2,6-dichlorophenolindophenol, which is
followed spectrophotometrically at 600 mμ. Results were cor-
rected for other substances capable of reducing the indophenol
by running controls in the presence of the specific succinic de-
hydrogenase 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 chymo-
trypsin 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 measure-
ments 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 cor-
rected for internal quenching with internal riboflavin standards.
The flavin content was calculated with respect to the fluores-
cence 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
Blendor in 450 ml of 0.20 m sucrose-0.0033 m CaCl2 for 3 min-
utes 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 CaCl2 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 x g for 7 minutes. The nuclear pellet was
washed once by resuspending it in 0.25 m sucrose-0.0030 m CaCl2
and centrifuged at 800 x g for 7 minutes. Finally, the pellet
was resuspended in 40 ml (final volume) of 0.25 m sucrose-0.0030 m
CaCl2, 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
nucleotides, including ATP, and lower metabolic activity, in-
cluding respiration and amino acid incorporation. This differ-
ence is due in part to damage induced by hypotonic shock.

Method 2. Nuclei were also prepared in 0.25 m sucrose-0.0030 m
CaCl2 as described previously (21). Such preparations cur-
rently 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
succrose (2.0, 2.1, or 2.2 m) containing 0.003 m CaCl2 and cen-
trifuged 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 CaCl2.
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
that the particular organelle has neither lost nor gained the sub-
ing the distribution of a water-soluble substance among the cell
is a measure of cytoplasmic contamination. Thymus nuclei, for
ways (3). Enzyme distributions usually reveal characteristic
plasm, so that an assay for characteristic cytoplasmic enzymes
differences in composition between the nucleus and the cyto-
tion isolated in nonaqueous media can be estimated in several
procedure."

nuclei in nonaqueous media is found in "Experimental Pro-
solvents prevent the exchange of water-soluble substances be-
tween the nucleus and cytoplasm during fractionation. A
resume of the methods and criteria of purity for the isolation of
organic solvents for density gradient centrifugation (4). Such
frozen, lyophilized tissue in the complete absence of water, with
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°.

<table>
<thead>
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<th>Pathway</th>
<th>Enzyme</th>
<th>Specific activity</th>
<th>Relative specific activity</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>Nuclei</td>
<td>Tissue</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ΔO.D./min/mg</td>
<td></td>
</tr>
<tr>
<td>A. Shunt</td>
<td>Glucose-6-P dehydrogenase</td>
<td>0.00504</td>
<td>0.0148</td>
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<td></td>
<td>6-PG  dehydrogenase</td>
<td>0.00184</td>
<td>0.00285</td>
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<td>B. Glycolysis</td>
<td>Aldolase</td>
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<td>Pyruvate kinase</td>
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<td>Lactic dehydrogenase</td>
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<td>0.503</td>
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<td>C. Citric acid cycle</td>
<td>Isocitric dehydrogenase</td>
<td>0.0081</td>
<td>0.0418</td>
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<td>Succinic dehydrogenase, pH 6.6</td>
<td>0.0011</td>
<td>0.0025</td>
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<td></td>
<td>Succinic dehydrogenase, pH 7.8</td>
<td>0.0012</td>
<td>0.0036</td>
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<td>Malic dehydrogenase</td>
<td>0.371</td>
<td>0.514</td>
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<td>D. Other</td>
<td>Alkaline phosphatase</td>
<td>0.00027</td>
<td>0.00492</td>
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</tbody>
</table>

* 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 CaCl₂, 0.1 ml of 1 m MgCl₂, and 2 ml of 0.00 m sodium phosphate buffer, pH 7.0. By son-
activating 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 sub-
stance during fractionation. Isolation of cell nuclei in isotonic sucrose solutions by the conventional techniques frequently re-\nsults in the loss of small molecules, such as acid-soluble nucleo-
tides,1 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 pro-
vided 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 be-
tween the nucleus and cytoplasm during fractionation. A
resume of the methods and criteria of purity for the isolation of
nuclei in nonaqueous media is found in "Experimental Pro-
cedure."

The extent of cytoplasmic contamination in the nuclear frac-
tion isolated in nonaqueous media can be estimated in several
ways (3). Enzyme distributions usually reveal characteristic differences in composition between the nucleus and the cyto-
plasm, 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 × 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 × 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 in-
avivated in the nucleus or cytoplasm during the course of iso-
lation. Thus, the N/T × 100 ratios observed might not reflect
the actual enzyme distribution in the fresh tissue. As a pre-
caution 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 meas-
ure would not preclude selective damage to organized enzyme
systems, such as those of the mitochondrion, which are in ac-
tivated 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 × 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
1 A. E. Mirsky, unpublished observations.
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 × 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 of N/T × 100 for all three dehydrogenases indicate nuclear localization (Table I). In addition, three metabolites of the citric acid cycle, a-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, 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 Titrations—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 nucleus suspensions is due mostly to mitochondrial contamination, since 95% CO-5% O₂ 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% O₂ (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 × 100) was only 9.9. In contrast, succinic dehydrogenase activity remained associated with the nuclei, with N/T × 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 × 100 in purified aqueous nuclei agreed very well with the N/T × 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.)
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. No direct evidence to support this idea is available yet, although nuclei isolated in nonaqueous media have an iron content of 0.0066% (4).

**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 Blender in 0.25 M sucrose-0.003 M CaCl\(_2\) for 4 minutes at 1000 r.p.m. and filtering through gauze and flannel. Nuclei were isolated in isotonic sucrose by pelletizing fresh tissue in a Waring Blender in 0.25 M sucrose-0.003 M CaCl\(_2\) for 4 minutes at 1000 r.p.m. and filtering through gauze and flannel. Nuclei were isolated in isotonic sucrose by

<table>
<thead>
<tr>
<th>Description of fraction</th>
<th>Sucrose oxidase</th>
<th>Relative activity</th>
<th>Succinic dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \mu \text{g O}_2/50 \text{ min/mg} )</td>
<td>( \Delta \text{O.D.} / \text{mg} \times 10^5 )</td>
<td>pH 6.6</td>
</tr>
<tr>
<td>Whole thymus homogenate</td>
<td>4.48</td>
<td>18.6</td>
<td>26.6</td>
</tr>
<tr>
<td>Nuclei</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolated in isotonic</td>
<td>2.01</td>
<td>44.0</td>
<td>11.8</td>
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<tr>
<td>sucrose</td>
<td></td>
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<td></td>
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<tr>
<td>Centrifuged, 2.0 M</td>
<td>0.51</td>
<td>11.3</td>
<td>8.8</td>
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<td>sucrose</td>
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<tr>
<td>Centrifuged, 2.1 M</td>
<td>0.49</td>
<td>10.9</td>
<td>7.4</td>
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<tr>
<td>sucrose</td>
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<td></td>
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<tr>
<td>Centrifuged, 2.2 M</td>
<td>0.43</td>
<td>9.0</td>
<td>7.6</td>
</tr>
<tr>
<td>sucrose</td>
<td></td>
<td></td>
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<tr>
<td>Cytoplasma†</td>
<td>10.5</td>
<td>35.0</td>
<td>54.0</td>
</tr>
</tbody>
</table>

* 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, dehydroascetic 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 dehydroascetic 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

<table>
<thead>
<tr>
<th>Subcellular fraction</th>
<th>pH</th>
<th>2,6-Dichlorophenolindophenol</th>
<th>1.0 mg/ml Phenazine methosulfate</th>
<th>1.0 mg/ml Phenazine methosulfate</th>
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<tbody>
<tr>
<td>A. Thymus mitochondria</td>
<td>6.6</td>
<td>512</td>
<td>587</td>
<td>605</td>
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<td></td>
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<td>7.6</td>
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<td>7.6</td>
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</table>

TABLE V

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 produce lactate and pyruvate from added glucose and convert glucose-6-C14 to CO2 (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 C14O2 (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

 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, phosphoglyceraldehyde 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-C14 to CO2 (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. Arrighi and Singer (36) have outlined more rigorous kinetic tests which increase the diagnostic significance of the phenaexine methsulfate-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). Warrinha and Giuditta (37) reported that fumarate reduction to succinate, catalyzed by an enzyme from Micrococcus lacticifficus, 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 freudenreichii.

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 dye 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.

Relation between Nuclear and Cytoplasmic Enzymes

Recently, Vocell and Bearn (41) 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 X 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.

REFERENCES


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