THE INTRACELLULAR DISTRIBUTION OF PENTOSE CYCLE ACTIVITY IN RABBIT KIDNEY AND LIVER*

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Horecker (1) has demonstrated the occurrence of a cyclic mechanism in yeast and animal tissues which can account for the oxidation of carbohydrate to carbon dioxide and water without participation of triosephosphate dehydrogenase or the enzymes of the tricarboxylic acid cycle. The term "pentose cycle" has been suggested (2) for this series of reactions previously called the "shunt" or "oxidative pathway," since the reactions occur in a cyclic manner and involve pentoses as key compounds. Knowledge of the presence of this cycle has been extended to include plants (3), bacteria (4), insects (5), and the wheat smut fungus (6). This cycle might function as an energy source and also as a means by which ribose, an important constituent of nucleic acids and certain coenzymes, can be synthesized or catabolized.

Several workers (7, 8) have demonstrated the nearly exclusive location within the mitochondria of many oxidative enzymes, particularly those involved in the tricarboxylic acid cycle, various electron carrier systems, and oxidative phosphorylation. The glycolytic enzymes for the anaerobic conversion of carbohydrate have been shown to be localized in the soluble portions of the cell (9). Since the reactions of the pentose cycle may conceivably be associated with either or several of these activities, their behavior in the centrifugal field seemed of interest.

Several experimenters (10–14) have compared the quantitative contribution of the pentose cycle and the glycolytic scheme (together with the Krebs cycle) to the oxidation of carbohydrate. Organisms which contain little or no Krebs cycle activity may obtain much of their energy and assimilate carbon by such a mechanism, as suggested by Scott and Cohen (15) for

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Escherichia coli. In this organism the rate of assimilation of glucose by the pentose cycle is considered similar to the combined rate of assimilation by glycolysis and the Krebs cycle.

Methods and Materials

Preparation of Subcellular Fractions—The fresh liver (divested of the gallbladder) or kidneys from decapitated rabbits were removed and packed in ice. The medulla of the kidney was removed and the cortical material used for the fractionation of kidney homogenates. The livers or kidneys were then cut into small pieces and homogenized in 6 volumes of a solution containing 0.001 M sodium Versenate and either (a) 0.25 M sucrose or (b) 0.001 M citrate in 0.9 per cent KCl. Homogenizing was usually carried out in a Waring blender for 30 seconds at half speed. The KCl preparations were generally used, since large amounts of sucrose greatly interfered in the colorimetric methods used for the pentose cycle sugars. The pH was constantly adjusted with 1 N KOH to 7.0 to 7.5 during the blending operation.

The material was then centrifuged according to the scheme presented in Diagram 1. An International refrigerated centrifuge with a high speed attachment was used for centrifugal forces up to 25,000 × g. A Spinco model L ultracentrifuge was used for higher speeds (for speeds up to 105,000 × g the No. 30 head, and for speeds up to 144,000 × g the No. 40.3 head). All operations were performed at 0–5°. For the kidney experiments, the mitochondrial fraction was washed three to five times with the appropriate isotonic solution before use. The citrate-oxidizing capacity was only slightly impaired, even over the 6 hour period required for centrifuging. Kidney preparations with sucrose and KCl showed similar activity, but liver mitochondria seemed more stable when prepared in sucrose, as suggested by Hogeboom et al. (17).

Rat livers were also tested. For these experiments, the tissues were homogenized in a Potter-Elvehjem glass homogenizer.

A Warburg apparatus was employed for the manometric experiments. Gassing was performed with a conventional manifold. A Bausch and Lomb spectronic 20 spectrophotometer was used for the colorimetric assays and a Beckman spectrophotometer for the studies of triphosphopyridine nucleotide reduction. The methods used to follow non-oxidative pentose cycle conversions have been described previously (5, 6).

Materials—The following materials were commercial preparations: glucose-6-phosphate (G-6-P), triphosphopyridine nucleotide (TPN), cytochrome c, and adenosine-5'-phosphate, all from the Sigma Chemical Company; ribose-5-phosphate (R-5-P), from the Schwarz Laboratories, Inc.; thiamine pyrophosphate (TPP) from Hoffmann-La Roche, Inc.; and
Diagram 1. Scheme for Fractionation of Rabbit Liver or Kidney by Differential Centrifugation

Approximately 100 ml. homogenate of kidney or liver

Centrifuged 10 min. at 600 X g

Residue (discarded) Supernatant ($S_{5000}$)

Recentrifuged 10 min. at 8500 X g

Residue (mitochondria) Supernatant ($S_{8500}$)

Resuspended in 25 ml. 0.25 M sucrose or 0.9% KCl

Recentrifuged 3 hrs. at 25,000 X g

Washed mitochondria Washings (washed 3-5 times as above)*

Residue ($R_{25,000}$) Supernatant ($S_{25,000}$)

Recentrifuged 2 hrs. at 105,000 X g

Residue (microsomes) Supernatant ($S_{105,000}$) (soluble fraction)

Recentrifuged 16 hrs. at 144,000 X g

Residue ($R_{144,000}$) Supernatant ($S_{144,000}$)

* Similar to the preparations of Green et al. (16) (with KCl), or of Hogeboom et al. (17) (with sucrose).
Armour's coenzyme concentrate from Armour and Company. N-Methyl-
phenazine sulfate, sedosan, and disodium Versenate were kindly supplied
by Dr. R. H. Burris, Dr. N. E. Tolbert, and Versenes, Inc.; 6-phospho-
gluconic acid (6-PGA) was prepared as before (5).

**Fig. 1**

**Fig. 2**

*Fig. 1. Pentose cycle activity in rabbit liver fractions (non-oxidative).* Each
tube contained 1 ml. of enzyme, 200 μmoles of tris(hydroxymethyl)aminomethane
buffer, pH 8.0, 100 γ of TPP, 5 μmoles of R-5-P. Total volume 7.6 ml.; temperature
37°. 1 ml. aliquots were removed at the times designated and pipetted into 1 ml.
of 10 per cent trichloroacetic acid. After centrifugation, 0.5 ml. aliquots were re-
moved for the determination of R-5-P, sedoheptulose, and hexose. The mitochondria
were resuspended twice in 0.9 per cent KCl. ○, R-5-P (mitochondria); ●, R-5-P
(S_{9000}); Δ, sedoheptulose (S_{9000}); ×, sedoheptulose (mitochondria).

*Fig. 2. Pentose cycle in the S_{8500}, S_{106,000}, mitochondrial, and microsomal (R_{106,000})
fracthns of rabbit kidney (non-oxidative).* Additions as for Fig. 1. 1 ml. of mito-
chondria contained 4 mg. of protein (washed three times with 0.9 per cent KCl); 1 ml. of S_{8500} contained 8 mg. of protein; 1 ml. of S_{106,000} contained 2.4 mg. of protein; 1 ml. of microsomes contained 6 mg. of protein. ○, R-5-P (mitochondria); ●, R-5-P (microsomes); Δ, R-5-P (S_{8500}); ●, hexose (S_{9000}); ▲, sedoheptulose (S_{9000}); ×,
R-5-P (S_{106,000}); ■, hexose (S_{106,000}); □, sedoheptulose (S_{106,000}).

**Results**

Citrate oxidation served as the criterion of mitochondrial activity, and
its oxidation was nearly complete. G-6-P and R-5-P were not oxidized
by the mitochondria; neither were citrate, G-6-P, and R-5-P by the super-
natant fractions (except S_{900} which contained the mitochondria). Recom-
bination of S_{8500} and the mitochondria resulted in limited oxidation of these
substrates. The lowered activity may be due in part to dilution of the
oxidative enzymes. Oxidation of G-6-P and R-5-P proceeded in the S_{600}
fraction in the presence of cytochrome c and was enhanced when N-methyl-
phenazine sulfate was used as an electron carrier. Limited oxidation of
these substances occurred with the S_{25,000} fraction only in the presence of the artificial electron acceptor.

*Intracellular Localization of Non-Oxidative Pentose Cycle Enzymes* The disappearance of R-5-P and formation of sedoheptulose and hexose with increasing time were used as evidence for the occurrence of the pentose cycle in the various cellular fractions. This procedure seemed justified, since the bulk of the pentose could be accounted for as sedoheptulose and hexose.

Fig. 1 illustrates the localization of the pentose cycle enzymes in rabbit liver. These are soluble after centrifugation at 8500 \( \times g \), whereas little or no activity is found in the mitochondria. In kidney, the pentose cycle activity remained soluble after 2 hours of centrifugation at 105,000 \( \times g \), as shown in Fig. 2. Similar results were obtained with the S_{25,000} fraction. Neither the mitochondria nor microsomes contained activity.

Quantitative recovery of non-oxidative activity in the cycle was sought next, after centrifugation at 600 \( \times g \), 25,000 \( \times g \), and 105,000 \( \times g \). Aliquots were analyzed after various periods of incubation of enzyme with R-5-P. The results for one incubation period (45 minutes) are reported in Table I. The results at other times were similar. It is apparent that all of the original activity was recovered in the supernatant fluid after centrifugation at 105,000 \( \times g \) for 2 hours.

In another experiment, the S_{100,000} fraction was centrifuged for 16 hours at 144,000 \( \times g \). The red-colored material present in the S_{100,000} fraction

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**Table I**

*Intracellular Localization of Pentose Cycle (Non-Oxidative) in Rabbit Kidney*

The reaction tubes contained 1 ml. of enzyme of the following fractions: S_{400}, 3.8 mg. of protein; S_{28,000}, 2.4 mg. of protein; S_{105,000}, 2.0 mg. of protein; the tubes also contained 200 \( \mu \)moles of tris(hydroxymethyl)aminomethane buffer (pH 8.0), 100 \( \gamma \) of TPP, 20 \( \mu \)moles of Mg, and 5 \( \mu \)moles of R-5-P. Total volume 7.7 ml.; temperature 37\( ^{\circ} \). 1 ml. aliquots were removed at various times and added to 1 ml. of 10 per cent trichloroacetic acid, centrifuged to remove protein, and assayed colorimetrically.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein</th>
<th>mg.</th>
<th>mg.</th>
<th>Total</th>
<th>( \mu )moles</th>
<th>( \mu )moles</th>
<th>Total</th>
<th>( \mu )moles</th>
</tr>
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<td>S_{400}</td>
<td></td>
<td>362</td>
<td></td>
<td>1.04</td>
<td>378</td>
<td>0.13</td>
<td>47</td>
<td>0.61</td>
</tr>
<tr>
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<td></td>
<td>228</td>
<td></td>
<td>1.6</td>
<td>365</td>
<td>0.21</td>
<td>48</td>
<td>1.0</td>
</tr>
<tr>
<td>S_{105,000}</td>
<td></td>
<td>190</td>
<td></td>
<td>1.93</td>
<td>370</td>
<td>0.25</td>
<td>47</td>
<td>1.3</td>
</tr>
</tbody>
</table>
sedimented, leaving a clear colorless solution above it. The colorless layer was carefully removed with a pipette and designated the $S_{144,000}$ fraction. The red layer was removed and designated the $R_{144,000}$ fraction. As shown in Fig. 3, it appears that the pentose cycle is contained in the lower red-colored layer.

![Fig. 3](image_url)

**Fig. 3.** Pentose cycle in the $S_{105,000}$, $S_{144,000}$, and $R_{144,000}$ fractions of rabbit kidney (non-oxidative). Additions as for Fig. 1. 1 ml. of $S_{105,000}$ contained 2.2 mg. of protein; 1 ml. of $S_{144,000}$ contained 1 mg. of protein; 1 ml. of $R_{144,000}$ contained 4.7 mg. of protein. Neither hexose nor sedoheptulose was formed with the $S_{144,000}$ fraction. ◦, R-5-P ($S_{105,000}$); ●, hexose ($S_{105,000}$); □, sedoheptulose ($S_{105,000}$); ○, R-5-P ($R_{144,000}$); Δ, hexose ($R_{144,000}$); ■, sedoheptulose ($R_{144,000}$); X, R-5-P ($S_{144,000}$).

![Fig. 4](image_url)

**Fig. 4.** Glucose-6-phosphate dehydrogenase in rabbit kidney tissue. The complete system consisted of 20 µmoles of tris(hydroxymethyl)aminomethane buffer, pH 8.0, 10 µmoles of nicotinamide, 0.25 µmole of TPN, 2 µmoles of G-6-P. Total volume 3.5 ml. Appropriate blanks without enzymes, TPN, or substrate were used. The cuvettes for $S_{8500}$ contained 0.8 mg. of protein; for $S_{25,000}$, 0.6 mg. of protein; for $S_{105,000}$, 1.2 mg. of protein; for microsomes ($R_{105,000}$), 3 mg. of protein. The times listed refer to the duration of centrifugation. ○, G-6-P ($S_{8500}$), 10 minutes; ●, G-6-P ($S_{25,000}$), 3 hours; □, G-6-P ($S_{105,000}$), 2 hours; X, G-6-P ($R_{105,000}$), 2 hours.

Experiments with rat livers yielded similar results in that no pentose cycle activity was found in the mitochondria, but resided in the $S_{25,000}$ fraction. Higher gravitational fields were not employed.

**Glucose-6-phosphate Dehydrogenase and 6-Phosphogluconic Acid Dehydrogenase**—The reduction of TPN by G-6-P or 6-PGA was used to localize these enzymes in the various fractions. The soluble nature of G-6-P dehydrogenase is demonstrated in Fig. 4. Equal amounts of dehydrogenase were added to both cuvettes. The turbidity of the $S_{800}$ fraction precluded
accurate recovery data at this point in the centrifugation scheme, and therefore data similar to those obtained for the non-oxidative conversions were not obtained. After centrifugation for 2 hours at 105,000 \( \times g \), no activity was found in the microsomes, but remained instead in the supernatant fluid. The results with 6-PGA were similar.

In another experiment at higher centrifugal forces, i.e., 144,000 \( \times g \) for 16 hours, as described previously, a sedimentation of the two dehydrogenases occurred as shown in Fig. 5. In addition, TPN was reduced by R-5-P, although at a slower rate than by G-6-P, but more rapidly than by 6-PGA. In the presence of R-5-P and the S\(_{164,000}\) enzymes, no reduction of TPN occurred.

The principal remaining step in the pentose cycle comprises the fructose-6-phosphate; fructose-1,6 diphosphate \( \rightarrow \) G-6-P conversions. Although these have not been examined directly, the oxidation of R-5-P and its anaerobic conversion to hexose indicate that the hexose isomerases accompany the remainder of the pentose cycle complex. Earlier studies (9) have demonstrated the soluble nature of glycolytic enzymes at somewhat lower centrifugal fields.

![Fig. 5. Reduction of TPN by G-6-P, R-5-P, and 6-PGA in rabbit kidney tissue.](image-url)
DISCUSSION

The apparently similar centrifugal behavior of the entire group of pentose cycle enzymes (including the oxidative steps) suggests the possibility of intimate association among the cycle members, or the presence of a small organelle containing these enzymes. The localization of the pentose cycle apart from the particulate electron carrier systems or the known oxidative phosphorylation apparatus increases the importance of determining how such multienzyme groups may be associated or related to one another.

SUMMARY

The pentose cycle enzymes (non-oxidative) of rabbit kidney and liver tissue have been shown to be soluble after centrifugation at 105,000 \( \times g \) for 2 hours, but they sedimented after centrifugation at 144,000 \( \times g \) for 16 hours. Glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase behaved in a similar manner.

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