Citric Acid Cycle Patterns in Certain Thyroid States*

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The “citric acid cycle” is now regarded as the final common pathway of metabolism serving as the primary route for the oxidation of metabolites. Likewise, the thyroid hormone is considered a major regulatory substance for biological oxidations. It, therefore, seemed logical to study possible quantitative alterations in the cycle acids in experimental hypo- and hyperthyroid states. The concentrations of the cycle acids in metabolically active tissues, liver and muscle, were determined in experimental hypo- and hyperthyroid and control rats.

**EXPERIMENTAL PROCEDURE**

Thirty, adult, male, albino rats were stabilized for 1 week on a basal purified diet composed of the following percentages; casein, 20; corn oil, 15; sucrose, 59; salt mixture (Wesson's formula without iodide), 4; haliver oil, 5 ml per kilo; and an adequate vitamin mixture (incorporated in part of the sucrose). The animals were then divided into a control group, Group 1 (6 animals), which continued to receive only the basal ration; a hypothyroid Group 2 (12 animals) which was fed the basal diet and received daily subcutaneous injections of 4 mg of the goitrogen Tapazole (1-methyl-2-mercaptoimidazole); and a dedicated thyroid-fed2 Group 3 (12 animals) which received this material at first as a supplement in a thiamine solution and later in the basal ration at a level of 50 mg per 100 g. All three groups were maintained for 6 weeks.

Two other groups of animals were studied.3 Group 4 consisted of seven commercially hypophysectomized rats purchased as seven-day-old animals, which continued to receive only the basal ration after the basal metabolism had reached -26 to -60%, they were given prophylactic calcium lactate and were injected daily with thyroxine for 3 weeks. Before being killed they were maintained on the purified basal ration for 2 weeks without medication. At the time of sacrifice, the 24-hour fasted animals were anesthetized with a phenobarbital preparation (40 mg per kg) injected subcutaneously. The animals were exsanguinated from the abdominal aorta; the blood transfused into a small test tube, and the serum used for determination of protein-bound iodine (1). The quadriiceps and gastrocnemius muscles, and the liver were immediately excised, quickly frozen with dry ice in individual containers, and stored in the frozen state.

The thyroid status of each animal was established by two or more criteria: protein-bound blood iodine titer, gross size of thyroid gland at time of sacrifice, evaluation of coded histological sections of thyroid gland,4 animal weight records, and basal metabolism data. The protein-bound blood iodine titer of the animals used for the hypothyroid tissue pools was <1 μg per 100 ml, whereas the range of a 50-ml serum pool from stock laboratory rats was 4.03 to 4.23 μg per 100 ml, and the range for sera of control Group 1 animals (low-iodide) was 1.53 to 6.55 μg per 100 ml. The average gain of the animals whose tissues were ultimately pooled was 56.6 g in Group 1, 58.0 g in Group 2, and 31.6 g in Group 3.

Based on thyroid status, the liver and muscle tissues to be pooled were selected. Two equivalent pools of 12 g each were prepared by rapidly slicing the frozen liver or muscle with a razor blade and allocating one-half of each slice to each pool. The tissue was weighed and transferred to a flask and allowed to thaw under acetone. It was then homogenized with acetone in a Potter-Elvehjem homogenizer and transferred to a calibrated 50-ml centrifuge tube. Concentrated hydrochloric acid was added dropwise to approximately pH 2.0. After storage overnight at 0°, to allow complete extraction of the cycle acids, the acid-acetone extract was centrifuged and aliquots were pipetted into separate tubes for subsequent chromatography.

The acetone present in the extract was evaporated by water pump suction of dry air through the sample, to a volume of 3 ml, and the residual aqueous extract was centrifuged to remove acid haze. The extract was filtered through a disk of Whatman No. 50 filter paper and 2.5 ml of clear straw-colored filtrate were collected in a centrifuge tube, equivalent to 5 g of the original tissue. Anhydrous acetone (Mallinkrodt or Merck) was added repeatedly to the filtrate until the extract was evaporated to dryness.

4 Appreciation is expressed to Dr. Ernest Gardner of the Department of Anatomy, Wayne State University, College of Medicine, for this evaluation.

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1 Appreciation is expressed to Dr. Carl A. Kuether of the Lilly Research Laboratories for the Tapazole used in this study.

2 Appreciation is expressed to Dr. Daniel McGinty of Parke Davis Research Laboratories for the dedicated thyroid used in this study.

3 Courtesy of Dr. Marian Barnhart and Dr. Paul Halick, Department of Physiology, Wayne State University, College of Medicine.
dryness by water pump suction. The dry film was redissolved in 2 ml of redistilled 10% tertiary amyl alcohol in chloroform, and the mixture was allowed to stand for at least 3 hours before chromatographing on silica gel as described earlier (2, 3), but with the use of 100 mesh silicic acid (Mallinckrodt). In addition, 0.1 ml of 0.4% resorcinol in chloroform was added to the collector tubes before fraction collection.

After the required number of 80-drop fractions (90 to 100 tubes) had been collected (approximately 3 days), the tubes were allowed to evaporate to dryness at room temperature; heated in an electric oven at 110° ± 3° for 1 hour, then aspirated by a five-fingered suction manifold for a total time of 60 minutes more to remove final traces of tertiary amyl alcohol vapor. At room temperature, 0.2 ml of concentrated sulfuric acid (Mallinckrodt) was added, and the fluorescent organic acid derivatives were developed by heating in a boiling water bath for 20 minutes (4). The fluorescence obtained was plotted against fraction number.

RESULTS

A typical liver chromatogram is shown in Fig. 1. The identity of the numbered peaks was established by prior placement of pure organic acids singly and in mixtures. An irregular peak at Fractions 12 to 16 was present in the liver tissue of the control animals (cf. diagonal shading). This peak was increased in the liver extract from the hypothyroid animals (crosshatched area) and was decreased in that from the desiccated thyroid-fed rats. This peak was initially designated "X". The average concentrations of the cycle acids found in liver and muscle by the fluorometric procedure are given in Table I. Each value is an average of the values from three to six chromatograms. The extent of increase of the "X" peak in the hypothyroid groups and decrease in the thyroid-fed group is evident. No other consistent differences were detectable between the control and hypothyroid groups. However, the levels of all of the cycle acids appear to be somewhat lower in the desiccated thyroid-fed group than in those of the controls. The values obtained with the pooled muscle tissue of the various groups paralleled those obtained with liver but were somewhat lower.

Identification of the "X" peak at Fractions 12 to 16 was next attempted. The evidence indicated that "X" was oxalacetic acid and not pyruvate per se.

1. Titration of Fractions 12 to 16 indicated that "X" was acidic.
2. Repeated placement of pure pyruvic acid on the silica gel column clearly affirmed that pyruvic acid was eluted in this system as a single peak far removed from the "X" position in question.
3. Previous studies (3) concluded that oxalacetate was eluted near Fractions 12 to 19, immediately following α-ketoglutarate.
4. When pure solutions of oxalacetic acid were measured by the 2,4-dinitrophenylhydrazine method (5), a 90% or better conversion to pyruvate could be demonstrated.
5. Determination of keto acids (as α-ketoglutarate and pyruvate) in aliquots of homogenized tissues by the 2,4-dinitrophenylhydrazine method (5) indicated that the total pyruvate content was greater in the hypothyroid pool.
6. When fractions of the "X" peak were trapped in 2,4-dinitrophenylhydrazine reagent during column collection, and measured by their conversion to pyruvate, levels of 6.30, 7.40, and 2.99 mg per 100 ml were found for control liver pool, Tapazole-injected animal liver pool, and desiccated thyroid-fed animal liver pool, respectively.
7. Direct determination of the oxalacetate content of experimental and control tissues by the borate complex procedure of Greenwood and Greenbaum (6) revealed that the oxalacetate level was higher in hypothyroid and lower in desiccated thyroid-fed rat livers, than in livers of control animals.
8. Addition of known oxalacetic acid to normal liver homogenates, and subsequent column chromatography, produced an augmentation of the "X" peak only.

Further studies, with the techniques of paper chromatography of the silica gel chromatographic fractions, and infrared spectra of the "X" peak confirmed its homogeneity and identification as oxalacetate. Duplicate hypothyroid tissue extracts were chromatographed in the usual manner (Columns 1 and 2) except that 2 drops of NaOH (1 mg/ml) were added to the collecting tubes. Standard keto acids were accorded this same treatment before paper chromatography. The sequence of tubes thought to con-

**Fig. 1.** Control liver chromatogram: 1, fat; 2, fumaric acid; 3, α-ketoglutaric acid; "X", oxalacetic acid; 4, lactic acid; 5, succinic acid; 6, pyruvic acid; 7, cis-aconitic acid; 8, malic acid; 9, isocitric acid; 10, citric acid.
Table I
Average concentration of organic acids in tissues of 24-hour fasted animals

<table>
<thead>
<tr>
<th>Acid</th>
<th>Liver, mg per 100 g frozen tissue</th>
<th>Muscle, mg per 100 g frozen tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group 1 (control)</td>
<td>Group 4*</td>
</tr>
<tr>
<td>&quot;X&quot; Peak (oxalacetic)</td>
<td>3.15</td>
<td>2.50</td>
</tr>
<tr>
<td>Citric</td>
<td>0.74</td>
<td>0.79</td>
</tr>
<tr>
<td>cis-Aconitic</td>
<td>0.61</td>
<td>0.16</td>
</tr>
<tr>
<td>Isocitric</td>
<td>0.45</td>
<td>0.40</td>
</tr>
<tr>
<td>α-Ketoglutaric</td>
<td>7.36</td>
<td>5.23</td>
</tr>
<tr>
<td>Succinic</td>
<td>0.60</td>
<td>1.05</td>
</tr>
<tr>
<td>Fumaric</td>
<td>1.06</td>
<td>1.21</td>
</tr>
<tr>
<td>Malic</td>
<td>0.33</td>
<td>0.97</td>
</tr>
</tbody>
</table>

* Group 4, hypophysectomized animals.
* Group 5, surgically thyroidectomized animals.
* Group 2, Tapazole-injected animals.
* Group 3, desiccated thyroid-fed animals.

Table II
Infrared spectra of keto acids

<table>
<thead>
<tr>
<th>Solute</th>
<th>Solutes in organic solvent*</th>
<th>Solutes in aqueous solvent†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvic acid</td>
<td>Broad peak, 5.8-5.9 μ</td>
<td>Broad peak, 7.0-7.2 μ</td>
</tr>
<tr>
<td></td>
<td>Sharp peaks, 7.2-7.4 μ</td>
<td>Sharp peak, 7.4 μ</td>
</tr>
<tr>
<td></td>
<td>Broad peak, 8.5-8.9 μ</td>
<td>Sharp peak, 8.5 μ</td>
</tr>
<tr>
<td>α-Ketoglutaric</td>
<td>Sharp peaks, 5.85-9.16 μ</td>
<td>Sharp peak, 9.2 μ</td>
</tr>
<tr>
<td></td>
<td>9.58 μ</td>
<td>Sharp peaks (KBr pellet method), 5.8-9.9 μ</td>
</tr>
<tr>
<td>Oxalacetic</td>
<td>Broad peak, 11.2 μ</td>
<td></td>
</tr>
<tr>
<td>Pooled chromatography Fractions 12-16</td>
<td>Sharp peak, 5.5 μ</td>
<td>Double peak centered at 5.85 μ and 6.10 μ</td>
</tr>
</tbody>
</table>

* 200 μg of solute in 10% tertiary amyl alcohol in chloroform; Perkin-Elmer model 112; 0.32-mm cell thickness for standards; 1-mm cell thickness for chromatography fractions.
† Transmittance evaluated from Parker (8) for 10% aqueous solutions of solute; Perkin-Elmer model 21; 0.053-mm cell thickness.

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Discussion
The foregoing results obtained on the tissues from adult rats suggest that there is no gross failure of the functioning of the tricarboxylic acid cycle in hypothyroid and hyperthyroid animals. However, there is a definite alteration in a keto acid which our studies indicate is oxalacetic acid. Why this acid is significantly elevated above the normal level in hypothyroid animals, and depressed in hyperthyroid animals, is not entirely clear at this time. Estabrook et al. (9) suggested on the basis of heart homogenate studies that thyroxine may, like calcium, act indirectly by lowering the concentration of oxalacetate, a potent inhibitor of succinic dehydrogenase. Clarke and Ball (10) found that thyroxine addition in vitro decreased oxalacetate (formation?) in fresh rat heart homogenates, and placed the site of thyroxine action at the malic dehydrogenase enzyme. The further observations of Wolff and Ball (11) also support an hypothesis that added thyroxine acts in heart homogenates to prevent oxalacetate production from reaching a level which would inhibit succinic dehydrogenase.

The present investigation indicates that not only is oxalacetate concentration decreased in vivo in liver and muscle of desiccated thyroid-fed rats but that oxalacetate accumulates in these same tissues of hypothyroid rats.

Summary
Determination of the citric acid cycle pattern in the liver of hypothyroid animals (hypophysectomized, surgically and chemically)
ically thyroidectomized) indicated no gross alteration in the functioning of the cycle. However, there was present a significant increase in a keto acid which column and paper chromatography, and infrared spectra studies indicate is oxalacetic. The amounts of the cycle acids in muscle of hypothyroid rats tend to parallel those found in liver.

A significant decrease in the oxalacetate concentration was found in the liver and muscle of desiccated thyroid-fed animals. The concentrations of the other cycle acids likewise tended to be lower than those of control animals. These observations suggest that the regulation of the oxalacetate concentration of tissues may be a specific point of metabolic activity of thyroxine.

REFERENCES

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