Urinary Excretion of Pyruvic Acid, \( \alpha \)-Ketoglutaric Acid, and Oxaloacetic Acid in Scurvy

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Scurvy is associated with abnormal carbohydrate metabolism and diminished production of insulin (1). Banerjee et al. (2) observed increased accumulation of citrate, malate, and lactate in the tissues of scorbutic guinea pigs. This was possibly due to the insulin insufficiency associated with scurvy since prolonged injection of insulin to scorbutic guinea pigs diminished the tissue concentrations of these acids. Banerjee et al. (3) also observed decreased succinic acid dehydrogenase and malic acid dehydrogenase activity in tissues of scorbutic guinea pigs, which came to the normal level after the scorbutic animals received prolonged injection of insulin. Increased accumulation of citrate in tissues of scorbutic animals supposedly due to a decreased activity of the enzyme or enzymes intimately concerned with citrate oxidation through the tricarboxylic acid cycle. It was, therefore, of interest to study the metabolism of the members of the tricarboxylic acid cycle in scurvy. In the present investigation, the urinary excretions of the keto acids, pyruvic acid, \( \alpha \)-ketoglutaric acid, and oxaloacetic acid, were estimated in the urine of normal and scorbutic guinea pigs before and after they were fed citric, succinic, and malic acids. As insulin has marked effects on the enzyme systems of the Krebs cycle (4) the effects of prolonged injection of insulin in scorbutic guinea pigs on the excretion of keto acids were also studied.

EXPERIMENTAL

Materials and Methods

Male guinea pigs, weighing from 250 to 300 g were fed with green grass, soaked gram, and the scorbutic diet (5) for 5 to 6 days. Those animals which grew well were selected and separated into several groups, each group consisting of one normal, one scorbutic, and one insulin-treated scorbutic guinea pig. The animals in each group were fed equal amounts of the scorbutic diet. The normal control was fed 5 mg of ascorbic acid daily. All the animals were fed 2 drops of a concentrate of vitamins A and D twice a week. Regular insulin (Lilly) was injected subcutaneously into the animal intended for insulin treatment with a dose increasing from 0.1 to 0.3 unit per 100 g body weight per day from the beginning of the second week. Twenty-four-hour urine samples were collected under toluene from individual animals to determine the basal excretion of pyruvic acid, \( \alpha \)-ketoglutaric acid, and oxaloacetic acid. Each animal was then fed at intervals citric acid (1 mmole per 100 g body weight), succinic acid (2 mmole per 100 g body weight), and malic acid (2 mmole per 100 g body weight) for two consecutive days and the urinary excretions of the different ketoacids were estimated (Table 1).

Pyruvic acid, \( \alpha \)-ketoglutaric acid, and oxaloacetic acid in the urine samples were converted into dinitrophenylhydrazones and separated by paper chromatography according to the method of El Hawary and Thompson (6). Twenty-four-hour urine samples were treated with 2 ml of 0.2% 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid, kept at 38° for 20 minutes, and extracted with 5-ml aliquots of ethyl acetate. The combined ethyl acetate phase was extracted four times with 2 ml of 10% sodium carbonate, the sodium carbonate extract was neutralized with cold concentrated hydrochloric acid, and extracted four times with 2 ml of ethyl acetate. This combined extract was evaporated to dryness under reduced pressure, the residue dissolved in 0.1 to 0.3 ml of 0.1 N sodium hydroxide, and treated with phosphate buffer (0.1 M; pH 7.2) until the red color of the solution disappeared. The total volumes of sodium hydroxide and phosphate buffer added were noted and 0.2 ml of the solution was chromatographed with n-butanol-ethanol-0.5 N ammonia (70:10:20) as the solvent. Ten \( \mu \)g of the synthetic hydrazones of the keto acids dissolved in 0.02 ml of dilute phosphate buffer were applied to paper strips and chromatographed simultaneously. The yellow spots were eluted with sodium carbonate, the eluate treated with sodium hydroxide, and the pink color estimated in a Klett-Summerson photoelectric colorimeter. Recovery experiments with these keto acids gave the following values: 90% for pyruvic acid, 88% for \( \alpha \)-ketoglutaric acid, and 95% for oxaloacetic acid. Spots were identified by running standard hydrazones of the keto acids simultaneously along with the unknown samples.

RESULTS

Excretion of Pyruvic, \( \alpha \)-Ketoglutaric, and Oxaloacetic Acid before and after Feeding of Citric Acid—After the feeding of citric acid to normal guinea pigs the urinary excretion of pyruvic acid did not change, excretion of \( \alpha \)-ketoglutaric acid increased, and oxaloacetic acid which was absent in normal urine, appeared. Scorbutic guinea pigs excreted increased amounts of pyruvic acid which was further enhanced after the feeding of citric acid. The urinary excretion of \( \alpha \)-ketoglutaric acid greatly diminished in scorbutic guinea pigs and did not rise after the feeding of citric acid. Scorbutic guinea pigs excreted measurable quantities of oxaloacetic acid and the excretion increased slightly after the feeding of citric acid. When the scorbutic guinea pigs were treated with insulin the urinary excretion of pyruvic acid was considerably lowered. The excretion, however, was higher than the excretion by the normal guinea pig. The \( \alpha \)-ketoglutaric acid excretion in the insulin-treated scorbutic animal was slightly higher than that of the scorbutic guinea pigs.

Excretion of Pyruvic Acid, \( \alpha \)-Ketoglutaric Acid, and Oxaloacetic Acid after Feeding Succinic Acid and Malic Acid—After the feed-
is possible that a defective operation of the Krebs cycle prevents vie acid increased in normal guinea pigs. The excretion was fur-
malic acid.

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In scurvy, the urinary excretion of pyruvic acid and oxalo-
acetic acid increased whereas that of α-ketoglutaric acid de-
creased in comparison to excretions by normal guinea pigs. It
is possible that a defective operation of the Krebs cycle prevents
the entry of pyruvic acid effectively into the cycle for oxidation
resulting in the accumulation of pyruvic acid in blood. Enor-
mous increase in the tissue content of lactic acid observed pre-
viously (2) is in consonance with the present result. Decreased
excretion of α-ketoglutaric acid is possibly due to the defective
operation of the Krebs cycle before the formation of the keto
acid.

After the feeding of citric acid to normal animals there was in-
creased excretion of α-ketoglutaric acid and oxaloacetic acid.
Pyruvic acid excretion did not change. As the Krebs cycle goes
in the forward direction, the load of citric acid goes to the forma-
tion of α-ketoglutaric acid and oxaloacetic acid and these are
subsequently excreted in larger quantities. Ingested citric acid
is possibly completely oxidized in the cycle and its metabolism
in the glycolytic cycle through the formation of pyruvate may not
be a preferable pathway. When citric acid was fed to scorbatic
animals a different metabolic picture was observed; the excretion
of α-ketoglutaric acid and oxaloacetic acid was only slightly in-
creased and pyruvic acid excretion was greatly enhanced. This
possibly indicates that citric acid could not go to the formation
of α-ketoglutaric acid due to a defect in the operation of the
Krebs cycle at a level which was below citric acid and above α-
ketoglutaric acid. In spite of this defect in the operation of the
cycle, there was increased excretion of oxaloacetic acid and pyru-
vic acid. It was possible that the enormous accumulation of
citric acid in scurvy might have caused shifting of the equilibrium
position of reactions towards malate formation which gave rise
to increased accumulation of oxaloacetate and pyruvate (7, 2).
The existence of the glyoxalate shunt in microorganism and plant
tissue which drives citrate to give rise to malate through isocitrate
and malate synthetase has been reported. Such a mechanism
with an ascendency in scurvy as a result of a defect in the Krebs
cycle might have been possible which could explain the accumula-
tion of malic acid, oxaloacetic acid, and pyruvic acid. But the
two key enzymes, isocitrase and malate synthetase, have not
been demonstrated so far in animal tissues.

When succinic acid and malic acid were fed to normal animals,
the excretion of the α ket acids were increased. Pyruvic acid
could be derived by decarboxylation of malic acid and oxaloacetic
acid. The other two keto acids could be derived from the in-
gested acids as the Krebs cycle goes in the forward direction.
When succinic acid and malic acid were fed to scorbatic guinea
pigs, the urinary excretion of pyruvic acid and oxaloacetic acid
were further increased and the urinary excretion of α-ketoglu-
taric acid which was lowered was only slightly increased. This
also shows that succinic acid and malic acid could not go to the
formation of α-ketoglutaric acid in scurvy possibly due to a defect
in the cycle at level above α-ketoglutarate. Banerjee et al. (3)
observed that the activities of succinic acid and malic acid de-
hydrogenases were moderately decreased in scurvy. In spite of
this decrease in the activity of these enzymes, increased excretion
of oxaloacetic acid by scorbatic guinea pigs could be observed.
The decrease in the activity of the enzymes in the tissue of sco-
butic guinea pigs ranged between 25 and 60%. Under conditions
of loading such as resorted to in the present investigation, it was
possible that in spite of the moderate decrease in the activity
of succinic and malic dehydrogenases in scurvy, the loads of succinic
acid and malic acid could give rise through mass action to con-
siderable amounts of their subsequent oxidative products. An
outstanding effect of scurvy seems to be the inability to form α-

<table>
<thead>
<tr>
<th>Substance fed</th>
<th>Group†</th>
<th>Pyruvic acid</th>
<th>α-Ketoglutaric acid</th>
<th>Oxaloacetic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before µg</td>
<td>After µg</td>
<td>Before µg</td>
</tr>
<tr>
<td>Citric acid (1 mmole/100 g)</td>
<td>Normal</td>
<td>176 ± 6.5</td>
<td>170 ± 7.6</td>
<td>205 ± 11.4</td>
</tr>
<tr>
<td></td>
<td>Scorbatic</td>
<td>249 ± 21.7</td>
<td>366 ± 14.1</td>
<td>161 ± 8.7</td>
</tr>
<tr>
<td></td>
<td>Insulin-treated</td>
<td>206 ± 9.3</td>
<td>311 ± 9.7</td>
<td>187 ± 7.9</td>
</tr>
<tr>
<td>Succinic acid (2 mmole/100 g)</td>
<td>Normal</td>
<td>161 ± 16.8</td>
<td>226 ± 21.6</td>
<td>200 ± 6.4</td>
</tr>
<tr>
<td></td>
<td>Scorbatic</td>
<td>252 ± 14.7</td>
<td>386 ± 9.1</td>
<td>154 ± 7.4</td>
</tr>
<tr>
<td></td>
<td>Insulin-treated</td>
<td>186 ± 6.7</td>
<td>275 ± 21.5</td>
<td>193 ± 12.1</td>
</tr>
<tr>
<td>Malic acid (2 mmole/100 g)</td>
<td>Normal</td>
<td>162 ± 9.8</td>
<td>235 ± 5.2</td>
<td>206 ± 7.0</td>
</tr>
<tr>
<td></td>
<td>Scorbatic</td>
<td>250 ± 13.2</td>
<td>308 ± 10.4</td>
<td>122 ± 6.0</td>
</tr>
<tr>
<td></td>
<td>Insulin-treated</td>
<td>193 ± 8.8</td>
<td>271 ± 6.5</td>
<td>179 ± 7.9</td>
</tr>
</tbody>
</table>

* Mean ± standard error.
† There were six animals in each group.

Table I

Twenty-four-hour urinary excretion of α-keto acids* by guinea pigs before and after they were fed Krebs cycle intermediates.
Excretion of Pyruvic Acid

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ketoglutaric acid from all precursors which show that the lesion or lesions involved may be quite sensitive. The results obtained in the present experiments are rather tentative and in no way very conclusive. Two important aspects which have not been taken into account are the possible action of intestinal microflora on the ingested acids and the functioning of the kidney.

The effect of insulin treatment to scorbutic guinea pigs was to reverse the excretion patterns of α-keto acids. The increased urinary excretion of pyruvic acid and oxaloacetic acid by scorbutic guinea pigs before and after they were fed Krebs cycle intermediates were considerably lowered after treatment with insulin. Likewise the urinary excretion of α-ketoglutaric acid, which was very low, increased after treatment of the deficient animals with insulin for a prolonged period of time. The whole effect of insulin treatment was to restore to normal the metabolism through the Krebs cycle to a great extent. The result is in support of the contention that insulin insufficiency associated with scurvy is responsible to a great extent for the deranged metabolism of carbohydrate through the Krebs cycle.

SUMMARY

1. The urinary excretion of pyruvic acid, α-ketoglutaric acid, and oxaloacetic acid was determined in normal, scorbutic, and insulin-treated scorbutic guinea pigs. These excretions were also studied after the animals were fed citric, succinic, and malic acids.

2. Normal guinea pigs excreted pyruvic acid. The excretion increased when the animals were fed succinic and malic acids. The administration of citric acid did not alter the urinary excretion of pyruvic acid. Scorbutic guinea pigs excreted increased amounts of pyruvic acid, and the excretion was further enhanced when the animals were fed citric, succinic, and malic acids. When the scorbutic guinea pigs were treated with insulin, the urinary excretion of pyruvic acid diminished.

3. Normal guinea pigs excreted α-ketoglutaric acid. The excretion increased when the animals were fed citric, succinic, or malic acids. The urinary excretion of α-ketoglutaric acid diminished when the animals developed scurvy. The feeding of citric, succinic, and malic acids to scorbutic animals did not change this excretion. Treatment of the scorbutic animals with insulin increased the urinary excretion of α-ketoglutaric acids.

4. Normal guinea pigs did not excrete oxaloacetic acid which, however, appeared in urine after the animals were fed citric, succinic, and malic acids. Scorbutic animals excreted oxaloacetic acid in urine both before and after the administration of these acids. Treatment of the scorbutic animals with insulin led to the diminution in the excretion of this acid.

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REFERENCES

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