Dickmann and Cloutier (1) observed that aconitase is stabilized and activated by ferrous iron and reducing agents such as ascorbic acid or cysteine. This led Takeda and Hara (2) to study the urinary excretion of citric acid and ketone bodies in scorbutic and \( \alpha,\alpha' \)-dipyridyl-injected guinea pigs after the animals were fed butyrate. These animals excreted increased amounts of citric acid and ketone bodies, possibly attributable to a decline of the ferrous iron concentration with subsequent decrease in aconitase activity. Banerjee et al. (3) observed increased tissue contents of citric, malic, and lactic acids in scorbutic guinea pigs and indicated a possible derangement in their metabolism through the tricarboxylic acid cycle. They observed, however, that the above defects were corrected when guinea pigs received daily injections of a small dose of insulin along with the scorbutogenic diet. Whereas the Japanese workers (2) considered that the decreased aconitase activity was a direct result of the lack of ascorbic acid which maintains the prosthetic Fe\(^{++}\) of the enzyme in the reduced state, Banerjee et al. (3) pointed out that the defect in citric acid metabolism in scurvy might be due as well to insulin insufficiency associated with scurvy (4). The present investigation was undertaken to study the urinary excretions of ketone bodies, citric acid, and malic acid in normal, scorbutic, insulin-treated scorbutic, and Fe\(^{++}\) and cysteine-treated scorbutic guinea pigs after the feeding of butyrate.

**EXPERIMENTAL**

Male guinea pigs, weighing 250 to 300 gm., were fed green grass and a scorbutogenic diet (5) for 6 days. Animals which grew well were selected. Each animal was then placed in a metabolism cage and fed ad libitum the scorbutogenic diet, 5 mg. of ascorbic acid per day, and 2 drops of a concentrate of vitamins A and D twice a week. After the collection of a 24-hour sample of urine under toluene, each animal was fed daily in three divided doses a neutral solution containing 25 per cent of butyric acid (1 ml./100 gm. of body wt.) for 3 consecutive days. Urine samples were collected over 24 hours. In view of the small volumes of urine (8 to 10 ml.) passed by each animal in 24 hours, the glass funnels of metabolism cages were washed with distilled water so as to collect traces of urine adhering to the funnel. The volume of the urine plus washings was made up to 50 ml. Ketone bodies, citric acid, and malic acid were estimated in the samples of urine.

After the above experiment, the animals were rendered scorbutic by the withdrawal of the ascorbic acid supplement. They were divided into three groups. Animals of one of the groups were injected with protamine zinc insulin (Lilly), one injection per day, in doses increasing from 0.1 to 0.3 unit/100 gm. of body weight from the second week of the scorbutic regime. To a second group of animals, intraperitoneal injections of a mixture of 0.5 ml. of 0.05 M Fe\(^{++}\) as ferrous ammonium sulfate and 0.5 ml. of 0.2 M cysteine hydrochloride were given from the 3rd week of the scorbutic regime. The animals of the third group served as untreated scorbutic controls. When the animals showed gross signs of scurvy, which were usually observed during the 4th week of the scorbutic regime, they were fed butyrate for 3 days. Twenty-four-hour urine samples, collected before and after the administration of butyrate, were analyzed for ketone bodies, citric acid, and malic acid.

Four normal guinea pigs, receiving the scorbutogenic diet and 5 mg. of ascorbic acid per day, were injected with insulin in the same way as the insulin-treated scorbutic guinea pigs. Urinary excretions of the above metabolites were determined before and after butyrate was fed.

Total ketone bodies were estimated as acetone by the method of Behre (6). Citric acid was determined according to the method of Speck et al. (7). Malic acid was estimated by the fluorometric method of Hummel (8).

**RESULTS**

Results are given in Tables I and II. The urinary excretion of ketone bodies increased enormously in all the groups after they were fed butyrate. These excretions did not differ markedly in the normal, scorbutic, and Fe\(^{++}\) and cysteine-treated scorbutic guinea pigs. The insulin treatment produced a significant decrease in the ketonuria in both normal and scorbutic animals.

The excretion of citric acid was considerably increased after the feeding of butyrate in the scorbutic and Fe\(^{++}\) and cysteine-treated scorbutic guinea pigs as compared with similar excretions by normal and insulin-treated scorbutic animals.

Scorbutic guinea pigs excreted increased amounts of malic acid after the feeding of butyrate. This excretion diminished significantly when the scorbutic animals were given injections of insulin or Fe\(^{++}\) and cysteine.

Prolonged treatment of normal animals with insulin did not affect the excretions of citric acid and malic acid but considerably lowered the excretion of ketone bodies.

**DISCUSSION**

Beatty and West (9) and Takeda and Hara (2) assumed that the ketosis in normal rats and guinea pigs after the administration of butyrate was due to the nonavailability of sufficient oxaloacetic acid to permit oxidation, via the tricarboxylic acid cycle, of acetyl CoA abundantly produced. This assumption was supported by...
The ketolytic effect of these substances was not observed, however, in alloxan diabetic rats (9) nor in scorbutic and α,α'-dipyridyl-injected guinea pigs (2). Beatty and West (9) supposed that condensation of oxaloacetate with acetyl-CoA could not proceed efficiently in the absence of insulin. Takeda and Hara (2) observed decreased aconitase activity and increased citrate excretion in scorbutic and α,α'-dipyridyl-injected guinea pigs fed butyrate along with citrate or malate. They concluded that ketonuria in these animals was not due to a defect in the condensing enzyme system. Rather, it was attributed to a decreased oxidation of citrate as a result of decreased aconitase activity.

The continuous increase in the urinary excretion of citric acid by scorbutic guinea pigs after the administration of butyrate perhaps suggests that the condensation of acetyl-CoA and oxaloacetate is not diminished in scurvy. The increased excretion might be due to decreased oxidation of citrate. The increase in the urinary excretion of malic acid under similar conditions might be due to reversible conversion of citric acid to malic acid through the condensing enzyme malic dehydrogenase system (10). Both normal and scorbutic guinea pigs, however, excreted increased amounts of ketone bodies in urine after they were fed butyrate.

Prolonged treatment of the scorbutic animals with insulin significantly reduced the urinary excretion of citric acid and malic acid. It is likely that exogenous insulin promotes the oxidation of citrate and malate through the normal operation of the Krebs cycle. The excretion of ketone bodies was distinctly lowered by the insulin treatment of the scorbutic animals. Exogenous insulin possibly promotes increased oxidation of ketone bodies by peripheral tissues, stimulates the production of more oxaloacetate through restoration of the normal operation of the Krebs cycle, or stimulates fatty acid synthesis. Normal animals, when fed butyrate, excreted an increased amount of ketone bodies. Ketonuria was considerably diminished when these animals were given injections of insulin. It seems that insulin released in normal guinea pigs was not sufficient to deal with the excessive amount of ketone bodies formed as a result of the feeding of butyrate.

Takeda and Hara (2) considered that the decreased aconitase activity in scurvy was brought about by the inability of the deficient animals to keep the ferrous iron cofactor of the enzyme in the reduced state. There are several reports of the activation of aconitase by Fe++ and cysteine (11, 12). The treatment of the scorbutic animals with Fe++ and cysteine, however, had no significant effect on the urinary excretion of ketone bodies and citric acid. This finding does not necessarily invalidate the possibility of the production of decreased aconitase in scurvy. The injection of Fe++ and cysteine might not be effective because of insufficient dose, oxidative destruction, or quantitative diminution of aconitase.

Insulin is necessary for the synthesis of proteins (13) and glutathione (14). The formation of enzymes, therefore, might be diminished in prolonged insulin deficiency. Banerjee et al. (15) observed significantly decreased activity of succinic, malic, and lactic dehydrogenases in scurvy. The activity of the enzymes was restored to normal after prolonged treatment of the animals with insulin. Takeda and Hara (2) observed that the addition of Fe++ and ascorbic acid to the enzyme preparation obtained from scorbutic animals could enhance the enzyme activity only to approximately 50 per cent of normal. They concluded that ascorbic acid depletion resulted in a qualitative decrease of the enzyme. It is possible that factors other than the lack of protection of the prosthetic Fe++ are operating in scurvy so as to cause a quantitative reduction in the aconitase activity. Prolonged insulin deficiency with its attendant effects on the synthetic processes is more likely to be the major factor in bringing about the decline in the activity of aconitase in scurvy.

### Table I

<table>
<thead>
<tr>
<th>Animals*</th>
<th>Ketone bodies†</th>
<th>Citric acid†</th>
<th>Malic acid†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmoles</td>
<td>µmoles</td>
<td>µmoles</td>
</tr>
<tr>
<td>Normal (6)</td>
<td>4.87 ± 0.436</td>
<td>14.80 ± 2.31</td>
<td>3.02 ± 0.26</td>
</tr>
<tr>
<td>Insulin-treated normal (4)</td>
<td>2.75 ± 0.0145</td>
<td>13.20 ± 2.104</td>
<td>3.56 ± 0.519</td>
</tr>
<tr>
<td>Scorbatic (6)</td>
<td>5.73 ± 0.511</td>
<td>38.87 ± 14.45</td>
<td>4.53 ± 0.903</td>
</tr>
<tr>
<td>Insulin-treated scorbatic (6)</td>
<td>4.92 ± 0.208</td>
<td>12.17 ± 3.15</td>
<td>3.08 ± 0.204</td>
</tr>
<tr>
<td>Fe++ + cysteine-treated scorbatic (6)</td>
<td>6.10 ± 0.245</td>
<td>15.91 ± 1.45</td>
<td>1.84 ± 0.342</td>
</tr>
</tbody>
</table>

* Figures in parentheses indicate the number of animals used. † The mean ± the standard error is given.

### Table II

<table>
<thead>
<tr>
<th>Animals*</th>
<th>Ketone bodies µmoles†</th>
<th>Citric acid µmoles†</th>
<th>Malic acid µmoles†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (6)</td>
<td>62.64 ± 4.473</td>
<td>46.99 ± 5.55</td>
<td>8.77 ± 0.004</td>
</tr>
<tr>
<td>Insulin-treated normal (4)</td>
<td>10.15 ± 0.975</td>
<td>45.46 ± 11.983</td>
<td>6.41 ± 1.143</td>
</tr>
<tr>
<td>Scorbatic (6)</td>
<td>79.39 ± 8.051</td>
<td>438.43 ± 85.03</td>
<td>20.92 ± 1.475</td>
</tr>
<tr>
<td>Insulin-treated scorbatic (6)</td>
<td>28.86 ± 3.056</td>
<td>68.36 ± 11.33</td>
<td>4.04 ± 0.544</td>
</tr>
<tr>
<td>Fe++ + cysteine-treated scorbatic (6)</td>
<td>55.87 ± 13.17</td>
<td>292.10 ± 50.65</td>
<td>6.13 ± 0.198</td>
</tr>
</tbody>
</table>

**Statistical analysis t values**

| Between normal and scorbutic | 1.81 | 4.59‡ | 7.02‡ |
| Between normal and insulintreated scorbutic | 6.05‡ | 1.73 | 4.50‡ |
| Between scorbutic and insulin-treated scorbutic | 5.86‡ | 4.31‡ | 10.75‡ |
| Between scorbutic and Fe++ + cysteine-treated scorbutic | 1.52 | 1.57 | 9.99‡ |

* Figures in parentheses indicate the number of animals used. † The mean ± the standard error is given. ‡ Significant at the 5 per cent level.
SUMMARY

1. Urinary excretion of ketone bodies, citric acid, and malic acid was studied in normal, scorbutic, insulin-treated scorbutic, Fe++ and cysteine-treated scorbutic, and insulin-treated normal guinea pigs before and after they were fed butyrate.

2. The excretion of ketone bodies increased considerably after the feeding of butyrate in all the groups of animals. Ketone bodies excreted in the urine by normal, scorbutic, and Fe++ and cysteine-treated scorbutic animals did not differ markedly. Insulin treatment produced a distinct decrease in ketonuria in both normal and scorbutic animals.

3. Citric acid and malic acid excretions were enormously increased in scorbutic animals after the feeding of butyrate. These excretions diminished when the scorbutic animals were treated with insulin. Fe++ and cysteine treatment of the scorbutic animals reduced the urinary excretion of malic acid but did not significantly affect the excretion of citric acid. Injection of insulin into normal guinea pigs did not affect the excretion of citric acid and malic acid.

4. The significance of these observations in relation to the operation of the tricarboxylic acid cycle in scurvy has been discussed.

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Studies on the Operation of the Tricarboxylic Acid Cycle in Scurvy
Sachchidananda Banerjee and W. K. Kawishwar


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