Finkelstein and Gold (1) administered itaconic acid and its sodium, magnesium, and calcium salts to adult cats, both acutely and chronically. They observed vomiting and diarrhea when single oral doses of 0.5 gm. per kilo or more of this compound or its salts were given. They attributed these reactions to local action in the gastrointestinal tract, since doses up to 1 gm. per kilo failed to produce signs of systemic toxicity. Oral doses of 5 gm. per kilo proved fatal after severe gastrointestinal disturbances, convulsions, and prostration. These results indicated that acute oral toxicity of itaconic acid and its salts is very low. Daily oral administration of 100 mg. per kilo of itaconic acid for 14 weeks failed to reveal toxicity, as indicated by the state of nutrition, blood picture, liver and kidney function, electrocardiogram, and histological examination of liver and kidney.

Since the cat is a carnivore and since Finkelstein and Gold utilized adult rather than growing cats, it was considered important to obtain confirmatory data for another species and with growing animals. The results to be reported are based on long term feeding in which itaconic acid was mixed with the food and fed to rats from the time they were weaned until time of sacrifice after 210 days on the diet.

Data are presented showing decreased rates of growth as the intake of itaconic acid was increased. It is well known that malonate competitively inhibits succinic dehydrogenase. Furthermore, Ackermann and Potter (2) have demonstrated that itaconate also competitively inhibits this enzyme. Since itaconic acid, like malonic acid, is structurally similar to succinic acid, it seemed possible that the mechanism of action could be one of competition between the analogue and the normal metabolite. Because an inhibition of succinate oxidation demonstrable in vitro might occur in vivo with a resultant increase in succinic acid excretion, as in the case of malonic acid (3), we have measured the excretion of succinic acid by the rabbit before and after oral administration of itaconic acid. The observed increase in urinary excretion of succinic acid in this experiment suggests
that decline in growth rate of the rats was due to faulty utilization of succinate because of inhibition of the succin oxidase system.

Methods

The toxic effects of continued oral administration of itaconic acid\(^1\) were studied by the drug-diet method. Groups of five female weanling rats (24 to 29 days old) from our own stock colony (4) were fed diets containing varying amounts of the acid for a period of 210 days. Control animals were fed the basal ration consisting, in percentage, of yellow corn-meal 73, linseed oil cake meal 10, alfalfa meal 2, crude casein 10, cod liver oil 3, bone ash 1.5, and sodium chloride 0.5. The experimental diets were prepared by thoroughly mixing the finely powdered itaconic acid with the basal ration. The experimental diets contained 0.125, 0.25, 0.5, 1.0, and 2.0 per cent itaconic acid. Each group of five rats was housed in a single cage and diet and water were available ad libitum. At weekly intervals the rats were weighed, the food consumption of each group was determined, and the animals were observed for any abnormalities. Autopsies were performed on rats surviving at the end of 210 days. Tissue sections stained with hematoxylin and eosin were examined.

The inhibitory effects of sodium itaconate in vitro were demonstrated by Warburg's "direct method." By means of the rat liver succinic dehydrogenase assay system of Schneider and Potter (5), experiments were designed to obtain the graphic representation of type of inhibition described by Ackermann and Potter (2). The data obtained in these experiments showed that curves relating the amount of enzyme to rate of oxidation are straight lines through the origin; the slope decreases with increasing concentration of inhibitor. Thus, competitive inhibition is indicated, as Ackermann and Potter (2) stated.

In addition, rat heart succinoxidase preparations were obtained essentially as described by Umbreit et al. (6). However, the muscle pulp was ground with sand in a few ml. of phosphate buffer just prior to use. After the addition of the total required amount of buffer, the sand and larger fragments were removed by low speed centrifugation of short duration. 2 ml. of this succinoxidase preparation were used per vessel. Both substrate and inhibitor were tipped in from the side arms at the same time after equilibration. Preliminary experiments indicated that earlier addition of the inhibitor did not modify the rate or degree of reaction. The temperature was 37.1°, the shaking rate 104 per minute, and the amplitude of shaking 3.5 cm. Readings were taken every 5 minutes for the first 75 minutes, dropping in frequency to every 15 to 20 minutes in the later stages of the reaction.

\(^1\) Obtained from the Northern Regional Research Laboratory, Peoria, Illinois.
To measure the effect of oral administration of itaconic acid on succinic acid excretion, two adult rabbits were used. Food and water were given ad libitum. 24 hour urine samples were collected in bottles containing 10 ml. of 20 per cent sulfuric acid as a preservative. After collection of a normal sample, 2 gm. of itaconic acid in 30 ml. of water were given by stomach tube. The amount is of the same order of magnitude on a weight basis as that ingested in a 24 hour period by the rats having reduced growth rates. No signs of toxicity were observed following this procedure.

The normal and the experimental urine samples were extracted continuously with ether for 13 hours. Shorter extraction periods failed to remove all of the succinic acid. Itaconic acid, which was extracted along with succinic acid from the experimental urine and which would interfere with the manometric determination of succinic acid, was destroyed in the extracts after evaporation of the ether by oxidation with potassium permanganate. This was done according to the method used by Krebs and Eggleston (7) to destroy malonic acid.

The oxidized residue was extracted with ether for 6½ hours, the ether was removed by evaporation, and the residue was brought to pH 7.0 to 7.4 with sodium hydroxide and phosphate buffer. After dilution to the proper range, a 1 ml. aliquot was taken for the manometric estimation of succinic acid. In these determinations, the technique for preparing the beef heart succinoxidase and the manometric procedure used were those described by Umbreit et al. (6).

Results

An inspection of Table I shows that in the chronic feeding experiments there was a definite decrease in growth rate as the intake of itaconic acid was increased, compared to the controls. With the exception of the groups receiving 0.125 and 0.25 per cent itaconic acid, the decrease in body weight was directly proportional to the increase of itaconic acid in the diet. A statistical evaluation of the average final body weights of the groups of rats receiving the supplements compared with the controls revealed that the reduced growth rates for the groups receiving 1.0 and 2.0 per cent itaconic acid were highly significant. Food consumption was quite uniform among the various groups; the rats exhibiting poor growth ingested about the same amount of food as did the controls. Postmortem examination for gross pathological changes did not reveal any specific abnormalities. Histological sections of the following tissues were made: heart, lung, liver, spleen, kidney, adrenal, pancreas, thyroid, ovary, and

2The histological preparations were interpreted by Dr. Alvin J. Cox, Jr., Department of Pathology, Stanford University School of Medicine, San Francisco, California.
ileum. Other than a few isolated inflammatory lung changes, no significant lesions were found.

TABLE I

<table>
<thead>
<tr>
<th>Diet</th>
<th>gm. per rat per day</th>
<th>Final body weight*</th>
<th>Significance of difference, P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.6</td>
<td>262 ± 4.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>0.125% itaconic acid</td>
<td>9.7</td>
<td>241 ± 5.4</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>0.25% &quot;</td>
<td>9.8</td>
<td>247 ± 6.5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>0.5% &quot;</td>
<td>10.0</td>
<td>237 ± 9.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>1.0% &quot;</td>
<td>9.8</td>
<td>224 ± 6.0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>2.0% &quot;</td>
<td>10.9</td>
<td>204 ± 3.7</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* Five rats per group, including the standard error, $\sqrt{\Sigma(d^2)/n(n-1)}$.

FIG. 1. Oxygen uptake of a rat heart succinoxidase preparation, in the presence of varying concentrations of sodium itaconate. The concentration of sodium succinate was 1.5 x 10^{-2} M. The molar concentrations of sodium itaconate appear on the curves. The arrow indicates the time at which substrate and inhibitor were tipped in from the side arms.

Since the concentration of itaconic acid in the diet had a marked effect on the growth of the rats, and since this effect could conceivably be attributed to an interference with succinate oxidation, a test was conducted
in vitro with increasing concentrations of sodium itaconate on the rat heart succinoxidase preparation. Representative curves are shown in Fig. 1. Increasing concentrations of itaconate decreased the rate of oxidation without modifying the final oxygen uptake, as is shown by the gradual approach of all curves to the level of total oxygen uptake occurring in the control vessel. After a 5 hour period of incubation, only the vessel containing the highest concentration of inhibitor had an oxygen uptake substantially less than the control. Total oxidation of succinate was observed, even when the molar concentration of the inhibitor was substantially greater than that of substrate. Fig. 1 shows that the oxidation of

**TABLE II**

*Succinic Acid Excreted by Rabbits in 24 Hour Periods before and after Oral Administration of 2 Gm. of Itaconic Acid*

<table>
<thead>
<tr>
<th></th>
<th>0-24 hrs.</th>
<th>24-48 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
</tr>
<tr>
<td>3.04</td>
<td>16.49</td>
<td></td>
</tr>
<tr>
<td>2.28</td>
<td>14.30</td>
<td>4.15</td>
</tr>
<tr>
<td>3.77</td>
<td>14.28</td>
<td></td>
</tr>
</tbody>
</table>
INHIBITORY EFFECTS OF ITACONIC ACID

succinate in the earlier stages is a linear function of time. Fig. 2 indicates that in this early stage of oxidation the oxygen consumption per unit of time decreases in a linear manner with the logarithmic increase in itaconate concentration.

The effect of the oral administration of itaconate on the succinic acid excretion in the rabbit is shown in Table II. Approximately 4 to 6 times as much succinic acid appears in the urine in the 24 hours after the administration of itaconate as in a 24 hour control urine. Krebs et al. (3) injected malonate into rabbits and obtained similar increases. Thus it appears that the inhibition of succinate oxidation demonstrated for the in vitro systems previously described also occurs in vivo when itaconic acid is administered orally. The formation of the enzyme-inhibitor complex impairs the utilization of the normal metabolite, resulting in excretion of this metabolite into the urine. Apparently the major portion of itaconic acid is removed within 24 hours, as indicated by the nearly normal amount of succinic acid excreted in the 24 to 48 hour urine collection. That the removal of itaconic acid is, at least in part, an excretion in the urine is shown by the markedly increased amount of permanganate required to oxidize the experimental samples as compared to the controls.

SUMMARY

Rats maintained on diets containing itaconic acid for 210 days showed no significant toxic effects other than restricted growth. In rats ingesting diets containing 1 or 2 per cent of this compound there was a statistically significant inhibition of growth, although the food intake was comparable to that of the controls.

The inhibitory effects of sodium itaconate in vitro on succinoxidase systems and the demonstrated increase in urinary excretion of succinic acid by adult rabbits following a large oral dose of itaconic acid suggest that impaired utilization of succinic acid was the cause of the decreased rate of growth.

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