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References and Notes

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Detection of a New Inhibitor of the Tricarboxylic Acid Cycle

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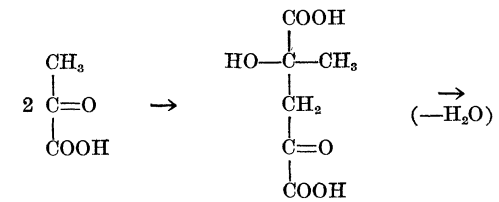
Comparison of different samples of sodium pyruvate (commercial and prepared in this laboratory) showed the presence of variable amounts of a substance that was correlated with an altered behavior of rat heart mitochondria in the oxidation of pyruvate (*1*). The mitochondrial suspension oxidized pure pyruvate completely when small amounts of malate were added, with an oxygen-to-pyruvate ratio of the theoretical value of 5, the tricarboxylic acid cycle being self-perpetuating and operating at an initial Q_{O_2} of between 800 and 1000. Each member of the cycle initiated the oxidation of pyruvate by acting as an oxalacetigen (*2*). However, using a pyruvate containing the impurity, a different pattern of metabolism was exhibited: (i) The cycle was no longer self-perpetuating and, for the utilization of all the pyruvate, an equimolar amount of malate was required; (ii) the oxygen-to-pyruvate ratio dropped to a value arund 3; (iii) the tricarboxylic acids (citrate, cis-aconitate, and isocitrate) no longer acted as oxalacetigens; and (iv) a ketonic substance accumulated as the pyruvate was utilized, the conversion being quantitative. The incorporation of pyruvate into the cycle was not affected by this impurity, as was indicated by the fact that no change was observed in the initial Q_{O_2}. The spectral absorption curve of the 2,4-dinitro-phenylhydrazone of the accumulated substance corresponded quite closely to that of the similar derivative of α-ketoglutarate, and the accumulated substance, therefore, was temporarily assumed to be α-ketoglutarate.

This impurity was found to be present in commercial pyruvic acid and in certain samples of sodium pyruvate prepared from triply redistilled pyruvic acid according to the standard procedure (*3*). Pyruvic acid, originally 99-percent pure, contained no more than 25 percent pyruvic acid after 1 to 2 years. When such pyruvic acid was redistilled under reduced pressure (10 mm-Hg), a fraction distilled over at a higher temperature (105° to 108°C) than the pyruvic acid fraction (55° to 58°C). The higher boiling fraction was a viscous fluid at room temperature from which a waxy crystalline mass slowly formed in the cold, and both liquid and solid material produced the same type of block in the cycle as was previously demonstrated with the impure samples of sodium pyruvate. Sodium pyruvate prepared from triply redistilled pyruvic acid sometimes contained the sodium salt of this impurity in sufficient amounts to produce marked cycle block. Depending on the purity of the original pyruvic acid and the conditions of crystallization of the sodium pyruvate, as much as 25 percent impurity was found in the final product.

A sample of sodium pyruvate containing the impurity was examined by paper chromatography, and it was found that the impurity migrated at approximately the same rate as the pyruvate, indicating that it was a monocarboxylic acid under these conditions. Pyruvate treated for 3 days with 1*N* hydrochloric acid inhibited the oxalacetigenic action of citrate in the mitochondrial preparation 65 percent. It was concluded that the impurity was formed from pyruvic acid. It is known that pyruvic acid is capable of slowly forming a dimer (γ-methyl-γ-hydroxy-α-ketoglutaric acid, I) and that this dimer may lose water to form the cyclic α-keto-γ-valerolactone-γ-carboxylic acid (II). The lactone, furthermore, is believed to exist in a keto-enol equilibrium (II, III). It was considered that



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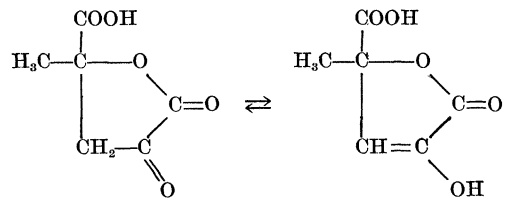

II III

any one of these substances might be involved in the mitochondrial block observed. We therefore prepared the lactone by a method (*4*) in which hydrochloric acid gas was passed for 9 days through a long column of pure, triply redistilled pyruvic acid; the final viscous liquid was allowed to stand over concentrated sulfuric acid in vacuum for 3 days, and a mass of waxy, highly hygroscopic, crystalline material was obtained. The prepared lactone was found to be a potent blocker of the tricarboxylic acid cycle in rat heart mitochondria and, when added with pure pyruvate, produced all the phenomena described here, namely, the failure of the cycle to be self-perpetuating, the inability of the tricarboxylic acids to act as oxalacetigens, and the accumulation of a substance temporarily identified as α-ketoglutarate. The inhibition of the ability of citrate and isocitrate to function as oxal-

acetigens was pronounced at concentrations of lactone as low as $1 \times 10^{-5} M$. The time curves of oxygen uptake during pyruvate oxidation illustrated the blocking action (which prevents the self-perpetuation of the cycle) of the impurity and the prepared lactone; the oxidation of pure pyruvate proceeded at a fairly steady level until completion, whereas with impure pyruvate or in the presence of the lactone the rate suddenly fell to a low level when the small quantities of malate had been utilized. Neither the oxidation nor the oxalacetogenic ability of succinate was inhibited by the lactone.

These results might be interpreted to mean that this potent inhibitor blocked some reaction between isocitrate and the α-ketoglutarate-succinate step in the cycle. The inhibition did not seem to be on the isocitric dehydrogenase, since there was good oxidation of isocitrate in the presence of impure pyruvate or the lactone, although no oxalacetate was formed and no pyruvate was oxidized. A photometric determination of TPN reduction by isocitrate as catalyzed by isocitric dehydrogenase in an acetone powder of rat heart mitochondria (*5*) showed that the lactone had no inhibitory effect in concentrations up to 5 mM.

Essentially complete conversion of 5 mM pyruvate to α-ketoglutarate was obtained when 1 mM lactone was present, and equally good conversion was seen from isocitrate. Since these results would point to a block on the α-ketoglutarate oxidase, we examined the effect of the lactone on this enzyme isolated from pig heart (*6*) by one of us (C.M.M.) with the help of D. R. Sanadi at the Institute for Enzyme Research, Madison. When α-ketoglutarate and the lactone were present in equimolar concentration, inhibition of 50 percent was observed. This inhibition would seem to be competitive, since increasing the concentration of the α-ketoglutarate reduced the inhibition. The oxalacetigenic ability of α-ketoglutarate in the mitochondria was inhibited even more potently than the oxidase preparation. The block in the cycle would, therefore, seem to be due to this competitive inhibition of the α-ketoglutarate oxidase. Blocking of the initiation of pyruvate oxidation by citrate or isocitrate, or of the normally operating cycle, would be much more complete owing to the low concentrations of α-ketoglutarate present under these circumstances.

Titration of the lactone demonstrated two acidic groups with $pK_1 = 2.35$ and $pK_2 = 6.95$. The first dissociation constant refers to the normal free carboxyl group, but pK_2 is too high for a carboxyl group and would argue strongly against the presence of the straight-chain dimer (where pK_2 would be expected to be between 4.5 and 5.0). Cleavage of the lactone ring near neutrality is unlikely, since rapid back titration showed identical values for the pK's and it is doubtful that ring closure would occur rapidly under these conditions. There remained the possibility that enolization takes place and that form III is prevalent around neutrality, the pK_2 referring to the enolic hydroxyl group whose acidic strength is increased by the surrounding groups and chelation with cations. Titration of diethyl-oxalacetate, which possesses a similar —CO—CO—CH_2— grouping showed that the pK of the enolic hydroxyl here was 7.6. Thus we assume that the inhibitor is present in aqueous solution as the lactone and around neutrality in the enolic form (III). Permanganate reduction indicated an active double bond in the prepared lactone at neutrality, again suggesting the enolic form.

Preliminary work on liver and kidney mitochondria demonstrated that the cycle is not blocked so readily by the lactone as in heart mitochondria. We are at present attempting to purify the lactone and related substances, investigating the more intimate mechanism of the inhibition, as well as studying the action of the inhibitor on the functional activity of rat cardiac muscle.

References and Notes

1. This investigation was supported by grants from the National Heart Institute, National Institutes of Health (H-1391C), and the Life Insurance Medical Research Fund, and aided by facilities supplied by the Allan Hancock Foundation.
2. The term *oxalacetigen* is used to designate a substance that, in the presence of the mitochondrial preparation, will form oxalacetate with which pyruvate may condense to enter the tricarboxylic acid cycle.
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Carbon Dioxide Utilization by Rabbit Liver

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During the investigation of labeled carbon dioxide fixation into the organic acids of liver homogenates, it was observed that such incorporation of the isotope in the liver of the rabbit differed from that of other species examined (*1–5*). Fixation was evident within the times described into a component of the acetone extracts insoluble in the solvent that readily dissolves the organic acids. Unlike any other species examined, the extent of the incorporation into the insoluble compound was as great and at times greater than that into such acids as succinic, fumaric, and malic.

Liver tissue from three adult rabbits was incubated with $NaHC^{14}O_3$ (*6*) under the conditions previously described (*5*). Incubations were for 10 min except where indicated. Acetone extracts were prepared. The chromatography and radioactive assays have been outlined in other experiments (*3, 5*). The paper chromatographic separations were those of Denison and Phares (*7*) and Benson *et al.* (*8*) and the tests for sugars followed the procedures of Dische (*9*).

The data of Table 1 indicate that the fixation of the