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THE METABOLISM OF VOLATILE FATTY ACIDS WITH RUMEN EPITHELIUM

IV. ON THE MODE OF PRESENCE OF T.C.A. CYCLE IN RUMEN EPITHELIUM

By

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Introduction

It is well known that aerobic cell is provided with a universal enzyme system called T.C.A. Cycle in which pyruvate is decomposed into CO_2 and H_2O and the cell obtains energy by conjugating with oxidative phosphoryration (1). It has been understood that the T.C.A. Cycle is related with the metabolism of fatty acids as one of the terminal pathways (2). As already reported on the rumen epithelium, the metabolism of propionate was related closely with T.C.A. Cycle, but the metabolisms of acetate and butyrate had no great relation with T.C.A. Cycle (3-5). In particular, as CO_2 formation from butyrate received no inhibition with malonate, the result led to that the complete oxidation of butyrate took place without the participation of a system to which malonate acted as an inhibitory agent (6, 7).

In view of that the ketone body is not formed from glucose, though it is formed from succinate and other materials belonged to T.C.A. Cycle, it could be said that the relation between the metabolic system of glucose and T.C.A. Cycle also has not been made clear (8).

Therefore, the investigation was made on the mode of the presence of T.C.A. Cycle in the rumen epithelium.

Methods and Materials

Tissues were obtained from cattle killed at the slaughter house. They were immediately put into a cold 1.15 per cent KCl solution. The experiments were begun within three hours after slaughtering.

Rumen epithelium could be separated easily from the muscle layer. One

gram (wet weight) of epithelium was put into an incubation flask, in which 10 ml of Krebs-Ringer phosphate buffer, pH 7.2, and various substrates were contained. The flask, after the contained solution was saturated with oxygen, was shaken in a water bath for three hours at 38°C. After incubation, the total ketone bodies, namely β-hydroxybutyrate, acetoacetate and acetone, were determined by Thin and Robertson's method (9). Volatile fatty acids and citrate were determined by Conway's diffusion method and Tahara's method respectively (10, 11). Oxygen uptake was measured by the Warburg manometer with the usual method (7).

Results and Discussion

1. Fluoroacetate inhibition in the rumen epithelium.

The condensation reaction of acetyl-CoA and oxalacetate is the first reaction for turning the T.C.A. Cycle. A precursor of acetyl-CoA and oxalacetate are, therefore, necessary to form citrate for rotating the T.C.A. Cycle. It is said that fluoroacetate, which does not act on the individual isolated enzyme but on multiple enzyme system in which fluoroacetate converted to fluorocitrate, inhibits the rotation of T.C.A. Cycle by working on aconitase as a competitive inhibitor and bring about the citrate accumulation (12, 13). Another work showed that fluoroacetate, the same as acetate, activated by the activation system, is condensed with citrate and exhibits an inhibitory action due to the formation of fluorocitrate in T.C.A. Cycle. Therefore, the accumulation of citrate in T.C.A. Cycle or inhibition of respiration in overall reaction caused by the addition of fluoroacetate means that three systems, activation system of acetate, condensation system in T.C.A. Cycle and respiration system to which aconitase belongs, couple with one other. In brief, these mentioned phenomena prove that T.C.A. Cycle acts as a terminal pathway of the respiration system in the cells.

Table 1. The effects of fluoroacetate on the citrate formation from pyruvate in rumen epithelium.

Additions	Citrate formed
—	0.7μM
Pyruvate	1.4
Oxalacetate	0.9
Fluoroacetate	4.5
Pyruvate + Fluoroacetate	5.0
Oxalacetate + Fluoroacetate	5.2
Pyruvate + Oxalacetate	3.1
Pyruvate + Oxalacetate + Fluoroacetate	6.3

One g of tissues were incubated in 10 ml of Krebs-Ringer phosphate buffer containing 100μM of pyruvate or 100μM of oxalacetate. Fluoro-acetate concentration was 0.025M/l.

First, investigation was made on pyruvate as the precursor of acetyl-CoA. The experimental results are shown in Table 1.

As the result, the addition of oxalacetate to pyruvate increased the formation of citrate a little. By the addition of fluoroacetate, a marked accumulation of citrate was seen even in the case of each substrate. Especially, the coexistence of pyruvate and oxalacetate showed the maximum accumulation of citrate.

Table 2. The effects of fluoroacetate on the citrate formation from acetate in rumen epithelium.

Additions	Citrate formed
—	0.3μM
Oxalacetate	1.0
Acetate	0.9
Fluoroacetate	5.4
Acetate + Fluoroacetate	5.8
Oxalacetate + Fluoroacetate	6.3
Acetate + Oxalacetate	1.6
Acetate + Oxalacetate + Fluoroacetate	7.0

One g of tissues were incubated in 10 ml of Krebs-Ringer phosphate buffer containing 100 μM of each substrate. Fluoroacetate concentration was 0.025 M/l.

As shown in Table 2, the application of acetate as the precursor of acetyl-CoA increased the formation of citrate a little, and a considerable amount of ctirate was accumulated with fluoroacetate inhibition.

Such a considerable accumulation of citrate with fluoroacetate inhibition signifies the presence of both donating reaction system to acetyl-CoA and the condensing reaction system of oxalacetate and acetyl-CoA in the rumen epithelium. As these systems are the basic ones for T.C.A. Cycle, the existence of them proves the presence of T.C.A. Cycle in the rumen epithelium.

2. *Malonate inhibition in the rumen epithelium.*

Malonate which was discovered as the inhibitor of succinoxidase is a well known inhibitor first used by Krebs for proving T.C.A. Cycle (14–16). In the rumen epithelium, malonate scarcely inhibited the consumption of acetate and the formation of ketone body from acetate, with a slight decrease of oxygen uptake, although the metabolism of propionate was inhibited considerably. The metabolism of butyrate did not receive any effect of malonate inhibition (5–7).

In this respect, experiments were made on the malonate inhibition to investigate the mode of presence of T.C.A. Cycle in the rumen epithelium.

With respect to the increases of oxygen uptake in the rumen epithelium by the additions of malate, citrate and succinate, which all belong to the members of T.C.A. Cycle, an investigation was made to determine the participation of the malonate inhibition system in the respiratory system of rumen epithelium.

The experimental results are shown in Tables 3, 4, and 5.

The increased oxygen uptakes caused by the addition of citrate, succinate and malate were remarkably decreased with the coexistence of malonate.

Table 3. The effect of malonate inhibition on the oxidation of citrate in rumen epithelium.

Additions	Oxygen uptake
—	51μl
Malonate	25
Citrate	92
Citrate + Malonate	46

Fifteen hundred mg of tissues were incubated in 2 *ml* of Krebs-Ringer phosphate buffer containing 10 μM of citrate. Malonate concentration was 0.025 *M/l.*

Table 4. The effect of malonate inhibition on the oxidation of succinate in rumen epithelium.

Additions	Oxygen uptake
—	81μl
Malonate	40
Succinate	142
Succinate + Malonate	37

Fifteen hundred mg of tissues were incubated in 2 *ml* of Krebs-Ringer phosphate buffer containing 10μM of succinate. Malonate concentration was 0.025 *M/l.*

Table 5. The effect of malonate inhibition on the malate oxidation in rumen epithelium.

Additions	Oxygen uptake
—	97μl
Malonate	51
Malate	133
Malate + Malonate	51

Fifteen hundred mg of tissues were incubated in 2 *ml* of Krebs-Ringer phosphate buffer containing 10μM of malate. Malonate concentration was 0.025 *M/l.*

As shown in Tables 6 and 7, the oxidation of glucose and pyruvate received a considerable malonate inhibition.

A drop of oxygen uptake of the epithelium caused by malonate inhibition when incubated with glucose, pyruvate, or members of T.C.A. Cycle, may indicate the participation of a system which was inhibited by malonate, probably T.C.A. Cycle, in the metabolisms of glucose, pyruvate and members of T.C.A. Cycle.

In the next experiment, whether this malonate inhibition combines with the succinate → fumarate system in the sequence of T.C.A. Cycle was examined.

Table 6. The effect of malonate inhibition on the oxidation of glucose with rumen epithelium.

Additions	Oxygen uptake
—	94μl
Malonate	56
Glucose	248
Glucose + Malonate	97

Fifteen hundred mg of tissues were incubated in 2 *ml* of Krebs-Ringer phosphate buffer containing 10 μM of glucose. Malonate concentration was 0.025 *M/l.*

Table 7. The effect of malonate inhibition on the oxidation of pyruvate with rumen epithelium.

Additions	Oxygen uptake
—	70μl
Malonate	31
Pyruvate	92
Pyruvate + Malonate	85

Fifteen hundred mg of tissues were incubated in 2 *ml* of Krebs-Ringer phosphate buffer containing 10μM of pyruvate. Malonate concentration was 0.025 *M/l.*

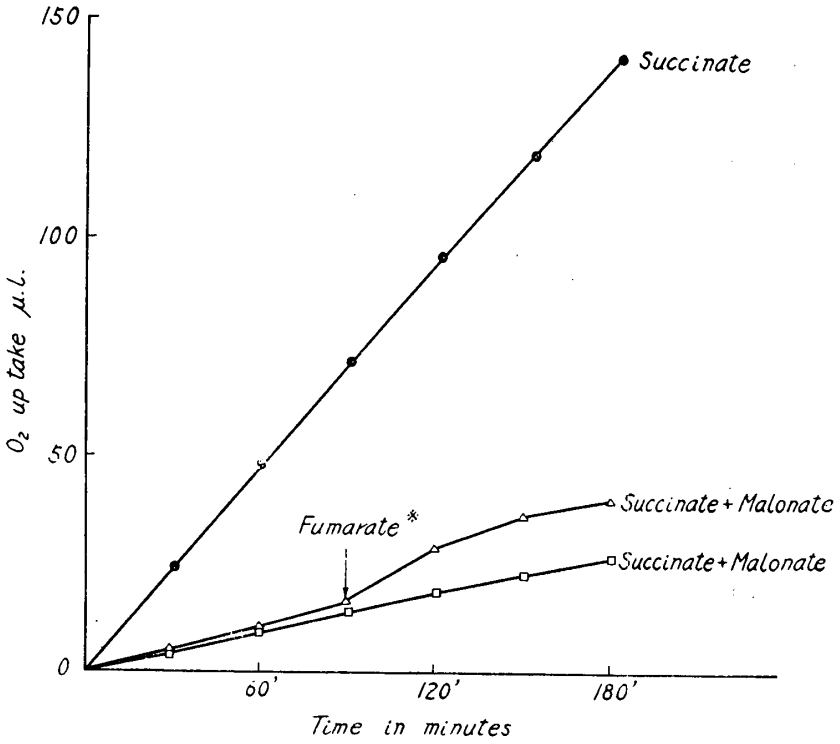

Fig. 1. Succinate → Fumarate system in rumen epithelium. 150mg of tissues were incubated in 2*ml* of Krebs-Ringer phosphate buffer containing 10μM of succinate.
※ Ten μM fumarate was added.
Malonate concentration was 0.025*M/l.*

During the period of the incubation of rumen epithelium with succinate as substrate and malonate as inhibitor, fumarate was added to the solution from the side arm of incubation flask. The experimental result is shown in Fig. 1.

As the result, the oxygen uptake of succinate inhibited with malonate was fairly recovered with the addition of fumarate. This may indicate that malonate inhibits the succinate → fumarate system in the series of T.C.A. Cycle.

Accordingly, it can be concluded that the succinate → fumarate system participate in the oxygen uptake system in the rumen epithelium when malate, citrate, succinate, glucose, pyruvate or propionate, each of them is inhibited its metabolism by malonate, is used as a substrate.

As mentioned already, it is known that succinate itself forms the ketone body and accelerates the ketone body formation from butyrate. In consideration of the significance on the process of ketone body formation from succinate, investigation was made on the effect of malonate on the ketone body production from succinate. The experimental result is shown in Table 8.

Table 8. The effects of malonate on the ketone body formation from succinate with rumen epithelium.

Additions	Butyrate used (A)	Ketone body formed (B)	(B)/(A) ×100
—	(1.7)μM	1.0μM	—%
Succinate	(2.1)	2.0	—
Butyrate	19.7	7.7	39.1
Malonate	(1.8)	1.4	—
Succinate + Malonate	(1.5)	1.0	—
Butyrate + Malonate	19.4	7.8	40.4
Butyrate + Succinate	22.3	12.2	54.8
Butyrate + Succinate + Malonate	20.1	7.8	38.8

The result showed that the ketone body formation from succinate was inhibited with malonate. Further, the accelerating action of succinate for the ketone body formation from butyrate disappeared with the addition of malonate. It is assumed that succinate is oxidized to give malate through succinate → fumarate system on which malonate effects as inhibitor and resulted malate forms ketone body *via* pyruvate. To prove this assumption, the following experiments were carried out.

Table 9. The effect of malonate inhibition on the ketone body formation from fumarate with rumen epithelium.

Additions	Ketone body formed
—	0.9μM
Malonate	1.2
Fumarate	1.8
Fumarate + Malonate	2.9

One g of tissues were incubated in 10ml of Krebs-Ringer phosphate buffer containing 100μM of fumarate. Malonate concentration was 0.025M/l.

As shown in Table 9, the amount of ketone body formed from fumarate did not decrease but rather increased with malonate inhibition. In other words, the fact that malonate inhibited the ketone body formation from succinate and did not inhibit its formation from fumarate signifies that the process of ketone body formation from succinate may pass through the succinate → fumarate system which malonate cut its pathway.

Table 10. The effect of malonate inhibition on the ketone body formation from malate with rumen epithelium.

Additions	Ketone body formed
—	1.21μM
Malate	2.31
Malonate	1.28
Malate + Malonate	3.75

One g of tissues were incubated in 10*ml* of Krebs-Ringer phosphate buffer containing 100μM of malate. Malonate concentration was 0.025*M/l.*

As shown in Table 10, the ketone body formation from malate did not decrease but increased with the addition of malonate. This was the same as in the case of pyruvate as shown in Table 11.

Table 11. The effect of malonate inhibition on the ketone body formation from pyruvate with rumen epithelium.

Additions	Ketone body formed	Lactate formed
—	1.3μM	1.8μM
Malonate	1.6	2.1
Pyruvate	3.1	10.6
Pyruvate + Malonate	4.5	7.2

One g of tissues were incubated in 10*ml* of Krebs-Ringer phosphate buffer containing 100μM of pyruvate. Malonate concentration was 0.025*M/l.*

In case of glucose, as shown in Table 12, ketone body formation increased with the addition of malonate. At that time, the consumption of glucose did not decrease greatly.

Table 12. The effect of malonate inhibition on the glucose metabolism with rumen epithelium.

Additions	Glucose consumed	Ketone body formed	Acetate formed
—	(1.2)μM	0.7μM	2.4μM
Malonate	(0.9)	1.1	2.2
Glucose	14.7	0.3	5.7
Glucose + Malonate	13.8	2.8	5.0

One g of tissues were incubated in 10*ml* of Krebs-Ringer phosphate buffer containing 100μM of glucose. Malonate concentration was 0.025*M/l.*

From the facts above mentioned, it is assumed that succinate is converted to pyruvate passing through the succinate → fumarate system and thus formed pyruvate gives rise to the formation of ketone body through the acetyl-CoA system.

The following two facts that the process of ketone body formation from pyruvate is almost the same as that from acetate and that small amounts of volatile fatty acids are detected during the period of the incubation of pyruvate lead to the assumption that acetate metabolizing system may participate with the ketone body formation system from pyruvate.

Summary

The mode of presence of T.C.A. Cycle in the rumen epithelium as the terminal metabolic pathway was confirmed.

1. The accumulation of citrate was found with fluoroacetate inhibition.
2. The oxygen uptake of the rumen epithelium was increased by the addition of the members of T.C.A. Cycle such as succinate and its increase was inhibited by malonate.
3. When succinate was used as the substrate, the inhibited oxidation with malonate was recovered with the addition of fumarate.

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