154. On the Mechanism of Thiamine Action. V. Intermediate Metabolism of Pyruvate.

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On the way in intermediate metabolism of pyruvate in what manner citrate will be made is as yet unknown. According to the studies by Green et al.¹⁾ and Mizuhara et al.²⁾ not only carboxylase but also free thiamine is capable of converting pyruvate into acetoin and carbonic acid. But by many other investigators it is considered that acetoin is only a byproduct in the pyruvate metabolism.

The diacetyl, by dehydration derived from acetoin, is then converted into acetic acid not only by Diacetylmutase³⁾ but also by free thiamine⁴⁾ and from the electronic theory, ketene might be formed as an activated intermediate product on the way from diacetyl to acetate. But according to the new investigation of Novelli and Lipmann⁵⁾ citrate can be synthesized from these acetylphosphate and oxalacetate derived from pyruvic acid and carbonic acid by a co-enzyme A and these reactions form the so-called tri-carboxylic acid cycle as follows. Out of these data, the following intermediate steps may be deduced:

To confirm the above described formula, pyruvate, diacetyl, acetate and oxalacetate were administered either with thiamine or alone to normal rabbits, and pyruvate, acetoin, acetate and citrate in the urine were estimated.

Methods.

Sodium pyruvate and oxaloacetic acid were prepared by the methods described in Manometric Techniques and Tissue Metabolism

(1949)⁶⁾. Diacetyl was prepared by Pechmann's⁷⁾ method. Acetoin was determined by Westerfeld's method⁸⁾ after distilling the urine after adding of iron chloride, and acetic acid colorimetrically by Oono's method⁹⁾, and citric acid by Krebs's method¹⁰⁾. Before the administration of these metabolites, these components of the normal urine were measured for two days as a control.

Experimental.

(I) The Influence of Thiamine upon the Intermediate Metabolism of Pyruvate.—The intravenous administration of thiamine 15 mg to a normal rabbit increases the amounts of acetoin and pyruvate, but decreases that of citrate in the urine. Data is shown in Table I(1).

On the contrary the injection of small amounts of thiamine increases the acetic acid in the urine as the data are shown in Table I(1). But other components in the urine such as pyruvic acid, acetoin and citrate remained by the administration of thiamine uninfluenced or slightly decreased.

Akabori¹¹⁾ demonstrated that the oxidation of acetoin to diacetyl will be carried out by the catalytic action of Lactoflavin. In the first case, see Table I(1), it has been occurred the increased excretion of acetoin by thiamine. Hence in the rabbit body it must be a relative hypovitaminosis of the flavin enzyme. The increased excretion of pyruvate by administration of thiamine may be due to the affinity between thiamine and pyruvate. It is already well known that free thiamine, when injected, is excreted very quickly in the urine. Hence it is possible that pyruvate, metabolite of sugar, will be excreted with the injected thiamine. In the second case (Table I(1)), the excretion of relatively small amounts of acetoin in the urine must be due to the increased oxidation of acetoin to diacetyl and diacetyl to acetic acid by the thiamine. Consequently a large quantity of acetic acid is excreted in the urine. From these facts it may be probably concluded that thiamine enzyme catalyzes the following two reactions in vitro;

The increased excretion of acetoin on the second day after the thiamine administration indicates that the free thiamine at first must be changed into an enzyme carboxylase before its activity in vitro will be produced.

(II) The Break Down of Pyruvate in the Animal Body.—The intravenous injection of sodium pyruvate 10 mg. per Kg. body

weight of rabbit causes the increased excretion of acetoin in the urine on the following day after injection. The data is shown in Table I(2).

By the injection of sodium pyruvate together with a small amount of thiamine large amounts of acetoin are excreted for three days after injection, but the excretion of acetic acid is increased for the first time after the excretion of acetoin tends to decrease (Table I(3)).

From these datas, it can be understood that the pyruvate is at first converted to acetoin and then this acetoin to acetic acid by the thiamine enzyme as above mentioned.

(III) The Break Down of Diacetyl in the Animal Body.—The injection of diacetyl into a rabbit causes the increased excretion of acetoin and acetic acid in the urine. The data is shown in Table I(4).

The following reaction, as indicated by Green and confirmed by Mizuhara et al. by using free thiamine, must hence take place in the animal body.

At the administration of diacetyl together with oxalacetate is increased the acetoin excretion in the urine, but acetic acid excretion rather decreased. The data is shown in Table I(5).

These break down of diacetyl in the rabbit body is stimulated by the presence of oxalacetate. This fact suggests the formation of citrate will take place by the condensation of oxalacetate and diacetyl or its decomposition product (ketene?), and the excretion of citrate in the urine must be increased. But on the contrary the amount of citric acid in the urine remains always constant, even when citrate is administered. It is well known that the excretion of citrate in the urine is influenced only by the acid-base equilibrium in the blood. Therefore the amount of citrate in the urine is not a quite reliable indicator in citrate metabolism.

(IV) The Excretion of Acetate in the Urine.—When sodium acetate is injected, the acetoin is increased in the urine, but the acetate has the tendency to decrease. Perhaps the splitting of diacetyl in animal body could be depressed, if the concentration of acetic acid in the blood and the excretion of acetoin in the urine to a certain extent are increased. The data is shown in Table I(6).

At the injection of sodium acetate together with oxalacetate a similar tendency to decrease in the urinary excretion of acetate

is observed, as shown in Table I(7). Hence the citrate formation from acetate and oxalacetate appears very doubtful. Perhaps the citric acid may be formed from the oxalacetate and ketene produced from diacetyl by the action of the thiamine enzyme. The data is shown in Table I(7).

(V) The Influence of Citrate upon its Urinary Excretion.—As shown in the 9 No (1-9) Table I below presented, the amount of citrate excreted remains always constant. In this case, however, the citrate was intravenously injected, and its amount excreted in the urine were estimated. As shown in Table I(8) the injection of citrate causes the increased excretion of pyruvate, acetoin and acetate in the urine. These datas indicate probably that the splitting of the intermediate metabolite especially diacetyl is depressed by the high concentration of citrate in the blood.

(VI) The Influence of Oxalacetate upon the Intermediate Metabolite of Pyruvate in the Urine.—When oxalacetate solution, three fourths neutralizing with potassium carbonate solution, is injected, the acetic acid in the urine decreased, but another urinary constituents remains unchanged.

Hence it may be deduced that oxalacetate is indispensable for splitting the diacetyl or acetate in the animal body.

The data is shown in Table I(9).

Table I.

Administered substances	Excreted substances in urine				
		Pyruvate	Acetoin	Acetate	Citrate
(1) B ₁ 15 mg. (7 mg./Kg. Body weight) 4.5 mg. (2 mg./Kg.)		+	+	±	—
		±	±	+	±
(2) Pyruvate (10 mg./Kg.)		—	+	±	±
(3) Pyruvate (10 mg./Kg.) + B ₁ (1 mg./Kg.)		±	+	+	±
(4) Diacetyl (10 mg./Kg.)		±	+	+	±
(5) Diacetyl (10 mg./Kg.) + Oxalacetate (10 mg./Kg.)		±	±	—	±
(6) Na-Acetate (10 mg./Kg.)		±	+	—	±
(7) Na-Acetate (10 mg./Kg.) + Oxalacetate (10 mg./Kg.)		±	+	—	±
(8) Na-Citrate (20 mg./Kg.)		+	+	+	±
(9) Oxalacetate (10 mg./Kg.)		±	±	—	±

+ Increase — Decrease ± Unchanged

Discussion.

From the studies by Green and by Mizuhara, the formula of reaction as to the intermediate metabolism of pyruvate may be shown as follows:

In 1939, the equation (9) has already been presented by Lipmann without its explanation. From the electronic point of view ¹²⁾ ketene has been postulated to be an activated two carbon fragments ($\text{CH}_3 \cdot \text{CO} \cdot \text{X}$). Then it is convenient to elucidate the formation of the intermediate products in pyruvate metabolism; citrate, acetate, acetylphosphate and acetoacetate.

Summary.

(1) The excretion of acetoin and acetic acid in the urine of normal rabbits is increased by the administration of thiamine.

(2) When pyruvate is injected into normal rabbits, acetoin in the urine for a while is increased.

(3) The injection of diacetyl increases the urinary excretion of acetic acid and acetoin, but that of diacetyl together with oxalacetate decreases the urinary excretion of acetate.

(4) The injection of acetic acid increases the urinary excretion of acetoin for a time.

(5) Injection of citrate causes the increased excretion of acetate, acetoin, and pyruvate in the urine, but citrate excretion remains always unaltered.

(6) Acetate excretion in the urine is decreased by the injection of oxalacetate.

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