CCCXV. THE EFFECT OF FUMARATE ON THE RESPIRATION OF LIVER AND KIDNEY TISSUE.

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THE interesting theory of Szent-Györgyi and co-workers [Annau *et al.*, 1935] that in respiration the system fumarate-oxaloacetate functions catalytically in transporting activated metabolite hydrogen to the cytochrome (Warburg-Keilin) system has received support in the experiments of Stare & Baumann [1936]. Using the manometric method with pigeon breast muscle and heart muscle (swine), the latter investigators observed the following:

"1. The oxygen uptake of tissues is increased by very small amounts of fumarate and the increase is much greater than can be accounted for by the oxidation of the fumarate itself.

2. Small amounts of added fumarate are detectable after prolonged contact with respiring tissue.

3. The increased respiration is normal, that is, the respiratory quotient is not materially changed by the addition of small amounts of fumarate.

4. Substances which yield fumarate on contact with tissue—succinate, malate, oxaloacetate—show a similar action to fumarate itself."

Recently Szent-Györgyi and co-workers [Annau *et al.*, 1936] have applied quantitative methods for the micro-estimation of oxaloacetic, pyruvic, and malic acids and have given additional evidence in support of their theory. They followed chemically the disappearance of oxaloacetic acid added to muscle and observed a simultaneous increase in malic acid, with only a very small increase in pyruvic acid. They are of the opinion that most of this pyruvic acid comes from the oxidation of a triose or triosephosphate and not from the decarboxylation of the added oxaloacetate. They have also shown chemically that oxaloacetate is formed in muscle tissue from added fumarate. These findings are of fundamental importance since they give chemical indication of the mechanism of the catalytic action of fumarate and related compounds in respiration.

The writer had the opportunity of spending some time in Prof. Szent-Györgyi's laboratory and of repeating and confirming the basic chemical estimations recently given. It is the object of this paper to report on manometric and chemical experiments which show the applicability of the fumarate-oxaloacetate catalytic theory to the respiration of the liver and kidney tissue of the rabbit.

Methods.

The measurements of oxygen consumption were made in Warburg manometers at a temperature of 37° and in a Ringer-phosphate buffer. As previously shown [Stare & Baumann, 1936] this solution is a more satisfactory medium than plain phosphate buffer to demonstrate the activity of fumarate and related compounds.

The rabbits were killed by decapitation. The liver and kidneys were immediately removed, washed briefly in ice cold water, ground in an ice-cold

mincer, and suspended in ice-cold Ringer-phosphate buffer in the concentration of 1 g. of tissue to 3 ml. of buffer. 2 ml. of this suspension were pipetted into each Warburg vessel. The volume of the fluid in the respiration vessel was always 4 ml.

For the chemical estimations the tissue suspension was pipetted into small Erlenmeyer flasks and diluted with water and various supplements to the same concentration as used in the respiration vessels. These flasks were agitated in a bath at 37° for periods from 0 to 20 min. and the reactions stopped by the addition of tungstic acid. The contents were then centrifuged and the clear protein-free liquid used for the chemical estimations. The details of the various chemical estimations are given by Straub [Annau *et al.*, 1936]. It should be mentioned that the method of estimating pyruvic acid is affected by acetone. Using the nitroprusside test acetone was estimated and its formation observed to be negligible during the short time of these experiments.

Results and discussion.

Figs. 1 and 2 show the effects of fumarate and malonate on the respiration of liver and kidney tissue. They are representative experiments from a total number of eight for each tissue. The general effects previously described with

Fig. 1. The effects of fumarate and of malonate on the respiration of rabbit liver. (Ringer-phosphate buffer—concentrations of fumarate and of malonate expressed as mg. added to 4 ml. volume in Warburg vessel.)

Fig. 2. The effects of fumarate and of malonate on the respiration of rabbit kidney. (Ringer-phosphate buffer—concentrations of fumarate and of malonate expressed as mg. added to 4 ml. volume in Warburg vessel.)

muscle tissue are observed, malonate inhibits respiration, fumarate “preserves” it, and when both are added the malonate inhibition is overcome and the tissue appears to respire normally. Whether or not fumarate plus malonate shows the same conserving action as fumarate alone depends upon the intensity

of the respiration, the duration of the experiment, and the absolute amounts of fumarate and malonate present.

Malonate is known to poison succinic dehydrogenase. As Szent-Györgyi has shown [Annau *et al.*, 1936] a small part of the oxaloacetic acid is reduced to succinate. In the presence of malonate this succinate is not oxidized to fumarate, and respiration is inhibited owing to a lack of essential fumaric acid. The "time" at which malonate inhibits respiration, therefore, depends upon when the available fumaric acid has been converted into succinate. This is dependent upon the intensity of the respiration, the duration of the respiration and the absolute amounts of fumaric and malonic acids present within the cell.

From Figs. 1 and 2 it is observed that without added fumarate, malonate inhibits within the first 5 min. of the experiment, showing that by that time the normal fumarate content is converted into succinate. When 1 mg. each of fumarate and malonate are added respiration proceeds normally for the first 15–20 min. By this time malonate inhibition is observed and it is probable that the added fumarate which has penetrated the cell has been inactivated by conversion into succinate. With larger amounts of fumarate a longer time is required for its conversion into succinate and the consequent appearance of malonate inhibition. Without added malonate, the conserving action of fumarate may be shown within such wide limits as 0.0001 to 0.02 *M*.

The extent of malonate inhibition is dependent to some extent on the amount added. In the experiments reported, a concentration of 1 mg. in 4 ml. was used and an inhibition of 25–30% obtained. If the concentration is increased to 4 mg. in 4 ml. approximately 50% inhibition is observed. This is probably a result of maintaining a higher concentration of malonate within the cell and thus more effectively blocking the succinic dehydrogenase.

The total concentration of the various dicarboxylic acids must not be too high or a general non-specific inhibition of respiration is obtained after the first 30–40 min. This appears to apply particularly to kidney tissue which seems to be more sensitive to such changes.

Oxaloacetate is very rapidly reduced by tissue and hence its concentration in the tissue is always low. Yet its formation can be demonstrated by the hydrazine fixation method as described by Banga [Annau *et al.*, 1936]. That liver and kidney tissue form oxaloacetate from fumarate was shown by the following experiment. To a small Erlenmeyer flask were added 2 ml. of the tissue suspension, 0.2 ml. of a saturated As_2O_3 solution, 5 mg. of fumarate, 10 mg. of hydrazine hydrochloride and water to a volume of 4 ml. The mixture was shaken in a bath at 37° for 15 min., and then 0.5 ml. each of 10% H_2SO_4 and 10% Na_2WO_4 were added. The contents were centrifuged and oxaloacetate was estimated in the filtrate. In terms of the fumarate added there was an increase of approximately 15% in oxaloacetate. Without added fumarate or hydrazine no oxaloacetate is detectable.

According to Szent-Györgyi [Annau *et al.*, 1935] oxaloacetic acid is reduced by metabolite hydrogen to fumarate. Krebs [1936] has also suggested that oxaloacetic acid may be reduced in the oxidation of pyruvic acid. Recently Laki [Annau *et al.*, 1936] has shown by micro-chemical estimation that oxaloacetic acid added to pigeon breast muscle rapidly disappears and that there is a simultaneous increase in the amount of the fumaric-malic equilibrium mixture. That these considerations likewise apply to liver and kidney tissue is shown in the following experiment. Into Erlenmeyer flasks were placed 10 ml. of the tissue suspension, 50 mg. oxaloacetic acid and water to 21 ml. volume. The flasks were shaken in a bath at 37° for periods of 0, 2, 5, 10 and 15 min. and at the end of the

periods 2 ml. each of 10 % H_2SO_4 and 10 % Na_2WO_4 were added. The mixture was centrifuged and oxaloacetic acid, pyruvic acid, acetone, and malic acids were estimated in the clear fluid. Figs. 3 and 4 show representative experiments of this type. It is seen that as the added oxaloacetate disappears there is a simultaneous increase in the malic acid sufficient to account for about 75 % of the oxaloacetic

Fig. 3.

Fig. 4.

Fig. 3. Liver tissue. The curves show the decrease in added oxaloacetic acid and the simultaneous increase in malic acid together with a slight increase in pyruvic acid.

Fig. 4. Kidney tissue. The curves show the decrease in added oxaloacetic acid and the simultaneous increase in malic acid together with a slight increase in pyruvic acid.

acid lost. Since fumarate and malate exist in tissue in an equilibrium mixture, the increase in the latter upon the addition of oxaloacetic acid does not necessarily imply that it is the primary reduction product of oxaloacetate. There is likewise a slight increase in the pyruvic acid content. This may come from oxidation of a triose as suggested by Szent-Györgyi [Annau *et al.*, 1936], or since the amount of oxaloacetic acid originally added was rather large it may be the result of decarboxylation of part of it. Acetone formation, as determined with the nitroprusside test, was very slight in the liver tissue during the time of these experiments and was absent in the kidney tissue.

SUMMARY.

Manometric and chemical studies on the effect of fumarate on the respiration of liver and kidney tissue were made. Fumarate increases the oxygen uptake of these tissues by conserving the initial rate of oxygen uptake. Malonate inhibits the normal respiration. With an adequate quantity of fumarate, respiration proceeds normally even in the presence of malonate. By quantitative chemical estimations it has been shown that fumarate added to liver or kidney tissue is oxidized to oxaloacetate. Likewise oxaloacetic acid added to these tissues is reduced, as indicated by the rapid disappearance of added oxaloacetic acid and the simultaneous appearance of a nearly equivalent amount of the fumaric-malic equilibrium mixture.

These facts suggest that the theory of fumaric catalysis of respiration, developed by Szent-Györgyi *et al.* for muscle tissue, applies also to liver and kidney tissue.

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