CCLXXXVIII. THE ROLE OF THE 4-CARBON DICARBOXYLIC ACIDS IN MUSCLE RESPIRATION.

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THE oxidation of dicarboxylic acids in muscle was studied by Battelli & Stern [1911; 1914], Thunberg [1918; 1923] and other workers, whilst Einbeck [1913; 1914] demonstrated the presence of succinic and fumaric acids in perfectly fresh muscle tissue.

The dynamic part played by these acids in muscle respiration was first studied by Mrs Needham [Moyle, 1924; Needham, 1927] who followed the changing concentrations of succinic, fumaric and malic acids in muscle under various conditions, finding that the total concentration of these acids rises in anaerobiosis and falls on oxygenation, and that the succinic acid maximum is renewed under anaerobic conditions from some source other than fumaric and malic acids.

An increased oxygen uptake on addition of fumaric acid to muscle after various degrees of washing was demonstrated by several workers [Thunberg, 1909, 1911; Meyerhof, 1919; Grönvall, 1924] and Gözsy & Szent-Györgyi [1934] also observed that the increased oxygen uptake after addition of fumaric acid to minced muscle tissue was inhibited by malonic acid. From these and other observations Szent-Györgyi *et al.* [1935] formulated a theory which, in its final published form, assigns to fumaric acid the role of an essential catalytic link in the chain of reactions which composes the chief respiratory system of muscle. Szent Györgyi *et al.* suggest that, in this system, the fumaric acid is oxidized by the Warburg-Keilin system to oxaloacetic acid and that this is reduced by the substrate dehydrogenase systems plus substrates to succinic acid which becomes reoxidized to fumaric acid.

In an attempt to confirm this theory the present experiments were divided into two sections.

(*a*) *With unwashed tissue.* Respiration experiments were carried out, a known quantity of fumaric acid being added to the suspension fluid of the tissue in some cases, but not to the controls. Accurate methods of estimation of small quantities of fumaric acid and its possible oxidation and hydration products were found and an attempt was made to draw up a balance sheet for each experiment showing the oxygen used in respiration, the fumaric acid remaining and the succinic, oxaloacetic, pyruvic and malic acids produced. Thus it could be seen whether any actual disappearance of fumaric acid occurred, or whether the acid was present at some stage of the fumarate-oxaloacetate-succinate-fumarate cycle, or as the hydration product malic acid.

(*b*) *With washed tissue.* Experiments were done in which cozymase, fumaric acid and a substrate were added to the washed tissue. Estimations were carried out as before, and a balance sheet was drawn up to see whether the fumaric acid was catalysing the oxidation of the added substrate or merely itself being consumed as a substrate for oxidation.

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Experimental methods.

The oxygen uptake was measured in Barcroft manometers, the CO_2 evolved being absorbed by frilled filter-papers soaked in 5% KOH. The suspension fluid was *M* phosphate buffer at *p*H 7, and the solutions of the acids added (neutralized with NaOH) were 0·1 *M*. The volumes of these solutions and water put into the vessels of the manometers were such that the final concentration of the fluid was 0·099 *M*, which is approximately isotonic with the muscle; this is important as Greville [1936] shows that the respiration of minced muscle varies with the tonicity of the suspension fluid.

The manometers were shaken in a water-bath, the temperature of which was thermostatically controlled at 37·5°.

Preparation of the muscle.

(*a*) *Unwashed tissue.* The pigeon breast muscle used in these experiments was prepared according to the directions given by Szent-Györgyi *et al.* [1935], everything being done as rapidly as possible after death and all the implements, vessels and solutions used being ice-cold. Three methods of weighing out the minced tissue were tried, and the following proved most satisfactory, allowing for the greatest speed in preparation of the tissue: 4 g. of the minced muscle were weighed out and transferred to an ice-cooled mortar in which they were mixed carefully with 26 ml. of buffer. The suspension was rapidly stirred and 3 ml. (containing 0·4 g. of muscle) were pipetted into each manometer vessel.

(*b*) *Washed tissue.* The breast muscle was prepared as before and the 4 g. of muscle were transferred to a flask and shaken vigorously with twenty times the volume of ice-cold distilled water. After 2 min. shaking the suspension was filtered through muslin and squeezed well. The muscle was returned to the flask and shaken with a fresh volume of ice-cold water, being allowed to stand for 5 min. in ice before filtration and squeezing. The washed tissue so obtained was transferred to a mortar, suspended in buffer and pipetted out into the manometer vessels in 3 ml. amounts as above.

Solutions to be added to the respiring tissue after equilibration of the manometers in the water-bath were put either into a side-tube of the manometer vessel or into a Keilin cup suspended in the vessel. The final volume of the suspension plus all additions was 4 ml. and a similar volume of buffer was put into the other cup of the manometer.

In early experiments the vessels were filled with air, but in more recent ones the apparatus was evacuated and filled with oxygen. Duplicate experiments were run simultaneously in every case, each duplicate consisting of three manometers with fumarate added to the respiring muscle and one without addition which acted as a control. Measurement of the rate of respiration was started 20 min. after death with unwashed tissue. The experiments were run for times varying from 1 to 2 hours.

At the end of the respiration experiments the vessels were detached from the manometers and the muscle was immediately precipitated by the addition of 0·8 ml. of 20% trichloroacetic acid (rendering the total concentration of trichloroacetic acid 4%). The filter-paper was removed from the central cup of the vessel and any remaining potash was blotted up with strips of dry filter-paper. The contents of the three vessels of each duplicate experiment, containing additions of fumarate or fumarate plus other substrate, were filtered together

through a small filter-paper into a glass evaporating dish, whilst the contents of the vessels containing the control muscle from each set of duplicates were also filtered together. The vessels were carefully rinsed with successive small quantities of 2% trichloroacetic acid which were then used to wash the muscle on the paper. The muscle was also ground up in a mortar with 2% trichloroacetic acid, being refiltered on the same paper. The final volume of the fluid in the evaporating dish after intensive washing of the vessels, mortar, muscle and paper, was about 30 ml. This was then used for the estimations described below.

The amounts of dicarboxylic acids and others to be estimated were generally between the limits 0·1–6 mg., as 2 mg. of fumaric acid were added to 400 mg. of tissue. Experiments were done to test each method, using the concentrations of the particular acid ranging between the limits, adding it to 0·4 g. of the minced muscle in phosphate buffer and proceeding as in the experiments above after measurements of the respiration. Results showed that the methods used were accurate to a degree which allowed the amounts of substances estimated after respiration experiments to be regarded as significant.

Estimation of the dicarboxylic acids.

The method used was a modification of that described by Needham [1927].

(*a*) *Fumaric and succinic acids.* The trichloroacetic acid extract from the muscle was evaporated on a boiling water-bath until the volume was reduced to less than 10 ml. This was transferred to a separating funnel and the evaporating dish was rinsed out with a few ml. of distilled water which were added to the bulk of the fluid. Four successive extractions with dry ether were made, the volumes used being 20, 15, 20 and 15 ml. The ether and fluid were shaken vigorously for 2 min. during each extraction and, after separation, the ether was filtered through a dry pleated filter-paper into a flask containing 5 ml. of distilled water, and the ether was distilled off. A drop of phenol red was added to the remaining solution and *N* NaOH was run in to bring its *p*H to 8 (deep pink to phenol red). 0·2 ml. of 25% barium acetate solution was added to precipitate any phosphate which had been extracted by the ether, the solution was transferred to a centrifuge-tube and the precipitate was spun down. The centrifugate was filtered through a small paper into a glass dish containing a few drops of dilute nitric acid to bring the *p*H of the filtrate just to the acid side (otherwise a film of barium carbonate formed on the surface and interfered with the silver precipitation). The precipitate in the centrifuge-tube was washed with successive small volumes of slightly alkaline distilled water (pink to phenol red) which had already been used to wash out the flask in which precipitation had occurred, and these washings were also filtered through the paper into the dish. The final volume was 20 ml. and to this 10 ml. of 97% alcohol were added. The *p*H was brought to 7 (orange to phenol red) and 2 ml. of 10% silver nitrate solution were added to precipitate the dicarboxylic acids present. The precipitate was allowed to stand for 5 min. to flocculate and then filtered through asbestos on a Gooch crucible. The dish was washed out with 15 ml. of 30% alcohol and the washings were filtered through the crucible. This was repeated several times. The asbestos mat was carefully detached with a glass rod from the crucible and transferred to a glass dish where it was suspended in a few ml. of very dilute nitric acid. The sides of the crucible were washed with a little more of the acid which was added to that in the dish. The suspension was titrated with 0·01 *M* potassium thiocyanate solution, iron alum in nitric acid being used as external indicator.

Test for accuracy of method.

	1	2	3
Fumaric acid added to muscle (mg.) (0·4 g. of muscle in each of 3 cups)	3·03	2·02	0·5
Fumaric acid found (after subtraction of amount of dicarboxylic acid already in muscle)	3·04	2·00	0·51

(*b*) *Malic acid.* The trichloroacetic acid extract (remaining after ether extraction) and the titrated alcoholic solution were each evaporated down to a small volume, neutralized to litmus with NaOH and made up to 8·4 ml. with distilled water in a graduated tube. The optical rotation of the solutions was measured with sodium light and each solution was evaporated to 4 ml. 4 ml. of a 14·2% ammonium molybdate solution and 0·4 ml. of glacial acetic acid were added, the solutions thoroughly mixed and allowed to stand in the dark for 2–3 hours [Auerbach & Kruger, 1923]. The rotation was again measured and the amount of malic acid was calculated as, under these conditions, the rotation changes 0·21° for each mg. of malic acid in the solution.

Test for accuracy of method.

	1	2	3
Malic acid added to muscle (mg.) (0·4 g. of muscle in each of 3 cups)	1·66	1·0	0·50
Increase in rotation	0·34°	0·22°	0·11°
Malic acid estimated (mg.) (after subtraction of amount already in muscle)	1·65	1·04	0·50

(*c*) *Pyruvic and oxaloacetic acids.* These acids were at first both estimated in the form of pyruvic acid, as Clift & Cook [1932, 2] showed that oxaloacetic acid and its 2:4-dinitrophenylhydrazone are decomposed quantitatively to the corresponding pyruvic acid and derivative when heated on a boiling water-bath. If the amounts of pyruvic acid found in the experiments were appreciable, a differentiation between the two acids would have been made in later work by the method of Ostern [1933] which estimates oxaloacetic acid only. The method of estimation of the pyruvic acid adopted was Szent-Györgyi's modification of the method of Case [1932] which is fully described by Needham & van Heyningen [1935].

Test for accuracy of method.

	1	2	3
Pyruvic acid added to muscle (mg.) (0·4 g. of muscle in each of 3 cups)	2·25	1·05	0·75
Pyruvic acid found (mg.) (after subtraction of any present in muscle)	2·25	1·01	0·75

(*d*) *Lactic acid.* Lactic acid was estimated by the method of Friedemann & Graeser [1933], the soluble carbohydrate being removed from the trichloroacetic acid extract by precipitation with copper sulphate and calcium carbonate.

(*e*) *Detection of the fumaric acid in the presence of succinic acid.* The dicarboxylic acid remaining after the experiments was shown to be fumaric acid by incubation with an enzyme preparation (kindly supplied by Dr D. E. Green) which distinguishes between fumaric and succinic acids. Details of this preparation and its mode of action are now in the press.

Experimental results.

(*a*) In the respiration experiments with unwashed tissue the contents of the manometer cups were:

	Buffer ml.	Fumarate solution ml.	Water ml.	Muscle g.
Experimental cups	2·6	0·2	0·8	0·4
Control cups	2·8	—	0·8	0·4

The oxygen uptake varied with the individual pigeons but the duplicates and controls agreed well. It appeared from the results of all experiments that, with phosphate buffer as suspension medium, the addition of 2–4 mg. of fumaric acid to 0·4 g. of minced muscle produced a stimulation of respiration, that of the muscle with added acid being always higher than that of the control from the beginning of the experiment (Fig. 1).

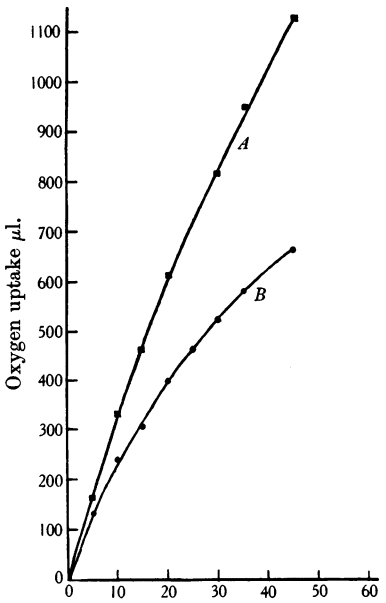

Fig. 1.

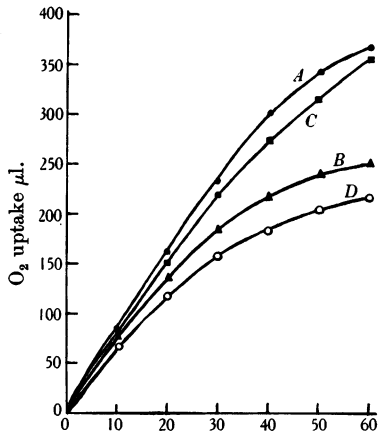

Fig. 2.

Fig. 1. O_2 uptake of minced muscle with and without added fumarate. *A*, ■—■, with added fumarate; *B*, •—•, control without added fumarate.

Fig. 2. O_2 uptake of washed minced muscle with addition of fumarate, lactate and fumarate plus lactate. *A*, •—•, with added fumarate; *B*, ▲—▲, with added lactate; *C*, ■—■, with added fumarate plus lactate; *D*, o—o, control without addition.

A similar effect was observed in experiments where 2–4 mg. of succinic acid were added to 0·4 g. of muscle under the same conditions.

Estimation of the fumaric and succinic acids remaining both in the experimental muscle and in the control showed that there was always a definite disappearance of some of the fumaric acid added to the experimental muscle. Estimations of the malic acid were carried out in each experiment to see if the

fumaric acid was to be found in this form. It was discovered that the amount of malic acid formed depended on the oxygenation of the tissue during the experiment, as practically no malic acid was found in samples taken from vessels filled with oxygen, whereas an appreciable quantity was found in those from vessels filled with air. When the fumaric acid equivalent of the malic acid found was added to the amount of fumarate estimated, there was still a deficit.

It was thought that the missing fumaric acid might be found in the form of the other two links of Szent-Györgyi's suggested cycle. If it had been present as succinate it would have been estimated with the fumaric acid in the silver precipitation method. The other possibility was oxaloacetic acid. It had been shown, in some early experiments in which the minced muscle was incubated aerobically with fumarate, 0·02 *M* arsenite solution, bisulphite and phosphate buffer, that a bisulphite-binding compound was formed in the flask containing added fumarate in excess of that in the flask containing control muscle. (Estimations of this bisulphite-binding compound were done by the methods of Clift & Cook [1932, 1] and of Case [1932].) Preliminary estimations of the oxaloacetic and pyruvic acids in the form of the 2:4-dinitrophenylhydrazone were therefore made on the trichloroacetic acid extracts from respiration experiments. The amounts of pyruvic acid found in the extracts from muscle with added fumarate were slightly in excess of those from the controls (of the order of 0·02 mg.), but none of them was large enough to be significant in the balance sheets.

It appears therefore that the missing fumarate was not to be found in the form of the other substances in the cycle or as pyruvic or malic acid. It is also seen, from the experiments with Green's enzyme preparation, that the remaining fumaric acid was present in its original form. The extra oxygen uptake of the tissue with added fumarate above the respiration of the control was in no case greater than the amount necessary for complete oxidation of the missing fumaric acid, but in every experiment there was still a portion of the fumaric acid untraced. The fate of this untraced fumaric acid may possibly be explained in the light of the experiments with washed tissue (see below).

Assuming that the extra oxygen was used for complete oxidation of fumaric acid, balance sheets of each experiment were drawn up as follows, the results in every case being essentially the same:

Table I.

	(1) With air			(2) With oxygen		
Experiment	(*a*) 3 cups	Control	(*b*) 3 cups	(*a*) 3 cups	Control	(*b*) 3 cups
Fumaric acid added (mg.)	5·73	—	5·73	6·26	—	6·26
Fumaric acid estimated (mg.)	1·59	0·45	1·61	2·56	0·41	2·37
"Extra fumaric acid" (above control)	1·14	—	1·16	2·15	—	1·96
Oxygen uptake (μl.)	2064	1416	2053	4192	3045	4180
Fumaric acid≡of "extra oxygen" (mg.)	1·12	—	1·1	1·97	—	1·96
Fumaric acid≡of malic acid (mg.)	1·64	—	1·65	—	—	0·12
Total "extra fumaric acid" (mg.)	3·9	—	3·91	4·12	—	4·04
Fumaric acid unaccounted for (mg.)	1·83	—	1·82	2·14	—	2·22

(*b*) In the respiration experiments with washed tissue the manometer vessels contained:

Cups	Cozymase ml.	Buffer ml.	Fumarate solution ml.	Lactate solution ml.	Water ml.	Muscle g.
(1) & (2)	0·3	2·3	0·2	0·2	0·6	0·4
(3) & (4)	0·3	2·5	0·2	—	0·6	0·4
(5) & (6)	0·3	2·5	—	0·2	0·6	0·4
(7) & (8)	0·3	2·7	—	—	0·6	0·4

The oxygen uptake was measured as before, and in every experiment it was found that the respiration with added fumarate was greater than that with added lactate (see Fig. 2).

Balance sheets were drawn up as before (Table II) and from these it can be seen that, in these concentrations, the fumaric acid is always used in preference to the lactic acid unless there is an added excess of lactic acid.

Table II.

Cups	(1) & (2)	(3) & (4)	(5) & (6)	Controls
Fumaric acid added (mg.)	4·04	4·04	—	—
Fumaric acid found (mg.)	2·10	1·99	0·44	0·19
"Extra fumaric acid" (mg.)	1·91	1·80	0·25	—
Lactic acid added (mg.)	3·76	—	3·76	—
Lactic acid found (mg.)	5·11	1·77	3·83	1·23
"Extra lactic acid" (mg.)	3·97	0·54	3·83	—
Oxygen uptake (μl.)	743	754	520	421
Fumaric acid≡of extra O_2 (mg.)	0·55	0·57	—	—
Fumaric acid≡of malic acid (mg.)	0·4	0·3	—	—
Lactic acid≡of extra O_2 (mg.)	—	—	0·13	—

(*c*) Experiments were also carried out in which 2 mg. of pyruvic acid were added to 0·4 g. of muscle to see if this would be oxidized as rapidly as fumaric acid and therefore constitute an intermediary product in the path of oxidation. In this concentration, however, pyruvic acid depresses the respiration of the minced muscle.

Discussion.

The above results (see Tables I and II) appear to indicate that fumaric acid added in the concentrations specified is utilized as the chief substrate for oxidation by the minced muscle. There is no evidence that fumaric acid in this concentration is acting as a catalyst for transference of oxygen to some other substrate in the muscle, as the missing fumaric acid is not to be found either as succinic or oxaloacetic acid, which are the other two links postulated in the cycle. The amount of fumaric acid added in each case was slightly less than that which Szent-Györgyi considers essential for prevention of diffusion of the muscle's fumarate into the surrounding medium and for the maintenance of the cycle. The effect obtained in phosphate buffer was that of raising the respiration rather than conserving it (see Fig. 1), and this does not agree with the results of Banga [1935], but does agree with the idea that the fumaric acid is being used as substrate rather than a catalyst for preservation of respiration.

The estimation of malic acid occurring in the tissue incubated with fumaric acid appears to indicate that the amount formed depends on the conditions of incubation. In oxygen no malic acid was found, whereas in air quite appreciable quantities were detected, although these did not appear to have any fixed relationship to the amount of fumaric acid present. Szent-Györgyi *et al.*, and originally Clutterbuck [1927], found that a ratio of fumarate: malate of 1 : 3 was

obtained in their experiments with muscle, but the above results indicate that this equilibrium cannot be applied indiscriminately to experiments under differing aerobic conditions. As, however, in the present case, both the fumaric and malic acids were estimated by separate methods, it was unnecessary to use the method of Szent-Györgyi *et al.* of multiplying the fumarate estimated by four to obtain a true "fumarate" value. The observation that malic acid does not accumulate under good aerobic conditions is in agreement with Needham [1927] who used succinate and obtained no accumulation of malic acid in well aerated muscle.

The slight increase in the amounts of oxaloacetic and pyruvic acids in muscle incubated with added fumaric acid was not large enough to be significant in the balance sheet, but it did indicate that the oxidation of fumaric acid might be through the path of oxaloacetic and pyruvic acids, or that the Szent-Györgyi cycle was working at a much smaller concentration of the various acids than he considers necessary in the muscle. The belief that oxaloacetic or pyruvic acid is an intermediary in the path of oxidation of fumaric acid was substantiated by the experiments of incubation of minced muscle with added fumarate in the presence of arsenite and bisulphite aerobically, when a bisulphite-binding compound was formed.

The depression of respiration by pyruvic acid in the concentration used has also been noted by other workers [Elliott, 1935], but this does not rule out pyruvic acid as an intermediary, as it is probable that the tissue is still capable of dealing continuously with small quantities of the acid as formed from precursors in the muscle.

The experiments with washed tissue and added lactate and fumarate reveal that fumaric acid in any appreciable concentration appears to spare other substrates even if these are present in equal concentration. It therefore appears probable that the fumaric acid unaccounted for in the experiments with unwashed tissue was used in preference to some other substrate oxidized by the control muscle.

Summary.

1. Methods of estimation of the various dicarboxylic acids involved in these experiments were worked out.

2. The addition of 2 mg. of fumaric acid to 0·4 g. of minced pigeon breast muscle suspended in phosphate buffer (*p*H 7) causes an elevation of the respiration above that of the tissue without addition. During this increased respiration some of the added fumaric acid disappears.

3. Estimations of oxaloacetic and pyruvic acids show a very slight rise in concentration after incubation of minced muscle with fumaric acid in oxygen. This may be due to the path of oxidation of fumaric acid being through oxaloacetic or pyruvic acid.

4. Estimations of malic acid indicate that the amounts of this substance accumulating in minced muscle with added fumaric acid depend on the degree of oxygenation during incubation.

5. It therefore appears that, under the conditions of the experiments and with the concentration of fumaric acid used (which is that suggested by Szent-Györgyi *et al.* as essential for maintenance of the level of fumaric acid in muscle for correct working of the catalytic cycle), the extra oxygen uptake is in no case greater than can be accounted for by oxidation of some of the fumaric acid which disappears.

6. From experiments with washed tissue it is seen that fumaric acid is oxidized in preference to lactic acid in an equivalent concentration.

7. The above results suggest that, under these conditions, fumaric acid is being utilized as a substrate for respiration and not as a catalyst for transference of oxygen to other substrates in the muscle.

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