XC. THE EFFECT OF CALCIUM ION ON TISSUE RESPIRATION; WITH A NOTE ON THE ESTIMATION OF OXALOACETIC ACID

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THAT the respiration of minced muscle is strongly inhibited by Ca^{++} in approximately "physiological" concentration was shown by Thunberg [1909, 1, 2], an observation confirmed and extended by others, including Meyerhof [1919], Holck [1934], Greville [1936] and Krebs & Eggleston [1938]. Ca^{++} inhibits the respiration of other tissues also, provided that they are minced or otherwise mechanically damaged [Warburg, 1914; Holck, 1934; Krebs & Eggleston, 1938]. The contrast between the effects of neutral salts on the respiration of minced pigeon breast-muscle and cerebral cortex slices supported the view that the effect of Ca^{++} with the former tissue is due to irreversible damage consequent on its penetration into the tissue [Greville, 1936]. That in minced tissues "Ca^{++} may reach intracellular enzymes to which they normally have no access" was considered also by Krebs & Eggleston [1938].

Although it has been found that the respiration in presence of fumarate was strongly inhibited by Ca^{++} [Greville, 1936], it is not certain whether this ion inhibits one or both of the central reactions of respiration, namely the formation and the removal of oxaloacetate [Annau *et al.* 1935; 1936; Laki *et al.* 1937; Krebs & Johnson, 1937]. Elliott & Elliott [1939] write: "Banga [1935] mentioned that in Ringer's solution the reduction of oxaloacetate to malate by muscle suspension was inhibited. This was probably due to the Ca in her Ringer's solution and her observation may help to fix the point of action of Ca." This remark suggested to the present writer that some hitherto unpublished experiments made by him on this matter may be of interest.

EXPERIMENTAL

Oxidation of fumarate. Following Banga [1935] the suspension of minced pigeon breast muscle in phosphate was shaken at 38° for 10–15 min. aerobically in the presence of arsenite, and then for a further 15 min. after the addition of fumarate. The mixture was deproteinized with trichloroacetic acid, this and all further operations being conducted in ice-cooled vessels. After filtration a weighed aliquot was brought to *p*H 4·5 with NaOH, using "4·5" indicator, and the oxaloacetic acid in it was estimated by the aniline citrate method described below. In this way the oxaloacetate formation was determined in the presence

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and absence of 0·0021 *M* Ca^{++} together with 0·0031 *M* K^+. A typical experiment was arranged as follows:

Vessel no. ...	1	2	3	4
Muscle 1 : 4 in 0·177 *M* phosphate, *p*H 7·3 (ml.)	1·5	1·5	1·5	1·5
As_2O_3, 0·1 *M* (ml.)	0·5	0·5	0·5	0·5
Fumarate, 0·02 *M* (ml.)	1·0	1·0	1·0	1·0
$CaCl_2$, 0·103 *M* (ml.)	—	—	0·08	0·08
KCl, 0·155 *M* (ml.)	—	—	0·08	0·08
NaCl, 0·9 % (ml.)	0·16	0·16	—	—
Water (ml.)	0·84	0·84	0·84	0·84
Time of incubation after fumarate addition (min.)	0	15	0	15

The results are summarized below, most of the figures giving the mean of duplicate observations. The "preformed" oxaloacetic acid (vessels 1 and 3) was never larger than 0·026 mg. In Exp. 5 the Ca^{++} and K^+ were added with the fumarate; otherwise they were present from the start. It is seen that in the presence of Ca^{++} and K^+ the oxaloacetate formation is strongly inhibited.

	Oxaloacetic acid formed (mg.)	
Exp. no.	Ca, K absent	Ca, K present
1	0·43	0·06
2	0·36	0
3	0·11	0·03
4	0·24	0·05
5	0·29	0·13

When the muscle was suspended in the $NaHCO_3$-containing salt solution used by Annau *et al.* [1935], but without the Ca, there was a good oxaloacetic acid formation from fumarate in the presence of arsenite. Ca^{++} addition (0·003 *M*) caused a strong inhibition, whether the flasks were filled with air, or with air containing 5 % CO_2.

Oxaloacetate removal. Banga's finding, referred to above, was obtained with the "semi-quantitative" Simon-Piaux nitroprusside test. In the experiments summarized below, minced pigeon breast muscle was shaken for 15 min. aerobically at 38° in the presence and absence of Ca^{++} and K^+ (concentrations as above), and then for a further 15 min. after the addition of 15 mg. neutralized oxaloacetic acid. Oxaloacetic acid was determined by the aniline citrate method before and after the second incubation. It will be seen that the oxaloacetate removal is inhibited by the added cations. The total amount of carbonyl compound present was also determined [Clift & Cook, 1932]; and it was found that at the end of the experiment the amount present was greater than the residual amount of oxaloacetic acid. The difference was presumed to be due to the presence of pyruvic acid [Banga & Szent-Györgyi, 1937], especially as the com-

Exp.	Wt. of minced muscle (g.)	Added cations	Oxaloacetic acid disappearance (mg.) (1)	"Pyruvic acid" formed (mg.) (2)	Minimum oxaloacetic acid removal by ways other than decarboxylation, from (1) and (2) (mg.) (3)
1	0·38	—	8·8	3·0	4·4
		Ca^{++}, K^+	4·8	2·3	1·3
2	0·25	—	3·3	1·3	1·3
		Ca^{++}, K^+	2·1	1·1	0·5
3	0·38	—	8·8	2·0	5·8
		Ca^{++}, K^+	6·3	1·9	3·4

pound formed was alkali-stable [Clift & Cook, 1932]. The pyruvate formation, which was probably due to decarboxylation of oxaloacetate, was not greatly affected by the Ca^{++} and K^+.

Succinate formation. Straub found that in Ringer solution the aerobic disappearance of fumarate in the presence of malonate was much less than in phosphate; under these conditions and in the latter medium Gözsy found succinate formation [Annau *et al.* 1935]. In the two experiments summarized below, an inhibition of succinate formation by 0·0018 *M* Ca^{++} with 0·0028 *M* K^+ was observed.

Time 30 min. Air. Minced muscle 1 g. 38°. Malonate 0·01 *M*. Fumaric acid 7 mg. (neutralized).

	Succinic acid formed (mg.)	
Exp.	Ca^{++}, K^+ absent	Ca^{++}, K^+ present
1	1·66	0·75
2	1·88	0·92, 0·81

The succinic acid was estimated as follows: after deproteinization with alcohol, the acidified solution was extracted with ether in a continuous extractor, the dry extract was autoclaved to remove malonate, and the succinic acid determined using a succinoxidase-containing dispersion obtained from pigeon breast muscle, which did not oxidize lactate, α-ketoglutarate or glycerophosphate [cf. Annau *et al.* 1935; Weil-Malherbe, 1937].

Estimation of oxaloacetic acid

In Ostern's [1933] method the CO_2 evolved when aniline reacts with oxaloacetic acid is determined in the Warburg apparatus. The analysis is performed at 5° in order to minimize the breakdown of the acid before the addition of the aniline. However, at this temperature the reaction is slow, becoming complete in 60–90 min. Two ways have been used to increase the amount of dissolved aniline and hence the speed of the reaction.

(1) *Citrate method.* Edson [1935] used aniline citrate in the manometric determination of acetoacetic acid at 25°. His technique can be applied to the estimation of oxaloacetic acid at 5°. The vessel is shaken for 10 min. before the addition of the aniline. If the solution originally contained much bicarbonate, the shaking should be continued for a test period of 5 min. Reaction is complete in 10–20 min. after addition of aniline citrate from the side-bulb. For the calculation of vessel constants it is necessary to know the solubility of CO_2 in the mixture in the vessel. For this $\alpha^{5°}_{CO_2}$ was found to be 1·15. If it is necessary to use rather more oxaloacetic acid solution in the vessel, the value $\alpha^{5°}_{CO_2}=1·19$ should be used for the mixture 3 ml. H_2O + 0·4 ml. 50 % citric acid + 0·4 ml. aniline citrate solution. Duplicate determinations never differed by more than 2·5 %. 97 % purity was indicated by the method for three different oxaloacetic acid preparations.

(2) *Alcohol method.* The bulb contains 0·2 ml. aniline previously mixed with 0·14 ml. conc. HCl. The main part contains 1·5 ml. absolute alcohol, 0·3 ml. acetate buffer (0·3 *N* Na acetate + 2·7 *N* acetic acid) and 0·86 ml. solution to be analysed. The thermo-barometer contains 0·86 ml. of water instead of solution. The analysis is carried out as in method (1). The reaction is complete in 10–15 min. at 5°. $\alpha^{5°}_{CO_2}=1·21$. The method gives the same results on oxaloacetic acid solutions as do Ostern's method and method (1).

Acetoacetic acid. The alcohol method does not distinguish between oxaloacetic and acetoacetic acids. The reaction with acetoacetic acid at 5° is complete in

10–15 min. (with the citrate method the reaction at 25° takes up to 70 min. [Edson, 1935]). With the citrate method at 5°, however, oxaloacetic acid can be determined in the presence of acetoacetic acid.[1] The CO_2 evolution with the latter is slow and fairly constant for a long time, and extrapolation to zero time will give the amount of oxaloacetic acid with reasonable accuracy (Fig. 1). Nevertheless it is possible that the alcohol method may also prove convenient on occasion.

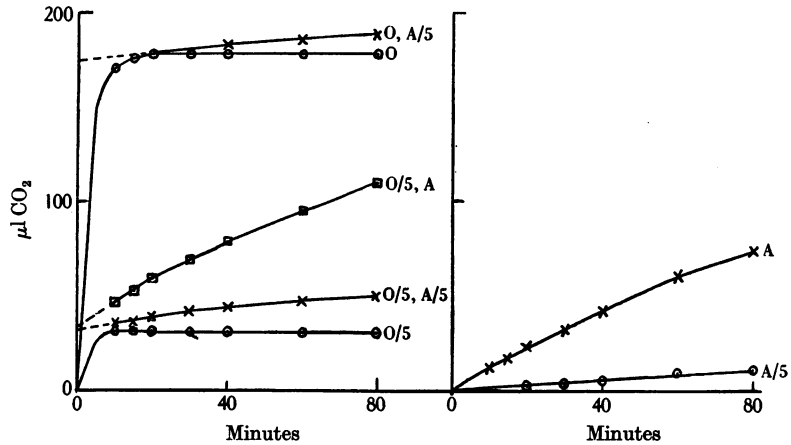

Fig. 1. A = 1 ml., A/5 = 0·2 ml., acetoacetic acid. O = 1 ml., O/5 = 0·2 ml., oxaloacetic acid. O/5, A/5 = 0·2 ml. oxaloacetic + 0·2 ml. acetoacetic acid. O/5, A = 0·2 ml. oxaloacetic + 1 ml. acetoacetic acid. O, A/5 = 1 ml. oxaloacetic + 0·2 ml. acetoacetic acid. (Oxaloacetic and acetoacetic acid solutions approx. 1 mg. per ml.)

Thermostat. A simple and inexpensive device serves to keep the thermostat at 5°. Water is transferred to the thermostat from a bucket containing ice by means of a small water-circulating pump. It returns through a syphon tube. The pump is driven by an electric motor which is switched on and off by a relay controlled by a mercury-toluene regulator in the thermostat. The occasional addition of a lump of ice to the bucket is the only attention necessary.

Summary

1. On the addition of Ca^{++} and K^{+} in physiological salt solution concentrations to a suspension of minced muscle, both the formation and the removal of oxaloacetic acid, and also the accumulation of succinic acid, are inhibited. Hence the inhibitory effect of Ca^{++} on the respiration of minced muscle cannot be localized in any particular enzymic reaction.

2. Rapid methods are given for the manometric estimation of oxaloacetic acid.

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[1] Elliott & Elliott [1939] have used Edson's method at 38° in order to determine oxaloacetic acid. At this temperature the method does not differentiate between oxaloacetic and acetoacetic acids.

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