XCIX. A QUANTITATIVE STUDY OF SUCCINIC ACID IN MUSCLE. II.

THE METABOLIC RELATIONSHIPS OF SUCCINIC, MALIC AND FUMARIC ACIDS.

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(*Received April 30th, 1927.*)

Introduction.

That the succinic acid content of fresh, chopped muscle tissue increases on incubation in buffer solutions of suitable p_H has been shown in a previous paper [Moyle, 1924]. It would obviously be of the greatest assistance towards elucidating the rôle of the succinic acid in the intact muscle if one could discover whether the succinic acid maximum reached *in vitro* is an equilibrium point or not; that is to say, whether the formation of more succinic acid is hindered by the accumulation of the acid. If this were the case, we might expect that, after removal of the accumulated acid by admission of oxygen, putting the system back under anaerobic conditions would lead to a renewal of the maximum from some unknown precursor.

When such experiments were performed, it was indeed found that after a period in nitrogen, followed by a period in oxygen involving decrease in succinic acid content, a return to nitrogen was accompanied by rise in concentration of this acid.

There is of course an alternative explanation to the one just put forward of the renewed succinic acid maxima: that the fumaric and malic acids formed in presence of oxygen [Batelli and Stern, 1911, 1; Einbeck, 1919] are themselves not oxidised or only very slowly; when anaerobic conditions again prevail these acids might be reconverted into succinic acid. As regards the possibility of the removal of fumaric and malic acids by oxidation, Batelli and Stern [1911, 2] showed that these acids, as well as citric acid, were oxidised to some extent by chopped muscle suspended in three times its weight of water, though the oxygen uptake is very much less rapid than in the oxidation of succinic to fumaric acid. Thorough washing they found to destroy the power of the tissues to bring about the former oxidations. Other workers [Thunberg, 1909, 1911, 1 and 2; Meyerhof, 1919; Grönvall, 1924] have observed increased oxygen uptake upon adding fumaric acid to muscle after various degrees of extraction. The oxidation of all these acids is inhibited by the presence of

inorganic salts [Batelli and Stern, 1911, 2], but with the fumaric and malic acids, if we may judge from the figures given for citric acid, not more than with the succinic acid.

When the alternate rise and fall in succinic acid content had been established, it therefore became necessary to determine the amounts of fumaric and malic acids present at the same times, and a method is described for estimating the malic acid, as well as the total quantity of the three acids together, in the same muscle sample. From these two values, as is explained later, a rough calculation may be made of the succinic and fumaric acids present. Using these methods, the experiments with alternate atmospheres were repeated, and it was found that the total content of the three acids does not remain constant, but rises and falls markedly with the succinic acid content. Simple interconversion of succinic acid on the one hand and the oxidation products malic and fumaric acids on the other, is not then the explanation of the results obtained.

The effect of alternate aerobic and anaerobic conditions on the succinic acid content.

The first result which emerged from the following experiments was the difficulty of obtaining complete disappearance of the succinic acid in oxygen, although, as is well known, succinic acid added to washed muscle is rapidly and quantitatively converted into fumaric acid [Fleisch, 1924].

Muscle removed from the neck of the bullock immediately after slaughtering was brought to the laboratory in a pan surrounded by a freezing mixture; a long, cylindrical muscle could be dissected out with little injury, and this, after further cooling, was minced and samples were weighed out. After the required time of incubation the flask was cooled, and an equal volume of alcohol was added (counting each g. of muscle as 1 cc. in the suspension). The succinic acid was estimated according to the method previously described.

Exp. 1. Four 50 g. lots were used, each suspended in 100 cc. of buffer solution at p_H 7·4. Two of the flasks also contained about 50 mg. each of succinic acid. One of these flasks and one control were connected to a nitrogen cylinder, the remaining two to an oxygen cylinder. All were left at 33° for 1·5 hours.

	Succinic acid in mg. per 50 g.
Control in oxygen	8·5
,, +succinic acid in oxygen...	28·8
Control in nitrogen	12·8
,, +succinic acid in nitrogen	54·4

It seemed possible that the conditions might be unfavourable for complete oxidation, either of the succinic acid to fumaric, or of the fumaric acid further (with consequent retardation of fumaric acid formation) owing to the inhibitory effect of the phosphate in the buffer and of too low a p_H (according to Ohlsson the optimum p_H is about 8·7). A buffer solution much less concen-

trated was therefore made up, following the directions given by Ohlsson [1921] for the buffer that he used with his enzyme preparation. The p_H of this solution was 9·0, and it was ascertained by means of the hydrogen electrode that the addition of 40 g. of beef to 500 cc. did not cause the value to fall below 7·47, even after several hours.

Exp. 2. 40 g. lots of beef were used, each suspended in 500 cc. of the dilute buffer; 50 mg. of succinic acid were added to each bottle and both were oxygenated at 37°.

	Succinic acid in mg. per 40 g.
After 40 mins.	28·2
After 60 mins.	28·8

It is clear that these precautions have not overcome the difficulty of incomplete removal, which must be due to some other factor than the effects of the medium already considered. For instance, it is possible that under the conditions of the experiment continuous production of succinic acid is taking place, while with washed muscle the precursor has been removed. Further attempts, therefore, to obtain complete disappearance were abandoned, and the experiments on anaerobiosis and oxygenation were begun.

In the remaining experiments described in this paper a somewhat different arrangement was used; the desired atmosphere was obtained by evacuating the flasks till the contents boiled, and then filling with the required gas until a little more than atmospheric pressure was reached. As it had been found that pigeon breast muscle gave a higher yield of succinic acid than any other kind tried, this muscle was used; four pigeons were generally taken for each experiment. After killing, bleeding and plucking, they were placed breast downwards on ice for a short time; then the two breast muscles were dissected off from each with as little injury as possible, further cooled in a covered glass dish packed in freezing mixture, and minced. As the succinic acid content varies considerably from one pigeon to another, very thorough mixing was essential before samples were weighed out. The anaerobic flasks were always evacuated before removal from the bath, and were opened with a side-tube dipping under alcohol, so that the contents were precipitated without admission of air. The oxygen flasks were shaken almost continuously.

Exp. 3. Temp. 33°. Phosphate buffer, p_H 9·0 (1·4 % Na_2HPO_4, $2H_2O$); two volumes used.

1.0 p.m.	All flasks filled with nitrogen.
2.0 „	First flask, N 1, removed; oxygen introduced into remaining four.
2.40 „	Second flask, O 1, removed; nitrogen introduced into the others.
3.20 „	Third flask, N 2, removed; oxygen introduced into remaining two flasks.
4.0 „	Fourth flask, O 2, removed; nitrogen introduced into the last flask.
5.15 „	Last flask, N 3, removed.

The results are shown in Fig. 1.

Exp. 4. Temp. 33°. The concentration of phosphate was halved and the volume used was doubled. All the periods were shortened (see Fig. 1). The results were very like those in Exp. 3.

Exp. 5. This experiment was similar to the last, except that at the end of

the first nitrogen period all the flasks but N 1 were opened and 20 cc. of 0·2 *N* sodium hydroxide were added to each. It was calculated that this should raise the p_H by about one unit, and the final p_H was found to be about 7·3 instead of below 7·0, as in the previous experiment. This change in the treatment does not seem to affect the results (Fig. 1).

The combined results of these three experiments seem to show that, as time goes on, the possibility of the complete removal of succinic acid in oxygen becomes progressively greater. When the second oxygen period falls, 3 hours after the beginning of the experiment, disappearance is almost complete. Renewed maxima in nitrogen are obtained, but the value in each is generally somewhat lower than in the previous one.

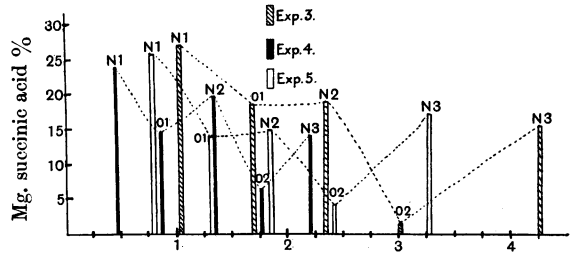

Fig. 1.

THE METHOD OF ESTIMATION OF MALIC ACID AND OF THE THREE ACIDS TOGETHER.

In the method used for the estimation of the total quantity of the three acids, they were quantitatively precipitated as the silver salts by adding $AgNO_3$ to a neutral solution in 36 % alcohol; the silver was then determined by titration with *N*/100 KCNS. After removal of the silver precipitate, the malic acid was estimated polarimetrically as the molybdenum compound, which, as is well known, shows an enormously increased rotation.

As two important difficulties were encountered in the working out of the method, it might be well to state the reason for some of the processes described.

(1) It was desirable to extract with ether before the silver precipitation to get rid of traces of amino-acids, purines, etc., and also the greater part of the phosphate (if the whole of the phosphate of the buffer is removed with barium, there is much loss by adsorption of the acids, especially succinic, on the barium phosphate precipitate). On account of the high partition coefficient of malic acid between water and ether (62 : 1) it was necessary to put the solution on to some water absorbent, and to extract this in a Soxhlet. As water absorbents, plaster of Paris, anhydrous sodium sulphate and fat-free filter paper strips were tried, but found useless because the malic acid, once adsorbed, could not be quantitatively removed. Only about 66 %, and sometimes much less, could be recovered when the pure acid was tested. In the presence of high concentrations of salts, *e.g.* ammonium sulphate, in the liquid adsorbed, the

recovery was much less; and when a muscle extract with added malic acid was tried, none of the acid could be recovered. Using silica gel[1], on the other hand, 90 % of the acid could be found again after 6 hours' extraction, when the pure acid was tried; and 84 % was recovered after 24 hours' extraction in the presence of phosphate and lactate in amounts comparable to those in a muscle sample. No explanation of these phenomena inhibitory to extraction can be offered; in the case of the lactate some indications were obtained that, even in the presence of silica gel, the malic acid only begins to be extracted when all or most of the lactic acid has been extracted, and for this reason the time of extraction was made lengthy.

(2) In the presence of so large a proportion of lactic acid, it was found that the usual precipitation methods for malic and succinic acids, especially the former, fail. Thus for succinic acid alone very good results were obtained for 10 mg. of the acid by precipitating the calcium salt in 87 % alcohol, and, after filtering and slight washing, dissolving the precipitate in water and estimating as the silver salt; but on addition of 150 mg. lactic acid, practically no precipitate was obtained on adding alcohol. Using the barium salts, in presence of 300 mg. lactic acid, 10 mg. succinic acid was precipitated to the extent of 70 % only. 300 mg. lactic acid almost entirely prevents the precipitation of 10 mg. malic acid whether as the calcium or the barium salt in 85 % alcohol.

Similar results were obtained for the silver methods. Precipitation in 36 % alcohol solution (3 cc. alcohol to 5 cc. water) had been adopted because, in this medium, at p_H 6·2–7·2 the three acids are quantitatively precipitated as the silver salts, whilst in aqueous solution only about 60 % of the malic acid is precipitated. When lactic acid was present, however, to the extent of 40 times the malic acid, only about 50 % of the latter was precipitated; the succinic acid precipitation was less affected. In order to overcome this difficulty, the practice was adopted of a preliminary silver precipitation in 75 % alcohol; in this way practically all the lactic acid is removed, and the precipitate obtained, which gives a high percentage of silver and probably contains basic salts, is dissolved and re-precipitated as described later. This double precipitation is unnecessary if the proportion of lactic to malic acid is not greater than 20 : 1, but, as we shall see, it is usually greater than this in muscle.

Complete removal of the lactic acid is also necessary for the polarimetric estimation of malic acid, as the rotation of the former acid is also markedly increased on addition of molybdenum.

The details of the method finally used were as follows. The 50 % alcoholic extract is strained from the muscle residue by pressing through muslin, and the muscle washed twice with 50 % alcohol. The extract is filtered through paper, neutralised, and evaporated *in vacuo* at 40°. When the volume is reduced to about 75 cc., the precipitated protein is filtered off and the filtrate

[1] The commercial name of the product used was "Super-Cel Hyflo," kindly supplied to me by the makers, the Celite Products Corporation.

evaporated on the water-bath to about 7 cc.; it is cooled on ice and 2 cc. of 50 % H_2SO_4 (by volume) added. The acid extract is now adsorbed on a silica gel, the dry powder is well mixed and ground, and placed in a Soxhlet thimble. After 1 hour's extraction with light petroleum to remove most of the fat, it is extracted for about 90 hours with ether.

When the extraction is finished, a little water is added, and the ether is distilled off. The warm aqueous solution is neutralised with saturated baryta solution to remove sulphate and phosphate. The neutralised solution should not be allowed to stand very long or remain very hot before filtration, on account of the risk of converting into a less soluble form the barium salts of the acids to be estimated. The barium precipitate is filtered off and well washed with hot water.

The filtrate is evaporated down to 10 cc., and 30 cc. 97 % alcohol are added. The alcoholic solution is adjusted to p_H 7·0 (using phenol red) and 1–2 cc. 10 % $AgNO_3$ solution is added. The silver precipitate contains all the succinic, malic and fumaric acids, but very little lactic acid. The precipitate is filtered off in a Gooch crucible, washed with 75 % alcohol, suspended in water with a few drops of 1 % sulphuric acid and treated with hydrogen sulphide. After removal of the silver sulphide by filtration and of the hydrogen sulphide by aeration, the solution is neutralised with sodium hydroxide and evaporated down to 20 cc. 12 cc. 97 % alcohol are now added, the solution is brought to p_H 7·0, and 10 % $AgNO_3$ is again added. The silver salts of the succinic, malic and fumaric acids are now quantitatively precipitated; in order to estimate their amount, the precipitate is ground with warm water containing a few drops of 50 % H_2SO_4, and the cold solution is titrated with 0·01*N* KCNS, using iron alum in nitric acid as outside indicator.

The silver thiocyanate is filtered off, and in the filtrate the malic acid is estimated polarimetrically as the molybdenum compound, according to the method of Auerbach and Kruger [1923]. The filtrate is neutralised and evaporated down to 8·4 cc., and its rotation taken in a 2 dm. tube. After concentration to 4 cc., 4 cc. of 14·2 % ammonium molybdate solution are added, and 0·4 cc. of glacial acetic acid; when the solution has stood in the dark for 2 to 3 hours, the increase in rotation is determined. Using the mercury green line, it was ascertained that 1 mg. of malic acid gave, under these conditions, a rotation of + 0·21°.

The method was tested by adding known amounts of the acids to muscle, suspended in two volumes of 1·4 % Na_2HPO_4, $2H_2O$, and some results, after subtracting the small quantities found in the control, are shown in Table I.

These figures have been arranged in a diagram (Fig. 2), and it will be seen that, with quantities of malic acid between 45 mg. and 8 mg. added to about 50 g. muscle, the amount recoverable falls off gradually from about 80 % to about 55 %; below 8 mg. only about 35 % is recoverable. The points marked *X* were obtained by the double precipitation method, which gives better results in these regions of low concentration. The low results obtained even

with this method for amounts below 8 mg. cannot be due to the effect of lactic acid, but are probably brought about by adsorption of the barium malate on the barium phosphate precipitate.

Table I.

Muscle used	Amount added	Amount found	% recovered
40 g. beef	43 mg. malic acid	34·0 mg.	79
40 „	21·5 „	13·1	61
40 „	10·75 „	5·8	54
45 „	30 „	24·0	80
45 „	50 „	37·5	75
45 „	10 „	3·7	37
50 „	10 „	3·4	34
50 g. pigeon	8·5 „	5·3	62
50 „	5·6 „	1·8	32
50 „	3·8 „	1·2	32
45 g. beef	30 mg. succinic + 30 mg. fumaric	42·0	70
45 „	„ „	42·0	70
50 g. pigeon	21·7 mg. succinic + 3·9 mg. fumaric	21·0	82
50 „	„ „	15·4	60
50 „	„ „	17·0	66

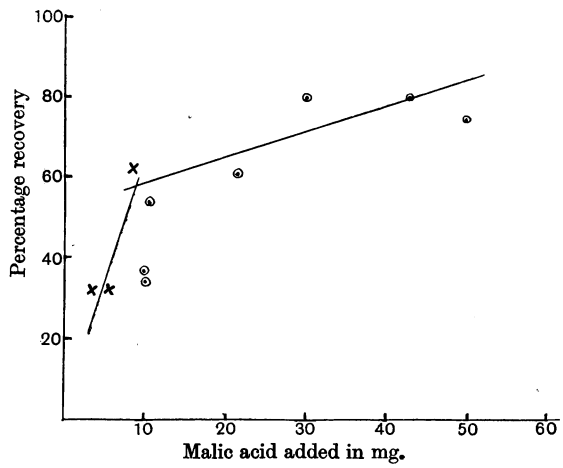

Fig. 2.

In order to test whether the silver precipitate obtained consisted only of succinate, malate and fumarate (one or more of these acids) the silver in weighed amounts was estimated.

(*a*) The silver precipitate obtained in nitrogen weighed 58·7 mg. and contained 65·1 % of silver; the calculated percentage, as the precipitate was found to contain 3·4 mg. of silver malate, is 64·7.

(*b*) The silver precipitate obtained from an initial sample weighed 54·5 mg., and this contained 64·1 % of silver. Allowing for the 5·7 mg. of silver malate contained in it, the calculated percentage was 64·5.

In this method the removal of hydroxy-acids with acid permanganate is avoided; there is therefore no risk, as in the previous method for succinic acid, of the breakdown of possible traces of 5-carbon chain acids to succinic acid in the course of the estimation.

THE EFFECT OF AEROBIC AND ANAEROBIC CONDITIONS ON THE MALIC ACID CONTENT AND ON THE TOTAL CONTENT OF THE THREE ACIDS.

Pigeon breast muscle was used in these experiments, and the arrangement was similar to that in Exps. 3, 4 and 5. As will be seen, the malic acid found per 50 g. muscle always lies below 8 mg., that is, in the region of only about 35 % recovery; in order, therefore, to compare the total amount of acids under the different conditions, this amount of malic acid is doubled, and thus can be compared with the amounts of succinic and fumaric acids found. Of the latter acids, as we have seen, about 70 % is recovered. The fumaric acid is reckoned as present to the extent of 50 % of the malic (according to Einbeck [1919] and Dakin [1922] this is roughly the ratio found at equilibrium when the acids are in the presence of the muscle enzyme). The succinic acid is found by difference.

Exp. 6. Two samples were ground immediately after removal from the animal with a mixture of equal volumes of ice-cold alcohol and ice-cold 0·7 % Na_2HPO_4, $2H_2O$; three other samples remained at 33° in four volumes of 0·7 % Na_2HPO_4, $2H_2O$, one in nitrogen for 3 hours, the second in oxygen for 1 hour and the third in oxygen for 3 hours.

	Malic acid in mg. per 100 g.	
	Found	Corrected
Initial (1)	9·7	19·4
„ (2)	12·0	24·0
In nitrogen	1	2
In oxygen 1 hour	1·5	3
„ 3 hours	1	2

Exp. 7. Temp. 37–39°; each muscle sample in two volumes of 1·4 % Na_2HPO_4, $2H_2O$.

11.45 a.m. Flasks filled with nitrogen.
1.45 p.m. N 1 removed; other flasks filled with oxygen.
2.45 „ O 1 removed; remaining flask filled with nitrogen.
3.45 „ N 2 removed.

In order to inhibit if possible the oxidation of malic acid whilst not interfering with the oxidation of succinic acid to malic acid, the addition of arsenic was tried; according to Batelli and Stern the latter oxidation is not markedly slowed until the concentration of arsenious acid reaches 1 : 1000, while the further oxidation of malic acid is inhibited at a concentration of 1 : 10,000. A fourth flask was therefore exhausted at 12.0 noon and filled with nitrogen; at 2.0 p.m. 15 mg. of arsenious acid (dissolved in a little 0·1 *N* sodium hydroxide) were added, and the atmosphere was changed to oxygen. The flask was repeatedly shaken till 3.0 p.m., when alcohol was added.

An initial sample, precipitated at once with alcohol and an equal volume of phosphate solution, was also worked up.

	Malic acid found in mg. %	Malic acid corrected in mg. %	Total silver in cc. KCNS %	Amounts of acids calculated in Ag ppt. in mg. %	Total acid with malic corrected in mg. %
Initial	6·3	12·6	37	6·3 malic 6·3 fumaric 12·2 succinic	31·1
N 1	1	2	80	1·0 malic 1·0 fumaric 47·8 succinic	50·8
O 1	2·4	4·8	40	2·4 malic 2·4 fumaric 20·6 succinic	27·8
N 2	Nil	Nil	72	44·6 succinic	44·6
With arsenic	2·0	4·0	32	2·0 malic 2·0 fumaric 16·4 succinic	22·4

Exp. 8. This experiment was like the previous one, except that hydrogen, passed slowly through two pyrogallol bottles, was used for obtaining the anaerobiosis. Temp. 35°; three 60 g. lots, each suspended in 130 cc. 1·4 % Na_2HPO_4, $2H_2O$. An initial sample was also worked up.

1.10 p.m. Flasks filled with hydrogen.
3.10 „ H 1 removed; oxygen introduced.
4.0 „ O 1 removed; hydrogen introduced.
4.45 „ H 2 removed.

	Malic acid found in mg. %	Malic acid corrected in mg. %	Total silver in cc. KCNS %	Amounts of acids calculated in Ag ppt. in mg. %	Total acid with malic corrected in mg. %
Initial	9·3	18·6	57·3	9·3 malic 9·3 fumaric 18·0 succinic	46
H 1	1	2	77	1·0 malic 1·0 fumaric 46·0 succinic	49
O 1	2	4	25	2·0 malic 2·0 fumaric 11·8 succinic	17·8
H 2	1·2	2·4	53	1·2 malic 1·2 fumaric 30·7 succinic	34·3

Exp. 9. In this experiment the method of double silver precipitation was used; in other details the procedure was the same as for Exp. 8, except that an extra hydrogen and an extra oxygen period were introduced.

1.0 p.m. Flasks filled with hydrogen.
3.0 „ H 1 removed; oxygen introduced.
3.35 „ O 1 removed; hydrogen introduced.
4.10 „ H 2 removed; oxygen introduced.
4.45 „ O 2 removed; hydrogen introduced.
5.20 „ H 3 removed.

	Malic acid found in mg. %	Malic acid corrected in mg. %	Total silver in cc. KCNS %	Amounts of acids calculated in Ag ppt. in mg. %	Total acid with malic corrected in mg. %
Initial	4	8	41·4	4·0 malic 4·0 fumaric 19·4 succinic	31·4
H 1	1	2	61	1·0 malic 1·0 fumaric 37·8 succinic	40·8
O 1	—	—	6	3·9 succinic	3·9
H 2	1	2	38	1·0 malic 1·0 fumaric 22·9 succinic	25·9
O 2	—	—	4	2·6 as succinic	2·6
H 3	—	—	12·4	8·1 as succinic	8·1

Exp. 10. The power of the minced muscle was tested as regards the oxidation of added succinic and malic acids in the later stages of incubation.

Three samples were kept in hydrogen for 2 hours; then one was removed and precipitated. Oxygen was introduced into the other two, and to one a solution containing 25·5 mg. of succinic acid and 25 mg. of malic acid (neutralised with sodium hydroxide) was added; both flasks were kept in the bath for an hour. Temp. 35°; two volumes of 1·4 % Na_2HPO_4, $2H_2O$ used.

	Malic acid found in mg. per 50 g.	Malic acid corrected	Total silver in cc. KCNS per 50 g.	Amounts of acids calculated in Ag ppt. mg. per 50 g.	Total acid with malic corrected in mg. per 50 g.
H 1	1·3	2·6	32·1	1·3 malic 1·3 fumaric 18·5 succinic	22·4
O 1	0·2	0·4	3·0	0·2 malic 0·2 fumaric 1·65 succinic	2·25
O + 25 mg. malic + 25·5 mg. succinic	5·8	11·6	20	5·8 malic 5·8 fumaric 2·1 succinic	19·5

Discussion.

Perhaps the most important result of this work has been the evidence gained of the removal in oxygen of part of the succinic acid (probably by transformation into carbon dioxide and water, or at any rate into a substance not precipitated by silver in 36 % alcohol) and the renewed formation of the acid in the absence of oxygen. The explanation which may be put forward for the present is that accumulation of the acid up to a certain point hinders further formation; possibly there is a back reaction according to the mass action law. In oxygen when the accumulation is removed renewed formation takes place, and *in vivo* we may suppose that a continuous production and removal go on. As to the nature of the precursor in the muscle, there is at present no information.

Two suggestions may be made as to the cause of the greater removal in oxygen of the three acids at later stages in incubation. One is that oxidation is in some way facilitated; the other that in the early stages production of the acids is more vigorous, falling off later. The latter explanation seems the more

likely. There is no reason to suppose that production ceases in oxygen, and that its activity falls off as time goes on is indicated by the progressively lower maxima on return to nitrogen. Further, according to Batelli and Stern [1911, 2], the power of the muscle to oxidise added malic and fumaric acids, far from becoming greater in course of time, falls off rapidly after 1 or 2 hours. In one experiment in the present work, out of 40 mg. added to 50 g. of muscle at the beginning of the third hour, only 21 mg. had been oxidised away by the end of the hour; the muscle therefore is very near the limits of its oxidising capacity, and the fact that the maximum in nitrogen can be practically completely removed in oxygen at this stage must not be taken as a sign that the muscle can do much more than this. It would be interesting to test the degree of oxidation of quantities of acid added at different times throughout incubation.

When this work was begun, nothing was known of the concentration of malic and fumaric acids occurring in muscle; the amounts of succinic acid under varying conditions had been ascertained [Moyle, 1924], and it was known from the work of Einbeck and of Dakin that, in the presence of the muscle enzyme, the equilibrium between fumaric and malic acids lay at about 70 % of the latter. But the equilibrium concentration under various conditions of succinic acid and its two oxidation products had never been investigated. It was hoped that possibly conditions might be found favourable to the oxidation of succinic acid to malic and fumaric acids, but unfavourable to the oxidation of the latter acids; in this way, by accumulation of these oxidation products, evidence might be obtained of succinic acid metabolism. In spite of the probability, however, that the oxidation of malic and fumaric acids falls off considerably an hour or two after the death of the animal, while the oxidation of added succinic acid can certainly go on vigorously for many hours, it has not so far been possible to obtain any accumulation of malic acid under aerobic conditions. Even the use of arsenious acid had no effect. This is probably because production of the acids falls off.

The disappearance, when the muscle is transferred to nitrogen, of the malic acid initially present shows that the change from succinic acid to malic is a reversible one. This was to be expected from the experiments of Thunberg [1925], of Batelli and Stern [1921] and of Ahlgren [1925] with added malic acid in the presence of methylene blue or of thionine.

The comparatively high concentration of malic acid in the fresh muscle is interesting; probably the more *post mortem* change were avoided, the higher would be the concentration found, as during the necessary manipulation the muscle suffers oxygen deprivation. In this connection, it is interesting to notice that Einbeck [1914] prepared fumaric acid from fresh beef; he obtained a yield of 2·8 mg. per 100 g. of muscle, but considerably more was probably present, as the method he used of silver precipitation in acid solution is not quantitative. The concentration of malic acid in the oxygenated samples never reaches that found in the initial sample; probably the conditions in the living muscle for production and oxidation of the acids cannot be, or have not yet been, successfully imitated *in vitro*.

SUMMARY.

1. A method has been worked out for the estimation of malic acid in muscle, and of the total amount of succinic, fumaric and malic acids taken together.

2. When minced muscle, suspended in buffer solution, is placed alternately under anaerobic and aerobic conditions, the succinic acid content rises in the nitrogen, falls in the oxygen, rises again in the nitrogen, and so on.

3. With similarly repeated changes of atmosphere, the total amount of succinic, fumaric and malic acids taken together rises in anaerobiosis and falls on oxygenation.

4. The malic acid content is highest in the fresh muscle (about 12–18 mg. per 100 g.); it falls to practically nothing in nitrogen, and on admission of oxygen it may rise, but very slightly.

5. When succinic acid disappears in oxygen it is oxidised further than to fumaric and malic acids; and when the succinic acid maximum is renewed in nitrogen, the latter acid is formed from some other source than reversibly from fumaric and malic acids.

6. The results also suggest that production of succinic acid is hindered by accumulation of the acid, and make possible the hypothesis that *in vivo* production and oxidation go on continuously.

I wish to express my sincere thanks to Sir F. G. Hopkins for his most kind interest and encouragement in this work.

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