55. THE OXIDATION OF PYRUVATE IN PIGEON BREAST MUSCLE

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PYRUVATE is very readily oxidized in animal tissues, yet little is known about the immediate products of its oxidation. Such oxidative reactions of pyruvate as are known to occur—dismutation, formation of succinate, acetate or ketone bodies—are side reactions whose significance varies from tissue to tissue: in no tissue can these reactions account for the total oxidation, and in some tissues, such as muscle or kidney, they account for even less than 20%.

We have tried to elucidate the oxidation of pyruvate in pigeon breast muscle by studying in detail the factors governing the oxidation. We find that fumarate acts as catalyst in the oxidation of pyruvate and that malonate breaks the catalysis when one molecule of pyruvate has reacted with one molecule of fumarate. The following reaction takes place in the presence of malonate:

(1) Fumarate + pyruvate + $2O_2$ = succinate + $3CO_2$ + H_2O.

In the absence of malonate this action is followed by the oxidation of succinate:

(2) Succinate + $\frac{1}{2}O_2$ = fumarate + H_2O.

The net effect of 1 and 2 is the complete oxidation of pyruvate:

Pyruvate + $2\frac{1}{2}O_2$ = $3CO_2$ + $2H_2O$.

In reaction 1 the succinate does not arise from fumarate by anaerobic reduction. It is formed from fumarate and pyruvate by a series of oxidative processes of the type formulated in the theory of the "citric acid cycle" [Krebs & Johnson, 1937, 1]. This theory is supported by the fact that a change of the experimental conditions directs reaction 1 in such a way as to yield citrate (about 15%) or α-ketoglutarate (about 50%) instead of succinate and CO_2. Up to the present the citric acid cycle is the only theory accounting for the experimental observations in pigeon breast muscle.

I. *Principle of the procedure*

Suspensions of minced muscle are shaken in Warburg manometer cups for periods varying between 20 min. and 4 hr., with and without pyruvate, fumarate, malonate or other substances. The disappearance of O_2 and pyruvate and the production of CO_2, succinate and other products are determined under varying conditions.

To make the quantities of the different metabolites directly comparable they are all expressed in terms of μl. gas, one μmol. being taken as equivalent to 22,400 μl.

Throughout the paper we write "fumarate" when in fact the system fumarate ⇌ *l*(+)malate is present, and "citrate" when in fact the system citrate ⇌ *cis*aconitate ⇌ *iso*citrate [see Johnson, 1939] is present.

Most of the results are presented in the form of tables, but of each series only a few experiments which we consider typical are included. The full experimental details are given in section XIX.

II. *Aerobic disappearance of pyruvate*

Minced pigeon breast muscle, suspended in phosphate saline and shaken in an atmosphere of O_2, removes added pyruvate with great rapidity. We find, for instance, that 4 ml. muscle suspension containing 267 mg. fresh muscle (or approximately 53 mg. dry tissue) remove 868 μl. or 3·4 mg. pyruvate in 1 hr. The rate of pyruvate utilization gradually decreases with time (Fig. 1 and Table 1). It ceases in the course of the third or fourth hour of the experiment, at the same time as does the respiration of the muscle. Under similar conditions the rate of the anaerobic removal of pyruvate, due to dismutation [Krebs & Johnson, 1937, 2] or to reactions with triosephosphate [see Meyerhof & Kiessling, 1935] amounts to no more than 5–10 % of the aerobic removal.

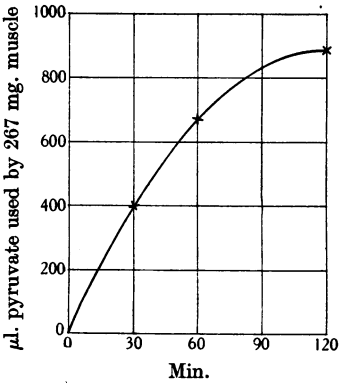

Fig. 1. Pyruvate utilization in minced pigeon breast muscle. (For details see Table 1.)

The best medium for experiments on the utilization of pyruvate is calcium-free phosphate saline. Plain phosphate buffer as used in previous experiments [Krebs & Eggleston, 1938] is definitely inferior, while phosphate buffer containing 0·01–0·06 M $MgCl_2$ is only slightly inferior to phosphate saline. Calcium when present in concentrations occurring in blood serum strongly inhibits the removal of pyruvate. These results are similar to those of Stare & Baumann [1936], Greville [1937] and Elsden [1939] who studied the effects of various media on the respiration of muscle. Examples and further experimental details are shown in Table 1.

Table 1. *Pyruvate utilization in pigeon breast muscle*

40°; O_2; 267 mg. fresh tissue in 4 ml. medium. The braces indicate sets of experiments carried out on the same muscle suspension and therefore directly comparable.

Medium	Period of incubation min.	Initial pyruvate in medium μl.	Final pyruvate in medium μl.	Pyruvate used μl.
Phosphate saline	120	1752	648	1104
" "	60	1716	974	742
" "	30	1708	1308	400
" "	60	1708	1030	678
" "	120	1708	820	888
Phosphate buffer (0·1 M; pH 7·4)	80	1734	1404	330
" " (0·1 M; pH 7·4) + 0·0025 M $MgCl_2$	80	1734	1184	550
" " (0·1 M; pH 7·4) + 0·005 M $MgCl_2$	80	1734	1120	614
Phosphate saline	80	1734	1005	729
Phosphate saline 0·0025 M $CaCl_2$	80	1734	1655	79

III. *O_2 consumption in the presence of pyruvate*

The O_2 consumption of muscle suspensions usually increases when pyruvate is added. The increase is small in the early periods of the experiment; it becomes more pronounced after 30 or 60 min. when the initial rate of respiration begins to fall in the control experiments. This fall is delayed by pyruvate as shown in Fig. 2 and Table 2.

Table 2. *O_2 consumption of minced pigeon breast muscle in the presence of pyruvate*

Suspension used	Conc. of pyruvate	μl. O_2 used after 30 min.	60 min.	120 min.
267 mg. muscle in 4 ml. phosphate saline	0·02 *M*	860	1665	2870
	0	815	1412	2165
267 mg. muscle in 4 ml. phosphate saline	0·02 *M*	1000	1885	—
	0	780	1270	—
267 mg. muscle in 4 ml. phosphate saline	0·01 *M*	890	1552	1725
	0	740	965	1015

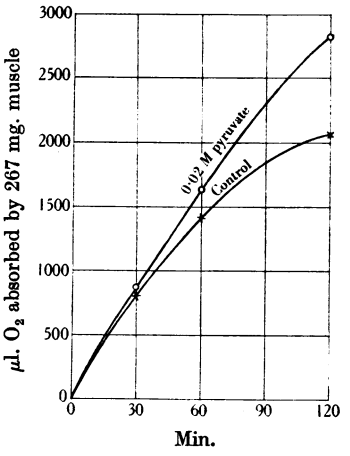

Fig. 2. O_2 consumption in the presence of pyruvate. (For details see Table 2.)

IV. *CO_2 production and respiratory quotient*

The CO_2 production increases even more than the O_2 uptake when pyruvate is added. Consequently the R.Q. rises, for example from 0·922 to 1·184 (Table 3).

Table 3. *Respiratory quotient in the presence of pyruvate*

4 ml. muscle suspension (267 mg. fresh muscle in phosphate saline; 40°); period: 60 min.; method of Warburg & Yabusoe [1924].

	No substrate added	0·02 *M* pyruvate added
μl. pyruvate used	—	742
μl. O_2 used	1270	1885
μl. CO_2 formed	1177	2237
R.Q.	0·922	1·184
Ratio $\frac{O_2 \text{ used}}{\text{Pyruvate used}}$	—	2·54
Ratio $\frac{CO_2 \text{ formed}}{\text{Pyruvate used}}$	—	3·01

The latter figure, being very close to 1·20 (i.e. the quotient calculated for the oxidation of pyruvate), suggests that pyruvate, if available, is the only substrate undergoing oxidation and this conclusion is borne out by the determination of pyruvate: for each mol. of pyruvate 2·54 mol. O_2 are absorbed (calculated 2·50) and 3·01 mol. CO_2 are liberated (calculated 3·0). These figures, it should be noted, represent the total gas exchange of the tissue. The extra gas exchange

caused by the addition of pyruvate is much too small to account for the removal of the pyruvate and we must therefore conclude that the added pyruvate competes for the O_2 with the substrates normally present in the tissue and suppresses their oxidation. In section XII we shall refer again to this inhibitory effect of pyruvate on the oxidation of other substrates when we discuss experiments in which pyruvate is used as an inhibitor of the oxidation of citrate and α-ketoglutarate.

V. *Effect of malonate*

Malonate inhibits the aerobic utilization of pyruvate (Table 4). The utilization is reduced to 8% by 0·025 *M* malonate and to about 23% by 0·001 *M* malonate. Most, if not all, of the removal of pyruvate remaining in the presence of 0·025 *M* malonate is due to anaerobic reactions which are not appreciably inhibited by malonate (see Table 4).

Table 4. *Effect of malonate on the removal of pyruvate*

4 ml. muscle suspension (267 mg. in phosphate saline); pyruvate added from side arm after 10 min., malonate added from the start; initial pyruvate concentration 0·02 *M*; 40°.

Malonate added final conc.	Gas	μl. pyruvate used after 1 hr.	μl. pyruvate used after 3 hr.
0	O_2	556	793
0·001 *M*	O_2	40	184
0·025 *M*	O_2	40	38
0	N_2	—	84
0·001 *M*	N_2	—	94
0·025 *M*	N_2	—	52

VI. *Effect of fumarate*

Fumarate abolishes the inhibitory effects of malonate. The extent of the fumarate effect depends on the relative concentrations of malonate and of fumarate: if the malonate concentration is low (approximately 0·001 *M*) its

Table 5. *Effect of fumarate on the removal of pyruvate at low and high concentrations of malonate*

4 ml. muscle suspension; 0·02 *M* pyruvate; pyruvate and fumarate added from side arm 10 min. after the addition of malonate.

Further substances added (final conc.)	Absolute quantity of fumarate in μl.	μl. pyruvate used after (min.) 30	60	120	180	μl. extra pyruvate used on addition of fumarate
Exp. I. Low malonate concentrations						
None	—	—	556	—	793	—
0·0025 *M* fumarate	56	—	672	—	928	135
0·001 *M* malonate	—	—	40	—	184	—
0·001 *M* malonate; 0·000625 *M* fumarate	56	—	436	—	492	308
0·001 *M* malonate; 0·00125 *M* fumarate	112	—	578	—	768	584
0·001 *M* malonate; 0·0025 *M* fumarate	224	—	666	—	928	744
Exp. II. High malonate concentration						
None	—	456	868	1028	1046	—
0·0025 *M* fumarate	224	452	740	1044	1005	—
0·025 *M* malonate	—	—	76	80	92	—
0·025 *M* malonate; 0·0025 *M* fumarate	224	168	244	292	313	221

inhibitory effect is completely removed by the addition of 0·0025 *M* fumarate. If the malonate concentration is high (approximately 0·025 *M*) addition of fumarate only restores the utilization of an equivalent quantity of pyruvate. These facts which are of decisive importance for the theory of pyruvate oxidation are illustrated by Table 5.

In the first experiment (Table 5) the inhibition caused by 0·001 *M* malonate is completely removed by 0·0025 *M* fumarate, but lower concentrations of fumarate have also great effects on the pyruvate utilization. This becomes clear when the quantity of fumarate present is compared with the effect produced: 1 mol. of fumarate causes an extra utilization of more than 5 mol. of pyruvate when the fumarate concentration is 0·000625 *M*.

In the second experiment (Table 5) where the malonate concentration is 0·025 *M* the addition of 224 μl. fumarate causes an additional pyruvate consumption of 221 μl. Further examples showing the equivalence of the amounts of fumarate added and pyruvate removed are given in Table 6. At high concentrations of fumarate the pyruvate removal is smaller than expected (because

Table 6. *Effect of fumarate on the disappearance of pyruvate in the presence of malonate*

O_2; 4 ml. muscle suspension; pyruvate added: 896 μl.

Concentration of malonate *M*	Time min.	μl. pyruvate used (in the absence of fumarate)	μl. fumarate added	μl. pyruvate used in the presence of fumarate	Extra pyruvate used / Fumarate added
0·0125	100	158	112	294	$\frac{136}{112}=1{\cdot}21$
0·0125	100	158	224	398	$\frac{240}{224}=1{\cdot}07$
0·0125	100	158	448	642	$\frac{484}{448}=1{\cdot}08$
0·025	80	27	56	78	$\frac{51}{56}=0{\cdot}91$
0·025	80	28	112	174	$\frac{146}{112}=1{\cdot}31$
0·05	120	130	224	299	$\frac{169}{224}=0{\cdot}75$
0·05	120	130	448	449	$\frac{319}{448}=0{\cdot}71$
0·05	120	130	896	499	$\frac{369}{896}=0{\cdot}41$
0·01	170	172	224	413	$\frac{241}{224}=1{\cdot}08$
0·01	170	172	448	592	$\frac{420}{448}=0{\cdot}94$
0·01	170	172	896	564	$\frac{392}{896}=0{\cdot}44$
0·01	115	99	56	165	$\frac{66}{56}=1{\cdot}18$
0·01	115	99	112	229	$\frac{130}{112}=1{\cdot}16$
0·01	115	99	224	380	$\frac{281}{224}=1{\cdot}25$
0·01	115	99	448	595	$\frac{496}{448}=1{\cdot}11$

of the gradual disappearance of the respiration in the experiments which makes it impossible to continue them over sufficiently long periods).

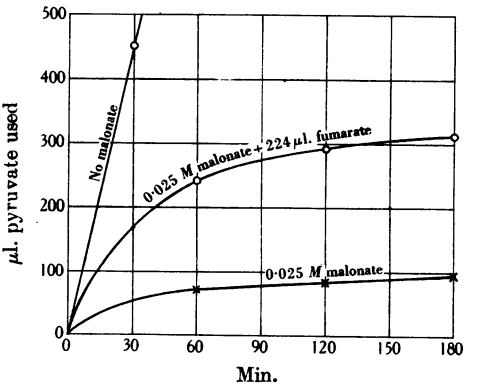

Fig. 3. Effect of malonate and fumarate on pyruvate utilization. Fumarate restores the utilization of an equivalent quantity of pyruvate. (For details see Table 6.)

The experiments recorded in Table 5 represent the two extreme, and therefore clear-cut types, of malonate inhibition. If intermediate concentrations of malonate (0·005 *M*) are used, a rapid oxidation of 1 mol. of pyruvate is followed by a slow disappearance of further molecules.

VII. *O_2 uptake in the presence of fumarate*

Szent-Györgyi has shown that fumarate increases the O_2 usage of muscle suspensions, especially in the presence of malonate. When the malonate concentration is low (0·001 *M*) the additional O_2 uptake caused by fumarate bears no quantitative relation to the amount of fumarate added. Fumarate acts like a catalyst and the extra O_2 uptake may therefore amount to a multiple of the quantity required for its complete oxidation. The position is however entirely different when the concentration of malonate is high (0·0025 *M*). In this case we find the additional O_2 uptake caused by fumarate to be proportional to the quantity of fumarate added. About 2–2½ mol. O_2 are taken up per mol. of fumarate (Table 7).

Table 7. *O_2 uptake in the presence of fumarate and malonate*

4 ml. muscle suspension.

Substrates added	μl. O_2 absorbed after					Extra O_2 absorbed / Fumarate added
Exp. I. (0·025 *M* malonate):	20 min.	40 min.	60 min.	80 min.		
None	69	107	129	156		—
896 μl. pyruvate	77	116	139	163		—
112 μl. fumarate	268	351	384	412		2·29
896 μl. pyruvate; 112 μl. fumarate	240	328	359	384		1·97
56 μl. fumarate	167	221	245	263		1·92
896 μl. pyruvate; 56 μl. fumarate	161	213	237	257		1·68
Exp. II. (0·01 *M* malonate):	20 min.	45 min.	65 min.	85 min.	115 min.	
869 μl. pyruvate	35	45	62	77	102	—
869 μl. pyruvate; 56 μl. fumarate	136	198	205	222	247	2·59
869 μl. pyruvate; 112 μl. fumarate	199	324	356	380	411	2·76
869 μl. pyruvate; 224 μl. fumarate	287	571	669	717	756	2·91
869 μl. pyruvate; 448 μl. fumarate	299	728	987	1180	1341	2·77

Since the complete oxidation of fumarate requires 3 mol. O_2 the oxidation of fumarate in the presence of 0·025 *M* malonate is incomplete. This incomplete oxidation, as will be shown later, can be formulated as follows:

(3) Fumarate + about $2O_2 + x \rightarrow$ succinate + about $2CO_2$,

where x is pyruvate, if available, otherwise an endogenous substrate such as lactate, or another triose derivative.

VIII. *Formation of succinate from fumarate*

As was first shown by Szent-Györgyi, succinate accumulates in respiring muscle poisoned by malonate. The amount of succinate formed is small when no substrate is added to the muscle; it increases slightly when pyruvate is added [Krebs & Johnson, 1937, 2; Weil-Malherbe, 1937] and rises very considerably when fumarate is added [Szent-Györgyi, 1935].

We found by varying the experimental conditions that the yield of succinate after addition of fumarate can be brought up to 80–100% (in terms of the fumarate added). The highest yields of succinate were obtained if fumarate and pyruvate were added together and if the solution was analysed after the absorption of approximately 2–3 mol. O_2 per mol. of fumarate. An example and further details are given in Table 8.

Table 8. *Formation of succinate from fumarate and pyruvate in the presence of malonate*

0·0125 *M* malonate; 4 ml. muscle suspension; O_2 in Nos. 1–8; N_2 in No. 9.

	1	2	3	4	5	6	7	8	9
Substrates added		840 µl. pyruvate	112 µl. fumarate	840 µl. pyruvate 112 µl. fumarate	224 µl. fumarate	840 µl. pyruvate 224 µl. fumarate	448 µl. fumarate	840 µl. pyruvate 448 µl. fumarate	840 µl. pyruvate 448 µl. fumarate
µl. O_2 absorbed after									
20 min.	52	57	286	286	415	462	445	424	Anaerobic conditions
40 min.	83	88	377	370	635	603	820	805	—
60 min.	100	107	416	406	731	689	1020	995	—
80 min.	114	121	—	—	—	—	1070	1035	—
µl. pyruvate found	—	716	—	562	—	450	—	278	732
µl. pyruvate used	—	124	—	278	—	390	—	562	108
µl. pyruvate used due to added fumarate	—	—	—	154	—	266	—	438	—
µl. succinate found	18	29	103	140	186	230	286	392	94
µl. succinate due to added fumarate	—	—	85	111	164	201	264	363	—
Fumarate added/ extra pyruvate used/ extra succinate formed				1/1·35/0·99		1/1·19/0·90		1/0·98/0·81	

In interpreting these findings one must bear in mind that a direct reduction of fumarate to succinate is inhibited by malonate (see Table 8, anaerobic experiment). Hence the conversion of fumarate into succinate in the presence of malonate cannot be explained by an anaerobic reduction and it follows that there must be a second mechanism (which is oxidative, and unaffected by malonate) whereby fumarate is converted into succinate.

Before discussing the nature of this mechanism, we report an experiment in which another of the end-products of reaction 3, viz. CO_2, was quantitatively determined.

IX. *CO_2 production in the presence of fumarate and malonate*

In this experiment the R.Q. was measured in the presence of malonate by Warburg & Yabusoe's [1924] method. At the same time the decrease in pyruvate and the increase in succinate were determined. The results are shown in Table 9.

Table 9. *R.Q., pyruvate usage and succinate production in the presence of malonate (0·025 M)*

4 ml. muscle suspension; 100 min.

No.	Substrates added	μl. O_2 used	μl. CO_2 formed	μl. pyruvate used	μl. succinate formed
1	224 μl. fumarate	686	736	—	149
2	886 μl. pyruvate	129	110	25	20
3	224 μl. fumarate + 896 μl. pyruvate	606	778	165	207
	Metabolism due to addition of 10^{-2} m*M* fumarate in the presence of pyruvate (obtained by deduction of No. 2 from No. 3)	476 ($2{\cdot}13 \times 10^{-2}$ m*M*)	668 ($2{\cdot}98 \times 10^{-2}$ m*M*)	140 ($0{\cdot}63 \times 10^{-2}$ m*M*)	187 ($0{\cdot}84 \times 10^{-2}$ m*M*)

Per mol. of fumarate added 2·13 mol. of O_2 and 0·63 mol. of pyruvate are used, and 2·98 mol. of CO_2 and 0·84 mol. of succinate are formed. These results together with those reported in the preceding sections may be summed up with reasonable approximation by the equation

(1) $\text{Fumarate} + \text{pyruvate} + 2O_2 = \text{succinate} + 3CO_2 + H_2O.$

The deviations from this equation which occur in some experiments may be explained either by side reactions, viz.

(4) $\text{Fumarate} + \frac{1}{2}O_2 \rightarrow \text{oxaloacetate} \rightarrow \text{pyruvate} + CO_2,$
(5) $\text{Lactate (or triose)} + \frac{1}{2}O_2 \rightarrow \text{pyruvate},$

or by the incomplete inhibition of succinic dehydrogenase. The latter would permit the oxidation of succinate and the repetition of reaction 1. This will occur when the concentration of malonate is comparatively low.

X. *Theory of the aerobic formation of succinate from fumarate*

At this stage it may be of use for the guidance of further experiments to discuss the theory of the reactions described in the preceding section. Szent-Györgyi [1935], who observed the aerobic formation of succinate from fumarate in the presence of malonate and realized that this could not be explained by the anaerobic reduction of fumarate, proposed the hypothesis of the "over-reduction" of oxaloacetate. He assumed that fumarate is first oxidized to oxaloacetate and that the latter is then "over-reduced" to succinate:

$$\text{Fumarate} \xrightarrow{+\frac{1}{2}O_2} \text{oxaloacetate.}$$
$$\text{Oxaloacetate} \xrightarrow{\text{"over-reduction"}} \text{succinate.}$$

"Over-reduction" is defined as a process by which oxaloacetate is directly reduced to succinate without the intermediation of fumarate or malate. Since this is an *ad hoc* hypothesis for which no evidence whatsoever has as yet been adduced, it cannot be regarded as a satisfactory explanation.

Another explanation is contained in the theory of the citric acid cycle [Krebs & Johnson, 1937, 1] which postulates the following reactions:

$$(6)\quad \text{Fumarate} \xrightarrow{+O} \text{oxaloacetate} \xrightarrow{+\text{pyruvate}+O} \text{citrate} + CO_2 \xrightarrow{+O} \alpha\text{-ketoglutarate} + CO_2 \xrightarrow{+O} \text{succinate} + CO_2.$$

The net effect of this series is equation 1.

When this theory was first proposed [Krebs & Johnson, 1937, 1] it was based on three different observations. The first showed that oxaloacetate yields succinate in the presence of O_2 and malonate, i.e. under conditions preventing the anaerobic reduction. The second demonstrated the formation of citrate from oxaloacetate under anaerobic conditions. The third showed that the oxidation of citrate to α-ketoglutarate and succinate is very rapid and that it is therefore permissible to ascribe to these substances the role of intermediate metabolites.

Of these three observations the first was the most important because it was incompatible with Szent-Györgyi's theory of the catalytic function of the system

$$\text{Fumarate} \rightleftharpoons \text{oxaloacetate}.$$

It postulated a new mechanism for the formation of succinate. The other two observations served to support the theory that the postulated reactions are those formulated in the citric acid cycle. In themselves these two observations did not constitute direct evidence in favour of the occurrence of the cycle in normal respiration, since they could also be interpreted as side reactions of minor importance. The first, however, proved conclusively that there is an oxidative reaction leading to the formation of succinate.

This is confirmed by the experiments reported in the preceding sections of this paper and summarized by reaction 1. The citric acid cycle is so far the only theory which explains reaction 1. At the same time there is no observation which contradicts the theory. Scheme 6 is further supported by the fact that the conclusions drawn from it are confirmed by experiment as will be shown in the next sections.

XI. *Succinate formation from further substances in the presence of malonate*

If scheme 6 is correct, the intermediate stages between fumarate and pyruvate on the one side, and succinate on the other, should all form succinate under the conditions in which reaction 1 is found to take place. That this is qualitatively the case is already known from earlier papers by Szent-Györgyi [1935], Martius & Knoop [1937] and Krebs & Johnson [1937, 1], but the previous experiments do not yet answer the quantitative aspect of this postulate, according to which the yields of succinate must be no less, and rates of its formation no lower than those found in the presence of fumarate and pyruvate. To fill in this gap we have carried out further experiments (Table 10) which show that the rates of the postulated reactions, as measured by the rates of O_2 absorption and the yields of succinate, are of the expected order. This of course does not prove that the reactions are actually intermediate stages. Theoretically it remains possible that there are alternative routes, but no other path is as yet supported by experimental evidence. We have tested those substances which might possibly be expected to be alternative intermediates in reaction 1, namely acetate, acetoacetate, aspartate, citramalate, citraconate, mesaconate, alanine, α-hydroxyglutarate, β-hydroxybutyrate, tiglate, crotonate, acrylate, and none of them

Table 10. *Succinate formation and O_2 uptake in the presence of malonate and various substrates*

267 mg. muscle in 4 ml. phosphate buffer (0·1 *M*; *p*H 7·4) containing 0·005 *M* $MgCl_2$ and 0·01 *M* malonate.

No.	Substrates added	µl. Succinate formed	µl. O_2 absorbed
1	None	37	118
2	224 µl. fumarate 896 µl. pyruvate	226	640
3	224 µl. oxaloacetate 896 µl. pyruvate	172*	439
4	224 µl. citrate	204	280
5	224 µl. α-ketoglutarate	—	214

* The lower yield of succinate in the presence of oxaloacetate can be explained by the side reaction 4.

was found to behave like an intermediate. Either they are not oxidized at all, or they fail to yield succinate in the presence of malonate. Little can therefore be said at present for alternative theories.

XII. *Formation of α-ketoglutarate and citrate*

To test the validity of scheme 6 in another and more direct way we tried to break this series of reactions at stages prior to the formation of succinate. This was achieved by making use of the competitive inhibition by pyruvate mentioned in section IV of this paper. Pyruvate, as was shown in section IV, is preferentially oxidized when several substrates are present; in other words it suppresses the oxidation of other substrates and an excess of pyruvate would therefore be expected to cause an accumulation of intermediates. The experiments show that this is the case if fumarate together with an excess of pyruvate is added. α-Ketoglutarate appeared in quantities reaching about 50 % of the maximum to be expected and citrate in quantities reaching about 15 %. The details describing the conditions under which these yields are obtained are given in Tables 11–14.

Table 11. *Effect of pyruvate on the accumulation of α-ketoglutarate in the presence of fumarate*

4 ml. muscle suspension; 60 min.; the yields are calculated in terms of fumarate added.

Substrates added	µl. O_2 used	µl. α-Ketoglutarate formed
None	993	0
0·01 *M* fumarate (=896 µl.)	1620	109 (12 % yield)
0·01 *M* fumarate; 0·01 *M* pyruvate	1460	204 (23 % yield)
0·01 *M* fumarate; 0·02 *M* pyruvate	1500	237 (26 % yield)

Table 12. *Time course of the accumulation of α-ketoglutarate in the presence of pyruvate and fumarate*

4 ml. muscle suspension; 0·0025 *M* fumarate (224 µl.); 0·02 *M* pyruvate; yields calculated as in Table 12.

Time min.	µl. O_2 used	µl. Succinate formed	µl. α-Ketoglutarate formed
30	740	0	114 (51 % yield)
60	1260	7	114 (51 % yield)
120	1670	17	118 (53 % yield)

Table 13. *Effect of fumarate concentration on the formation of α-ketoglutarate*

4 ml. muscle suspension; 0·03 *M* pyruvate; 60 min.

Conc. of fumarate	μl. fumarate added to 4 ml.	μl. α-ketoglutarate formed
0·0025 *M*	224	132 (59% yield)
0·005 *M*	448	174 (39% yield)
0·01 *M*	896	272 (30% yield)

Table 14. *Formation of citrate in the presence of pyruvate and fumarate*

4 ml. muscle suspension; O_2.

Substrates added	Time of incubation min.	μl. citrate found	Yield of citrate as % of fumarate added
None	60	~0	—
0·02 *M* pyruvate	60	24	—
0·02 *M* pyruvate; 0·01 *M* fumarate	60	49	5·5
0·02 *M* pyruvate	40	4	—
0·02 *M* pyruvate	40	17	—
0·02 *M* pyruvate; 0·01 *M* fumarate	40	55	6·1
0·02 *M* pyruvate	90	7	—
0·02 *M* pyruvate; 0·0025 *M* fumarate	90	38	17·0
0·02 *M* pyruvate; 0·005 *M* fumarate	90	57	12·7
0·005 *M* fumarate	90	14	—

That fumarate must be added together with pyruvate becomes obvious when the nature of the "preferential oxidation" of pyruvate is considered in detail. The "preferential" oxidation applies in the first instance to the primary step of the oxidation, that is, according to the citric acid cycle, the reaction

$$\text{Oxaloacetate} + \text{pyruvate} \xrightarrow{+O} \text{citrate}.$$

This reaction will come to a standstill when all available oxaloacetate is used up. After this such other substrates as are available, for instance citrate, will undergo oxidation, and pyruvate will not be attacked again before the cycle has been completed. Since the concentration of oxaloacetate (or its precursors) is low in normal muscle, no appreciable accumulation of intermediates can be expected: pyruvate is oxidized to completion (Table 3). Only after addition of oxaloacetate (or its precursors) can accumulation of citrate and α-ketoglutarate take place. The experimental results are thus in perfect agreement with the theory.

XIII. *Aerobic formation of succinate from fumarate in the absence of added pyruvate*

Even when no pyruvate has been added to the tissue, fumarate yields, though in smaller quantities, succinate (Table 8), α-ketoglutarate (Table 11) and citrate (Table 14). We assume that these substances are formed in the same way, i.e. by reaction 1 in the presence and in the absence of added pyruvate and that the pyruvate required in reaction 1 is formed in the tissue from carbohydrate derivatives. Pyruvate is in fact formed with great rapidity when oxaloacetate is added anaerobically, as discovered by Szent-Györgyi [1936], according to the reaction:

(7) Oxaloacetate + "triosephosphate" → fumarate + pyruvate.

Recent work makes it probable that "triosephosphate" is 1:3-glyceraldehyde diphosphate [Negelein & Brömel, 1939].

The somewhat lower yield of succinate, referred to above, is probably due to the side reaction 4. This side reaction which causes a loss of the C_4-chain will be prevented when pyruvate is present in excess, as the pyruvate will react with the oxaloacetate at once when it is formed.

XIV. *The role of pyruvate oxidation in normal respiration*

The significance of reaction 1 depends on the part which it plays in the normal respiration of the tissue. It is therefore of interest to examine the relations between pyruvate oxidation and normal respiration; if the former is part of the latter, factors controlling the oxidation of pyruvate should also play a part in the normal respiration. Among these fumarate and malonate are the most conspicuous factors. It is already known from Szent-Györgyi's work that the normal respiration of pigeon breast muscle, like the oxidation of pyruvate, is inhibited by malonate and promoted by fumarate. At low concentrations of malonate (0·001 *M*) added fumarate acts as a catalyst and abolishes the malonate inhibition [see also Stare & Baumann, 1936]. Further experiments were however necessary to investigate the effect of fumarate quantitatively, especially at high concentrations of malonate. We find again a complete analogy between normal respiration and pyruvate oxidation: both reactions are *temporarily* restored by added fumarate. The period of the restoration is proportional to the quantity of fumarate added; it ends in the case of normal respiration when about 2·5 mol. per mol. of fumarate are absorbed. This is shown in Table 15 and in Fig. 4. It

Table 15. *Effect of fumarate on the O_2 uptake in the presence of malonate*

4 ml. muscle suspension; malonate concentration in Exp. I: 0·0125 *M*; in Exp. II: 0·025 *M*.

		Exp. I			
Quantity of fumarate added (μl.)	—	112	224	448	0 (No malonate)
μl. O_2 absorbed after					
10 min.	45	187	265	230	270
20 min.	77	304	516	530	540
40 min.	118	409	735	970	1065
70 min.	146	494	854	1255	1440
90 min.	164	526	915	1332	1650
100 min.	171	542	936	1368	1720
μl. succinate formed	32	132	161	262	0
		Exp. II			
Quantity of fumarate added (μl.)	—	56	112	224	—
μl. O_2 absorbed after					
20 min.	44	157	238	294	—
40 min.	72	206	320	501	—
60 min.	97	237	360	587	—
100 min.	138	296	440	665	—
μl. extra O_2 absorbed on addition of fumarate	—	148	302	527	—
$\frac{\text{Extra } O_2}{\text{Fumarate}}$	—	2·65	2·69	2·36	—

will be seen that the curve of the O_2 uptake bends sharply after the absorption of approximately 2·5 mol. of O_2 but continues to be a little steeper than the control without fumarate. The theory of malonate inhibition (see later) explains

this behaviour. The last column of Table 15 shows that the greater part of the added fumarate is converted into succinate in the course of the oxidation. It is further of importance that the rate of the restored oxidation is of the same order as that of normal respiration.

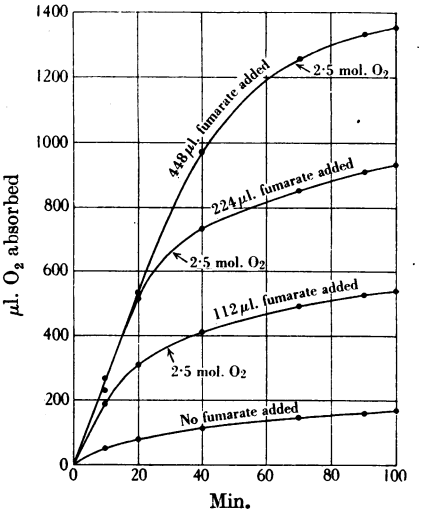

Fig. 4. Effect of fumarate on the O_2 uptake in the presence of malonate (0·0125 *M*). (For details see Table 15.)

The facts thus indicate that the oxidation of pyruvate, by way of reaction 1, is part of the normal respiration in pigeon muscle. It is of course the accepted view that carbohydrate is the chief substrate in muscle respiration and that pyruvate is an intermediate in the oxidation of carbohydrate, but it is a different matter whether the specific reaction 1 is part of the normal respiration. The comparison of this reaction and the total respiration permits the conclusion that reaction 1 represents in fact the bulk of the normal carbohydrate respiration. The latter only comprises two further reactions, namely:

$$C_3H_6O_3 \text{ (lactate, or triose or triosephosphate)} + O \rightarrow \text{pyruvate}$$

and

$$\text{Succinate} + \tfrac{1}{2}O_2 \rightarrow \text{fumarate}.$$

XV. *The evidence in favour of the citric acid cycle*

On the grounds of the facts discussed in section VIII we postulated that there is in muscle tissue an oxidative process whereby fumarate and oxaloacetate are converted into succinate. It has now been demonstrated that this process can be directed in such a way as to yield up to 50% α-ketoglutarate or up to 15% citrate. The high yield of the α-ketoglutarate must be considered as direct proof of its intermediate formation in reaction 1. As regards the intermediate formation of citrate the evidence is not so weighty, as the maximum yields could not be raised above 15%. It may therefore be argued that only part of reaction 1 passes through the stage of citrate. This is however an unconvincing argument, since incomplete yields must be expected owing to the rapid oxidation of the intermediates under the experimental conditions. We therefore consider the evidence in favour of the citric acid cycle as complete as can be expected for this type of reaction scheme.

The theory is further supported by the fact that certain carbohydrate derivatives and the members of the citric acid cycle are the only substances (apart from aspartate and glutamate) which are known to be readily oxidized in muscle tissue, although more than 80 substances have been tested by various workers for their oxidation since Thunberg [1910] realized that this method may provide a suggestion as to whether a substance is an intermediate metabolite.

It is also of interest to recall that Batelli & Stern [1911] concluded from their experiments as early as 1911 that the oxidations of citrate, fumarate and malate form part of the chief respiratory process in muscle tissue. The metabolic relations between carbohydrate and these acids were necessarily obscure to Batelli & Stern, but the correctness of their conclusion is fully borne out by modern development.

When the theory was first formulated the compound reacting with oxaloacetate to form citrate was provisionally termed "triose" or "carbohydrate derivative" [Krebs & Johnson, 1937, 1]. In the second paper [Krebs, 1937, 1] "pyruvic acid (?)" was inserted. We can now omit the query since it has been shown that (*a*) pyruvate reacts in preference to other substances, (*b*) that such other substances as may react can be oxidized to pyruvate in the tissue.

At the present stage, it may be pointed out, the theory is no more than a skeleton scheme. Many details remain to be worked out, e.g. those concerning the synthesis of citrate or the role of phosphorylations. Some other details concerning further intermediates (*iso*citrate, *cis*aconitate, malate) and the role of the coenzymes have for brevity's sake been omitted from our considerations.

XVI. *Theory of the malonate inhibition*

Malonate is known to inhibit succinic dehydrogenase specifically [Thunberg, 1910; Quastel & Wooldridge, 1928; Gözsy & Szent-Györgyi, 1934]. The inhibition is competitive, i.e the degree of inhibition does not depend on the absolute concentration of malonate, but on the ratio succinate/malonate. Direct proof of the specificity of the inhibition in muscle tissue is the accumulation of succinate in the solutions. We find an inhibition of about 50 % when the above ratio is 9·5. The concentration of succinate (including its precursors) in our muscle suspensions is below 0·0005 *M* when no substrate has been added (as measured by the amount of succinate accumulated in the presence of malonate). Hence 0·001 *M* malonate is expected to inhibit respiration more than 90 %. When 0·0025 *M* fumarate is added (see Table 4) this will gradually be converted into succinate and the malonate inhibition of succinic dehydrogenase, though still about 50 %, will be sufficiently overcome to provide the fumarate required for the continuation of the cycle at the full rate. If high concentrations of malonate (0·025 *M*) are employed, the succinate formed will not be able to overcome the malonate barrier sufficiently and when all the added fumarate is used up, the respiration will continue at a greatly reduced rate, depending on the amount of succinate oxidized. This explains the sharp bend in the curves of Fig. 4.

XVII. *Szent-Györgyi's cycle*

The theory of the citric acid cycle is not contradictory of but supplementary to Szent-Györgyi's theory, according to which the system:

$$\text{Fumarate} \rightleftharpoons \text{oxaloacetate},$$

acts as a hydrogen carrier in carbohydrate oxidation. The bases of this theory are the following observations of Szent-Györgyi and his collaborators:

1. The C_4-dicarboxylic acids promote respiration catalytically, i.e. without being used up.

2. Respiration is inhibited by malonate.

3. Fumarate restores the respiration in the presence of malonate (as shown in this paper the restoration is incomplete when the malonate concentration is high).

4. Fumarate is rapidly oxidized to oxaloacetate, and oxaloacetate is rapidly reduced to fumarate.

5. "Triose" is the hydrogen donator for the reduction of oxaloacetate. "Triose" undergoes oxidation to pyruvate.

These facts (no. 3 with the important limitation stated above) have all been established beyond doubt. On the other hand it must be remembered that Szent-Györgyi never analysed in detail the mechanism of the oxidation of pyruvate in muscle tissue. In discussing the fate of the "hydrogen atoms of the substrates" he does not concern himself with the earlier stages of the hydrogen transport, i.e. with those reactions through which the hydrogen is released from the carbohydrate molecule (with the exception of reaction 7). The citric acid cycle thus fills a gap left open in Szent-Györgyi's work.

The oxidation of "triose" to pyruvate is certainly not the only reaction in which Szent-Györgyi's system acts as a hydrogen carrier. Several observations suggest that it plays a part in the reactions leading to the formation and oxidation of citrate. This remains to be investigated. The decisive argument against the view that Szent-Györgyi's theory fully explains the catalytic effects of fumarate is the fact that fumarate does not completely remove the malonate inhibition, but only restores a fraction of the respiration equivalent to the amount of fumarate added.

XVIII. *The citric acid cycle in other tissues*

Preliminary experiments on other tissues show that the citric acid cycle occurs in heart muscle and in kidney, and possibly in liver, but it seems that other mechanisms also play a significant role in the two latter tissues. It is doubtful however whether the citric acid cycle occurs in brain [cf. Banga *et al.* 1939] or in testis.

Experiments which show the occurrence of the citric acid cycle in cat kidney are also contained in a recent paper of Breusch [1939] who found a rapid synthesis of citrate from oxaloacetate and pyruvate under anaerobic conditions, and a rapid removal of added citrate under aerobic conditions.

XIX. *Experimental details*

1. *Muscle suspensions.* Pigeon breast muscle was cooled on ice immediately after the death of the animal and minced in the Latapie mill. Unless otherwise stated 4 g. (fresh weight) of muscle were suspended in 45 ml. ice-cooled phosphate saline, made up by mixing 1000 ml. 0·9% NaCl, 40 ml. 1·15% KCl, 10 ml. 3·84% $MgSO_4, 7H_2O$ and 300 ml. phosphate buffer, *p*H 7·4. The latter was prepared by dissolving 17·8 g. $Na_2HPO_4, 2H_2O$ and 20 ml. *N* HCl in 1 l. This medium, in accordance with the results of Stare & Baumann [1936] and of Greville [1937], was found to give higher metabolic rates than phosphate buffer + NaCl [Krebs & Eggleston, 1938] or phosphate buffer containing 0·006 *M* $MgCl_2$ [Greville, 1937].

3 ml. muscle suspension, measured with a wide-mouthed pipette, were placed in a conical manometric cup provided with a side arm and a centre chamber. Further additions (substrates, inhibitors, water) amounted to 1 ml., so that the final suspension contained 1 part of muscle in 15 parts of medium.

This degree of dilution was found to be the optimum for the work reported in this paper. Acidic substances were added in the form of neutral Na salts.

2. *Determination of pyruvate.* At the end of the experimental period 1 ml. 3*M* acetate buffer, *p*H 5·0, was added to 4 ml. muscle suspension, to stop further reactions and to adjust the *p*H. Pyruvate was then determined manometrically by the carboxylase method in an aliquot of the filtrate [see Westerkamp, 1933; Krebs & Johnson, 1937, 2]. The results are expressed in μl. CO_2 liberated from pyruvate in the presence of carboxylase.

3. *Determination of the O_2 uptake.* The centre chamber of the manometric cup contained 0·2 ml. 2*N* NaOH (unless otherwise stated), so that the O_2 uptake could be measured by the "direct" method. Since the O_2 uptake was generally very rapid, the rate of shaking was fast, 70–80 periods per min., amplitude 12 cm. Frequently the O_2 uptake exceeded the quantities which can be directly measured on the 300 mm. manometer scale. In these experiments a reading was taken when the level of the manometric fluid approached the bottom of the scale and sufficient air was then admitted to raise the pressure to the top of the scale. After a few minutes' equilibration a new reading was taken. The O_2 usage between these two readings was calculated by interpolation.

If the O_2 uptake was to be measured in the presence of added substrates, the latter were generally added from the side arm after the first reading.

4. *CO_2 production and respiratory quotient.* To determine the R.Q. the method of Warburg & Yabusoe [1924] requiring 3 cups (including 2 with two side arms) for each determination was used.

5. *Malonate inhibition.* The inhibitory effect of malonate has a certain induction period, especially when the concentration of malonate is low. To eliminate the lag, the tissue was incubated with malonate before the substrate was added, generally for 10 min. In filling the cups malonate was added directly to the muscle suspension whilst the substrate was placed in the side arm and mixed with the tissue after the first reading, i.e. after having been shaken in the water bath for 10 min.

6. *Succinate determination in the presence of malonate.* Succinic acid was determined manometrically with the aid of succinic dehydrogenase [Szent-Györgyi & Gözsy, 1936; Krebs, 1937, 2]. The muscle suspension was washed into a measuring cyclinder with water, 1 ml. 10% Na_2WO_4 and 1 ml. 0·8*N* H_2SO_4, made up to 15 ml. and filtered. An aliquot of the filtrate (about 12 ml.) was poured into a Kutscher-Steudel extractor together with 2 ml. 3% $KMnO_4$, 1 ml. 50% H_2SO_4, 5 ml. of a solution containing 50 g. $MnSO_4, 7H_2O$, 140 g. Na_2SO_4 and 15 ml. conc. H_2SO_4 per litre and placed for 5 min. in a boiling water bath. This serves to destroy the malonate which interferes with the determination of succinic acid. At the same time this treatment converts α-ketoglutarate into succinate and the results obtained are therefore the sum of succinate + α-ketoglutarate. Separate determinations of α-ketoglutarate and succinate [see Krebs, 1938] showed that at least 90% of the "succinate" found by the above method is actually succinate when malonate is used as an inhibitor. The conclusions drawn from these experiments are not affected by the fact that "succinate" included a certain fraction of α-ketoglutarate.

7. *Determination of succinate and α-ketoglutarate in the absence of malonate.* When both succinate and α-ketoglutarate were to be determined in the absence of malonate (section XII) duplicate experiments were set up, one for each analysis. In the first, succinate was determined after ethereal extraction from the deproteinized solution. The second cup was treated with $KMnO_4$ before the ethereal extraction, as described in the preceding paragraph. The results of the

succinate determination represent in this case the sum of succinate and α-ketoglutarate.

8. *Determination of citrate.* The method of Pucher *et al.* [1936] was used with the precautions described previously [Krebs & Johnson, 1937, 1]. Dioxan was used in place of pyridine as the colour stabilizer [Johnson, 1939].

Summary

1. Added pyruvate is readily oxidized by minced pigeon breast muscle. The oxidation of other substrates is inhibited when an excess of pyruvate is present. This inhibition is a "competitive inhibition".

2. The oxidation of pyruvate is inhibited by malonate.

3. Fumarate removes the malonate inhibition. The removal is complete when the malonate concentration is relatively low (0·001 *M*), but is incomplete when the malonate concentration is higher (0·025 *M*). In the latter case each molecule of added fumarate causes the removal of 1 mol. of pyruvate, whilst 2 mol. of O_2 are absorbed and 3 mol. of CO_2 produced, according to the equation:

(1) Pyruvate + fumarate + $2O_2$ = succinate + $3CO_2$ + H_2O.

4. The succinate formed in reaction 1 cannot arise by anaerobic reduction since this reaction is inhibited by malonate. Thus there must be a second route leading from fumarate to succinate which is oxidative and unaffected by malonate.

5. If an excess of pyruvate is added, together with fumarate, reaction 1 yields citrate, or α-ketoglutarate, instead of succinate:

(8) Pyruvate + fumarate + O_2 → α-ketoglutarate (yield up to 50 %).

(9) Pyruvate + fumarate + O_2 → citrate (yield up to 15 %).

6. When no pyruvate, but fumarate, is added to muscle in the presence of 0·025 *M* malonate, a reaction similar to 1 takes place:

(10) Fumarate + triose equivalent + $2\frac{1}{2}O_2$ = succinate + $3CO_2$ + $2H_2O$.

7. Reactions 1 and 10 represent the major part of the normal respiration in pigeon breast muscle.

8. Szent-Györgyi's theory of hydrogen transport by the system fumarate ⇌ oxaloacetate is accepted for the conversion of triose into pyruvate, the only reaction for which it has been proved. It is probable that this system also acts as a hydrogen carrier in the reactions which lead to the formation and to the breakdown of citrate. The theory fails however to explain the oxidation of pyruvate, because it does not account for the oxidative formation of succinate from fumarate and for the stoichiometric relations shown in reaction 1.

9. All observations are explained by the theory of the citric acid cycle which is not contradictory of but supplementary to Szent-Györgyi's theory. Reaction 1 shows that a series of reactions of the type formulated in the citric acid cycle occurs. The theory is directly supported by reactions 8 and 9. Whilst there is no doubt that the major part of muscle respiration goes through the citric acid cycle, the possibility of an alternative reaction is not excluded. This possibility is however purely theoretical and so far without any experimental support.

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