CCXIX. THE FATE OF OXALOACETIC ACID IN DIFFERENT ORGANS

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SZENT-GYÖRGYI and collaborators [1935] have shown that oxaloacetic acid (in this paper referred to as OAA) plays an essential part in the system of C_4 -dicarboxylic acids concerned with the transport of H from the donators to O_2 . OAA, by accepting H, is reversibly hydrogenated to *l*-malic acid (very likely by the yellow enzyme of Warburg) [Banga, 1937, 1, 2; Laki, 1937], and dehydrogenated back to OAA by malic dehydrogenase.

Apart from the hydrogenation to *l*-malic acid, the aqueous solution of OAA spontaneously decomposes slowly into pyruvic acid (referred to as PUA in this paper) and CO_2 , and in tissues it undergoes several other reactions. Decarboxylation of OAA is increased in presence of either surviving or heated tissue. Further, OAA and glutamic acid undergo rapid conversion in the tissue into α -ketoglutaric acid and aspartic acid [Szent-Györgyi *et al.* 1935; Braunstein & Kritzmann, 1937]. Knoop & Martius [1936] proposed a theory of the probable formation of citric acid in tissues from OAA and a substance containing three carbon atoms. A year later Orten & Smith [1937] discovered that in fact the injection of large amounts of C_4 -dicarboxylic acids brought about an increased excretion of citric acid in urine. This citric acid must have been formed as an intermediary product of the C_4 -dicarboxylic acids. On this Krebs & Johnson [1937] based a theory of the breakdown of trioses, thus combining in a cycle the system of C_4 -dicarboxylic acids of Szent-Györgyi with the reactions of formation and breakdown of citric acid according to Knoop & Martius. In my recent work on pigeon muscle [1937, 1] I was unable to confirm the formation of citric acid from OAA, and therefore I could not accept the citric acid cycle as a mechanism of metabolism in the muscle.

As in these fundamental questions there are two opposite views which need to be elucidated, it seemed advisable to make quantitative investigations to decide if the sum of the hitherto known breakdown products of the OAA corresponds within the limit of error to the added amount of OAA; it could then be concluded that other reactions and products of metabolism than those investigated could only play a secondary part.

For this purpose I have quantitatively investigated the fate of OAA in many tissues of warm-blooded animals by estimation of the following products originating from OAA: PUA, lactic acid, citric acid, *l*-malic acid.

In order to account for that part of the lactic acid which is formed from PUA, I was obliged first to examine the spontaneous formation and destruction of lactic acid in the various tissues. Experiments to determine the total amount of carbohydrate which might have been resynthesized from lactic acid did not yield any results, as no relation could be found between the total carbohydrate and the addition of OAA.

The total balance (of PUA, *l*-malic acid, lactic acid and, in kidney, also citric acid) gave in kidney, lung, spleen, placenta and peripheral nerves a practically complete recovery of added OAA. In these organs OAA and the above-mentioned substances, under my conditions, virtually do not undergo any other important reactions than those referred to.

In muscle (pigeon, cat) I found a residue of only 70 %, in liver 50 % and in brain 20 %, calculated as the sum of the above-mentioned substances. It seems that in these organs the loss is due to other changes and reactions of formed PUA, leaving dismutation according to Krebs or total combustion of the formed lactic acid as alternatives.

Following these balance investigations I investigated the various enzymes taking part in the metabolism of OAA. In the first place the enzymic hydrogenation of OAA to *l*-malic acid, secondly the enzymic condensation of OAA and PUA to citric acid in the kidney, thirdly, the transamination of glutamic acid with OAA and, finally, the decarboxylation of OAA to PUA were investigated.

EXPERIMENTAL

All experiments were performed on the organs of cats and pigeons; the animals were killed by a blow on the head and subsequently bled. The organs were instantly taken out, minced in the Latapie mill, weighed and the minced tissue instantly transferred into the already prepared solutions.

Every investigation was started 3–5 min. after the death of the animal except in the case of peripheral nerve, the separation of which (2.5 g. per animal) took about 15 min. Because of the insufficient quantity of nerve tissue obtained after passage through the Latapie mill, I had to resort to mincing the nerves with a pair of fine scissors.

As I found that the same organs of different animals showed metabolic deviations up to 100 %, all balance investigations were always carried through in one experiment. In those cases where the weight of one organ proved to be insufficient, another or several others of the same kind were used, care being taken to kill the animals at the same time and to mix the minced tissues thoroughly.

As preliminary experiments, to be described later, had shown the following conditions to give the most satisfactory results, these conditions were maintained: 5 g. minced tissue, suspended in 30 ml. Ringer-phosphate solution [Banga & Szent-Györgyi, 1937] containing 50 mg. OAA (neutralized in not more than 0.5 % solution), were shaken in 200 ml. Erlenmeyer flasks under aerobic conditions, the temperature of the thermostat being kept at 38°. Small glass funnels prevented evaporation.

After 2 hr. shaking, the precipitation of proteins was carried through with trichloroacetic acid for the estimation of citric acid, while for all other substances tungstate was used. Quantitative tests for OAA, PUA, *l*-malic acid, lactic acid and citric acid were then performed.

The analytical methods used were the following: OAA, PUA and *l*-malic acid were determined according to Szent-Györgyi & Straub [Szent-Györgyi *et al.* 1935], lactic acid according to Friedemann *et al.* [1927] in the apparatus of Fuchs [1933], citric acid gravimetrically after Cometiani [1931], as described by Breusch [1937, 1]; in the present study a modification of the latter method was used, in which the precipitate of pentabromoacetone after filtration on a glass filter was dried in a non-evacuated desiccator over P_2O_5 at room temperature.

The OAA used was synthesized according to Wohl & Österlin [1901] and melted at 150–152°.

To allow for the absorption by precipitated protein of the estimated substances, a correction of 20 %, added to the found values, was made according to the findings of Straub [cf. Szent-Györgyi *et al.* 1936]. Repeating these investigations, I found that the loss due to adsorption was also 20 % in the case of the dilutions used here, with deviations of ± 5 %.

In order to find out whether lactic acid was formed at all after addition of OAA it was necessary to test the formation of lactic acid with and without OAA. Furthermore, it had to be tested whether and to what extent lactic acid added to tissue was oxidized.

The results are shown in Tables I and II.

Table I. *Tissue incubated without addition of lactic acid*

Organ	Found mg. lactic acid	
	Initial	After 2 hr.
Muscle, cat	18	32
Muscle, pigeon	27	45
Liver	5	8.8
Kidney	6	4.4
Spleen	8	10
Lung	7	8
Placenta	6	11.4
Brain	11	9.7
Nerve (periph.)	5	3.3
Pancreas	5	5

Table II. *Tissue incubated with addition of 30 mg. lactic acid*

Organ	Found mg. lactic acid	
	Initial	After 2 hr.
Muscle, cat	44	60
Muscle, pigeon	56	74
Liver	34	32
Kidney	31	33
Spleen	34	37
Lung	33	33
Placenta	31	32
Brain	34	31
Nerve (periph.)	33	34
Pancreas	—	—

No organ except muscle shows a considerable spontaneous increase of lactic acid. Little added lactic acid disappears from any organ. Special experiments showed that addition of fumaric acid (which originates from OAA in the metabolism of some tissues) does not change the balance of lactic acid to any appreciable degree. Within the limit of error under these conditions the values are the same with and without addition of fumaric acid.

In Table III column 2 shows the amount of OAA still present after 2 hr. incubation, column 3 the formed fumaric+*l*-malic acid; column 4 the citric acid; column 5 the PUA formed. Column 6 contains the total lactic acid; column 7 the lactic acid formed without addition of OAA (see Table I); column 8 the lactic acid formed from OAA, and column 9 the sum of all products found, calculated as OAA. Column 10 gives the percentage of OAA recovered from added OAA.

According to the rapid equilibrium between *l*-malic and fumaric acid in the presence of fumarase in every tissue [Breusch, 1937, 2] the equivalent of fumaric

Table III. *Quantities (mg.) present after 2 hr. incubation*

1	2	3	4	5	6	7	8	9	10
Organ	OAA	Fumaric + <i>l</i> -malic acid	Citric acid	PUA	Total lactic acid	Lactic acid Table I	Lactic acid from OAA	Sum as OAA	% recovery OAA
Liver	0	17.1	0	2.4	12.6	8.8	3.8	26.3	52.6
Kidney	0	25.3	10	1.0	8	4.4	3.6	44.0	88.0
Muscle, pigeon	0	15.2	0	3	56	45	11	35.5	71.0
Muscle, cat	0	6.7	0	10	42	32	10	36.6	73.2
Brain	0	5.2	0	3.5	8.5	9.7	0	10.3	20.6
Pancreas	0	6.5	0	20.0	7	5	2	39.3	78.6
Lung	0	0	0	28	10	8	2	45.0	90.0
Spleen	0	0	0	31	12.2	10	2.2	48.8	97.6
Placenta	0	0	0	27.6	12.4	11.2	1.2	42.4	84.8
Nerve (periph.)	0	0	0	28.3	5.4	3.3	2.1	45.6	91.2

acid formed was added to the value of *l*-malic acid. The same procedure was used in the case of citric acid, corresponding to the equilibrium ratio of the enzyme aconitase [Martius, 1938], citric acid/*isocitric* acid = 9/1.

The result of these investigations is as follows. All organs accelerated the decarboxylation of OAA. In all tissues OAA completely disappeared after 2 hr.

Only five of the organs investigated, i.e. muscle, liver, kidney, pancreas and brain, are able to hydrogenate OAA to *l*-malic acid. In these five organs only, is this step of redoxiation of the C₄-dicarboxylic system used for the transport of H. In brain I did not always find a reduction of OAA; some brains used immediately after death did not show a measurable formation of *l*-malic acid.

Thus the relation is revealed, that a tissue either has or has not at all the ability to reduce OAA and PUA. This result establishes connexion with results of Green *et al.* [1937], who found that the oxidoreduction between PUA and OAA on the one hand, and C₃-compounds on the other, is brought about by the same group of mutases.

The brain stands midway between these two groups of organs; it combines a poor capacity for reducing OAA with a great ability to metabolize PUA [Peters, 1936; Peters & McGowan, 1937; Long, 1938; Barron & Lyman, 1939; Annau & Mahr, 1937; Weil-Malherbe, 1937; McGowan, 1937; Elliot *et al.* 1937; etc.]; in this case only a very small part of the PUA disappearing is recovered as lactic acid. PUA also disappeared in liver and kidney without giving rise to detectable amounts of lactic acid [cf. Deutike & Jens, 1938; Annau & Mahr, 1937].

In confirmation of my previous work [Breusch, 1937, 1] I could find no ability to synthesize citric acid in any kind of muscle; namely breast and stomach muscle of pigeons and skeletal and heart muscle of cats. It was only in kidney that after incubation with OAA I observed formation of citric acid. After addition of larger amounts of OAA and PUA together and incubating under anaerobic conditions in some organs (liver, brain and lung) a small amount of citric acid could be detected. This will be discussed in the section on citric acid formation. Even under these most favourable conditions I did not find any citric acid formation in any of the muscles examined.

As can be seen from column 9 of Table III, within the limit of analytical error, all metabolites of OAA originating from the metabolism of the following organs can be recovered: kidney, lung, placenta, spleen and peripheral nerves, giving thus a practically quantitative balance of the decomposition of OAA. Other reactions of OAA which may be found in future can be regarded as of minor importance in these organs.

No such quantitative result is revealed by the analysis in brain (loss of 80 %), in liver (loss of 50 %) and in muscle (loss of 30 %). In these organs a part of the PUA, or of the lactic acid formed from the latter, seems to be totally burned or to be transformed into other products.

The reduction of OAA to l-malic acid

Banga [1937, 1] was the first to try to isolate the complex of enzymes transporting the H from the donator to OAA. By extracting the fresh minced muscle with ice-cold water, centrifuging the extract, and precipitating with ice-cold acetone she succeeded in isolating this complex and found it to be highly sensitive. A somewhat modified method was employed by Green *et al.* [1937].

I found that the system responsible for reduction of OAA is nearly quantitatively extracted by ice-cold alkaline phosphate solution and that it can be precipitated by saturating with ammonium sulphate.

The method is as follows: immediately after the death of the animal 50 g. pigeon muscle are minced in an ice-cold Latapie mill, the minced tissue ground in an ice-cold mortar with 200 ml. ice-cold 1 % Na_2HPO_4 for 10 min. and then pressed through a cotton cloth. The impure filtrate is centrifuged for 10 min. in ice-cold tubes, and the supernatant then filtered through cotton-wool. The almost clear filtrate is saturated with crystallized ammonium sulphate and the precipitated protein filtered in the ice-box through two filter papers (3 hr.). After one washing with sat. ammonium sulphate the filter with the wet precipitate is dried by squeezing it out between filter papers. The whole precipitate is dissolved in Ringer-phosphate solution and freed from the small insoluble residue by filtration.

This solution is stable in the ice-box for about 6–10 hr. It is virtually free from donators; and incubation with OAA alone does not yield *l*-malic acid. If the protein precipitate is not washed with sat. ammonium sulphate it still contains sufficient coenzyme; but after it has been washed, coenzyme must be added in order to obtain reduction to *l*-malic acid after addition of a donator.

Banga [1937, 1] found hexosediphosphate and monophosphate, Green *et al.* [1937] triosephosphate to be effective H donators, as well as glycerophosphate. I can confirm this, but in my enzyme preparation glycerophosphate was not dehydrogenated. Further, I found phosphoglyceric acid and, to a small degree, dihydroxyacetone to be effective donators. Glucose, fructose, lactic acid, phospholactic acid, PUA, glycerol, glycerophosphate (α - and β -), β -hydroxybutyric acid, *isocitric* acid, α -ketoglutaric acid and α -hydroxyglutaric acid did not show any donator activity; *dl*-glyceraldehyde even inhibited.

The *pH* optimum of OAA-reduction I found to be in the range of *pH* 8–8.5. For examining the influence of dilution with Ringer-phosphate solution on the minced tissue suspension the conditions given in Table IV were used.

Table IV

10 g. of minced breast muscle of pigeons and 100 mg. neutralized OAA in 10 ml. Ringer-phosphate solution were shaken at 38° for 1 hr. under aerobic conditions; the dilution by further addition of Ringer-phosphate can be seen below.

Dilution	×2	×3	×4	×5	×6	×7	×8
Found <i>l</i> -malic acid (mg.)	10	16	19	18	15	13	7

The amount of *l*-malic acid formed is practically unaffected by as much as 7-fold dilution.

The influence of the quantity of added OAA on the formation of *l*-malic acid is shown in Table V.

Table V

Conditions: 5 g. minced kidney tissues of cats; addition of 5, 10, 15, 20, 30, 50 mg. neutralized OAA in 20 ml. Ringer-phosphate solution; 1 hr. anaerobically with shaking; at 38°.

Addition mg. OAA	5	10	15	20	30	50
Fumaric + <i>l</i> -malic acid formed (mg.)	4.3	8.0	10.5	11.0	17.0	25.3
As % of added OAA	86	80	70	55	57	50

With increasing concentration the percentage amount of fumaric + *l*-malic acid formed decreases.

As already shown by Szent-Györgyi and collaborators, the reduction of OAA is a reaction of the zero type; i.e. even minimum concentrations of OAA are metabolized.

As, under physiological conditions, the concentration of OAA is very low (it cannot be detected and must, therefore, be lower than 1 mg. per 100g.), practically

all is reduced; only by adding amounts of OAA far in excess of the physiological limits do decarboxylation to PUA and, in kidney, condensation to citric acid, come into action.

Citric acid formation from OAA

Since I could not confirm the establishment of the citric acid cycle of Krebs & Johnson [1937] as a main step in tissue metabolism [Breusch, 1937, 1], it seemed important to undertake a closer investigation into the citric acid formation in tissues.

As can be seen in Table III, it is only minced kidney which, incubated with OAA alone, can form citric acid; while anaerobically in the presence of larger amounts of OAA and PUA, liver, brain and lung also form small amounts of citric acid. Muscle under the same conditions did not yield any analysable amount of citric acid.

According to the theory of Knoop citric acid is formed by condensation of OAA and PUA and subsequent oxidation of one C-atom. I therefore investigated which C₃- or C₂-compounds when incubated together with OAA and kidney tissue gave a higher value for citric acid than OAA alone. Among all the compounds tried, I only found PUA, glyceric acid, dihydroxyacetone and, to a very small degree, phosphoglyceric acid to be effective (Table VI).

Table VI

The conditions used were as follows: to 5 g. minced kidney tissue and 15 ml. Ringer-phosphate are added 100 mg. OAA and 50 mg. of the acid to be tested, neutralized in 15 ml. Ringer-phosphate solution. With 100 mg. OAA alone and minced kidney tissue I found in 2 hr., aerobically, 12 mg. citric acid; the Table shows the increase of citric acid formation due to the addition of the various substances:

Additions	Increase of citric acid formation %	Additions	Increase of citric acid formation %
Glycerophosphate, α - and β -	0	Glyceric aldehyde	Inhibition
Lactic acid	0	β -Hydroxybutyric acid	0
Phospholactic acid	0	Glyceric acid	100
Acetic acid	0	Pyruvic acid	200
Acetaldehyde	0	Dihydroxyacetone	100
Ethanol	0	Phosphoglyceric acid	50
Glycolaldehyde	0	Glycerol	0

These results confirm the theory of Knoop & Martius [1936], according to which citric acid formation from OAA and PUA happens not only *in vitro* but also in the organism. Dihydroxyacetone, glyceric acid and, to a lesser extent, phosphoglyceric acid, also have this ability: whether directly or after transformation into PUA remains unsolved.

No other acid can replace OAA in the formation of citric acid. Under the conditions of my experiments all the substances examined without addition of OAA were unable to form citric acid: succinic acid, fumaric acid, *l*-malic and *dl*-malic acid, β -hydroxybutyric acid, butyric acid, maleic acid, glutamic acid, α -ketoglutaric acid and all substances with 2 or 3 C-atoms known to be normally present in tissue.

As I did not find any of the C₄-dicarboxylic acids other than OAA effective, it seems certain that, in the experiments of Orten & Smith [1937] concerning the transformation of injected C₄-dicarboxylic acids into citric acid excretable in the urine, these reactions must have passed through the stage of transformation into OAA.

The same applies to the investigations of Simola [1938], who found that injected β -hydroxybutyric acid causes an increased citric acid excretion in urine. In a paper of Kühnau [1928], which has unfortunately aroused insufficient attention, this author has shown that liver partly transforms β -hydroxybutyric acid into C_4 -dicarboxylic acids.

The failure to obtain citric acid by incubating kidney with fumaric + *l*-malic acids is probably due to the fact that under my conditions the capacity for re-oxidizing *l*-malic acid into OAA is slower than the reaction leading to the formation of *l*-malic acid from OAA. Thus a sufficiently high concentration of OAA was never reached to yield citric acid.

The possibility of isolating a substance from tissue depends on the ratio of enzymic velocities. A substance will accumulate if the synthesizing enzymes act more rapidly than the catabolizing ones. This may be the case in muscle, where, in contradiction to the citric acid cycle of Krebs, I could not detect citric acid formation; here citric acid is perhaps more quickly broken down than formed. I therefore investigated the ability of different organs to consume citric acid (Table VII).

Table VII

Conditions: 5 g. minced tissue and 30 mg. citric acid in 30 ml. Ringer-phosphate solution; (a) immediately analysed, (b) after 2 hr. aerobic incubation with shaking; at 38°.

Organ	Immediately analysed mg. citric acid	After 2 hr. incubation mg. citric acid	Disappearance %
Liver	26	0	100
Muscle, cat	25	21	16
Kidney	26	12	58
Brain	27	22	19
Lung	27	23	16
Spleen	25	22	12

In all the organs 1/6 to 1/7 of added citric acid disappears; this is [Breusch, 1937, 1] due to a balance between citric and *isocitric* acids. This was confirmed by Martius [1939], who by exact analysis with plant aconitase found the equilibrium to be at 10 % *isocitric* acid and 90 % citric acid. The enzyme "aconitase" is not identical with fumarase [Breusch, unpublished; Martius, 1938; Johnson, 1939; da Cunha & Jacobsohn, 1939]. Johnson found the balance to be at 80 % citric acid, 16 % *isocitric* acid and 4 % aconitic acid; a value better corresponding to those obtained by myself than to those of Martius.

It is only in liver and kidney, that citric acid disappears to a far greater amount than that corresponding to this equilibrium.

Though muscle proves in the Thunberg test to have a great ability to dehydrogenate *isocitric* acid, it is not able to break down citric acid to an appreciable extent.

In pigeon muscle the breakdown of citric acid is variable and seems to be dependent on the season; I found that in summer about 20 %, in winter 30–40 %, citric acid disappeared.

In confirmation of these results Martensson [1938] showed in perfusion experiments, using the cat's hind leg, that in the living muscle also, citric acid practically does not disappear from the perfusion fluid; on the contrary small amounts (of the order of μ g.) are formed. Contrary to the behaviour of muscle, in the perfused liver and kidney large quantities of citric acid disappear, thus exactly corresponding to my results.

As has been proved by two completely different methods, muscle neither

forms nor breaks down appreciable amounts of citric acid. Therefore the inability to find citric acid in muscle after incubation with OAA cannot be due to a quick combustion of citric acid first formed.

To inhibit the combustion of citric acid, perhaps already formed, the experiments were repeated under the most favourable anaerobic conditions adding OAA and PUA to various tissues. Condensation of OAA with PUA into citric acid occurs in kidney tissue anaerobically also; the removal of the extra C-atom, formed during condensation, seems to be brought about either by dismutation with a keto-acid according to Krebs; or the condensation product is also oxidized to pentabromacetone during analysis and thus estimated as citric acid.

Under these conditions different organs show a small ability to condense and forming citric acid. Even under these most favourable conditions all kinds of muscle show no citric acid formation.

Table VIII

Conditions: 5 g. fresh minced organ were put with 15 ml. Ringer-phosphate solution into a cylindrical evacuable vessel, fitted with a ground end and stopcock. In a small inner tube, standing in the large vessel, were placed 100 mg. OAA and 50 mg. PUA, precisely and separately neutralized to litmus, in 15 ml. Ringer-phosphate. The whole was closed and evacuated for 3 min. with a motor-oil pump. Then the stopcock was closed, the acids mixed *in vacuo* with the tissue suspension by turning and the whole anaerobically incubated for 1 hr. at 38° and then instantly deproteinized with trichloroacetic acid. A control with the acids and Ringer-phosphate solution alone was treated under the same conditions.

The different organs yielded the following synthesis:

Organ	Kidney	Liver	Pigeon muscle	Cat muscle	Lung	Brain	Spleen	Placenta	Intestine
mg. citric acid formed	28	2	0	0	1	1	0	0	0

Table VIII shows that, even under these most favourable conditions, only kidney synthesizes citric acid to a large extent; liver, brain and lung do so to a small extent.

To see which of the competing reactions of OAA, the reduction to *L*-malic acid or the condensation to citric acid, has the advantage in kidney tissue, I investigated (Table IX) the dependence on the concentration of added OAA of the formation of *L*-malic acid and citric acid.

Table IX

Conditions: 5 g. minced kidney and varying amounts of OAA, neutralized in 30 ml. Ringer-phosphate solution, shaken 1 hr. anaerobically at 38°.

Added amount of OAA mg.	Found <i>L</i> -malic + fumaric acid mg.	Found citric acid mg.
5	4.3	0
10	9.0	0
15	10.5	0
20	12.0	0
30	17	1
50	29	2
100	40	12
200	45	27

Under physiological conditions the reduction of OAA to *L*-malic acid happens practically quantitatively, as already shown by Szent-Györgyi & Banga. Only with concentrations of OAA far beyond the physiological content of tissue is

citric acid formed in kidney by condensation of OAA and the PUA which is formed from it.

For metabolism, that is the transport of H from trioses to cytochrome, it seems that only the reduction of OAA to *l*-malic acid and not the formation of citric acid is important, the latter according to my results only happening in the presence of a surplus of OAA.

The formation of citric acid in kidney seems to be a means by which the organism is able to get rid of a surplus of the C_4 -dicarboxylic acids and to excrete them; since the C_4 -dicarboxylic acids are not themselves excretable in the urine [Flaschenträger & Bernhard, 1929; 1937; Balassa, 1937]. Citric acid is a constant constituent of urine; everyone excretes 100–300 mg. per day [Östberg, 1931; Fürth *et al.* 1934; Sherman *et al.* 1936].

The formation of citric acid in the kidney appears to be a mechanism by which the level of C_4 -dicarboxylic acids in the tissues is kept constant, since these C_4 -dicarboxylic acids, according to our increasing knowledge, are the most important carriers of H from the trioses to cytochrome. Furthermore, the formation of citric acid in kidney seems to be a mechanism by which the organism synthesizes α -ketoglutaric acid (a breakdown product of citric acid) which, according to Braunstein & Kritzmann [1937], plays an important part as carrier of NH_3 in the formation and breakdown of amino-acids.

By these experiments the results of Orten & Smith [1937], who found an increase of citric acid excretion after injection of the various C_4 -dicarboxylic acids, are elucidated as a mechanism for the removal of surplus C_4 -dicarboxylic acids.

I have attempted to determine the properties of the enzyme forming citric acid in the kidney. The pH optimum is at the neutral point. The dependence of citric acid formation on dilution is shown by the following experiment.

Table X

Conditions: 5 g. minced kidney and 100 mg. neutralized OAA in 5, 10, 20, 40 and 100 ml. Ringer-phosphate solution were shaken aerobically for 1 hr. at 38°.

Ringer-phosphate (ml.)	5	10	20	40	100
Dilution of tissue	$\times 2$	$\times 3$	$\times 5$	$\times 9$	$\times 21$
Citric acid formed (mg.)	19	18	19	20	10

The amount of citric acid formed under the conditions of the experiment is independent of dilution up to a $\times 9$ dilution.

The citric acid formation is not inhibited by $M/15$ NaF or by $M/1000$ HCN. $M/2000$ arsenious acid inhibits it 50 %, $M/750$, 70 %. $M/100$ HCN and $M/750$ iodacetate inhibit 100 %.

It is very difficult to bring the condensation system into solution. Neither alkaline, neutral, nor acid phosphate solutions are effective. The enzyme is destroyed by heating in the water-bath. I propose to call this enzyme "citrogenase".

Condensation of OAA with glutamic acid

Added OAA disappears very quickly from tissue after addition of *l*(+)-glutamic acid, as found by Szent-Györgyi & Banga. It is very likely that this reaction occurs by the transaminating enzyme of Braunstein & Kritzmann [1937, 1939], yielding aspartic and α -ketoglutaric acids from OAA and glutamic acid. I investigated (Table XI) the capacity of some organs to catalyse this reaction.

Table XI

Conditions: To 2 g. minced organ in 5 ml. Ringer-phosphate 25 mg. OAA and 50 mg. *l*(+)-glutamic acid, neutralized in 10 ml. Ringer-phosphate were added; the mixture was incubated for exactly 10 min. at 38°, then deproteinized with tungstate and OAA determined colorimetrically. In control experiments without glutamic acid decarboxylation and reduction of OAA were determined and subtracted.

The following Table shows how many mg. of OAA are metabolized under these conditions in 10 min. by 1 g. wet tissue.

Cat muscle	Liver	Kidney	Lung	Cat embryo muscle	Brain	Washed erythrocytes
7 mg.	5 mg.	8 mg.	8 mg.	5 mg.	7 mg.	1 mg.

Unlike the other enzymes this enzyme is present in nearly the same amount in all organs. Erythrocytes contain only one-seventh as much as the other organs; serum none.

For the isolation of the enzyme lung tissue is especially suitable because of its low content of dehydrogenases [Breusch, 1937, 2].

50 g. of fresh minced lung of cats were allowed to stand for 30 min. in 200 ml. Ringer-phosphate solution, stirring from time to time. After centrifuging, the red-brown fluid was filtered through cotton-wool, precipitated with an equal volume of saturated solution of ammonium sulphate and filtered through filter paper in an ice-box. The whole active principle goes into the filtrate. The clear filtrate is saturated with ammonium sulphate, and the second newly formed precipitate filtered on filter paper in an ice-box. The precipitate is dried by pressing it between filter paper and dissolved in a Ringer-phosphate solution. A further purification can be obtained by repeated precipitation with ammonium sulphate up to 66% saturation. By repeated precipitation with ammonium sulphate the activity decreases rapidly, perhaps owing to the elimination of a coenzyme.

In watery solution the enzyme is active at room temperature for about 24 hr., in the ice-box for some days. The *pH* optimum lies at the neutral point. Activity is destroyed by heating to 100°. The enzyme is not inhibited by *M*/200 arsenious acid, or by 0.1*M* NaF and *M*/1000 iodoacetic acid. *M*/1000 HCN inhibits 40%.

The transamination is only given by free glutamic acid; glutathione, containing as an end group glutamic acid with a free amino group, does not react. As von Euler *et al.* [1939] showed, not only *l*(+)-glutamic acid, but also, to a smaller extent, *d*(-)-glutamic acid reacts.

Decarboxylation of OAA to PUA

Mayer [1914] first found that tissues contain a substance which is extractable by water, is resistant to heating and accelerates the breakdown of OAA to PUA. After having found in preliminary experiments that the whole of the active substance goes into the extract during boiling, I quantitatively investigated the ability of different organs to decarboxylate OAA to PUA.

10 g. of minced organ in 10 ml. Ringer-phosphate solution were boiled for 20 min. in a water bath, cooled, centrifuged and filtered. Of the filtrate 25 ml. were incubated at 38° with 50 mg. OAA, neutralized in 10 ml. H₂O. Every 5 min. 5 ml. were analysed for OAA. The control was done with Ringer-phosphate and OAA alone.

Under these conditions I found the power of decarboxylation to be the same for liver, muscle, kidney and brain. Extract from 1 g. of fresh tissue at pH 7.7 is able to decarboxylate 1.5–3 mg. OAA in addition to the spontaneous decarboxylation of OAA. This ability varies up to 100 % in the corresponding organs of different cats.

According to the theories of Langenbeck [1936] on enzyme models it could perhaps be expected that the carboxylase activity might be due to an amine. I therefore investigated the ability of a number of biologically important amines to effect this additional decarboxylation of OAA.

To 40 mg. neutralized OAA in 25 ml. Ringer-phosphate were added 2 mg. of the neutralized substance in 5 ml. H_2O ; incubation was at 38° and a part was analysed after every hour.

Under these conditions I found the following substances to be ineffective: adenosine, adenylic acid (from yeast and from muscle), vitamin B_1 , histamine, acetylcholine and diethylamine. A low rate of decarboxylation was shown by adenine (20 % increase) and methylamine (80 % increase).

Furthermore, I examined the influence of pH on the spontaneous decarboxylation of OAA in watery solution.

The conditions were as above; to the solution were added 5 ml. 1 % HCl, 5 ml. H_2O and 5 ml. 1 % NaOH to the respective vessels.

In acid solution decarboxylation happens about twice as quickly as in alkaline and is about 50 % more rapid than in neutral solution (Fig. 1).

Fig. 1. Decarboxylation of oxaloacetic acid at 38° .

Wieland [1924], by measuring the CO_2 production, found no detectable decarboxylation during 2 hr. at room temperature. With this method I too found no decarboxylation: analysing the amount of OAA directly however in every case decrease of OAA was found; analysing also for PUA an equivalent increase was noted.

While neutralizing OAA in concentrated solutions, as already shown [Breusch, 1937, 1], citric acid is formed up to 10% yield by autocondensation. As Knoop has proved that citric acid is formed by shaking OAA and PUA in very weakly alkaline solution with air, it is always necessary in experiments with tissue and OAA to allow for this error by blank experiments with OAA and without tissue under the same conditions.

The neutral salts of the two isomerides of OAA, hydroxyfumaric and hydroxymaleic acid, show no difference in their behaviour in presence of tissue.

The order of loss of activity of the different enzymes

Immediately after the death of an animal there begins (under usual conditions) a slow destruction of the enzymes taking part in the metabolism of the cell.

To obtain a quantitative idea of this disturbance I investigated the stability of the enzymes under the conditions used in this paper.

5 g. of minced kidney were allowed to stand in 25 ml. Ringer-phosphate solution at 18 and 38°. After 1, 2, 3, 4 and 6 hr. the substrate to be investigated was neutralized in 5 ml. of Ringer-phosphate solution, added to the minced tissue suspension and incubated for 30 min. at 38°. The suspension was then deproteinized and analysed.

The ordinates in Figs. 2 and 3 give the amounts of substrate in mg. metabolized by 1 g. of wet tissue in 10 min.; the abscissae give the times of incubation after the death of the animal and mincing the tissue.

Fig. 2. At 18°.

Fig. 3. At 38°.

The enzyme of OAA reduction is the most easily destroyed; that responsible for condensation of citric acid is destroyed nearly as quickly. At 38° destruction is about five times more rapid than at 18°.

The enzyme which condenses OAA with glutamic acid is more resistant. The thermostable substance which accelerates decarboxylation of OAA does not disappear to a perceptible extent under my conditions.

SUMMARY

The quantitative fate of added oxaloacetic acid has been investigated in incubation experiments, using different freshly minced tissues of cats and pigeons.

In spleen, lung, placenta and peripheral nerves added oxaloacetic acid is recovered almost quantitatively as pyruvic acid; in kidney almost quantitatively as a mixture of *L*-malic acid, pyruvic acid, lactic acid and citric acid. Deficiencies are found in muscle (about 30%), liver (50%), and brain (80%).

Spleen, lung, placenta and peripheral nerves, like embryonic tissue and tumour tissue, are unable to hydrogenate oxaloacetic acid to *l*-malic acid; they merely decarboxylate the oxaloacetic acid to pyruvic acid. As they are also virtually unable to reduce pyruvic acid to lactic acid, nearly the whole oxaloacetic acid is recovered in these organs as pyruvic acid.

Muscle, liver, kidney and pancreas reduce oxaloacetic acid to *l*-malic acid, at the same time decarboxylating a part of the oxaloacetic acid to pyruvic acid and reducing this partly to lactic acid.

Brain hydrogenates oxaloacetic acid only very little. Its main action is decarboxylation and only a small part of the pyruvic acid is reduced to lactic acid.

Kidney, and to a small extent liver, brain and lung, condense oxaloacetic acid with pyruvic acid (also with glyceric acid and dihydroxyacetone) to give citric acid.

Muscle has, in confirmation of my previous work and contrary to the citric acid cycle theory of Krebs, no ability to form citric acid. It is further shown, in confirmation of perfusion experiments of Martensson, that muscle, unlike liver and kidney, has only slight ability to break down citric acid; again contrary to the cycle theory.

A new method for extraction of the enzyme which reduces oxaloacetic acid to *l*-malic acid is described. Its *pH* optimum is in the alkaline region. This enzyme is very unstable, needs coenzyme and is destroyed by heating.

The enzyme which combines pyruvic acid (to a smaller extent also glyceric acid and dihydroxyacetone) with oxaloacetic acid to citric acid in kidney, is destroyed by heating and its *pH* optimum is at the neutral point. No muscles examined (skeletal, heart and stomach of cat and pigeon), contain detectable amounts of this enzyme. I propose to call the new enzyme citro-genase.

In the competition between the reduction of oxaloacetic acid to *l*-malic acid and the formation of citric acid in kidney, the hydrogenation clearly has the advantage, as postulated by the theories of Szent-Györgyi concerning the C_4 -dicarboxylic acids as H transporters.

The citric acid formation seems to be chiefly an elimination process and happens only above a certain threshold of oxaloacetic acid; by this process the body seems to dispose of any surplus of C_4 -dicarboxylic acids, which are not excretable in the urine, whilst citric acid is readily excreted.

The enzyme for condensation of oxaloacetic acid with glutamic acid can be extracted from tissues by phosphate; it is possible to purify it by precipitation with ammonium sulphate; it is obtained in the fraction between 50 and 66 % saturation.

It is much more stable than the first two enzymes; the *pH* optimum is at the neutral point. It is destroyed by heating. Unlike the other enzymes this enzyme is found in nearly the same amount in all organs.

The thermostable substance which accelerates the decarboxylation of oxaloacetic acid to pyruvic acid is also present in all organs to about the same amount.

The order of spontaneous inactivation of these enzymes in minced kidney tissue was investigated. The enzyme whose activity disappears first is that which reduces oxaloacetic acid to *l*-malic acid; the next that condensing oxaloacetic acid with pyruvic acid to form citric acid; 5–10 times later the enzyme condensing oxaloacetic acid with glutamic acid. The thermostable substance which accelerates the decarboxylation of oxaloacetic acid to pyruvic acid remains unchanged.

The spontaneous decarboxylation in aqueous solution of the oxaloacetic acid used was examined in relation to acidity or alkalinity. In acid solution decarboxylation is twice as fast as in alkaline and 50 % more rapid than in neutral solution.

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