XLIV. STUDIES ON BRAIN METABOLISM

II. FORMATION OF SUCCINIC ACID

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INTRODUCTION

No undisputed and generally recognized explanation exists at present of the occurrence in almost every living tissue of succinic acid and succinic dehydrogenase, its oxidizing enzyme. Interest has recently been focused again on this problem by the work of Szent-Györgyi and his collaborators [Gözszy & Szent-Györgyi, 1934; Annau *et al.*, 1935; 1936]. In the view of Szent-Györgyi's school succinic acid and succinic dehydrogenase are parts of a catalytic system engaged in the transport of hydrogen from substrates to the "Atmungsferment" (cytochrome-indophenol oxidase) of the cell. Succinic acid is, according to this theory, a catalyst and not a substrate. This claim is opposed by strong evidence suggesting that succinic acid is an intermediate in the metabolism of carbohydrates and of certain amino-acids. Pasteur [1858] wrote: "J'ai trouvé que l'acide succinique était un des acides normaux de la fermentation alcoolique, c'est-à-dire que jamais il n'y avait fermentation alcoolique sans qu'il y eût production *aux dépens du sucre* d'une quantité d'acide succinique très notable." Thus Pasteur clearly recognized succinic acid as an intermediate in the carbohydrate metabolism of yeast. Under the influence of the work of Ehrlich [1909], who demonstrated the formation of succinic acid from glutamic acid by yeast, the view prevailed for some time that the succinic acid formed during alcoholic fermentation was derived from the tissue protein via glutamic acid. Mrs Needham [1930] demonstrated the formation of succinic acid from glutamic and aspartic acids in mammalian muscle; but, at least as far as micro-organisms, such as bacteria, yeast and fungi, are concerned, there is ample evidence that succinic acid can be formed from carbohydrates or their cleavage products too [Stephenson, 1930; Raistrick, 1932; Bernhauer, 1934; Kluyver, 1935, *Annual Reviews of Biochemistry*, 1932-6].

Although the mechanism of succinic acid formation is still the subject of controversy, recent work seems to provide an experimental basis for the Thunberg-Wieland theory according to which succinic acid arises from oxidative condensation of two molecules of acetic acid. Thus Wieland & Sonderhoff [1932] and Sonderhoff & Thomas [1936] have demonstrated succinic acid formation from acetic acid by yeast, and the same was shown for various species of fungi by Butkewitsch & Fedoroff [1929; 1930, 1] and by Chrzyszcz & Tiukow [1930]. Related precursors of succinic acid were found to be alcohol [in yeast: Wieland & Wille, 1933; in fungi: Butkewitsch & Fedoroff, 1930, 2; Chrzyszcz *et al.*, 1932], lactic acid [in yeast: Wieland *et al.*, 1933] and glycerol [in propionic acid bacteria: Wood & Werkman, 1936].

There is, however, hardly any evidence at present which would allow the application of this scheme to mammalian tissues. Mrs Needham [1927] showed

that muscle contains a certain amount of preformed succinic acid which decreases during aerobic incubation and again reaches the original level in anaerobiosis. This reappearance of succinic acid is due partly to reduction of fumaric and malic acids and partly to continuous production from unknown precursors. Later [1930] she showed that glutamic and aspartic acids could give rise to succinic acid in muscle whereas acetic acid gave negative results. Clutterbuck [1927] was unable to find increased formation of malic acid from possible precursors, e.g. acetic and α -ketoglutaric acids, in muscle and liver pulp. Quastel & Wheatley [1932] found no increased formation of malic acid from lactic acid in minced brain. Toeniessen & Brinkmann [1930], on the other hand, succeeded in demonstrating the formation of succinic acid from pyruvic acid perfused through the hind limbs of a rabbit in the presence of a limited oxygen supply, whereas they failed to observe a similar production of succinic acid from acetic acid. No acetic acid could be found after perfusion with pyruvic acid, but formic acid was present. Since the latter was obtained also in control experiments without pyruvic acid, the conclusion that it was derived from pyruvic acid seems to be doubtful. The authors suggested a condensation of 2 mol. of pyruvic acid to $\alpha\alpha'$ -diketoadipic acid which by hydrolysis would yield 1 mol. of succinic and 2 mol. of formic acid.

While this work was in progress, a preliminary note was published by Krebs [1936] stating that acetic and succinic acids had been identified amongst the products of the anaerobic oxidation of pyruvic acid.

Elliott & Schroeder [1934] and Elliott *et al.* [1935] have shown that succinic, fumaric and malic acids are oxidized in kidney slices with sufficient velocity to be intermediates in pyruvic acid oxidation, in contrast to acetic acid which is only slowly oxidized. If the same methods are applied to brain slices, however, it appears (Weil-Malherbe, unpublished) that, although succinic acid is readily oxidized to fumaric or malic acid, its further oxidation, as judged by the disappearance of acid groups and by the R.Q., proceeds only very slowly. The slow oxidation of fumaric and malic acids by brain tissue led Quastel & Wheatley [1932] to reject Toeniessen & Brinkmann's scheme for brain. If, however, the formation of succinic acid from pyruvic acid in brain could be proved, these conclusions would have to be reconsidered and the slow oxidation of added malic acid would have to be explained in a different way. That brain tissue is rich in active malic dehydrogenase has been shown by Green [1936, 2] and by my own experiments. The problems arising out of this discrepancy will be discussed in a separate paper where it will be shown that the conditions are different if succinic acid is formed from pyruvic or α -ketoglutaric acid or if it is added as such.

The starting point of the present paper was the observation [Weil-Malherbe, 1936, 1] that brain slices can synthesize glutamine from pyruvic acid and ammonia. α -Ketoglutaric acid was supposed to be an intermediate in this reaction, since (1) it yields glutamine under similar conditions, (2) it is formed by deamination of glutamic acid in brain, a reaction which must be regarded as the reversal of glutamic acid synthesis. α -Ketoglutaric acid thus appears to arise in the course of pyruvic acid oxidation. It was to be expected that the next step would be its conversion into succinic acid, as shown for yeast by Neuberg & Ringer [1915]. The importance of α -ketoglutaric acid as an intermediate is supported by the fact that it is readily oxidized by brain slices and by many other tissues to CO_2 and H_2O and further by the existence of an enzyme which brings about its reversible reduction to *l*- α -hydroxyglutaric acid [Weil-Malherbe, 1936, 3].

EXPERIMENTAL

Method of succinic acid estimation. In view of the easy preparation of active and specific succinic dehydrogenase the use of this enzyme for the determination of small amounts of succinic acid at once suggested itself. A method based on this principle has been devised by Gözsy [Annau *et al.*, 1935] who uses a suspension of washed pigeon breast muscle. Equal amounts of the solution to be analysed are incubated with the muscle preparation in presence and in absence of malonic acid and the amount of succinic acid is calculated from the difference of the oxygen uptakes. This method has obvious disadvantages: (1) a muscle suspension, even if well washed, has still a considerable oxygen uptake, so that small differences of weight introduce relatively large errors; (2) the specificity of such a preparation is an uncertain and variable factor, since it depends largely upon the removal of coenzymes which may sometimes be incomplete; (3) the assumption is made that malonic acid inhibits succinic dehydrogenase specifically. As Gözsy emphasizes himself, the inhibition is not 100 % with muscle pulp, but he should have added that on the other hand the oxygen uptake is considerably depressed even in absence of succinic acid.

It is desirable to work with a standardized enzyme preparation whose properties are exactly known and reproducible. It should be highly specific and purified. It should be stable for a long time, if possible in a dry state. This would allow the use of the same enzyme preparation for many experiments and would dispense with the cumbersome preparation of fresh enzyme for each experiment.

Preparation of enzyme. An enzyme preparation which fulfils most of these requirements is obtained in the following way.

Fresh ox heart is freed from fat and connective tissue, chopped and minced. The mince is washed with cold tap water in a large basin until the wash water is colourless. About 5 washings are required, the wash water being decanted every time as completely as possible. The washed mince is squeezed out and ground very thoroughly with sand in a mortar, until it forms a thin, homogeneous paste. The pulp is pressed out in a hand-press and the liquid is rejected. About 400 ml. of 5 % $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ are now added to the tissue [Ohlsson, 1921], thoroughly mixed and left for $\frac{1}{2}$ hour. On pressing a turbid solution is obtained which is rich in succinic dehydrogenase. The solution is brought to pH 4.6 [Ogston & Green, 1935] by addition of 200–300 ml. of $\frac{1}{3}$ M acetate buffer of this pH. The precipitate is centrifuged, washed once with distilled water and dried *in vacuo*. The dry preparation, if kept *in vacuo*, can be stored for several months without serious loss of activity. For use it is ground with 10 vol. of 0.1 M phosphate buffer of pH 7.4 and the homogeneous emulsion is pipetted into the side-bulb of the manometer vessel whence it is tipped after thermal equilibrium is reached. The oxygen uptake of the enzyme preparation itself is negligible.

Choice of carrier. Succinic dehydrogenase, succinic acid and oxygen cannot react unless a suitable "carrier" is present [Ogston & Green, 1935]. Ogston & Green showed that cytochrome and methylene blue were active as carriers with their preparations of succinic dehydrogenase, cytochrome being by far superior. The same applies to our dry preparation. It follows that it contains an active indophenol oxidase which, hitherto, could not be obtained in a dry state. The presence of active indophenol oxidase can be demonstrated by the oxidation of *p*-phenylenediamine (Table I). The laborious preparation and perishable nature of cytochrome render it unsuitable for routine analyses. The

Table I. *Activity of indophenol oxidase in the dry preparation from ox heart*

	1	2	3
<i>M</i> /10 <i>p</i> -phenylenediamine	—	2.0 ml.	2.0 ml.
Water	2.0 ml.	0.5 ml.	—
10% enzyme suspension	0.5 ml.	—	0.5 ml.
$\mu\text{l. O}_2$ uptake in 20 min.	0	11	206

most suitable carrier was found to be brilliant cresyl blue. Pyocyanine is entirely inactive. Whether this is explicable merely on potentiometric grounds is doubtful. Table II shows a comparison of the activities of different carriers.

Table II. *Comparison of different carriers*

2 ml. of *M*/10 succinate and 0.5 ml. of 10% enzyme suspension in each vessel

	$\mu\text{l. O}_2$ uptake in 2 hours
0.2 ml. of cytochrome solution [Keilin, 1933]	438
0.5 ml. of 0.5% brilliant cresyl blue	296
0.5 ml. of 0.5% methylene blue	197
0.5 ml. of <i>M</i> /100 pyocyanine	0

Technique. For the estimation of succinic acid 2 ml. of the neutral solution are pipetted into the main part of the manometer vessel, 0.5 ml. of 0.5% brilliant cresyl blue is added and 0.5 ml. of the 10% enzyme suspension is placed

Fig 1. I, Oxygen uptake of 0.59 mg. succinic acid (theoretically: 56 $\mu\text{l. O}_2$). II, Oxygen uptake of 1.18 mg. succinic acid (theoretically: 112 $\mu\text{l. O}_2$).

in the side-bulb. The inner cup contains 0.2 ml. of 10% NaOH. As thermobarometer a vessel is used which contains enzyme and carrier like the other vessels but 2 ml. of distilled water in the main part. Any oxygen uptake by the enzyme itself is thus allowed for. The estimation is carried out in Warburg manometers at 37.5°, the gas space containing air. The oxygen uptake reaches

100 % of the theoretical value, i.e. $1/2 \text{ O}_2$ for $1 (\text{CH}_2\text{COOH})_2$, in the course of 1-3 hours (Fig. 1). In a few cases the manometers have been left in the thermostat overnight without the correct result being impaired.

In the first experiments the tissue was extracted repeatedly with a total quantity of 50 ml. of 4 % trichloroacetic acid. The recovery of added succinic acid was 80 % only. Later it was found that better results could be obtained when the tissue suspension was made up with water to 6 or 8 ml., thoroughly mixed, centrifuged and the measured supernatant fluid used for further procedures. The recovery with this method amounted to 90-95 %.

Specificity of the enzyme. In Table III a number of substrates are enumerated which were examined with the enzyme preparation in the usual arrangement. With three of them a slight oxygen uptake was observed, which was, however, small compared with that of succinic acid. These substrates are: *l*- α -hydroxyglutaric

Table III. *Specificity of succinic dehydrogenase preparation*

2 ml. of *M*/10, neutralized substrates, 0.5 ml. of 10 % enzyme suspension and 0.5 ml. of 0.5 % brilliant cresyl blue per vessel

	$\mu\text{l. O}_2$ 2 hours		$\mu\text{l. O}_2$ 2 hours
Acetic acid	0	Hexosemonophosphate	0
<i>l</i> (+) Alanine	0	Hexosediphosphate	0
<i>dl</i> -Alanine	0	<i>dl</i> - β -Hydroxybutyric acid	0
<i>l</i> (-) Aspartic acid	2	<i>l</i> - α -Hydroxyglutaric acid	10
<i>dl</i> -Aspartic acid	2	α -Ketoglutaric acid	0
Citric acid	0	Lactic acid	0
Fumaric acid	0	<i>L</i> -Malic acid	0
<i>l</i> (+) Glutamic acid	0	Pyruvic acid	0
<i>d</i> (-) Glutamic acid	45	Phosphohexonic acid	0
α -Glycerophosphate	29	Succinic acid	311
β -Glycerophosphate	0		

acid, α -glycerophosphate and *d*(-) glutamic acid. With *l*- α -hydroxyglutaric acid the total of the small oxygen uptake occurred in the first 30 min. It is not probable, however, that this is due to contaminating succinic acid since similar results were obtained with different concentrations. It is interesting to note that our preparation contains a glutamic acid deaminase which attacks *d*(-) glutamic acid only. The same change of optical specificity has taken place on purification of the enzyme here as was described previously for the glutamic acid deaminase of brain [Weil-Malherbe, 1936, 1]. This is fortunate from the point of view of the succinic acid estimation since it is not likely that *d*(-) glutamic acid will occur in biological material. The only serious objection with regard to specificity is the presence of α -glycerophosphate dehydrogenase. This was to be expected from the work of Green [1936, 1] who demonstrated the close resemblance of this enzyme to succinic dehydrogenase. The oxygen uptake with α -glycerophosphate is, however, less than $1/10$ of that with succinic acid, in agreement with Green's observation that heart is a poor source of the enzyme. It is highly improbable, however, that α -glycerophosphate caused any error in the experiments to be described. It is not likely to arise from the substrates mainly used in this research. Furthermore, in a large proportion of experiments the solution was heated with acid to 130° in the autoclave for 1 hour before the succinic acid estimation was carried out; it can be assumed that glycerophosphate is hydrolysed by this treatment.

Inhibition of succinic dehydrogenase. In agreement with earlier observations it was found that malonic acid inhibits the enzyme completely, whereas maleic

acid does not inhibit at all. An unexpected inhibitor of succinic dehydrogenase was found to be α -ketoglutaric acid. This substance, if used in equal concentration with succinic acid, causes an inhibition of 40–50%. This effect of α -ketoglutaric acid is very specific and is not observed either with other keto-acids, such as pyruvic or 2-ketogluconic acid, or with other substances of related constitution, e.g. hydroxyglutaric or glutamic acid. Purification via the barium salt had no influence on the inhibiting effect. A further observation can be regarded as a definite indication that this inhibition is actually due to α -ketoglutaric acid and not to an impurity: with *d* (–) glutamic acid a small inhibition can be seen which increases with time. This must be attributed to the formation of small amounts of α -ketoglutaric acid from *d* (–) glutamic acid by the action of the glutamic acid deaminase.

Both pyocyanine and phenosafranine inhibit the enzyme considerably and to the same extent (Table IV).

Table IV. *Inhibition of succinic dehydrogenase*

2 ml. of *M*/20 succinate, 0.5 ml. 10% enzyme and 0.5 ml. 0.5% brilliant cresyl blue per vessel

Addition	μ l. O ₂ in 60 min.	% inhibition
O	182	—
<i>M</i> /20 α -Ketoglutaric acid	82	55
<i>M</i> /20 <i>d</i> (–) Glutamic acid	155	15
<i>M</i> /350 Pyocyanine	104	43
<i>M</i> /350 Phenosafranine	103	43.5

It is not permissible to draw any conclusions from these observations with purified enzyme as to the behaviour of succinic dehydrogenase in the intact or minced tissue, as will be shown later. This is especially obvious in the case of α -ketoglutaric acid which is readily oxidized by both brain slices and mince without showing any sign of toxic action.

RESULTS

In experiments where an accumulation of succinic acid is aimed at, it is necessary to inhibit its further oxidation. Two methods were used: (1) anaerobic incubation, (2) poisoning of succinic dehydrogenase with malonic acid. Pyruvic, acetic and α -ketoglutaric acids were examined as possible precursors.

Anaerobic formation of succinic acid

It is well known that biological oxidations can proceed anaerobically to a certain extent, at the expense of hydrogen acceptors which are reduced simultaneously. Under these conditions, the reduced forms of reversible oxidation-reduction systems, succinic acid, amongst others, will accumulate. The anaerobic oxidation of pyruvic acid is accomplished at the expense of other pyruvic acid molecules which are reduced to lactic acid. If α -ketoglutaric acid is oxidized anaerobically to succinic acid, an equivalent amount of *l*- α -hydroxyglutaric acid is formed. The quantitative aspect of these problems will be dealt with in a separate paper. The object of the present communication is merely the qualitative demonstration of succinic acid formation.

The anaerobic experiments were done with slices of guinea-pig brain and with minced brains obtained from the slaughter house. The slices of guinea-pig brain were suspended in 2 ml. of bicarbonate saline containing different substrates and were incubated for 2 hours in an atmosphere of N₂/5% CO₂. Before

the manometers were removed from the thermostat 0.2 ml. of 10% H_2SO_4 was tipped from the side-bulb. The tissue was taken out, dried and weighed, and the manometer vessels were put in a boiling water-bath for 5 min. to destroy any succinic dehydrogenase which might have passed into solution. The contents were neutralized and the side-bulbs of the vessels well rinsed. After addition of carrier and enzyme, succinic acid estimation was carried out in the manner already described. The formation of succinic acid (Table V) is very small from α -ketoglutaric acid, a little larger from pyruvic acid and from glucose. Addition of glucose to pyruvic or ketoglutaric acid increases the formation of succinic acid beyond a mere summation. The reason is perhaps that glucose provides additional hydrogen acceptors.

Table V. *Anaerobic succinic acid formation by guinea-pig brain slices*

Addition	$Q_{\text{succinic acid}}^*$
0	0
0.2% Glucose	0.17
<i>M</i> /50 Pyruvate	0.34
<i>M</i> /50 Ketoglutarate	0.09
0.2% Glucose + <i>M</i> /50 pyruvate	1.18
0.2% Glucose + <i>M</i> /50 ketoglutarate	0.33
0	0
0.2% Glucose	0.12
<i>M</i> /50 Pyruvate	0.44
<i>M</i> /50 Ketoglutarate	0.08
<i>M</i> /50 Acetate	0
0.2% Glucose + <i>M</i> /50 pyruvate	0.82
0.2% Glucose + <i>M</i> /50 ketoglutarate	0.48
0.2% Glucose + <i>M</i> /50 acetate	0

* $Q_{\text{succinic acid}}$ = succinic acid/mg. dry weight/hour.

These experiments with slices were not entirely satisfactory, since the quantity of succinic acid formed was very small and amounted often only to a few $\mu\text{l.}$ of O_2 taken up. Experiments with minced brain yielded amounts of succinic acid large enough to allow manometer readings which were outside the limits of error. Brain is a material especially suitable for "Brei" experiments, since it can be made into a sufficiently mobile and homogeneous paste with very little dilution. In our experiments with minced brain the final proportion of solution to tissue did not exceed 1 : 1. The use of the tissue in this highly concentrated form is necessary, as the tissue loses its power of oxidizing keto-acids with increasing dilution. The procedure was usually as follows: the cortical parts of the brain obtained freshly from the slaughterhouse were minced and two parts of it were ground in a mortar with a little sand and 1 part of *M*/50 phosphate buffer *pH* 7.4, to which other components (substrates or poisons) could be added as required. 3 ml. of the thick fluid were then pipetted into a manometer vessel and 1 ml. of water or solution as required was added. The vessel contained therefore 2 g. of tissue in 2 ml. of solution. The anaerobic experiments of this kind were carried out in pure N_2 . After 2 hours' incubation at 37.5° the vessels were quickly put in a boiling water-bath, the contents were rinsed with 3 ml. of water into centrifuge-tubes, thoroughly mixed with a glass rod and centrifuged. An aliquot of the supernatant fluid was deproteinized with 1/10 vol. of 30% trichloroacetic acid, the filtrate was heated in a boiling water-bath for $\frac{1}{2}$ hour or until the excess trichloroacetic acid was decomposed and was then ready for the succinic acid estimation.

The amounts of succinic acid which were found in these experiments (Table VI) are expressed in $\mu\text{l.}$, i.e. the volume of a certain quantity of succinic

Table VI. *Anaerobic succinic acid formation by minced brain*

μl. succinic acid formed upon incubation with													
Species	O	M/5 pyruvate			M/5 acetate			M/8 keto-glutarate			1.25% glucose		
		μl. suc-cinic acid	% in-crease	μl. suc-cinic acid	% in-crease	μl. suc-cinic acid	% in-crease	μl. suc-cinic acid	% in-crease	μl. suc-cinic acid	% in-crease	μl. suc-cinic acid	% in-crease
Ox	100	280	180	108	0	255	155	93	0	257	157	—	—
	108	196	82	93	0	256	135	—	—	—	—	—	—
Horse	72.5	122	69	67	0	265	268	69	0	285	290	68.5	0
	84	194	131	64	0	140	67	—	—	—	—	—	—

acid in gaseous form (118 mg. = 22400 μl.). The increase of succinic acid in the samples which were incubated with pyruvic or ketoglutaric acid is evident. Glucose does not cause any increased succinic acid formation, nor does it increase the formation from pyruvic or ketoglutaric acid.

It is remarkable that increased succinic acid formation from acetic acid was never observed in these experiments, either with slices or with mince, in presence or in absence of glucose.

Aerobic succinic acid formation

No increase of succinic acid was found after aerobic incubation of either slices or mince with any of the substrates used, owing to the activity of the tissue's own succinic dehydrogenase. An attempt was therefore made to poison this enzyme with malonic acid which, according to Szent-Györgyi and collaborators, inhibits it specifically. This inhibitor had to be removed before the succinic acid estimations were carried out. To this end the solution, after deproteinizing and after addition of enough H_2SO_4 to give a final acidity of 2*N*, was heated for 1 hour at 130–135° in an autoclave. Succinic acid is quite stable under these conditions, but malonic acid is completely decomposed. The solution, after autoclaving, was neutralized to litmus with baryta, the precipitate of $BaSO_4$ filtered off, and succinic acid was estimated in the usual way. α -Ketoglutaric acid is partly converted into succinic acid by autoclaving. It has therefore to be removed previously in the following way: a slight excess of *M* bisulphite and enough H_2SO_4 to make the solution 2*N* are added and the solution is extracted with ether in a continuous extraction apparatus overnight. The bisulphite-keto-acid compound which is insoluble in ether is left behind whereas succinic and malonic acids pass into the extract. The ether is distilled off, the residue taken up with water, acidified and autoclaved. This procedure was sometimes carried out with all solutions whether they contained α -ketoglutaric acid or not. But after it was seen that the results in absence of α -ketoglutaric acid did not differ whether the ether extraction was done or not the latter was in most cases restricted to the solutions which contained α -ketoglutaric acid. The removal of α -ketoglutaric acid also reduces the time necessary for the succinic acid estimation, which, in the anaerobic experiments, had to be extended to 2–3 times its normal duration owing to the inhibiting effect of ketoglutaric acid on succinic dehydrogenase.

Although the purified enzyme preparation is completely inhibited by malonic acid, it was soon realized that the position is more complex when malonic acid is

added to the minced tissue. It was found that there exists a critical concentration of malonic acid, above and below which less succinic acid or none accumulates (Table VII). This concentration is, for the concentration of mince used, i.e. one

Table VII. *Influence of different concentrations of malonic acid on the accumulation of succinic acid by minced brain*

Species	Addition	$\mu\text{l. succinic acid}$	% increase
Ox	<i>M/5</i> Malonic acid	171	—
	<i>M/5</i> Malonic + <i>M/5</i> pyruvic acid	167	0
	<i>M/20</i> Malonic acid	106	—
	<i>M/20</i> Malonic + <i>M/5</i> pyruvic acid	214	100
	<i>M/40</i> Malonic acid	130	—
	<i>M/40</i> Malonic + <i>M/5</i> pyruvic acid	180	39
Horse	<i>M/5</i> Malonic acid	103	—
	<i>M/5</i> Malonic + <i>M/8</i> ketoglutaric acid	124	20
	<i>M/20</i> Malonic acid	42	—
	<i>M/20</i> Malonic + <i>M/8</i> ketoglutaric acid	276	560
	<i>M/40</i> Malonic acid	53	—
	<i>M/40</i> Malonic + <i>M/8</i> ketoglutaric acid	314	495

part of tissue in one part of solution, *M/20–M/50*. In experiments with slices of guinea-pig brain I was unable to find any concentration of malonic acid within the range from *M/250* to *M/25* which would have brought about any accumulation of succinic acid, although there was a marked inhibition of respiration with the higher concentrations. In order to analyse these results in more detail, the R.Q. of α -ketoglutaric acid oxidation in presence of different concentrations of malonic acid was measured. An accumulation of succinic acid should be reflected in a rise of R.Q. which is 1.25 for complete oxidation. It should rise to 2.0 in the case of a 100 % inhibition of succinic dehydrogenase and to 1.33 in the case of a 50 % inhibition. The position is less favourable in the case of pyruvic acid since the figures lie closer together. They are 1.20 for complete oxidation, 1.33 for 100 % inhibition and 1.23 for 50 % inhibition of succinic dehydrogenase. Since the R.Q. in presence of pyruvic acid is usually above the theoretical value (1.25–1.28) any significant increase cannot be expected and is in fact not observed. The R.Q. of ketoglutaric acid oxidation, however, is markedly raised (Table VIII), but only with those concentrations of malonic

Table VIII. *R.Q. of minced guinea-pig brain in presence of α -ketoglutaric acid and different concentrations of malonic acid*

In each vessel 0.4 g. of tissue and 0.4 ml. of solution. Method: Dickens & Šimer [1930].
60 min. incubation

Addition	$\mu\text{l. O}_2$	$\mu\text{l. CO}_2$	R.Q.
<i>M/10</i> Ketoglutaric acid	352	347	0.99
<i>M/5</i> Malonic + <i>M/10</i> ketoglutaric acid	142	133	0.94
<i>M/8</i> Ketoglutaric acid	238	244	1.02
<i>M/20</i> Malonic + <i>M/8</i> ketoglutaric acid	161	203	1.26
<i>M/10</i> Ketoglutaric acid	305	309	1.01
<i>M/20</i> Malonic + <i>M/10</i> ketoglutaric acid	139	185	1.33
<i>M/40</i> Malonic + <i>M/10</i> ketoglutaric acid	198	254	1.28

acid which lead to accumulation of succinic acid. From the increase of R.Q., an inhibition of succinic dehydrogenase of about 50 % can be estimated.

No change of R.Q. was observed when slices of guinea-pig brain were poisoned with malonic acid (Table IX), in complete agreement with the negative attempts to accumulate succinic acid.

Table IX. R.Q. of guinea-pig brain slices in presence of pyruvic or α -ketoglutaric acid and different concentrations of malonic acid. Phosphate saline

Addition	Q_{O_2}	Q_{CO_2}	R.Q.
<i>M</i> /50 Ketoglutaric acid	-9.1	9.7	1.06
Do. + <i>M</i> /200 malonic acid	-8.5	9.0	1.05
Do. + <i>M</i> /100 malonic acid	-7.8	8.3	1.06
Do. + <i>M</i> /25 malonic acid	-4.4	4.7	1.07
<i>M</i> /50 Pyruvic acid	-8.8	11.2	1.27
Do. + <i>M</i> /100 malonic acid	-8.1	10.1	1.25
Do. + <i>M</i> /25 malonic acid	-4.9	6.3	1.29

In Table X the results of a number of experiments with minced brain poisoned with *M*/20 malonic acid are presented. They show a clear and invariably large increase of succinic acid upon incubation with α -ketoglutaric

Table X. Accumulation of succinic acid in minced brain in presence of *M*/20 malonic acid

		μ l. succinic acid formed upon incubation with							
		0	<i>M</i> /5 pyruvate		<i>M</i> /8 ketoglutarate		<i>M</i> /5 acetate		
No.	Species	μ l. succinic acid	μ l. succinic acid	% increase	μ l. succinic acid	% increase	μ l. succinic acid	% increase	
1	Sheep	180	380	111	480	166	160	0	
2		108	306	183	270	150	218	101	
3		122	265	117	310	154	113	0	
4	Ox	158	353	123	418	165	226	43	
5	Pig	153	252	65	330	116	225	47	
6		184	530	188	444	141	506	175	
7	Horse	164	404	146	620	278	370	125	
8		148	295	100	410	177	220	48	
9		89	340	282	386	334	130	46	
10		81	78	0	320	295	84	0	
11		65	428	516	363	458	60	0	

acid, and also, with one exception (Exp. 10), with pyruvic acid. The surprising feature of these experiments is, in contrast to the negative results of the anaerobic experiments, the increase of succinic acid which is sometimes found after incubation with acetic acid. Although this result is erratic and less striking than with the other two acids, it is nevertheless significant.

The observation that pyocyanine inhibits succinic dehydrogenase suggested the use of this substance for accumulation of succinic acid. It was extracted from the neutral solution with chloroform before the succinic acid estimations were carried out. Pyocyanine seems to inhibit strongly the enzymes which bring about oxidative decarboxylation of keto-acids, but it neither increased the R.Q. nor did it bring about an accumulation of succinic acid. This is, in addition to α -ketoglutaric and malonic acids, the third example of disagreement of results obtained with the purified enzyme and with tissue.

Demonstration of the production of a permanganate-resistant volatile acid from pyruvic acid

Toeniessen & Brinkmann's scheme assuming the condensation of 2 mol. of pyruvic acid to $\alpha\alpha'$ -diketoadipic acid was based on two claims: (1) that acetic acid does not yield succinic acid in the animal body, (2) that no acetic acid is

formed from pyruvic acid. The first of these claims has been disproved at least in some cases, although the erratic and quantitatively small nature of the observation must be admitted. This result called for re-examination of the second claim also.

The method used for estimation of acetic acid is based on a steam distillation *in vacuo*, carried out in the Parnas apparatus for ammonia estimations. The volatility of the acid is increased when it is distilled from a concentrated salt solution [Virtanen & Pulkki, 1928]. Precautions are necessary to prevent the distillation of pyruvic acid. Lactic acid is volatile to a small extent, but the quantity of distilled acid does not increase in proportion to the concentration [Virtanen & Pulkki, 1928]. The distillate which is collected under a pressure of 12 mm. Hg, is free from CO_2 and can be titrated with CO_2 -free $N/100$ NaOH against phenolphthalein in the cold.

The procedure is as follows: to the solution which has been separated from the tissue by centrifuging is added 1 ml. of a solution containing 0.7 ml. of pure phenylhydrazine in 10 ml. of 5% phosphoric acid (this applies to solutions containing 30–40 mg. of pyruvic acid, as was the case in these experiments). After 1 hour at 0° the precipitate of the hydrazone and of proteins is filtered off, and 1 ml. of syrupy phosphoric acid is added to the filtrate. The solution is made up to roughly 10 ml. and is quantitatively transferred to the bulb of the Parnas apparatus. 4 g. of NaH_2PO_4 are added in order to increase the ionic strength of the solution and the distillation is started. Exactly 25 ml. of distillate are collected in a calibrated tube. It was found that under these conditions and with the particular apparatus used 80% of added acetic acid passed over in 25 ml. of distillate. This proportion was constant for the concentrations of acetic acid examined, i.e. 0.3–3 mg./10 ml. After each estimation distillation was continued until the last traces of acid had disappeared from the distillate.

Table XI. *Formation of volatile acid upon incubation of minced ox brain with pyruvic acid for 2 hours. 2 g. of tissue in 2 ml. of solution*

	Addition	ml. $M/100$ NaOH titrated		Difference	$\mu\text{l.}$ volatile acid, calculated as acetic acid
		Before incubation	After incubation		
In O_2	O	0.38	0.38	0	0
	$M/5$ pyruvic acid	0.38	0.65	0.27	75
In N_2	O	0.38	0.34	0	0
	$M/5$ pyruvic acid	0.38	0.70	0.32	90

It will be seen from Table XI that a small but definite quantity of volatile acid is found after aerobic and anaerobic incubation of minced brain with pyruvic acid. This acid does not reduce KMnO_4 and is therefore probably acetic acid, although it has not yet been identified by other reactions.

DISCUSSION

The evidence which has been presented confirms the observation of Toeniessen & Brinkmann that succinic acid is formed from pyruvic acid in the animal body and reveals a close similarity of the oxidative metabolisms of micro-organisms and higher animals. We can therefore safely regard succinic acid as a substrate of intermediary metabolism. Innes [1936] came to the same conclusion concerning fumaric acid. She found with minced muscle that the extra oxygen uptake upon addition of fumaric acid was accounted for by a corresponding

disappearance of this acid. It is therefore probable that the further oxidation of succinic acid passes through the stages of fumaric, malic and oxaloacetic acids; pyruvic acid is formed by decarboxylation of the latter and the oxidative cycle starts again.

One of the arguments put forward by Szent-Györgyi in support of his theory is the assumption that the depression of respiration in presence of malonic acid is due to a specific inhibition of succinic dehydrogenase. Our experiments have shown that there is no strict correlation. The only certain indication of a specific inhibition of succinic dehydrogenase is the accumulation of succinic acid from a precursor. But, although an inhibition of respiration was observed, no succinic acid accumulated when brain slices were incubated with malonic acid. The oxidation of succinic acid was therefore inhibited only in proportion to the inhibition of respiration generally. Succinic dehydrogenase seems to be protected from malonic acid in the structurally intact tissue. The behaviour of minced brain shows more resemblance to that of the purified enzyme. Greville [1936] has already pointed out that the effects of malonate are greater the more unphysiological the experimental conditions are. With minced brain, we see a certain specific inhibition of succinic dehydrogenase, as indicated by an accumulation of succinic acid, although it is much less powerful than with the purified enzyme. With increasing concentration of malonic acid however this specificity disappears again. This means that other enzyme systems are affected too and therefore the formation of succinic acid is as much inhibited as is its disappearance. The conversion of α -ketoglutaric acid into succinic acid involves only one reaction: an oxidative decarboxylation. This process must therefore be inhibited by malonic acid to the same degree as succinic dehydrogenase, if no succinic acid accumulates from α -ketoglutaric acid. Other effects of malonic acid have recently been described: increased formation of acetone bodies [Annau *et al.* 1935; 1936], inhibition of aerobic breakdown of acetoacetic acid [Quastel & Wheatley, 1935] and inhibition of malic dehydrogenase [Green, 1936, 2]. The effects of malonic acid are therefore complex and cannot all be attributed to a primary and specific inhibition of succinic dehydrogenase.

The mechanism of succinic acid formation from pyruvic acid is still an unsolved problem. Although the arguments put forward by Toeniessen & Brinkmann in support of their scheme were shown to be ill-founded, the reasons which were adduced in an earlier section in favour of α -ketoglutaric acid as an intermediate are still unshaken. To this can be added the results of our experiments showing a certain parallelism of succinic acid formations from both pyruvic and α -ketoglutaric acids. Whether α -ketoglutaric acid is formed by condensation of 2 mol. of pyruvic acid with subsequent decarboxylation [Weil-Malherbe, 1936, 1] or by condensation of 1 mol. of pyruvic acid with 1 mol. of acetic acid [Krebs, 1936], is at present a matter of speculation. Experiments in which pyruvic + acetic acids were used did not show any increased succinic acid formation compared with that from pyruvic acid alone. Attempts to poison the oxidative decarboxylation without affecting the condensing mechanism were equally unsuccessful. It is quite possible that condensation and decarboxylation are in some way coupled together. It may be that an especially reactive unstable intermediate, e.g. the ketene which was recently postulated [Weil-Malherbe, 1936, 2], plays an important role in the reaction.

Although it is very probable that α -ketoglutaric acid is an intermediary stage in the series of reactions which lead from pyruvic to succinic acid, the existence of a second, less important pathway is not excluded: the formation of succinic acid by the condensation of 2 mols. of acetic acid, as formulated by the original

Thunberg-Wieland theory. This mechanism seems to be the rule in micro-organisms, and the sporadic observations of succinic acid formation from acetic acid in our experiments can hardly be explained differently.

SUMMARY

A method of succinic acid estimation based on the action of a specific succinic dehydrogenase preparation from ox heart is described. The properties of this enzyme preparation have been studied in some detail. The only objection which can be made as to specificity is the presence of α -glycerophosphate dehydrogenase. The activity of this enzyme is, however, more than ten times smaller than that of succinic dehydrogenase. A glutamic acid deaminase which is also present only attacks the non-natural isomeride. Succinic dehydrogenase is inhibited by α -ketoglutaric acid, pyocyanine and phenosafranine.

The formation of succinic acid from pyruvic and α -ketoglutaric acids is demonstrated anaerobically with brain slices and with minced brain. Aerobically, in experiments with minced brain poisoned with malonic acid, pyruvic, α -ketoglutaric and, in some cases, acetic acids were found to yield succinic acid. Under the same conditions the R.Q. of ketoglutaric oxidation is raised, indicating a specific inhibition of succinic dehydrogenase of roughly 50 %. Malonic acid does not lead to succinic acid accumulation with brain slices, and with mince only if used in certain concentrations. Small amounts of a volatile acid, probably acetic acid, are formed from pyruvic acid by minced brain. The significance of these observations and the possible mechanism of succinic acid formation are discussed.

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