LXXIII. EXPERIMENTS ON THE FORMATION OF SUCCINIC ACID IN THE BODY.

PART I. THE DETERMINATION OF SUCCINIC ACID AND ITS FORMATION IN MUSCLE AND LIVER PULP.

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(*Received April 4th, 1927.*)

In several recent publications [*e.g.* Ascher, 1925; Burn and Marks, 1926] considerable support has been given to the view that the liver normally, to some extent, converts fat into reducing sugar, and that this process becomes increasingly important when the animal is deprived of its glycogen supplies. Nothing is yet known, however, of the chemical mechanism of such conversion, nor does the theory of β-oxidation of fats readily provide an obvious mechanism.

Two suggestions have been made. It has been shown [Clutterbuck and Raper, 1926] that acetoacetic acid on oxidation with hydrogen peroxide, which, according to Dakin [1908, 1, 2, 3; 1910], simulates certain physiological oxidations, yields α-hydroxyacetoacetic acid. It was therefore suggested that in the liver acetoacetic acid, known to arise in the metabolism of fat, might give rise successively to α-hydroxyacetoacetic acid, diketobutyric acid and finally, by loss of carbon dioxide, to methylglyoxal, which is readily converted by glyoxalase [Dakin and Dudley, 1913, 1914] to lactic acid, and to glucose.

Consideration of the chemistry of muscle leads to the second suggestion. It has been known for a long time [Einbeck, 1913, 1914] that fresh muscle extracts contain considerable amounts of succinic acid, which has not arisen from putrefaction as previously suggested [Wolff, 1903], and the amount in such extracts has recently been determined [Moyle, 1924]. Muscle tissue also contains a very powerful enzyme which acts on succinic acid [Thunberg, 1909; Batelli and Stern, 1911], and is remarkably specific. The enzyme, according to Batelli and Stern, converts succinic acid into optically inactive malic acid, but their identification of the acid was not very convincing. Einbeck [1914] regarded fumaric acid and not malic acid as the sole product, but later [1919] he showed that the enzyme first converted succinic to fumaric acid and then by a balanced reaction to a mixture of fumaric (25 %) and malic (75 %) acids. Dakin [1922] showed that fumaric acid, reacting with muscle

pulp, formed exclusively *laevo*-malic and not the inactive acid, and he pointed out that this asymmetric synthesis was of additional interest from the fact of the stereochemical similarity of *l*-malic and *d*-lactic acids. The succinic acid formed *in vivo* may, therefore, represent an intermediate stage of a special type of sugar formation by way of fumaric, malic and lactic acids. It is known that malic acid yields glucose in the diabetic organism and that succinic acid gives "extra glucose" in phloridzinised animals [Ringer, Frankel and Jones, 1913].

Nothing is known as to the origin of succinic acid in the body. Although succinoxidase is so very powerful, specific and widely distributed, yet its only function appears at the present time to be that of dealing with any small amounts of succinic acid supposed to arise from glutaminic acid during the metabolism of proteins. It is interesting to enquire whether succinic acid might not arise by direct oxidation of fatty acids in the body. During oxidations *in vitro* with hydrogen peroxide, formation of succinic acid very often occurs. Thus butyric acid [Cahen and Hurtley, 1917] gives, besides acetoacetic acid, a considerable amount of succinic acid. Also caproic, oenanthic, caprylic, myristic, palmitic and stearic acids [Clutterbuck and Raper, 1925] give, besides the β-oxidation product, γ-keto-acids; and the first three, δ-keto-acids also. These on further oxidation form succinic acid. Thunberg has also suggested that acetic acid may yield succinic acid *in vivo*

$$2CH_3.COOH \rightarrow COOH.CH_2.CH_2COOH + H_2O.$$

Knoop and Gehrke [1925] oxidised acetic acid and acetone by standing at the ordinary temperature for a long time with hydrogen peroxide, and although but little oxidation of acetic acid occurred, they obtained from acetone considerable amounts of a mixture of tartaric, malic and succinic acids. It has also been shown [Clutterbuck and Raper, 1926] that acetoacetic acid gives on oxidation with hydrogen peroxide, besides α-hydroxyacetoacetic acid, a considerable amount of acetonylacetoacetic acid and a little ethyl isocarbo pyrotritartrate, and it was suggested that these had arisen from the intermediate formation of diacetylsuccinic acid. It was considered that possibly diacetylsuccinic acid might similarly arise from acetoacetic acid *in vivo*

$$2CH_3COCH_2COOH \rightarrow \begin{matrix} CH_3COCH.COOH \\ | \\ CH_3COCH.COOH \end{matrix}$$

and give rise on hydrolysis to succinic acid.

Kay and Raper [1922] injected atropic acid into dogs and isolated a small amount of succinic acid from the urine. It would seem almost impossible that this acid could have been formed from the substance injected. Atropic acid, however, on injection caused some damage to the kidney, as evidenced by haemoglobinuria, and the explanation of the appearance of succinic acid possibly lies in this fact. This question is being further investigated.

The above evidence has led the author to embark upon a study of the

formation of succinic acid with the object of throwing some light on its origin. The method of Moyle [1924] gives excellent results with very small amounts of the acid. It is, however, rather long and tedious, and is not suitable for our purpose on account of the extremely great activity of succinoxidase, which is capable of disposing of large amounts of succinic acid in a short time. It seemed better, therefore, to attempt to follow the production of this acid not directly but in terms of the *l*-malic acid arising from it by the action of the enzyme, the malic acid being not readily further attacked in experiments with the isolated tissue and being rapidly determinable by means of its optical rotation. In the present paper, a polarimetric method of this kind is described. It has been used to follow the conversion of succinic and fumaric acids to malic acid, and the effect on these reactions of the addition of cyanide has been determined. It has also been adapted to the detection of smaller amounts of succinic acid and, in order to test the above suggestions, it was applied to determine whether succinic acid was produced in liver and muscle pulp when the following substances were added: sodium acetate, acetoacetate, α-ketoglutarate, δ-ketohexoate, diacetylsuccinate and acetone. It was found that although liver and muscle, under the conditions of the experiments, were able very readily to oxidise (*i.e.* dehydrogenate) succinic acid, yet they were not able to oxidise any of the above substances in the way that hydrogen peroxide could do. Thus sodium acetate was not attacked, acetone, aceto-acetic and diacetylsuccinic acids did not give rise to succinic acid, δ-ketohexoic acid, which was obtained by oxidation of hexoic acid with hydrogen peroxide and which very readily gave succinic acid on oxidising further, did not give an amount detectable by the method, and α-ketoglutaric acid, which may be expected to yield succinic acid in the intact animal, did not do so in these experiments. The *l*-malic acid, formed from succinic acid, also remains as such, although in the intact animal it is supposed to lose carbon dioxide yielding lactic acid, since it yields glucose in the diabetic animal. Similar differences between results under experimental conditions and in the intact animal are found when the liver is perfused. Raper and Smith [1926] found that only 80 % of the theoretical acetone could be recovered from perfused butyric acid, and concluded that the liver oxidised the remaining 20 % without the appearance of acetone bodies. On the other hand, Snapper and Greenbaum [1927] found that when either acetoacetic or β-hydroxybutyric acid was perfused, the latter was absorbed by the liver tissue, the amount contained by the liver being sometimes as great as six to eight times the amount expected from the acetone content of the blood. Taking this absorption into account, the amount of these acids further oxidised is very small indeed. The perfused liver, however, in that it has been shown to attack a large number of amino-acids, does appear to be much more powerfully oxidising than the isolated liver without its blood supply. It is proposed therefore to investigate the effect of perfusion of some of the substances referred to above through the surviving liver.

Experimental.

The immediate purpose of the experiments was to find out what substances, on addition to liver and muscle pulp, could result in the formation of succinic acid. Such substances, incubated with the pulp in presence of oxygen, should give rise to *l*-malic acid and be determinable, therefore, in virtue of the resulting change in rotation. In order to decide whether the formation of succinic acid could be detected in this way, a method was first devised for following the changes in rotation obtained when succinic acid is added to liver and muscle pulp.

Detection of succinic acid, added to muscle and liver pulp.

The method is an adaptation of that used by Dakin [1922] with fumaric acid, and depends on the fact that the normal small rotation of *l*-malic acid in water, $[\alpha]_D^{20°} - 1{\cdot}7°$, is increased in presence of uranium acetate to $[\alpha]_D^{20°} - 482°$. The muscle and liver used in these experiments were obtained from rabbits under sterile conditions. After killing the animal, the fur near the line of incision was removed, the animal washed with water and lysol, the skin carefully retracted and clipped back, and the back and leg muscles excised, using sterilised instruments. The dishes, mincer, container and pipettes were also sterilised. In each experiment, 5 g. of succinic acid were neutralised to litmus, diluted to 500 cc. and added to 100 g. minced sterile muscle or to 30 g. minced liver, from which the gall bladder and main portal tracts had been removed before mincing. The material was placed in a sterilised bottle (capacity about 1200 cc.) and oxygenated by passing in the gas through a cotton wool plug and sterilised tube, until the whole of the air was displaced with oxygen. The bottle was then closed with a rubber bung, wired and rotated in a thermostat at 38°. It was opened hourly for re-oxygenation and removal of samples. Each sample (25 cc.) was heat-coagulated on a boiling water-bath, 10 g. solid uranium acetate were added, made up to 100 cc., cooled, filtered and transferred to a polarimeter tube (4 decimetre) and the rotation determined. If the filtered solution was allowed to stand, occasionally a very slight opalescence appeared which could not be removed by filtration and which made it difficult to obtain a reading. Such solutions on standing longer, especially in sunlight, deposited a small amount of a flocculent precipitate and on filtration were clear. In two experiments also in which much larger amounts of a liver containing a considerable amount of glycogen were used, the glycogen opalescence made readings difficult.

The method was first applied to the incubated pulp without addition of sodium succinate. The zero reading was quite constant for different samples of muscle, and the change in rotation during 6 hours' incubation either nil or slightly dextro (+ 0·2°).

The changes in rotation in one of a number of experiments in which

5 g. succinic and 5 g. fumaric acids (as sodium salts) respectively had been added to 100 g. minced sterile muscle were as follows:

	5 g. succinic acid added			5 g. fumaric acid added		
Muscle sample	Time hours	Rotation	*l*-Malic acid formed (g.)	Time hours	Rotation	*l*-Malic acid formed (g.)
1	0	0·0 °	0·0	0	0·0 °	0·0
2	1	−0·30	0·36	1	−1·22	1·46
3	2	−0·63	0·75	2	−1·96	2·34
4	3	−0·88	1·05	3	−2·64	3·15
5	4	−1·18	1·41	4	−2·82	3·36
6	5	−1·42	1·69	5	−2·90	3·46
7	6	−1·64	1·96	6	−2·90	3·46
8	7	−1·83	2·18			
9	8	−2·01	2·40			
10	10	−2·36	2·82			
11	11	−2·40	2·86			
12	12	−2·40	2·86			
13	13	−2·40	2·86			

In calculating the *l*-malic acid formed, a small correction was applied for the volume of water contained respectively by the muscle and liver.

Replacing muscle by 30 g. liver, the following results were obtained:

	5 g. succinic acid added			5 g. fumaric acid added		
Liver sample	Time hours	Rotation	*l*-Malic acid formed (g.)	Time hours	Rotation	*l*-Malic acid formed (g.)
1	0	0·0 °	0·0	0	0·0 °	0·0
2	1	−0·59	0·64	0·5	−1·53	1·65
3	2	−1·22	1·32	1·0	−2·46	2·65
4	3	−1·90	2·05	1·25	−2·84	3·06
5	4	−2·58	2·79	1·50	−3·02	3·26
6	5	−3·12	3·37	1·75	−3·20	3·45
7	6	−3·28	3·54	2	−3·27	3·52
8	7	−3·28	3·54	3	−3·27	3·52

The experiments with 100 g. sterile muscle and 5 g. succinic and fumaric acids (as sodium salts) were then repeated with sufficient potassium cyanide added to make the solution 0·03 % KCN. The results are as follows:

	5 g. succinic acid added			5 g. fumaric acid added		
Muscle sample	Time hours	Rotation	*l*-Malic acid formed (g.)	Time hours	Rotation	*l*-Malic acid formed (g.)
1	0	0·0 °	0·0	0	0·0 °	0·0
2	2	−0·62	0·74	1	−1·22	1·46
3	3	−0·64	0·76	2	−1·94	2·31
4	4	−0·64	0·76	3	−2·58	3·08
5	6	−0·64	0·76	4	−2·79	3·32
6	7	−0·64	0·76	5	−2·84	3·38
7	—	—	—	6	−2·84	3·38

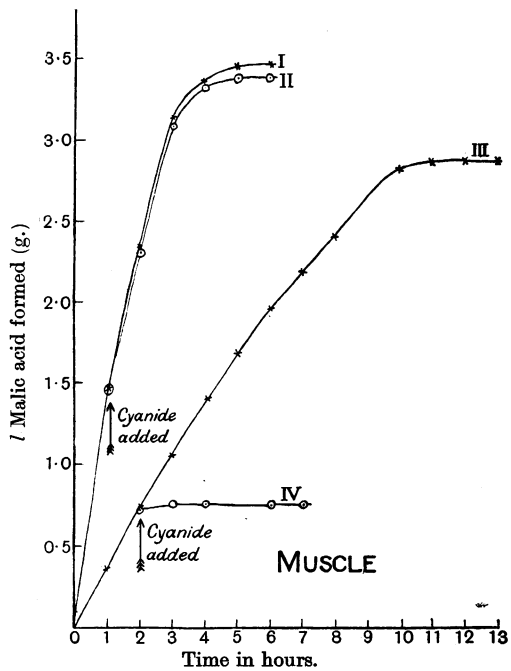
The cyanide was added, in the succinic acid experiment, after 2 hours and, in the fumaric acid experiment, after 1 hour had elapsed from the beginning of the experiment.

When these results are plotted, a number of interesting observations may be made.

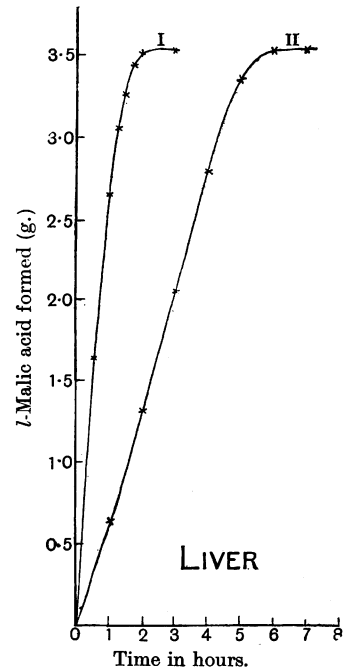
(1) Rabbit liver, weight for weight, is about eight times as active as rabbit muscle in the conversion of *both* succinic *and* fumaric acids to malic acid.

(2) The power of the liver enzyme to attack succinic and fumaric acids is remarkably great, 30 g. of liver converting 5 g. of succinic acid to the equilibrium mixture in 6 hours, and 5 g. fumaric acid in 2 hours. This will render isolation of these acids in the body an extremely difficult task. The velocity of the oxidation reaction is obviously much less than that of the subsequent addition of water and not *vice versa*, as has often been supposed.

(3) The succinic acid reaction with muscle does not attain quite to the same equilibrium as the fumaric acid reaction. This is probably due to the longer time required in the former, with resulting onset of bacterial infection, which often begins to make itself evident after 10–12 hours.



I. Fumaric acid + muscle.
II. Fumaric acid + muscle + 0·03 % KCN.
III. Succinic acid + muscle.
IV. Succinic acid + muscle + 0·03 % KCN.



I. Fumaric acid + liver.
II. Succinic acid + liver.

(4) Cyanide (0·03 %) completely stops the succinic acid reaction but has no effect on the fumaric acid reaction. The latter is therefore a simple addition of the elements of water and not an oxido-reduction, and the action of cyanide is strictly localised to the oxidation phase.

The curves above are readily reproducible with different samples of muscle and liver, the slight differences affecting the equilibrium point rather than direction of the curves.

Isolation and identification of the l-malic acid produced.

The *l*-malic acid formed by the oxidation of succinic acid with tissue pulp was isolated and identified in the following way. The resulting oxidation mixture was heated in a boiling water-bath to coagulate the proteins and filtered through muslin. The coagulated protein was washed twice in hot water and again filtered through muslin. The combined filtrate and washings were then filtered through papers and the filtrate evaporated under reduced pressure to about 200 cc., a concentrated solution of lead acetate (20 g.) added and then a little ammonia until the liquid was just alkaline, and finally about one-third the volume of alcohol, and the whole allowed to stand overnight. The precipitate containing malic and fumaric acids was filtered off, washed with a little cold water, decomposed with a slight excess of sulphuric acid and filtered. The filtrate was made alkaline with barium hydroxide and the precipitated phosphate and fumarate filtered off. The filtrate was then neutralised, the malic acid precipitated as the silver salt and recovered by passing hydrogen sulphide into its suspension in water, filtered and evaporated. The crude acid was then dissolved in a little water and extracted with butyl alcohol under reduced pressure by Dakin's [1920] method. For final identification, the *l*-malic acid was converted into the cinchonine salt, M.P. 197–198°, and shown to be identical with a synthetic sample obtained by resolving inactive malic acid by means of cinchonine [Dakin, 1924]. The mixed melting point and rotations were identical with those obtained by Dakin.

Limits of the method. In the preceding experiments, a 1 % solution of succinic and fumaric acids had been used. It was next attempted to find out if the presence of much smaller amounts of succinic acid could be detected by this rotational method. In this series of experiments, 100 g. muscle or 30 g. liver were added to 500 cc. of a neutralised 0·1 % solution of succinic and fumaric acids, and, for heat-coagulation, a sample of 100 cc. was removed, heat-coagulated, uranium acetate added and the rotation found as before. The following are typical results with muscle:

Acid	Time (hours)	Change in rotation	*l*-Malic acid formed (g.)
Succinic	6	−0·90°	0·27
Fumaric	6	−1·12	0·33

These results obtained with 0·1 % solutions of the acids compare very well with the equilibrium values obtained previously with 1 % solutions, for succinic acid 2·86, and for fumaric acid, 3·46. The above figures show that although the method has only been used for detection of amounts of succinic acid of this order, yet it is capable of greater delicacy by taking larger samples or by using smaller changes in rotation.

Application of the method. The method, in the adapted form, was then used in attempts to detect the formation of succinic acid in liver and muscle pulp from the following possible precursors, viz. acetone, sodium acetate, α-ketoglutarate, acetoacetate, diacetylsuccinate, and δ-ketohexoate. The sub-

stances in 500 cc. of water were added to the muscle or liver pulp (in place of the succinate) and rotated in oxygen for 6 hours, reoxygenating hourly. The difference in optical rotation at the beginning and end of the period was in every case slightly dextro (as in a blank determination). It would appear, therefore, that although the above substances (except diacetylsuccinate) have been shown to yield succinic acid with hydrogen peroxide, they are either not similarly oxidised by muscle and liver pulp under the conditions of these experiments, or the amount of succinic acid formed is so small that it cannot be detected by the method.

Preparation of δ-ketohexoic acid. [See Clutterbuck and Raper, 1925.]

Preparation of sodium diacetylsuccinate. Ethyl diacetylsuccinate was first obtained by the action of iodine on ethyl sodioacetoacetate in ether solution by the method of Rugheimer [1874], and converted into ethyl isocarbopyrotritartrate by the method of Knorr and Haber [1894], 10 g. of ethyl diacetylsuccinate yielding only 3·85 g. of the recrystallised product. This substance was converted into sodium diacetylsuccinate just before use.

Ethyl isocarbopyrotritartrate (6·8 g.) was warmed with 54·4 g. of a 20 % solution of sodium hydroxide until the solution became clear, and boiled for 6½ minutes. It was cooled under the tap and in iced water, and then added to 13·6 cc. of sulphuric acid in 102 cc. of water. The free isocarbopyrotritartaric acid was filtered off, washed, dried and dissolved in the theoretical amount of sodium hydroxide by heating for 1 minute. The sodium diacetylsuccinate thus formed was made up to 500 cc. and added to the liver pulp as before.

Preparation of α-ketoglutaric acid. By condensation of ethyl oxalate and succinate in presence of sodium ethoxide, an ester is obtained [Wislicenus, 1889], which according to Blaise and Gault [1908], on boiling with hydrochloric acid and evaporating, yields α-ketoglutaric acid. On attempting to obtain the acid by this method, by evaporating on the water-bath, a viscous syrup was obtained which, as Gabriel [1909] showed, readily reacts with hydrazine and must contain a considerable amount of α-ketoglutaric acid; yet it was difficult to obtain a pure specimen by recrystallisation. The method of obtaining the pure acid was as follows. Ethyl oxalylsuccinate was first prepared by the method of Wislicenus and Waldmüller [1911]. Potassium (13·4 g.) was added to a mixture of absolute, dry ether (150 g.) and absolute alcohol (40 g.) distilled over sodium and the whole warmed under a reflux until dissolved and then cooled. Pure oxalic ester (50 g.) was then added and after 10 minutes pure succinic ester (59·5 g.) poured in with shaking. The potassium salt of the double ester began to separate immediately and after standing a few hours the solution had set to a solid mass. The ether-alcohol and any unchanged ester were squeezed out of the solid mass by means of a press, the solid again shaken with ether and the ether similarly removed.

The potassium salt was then suspended in water, acidified and the ester

extracted with ether, the ethereal solution dried over sodium sulphate, and the ether removed. The ester was then boiled for 2 hours under reflux with six times its weight of diluted hydrochloric acid (1 in 2) and evaporated *in vacuo* (1 mm.) from a water-bath completely to dryness, when almost pure α-ketoglutaric acid set solid in the distilling flask. If the evaporation is not carried out *in vacuo*, other products, probably of condensation, arise and cause the difficulty in obtaining a pure product by recrystallisation. The product was dissolved in a little warm acetic acid and allowed to stand. After several hours, a little acid had separated and this was filtered off and the solution stirred and allowed to stand overnight. The bulk of the acid now separated and this second crop was practically pure and melted at 110–112°. It was once more recrystallised from acetic acid and melted at 112°. It readily gave a semicarbazone, M.P. 220°.

SUMMARY.

(1) A polarimetric method for following the conversion of succinic acid to fumaric and malic acids is described.

(2) Using the minced organs, rabbit liver is eight times as active, weight for weight, as rabbit muscle in bringing about the conversion *both* of succinic to fumaric acid and of fumaric to malic acid.

(3) Using the method, with the minced organs, no production of succinic acid from the following possible precursors could be detected: acetone, sodium acetate, acetoacetate, α-ketoglutarate, δ-ketohexoate and diacetylsuccinate.

(4) Cyanide, while inhibiting completely the change from succinic to fumaric acid, has no action on the conversion of fumaric to malic acid. The latter change is therefore not an oxido-reduction process but a simple addition of the elements of water.

In conclusion, the author desires to thank Professor H. S. Raper for helpful suggestions and criticism, and Mr O. N. Jones for considerable assistance in the experimental part of this work.

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