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Knowledge of the specificity of dehydrogenases.

by T. Thunberg.

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I. The specificity of succinic dehydrogenase activation.

I first raised the question of specificity in reports on a group of enzymes discovered by Wieland which I published in 1916, 1917 and 1920 (1, 2, 3) under the newly introduced name of dehydrogenases. In the second paper I stated that an enzyme present in the animal organism, succinic dehydrogenase, which activates the hydrogen of succinic acid, shows no hydrogen-activating powers in connection with about 50 organic acids and other organic substances listed in my article. Succinic dehydrogenase had a certain hydrogen-activating effect only on methyl-succinic acid (pyrotartaric acid). In the third paper I returned to the question of the specificity of dehydrogenases. Based on experiments discussed therein, I expressed the opinion that dehydrogenases are "enzymes of rather distinct specificity." I elaborated this concept further in these words: "This does not mean, of course, that specificity is absolute." I therefore disassociated myself expressly from the concept occasionally ascribed to me in the pertinent literature, that an unconditional specificity is characteristic of dehydrogenases. Nor do other parts of my writings contain expressions in this spirit. Naturally I did not intend to deny the possibility that future studies may uncover an enzymatic specimen of this type which possesses such specificity. At any rate, it ought to be easier to furnish disproof of the absolute specificity of an enzyme than it would be to offer proof thereof. Even though a large number of tested substances had proved inaccessible to the hydrogen-activating effect of a certain enzyme, there is a possibility that such an influence exists in connection with an as yet untested substance.

The observation that succinic dehydrogenase, in spite of its high specificity, not only affects succinic acid, but also methyl-succinic acid, prompted me to examine other alkylated succinic acids in this respect. Since such materials were not available commercially, I asked Dr. Erik Larsson to prepare the following: dimethyl-succinic acid in the fumaroid form with a high melting point; methylethyl-succinic acid in its fumaroid form with high m.p. as well as in its maleinoid form;

diethyl-succinic acid, which was obtained not in its homogeneous form, but as a mixture of the racemic and meso forms. These substances must not be considered completely pure, since their melting points do not quite coincide with those given in the literature for pure substances. The significance of this circumstance will be discussed after the description of results of attempts to employ these materials as donaters in the presence of succinic dehydrogenase.

The solution of succinic dehydrogenase used in our tests was prepared as follows:

100 g of commercial well-ground horse meat were repeatedly washed with small amounts of a boric acid - sodium chloride solution (1% boric acid, 0.25% NaCl). Washing was repeated until the meat had turned white. The meat, freed of the wash fluid by pressure, was then ground in the mortar together with 150 cc of a solution of sodium phosphate - boric acid (1.2% Sorensen's sodium phosphate, $Na_2HPO_4 + 2\ H_2O$, 1% boric acid). After the meat had rested for 30 minutes, it was vigorously centrifuged for 20 minutes. The opalescent upper layer of separated fluid represents the succinic dehydrogenase solution used in our tests.

Washing and extraction of the muscular mass with solutions containing boric acid were motivated by the desire to eliminate the effect of bacteria as much as possible. The technique is the result of experience gained with succinic dehydrogenase solutions, some of which were prepared without addition of boric acid to the wash fluid or the extraction fluid, some with such an additive. The variable results obtained with dehydrogenase solutions without addition of antiseptics were replaced by constant results after the introduction of boric acid.

Major tests were always accompanied by simultaneous controls.

In the examination of a possible hydrogen-activating effect of succinic dehydrogenase solution on alkylated succinic acids, I utilized my methylene blue method (cf., for example, its preparation in Oppenheimer, The Ferments and their Effects, 5th ed., Vol. 3, Leipzig 1929, p. 1118).

Tests were initiated with one repetition under conditions of optimal control, aimed at the demonstration of hydrogen-activating action by succinic dehydrogenase on methyl-succinic acid (pyrotartaric acid).

The following record will illuminate the experiments.

Test 1. 19 October 1931.

Methylene blue solution (methylene blue medical "Merck") 1:30000.

Succinic acid, 118 mg were neutralized with n/2 KOH, after which the solution was diluted to 10 cc. (Succinic acid and all other acids used

in these tests were neutralized without indicators, since indicator solutions occasionally act deleteriously on the decoloration process.)

Pyrotartaric acid, 132 mg were neutralized with n/2 KOH and diluted to 10 cc.

Succinic dehydrogenase solution, prepared according to the description given above.

Distilled water.

Now seven tubes are charged according to Table 1, evacuated and placed in a water bath of 35°C. Succinic dehydrogenase was added to each tube just before evacuation. Substances to be tested for possible donator action (succinic acid and pyrotartaric acid) were used as m/10 solutions.

Table 1 (19 October 1931)

Tube #	1	2	3	4	5	6	7
Meth. blue 1:30000, cc	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Pyrotartaric acid, cc		0.1	0.2				
Succinic acid, cc					0.1	0.2	
Succinic dehydrogenase, cc	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Distilled water, cc	0.4	0.3	0.2	0.4	0.3	0.2	0.4
Decoloration time, minutes	>60	15	16	>60	3	3	>60

As evident from Table 1, addition of succinic acid in said quantities reduces decoloration time in the system to 3 minutes. Admixture of corresponding amounts of pyrotartaric acid, while reducing decoloration time distinctly, does not accelerate beyond 15 minutes.

The ability of pyrotartaric acid to act as hydrogen donator in the presence of succinic dehydrogenase is shown also in the next test, in which the amount of pyrotartaric acid was varied within much wider limits than in the previous experiment. Variations were achieved by preparation of pyrotartaric solutions at concentrations m/10, m/50 and m/250. These solutions were added to various tubes in amounts ranging from 0.1 to 0.4 cc.

Test 2. (6 April 1932)

Methylene blue solution 1:50000.

Pyrotartaric acid, 26.4 mg neutralized with n/2 KOH, diluted to 2 cc = Br = m/10.

1 cc Br + 4 cc distilled water = Br/5 (= m/50),
1 cc Br/5 + 4 cc distilled water = Br/25 (= m/250).

Succinic dehydrogenase, prepared on the preceding day according to instructions.

Table 2 (6 April 1932)

Tube #	1	2	3	4	5	6	7
Meth. blue 1:50000, cc	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Pyrotartaric acid Br, cc							
" Br/5, cc						0.1	0.2
" Br/25, cc		0.1	0.2	0.4			
Succinic dehydrogenase, cc	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Distilled water, cc	0.4	0.3	0.2		0.4	0.3	0.2
Decoloration time, minutes	>180	133	90	81	>180	73	62

Tube #	8	9	10	11	12	13
Meth. blue 1:50000, cc	0.5	0.5	0.5	0.5	0.5	0.5
Pyrotartaric acid Br, cc			0.1	0.2	0.4	
" Br/5, cc	0.4					
" Br/25, cc						
Succinic dehydrogenase, cc	0.5	0.5	0.5	0.5	0.5	0.5
Distilled water, cc		0.4	0.3	0.2		0.4
Decoloration time, minutes	46	>180	49	49	49	>180

The shortest decoloration time achieved by addition of pyrotartaric acid to the enzyme system represents a considerably longer period than that listed in test 1, possibly because the succinic dehydrogenase solution used in test 2 was older. At any rate, this test also confirms the capability of pyrotartaric acid to act as donator substance. The curve of decoloration times plotted against the quantity of added donator substance is of the same type as that of the majority of donators studied to date. Initially the time shows a rapid drop in the presence of increased donator substance, after which it approaches a minimal value at a slower rate.

Thus the ability of pyrotartaric acid to occur as hydrogen donator under the influence of a succinic dehydrogenase solution has been confirmed. It is quite impossible that a specific pyrotartaric acid dehydrogenase is involved, different from succinic dehydrogenase, at least as long as the occurrence of pyrotartaric acid is not demonstrable in the animal organism.

The reaction product resulting from pyrotartaric acid upon dehydrogenation has not been isolated. In all probability it is methylfumaric acid (mesaconic acid).

Since every dehydrogenase not only activates hydrogen in the substrate's molecule to which it is adjusted, but also the molecule remaining after donation of activated hydrogen (the residual molecule), succinic dehydrogenase should also activate mesaconic acid and, in the presence of activated hydrogen in the solution, should effect a reduction of mesaconic acid to pyrotartaric acid, analogous to the influence of succinic dehydrogenase on fumaric acid. The extent to which dehydrogenation of methyl-succinic acid assert themselves in a specific case ought to depend on the solution's redox potential.

It would be interesting to know whether methyl-fumaric acid is influenced by aspartase or fumarase in a manner analogous to the interaction between fumaric acid and the corresponding enzyme.

While it was shown that pyrotartaric acid may enter the system methylene blue - succinic dehydrogenase as donator, a clear donator effect could not be demonstrated for the remaining alkyl derivatives of succinic acid mentioned in the introduction. Neither fumaroid dimethyl-succinic acid nor the low or high-fusing form of methylethyl-succinic acid, or diethyl-succinic acid as a mixture of the racemic and meso forms (in which form it was obtained) affected methylene blue decoloration in the system succinic dehydrogenase - methylene blue, at least not in a distinct manner. This circumstance shall be illustrated by data of two tests.

In the tests recorded below, the alkyl derivatives of succinic acid, which were to be examined for their possible donator action, were used in m/10 solutions. Each substance was employed in quantities of 0.1, 0.2 and 0.4 cc. Abbreviations given in the tables are readily interpreted. The tests include determination of decoloration rates of the system upon addition of variable amounts of a weak succinate solution. Results showed strong activating powers of succinic dehydrogenase solution with respect to succinic acid.

Table 3 (1 April 1932)

Tube #	1	2	3	4	5	6
Meth. blue 1:50000, cc	0.5	0.5	0.5	0.5	0.5	0.5
m/10 high m.p. dimethyl-succinic acid, cc		0.1	0.2	0.4		
m/10 low m.p. methylethyl-succinic acid, cc						0.1
m/250 succinic acid, cc						
Succinic dehydrogenase, cc	0.5	0.5	0.5	0.5	0.5	0.5
Distilled water, cc	0.4	0.3	0.2		0.4	0.3
Decoloration time, minutes	> 180	> 180	> 180	> 180	> 180	> 180

Tube #	7	8	9	10	11	12	13
Meth. blue 1:50000, cc	0.5	0.5	0.5	0.5	0.5	0.5	0.5
m/10 high m.p. dimethyl-succinic acid, cc							
m/10 low m.p. methylethyl-succinic acid, cc	0.2	0.4					
m/250 succinic acid, cc				0.1	0.2	0.4	
Succinic dehydrogenase, cc	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Distilled water, cc	0.2		0.4	0.3	0.2		0.4
Decoloration time, minutes	>180	175	>180	8	8	7	>180

Table 4 (1 April 1932)

Tube #	1	2	3	4	5	6
Meth. blue 1:50000, cc	0.5	0.5	0.5	0.5	0.5	0.5
m/10 high m.p. methylethyl-succinic acid, cc		0.1	0.2	0.4		
m/10 diethyl-succinic acid, cc						0.1
m/250 succinic acid, cc						
Succinic dehydrogenase, cc	0.5	0.5	0.5	0.5	0.5	0.5
Distilled water, cc	0.4	0.3	0.2		0.4	0.3
Decoloration time, minutes	>120	>120	>120	100	>120	>120

Tube #	7	8	9	10	11	12	13
Meth. blue 1:50000, cc	0.5	0.5	0.5	0.5	0.5	0.5	0.5
m/10 high m.p. methylethyl-succinic acid, cc							
m/10 diethyl-succinic acid, cc	0.2	0.4					
m/250 succinic acid, cc				0.1	0.2	0.4	
Succinic dehydrogenase, cc	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Distilled water, cc	0.2		0.4	0.3	0.2		0.4
Decoloration time, minutes	>120	>120	>120	8	8	7	>120

The listed decoloration times do not exclude the possibility of a weak donator effect of the alkyl derivatives of succinic acid under discussion. It may be maintained, however, that the donator action of these derivatives, if it exists, is extremely weak and has not been demonstrated to date despite the great sensitivity of our method.

We mentioned earlier that the alkyl derivatives used terminally may have contained impurities. This circumstance could affect test results if the added substances are capable of accelerating decoloration of the methylene blue - dehydrogenase system. In that case such a positive effect could be ascribed to impurities. The absence of such an effect indicates that the tested preparations are unable to function as donators.

II. The specificity of succinic dehydrogenase fixation.

As known from earlier studies, the specificity of dehydrogenases is expressed partly in the ability to fix certain substances, partly in the hydrogen activation of the substances. Fixation of the substrate of the appropriate enzyme group to the enzyme ought to be a necessary prerequisite for hydrogen activation. On the other hand, substrate fixation apparently is possible without subsequent hydrogen activation. The enzyme therefore shows greater specificity in its hydrogen activation than in its fixing power. Such substances, which are fixed to the enzyme without being activated by it, have an inhibiting effect on the ability of dehydrogenases to activate true hydrogen donators, a circumstance which permitted the discovery of fixation without activation.

Frequently the substances which are fixed to the dehydrogenase molecule without being activated are closely related chemically to the true donators. This is clearly indicated by experience gained with succinic dehydrogenase. Thus the inhibiting effect exerted on the activating power of succinic dehydrogenase vis-a-vis succinic acid by malonic acid and oxalic acid (cf. Quastel and Whetham 4, Quastel and Wooldridge 5) is best explained with reference to the position of malonic acid and oxalic acid opposite succinic acid in the chemical system. There is competition for the dehydrogenase molecule between succinic acid and malonic acid, if both substances are present in a solution of succinic dehydrogenase.

I was tempted to expand these studies to other dicarbonic acids. Tests with malonic acid and oxalic acid were first repeated.

The following test shows how decoloration of methylene blue in the system methylene blue - succinic dehydrogenase - succinic acid proceeds in the absence and presence of oxalate.

Test 3 (9 April 1932)

Methylene blue 1:50000 (= Mb)
Potassium succinate, 39 mg dissolved in 2 cc water (= B)
1 cc B + 24 cc water = B/25
Potassium oxalate, 66 mg dissolved in 4 cc water (= Ox).
Succinic dehydrogenase
Temperature in the water bath 35°C.

Each tube was charged with 0.5 cc Mb, 0.5 cc dehydrogenase and enough water to raise the total volume to 1.8 cc. Table 5 reflects only the amounts of succinate and oxalate solutions (in cc) added to the tubes, and the resultant decoloration times.

Table 5 (9 April 1932)

Tube #	B/25	Oxalate	Decoloration time in min.	Tube #	B/25	Oxalate	Decoloration time in min.
1	-	-	> 120	10	0.1	0.1	13
2	0.1	-	9	11	0.4	0.2	16
3	0.2	-	9	12	0.2	0.2	18
4	0.4	-	8	13	0.1	0.2	22
5	-	0.1	> 120	14	0.4	0.4	22
6	-	0.2	> 120	15	0.2	0.4	25
7	-	0.4	> 120	16	0.1	0.4	31
8	0.4	0.1	9	17	-	-	> 120
9	0.2	0.1	11				

The test shows initially that decoloration time is rapid in a solution containing succinic dehydrogenase and methylene blue, when only succinate is added. In the case under discussion the decoloration time was less than 10 minutes. No decoloration is obtained if oxalate alone is added. The tube series 8 through 16 indicates that inhibition results when the system Mb - succinic dehydrogenase is charged simultaneously with succinate and oxalate. Inhibition is barely apparent in tube 8, where the ratio between oxalate and succinate favors the latter, and most evident in tube 16, where the ratio favors oxalate. Note, however, that oxalate solution was used in a potency of m/10, while succinate was concentrated only m/250. The true molar relation between succinate and oxalate therefore had fluctuated between 1:6.25 and 1:100.

Inhibition of Mb decoloration is even more distinct in a succinate - succinic dehydrogenase solution, if malonate is added (cf. Table 6). Malonate solution was concentrated at m/10 (72 mg potassium malonate dissolved in 4 cc water). The remaining factors of this test were identical with the experiment using oxalate.

Table 6 (11 April 1932)

Tube #	B/25	Malonate	Decoloration time in min.	Tube #	B/25	Malonate	Decoloration time in min.
1	-	-	> 420	10	0.1	0.1	160
2	0.1	-	11	11	0.4	0.2	140
3	0.2	-	10	12	0.2	0.2	160
4	0.4	-	9	13	0.1	0.2	240
5	-	0.1	> 420	14	0.4	0.4	160
6	-	0.2	> 420	15	0.2	0.4	240
7	-	0.4	> 420	16	0.1	0.4	360
8	0.4	0.1	90	17	-	-	> 420
9	0.2	0.1	140				

The following tables show that, among dicarbonic acids with long, unbranched carbon chains most closely related to succinic acid, glutaric acid has a weak inhibiting effect on Mb decoloration, while that of adipinic acid is even smaller.

Table 7 (11 April 1932)

Tube #	B/25	Glutaric acid	Decoloration time in min.	Tube #	B/25	Glutaric acid	Decoloration time in min.
1	-	-	> 180	10	0.1	0.1	15
2	0.1	-	10	11	0.4	0.2	14
3	0.2	-	9	12	0.2	0.2	15
4	0.4	-	9	13	0.1	0.2	-
5	-	0.1	> 180	14	0.4	0.4	18
6	-	0.2	> 180	15	0.2	0.4	20
7	-	0.4	> 180	16	0.1	0.4	21
8	0.4	0.1	13	17		-	> 180
9	0.2	0.1	14				

Table 8 (12 April 1932)

Tube #	B/25	Adipinic acid	Decoloration time in min.	Tube #	B/25	Adipinic acid	Decoloration time in min.
1	-	-	> 180	10	0.1	0.1	12
2	0.1	-	11	11	0.4	0.2	11
3	0.2	-	10	12	0.2	0.2	13
4	0.4	-	10	13	0.1	0.2	14
5	-	0.1	> 180	14	0.4	0.4	14
6	-	0.2	> 180	15	0.2	0.4	16
7	-	0.4	> 180	16	0.1	0.4	18
8	0.4	0.1	10	17	-	-	> 180
9	0.2	0.1	12				

Of the alkyl derivatives of malonic acid, we examined dimethyl-malonic acid, ethyl-malonic acid, diethyl-malonic acid and allylmalonic acid for a possible inhibiting effect on the 3-substance system discussed here. While these substances do show a certain inhibiting action, it cannot be compared with that of malonic acid proper.

Table 9 (13 April 1932)

Tube #	B/25	Dimethyl malonate	Decoloration time in min.	Tube #	B/25	Dimethyl malonate	Decoloration time in min.
1	-	-	>180	10	0.1	0.1	7
2	0.1	-	6	11	0.4	0.2	7
3	0.2	-	6	12	0.2	0.2	8
4	0.4	-	5	13	0.1	0.2	8
5	-	0.1	>180	14	0.4	0.4	8
6	-	0.2	>180	15	0.2	0.4	10
7	-	0.4	>180	16	0.1	0.4	12
8	0.4	0.1	6	17	-	-	>180
9	0.2	0.1	7				

Table 10 (14 April 1932)

Tube #	B/25	Ethyl malonate	Decoloration time in min.	Tube #	B/25	Ethyl malonate	Decoloration time in min.
1	-	-	>180	10	0.1	0.1	13
2	0.1	-	6	11	0.4	0.2	12
3	0.2	-	8	12	0.2	0.2	14
4	0.4	-	6	13	0.1	0.2	18
5	-	0.1	>180	14	0.4	0.4	18
6	-	0.2	>180	15	0.2	0.4	20
7	-	0.4	>180	16	0.1	0.4	22
8	0.4	0.1	9	17	-	-	>180
9	0.2	0.1	11				

Table 11 (14 April 1932)

Tube #	B/25	Diethyl malonate	Decoloration time in min.	Tube #	B/25	Diethyl malonate	Decoloration time in min.
1	-	-	>180	10	0.1	0.1	14
2	0.1	-	9	11	0.4	0.2	13
3	0.2	-	9	12	0.2	0.2	14
4	0.4	-	7	13	0.1	0.2	16
5	-	0.1	>180	14	0.4	0.4	18
6	-	0.2	>180	15	0.2	0.4	21
7	-	0.4	>180	16	0.1	0.4	24
8	0.4	0.1	11	17	-	-	>180
9	0.2	0.1	12				

Table 12 (14 April 1932)

Tube #	B/25	Allyl malonate	Decoloration time in min.	Tube #	B/25	Allyl malonate	Decoloration time in min.
1	-	-	>180	10	0.1	0.1	19
2	0.1	-	8	11	0.4	0.2	15
3	0.2	-	6	12	0.2	0.2	18
4	0.4	-	6	13	0.1	0.2	23
5	-	0.1	>180	14	0.4	0.4	21
6	-	0.2	>180	15	0.2	0.4	28
7	-	0.4	>180	16	0.1	0.4	34
8	0.4	0.1	11	17	-	-	>180
9	0.2	0.1	15				

A study of the manner in which addition of pyrotartaric acid influences the rate of decoloration in the system Mb - dehydrogenase - succinate is very instructive, since pyrotartaric acid (methyl-succinic acid) can function as donator in the presence of succinic dehydrogenase. This substance may therefore be expected to furnish two effects: First, displacement of succinic acid from the dehydrogenase molecule, secondly, a donator function. In combinations listed in the table below, the result was a reduction in the rate of decoloration.

Table 13 (7 April 1932)

Tube #	B/25	Pyro-tartarate	Decoloration time in min.	Tube #	B/25	Pyro-tartarate	Decoloration time in min.
1	-	-	>180	10	0.1	0.1	17
2	0.1	-	13	11	0.4	0.2	18
3	0.2	-	12	12	0.2	0.2	20
4	0.4	-	12	13	0.1	0.2	22
5	-	0.1	60	14	0.4	0.4	25
6	-	0.2	60	15	0.2	0.4	27
7	-	0.4	60	16	0.1	0.4	30
8	0.4	0.1	16	17	-	-	>180
9	0.2	0.1	17				

We may conclude that slower Mb decoloration upon addition of pyrotartaric acid to the system Mb - succinic dehydrogenase, compared to Mb decoloration upon addition of succinic acid to the same system, is due to a difference in hydrogen activation rather than to dissimilarities in fixation of substrate.

We reported above that among alkyl derivatives of succinic acid examined for their ability to function as hydrogen donators in the system Mb - succinic dehydrogenase, only pyrotartaric acid gave evidence of donator action, while no such activity was found in connection with dimethyl-succinic acid, methylethyl-succinic acid and diethyl-succinic acid. The absence of donator action in the case of these substances may

be due either to the circumstance that they cannot be attached to succinic dehydrogenase or that they are fixed to the said enzyme, but do not participate in hydrogen activation. In the event the explanation is to be sought in absent hydrogen activation despite fixation to the enzyme, inhibition of Mb decoloration in the system Mb - succinic dehydrogenase - succinic acid can be expected. As evident from Tables 14-16, inhibition does indeed take place, although it is weak. For this reason these alkyl derivatives of succinic acid should not possess great affinity for succinic dehydrogenase.

Table 14 (15 April 1932)

Tube #	B/25	Dimethyl succinate m.p.182°C	Decoloration time in min.	Tube #	B/25	Dimethyl succinate m.p.182°C	Decoloration time in min.
1	-	-	> 180	10	0.1	0.1	12
2	0.1	-	10	11	0.4	0.2	14
3	0.2	-	10	12	0.2	0.2	14
4	0.4	-	12	13	0.1	0.2	15
5	-	0.1	>180	14	0.4	0.4	16
6	-	0.2	>180	15	0.2	0.4	18
7	-	0.4	>180	16	0.1	0.4	18
8	0.4	0.1	11	17	-	-	>180
9	0.2	0.1	12				

Table 15 (15 April 1932)

Tube #	B/25	Methylethyl succinate m.p.167°C		Decoloration time in min.	Tube #	B/25	Methylethyl succinate m.p.167°C	Decoloration time in min.
1	-	> 180	-	>180	10	0.1	0.1	10
2	0.1	10	-	10	11	0.4	0.2	13
3	0.2	10	-	10	12	0.2	0.2	13
4	0.4	11	-	11	13	0.1	0.2	14
5	-	> 180	0.1	>180	14	0.4	0.4	15
6	-	> 180	0.2	>180	15	0.2	0.4	13
7	-	> 180	0.4	>180	16	0.1	0.4	15
8	0.4	11	0.1	11	17	-	-	>180
9	0.2	11	0.1	11				

Table 16 (15 April 1932)

Tube #	B/25	Methylethyl succinate m.p.98°C	Decoloration time in min.	Tube #	B/25	Methylethyl succinate m.p.98°C	Decoloration time in min.
1	-	-	> 180	10	0.1	0.1	16
2	0.1	-	12	11	0.4	0.2	17
3	0.2	-	12	12	0.2	0.2	16
4	0.4	-	11	13	0.1	0.2	17
5	-	0.1	> 180	14	0.4	0.4	17
6	-	0.2	> 180	15	0.2	0.4	19
7	-	0.4	> 180	16	0.1	0.4	20
8	0.4	0.1	13	17	-	-	> 180
9	0.2	0.1	15				

III. Knowledge of the specificity of malic dehydrogenase.

Studies of muscular dehydrogenases published by me in 1917 (2) already demonstrated the presence in the musculature of a dehydrogenase adjusted to malic acid. While investigating the dehydrogenases of various types of seed, I soon found that malic dehydrogenase is one of the most prevalent enzymes in the seed's dehydrogenase system.

In my previous experiments with malic dehydrogenase I used both natural L-malic acid and synthetic D,L-malic acid. L-malic acid proved to be a better hydrogen donator. Since inactive malic acid is held to be a compound of both enantiomorphic configurations, it was likely that D-malic acid is less effective as donator substance than the natural, levorotatory form. One must even consider the possibility that D-malic acid is completely ineffectual. An investigation of this problem was initiated; its results are described below.

The D-malic acid used by me was synthesized and made available by Prof. Bror Holmberg of the Stockholm Technical Institute. He informed me that the preparation was produced by cleavage of inactive acid with (-)-phenethylamine and purified by precipitation from acetone solution with benzene. Prof. Holmberg also furnished L-malic acid purified in the same manner. With reference to the rotatory power of these samples, Prof. Holmberg writes that L-malic acid shows $(\alpha)_D = -419^\circ$ in solution with ammonia and uranylnitrate, and that D-malic acid indicates $+419^\circ$ under identical conditions. Aside from the prefix, rotatory power was identical.

I tested the action of D- and L-malic acid as donator substance in connection with a number of seeds which are known to exert a strong hydrogen-activating effect in the presence of L-malic acid. Seeds of the following plants were studied: Citrus aurantium dulc. (orange), Corydalis nobilis, Cucumis sativa (cucumber), Poinciana regia and Sicyos angulata.

Malic acids were first prepared in solutions of m/10 by neutralizing 27 mg and diluting to 2 cc. Solutions were made without indicators and their solvents, e.g., alcohol. Dilution at ratios 1/4 and 1/24 produced solutions in concentrations m/50 and m/250.

Malic dehydrogenase solutions used in our tests were prepared by treating husked seeds with an appropriate amount of 0.87% K_2HPO_4 solution. The seed substance is thoroughly mixed with the phosphate solution and extracted for 30 minutes under continuous stirring. The resultant emulsion is strongly centrifuged for 20 minutes. The centrifuged tube is then placed in ice water. Treatment of fatty seeds produces a cream-like upper layer, which is removed. The supernatant fluid is somewhat opanlescent and contains the effective dehydrogenase. In the test described here we used a 5-fold quantity (by weight) of potassium phosphate solution for the procurement of enzymatic extract; an exception was made in the case of citrus extract, where we used a 15-fold amount. Mb solution in a concentration of 1:50000 was used as hydrogen acceptor. The decoloration process was studied in a water bath of 35°C.

Tests were carried out in a series of 14 vacuum tubes, each of which was charged with 0.5 cc Mb solution, 0.2 or 0.4 cc of each of the various dilutions of malic acid, 0.5 cc seed extract and enough distilled water to make a total volume of 1.4 cc per tube. The extract had been freshly prepared. Each tube was charged with extract just prior to evacuation and placement in the water bath.

The resultant decoloration times are given in the following tables.

Table 17 (13 January 1932) Cucumis sativa

Micrograms malate per tube	Decoloration time in min. D-malate	L-malate	Micrograms malate per tube	Decoloration time in min. D-malate	L-malate
-	55	59	1080	48	15
108	55	28	2700	48	14
216	54	24	5400	46	16
540	53	17	-	59	58

Table 18 (14 January 1932) Sicyos angulata

Micrograms malate per tube	Decoloration time in min. D-malate	L-malate	Micrograms malate per tube	Decoloration time in min. D-malate	L-malate
-	43	42	1080	40	7
108	44	25	2700	40	5
216	42	18	5400	41	3
540	42	10	-	42	44

Table 19 (15 January 1932) Citrus aurantium dulc.

Micrograms malate per tube	Decoloration time in min. D-malate	L-malate	Micrograms malate per tube	Decoloration time in min. D-malate	L-malate
-	24	23	1080	22	10
108	20	18	2700	25	8
216	22	16	5400	29	7
540	22	12	-	23	22

Table 20 (15 January 1932) Poinciana regia

Micrograms malate per tube	Decoloration time in min. D-malate	L-malate	Micrograms malate per tube	Decoloration time in min. D-malate	L-malate
-	13	13	1080	13	6
108	11	12	2700	14	5
216	13	10	5400	14	4
540	13	9	-	13	13

Table 21 (14 February 1932) Corydalis nobilis

Micrograms malate per tube	Decoloration time in min. D-malate	L-malate	Micrograms malate per tube	Decoloration time in min. D-malate	L-malate
-	93	91	1080	89	-
108	91	37	2700	92	5
216	89	31	5400	99	5
540	89	11	-	91	-

As indicated by the tables, there is a pronounced difference between L- and D-malic acid. While L-malic acid is a distinct hydrogen donator, the ability of D-malic acid to function as donator is uncertain or weak.

The difference between the two malic acids is evident from the accompanying graphic illustrations which reflect the decoloration times in seed extracts of Cucumis sativa and Corydalis nobilis in relation to different amounts of malic acid with opposite configurations. While extracts from these seeds activated L-malic acid vigorously, D-malic acid is a weak hydrogen donator for Cucumis and has no effect whatever on Corydalis. Incidentally, side reactions are always possible if the decoloration effect is weak.

Literature

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