XIV. ON THE REDUCTION OF METHYLENE BLUE BY TISSUE EXTRACTS.

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THUNBERG [1909], in the course of an extensive study of the influence of various substances on the gas-exchange of surviving frog's muscle, found that previous treatment of the muscle with succinic acid caused a lowering of the respiratory quotient, in part due to diminished output of carbon dioxide, and, in part, to increased oxygen intake. If, however, the muscle was first subjected to a prolonged washing with water (a procedure which Batelli and Stern [1910] had found to cause a very considerable lowering of the gaseous exchange, the normal exchange being restorable in great measure by the addition of the aqueous extract, due, according to these workers, to the presence in the water-extract of an unisolated substance to which they gave the name "Pnein") and, after this washing, immersed in succinic acid, the gaseous exchange and particularly the consumption of oxygen would be considerably increased. Citric, malic, and fumaric acids were found also to increase the gas-exchange of this water-extracted muscle.

It is now well-known that many animal and vegetable tissues possess the property of reducing methylene blue, a reaction which makes itself apparent in the absence of oxygen by the formation of the colourless form of the pigment. As shown by Harden and Norris [1915] for dried yeast and rabbit muscle, and since then by various workers for many other tissues, this reducing property is removable to a greater or lesser extent by more or less prolonged washing of the tissue with water. Thunberg [1916], influenced by his previous results on the effects on the gas-exchange, showed that the addition of succinic acid to muscle, which had had its power of reducing methylene blue removed to a great extent by thorough washing, would restore its capacity for decolorising the pigment. The reaction was accompanied by the disappearance of the succinic acid and its replacement by fumaric acid, as shown by Einbeck [1913].

In a recent paper, Thunberg [1920] shows that many other substances besides succinic acid may act in a similar manner, and concludes that such

substances are to be considered as intermediary products of metabolism. From these results and the fact that such reactions are not influenced by cyanides, Thunberg concludes that here we have strong evidence in favour of Wieland's hypothesis that the cause of many biological oxidations is to be sought, not in the activation of oxygen but in the activation of hydrogen, the hydrogen being transferred from one substance to another by means of an enzyme. These enzymes have been given by Thunberg the generic name of "Hydrogentransportases" or "Dehydrogenases." In the succinic acid reaction the "Succinodehydrogenase" removes hydrogen from the "Donator," succinic acid, and transfers it to the "Acceptor," in this case, the methylene blue. In living tissues, of course, the acceptor for the removed hydrogen is oxygen, resulting in the formation of water. According to such a conception, the oxygen inspired and transported to the tissues is not concerned in the direct oxidation of the metabolic products, but in the removal of the hydrogen which has been taken from these substances by means of the dehydrogenising enzymes.

The question was raised by Thunberg [1920] as to whether each donator possesses its own specific dehydrogenase, or whether the indirect oxidation of these substances is to be considered as due to the activities of a common enzyme. The results of his experiments on freezing and warming the muscle-mass to various temperatures appeared to indicate that succinic, glutaric, and hydroxyglutaric dehydrogenases, at least, are specific for these substances respectively, a result which is supported by his finding that while *l*-tartaric acid will act as a donator *d*-tartaric acid will not.

The following experiments were conducted primarily with the object of obtaining evidence on the question of the specificity of the enzymes.

In order to obtain uniformity of results, an extract, prepared according to the method described by Ohlsson [1921], by shaking the washed muscle for one hour with a solution of K_2HPO_4 of one-fifteenth molar strength, was employed. 1 cc. of such an extract was measured into a Thunberg vacuum tube, suitable amounts of methylene blue and donator substance added, and a sufficient quantity of water to make the volume in each tube of the experiment the same. The ground stopper, smeared with a mixture of bees'-wax, resin, and vaseline was inserted, the tube evacuated under water with an ordinary water-pump until the contents boiled when warmed with the hand, and then placed in a thermostat at 35°, the time for complete decolorisation of the methylene blue being noted.

Experiments were first conducted to determine which substances would act as donators to such a phosphate extract; only those substances which had been found by Thunberg to act as efficient donators in working with unextracted washed muscle, were tried. All acids were used in the form of carefully neutralised solutions of their potassium salts.

The following table gives the results obtained: (A), with an extract made from ox-muscle; (B), with a rabbit's muscle extract; and (C), with an extract

made from rabbit's liver. The figures given are calculated according to the following formula introduced by Thunberg [1921]:

$$I = 100 \left[\frac{1}{A} - \frac{1}{B} \right]$$

(where A is the time required for complete decolorisation in the presence of a donator, and B the time for complete decolorisation when the muscle is acting alone).

The values of I give some idea of the relative efficiency of the various donators. Taking into consideration the possible errors due to leakage and bacterial action, it is doubtful if substances with a value below unity should be considered as hydrogen-donators.

Donator	A	B	C
Succinic acid ...	14	9	22
Methylsuccinic acid ...	4	3	—
α -Hydroxyglutaric acid ...	2.8	1.3	1.5
α -Ketoglutaric acid ...	2.4	4	2.2
Lactic acid ...	0.1	2	1.4
Tartronic acid ...	0.05	1	2
Formic acid ...	0	0.4	3.4
Acetic acid ...	0	0.2	0.7
Oxalic acid ...	See below	0.6	0.7
Glutaric acid ...	0	0.6	0.5
α -Hydroxybutyric acid ...	0.05	0.5	0
β -Hydroxybutyric acid ...	0.05	0.5	0.7
<i>l</i> -Tartaric acid ...	0	0.7	—
<i>d</i> -Tartaric acid ...	0	0.5	—
Glutamic acid ...	0	0.5	1.1
Propionic acid ...	0	0	3
Citric acid ...	0	0	1.4
Alanine ...	0	0	0.3
Butyric acid ...	0	0	0
Capronic acid ...	0	0	0
β -Ketoglutaric acid ...	0	0	0
Dimethylsuccinic acid ...	0	0	—
Methylethylsuccinic acid ...	0	0	—
Diethylsuccinic acid ...	0	0	—

Fumaric acid, malic acid, and maleic acid prevented decolorisation taking place at all in the case of both ox-muscle extract and rabbit-muscle extract, while with oxalic acid the decolorisation time in the case of ox-muscle extract was longer than in the absence of any donator substance.

The first experiments were those performed with extracts made from ordinary butcher-meat, but, as the age of such meat was always doubtful, extracts made from the muscles of a rabbit, killed immediately before the experiment, were afterwards employed. Considerable difficulty was at times experienced in obtaining an efficient extract from rabbit muscle, a difficulty which, sometimes but not always, could be surmounted by grinding the washed muscle with a little powdered glass in a mortar before extraction with the phosphate solution. A too prolonged grinding with glass powder was found to diminish the number of substances which would act as donators; this is in accordance with the observation of Harden and Norris that a too fine division of the muscle leads to the removal of the enzyme. Too pro-

longed washing of the muscle also diminished the number of substances acting as donators. Thunberg's finding that the succinic reaction was by far the most resistant was confirmed.

An attempt was now made to determine whether each donator possessed its own dehydrogenase, *i.e.* whether a number of enzymes, each specific for its own dehydrogenising reaction, were present, by the following method.

Keeping the amount of methylene blue constant, and varying the concentration of donator present, that concentration which would cause complete decolorisation in the shortest time was determined for each of two donators. Then, combining these amounts of donators in the one tube, it was presumed that the time required for complete decolorisation would be less than the shorter of the two times previously obtained if two specific enzymes were in action, while, if there were only one common enzyme, the time would be the same or slightly longer. As succinic, α -hydroxyglutaric, and α -ketoglutaric acids were found to be the most constant donators, these acids were used for such experiments.

The following gives an example of such an experiment carried out with an extract prepared from rabbit-muscle:

Determination of the concentration of succinic acid causing decolorisation in the shortest time.

Mg. of potassium succinate in total volume of 3 cc.	Decolorisation time minutes	Mg. of potassium succinate in total volume of 3 cc.	Decolorisation time minutes
0.024	Not within time of control with muscle alone	1.062	6.5
		1.18	7
0.047	10	1.77	8
0.071	8.5	2.36	10.5
0.094	8	2.95	11.5
0.118	8	3.54	12.5
0.236	7.5	4.72	12.5
0.354	7.5	5.9	13
0.472	7	7.08	14
0.590	7	8.26	16
0.708	6.5	9.44	17.5
0.826	6.5	10.62	20
0.944	6.5	11.8	22

Determination of the concentration of α -ketoglutaric acid causing decolorisation in the shortest time.

Mg. of potassium ketoglutarate in total volume of 3 cc.	Decolorisation time minutes	Mg. of potassium ketoglutarate in total volume of 3 cc.	Decolorisation time minutes
0.292	Not within time of a control with muscle alone	2.92	42
0.584		3.65	40
0.876		4.38	43
1.168	137	7.30	34
1.46	120	14.6	12
2.19	78		

Higher concentrations could not be tried owing to the small amount of ketoglutaric acid available.

When the optimum amount of succinic acid was now combined with the highest concentration of α -ketoglutaric acid, the time required for complete decolorisation was no shorter, in fact, slightly longer than the shortest time for succinic acid acting alone.

Combination of succinic and α -ketoglutaric acids.

	Mg. in total volume of 3 cc.	Decolorisation time minutes
Potassium succinate	0.826	7
„ ketoglutarate	13.59	

A similar experiment was carried out with an extract made from calf-muscle, the results of which are summarised below.

Concentration of donator. Mg. in total volume of 3 cc.

Potassium succinate	Potassium α -hydroxyglutarate	Potassium α -ketoglutarate	Decolorisation time minutes
0.029	—	—	Not within time of a control with muscle alone
0.047	—	—	74
0.118	—	—	7.5
—	13.3	—	61
—	—	13.1	20
0.029	13.3	—	19
0.047	13.3	—	11
0.118	13.3	—	7.5
0.029	—	13.1	23
0.047	—	13.1	22
0.118	—	13.1	34

From such experiments it would appear that:

(1) As the concentration of donator substance is increased, the time for complete decolorisation of a fixed quantity of methylene blue diminishes extremely rapidly, and further increase of donator beyond this optimum value causes a lengthening of the decolorisation time.

(2) The optimum time for α -ketoglutaric and α -hydroxyglutaric acids appears to be in very high concentration.

(3) Very slight changes in the concentration of donator may produce very great differences in the decolorisation time; when the concentration is very low there seems to be even inhibition of the process of decolorisation by the muscle itself. This is seen in the cases of succinic acid in a concentration of 0.024 mg. and α -ketoglutaric in concentrations of 0.292, 0.584 and 0.876 mg.

(4) It is hardly to be expected that a comparatively slow reaction such as that of α -hydroxyglutaric or α -ketoglutaric acid would show much detectable effect on a rapid reaction like that of succinic acid.

(5) It would appear from the results of such experiments that the optimum time for reduction in the presence of succinic acid cannot be diminished, while, provided that the combined reaction be an active one, the optimum time for decolorisation is prolonged, just as in the case where the concentration of succinic acid alone is increased.

The finding that low concentrations of substances acting as donators would inhibit the spontaneous decolorisation by the muscle suggested that malic acid, which hitherto had always been found to prevent decolorisation, might, in suitable concentration, act as an activator for the process. On investigation of this point it was found that, for extracts made from rabbit-muscle and ox-muscle: (a) the inhibiting action of malic acid on the reduction of the methylene blue was exhibited in concentrations as low as 0.2 cc. of *M*/100 solution of the acid in a total volume of 3 cc.; (b) at certain high concentrations [about 0.4 cc. of *M* solution in a total volume of 3 cc.] there was undoubted but incomplete decolorisation; (c) in accordance with observation (b), when various concentrations of malic acid were combined with the optimum concentration of succinic acid, complete decolorisation could be obtained at two stages in the series, first, if the concentration of malic acid were very small, and, secondly, some decolorisation could be obtained with very high concentrations of the malic acid.

The results suggested further that some of the substances tried in only one concentration and hitherto classed as not donators, might act as such if the concentrations were suitable. Glutaric, glutamic, *l*-tartaric, acetic, and fumaric acids were all tried in varying concentration; in no case was the decolorisation time shorter than that of a control with muscle alone, but in concentrations higher than 0.1 cc. of *M* solution in a total volume of 3 cc., inhibition of the spontaneous reduction by muscle alone was a general effect.

The strong inhibiting action of fumaric acid on the succinic acid reaction suggested that its combination with other donators might yield some information on the specificity of the enzymes. Fumaric acid was found to inhibit, to a certain extent, the decolorisation in the presence of both α -hydroxyglutaric and α -ketoglutaric acids.

These methods of investigation were now abandoned for two reasons. As previously stated, it is not to be expected that the slower reactions would show much effect on the more rapid ones, and, secondly, an investigation of the times for complete decolorisation of the *same* quantity of methylene blue by *different* concentrations of donator does not give a true indication of the course of the reaction. This will be apparent from a closer study of figures taken from two experiments where succinic acid was used as donator substance.

Experiment A. Optimum time—6.5 minutes—obtained when 0.708 mg. of succinic acid was present. The hydrogen removed from the succinic acid and transferred to the methylene blue [as calculated from the amount of methylene blue decolorised] was 2.6 % of the total hydrogen removable from all the succinic acid present, provided it all changed to fumaric acid. This represents a reduction at the rate of 0.4 % per minute.

In the same experiment a time *longer* than the optimum—8.5 minutes—was obtained when 0.071 mg. succinic acid was present. The hydrogen removed in this case equals 25 % of the total removable or a reduction at the rate of 3 % *per minute*.

Experiment B. Optimum time—13 minutes—obtained when 0.708 mg. succinic acid present, a reduction of 1.6 % of the total, or reduction at the rate of 0.13 % per minute.

In the same experiment 102 minutes were required when 0.024 mg. succinic acid were present. This represents a reduction of 51 % of the total or at the rate of 0.5 % per minute.

It was therefore thought that a better method would be to vary the quantity of methylene blue present, while keeping the concentration of donator substance constant. The times for complete decolorisation could then be compared with the amounts of methylene blue decolorised, which, presumably, represents the transportation of a certain amount of hydrogen. Such a comparison would then give a truer picture of the course of the reaction. The method, it must be remembered, will not give absolutely accurate results, because, the degree of evacuation of the tubes may not be originally quite the same in each case, and, while tubes that had obviously leaked were discarded, a small undetectable leakage may occur which will materially alter the result, especially where the experiment is of long duration; further it is impossible to determine with absolute accuracy when the contents of a tube are completely decolorised. The effect of these errors is, of course, diminished by the large number of observations made.

Extracts prepared from rabbit liver were employed for the following experiments; because such extracts react with a larger number of donators. In the case of liver extracts, it is more difficult to reduce by washing the activity of the decolorisation by the tissue alone, but a sufficient differentiation can be obtained between the spontaneous decolorisation and the decolorisation in the presence of a donator.

Series of tubes were prepared with the same amount of enzyme and the same concentration of donator in each, but with gradually increasing quantities of methylene blue. The times for complete decolorisation were then compared with the quantity of methylene blue decolorised, and hence the amount of hydrogen transported, in each case. The following table shows the series of quantities employed in conducting such an experiment with lactic acid as donator.

Decolorisation in absence of any donator.

Extract 1 cc.

Hydrogen removed as calculated from meth. blue decolorised. Mg. $\times 10^3$	0.098	0.196	0.391	0.782	1.563	3.125
Time for decolorisation (minutes)	12	21	48	106	217	Not decol. in 5 hours

Decolorisation with succinic acid as donator.

Extract 1 cc.; succinic acid as K salt, 0.4 cc. *M*/10 solution in total vol. of 3 cc.

Hydrogen removed as calculated from meth. blue decolorised. Mg. $\times 10^3$	0.098	0.196	0.391	0.782	1.563	3.125	6.250
Time for decolorisation (minutes)	4	7.5	13.5	23.5	37	51	77

Decolorisation with lactic acid as donator.

Extract 1 cc.; lactic acid as K salt, 0.4 cc. <i>M</i> /10 solution in total vol. of 3 cc.						
Hydrogen removed as calculated	0.098	0.196	0.391	0.782	1.563	3.125
from meth. blue decolorised.						
Mg. $\times 10^3$						
Time for decolorisation (minutes)	6.5	12	24	67	118	Not decol. in 5 hours

Decolorisation with both succinic and lactic acids as donators.

Extract 1 cc.; succinic acid as K salt, 0.4 cc. <i>M</i> /10 solution in total vol. of 3 cc.; lactic acid as K salt 0.4 cc. <i>M</i> /10 solution in total volume of 3 cc.						
Hydrogen removed as calculated	0.098	0.196	0.391	0.782	1.563	3.125
from meth. blue decolorised.						6.250
Mg. $\times 10^3$						
Time for decolorisation (minutes)	5	8	15	24.5	37.5	49
						82

Decolorisation with double quantity of succinic acid.

Extract 1 cc.; succinic acid as K salt, 0.8 cc. <i>M</i> /10 solution in total vol. of 3 cc.						
Hydrogen removed as calculated	0.098	0.196	0.391	0.782	1.563	3.125
from meth. blue decolorised.						6.250
Mg. $\times 10^3$						
Time for decolorisation (minutes)	8	13	22	31.5	43.5	67
						105

These results are presented graphically in graph I. A similar series of observations was made for each of the following: citric acid (graph II); α -keto-glutaric acid (graph III); formic acid (graph IV); and tartronic acid (graph V).

Graph VI shows the rapidity of decolorisation of methylene blue with four different extracts in the absence of any added donator. Owing to the comparative slowness of this spontaneous reaction, this graph is drawn to a different scale from that of the preceding ones.

From a survey of these graphs it will be noted that:

- (1) The graph of the rate of decolorisation by muscle alone, *i.e.* in the absence of any added donator, is approximately a straight line.
- (2) In the presence of a donator substance the graph of the decolorisation-rate does not resemble that of an ordinary enzymic reaction.
- (3) The greater the quantity of methylene blue present the more rapid the decolorisation. This effect is shown to an extreme degree in the case of citric acid where the tubes with least methylene blue took somewhat longer to decolorise than those with slightly greater amounts.

Decolorisation with citric acid as donator.

Extract 1 cc.; citric acid as K salt, 0.4 cc. <i>M</i> /10 solution in total vol. of 3 cc.						
Hydrogen removed as calculated from	0.098	0.196	0.391	0.782	1.563	3.125
meth. blue decolorised. Mg. $\times 10^3$						
Time for decolorisation (minutes)	16	11.5	11	24	52	105

In this case the liver was rapidly washed in order to preserve the effect of citric acid as a donator, and hence the spontaneous decolorisation by the muscle may have been taking a comparatively large part in the reaction.

Graph I

Graph II

(4) All the graphs are similar in this respect, that combination of another donator with succinic acid gives a curve tending towards the same form as that given when the concentration of succinic acid is increased.

CONCLUSIONS.

1. The hypothetical enzyme acting on the various donators is the same for all, though acting in different degrees with different donators, and being able to discriminate between very closely related substances, *e.g.* α - and β -ketoglutaric acids.

2. The reduction of methylene blue by the muscle alone is a process differing from the reduction which occurs in the presence of a donator substance like succinic acid; the graphs are of different form and it has several times been observed that, as the extract ages, the spontaneous reduction may increase in rapidity while there is little change or a diminution in the rapidity of decolorisation under the influence of a donator.

3. The form of the graphs obtained, both for donator reduction and spontaneous reduction, do not resemble the graphs of ordinary enzyme reactions, and hence it might be suggested that methylene blue does something more than merely play the passive rôle of hydrogen acceptor. In view of the fact that the greater the amount of methylene blue present in the test, the higher the proportional speed of the reaction, it is perhaps possible that the methylene blue actually takes some part in the various changes.

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