CCXXXVIII. THE INFLUENCE OF THIOL GROUPS IN THE ACTIVITY OF DEHYDROGENASES. II WITH AN ADDENDUM ON THE LOCATION OF DEHYDROGENASES IN MUSCLE

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It was shown [Hopkins & Morgan, 1938] that when washed tissues are incubated anaerobically for adequate periods with solutions of glutathione (GSSG) at pH 7.4 the succinic dehydrogenase is completely inactivated. On the other hand the original activity of the enzyme is fully regained when the preparations so treated are subsequently re-incubated with the reduced form of glutathione (GSH). In the case of a second enzyme, the α -glycerophosphate dehydrogenase, the activity is wholly unaffected by the treatment described. Hopkins and Morgan suggested that these facts show that a thiol group in its structure is essential for the activity of the former enzyme, but not at all for that of the latter.

In experiments now to be described these observations have been confirmed and extended. A number of other dehydrogenases have been studied, but among them the succinic dehydrogenase was the only one found to respond to the influence of glutathione. The influence on dehydrogenases of other substances known or supposed to inhibit the action of hydrolytic enzymes by combining or reacting with SH groups in their structure has been investigated, and also the influence of these and other inhibitors in protecting the succinic dehydrogenase from the action of glutathione. Preliminary experiments will be described which seem to bear on the location of dehydrogenases in muscle fibres.

EXPERIMENTAL

Methods

In most of the experiments washed muscles from the hind legs of rabbits were employed, but in a few the enzyme preparations were made from other tissues. The muscles, immediately after their removal from the animal, were passed through a Latapie mincer and washed thrice with distilled water. Occasionally they were subsequently finely ground with sand in a mechanical mortar. The effect of various forms of treatment on the activity of the enzymes was in all cases determined by the anaerobic methylene blue technique; but in the cases of the succinic, glycerophosphate and lactic enzymes the degree of activity was also determined spectroscopically by observing the rate at which a preparation, in the presence of a substrate, reduced cytochrome *c*. In some experiments with succinic dehydrogenase the rate of O₂ uptake in the presence of cytochrome and substrate was measured.

In the methylene blue experiments the procedure was made as nearly as possible the same in all experiments, so that usually in the individual protocols only the reduction times need be reported. In general 0.5 g. washed muscle was employed at this stage, or its calculated equivalent when, as a result of treatment, the tissue had gained or lost water. Small Thunberg tubes were used, and each tube contained, in addition to the tissue, 1 ml. phosphate buffer at pH 7.4 and 1 ml. water. To this was added 0.5 ml. $M/2$ substrate, the final concentration of the latter being therefore $M/12$. Incubation was always carried out *in vacuo* at 37°.

In all experiments which involved incubating the tissue with glutathione or other substances, the control sample used in the comparison of reduction times was itself incubated simultaneously in buffer solution without additions. The method of incubation was that described in the previous paper. The amount of methylene blue employed was uniformly 0.25 ml. of $M/1000$ solution.

I. THE SUCCINIC DEHYDROGENASE

That this enzyme can suffer complete loss of activity by adequate treatment with GSSG, and that its activity is restored on subsequent exposure to GSH solutions, has been repeatedly confirmed during our experiments. Since, as was pointed out in the previous paper, these reactions involve equilibria the complete removal of activity calls for more than one exposure to the GSSG solutions, though its complete restoration usually requires one exposure only to solutions of GSH of sufficient concentration. In many of the following experiments complete inactivation was not found necessary; wide changes in the degree of activity gave the information sought.

The suggestion has been made to us that the effect of GSSG might not involve an oxidation of SH groups as was assumed in the previous paper to occur, but might follow on the lines of some other inhibitions of enzyme activity. The constitution of the disulphide peptide is such that it might enter into a reversible association with the enzyme of a kind which would prevent essential groups in the structure of the latter from exerting their normal influence.

Such a view, unlikely from the general evidence discussed by Hopkins & Morgan [1938], seems to be disproved by experiments of the following simple kind. The process of inactivation, it will be shown, involves a reaction which has a prominent time factor and a somewhat high temperature coefficient, while its velocity increases continuously through a wide range of increase in the concentration of GSSG. In these respects it differs entirely of course from inhibition by agents which may be assumed to act by simply blocking the enzyme surface; e.g. malonic acid (see below).

Exp. 1. Latapie-minced and well-washed muscle tissue in portions of 0.5 g. was incubated anaerobically for 2 hr. at pH 7.4 with varying concentrations of GSSG. The samples were filtered off on a linen filter, well washed, and their reduction times determined as described above.

<i>Exp. 1</i>	Reduction times
Original tissue	10 min.
Incubated with $M/100$ GSSG	17 min.
Incubated with $M/50$ GSSG	25 min.
Incubated with $M/10$ GSSG	2 hr. 30 min.

It is seen that the reduction time (inactivation of the enzyme) increases continuously with the concentration of GSSG.

Exp. 2 demonstrates the effect of incubation time on the degree of inactivation. Equal portions of washed muscle were incubated *in vacuo* for various times with 0.8 ml. $M/5$ GSSG, then filtered off and washed. One sample after incubation for 2 hr. was incubated for a second 2 hr. with a fresh supply of GSSG.

Exp. 2

	Reduction times
Original tissue	16 min.
Incubated for 25 min.	23 min.
Incubated for 60 min.	46 min.
Incubated for 2 hr.	1 hr. 30 min.
2 hr. sample re-incubated	∞

Exp. 3 shows the effect of temperature on the process of inactivation. Samples of muscle (0.5 g.) treated as before were incubated, with and without GSSG ($M/10$), for 4 hr. at different temperatures. Washed as usual.

Exp. 3

Temperature	Reduction times
20° control (no GSSG)	12 min.
with GSSG	15 min.
30° control	12 min.
GSSG	56 min.
40° control	12 min.
GSSG	2 hr. 40 min.

These three experiments show that inactivation by GSSG involves a progressive chemical reaction with a somewhat high temperature coefficient.

Cu as an inhibitor of the succinic dehydrogenase

It is well known that Cu reacts with SH groups and if the activity of the dehydrogenase calls for the integrity of such groups in its structure, it is to be expected that its presence will inhibit activity. This is the case. Quastel & Wooldridge [1927] found that treatment with CuSO_4 inhibited the succinic enzyme as well as some other dehydrogenases in the case of *B. coli* but we have found no reference to the effects of the metal on the enzyme of animal tissues.

Exp. 4. To equal samples of washed muscle, treated as usual and suspended in buffer solution, CuSO_4 was added to give the concentrations of Cu shown in the following table. The reduction times were determined immediately after the addition.

Exp. 4

Cu added $\mu\text{g.}$	Reduction times min.
0	10
5	11
10	12
40	15
100	80

It will be seen that with the amount of tissue used in this as in other experiments (0.5 g.) the metal has relatively little effect on the enzyme up to a concentration of $40\mu\text{g.}$ in 3 ml. solution. With $100\mu\text{g.}$ it becomes large.

These results bear on those of the earlier experiments of Hopkins and Morgan and on some of our own. By far the most convenient method for preparing GSSG from GSH is that of Pirie [1931] in which the latter is oxidized by H_2O_2 in the presence of small amounts of Cu. The oxidized product so made which has been chiefly used in this laboratory contains traces of Cu. This was ignored in the previous paper chiefly because it was found that cystine and cysteine absolutely free from Cu could in essentials replace glutathione in experiments described. It seemed to us desirable nevertheless to study the effect of these small amounts of the metal. The amount of Cu present in the preparations of GSSG was determined by the method of Callan & Henderson as developed by Haddock & Evers [1932] and found to be $10\mu\text{g.}$ in 100 mg. (1 in 10,000). The presence of this amount as shown by experiments described below is not without effect on the apparent rate of enzyme inactivation by GSSG. It is sure however that the action of glutathione and that of the metal are distinct in kind. We have found that inactivation of the enzyme by GSSG occurs typically in the complete absence of Cu (Exp. 8).

Exp. 5 again illustrates the effect on the enzyme activity of small amounts ($10\mu\text{g.}$) and of larger amounts ($100\mu\text{g.}$) of Cu, and shows that in the case of the smaller amounts incubation does not increase the effect of the metal. This is exerted immediately. Of the tissue 0.5 g. was in 3 ml. buffer solution as usual. With each concentration of metal the reduction time was determined on one sample immediately after adding the metal. Two other samples in each case were incubated in its presence for two successive periods of 3 hr., the tissue being washed in between. One of the latter samples was then incubated with $M/10$ GSH solution. All reductions with succinate as substrate.

<i>Exp. 5</i>		Reduction times
No Cu		
Control untreated		12 min.
$10\mu\text{g.}$ Cu		
Determined immediately		14 min.
Incubated		13 min.
Incubated, then GSH		12 min.
$100\mu\text{g.}$ Cu		
Determined immediately		45 min.
Incubated		5 hr.
Incubated, then GSH		17 min.

The effect of $10\mu\text{g.}$ Cu, the amount contained in 0.1 g. of the GSSG preparation described above (and therefore the amount usually present in our experiments with that preparation), though small, has been always observed and was of the same order in other experiments. It is noteworthy that with ten times this amount of metal, though marked diminution of the enzyme activity occurs at once, the effect increases with time. This circumstance will receive later reference. Subsequent exposure to the influence of GSH always restores the activity of the Cu-treated enzyme though usually not quite completely.

Experiments were carried out to determine to what extent the small amount of Cu in GSSG preparations prepared as described above is concerned in the inactivation observed when enzyme preparations are exposed to them.

Exp. 6. Dithiodiethyl carbamate is well known to form a complex with Cu. The following experiment was made to show (1) whether the carbamate has by itself any effect on the enzyme, (2) how far it prevents the effect of added Cu on its activity and (3) to what extent its presence affects the rate at which the activity

disappears when enzyme preparations are exposed to solutions of GSSG. 1 g. Latapie-minced washed muscle was incubated for 3 hr. in solutions at pH 7.4 containing the various additions mentioned. 2 ml. 0.1% carbamate were employed and in each case the total volume was 5 ml. After incubation the tissue samples were filtered off, washed and the reduction times determined as usual.

Exp. 6

	Reduction times
Control (muscle alone)	11 min.
With carbamate only	11 min.
With 10 μ g. Cu	14½ min.
+ carbamate	11 min.
With GSSG (0.1 g. = 10 μ g. Cu)	5 hr.
+ carbamate	3 hr. 50 min.

It will be seen that the carbamate had by itself no influence on the enzyme, while it protected it from the inhibitory effect of the added Cu. Its presence reduced the reduction time of tissue incubated with GSSG from 300 min. to 220 min. The effect of the metal seems to be in some sense additive.

It was felt desirable to add to the evidence provided by carbamate inhibition by using, if possible, GSSG preparations wholly free from Cu. We have found that if preparations containing such amounts of the metal as those made by Pirie's method are shaken in solution with an adequate amount of freshly washed minced muscle, the Cu is completely removed by adsorption on the tissue, or reduced to amounts too small to be demonstrated.

Exp. 7 was carried out with GSSG thus freed from Cu, and its effect on the enzyme compared with that of an equal concentration of the original preparation.

Exp. 7

	Reduction times
Muscle incubated without GSSG	5 min. 20 sec.
Incubated with the original preparation of GSSG	72 min.
Incubated with Cu-free GSSG	56 min.

The reduction time is again larger in the presence of the Cu.

In Exps. 6 and 7 the tissue was exposed only once to the GSSG solution. This, as has been frequently pointed out, is never adequate, at least with reasonable concentrations, to remove the enzyme activity completely.¹

We wished therefore to show whether with adequate exposure the activity could be completely removed by a Cu-free preparation. This was the case, or nearly the case, in Exp. 8. The copper was removed by shaking 30 ml. *N*/10 GSSG at pH 7.4 with 2.25 g. of well-washed muscle. The tissue was filtered off after 5 min. Two samples of 1 g. tissue, prepared as usual, were incubated for two successive periods of 3 hr. each with 5 ml. *M*/10 Cu-free GSSG. A third equal sample was similarly treated but with the original GSSG preparation, and a fourth in the presence of 10 μ g. Cu alone. All samples were prepared for the reduction time determinations in the customary way.

The Cu-free preparation reduced somewhat more rapidly than the original, but the difference was slight. The experiment showed clearly that contact with

¹ The plan and extent of our experiments have been sometimes limited by the available supply of glutathione. Over 50 g. have been used in this research. All has been prepared in this laboratory.

GSSG when free from the metal can completely remove the activity of the dehydrogenase.

<i>Exp. 8</i>		Reduction times
Control (muscle incubated alone)		10 min.
Incubated with Cu-free GSSG		8 hr. +
	+ carbamate	8 hr. +
Incubated with original GSSG		8 hr. +
Incubated with 10 μ g. Cu		14 min.

Other inhibitors influencing SH groups

Morgan & Friedmann [1938, 1] having shown that maleic acid by reacting with SH groups forms stable addition compounds with thiolacetic acid, cysteine and glutathione, showed further [1938, 2] that it reacts with the fixed SH groups of proteins and therefore inhibits such enzymic activities as call for the integrity of these groups. Hopkins & Morgan [1938] having shown that the activity of the succinic dehydrogenase comes into this category, the first-mentioned investigators were led to study the effect of maleic acid upon it, finding that well-marked inhibition occurs [Morgan & Friedmann, 1938, 2]. They had previously found however that interactions of thiols with the acid reach equilibria and do not proceed to completion. The following experiments confirm and extend their observations in the case of the succinic enzyme.

Exp. 9 involved a comparison between maleic and fumaric acids. Of a washed muscle preparation 3 equal portions were incubated overnight, alone in phosphate buffer (control), in *M*/10 maleic acid and in *M*/10 fumaric acid respectively, all at pH 7.4. Each portion was then submitted to the same treatment for a second time. They were then well washed at the centrifuge and their reduction times in the presence of succinate determined.

	Reduction times (min.)	
	Once incubated	Twice incubated
Control	12	12
Maleic acid	27	53
Fumaric acid	13	12

It is seen that the inhibition by maleic acid is marked but involves a slow reaction not going to completion. Fumaric acid has no effect upon the enzyme.

Rapkin [1933] has shown that iodoacetic acid reacts with the SH group of proteins, and its influence as an inhibitor of the dehydrogenase was discussed by Hopkins & Morgan [1938]. We have confirmed their results in finding that its action on the enzyme is a relatively slow process, increasing at any given concentration with the time of incubation. We have ourselves found that its effects are irreversible, not removed by washing the enzyme preparation or under the influence of GSH. In one experiment for instance involving the customary technique we found that an untreated preparation with a reduction time of 12 min. gave, after incubation with *M*/25 iodoacetic acid for 1 hr., one of 120 min.; and when afterwards incubated with GSH, 130 min. In another experiment, in which the control time was 12½ min., the preparation after exposure to iodoacetic acid required 91 min. although it was first very thoroughly washed. The effects are strictly irreversible.

Protection of the dehydrogenase from the influence of GSSG

Malonic acid is well known as an inhibitor of the activity of succinic dehydrogenase and must therefore enter into some effective association with its active structure. We therefore investigated its ability to protect the enzyme from oxidation by GSSG. In experiments for this purpose it is important to know that malonate can be easily removed from its association with the enzyme by washing the tissue preparations which have been exposed to solutions containing it. Unless the concentrations are high this is the case after relatively few washings as shown by the following experiments. Two 0.5 g. portions of a muscle preparation were placed in 2 ml. *M*/10 malonic acid. They were incubated anaerobically for $\frac{1}{2}$ hr., filtered off, and, in one case, the tissue was washed thrice on a linen filter before the reduction times were determined and in the other left unwashed. In the latter case the presence of the malonic acid on the surface naturally inhibited the enzyme. The control was an equal portion of the tissue incubated in buffer solution alone. The following reduction times were obtained. Control, 15 min.; treated with malonic acid and not washed, 61 min.; washed sample, 17 min. It is seen that the washed sample was nearly as active as the original. Although in some experiments we have first incubated the enzyme preparation with the malonic acid its association with the enzyme is in fact established immediately (Exp. 11).

The following experiments show that malonic acid can very completely protect the enzyme from oxidation by GSSG.

Exp. 10 shows that protection occurs with very low concentrations. Latapie-minced washed muscle in portions of 0.5 g. was incubated for 2 hr. under the usual conditions with *M*/10 GSSG, respectively unprotected and protected by the presence of malonic acid in various concentrations. The following reduction times were obtained.

Exp. 10

	Reduction times
Original tissue untreated	9 min.
Incubated with GSSG unprotected	2 hr. 30 min.
with malonate <i>M</i> /10	15 min.
" <i>M</i> /50	10 min.
" <i>M</i> /100	11 min.
" <i>M</i> /500	11 min.
" <i>M</i> /1000	13 min.
" <i>M</i> /5000	17 min.

It is clear that a large degree of protection is afforded by very low concentrations of malonate and that the effect decreases only slowly with a fall in these. It is probable that in the above experiment the tissue from the strongest (*M*/10) solution was not quite adequately washed and the small amount of malonic acid still adhering somewhat increased the reduction time.

Exp. 11 carried out with the same tissue preparation as in Exp. 10 shows that the formation of the association between malonic acid and the enzyme is a rapid process scarcely affected by a time factor. The tissue in *M*/50 malonic acid was incubated in Thunberg tubes with hollow turn-over stoppers containing *M*/10 GSSG. The latter was emptied into the tubes at successive intervals during incubation which in each case was afterwards continued for 2 hr. Tissue samples were filtered off and washed free from malonic acid before the reduction experiments were carried out.

Exp. 11

GSSG solution added	Reduction times	
	min.	sec.
At once	13	30
After 5 min.	12	30
After 10 min.	14	45
After 30 min.	14	40

Previous incubation is seen to have but little effect on the degree of protection from the influence of the GSSG. As shown in the protocol of Exp. 10 the reduction time of this preparation after exposure to the GSSG solution alone for 2 hr. unprotected was 2 hr. 30 min.

The following experiments illustrate further the efficiency of malonic acid in protecting the enzyme from the influence of GSSG.

Exp. 12. In this equal samples of a muscle preparation were incubated for two successive periods of 3 hr. each respectively in $M/10$ GSSG alone and in the same with $M/10$ malonic acid present. A control sample was, as in all experiments, incubated for similar periods in the buffer solution alone. After washing the following reduction times were obtained:

Exp. 12

	Reduction times
Control	5 min. 45 sec.
Incubated with GSSG alone	5 hr. +
In presence of malonate	8 min.

Exp. 13. In this the muscle tissue used was passed twice through the Latapie mincer with the intention of increasing its permeability. The experiment was otherwise essentially the same in plan as the foregoing but, in the incubations, while the concentration of GSSG was the same, that of the malonic acid was only $M/50$. The incubations were two, of 4 hr. each. The tissues were very thoroughly washed before the reduction times were compared.

Exp. 13

	Reduction times
Control	8 min.
Incubated with GSSG alone	5 hr. 30 min.
In presence of malonate	9 min. 30 sec.

Of *Exp. 13a* it need only be said that the procedure was similar, and the muscle preparation the same as in Exp. 13, but the incubation times were shorter and the enzyme therefore not completely inactivated by GSSG. The reduction times were as follow: control, 9 min.; after GSSG alone, 4 hr.; after GSSG plus malonate, 9 min.

The influence of substances, other than malonic acid, capable of protecting the succinic enzyme from the influence of GSSG was studied. Other dibasic acids displayed this property more definitely than other substances tried, among them, as might be expected, succinic acid itself, but also fumaric and malic; pyrophosphoric protects though generally less efficiently than the others.

The results of *Exp. 14* demonstrate such effects. A muscle preparation was incubated for 4 hr. with GSSG solution alone, and also in the presence of the substances shown. All samples were thoroughly washed.

Exp. 14

	Reduction times
GSSG alone	3 hr. +
With succinate <i>M</i> /10	4 min.
With fumarate <i>M</i> /10	3 min.
With malonate <i>M</i> /10	4 min.
With pyrophosphate <i>M</i> /50	4 min.

In this experiment, in which the enzyme preparation was exceptionally active, protection by all four substances was very complete. Other experiments have confirmed these results though, as stated, pyrophosphate has usually proved somewhat less efficient than the others.

It seemed worth while to try the effect of a greater variety of possible protectors. In the following (Exp. 15) the GSSG and the other substances were all employed in *M*/10 solution. The incubation period was 4 hr.

Exp. 15

	Reduction times
Original tissue (untreated)	10 min.
GSSG alone	3 hr.
With addition of:	
Acetate	4 hr. +
Phenylacetate	4 hr. +
Malonate	13 min.
Hydroxymalonate	20 min.
Lactate	2 hr.
Glucose	1 hr.
Sucrose	2 hr.

The monobasic acids other than lactic afford no protection. The reason for the definite but relatively smaller protection by lactic acid and glucose lacks explanation. Hydroxymalonic is nearly as efficient as malonic acid itself. Except for these two, none of the substances tried is an inhibitor of the enzymic activity.

Various authors have shown that the most efficient inhibitors of the succinic dehydrogenase activity are dicarboxylic acids [Quastel & Wooldridge, 1928; Potter & Elvehjem, 1937; Leloir & Dixon, 1937]. Two acidic groups seem to enter into special relations with the enzyme. It was to be expected that inhibitors will also protect the enzyme from oxidation.

Protection from the influence of GSH

It was desirable to discover whether substances which protect from oxidation by GSSG also protect from subsequent reduction by GSH. In the following experiment a muscle preparation was first incubated twice with GSSG, and the succinic enzyme present proved to be completely inactivated. The tissue was then divided into portions of 0.6 g. each, and these were incubated, one sample with GSH alone, and others with the substances indicated below. The GSH was in *M*/10 solution, buffered as usual. It was placed in the hollow stoppers of Thunberg tubes and added after the samples of oxidized tissue had been incubated anaerobically for $\frac{1}{2}$ hr. with the materials under study. The samples were then washed and their reduction times determined.

Succinic and fumaric acids inhibit the reduction to some extent; the others not at all. That malonic acid protects from GSSG so completely and from GSH not at all suggests structural relations which may prove instructive.

Exp. 16

	Reduction times min.
Control (tissue untreated)	12
Oxidized by GSSG	∞
Re-reduced by GSH alone	12
GSH + succinic acid	19½
+ malonic acid	13
+ fumaric acid	17
+ pyrophosphoric acid	12½

Protection of the enzyme thiol groups from the influence of GSSG

When the nitroprusside test is used to compare the concentration of SH groups in active and inactivated preparations respectively it should be realized that a very low intensity in the test is compatible with activity.

If SH groups are necessary for the activity of the succinic dehydrogenase, and for this there seems to be strong and cumulative evidence, it is clear that malonic acid and other protectors of the enzyme activity must prevent that group from being oxidized by GSSG. It is by no means easy to prove directly that this is the case, nor is it perhaps easy to picture a mechanism for such protection. Malonic acid, for instance, apparently establishes no special relations with thiol groups, so that the protection must in any case be indirect.

So long as we remain unable to isolate the enzyme, or obtain preparations in which it is present in higher relative concentrations than in whole tissues merely washed, it is somewhat difficult to employ the nitroprusside reaction in proof that the SH groups proper to the enzyme itself have or have not been protected. They must represent but a minute portion of the thiol groups present in the tissue as a whole. That the active surface of the specific enzyme compared with the surfaces presented by disintegrated muscle must be exceedingly small cannot be doubted, and this seems to be illustrated by the minute concentration in which malonic acid can protect the enzyme (Exp. 10).

If the nitroprusside reaction given by a tissue sample which has been exposed unprotected to GSSG be compared with that given by a corresponding sample protected by malonic acid, though the activity of the enzyme is nearly lost in the former and intact in the latter, the reaction may be slight in both cases and a difficulty may be felt in deciding whether there is a significant difference between them. It is however easier to distinguish if the two samples be extracted with trichloroacetic acid and the test applied to the solution. It can be said definitely that in our experiments, though the difference may have been slight in some cases, it has always been more intense in a protected sample. It seemed desirable however to obtain some degree of quantitative evidence for this, and we have succeeded in doing so by incubating anaerobically with solutions of GSSG the samples to be compared. These were then titrated with very weak standard I_2 in the presence of KI in order to determine the amount of GSH produced by the interaction between the protein SH groups in the tissue preparation and the GSSG in solution. The quantities involved are doubtless too small for accuracy, but they allow of comparisons which are significant.

In each of two experiments sufficient of a preparation of muscle was made to carry out the above treatment, as well as determinations of enzyme activity. It was incubated with GSSG anaerobically in large Thunberg tubes for adequate periods, with and without the presence of malonate. The respective reduction times showed, in each experiment, that in the absence of protection the enzyme activity had nearly disappeared, somewhat more completely in the first experi-

ment than in the second. It was fully preserved in the presence of malonate. After incubation as above the evacuated tubes were opened under a solution of trichloroacetic acid, the tissue was filtered off and the filtrates titrated with $N/5000$ I_2 . The end points could be observed without difficulty. The following I_2 equivalents are calculated in each case for 5 g. tissue preparation.

	ml. $N/5000$ iodine	
	Exp. 1	Exp. 2
Incubated with GSSG alone	1.2	7.8
Incubated with malonate present	16.0	19.5

There seems to be no doubt that the enzyme SH groups are protected by malonic acid in some specific way.

We may add that in an occasional experiment when treatment of the tissue with GSSG had made the nitroprusside reaction completely negative the enzyme was found to be inactive. This result is relatively easy to obtain, as Hopkins & Morgan [1938] found, when heart muscle is the source of the enzyme. With the less permeable preparations from skeletal muscle the treatment must be prolonged and repeated. That the enzyme structure nevertheless remains intact save for the disappearance of the SH groups may be shown by subsequent treatment with GSH when the activity is fully restored.

Oxidation of SH groups by alloxan

It was shown by Purrr [1935] that papain and cathepsin are inactivated by alloxan owing to the fact that it oxidizes SH groups in the structure of these enzymes. We have tested its effect on succinic dehydrogenase and find that in adequate concentration it inactivates it, the effect being reversible (though apparently not completely) under the influence of GSH. The presence of succinic acid protects from oxidation by alloxan, but malonic gives relatively very little protection. The following experiments illustrate these statements. It should be here remarked that the nitroprusside test shows that alloxan oxidizes the SH groups of a muscle preparation very quickly, even in low concentrations ($M/100$, $M/50$). When used in $M/10$ solution it gives the familiar pink colour with the muscle protein which has been recently studied by Lieben & Edel [1932].

In the following experiment a muscle preparation, made as usual, was incubated for 30 min. with $M/100$ and $M/50$ alloxan. Samples were well washed and their reduction times determined. The activity of the α -glycerophosphate enzyme was determined at the same time, so it is convenient to give the results here. Other samples of the oxidized material were washed and then incubated for $1\frac{1}{2}$ hr. with $M/10$ GSH, again washed thoroughly and submitted to reduction tests. In the protocol SD=the succinic and GLD=the glycerophosphoric enzyme.

<i>Exp. 17</i>		Reduction times
		min.
Control untreated	SD	15
	GLD	8
Alloxan $M/50$	SD	50
	GLD	8
Alloxan $M/100$	SD	56
	GLD	8
GSH after alloxan $M/50$	SD	20
	GLD	8
GSH after alloxan $M/100$	SD	22
	GLD	8

The glycerophosphoric enzyme is seen to be unaffected by alloxan at this concentration. GSH reverses its effect upon the succinic enzyme. Other experiments have given similar results. The following shows that the alloxan reaction is progressive with time. The preliminary treatment of the samples was the same as in the last case.

<i>Exp. 17a</i>			
	Control	<i>M</i> /50 alloxan filtered off immediately	<i>M</i> /50 alloxan incubated 30 min.
Reduction times (min.)	SD 11	15	60
	GLD 4½	4½	4½

Finally the following shows that as stated above the protection of the enzyme by succinic and malonic acids from oxidation by alloxan differs in an interesting way from the protection they afford from oxidation by GSSG. Succinic acid affords a considerable degree of protection, malonic very little.

	Reduction times (min.)
Control	10
Alloxan alone	35
Alloxan + malonate	26
Alloxan + succinate	14

Alloxan in concentrations of the order of *M*/10 has effects on the enzyme which must be additional to the oxidation of thiol groups, and when incubated with it all the dehydrogenases we have studied lose much of their activity.

II. OTHER DEHYDROGENASES

We have conducted experiments, precisely similar in kind to those of the previous section, with the α -glycerophosphate, lactic and malic dehydrogenases; also with the aldehyde mutase and alcohol dehydrogenase of horse liver (prepared by the method of Dixon & Lutwak-Mann [1937]) and with two enzymes of the oxidase type, namely the cytochrome (indophenol) oxidase and the xanthine oxidase. None of these enzymes is appreciably influenced by glutathione. Consistent with this is the circumstance that their activity, except for that of the liver aldehyde mutase, is not affected, or affected to a small extent only, by inhibitors which are believed to act by suppressing the influence of thiol groups.

The experiments with the succinic dehydrogenase have been described in detail and those of the present section were essentially similar in all respects. The evidence can therefore be more briefly presented. To the lactic and malic enzymes cozymase was of course added when the reduction times were determined.

Effects of incubation with GSSG and GSH

In the following table the results of several experiments are collected.

Enzyme	Reduction times (min.)			
	Original	GSSG alone	GSSG with malonate	GSH
Glycerophosphate	6	6½	6	6
"	10	10	10	10
Lactic	3	5	—	—
"	3½	5	4½	5½
"	4½	7	7	—
Lactic (from horse liver)	20	21	—	22
Malic	6½	8½	—	—
"	9	13	—	—

In all cases the influence of GSSG was absent or negligible.

Effect of Cu

The relatively small effect of Cu on the lactic dehydrogenase is illustrated in the following experiment. The preparation was not incubated with the metal but the effect was tested immediately after its addition.

	Reduction times min.
Control (+ cozymase and lactate)	3½
With 5 µg. Cu	4
With 50 µg. Cu	6
With 5 µg. Cu (+ dithiodiethylcarbamate)	3½
With 50 µg. Cu (+ dithiodiethylcarbamate)	3½

In the cases of the lactic and malic enzymes the differences between the effect of incubation with the GSSG preparation containing 10 µg. Cu in 100 mg. of GSSG (see previous section) and that with the Cu-free preparation were compared in exactly similar circumstances. The results should be compared with Exp. 7 in which the same tissue was employed.

	Reduction time (min.)	
	With lactate	With malate
Tissue incubated alone	2½	6
With GSSG containing Cu	5½	8
With GSSG Cu-free	2½	7½

In one experiment with the glycerophosphate enzyme 10 µg. Cu were added to two equal portions of a tissue preparation, treated otherwise as usual. In one case the reduction time was taken immediately; in the other the tissue was first incubated with the metal for 3 hr. The control was reduced in 7 min.; the sample with Cu, unincubated, 8 min.; the incubated sample 10 min.

Effects of maleic and iodoacetic acids

The following experiments illustrate the absence of appreciable inhibition due to maleic (and fumaric) acids. The results were confirmed in other cases.

Enzyme	Reduction times (min.)		
	Controls	Maleic acid	Fumaric acid
Lactic	3	3½	3
Glycerophosphate	4	4	4
Malic	14½	15½	14½

The following show that the same is true of iodoacetic acid even after incubation with *M*/50 solution for 1 hr.

Enzyme	Reduction times (min.)	
	Control	Incubated with I.A.
Lactic	3½	4
"	3½	3½
Glycerophosphate	7	10

In this group of dehydrogenases, as will be seen from the various protocols given in this section, the influence of glutathione is absent, or, in comparison with the case of the succinic enzyme, negligible. The glycerophosphate enzyme has always shown remarkable resistance to the effects of the treatment involved in our experiments, its reduction times remaining constant at all stages; the lactic and malic enzymes have proved somewhat less stable but it will be seen

that their resistance to glutathione is definite enough. Consistent with this, in proof that thiol groups are not essential to their activity, is the circumstance that these enzymes are much less affected by Cu than is the succinic enzyme, and, unlike it, are not affected by the irreversible effects of maleic and iodoacetic acids.

The influence of glutathione on two other enzymes was tested, namely the aldehyde mutase and the alcohol dehydrogenase from horse liver. In neither case did exposure to GSSG affect the activity though the mutase has been said to be sensitive to iodoacetic acid [Dixon & Lutwak-Mann, 1937].

Oxidases

Experiments made with the Cu-containing preparation of GSSG described in the previous section led us at first to believe that the xanthine "oxidase" resembled the succinic enzyme in its relations with glutathione. This however proved to be due to its great sensitiveness to Cu, a property which had been previously recorded by others [cf. Andersson, 1936]. Small amounts of Cu equivalent to that contained in the glutathione removed the enzyme activity to an equal degree. The effect of Cu is however reversible and completely removed by treatment with GSH. It would seem that this enzyme may prove not to be a typical oxidase, but at the moment it may be left with the title.

The cytochrome (indophenol) oxidase was also studied. Its activity was measured by using phenylenediamine as a substrate and also by testing spectroscopically its ability to oxidize cytochrome. Its activity was found to be entirely resistant to the influence of GSSG.

CYTOCHROME-REDUCTION METHOD FOR DETERMINING ENZYME ACTIVITY

It is of interest in the case of those dehydrogenases which reduce the pigment to observe with a spectroscope the rate at which the prominent bands of reduced cytochrome *c* appear when the oxidized form is added to a solution containing

Enzyme preparations treated with following substances	The spectrum of reduced cytochrome <i>c</i> on adding the substrate became:					
	Just visible after			Fully developed after		
	SD	GLD	LD	SD	GLD	LD
Control untreated	At once	At once	At once	50 sec.	20 sec.	25 sec.
GSSG	No bands at any time	40 sec.	50 sec.	No bands	1 min.	3 min.
„ then GSH	At once	At once	1 min.	20 sec.	20 sec.	2 min.
„ Cu-free	No bands at any time	—	—	No bands	30 sec.	1 min.
„ + malonate	2 min.	—	—	4 min.	—	—
„ + succinate	5 min.	—	—	7 min.	—	—
„ + pyrophosphate	8 min.	—	—	13 min.	—	—
Cu high concentration	40 min.	—	5 min.	Several hours	—	10 min.
Cu low concentration	2 min.	1 min.	2 min.	4 min.	2 min.	5 min.
Alloxan <i>M</i> /50	60 min.	1 min.	—	2 hr.	3 min.	—
Iodoacetic acid <i>M</i> /80	22 min.	2 min.	2 min.	1 hr.	3 min.	15 min.
Iodoacetic* acid (muscle <i>in vivo</i>)	1 hr.	—	—	Several hours	—	—
Maleic acid	17 min.	—	—	30 min.	—	—

* In this experiment iodoacetic acid was injected into the lymph sac of frogs. One hind limb was removed before the injection as a control, and the motor nerves to the muscles of the other limb were cut. When rigor was evident in the forelimbs the second limb was removed. The muscles in each case were then ground up with a little water and washed till free from haemoglobin, and the succinic dehydrogenase activity determined spectroscopically. In the control the cytochrome bands were fully established in 30 min.

an enzyme and its specific substrate. It forms a check on the methylene blue results and is a convenient and rapid method for deciding whether an enzyme is present or absent, giving also approximate information as to the degree of its activity. It will be seen on examining the above table that results obtained with the methylene blue method are confirmed in every instance. The method of observation was of the simplest. A test tube containing the enzyme preparation, suspended in phosphate buffer at pH 7.4 together with a moderate concentration of oxidized cytochrome *c*, was clamped before the slit of a fixed straight vision spectroscope with an adequate light source.¹ A stopwatch was used and the times taken from the moment of adding the substrate (1) to the first observable appearance of the bands, and (2) to the establishment of their maximum intensity. The descriptions given in column 1 of the table will be understood from what has gone before.

The following is an experiment in which was measured the O_2 uptake by systems containing tissue preparations which had been previously treated with GSSG, unprotected and protected. One sample after oxidation was re-reduced with GSH. The substrate was succinate, and cytochrome *c* was added to the system. The necessary cytochrome oxidase was present in sufficient concentration in the muscle preparations. Barcroft manometers: O_2 uptakes are given in $\mu l.$ per hr.: reduction times for the corresponding samples.

Previous treatment of tissue	O_2 uptake	Reduction times
Original	280	11 min.
Incubated with:		
GSSG alone	0	7 hr. +
„ with malonate	210	14 min.
„ with succinate	190	19 min.
„ with pyrophosphate	140	16 min.
„ then GSG	310	10 min.

It will be seen that as measures of enzyme activity the two methods are in close correspondence.

ADDENDUM

Location of the dedydrogenase in muscle

We have found that if muscle tissue minced with the Latapie is thoroughly extracted with salt solutions as for the extraction of myosin etc., the extraction being continued until no trace of protein is present in a final extract, the fragmented fibres, of which the majority when examined under the microscope are seen to be severed transversely, still show cross striation and—as we have repeatedly observed—typical birefringence of the discs. That cross striation remained intact after continued extraction with NH_4Cl was observed by Danelewsky [1882]. That author believed—perhaps on not very convincing evidence—that a relatively high proportion of this “framework” in their fibres was characteristic of muscles with the greatest functional activity. We have found that muscle tissue after exhaustive extraction with salts still contains succinic, α -glycerophosphoric, lactic and malic dehydrogenases in relatively high concentrations.

The saline extracts, unless they obviously contain dispersed colloidal particles, are found to display no dehydrogenase activity. The later extracts of a series of extractions can be obtained, after centrifuging, perfectly clear, and these are quite free from these enzymes though in the extracted residue their concentrations are high. If the completely extracted tissue be now treated with, say, 10 times its volume of $N/100$ HCl, more protein is extracted and the final residue

¹ The experiments with the lactic dehydrogenase were carried out anaerobically.

becomes gelatinous. It can however be centrifuged off in a high speed centrifuge and is then found to be inactive, as is also the protein extracted by the acid. The framework structure of the fibres immediately disappears on treatment with HCl.

It is true, to judge from methylene blue reductions, that the concentrations of the enzymes when compared with those in the original tissue are not increased proportionately with the removal of so large a proportion of the muscle substance (some 85 % of the total muscle proteins), so that what happens during the prolonged treatment is not entirely clear. The facts stated may be illustrated by the following experiment, typical of several made.

Latapie-minced muscle (25 g.) was first extracted four times with 200 ml. NaCl (10 %) and centrifuged after each extraction; the second extract stood overnight in the refrigerator. It was then extracted thrice with LiCl [Bate Smith, 1935; 1937]. The last extract contained no trace of protein. The final residue in which the fibres showed typical cross striation was washed and then centrifuged until freed as far as possible from water. Equal weights (0.5 g.) were used in determining the following reduction times, but the equivalence of this weight with that of the original minced muscle was not accurately known.

	Reduction times		
	Control	Incubated GSSG	Re-incubated GSH
Dehydrogenase			
Succinic	12 min. 30 sec.	5 hr.	10 min.
α -Glycerophosphate	6 min.	6 min.	6 min.
Lactic	5 min. 30 sec.	11 min.	—
Malic	8 min. 40 sec.	13 min.	—

It will be seen that the enzymes in the extracted tissue show the same relations as those always observed with tissue when simply washed with water.

In a special experiment in every way similar to this the enzyme activity of the saline extracts was examined. The order of the extractions was the same as in the above. A sample of the fourth NaCl extract and one of the first LiCl extract were dialysed and in each case the precipitate which separated was centrifuged off, washed and tested for enzyme activity. The precipitate from the NaCl extract contained none of the succinic but very small amounts of the glycerophosphate enzyme and doubtful traces of the others. That from the LiCl extract was practically free from any activity. All the enzymes were present in the residue in concentrations comparable with those above. The supernatant solutions in the dialyser were free from activity.

It seems clear from the recent work of Weber [1934], Edsall & von Muralt [1930], Bate Smith [1935] and others that the protein which resists salt-extraction is mainly myosin itself, protected in some way from removal. In the case of Latapie-minced muscle it seems unlikely that this is due to the impermeability of the membranes, as the fibres are almost all broken across. Bate Smith [1937], who confirmed the presence in muscle of "globulin α " a protein first described by Meyer & Weber, is of opinion that some of this is associated with myosin in the residue after salt extraction (personal communication). It would be of interest, as he suggests, if, while myosin molecules form the basis of the contractile substances as is now generally admitted, the enzymes were associated with the above globulin which is in close relation with the myosin.

The circumstance that during and after the salt extraction the dehydrogenases seem to remain confined to the residual structural framework, while the degree of consequent increase in their concentration is not what would be

expected, calls for explanation. It is conceivable that denaturation changes during the extractions may be in part responsible for this.

In any case it is of interest to know that enzymes controlling energy-yielding reactions exist in this close relation with the structural framework of the fibres.

DISCUSSION

The experiments in this paper have fully confirmed those of Hopkins & Morgan in showing that the activity of the succinic dehydrogenase is completely removed when preparations containing it are incubated with GSSG and fully restored on subsequent incubation with GSH. Those authors showed that, in contrast, the α -glycerophosphate dehydrogenase is wholly unaffected by this treatment. We have extended the list of resistant enzymes by the inclusion of the lactic and malic dehydrogenases, the alcohol dehydrogenase and the aldehyde mutase from horse liver, xanthine oxidase and cytochrome oxidase. Rapkine [1938] has recently shown that the triosephosphate mutase from muscle may be inactivated by GSSG; but, to judge from our results, the succinic enzyme seems likely to prove the only typical dehydrogenase which undergoes this inactivation, and therefore probably the only one which requires for its activity the integrity of the SH groups in its specific protein structure. We have added to the evidence for the importance of such groups in the case of that enzyme by showing that Cu, maleic acid and iodoacetic acid, which are known or believed to react with thiol groups, are inhibitors of the enzyme, while having little or no effect on the activity of the other dehydrogenases tested. Of the above, Cu inhibits reversibly; the two acids irreversibly.

Nearly all our experiments have been made on skeletal muscle tissue, but Hopkins & Morgan showed that, so far at least as the effects of glutathione are concerned, preparations of the enzyme from cardiac muscle, kidney and liver behave identically. The evidence that these effects are due to oxidation and re-reduction of thiol groups is in any case strong, but we have perhaps added to it by showing that the inactivation by GSSG involves a progressive chemical reaction with a somewhat high temperature coefficient (Exps. 1, 2, and 3). If the inactivation were due merely to the formation of some reversible association of GSSG with the enzyme in which the latter is inactive but which dissociates under the influence of GSH (this being perhaps possible though unlikely) its kinetics would not be of this kind.

Alloxan, as an oxidant of SH groups, when in relatively low concentrations ($M/50$, $M/100$) inactivates the succinic dehydrogenase. In contrast, at such concentrations it has no effect at all on the α -glycerophosphate enzyme (Exp. 17). At high concentrations ($M/10$), when it produces the characteristic pink colour with the tissue proteins, it exercises a less specific destructive effect on all the enzymes we have tested. If the concentrations used are of the lower order its effects on the succinic enzyme are reversed by reduction with GSH; otherwise the reactivation is only partial.

Cu is well known to react with thiol groups and it was to be expected that an enzyme of which the activity calls for the integrity of such groups in its structure would be inhibited by the presence of the metal. It proved easy to show that this is true of the succinic dehydrogenase. With adequate Cu concentrations the inhibition is complete and immediate. With tissue preparations however, such as have been generally used by others and by ourselves in studies of this enzyme, certain difficulties arise in points of detail, and these were evident when the effects of varying the concentration of Cu were being studied.

Since Thunberg [1909] first demonstrated the existence of this dehydrogenase most experiments made with it, as well as those with other "insoluble" dehydrogenases, have involved the use of whole tissues which were merely minced (or sometimes more completely disintegrated) and then thoroughly extracted with water. In any such preparation the specific protein of any one enzyme is in association with those of other enzymes, and, of course, also with a relatively very large proportion of indifferent tissue proteins. In numerous experiments this circumstance has proved to be of no consequence, as the literature of the subject shows. When, however, the significance of some detail in the specific enzyme protein is to be determined difficulties may intrude. When for instance Cu is added to such a tissue preparation it must be expected to react with the SH groups of the indifferent tissue proteins present no less than with those proper to the enzyme, and, of course, the number of the former present must be relatively very great. Only a small fraction of the metal added may therefore be effective. An added amount which, were it possible to work with the enzyme in a concentrated form, might produce strong inhibition may with such tissue preparations have little effect. This was the case with the lower concentrations of Cu employed in our experiments (10–20 $\mu\text{g.}$ per g. tissue). Inhibition even with these was always to be observed, but it was slight.

With adequate concentrations of Cu, which are relatively large (in the conditions of our experiments 300 $\mu\text{g.}$ for each g. muscle tissue), inhibition, as already stated, is immediate and complete. With intermediate concentrations (e.g. 100 $\mu\text{g.}$) it might seem from Exp. 5 that a time factor is involved in the process. This however is unlikely and it would seem rather that the nature of the enzyme preparation is again involved, and that a permeability factor intervenes. While with such concentrations the amount of Cu reaching the dehydrogenase itself may be adequate to induce noteworthy inhibition, it yet takes an appreciable time after its addition to the washed tissue to reach the enzyme surface.

These various relations seem to call for comment, especially as they bear in a general way on the customary use of such tissue preparations. They do not however obscure the proof that the activity of the dehydrogenase can be completely inhibited by Cu. On the other hand our experiments have shown that the lactic, malic and α -glycerophosphate dehydrogenases, in keeping with their resistance to oxidation by GSSG, are affected to a very much smaller extent by the presence of the metal.

We had occasion in the experimental section of this paper to point out that the GSSG used by Hopkins & Morgan [1938] and by ourselves in most experiments, owing to the method of its preparation contained a trace of Cu. The relative slight effect of this on the rate with which such preparations inactivate the succinic enzyme is discussed in the earlier section. That inactivation proceeds typically under the influence of GSSG in the complete absence of Cu is shown in Exp. 8.

It should be noted that inhibition by Cu is reversible and is removed when the enzyme preparation is subsequently incubated with GSH (e.g. Exp. 5).

We have confirmed the results of Morgan & Friedmann who having found that maleic acid and SH-compounds interact to form stable addition compounds, showed that the acid also reacts with the SH groups of proteins and thus inhibits the activity of succinic dehydrogenase. This inhibition is not complete, as an equilibrium is earlier reached. We have found however that maleic acid fails to inhibit appreciably the activities of the lactic, glycerophosphate and malic enzymes, and the same is true—at least under the conditions of our experiments—of iodoacetic acid.

It seemed probable that substances which inhibit the succinic dehydrogenase must enter into such relations with the active structures of the enzyme as would make them capable of protecting it from oxidation. Malonic acid proved to be very effective in such protection. A tissue preparation exposed to GSSG and malonic acid is found, after being washed free from the acid, to have lost none of its original activity. The protection is exerted with very low concentrations (Exp. 10), much lower than those necessary for the inhibition of the succinic dehydrogenase.

Other efficient protectors are the dicarboxylic acids, succinic acid itself, for instance, and fumaric acid. This is in keeping with what is known as to the nature of the compounds which most readily inhibit the enzyme; two acidic groups being usually necessary. Pyrophosphoric acid also protects with somewhat less efficiency. It is of interest to know that Rapkine (personal communication), studying the inhibition of yeast fermentation by iodoacetic acid, has found that the above-mentioned dicarboxylic acids (though also lactic and glycollic acids) prevent this inhibition. He has come to the conclusion that this is because they may protect thiol groups which at some stage are essential for the fermentation process.

As bearing further on the proof that SH groups are essential to the activity of the succinic enzyme we endeavoured to obtain direct evidence that these groups remain intact when preparations are protected on the above lines. This again is made difficult by the circumstance that these groups, as present in the indifferent tissue proteins, are so greatly in excess of those which can belong to the enzyme itself. Some evidence for such protection which seems satisfactory has however been obtained and is described above.

With the evidence now available the significance of thiol groups in the activity of the succinic dehydrogenase scarcely admits of a doubt. In this respect the enzyme stands alone among the typical dehydrogenases so far studied, and it seems probable that its case is unique. It is remarkable that it thus shares a character with certain hydrolases while differing from enzymes with functions analogous with its own. This will add interest to future endeavours to discover the mechanism of its action.

SUMMARY

While the succinic dehydrogenase is inactivated by incubation with GSSG and its activity restored by subsequent incubation with GSH this treatment has no effect on the other dehydrogenases studied.

Alloxan in low concentrations, as an oxidant of SH-groups, inactivates the succinic enzyme but not the other dehydrogenases.

The succinic enzyme is inactivated by Cu and by maleic and iodoacetic acids; substances which react with SH groups. They have little or no effect on the other enzymes tested.

Malonic, succinic and fumaric acids protect the succinic dehydrogenase from the influence of GSSG.

The experiments described support the view that the succinic dehydrogenase requires for its activity the integrity of SH groups in its structure while this is not the case with other typical dehydrogenases.

When muscle tissue is exhaustively extracted with salt solutions dehydrogenases remain active in the residual structure of the fibres, which still shows cross striation and birefringence.

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Note added on 10 October, 1938. After the paper had been read in proof our attention was called to a publication by v. Euler and Hellström (*Ark. Kemi Min. Geol.* 1938, **13** B, 1) in which it is shown that oxidation by ferricyanide reduces the activity of the succinic dehydrogenase which reduction by hydro-sulphite increases. This paper has now appeared in the current number of *Hoppe-Seyl. Z.* (**255**, 159).