CXVI. MALEIC ACID AS INHIBITOR OF ENZYME REACTIONS INDUCED BY SH-COMPOUNDS

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It has been shown recently [Morgan & Friedmann, 1938] that maleic acid and SH-compounds interact with the formation of well defined, stable addition compounds. We have applied this reaction to the study of some enzyme reactions which are held to be induced by SH-compounds. We have found, in every case examined, that maleic acid inhibits enzyme reactions of this kind or under other conditions decreases their speed.

SH-compounds play a role in enzyme reactions in two forms, as coenzymes (A) and as active groups of some enzymes (B). The following paper gives examples of the inhibiting effect of maleic acid in these two classes of enzyme reactions.

A. INHIBITION OF THE COENZYME ACTIVITY OF GLUTATHIONE BY MALEIC ACID

Preliminary experiments

Interaction of maleic acid with glutathione in physiological concentrations. The SH-compound mainly responsible for the activation of some enzyme reactions is glutathione [see Bersin, 1935]. Determinations of glutathione in animal tissues indicate that interaction with maleic acid must be shown to occur at a concentration of the order of $M/200$ GSH to be within physiological concentrations. As in our previous paper [Morgan & Friedmann, 1938] the interaction of maleic acid with GSH was described only for the concentrations $M/10$ and $M/12.5$ for both reactants, the interaction of maleic acid with $M/200$ GSH had to be investigated. Fig. 1 gives the results obtained.

It will be seen from Fig. 1 that the interaction of $M/200$ GSH with $M/200$ maleic acid (curve I) gives after incubation for 8 hr. at 37° a loss of GSH of 36%, whilst with $M/50$ maleic acid (curve II) the loss is 88%. The reaction does not go to completion in 8 hr. The experiment gives the working conditions suitable for detecting the interaction of maleic acid with GSH in physiological concentrations: 4 mol. maleic acid are required for 1 mol. GSH.

Interaction of maleic acid with glutathione in minced tissues. We now investigated whether the interaction of maleic acid with glutathione proceeds in a physiological medium and whether the analytical methods applied were suitable for the detection of this reaction. We incubated, therefore, maleic acid with pig liver and ox liver. For determination of the GSH in the tissue ascorbic acid was estimated separately in each sample with Tillmann's reagent, using the micro-method of Birch *et al.* [1933]. The iodine value of the ascorbic acid, calculated from the amount so found, was deducted from the total iodine figure, thus giving the iodine value corresponding to glutathione. The method used and the values obtained are described for pig's liver.

The liver was obtained fresh from the slaughter-house and was minced in a Latapie mincer. 10 g. were extracted with 20 ml. of 10% trichloroacetic acid and the GSH content of the extract was found to be 20 mg. GSH, equivalent to 0.81 ml. $M/12.5$ maleic acid.

Fig. 1.

Fig. 2.

Fig. 1. Curve I. $M/200$ glutathione and $M/200$ maleic acid. Curve II. $M/200$ glutathione and $M/50$ maleic acid.

Fig. 2. Ox liver. Curve I. Maleic acid added in equivalent proportion to the amount of glutathione in the sample of liver. Curve II. Maleic acid added in proportion of 4:1.

For the experiment with maleic acid two sets of vacuum tubes were used, set A containing equimolecular proportions of GSH and maleic acid (0.81 ml. $M/12.5$) and set B containing 4 times as much of the latter. The Latapie minced liver was first weighed (10 g.) and washed into the vacuum tubes with Sørensen's phosphate buffer, 4 ml. pH 7.4. After dilution with water to make 10 ml. total liquid added, the tubes were placed in a water bath at 37° for 5 min. The maleic acid solution was then added, the tubes were evacuated for 1 min. and replaced in the water bath. The time was taken from the addition of the maleic acid.

Table I gives the results of this experiment.

Table I

Set A: maleic acid/GSH=1/1. Set B: maleic acid/GSH=4/1.

	Time min.	Total iodine ml.	Ascorbic iodine ml.	Glutathione iodine ml.
Control ...		10.2	3.7	6.5
A set	12	8.8	3.7	5.1
B set	12	7.4	3.7	3.7
A set	36	8.4	3.7	4.7
B set	36	5.6	3.3	2.3
A set	60	8.3	3.7	4.6
A set	120	8.2	3.7	4.5
B set	120	4.4	3.0	1.4
A set	240	7.9	3.5	4.4
B set	240	3.8	2.7	1.1

The figures recorded in Table I, last columns, demonstrate that the gradual interaction of maleic acid with GSH proceeds in a physiological medium such as pig's liver. The reaction is more rapid with the higher concentration of maleic acid, but does not go on to completion. The same result was obtained with ox liver as shown in Fig. 2.

Inhibition of coenzyme activity of glutathione by maleic acid

Glyoxalase and maleic acid. Lohmann [1932] has shown that glutathione (GSH) acts as coenzyme for glyoxalase (E). The influence of maleic acid on glyoxalase activity was therefore studied.

The enzyme was made from ox liver as described by Lohmann. Methylglyoxal (Mgl) was prepared by the method of Riley *et al.* [1932]; 10 mg. were dissolved in 1 ml. water and the pH adjusted to pH 7.4 with a little solid NaHCO_3 . The maleic acid solution (M) contained in 0.5 ml. 27.84 mg. maleic acid neutralized to pH 7.4 (concentration $M/12.5$). 0.3 ml. of the glutathione solution was equivalent to 0.0614 mg. GSH. Barcroft differential manometers were used for measuring the CO_2 output. They were filled in the following way:

	Control		Experiment	
	Left ml.	Right ml.	Left ml.	Right ml.
E	1.0	1.0	1.0	1.0
GSH	0.3	0.3	0.3	0.3
H_2O	1.5	1.7	1.0	1.2
M	—	—	0.5	0.5
Mgl	0.2	—	0.2	—
(in Keilin cup)				

The manometers were evacuated and filled with N_2 containing 5% CO_2 . The methylglyoxal was tipped in 5 min. after equilibrium had been reached in the bath at 37° . The results are shown in Fig. 3.

It will be seen from Fig. 3 that maleic acid inhibits the coenzyme activity of glutathione for glyoxalase.

Inhibition of anaerobic glucose breakdown by maleic acid.¹ Geiger [1935] has observed that glutathione increases lactic acid formation of mammalian muscle from glucose as much as 20 times. Large increases of this kind were also found with extracts of brain, liver, heart muscle and uterus. The failure of Meyerhof & Kiessling [1936] to confirm these results was explained by Needham & Lehmann [1937] to be due to the fact that Meyerhof & Kiessling used muscle extract incapable of breaking down glucose except when shunted into the phosphorylation path by hexokinase. A list was given by Haarmann [1932] of those tissues which need no hexokinase for glycolysis. Needham & Lehmann [1937] added the suggestion that just those tissues (brain, retina, tumour, embryo etc.) which have a high anaerobic glycolytic rate have, among other properties, a more or less strong preference for glucose as substrate without addition of hexokinase.

As tissues having no hexokinase, brain tissues were chosen. The anaerobic breakdown of glucose was investigated in the presence and in the absence of maleic acid.

Exp. 1. 7 rat brains (wet weight 14 g.) were ground in an ice-cold mortar with continuous addition of ice-cold water until the ratio tissue: water reached 3:2.

¹ Experiments carried out by Dr H. Lehmann.

Fig. 3. Curve I. Glyoxalase, methylglyoxal and glutathione. Curve II. Glyoxalase, methylglyoxal, glutathione and $M/12.5$ maleic acid.

Two sets of experiments were performed in Thunberg tubes. Each tube of set A contained 1.5 ml. brei, diluted 1:1 with a solution of 4.5 mg. glucose in phosphate buffer (Sørensen, pH 7.2) and sodium maleate, giving a final concentration of $M/12.5$ maleic acid and $M/45$ phosphate. In set B the maleate solution was replaced by water. The Thunberg tubes were evacuated, incubated at 37° and investigated at intervals. The contents were deproteinized with 5 ml. 4% trichloroacetic acid. In the neutralized filtrates the reducing substances were estimated according to Hagedorn & Jensen. The results are given in Fig. 4.

Fig. 4.

Fig. 5.

Fig. 4. Curve I. Rat brain, glucose and maleic acid. Curve II. Rat brain and glucose.

Fig. 5. Curve I. Rat brain, glucose and maleic acid. Curve II. Rat brain and glucose.

Fig. 4 shows the decrease of reducing substances expressed as glucose in presence or in absence of maleic acid. The glucose value at 0 min. is taken as 100% and corresponds to 4.71 mg. glucose. As only 4.5 mg. glucose were added 0.21 mg. glucose was present in the brei. This value may include reducing substances other than glucose. As the turnover of glucose in absence of maleic acid was after 100 min. 1.54 mg. glucose and in presence of maleic acid 0.47 mg. this possible error may be disregarded. In Exp. 1 the inhibition of the anaerobic glucose breakdown by maleic acid amounted to 70%. The inhibition, although not complete, stopped the further breakdown of glucose.

Exp. 2 gave different results. 4 rat brains (wet weight 8 g.) were ground in an ice-cold mortar with continuous addition of ice-cold $M/30$ phosphate buffer, pH 7.2, until the ratio brei:buffer reached 4:5. The Thunberg tubes were filled, incubated and their contents deproteinized as described in Exp. 1 (final concentration of maleic acid $M/12.5$, of phosphate buffer $M/82$). In the deproteinized samples the amount of reducing substances was estimated by heating with alkaline ferricyanide and titrating the ferrocyanide formed with cerium sulphate, using xylene cyanole FF as indicator. The method follows those of Miller & Van Slyke [1936] and of Giragossintz *et al.* [1936]. We are grateful to Mr S. W. Cole for the working details.

The original brei contained reducing substances equivalent to 0.65 mg. glucose. Fig. 5 gives the results of this experiment.

In Exp. 2 the inhibition by maleic acid of the anaerobic glucose breakdown amounted to 48% after 1 hr. In contrast to Exp. 1 maleic acid did not stop the

further glucose breakdown. Thus the inhibitory effect vanished as time went on. After 4 hr. only 14% inhibition could be found.

B. MALEIC ACID AS INHIBITOR OF ENZYME ACTIVITY DUE TO FIXED SH-GROUPS

Interaction of fixed SH-groups of proteins with maleic acid

Proteins containing fixed SH-groups such as unwashed and washed rabbit muscle, thermostable muscle powder, thermostable liver powder and egg albumin denatured by heat were ground to a fine paste and brought into reaction with maleic acid.

The unwashed rabbit muscles were minced in a Latapie mill and incubated in presence and in absence of maleic acid at 37°, pH 7.4. The control tubes contained 0.5 g. unwashed rabbit muscle, 2 ml. Sørensen's phosphate buffer, pH 7.4, 2 ml. water and one drop CHCl_3 . In the tubes containing maleic acid the water was replaced by 2 ml. maleate solution, giving a final concentration of $M/12.5$ maleic acid. A separate part of the untreated minced rabbit muscle from this experiment was washed 6 times with distilled water. The thermostable muscle powder and the thermostable liver powder were prepared according to Hopkins & Dixon [1922] and Hopkins [1925]. At intervals the tubes were tested by the nitroprusside reaction. In the absence of maleic acid none of the preparations used showed after 20 hr. incubation any appreciable reduction in the intensity of the reaction. In the presence of the maleic acid the reaction had by then wholly disappeared from the thermostable tissue powders and in the case of the muscle, washed and unwashed, it had become very faint. That the disappearance was slower in the latter was doubtless due to its relative impermeability.

It is clear that proteins containing SH-groups react with maleic acid, and the reaction involves the disappearance of these groups.

Inhibition of succinodehydrogenase by maleic acid

The inhibition of muscle respiration by maleic acid was shown by Thunberg [1911]. It was examined by Lipschitz & Gottschalk [1921], in great detail by Grönvall [1924] and more recently by Gözsy & Szent-György [1934]. Laki & Szent-György [1935] observed that the inhibition of succinodehydrogenase by maleic acid is only striking when the molar concentration of the maleic acid is greater than the molar concentration of the succinic acid. They suggested that maleic acid acts at the surface of the enzyme. We reinvestigated the action of maleic acid on succinodehydrogenase. The striking resemblance of the inhibition observed to the reactions described in this and our previous paper is illustrated by the following experiment.

Succinodehydrogenase prepared according to Ogston & Green [1935] was kindly supplied to us by Dr Green. The maleate solution contained in, 5 ml. water, 0.2784 g. maleic acid, neutralized to pH 7.4 with NaOH; the succinate solution had 0.270 g. sodium succinate in 10 ml. water. The buffer used was Sørensen's phosphate buffer, pH 7.4. The O_2 uptake was measured in Barcroft differential manometers at 37°. Two experiments were performed, after incubation of maleic acid with succinodehydrogenase for 15 min. and 3 hr. respectively. The succinate was tipped from a Keilin cup at 0 min. The flasks were filled as shown:

	Experiment		Control	
	Left	Right	Left	Right
Enzyme	0.5	0.5	0.5	0.5
Buffer	1.0	1.0	1.0	1.0
Water	1.0	0.8	1.5	1.3
Maleate	0.5	0.5	—	—
Succinate	—	0.2	—	0.2

Fig. 6 shows the results.

After incubation of succinodehydrogenase with maleic acid for 15 min. (curve II) the inhibition of O_2 uptake amounted to 40%, after incubation for 3 hr. it was 48.5%. In both experiments the interaction of maleic acid with succinodehydrogenase stopped the further O_2 uptake.

Fig. 6. Curve I. Succinodehydrogenase and succinic acid. Curve II. Succinodehydrogenase, succinic acid and $M/12.5$ maleic acid (incubation for 15 min. at 37°). Curve III. Succinodehydrogenase, succinic acid and $M/12.5$ maleic acid (incubation for 3 hr. at 37°).

Activation of papain and its inhibition by maleic acid

Bersin [1933] has shown that the number of fixed SH-groups of papain determines its activity. As the papain preparations contain not only fixed SH-groups but also fixed SS-groups an activation of papain can be obtained by processes which increase the fixed SH-groups and diminish the fixed SS-groups. This can be done mainly in two ways: with HCN [Mauthner, 1912; Grassmann, 1931] or following Hopkins's method [1925] by the reduction of SS-groups with SH-compounds such as glutathione [Grassmann, 1932]. The inhibition of activity brought about by maleic acid was therefore separately investigated for papain activated by HCN and by GSH.

(a) *Activation of papain by HCN and its inhibition by maleic acid.* The following solutions were prepared:

1.0 g. commercial papain (B.D.H.) was shaken for 1 hr. with 20 ml. water and filtered. The HCN solution was obtained by bringing a 1% KCN solution to pH 4.6 with HCl. 0.464 g. maleic acid was brought to pH 4.6 with NaOH and the solution made up to 10 ml. The gelatin solution contained 7% gelatin. The buffer was acetate buffer of pH 4.6.

Two conical flasks were filled with the reactants as follows: 5 ml. papain, 10 ml. acetate buffer, 5 ml. HCN, 20 ml. gelatin. In addition, flask 1 had 10 ml. water, flask 2 10 ml. maleic acid.

Before the addition of the gelatin to flasks 1 and 2 and of the maleate to flask 2, the papain was activated by incubation with HCN for 2 hr. at 37° . After this time maleate was added to flask 2 and gelatin to flasks 1 and 2. 5 ml. were withdrawn at intervals, HCHO was added and the solution titrated with 0.01 N NaOH to phenolphthalein.

In a second experiment the papain was again activated with HCN for 2 hr. at 37° . After activation maleic acid was added and the mixture incubated for further 16 hr. at 37° . The third experiment was a duplicate of the second experiment.

The results are given in Fig. 7.

After 2 hr. incubation of papain with HCN the activation of papain was inhibited to the extent of 32% by maleic acid (curve II). The activated papain, incubated for a further 16 hr. with maleic acid showed a diminution of activity of 68 and 70% respectively (curves IIIa and IIIb).

Fig. 7.

Fig. 8.

Fig. 7. Curve Ia. Papain, HCN and gelatin. Curve Ib. Papain, HCN and gelatin (incubated for 16 hr. at 37°, control of curves IIIa and IIIb). Curve II. Papain, HCN, gelatin and *M*/12.5 maleic acid (not incubated after activation). Curve IIIa and IIIb. Papain HCN, gelatin and *M*/12.5 maleic acid (incubated for 16 hr. at 37° with maleic acid after activation).

Fig. 8. Curve I. Papain and *M*/250 glutathione. Curve II. Papain, *M*/250 glutathione and *M*/12.5 maleic acid (not incubated after activation). Curve III. Papain, *M*/250 glutathione and *M*/12.5 maleic acid (incubated 16 hr. at 37° with maleic acid after activation).

(b) *Activation of papain by glutathione and its inhibition by maleic acid.* The glutathione solution (*M*/10) and the maleate solution (0.464 g. maleic acid in 13 ml.) were brought to pH 4.6 with NaOH. The other solutions had the compositions described above. The final concentration of glutathione was *M*/250 and of maleic acid *M*/12.5.

Three conical flasks were filled with the reactants as follows: 5 ml. papain, 10 ml. acetate buffer, 2 ml. GSH, 20 ml. gelatin. In addition, flask 1 contained 13 ml. water, flasks 2 and 3 had 13 ml. maleate.

Before the addition of gelatin to flasks 1, 2 and 3 and of maleic acid to flasks 2 and 3 the papain was activated by incubation with GSH for 2 hr. at 37°. After this time maleic acid was added to flask 2 and gelatin to flasks 1 and 2. Maleic acid only was added to flask 3 and the mixture incubated for a further 16 hr. at 37°. Then gelatin was added to flask 3. 5 ml. were withdrawn at intervals and titrated according to Sørensen. Fig. 8 shows the results of this experiment.

After 2 hr. incubation of papain with GSH the activity of papain was diminished 37% by maleic acid (curve II). The activated papain incubated for 16 hr. with maleic acid at 37° showed a decrease of activity of 88% (curve III).

DISCUSSION

In order to apply the reaction of maleic acid with SH-compounds to enzyme reactions the chemical basis of this reaction had to be broadened in two directions. It was necessary to study the interaction of maleic acid with GSH in physiological concentrations and further to investigate the reactivity of compounds with fixed SH-groups. The first set of experiments showed that although the reaction occurs it does not come to completion in physiological concentrations, and the second group of experiments indicated a remarkable difference of reactivity for the different SH-containing proteins. This second result is in agreement with well-

known facts. Baumann [1885] met with similar differences in the study of the reaction between pyruvic acid and different mercaptans, Michaelis & Schubert [1934] mention the differences of reactivity of the SH-groups according to the structure of the molecules of which they form a part, Smythe [1936] observed the greater reactivity of cysteine than of thiolacetic acid with iodoacetic acid, a difference we found again in the interaction of these compounds with maleic acid. Thus it is to be expected that the application of the maleic acid reaction to enzymic processes induced by SH-groups will give different results in every case investigated. In addition to this difference from enzyme to enzyme or coenzyme to coenzyme a serious complication arises when one changes from homogeneous to non-homogeneous media. Meyerhof [1937] has recently drawn attention to this important point. In a non-homogeneous medium constant results with the same enzyme system are not to be expected. Thus in pig's liver the reaction, although inhibited, does not come to an end whilst in ox liver the reaction is stopped.

Reviewing from these points of view the different enzyme reactions investigated we see that although the coenzyme activity of glutathione is strongly inhibited by maleic acid, the lactic acid formation from methylglyoxal is not stopped completely (Fig. 3). As at the concentrations used the interaction of maleic acid with glutathione is not complete some glutathione must remain uncombined, and this might suffice to keep the reaction going, it being assumed that glutathione is the only activator of glyoxalase. In the two experiments dealing with the activation of the anaerobic glucose breakdown we find inhibition with maleic acid in both, but while in one experiment (Fig. 4) the reaction is stopped, in the second (Fig. 5) it is not. The same inconsistency of results was found in the study of succinodehydrogenase, the activity of which could be diminished and stopped in some experiments, whilst other experiments showed partial inhibition without complete arrest. As both reactions proceed in a non-homogeneous medium this medium may be the cause of the inconsistency although other unknown factors may interfere.

In the maleic acid effect with papain activated either by HCN or by glutathione the inhibitions were 70 and 88% respectively after incubation for 16 hr. at 37° with maleic acid. The slight rise at the beginning of the experiments (Figs. 7 and 8) reminds one of similar observations of Bergmann [1937].

For all but one of the enzyme reactions we investigated it is now generally believed that SH-groups are necessary. The exception is the succinodehydrogenase. Thus the question arises whether inhibition of an enzyme reaction by maleic acid is necessarily due to its interaction with SH-groups. This conclusion, although indirect, would be safe if maleic acid were shown to react exclusively with SH-groups. Until now we have found no other groups reacting with maleic acid under our experimental conditions, but this might change in the course of further investigations. Maleic acid inhibition of an enzyme reaction suggests that SH-groups may play a role in this reaction. Fortunately a direct way is available for checking such a suggestion, Hopkins's SS, SH-reaction. This reaction applied recently by Hopkins & Morgan [1938] to succinodehydrogenase gave conclusive evidence that the SH-groups of succinodehydrogenase are among those groups which are essential for the activity of this enzyme. In agreement with these results succinodehydrogenase is inhibited by maleic acid as shown in this paper.

The advantage of the maleic acid reaction in the study of enzyme reactions seems to us to be that it supplements the sometimes vague views concerning displacements at the enzyme surface with a definite chemical conception based

on the reactivity of maleic acid with thiol-compounds. The material carefully collected by Grönvall [1924] concerning the manifold biological reactions influenced by maleic acid indicates a large field of further reactions in which thiol compounds may play a role.

SUMMARY

Maleic acid interacts with $M/200$ glutathione. The reaction can be detected in a physiological medium such as pig or ox liver. The reaction is not complete.

Maleic acid interacts with proteins containing fixed SH-groups.

The activation of glyoxalase and of the anaerobic glucose breakdown by glutathione is decreased by maleic acid.

The activities of succinodehydrogenase and of papain are decreased by maleic acid.

The inhibition of the enzyme reactions investigated is connected with the interaction of maleic acid with thiol groups.

The conclusions to be drawn from the maleic acid reaction in its application to enzyme reactions are discussed.

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