C. INTERACTION OF MALEIC ACID WITH THIOL COMPOUNDS

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It was observed by one of us (E. J. M.) that the titratable SH of glutathione was greatly decreased by incubation with maleic acid in absence or in presence of oxygen at pH 7.4 and 37° (but not with fumaric acid). Thiolacetic acid and cysteine behaved similarly to glutathione. The results contained in this paper show that the disappearance of the SH-groups is due to the circumstance that each of the three substances studied yields an addition compound with maleic acid in the formation of which these groups are involved. The kinetics of such reactions show points of special interest.

Titration experiments

The following solutions were prepared: (1) a 0.2 *M* solution of the Na salt of the unsaturated acid (A); (2) a solution of the Na salt of the thiol compound such that 1 ml. was equivalent to 40 ml. 0.01 *N* iodine, i.e. 0.4 *M* with respect to SH (B). For experiment *x* ml. of A and *y* ml. of B were placed in each of a series of Thunberg tubes together with Sørensen's phosphate buffer, pH 7.4 (1 ml. for every 4 ml. final volume) and water to the required volume. The tubes were evacuated and incubated at 37°.

Fig. 1.

Fig. 1. Final concentration *M*/50 thiolacetic and *M*/50 maleic acid.

Fig. 2.

Fig. 2. Curve I, final concentration *M*/12.5 thiolacetic and *M*/50 maleic acid.
Curve II, final concentration *M* 12.5 maleic and *M*/50 thiolacetic acid.

At successive intervals a tube was removed and its contents analysed iodimetrically for SH after acidification with trichloroacetic acid.

The interaction of maleic and thiolacetic acids is illustrated in Figs. 1 and 2 in which residual SH concentration in terms of ml. 0.01 *N* iodine is plotted against time. It will be seen that with equimolar concentrations of the reactants 50 % of the thiolacetic acid disappears (Fig. 1); with a molar ratio of thiolacetic to maleic acid of 4 : 1 on the other hand the disappearance of thiolacetic acid is only 15 % (Fig. 2, curve I) whilst with a ratio of 1 : 4 its removal is almost quantitative (Fig. 2, curve II).

Fig. 3 illustrates the comparative interactions of equimolar solutions of maleic and thiolacetic acids (Curve II), maleic acid and glutathione (Curve III), and maleic acid and cysteine (Curve V); it will be seen that thiolacetic acid and glutathione behave similarly whilst cysteine is more completely removed under these conditions. Curve IV shows that cyanide does not affect the reaction. No interaction was observed on substituting for maleic acid any of the following: fumaric acid (Fig. 3, curve I), citraconic, mesaconic, *cis*- and *trans*-cinnamic and α -phenyl- β -styrylmaleic [Friedmann & v. Heyningen, 1936] acids.

Fig. 3. Final concentrations. Curve I, *M*/12.5 thiolacetic and *M*/12.5 fumaric acids. Curve II, *M*/12.5 thiolacetic and *M*/12.5 maleic acids. Curve III, *M*/12.5 glutathione and *M*/12.5 maleic acid. Curve IV, *M*/12.5 thiolacetic acid, *M*/12.5 maleic acid and *M*/1000 KCN. Curve V, *M*/12.5 cysteine and *M*/12.5 maleic acid.

Polarimetric experiments

M/5 solutions were prepared of the Na salts of glutathione and of various unsaturated acids, the solutions containing 2 ml. of phosphate buffer, pH 7.4, in a total volume of 10 ml. For the experiments equal volumes of the solution of glutathione and that of one of the unsaturated acids were mixed and compared polarimetrically ($H_{g\text{green}}$, 2 dm. tube, 21.5°) with controls consisting of glutathione solution mixed with an equal volume of a 1 : 5 dilution of Sørensen's phosphate buffer, pH 7.4.

Fig. 4, curve I illustrates the interaction of maleic acid and glutathione whilst curve II of this figure again demonstrates the inability of fumaric acid to replace maleic acid in the reaction; similar failure to react was observed with *cis*- and *trans*-cinnamic acids.

Fig. 4. Curve I, *M*/10 glutathione and *M*/10 maleic acid. Curve II, *M*/10 glutathione and *M*/10 fumaric acid. *t* 21.5°.

Kinetic experiments

In Table I are given the results of an attempt to study the kinetics of the interaction of maleic acid and glutathione in equimolar proportions. The solutions were prepared as described above and the experiments were carried out in open tubes, the reactants being brought to the bath temperature before mixing and the reaction being followed iodimetrically. The decrease of the reaction constants with time indicates a complex reaction, a supposition which is confirmed by examination of the reaction products.

Table I. *M*/10 glutathione and *M*/10 maleic acid

Time min.	Titratable SH 0.01 <i>N</i> I ml.	Decrease of titratable SH 0.01 <i>N</i> I ml.	K' $\frac{1}{t} \log \frac{a}{a-x}$	$K'' \times 10^3$ $\frac{x}{t(a-x)a}$
A. Temp. 20°				
0	20.0	—	—	—
2	19.4	0.6	0.66	0.773
4	18.8	1.2	0.67	0.798
8	18.0	2.0	0.57	0.694
16	16.4	3.6	0.54	0.686
32	14.5	5.5	0.44	0.593
64	11.2	8.8	0.40	0.614
B. Temp. 30°				
0	20.0	—	—	—
2	18.8	1.2	1.34	1.596
4	17.8	2.2	1.265	1.545
8	16.25	3.75	1.128	1.442
16	14.3	5.7	0.910	1.246
32	12.1	7.9	0.671	1.020
64	9.4	10.6	0.504	0.881

Products of the reaction

For separation of the reaction products use was made of the facts that unchanged thiol compound and the reaction product were precipitated by mercuric sulphate whilst the thiol compound alone was precipitated by cadmium acetate.

Maleic acid and thiolacetic acid.

Isolation and properties of reaction product. A solution of maleic acid (9.282 g.), neutralized with *N* NaOH to pH 7.4, and diluted to 400 ml. was mixed with one of thiolacetic acid (7.149 g.), similarly neutralized, in water (200 ml.), and 400 ml. of water in a Büchner flask. The flask was evacuated and incubated for 22 hr. at 37° after which 3.395 g. unchanged thiolactic acid (47.5%) remained.

The solution was acidified with H_2SO_4 to Congo red and precipitated with HgSO_4 , avoiding excess. The Hg-precipitate was collected and decomposed with H_2S as usual. After removing H_2S with a stream of hydrogen and centrifuging HgS , the supernatant fluid was made up to 990 ml. (iodine equivalent unchanged), and treated with cadmium acetate (10%) avoiding excess; the filtrate from the cadmium acetate precipitate was reprecipitated with HgSO_4 . After decomposing the precipitate with H_2S and removing H_2SO_4 from the filtrate quantitatively with $\text{Ba}(\text{OH})_2$, the solution was concentrated under reduced pressure, transferred to a crystallizing dish and evaporated *in vacuo* over H_2SO_4 . A white, crystalline, uniform product (8.4621 g., i.e. theoretical yield allowing for the thiolacetic acid recovered) remained, consisting of rosettes of needles. It was recrystallized from a little water and analysed after drying *in vacuo* over P_2O_5 at 40°. (Found: 34.7; H, 4.0; S, 15.1%. $\text{C}_6\text{H}_5\text{O}_6\text{S}$ requires C, 34.6; H, 3.9; S, 15.4%.) m.p. 140.5° (uncorr.) after softening at 135°.

The substance contains no water of crystallization. It is readily soluble in cold water, methanol, ethanol and acetone, less soluble in cold ether; ethyl acetate and glacial acetic acid dissolve it sparingly in the cold, better on warming. Chloroform, benzene, toluene, xylene and light petroleum (b.p. 50–60°) scarcely dissolve it, even on heating.

Preparation, analysis and reactions indicate that the new substance is an addition compound of thiolacetic and maleic acids, of the formula

In accordance with this structure it titrates as a tricarboxylic acid: 0.0416 g., required 6.0 ml. 0.1 *N* NaOH to phenolphthalein, giving equiv. 208.0: $\text{C}_6\text{H}_5\text{O}_6\text{S}$ titrating as a tribasic acid requires 208.1.

Further evidence for the assumed constitution is provided by alkaline hydrolysis of the substance. Molecular proportions of the addition compound, *N* NaOH and benzyl chloride were heated at 70°, transferred to a thermos flask and shaken for 8 hr. The acidified solution deposited an oily precipitate which was discarded. The filtrate on evaporation at room temperature gave a crystalline mixture which was dissolved in alkali, extracted with ether in order to remove traces of dibenzylsulphide, acidified and again extracted with ether. This ethereal extract gave, after removal of the solvent, needles and plates which could be separated mechanically under a lens or better by extraction with benzene which dissolved the plates. The undissolved needles were recrystallized from water and proved to be fumaric acid. m.p. 280° (uncorr.). (Found: C, 41.6; H, 3.6%. $\text{C}_4\text{H}_4\text{O}_4$ requires C, 41.4; H, 3.5%. 0.0116 g. neutralized 2 ml. 0.1 *N* NaOH to phenolphthalein; $\text{C}_4\text{H}_4\text{O}_4$ (dibasic) requires 2 ml.).

The evaporated benzene solution gave a crystalline substance which, after purification by sublimation *in vacuo*, and recrystallization from dilute alcohol, softened at 59° and melted at 62°. Gabriel [1879] gives the m.p. of benzylthiolacetic acid as 58–59°; Andreasch [1897] gives 60°. (Found: C, 59.2; H, 5.5; S, 17.4%. $C_9H_{10}O_2S$ requires C, 59.3; H, 5.5; S, 17.6%. 0.0182 g. neutralized 1.0 ml. 0.1 *N* NaOH to phenolphthalein; $C_9H_{10}O_2S$ requires 1.0 ml.)

Isolation of thiolacetic acid. The reaction solution from a similar experiment with maleic and thiolacetic acids (residual thiolacetic acid by titration = 3.22 g.) was completely precipitated with cadmium acetate; the precipitate was collected and decomposed as usual and the filtrate evaporated in a vacuum desiccator over H_2SO_4 ; the residue was taken up in ether and the ether solution dried and evaporated, yielding 2.6 g. (81%) of thiolacetic acid, b.p. 108–109°/14 mm. The loss is due to partial volatilization in the desiccator.

Isolation of fumaric acid. The filtrate and washings from the $HgSO_4$ precipitation of the reaction solution obtained as above were freed from mercury with H_2S and concentrated under reduced pressure to about 100 ml.; on keeping in a refrigerator 3.965 g. of colourless needles were obtained which gave three successive crops from hot water (2.615, 0.769, 0.038 g.) all having m.p. 280°, not depressed by admixture with fumaric acid. (Found: C, 41.6; H, 3.7%. $C_4H_4O_4$ requires C, 41.4; H, 3.4%.) Since 4.25 g. maleic acid correspond to the 3.22 g. thiolacetic acid found not to have reacted, the yield of fumaric acid is 93.3%.

Maleic acid and cysteine

2.428 g. cysteine were dissolved in 200 ml. *M*/2.5 maleic acid and neutralized with *N* NaOH to pH 7.4, the final concentrations thus being *M*/2.5 maleic acid and *M*/10 cysteine. After evacuation, the solution was kept for 8 hr. at 37° and then at room temperature overnight. In the morning only a faint nitroprusside reaction remained. The solution was precipitated with $HgSO_4$, and the precipitate collected and decomposed as usual; the filtrate was evaporated *in vacuo*, yielding 4.728 g. (calculated 4.749 g.) of an amorphous product. For analysis the substance was dissolved in water, the solution frozen and dried by Pirie's method [1931] which gave a friable product. It was finally dried at 40° over P_2O_5 . (Found: C, 35.8; H, 4.95; N, 6.0; S, 14.7%. $C_7H_{11}O_6NS$ requires C, 35.5; H, 4.6; N, 5.9; S, 13.5%. 0.0474 g. neutralized 4.0 ml. 0.1 *N* NaOH to phenolphthalein, giving equiv. wt. 237; $C_7H_{11}O_6NS$ titrating as a dibasic acid has equiv. wt. 237.05.)

The substance softens at 102° and melts at 134–135° (decomp.). By analogy with the product of reaction between maleic and thiolacetic acids the constitution of the new compound can be assumed to be:

Maleic acid and glutathione

50 ml. of *M*/5 maleic acid and 50 ml. of approximately *M*/5 glutathione, both neutralized with *N* NaOH to pH 7.4, were mixed and kept under vacuum at 37° for 8 hr.; 1.166 g. glutathione remained (determined iodimetrically).

The $HgSO_4$ precipitate, worked up as before, gave a hygroscopic substance (2.402 g.; calculated 2.410 g.). It seems to contain water of crystallization which can be removed only with difficulty. (Found: N, 8.8; S, 6.5%. $C_{14}H_{21}O_{10}N_3S \cdot 3H_2O$ requires N, 8.8; S, 6.5%. After drying *in vacuo* over H_2SO_4 found: C, 37.2; H, 5.6%. $C_{14}H_{21}O_{10}N_3S \cdot 1\frac{1}{2}H_2O$ requires C, 37.3; H, 5.3%. Dried 14 days *in vacuo* over P_2O_5 , 0.0423 g. neutralized 3.1 ml. of 0.1 *N* NaOH to phenolphthalein,

corresponding to equiv. wt. 409.1, the theoretical value for $C_{14}H_{21}O_{10}N_3S$, titrating as a tribasic acid being 423.2.)

The substance dried over P_2O_5 softens at 122° and melts at 151 – 152° (decomp.). The analysis indicates a composition analogous to the addition compounds described above, and the compound may be formulated:

The cadmium precipitate, decomposed as before, gave glutathione in nearly theoretical yield (1.163 g.). It was identified by its m.p. 190° (decomp.), its iodine value and by analysis. (Found: N, 13.4; S, 10.3%. $C_{10}H_{17}N_3SO_6$ requires N, 13.4; S, 10.5%.)

The $HgSO_4$ filtrate gave 0.474 g. fumaric acid (calc. 0.499 g.).

Rearrangement of maleic acid to fumaric acid during the reaction

Fumaric acid has been shown above to be a reaction product of maleic and thiolacetic acids and of maleic acid and glutathione. The truth of this interpretation had to be checked since Skraup [1891] has shown that the metal salts of maleic acid when treated with H_2S give fumaric as well as maleic acids. We therefore investigated the degree of rearrangement due to the manipulations during the isolation of maleic acid in our experiments.

3 g. maleic acid, 0.97 g. Na_2SO_4 , 20 ml. 10% cadmium acetate, 20 ml. $HgSO_4$, 3 ml. glacial acetic acid and 30 ml. conc. H_2SO_4 in 1500 ml. distilled water were kept at room temperature for 4 days. The solution was treated with H_2S and worked up for unsaturated acids as above. The fumaric acid isolated amounted to 0.1098, i.e. only 3.6% of the maleic acid employed.

Conclusive evidence that fumaric acid is a true product of reaction formed during the interaction of maleic acid with certain thiol-compounds is provided by its direct isolation as follows. The reaction solution prepared as usual from glutathione (0.5986 g.) and maleic acid (0.232 g.) had lost 0.3530 g. glutathione, equivalent to 0.1309 g. maleic acid; thus 0.89 g. maleic or fumaric acid was contained in the remaining 22 ml. of solution. The latter was acidified with 5.3 ml. $N H_2SO_4$ and extracted with ether for 8 hr. The ether extract gave after evaporation from a water bath not above 40° a crystalline residue (0.76 g.) which, after recrystallization from hot water proved to be pure fumaric acid, m.p. 280° . (Yield 92.8%.)

In a control experiment in which glutathione solution was replaced by water the ether extract gave 0.207 g. maleic acid (89.2%).

For the interpretation of the results it seemed necessary to investigate whether the observed conversion of *cis*-acid into *trans*-acid was connected with addition of the thiol groups to the double bond or whether the mere presence of a thiol compound suffices to induce the rearrangement. Since *cis*-cinnamic acid has been shown not to react with glutathione the incubation of *cis*-cinnamic acid with glutathione was examined.

cis-Cinnamic acid (m.p. 58° , 0.296 g. in 10 ml.), glutathione (0.614 g. in 5 ml.), both neutralized to pH 7.4, and 5 ml. of water, were incubated together *in vacuo* at 37° for 6 hr.; the solution was then acidified with 4 ml. $N H_2SO_4$ and extracted continuously with ether for 8 hr. The ethereal extract, dried with Na_2SO_4 , gave a residue which was treated with aniline [cf. Liebermann, 1890, 1891]. The aniline salt so obtained was washed with light petroleum and dried (0.37 g.). It melted at 83 – 84° . The aniline salt was suspended in water, decomposed with $N HCl$ and the free acid extracted with ether. The ethereal extract, washed with

water and dried with Na_2SO_4 gave, after removal of the solvent *in vacuo*, a residue which was dissolved in light petroleum, B.P. under 40° . By slow concentration three fractions were obtained (0.130; 0.062; 0.021 g.) which were identical. They had M.P. 58° , not depressed in mixture with *cis*-cinnamic acid. Yield of pure *cis*-cinnamic acid 72%.

It is clear therefore that a thiol compound which cannot be added to the double bond of an unsaturated *cis*-acid is unable to induce the rearrangement from the *cis*- to the *trans*-configuration under the experimental conditions described.

*Yield of the end products of the reaction between maleic acid
and thiol compounds*

Over the concentration range so far studied ($M/50$ – $M/10$) the yield of the addition product of maleic and thiolacetic acids increases with increasing concentrations. In order to determine the maximum yield of the addition product we therefore investigated the interaction at $M/2$ concentration which is the highest compatible with the solubility of sodium maleate. M maleic acid (Kahlbaum) (40 ml. with 20 ml. 4*N* NaOH, 8 ml. phosphate buffer pH 7.4 and 12 ml. water) and M thiolacetic acid (B.D.H.) (40 ml. with 10 ml. 4*N* NaOH 8 ml. buffer and 22 ml. water) were warmed to 37° and mixed in vacuum tubes which were at once evacuated, each tube containing 4 ml. of the mixed solution. The tubes were incubated at 37° and removed at intervals for titration. The results are given in Table II.

Table II. $M/2$ maleic and $M/2$ thiolacetic acids at 37°

Time	0.1 <i>N</i> iodine ml.
Initial	20
15 min.	9.0
30 min.	7.8
45 min.	7.2
1 hr.	6.9
2 hr.	6.2
3 hr.	5.8
4 hr.	5.7

The reaction is complete after 3–4 hr., 72.2% of the addition compound having been formed. The experiment shows therefore that by increasing the concentrations of the reactants from $M/10$ to $M/2$ the yield of the addition compound is still further increased.

The yield of fumaric acid, formed during the reaction, has so far been determined only for $M/10$ concentrations of each reactant. For the interpretation of the reactions involved it seemed desirable to study the rearrangement (a) at $M/50$ for both reactants and (b) at a concentration of maleic acid compensating the loss of maleic acid by its rearrangement to fumaric acid. The latter conditions are fulfilled by the interaction of $M/12.5$ maleic acid with $M/50$ thiolacetic acid.

(a) 2.320 g. maleic acid and 1.842 g. thiolacetic acid were neutralized and made up to 100 ml.; 5 ml. required 10 ml. of 0.01 *N* iodine. The mixture was incubated for 15 hr. at 37° ; 5 ml. of the incubated solution required 4.3 ml. 0.01 *N* iodine, equivalent to 43% of the original thiolacetic acid. The HgSO_4 precipitate of this solution, worked up as above, gave 0.8215 g. fumaric acid. Allowing for the solution used for titration this corresponds to formation of 35.8% fumaric acid as against a calculated amount of 42.8%.

(b) 9.282 g. maleic acid and 1.842 g. thiolacetic acid were neutralized and made up to 100 ml. The mixture was incubated for 17 hr. at 37°; 20 ml. of the incubated solution required no iodine. Thus the thiolacetic acid was completely removed during the reaction. The HgSO_4 precipitate gave 1.8176 g. or 19.6% fumaric acid, and 5.0973 g. or 54.9% maleic acid both corrected for samples removed. As 2.3205 g. or 25% maleic acid were used for the formation of the addition compound 9.2314 g. or 99.5% of the maleic acid is accounted for.

The results showing the yields of the end products from the interaction of maleic acid with thiolacetic acid under different molar concentrations of the reactants are summarized in Table III.

Table III

Exp. no.	Concentrations of the reactants		Time of incubation hr.	End products				
				Thiolacetic acid		Maleic acid		Fumaric acid formed from maleic acid %
				In addition compound %	Free %	In addition compound %	Free %	
1	<i>M</i> /2	<i>M</i> /2	4	72.2	27.8	72.2	—	—
2	<i>M</i> /10	<i>M</i> /10	19	65	35	65	0	34.2
3	<i>M</i> /12.5	<i>M</i> /12.5	4	50	47	53	—	—
4	<i>M</i> /12.5	<i>M</i> /12.5	22	55	45	55	—	—
5	<i>M</i> /50	<i>M</i> /50	24	54.5	45.5	54.5	—	—
6	<i>M</i> /50	<i>M</i> /50	15	57	43	57	0	Found 35.8 Calc. 42.8
7	<i>M</i> /12.5	<i>M</i> /50	5	98.8	1.2	24.7	54	—
8	<i>M</i> /12.5	<i>M</i> /50	17	100	0	25	—	19.6
9	<i>M</i> /50	<i>M</i> /12.5	5	15	85	60	—	—

DISCUSSION

The results of the interaction of maleic acid with thiol compounds (at *M*/12.5 concentration) vary with the individual thiol compounds employed. The addition compound from cysteine is obtained in a yield of 93.5%, whilst that from thiolacetic acid and glutathione is formed in yields of 55 and 56.5% respectively (Fig. 3). Furthermore during the interaction of thiolacetic acid and glutathione with maleic acid a considerable amount of maleic acid is rearranged to fumaric acid.

The simpler case is obviously the reaction between cysteine and maleic acid, where in view of the large yield of the addition compound (93.5%), the interaction can be formulated as a simple addition reaction of cysteine and maleic acid:

The greater reactivity of cysteine as compared with those of thiolacetic acid and glutathione is analogous with the similar behaviour of these substances towards iodoacetic acid [Smythe, 1936].

The interpretation of the interactions of maleic acid with thiolacetic acid and with glutathione is more complicated. Table III shows that within the range of concentrations *M*/50–*M*/12.5 the reactions proceed on very similar lines, 54–57% of the theoretical yield of the addition compound being formed and 43–47% thiolacetic acid remaining uncombined after incubation. At higher concentrations the yield of the addition compound increases. This is shown in Fig. 5 for the concentrations *M*/50, *M*/12.5 and *M*/2.

Thus at higher concentrations the reaction resembles more and more the simple addition demonstrated in the case of cysteine with maleic acid.

Fig. 5. Curve I, $M/2$ thiolacetic and $M/2$ maleic acids. Curve II, $M/12.5$ thiolacetic and $M/12.5$ maleic acids. Curve III, $M/50$ thiolacetic and $M/50$ maleic acids.

At concentrations between $M/12.5$ and $M/50$ the interpretation has to account for the rearrangement of maleic acid to fumaric acid. The simplest formulation would be:

which implies that, for non-equimolecular proportions of reactants, the ratio of fumaric acid, formed by rearrangement, to maleic acid in the addition compound ought to be 1:1 and that for the experiment with $M/12.5$ maleic acid and $M/50$ thiolacetic acid the ratio of fumaric acid: combined maleic acid: free maleic acid should be 1:1:2. Actually, as will be seen from Table III, the amounts of fumaric acid found are considerably lower than required by these ratios.

This observation can be explained by the assumption that the simple addition reaction which is predominant at higher concentrations takes place to some extent also at lower concentrations. Two main reactions thus proceed simultaneously: (1) simple addition and (2) addition plus rearrangement. The question whether low concentrations of the reactants favour the second reaction as suggested by Exp. 6, Table III, will be investigated later.

The reaction described in this paper is a new example of the well established fact that reagents which are able to rearrange an ethylenic linkage can form addition compounds at the double bond. The reaction described can be applied to the investigation of biochemical processes in which SH-compounds play an important role. Work in this direction has already been done, and is continuing. In particular experiments on the succinodehydrogenase of muscles, on papain, on glyoxylase and on the non-phosphorylating breakdown of glucose which is activated by glutathione, have been carried out. The results obtained will shortly be described.

SUMMARY

Maleic acid reacts with thiol compounds with the formation of addition compounds. In the case of thiolacetic acid and glutathione, maleic acid is rearranged simultaneously to fumaric acid.

The addition compounds of maleic acid with thiolacetic acid, cysteine and glutathione have been isolated.

The mechanism of the reactions involved has been examined and discussed.

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