Reaction of Glutathione with Conjugated Carbonyls

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1. GSH reacts with conjugated carbonyls according to the equation: $GSH + R-CH=CH-COR \rightleftarrows R-CH(SG)-CH_2-COR$. The forward reaction follows second order, the reverse reaction first order kinetics. It is assumed that this reaction reflects best the ability of conjugated carbonyls to inactivate SH groups in biological systems.

2. The rate of forward reaction increases with pH approx. parallel with α_{SH}. Besides OH^- ions also proton donors (*e. g.* buffers) increase the rate. The catalytic effect of pH and buffer is interpreted in view of the reaction mechanism.

3. The equilibrium constants as well as the rate constants for forward (k_1) and reverse reaction show an extreme variation depending on the carbonyl structure. Acrolein and methyl vinyl ketone (k_1 = 120 and 32 mol^{-1} sec^{-1}, resp.) react more rapidly than any other carbonyl to give very stable adducts (half-lives for reverse reaction 4.6 and 60.7 days, resp.). Somewhat less reactive are 4-hydroxy-2-alkenals and 4-ketopentenoic acid (k_1 between 1 and 3 mol^{-1} sec^{-1}), but they also form very stable adducts showing half-lives between 3.4 and 19 days. All other carbonyl studied react either very slowly (*e. g.* citral, ethly crotonate, mesityl oxide, acrylic acid) or form very labile adducts (crotonal, pentenal, hexenal, 3-methyl-butenone). Comparing biological activities of conjugated carbonyls their reactivity towards HS (k_1) and the stability of the adducts must be considered.

Introduction

It was reported that some α,β-unsaturated aldehydes possess anticancer [1-9], antiviral [10-12] and antimicrobial [13-19] activity and inhibit protein synthesis [20, 21], nucleic acid synthesis [20-70], glycolysis [20, 25] and mitochondrial respiration [20, 25-27]. It was assumed by several authors that the reaction of the aldehydes with biologically important sulfhydryl groups plays a significant role in the mechanism by which these compounds exert their biological activity [15, 20, 24, 25, 28-30]. Conjugated aldehydes generally react with thiols by 1,4-addition forming Michael type adducts [31-33]. Little is known about the relation between the reactivity of such aldehydes towards thiols and their biological activity. In order to gain more information on this subject, we have investigated the chemical reactivity of a number of α,β-unsaturated aldehydes and some other unsaturated carbonyls tomards glutathione. We report in this paper rate constants, equilibrium constants and some aspects of the mechanism of these reactions. The results show that the different ability of unsaturated aldehydes to effect biological systems is reflected to some extent by their affinity towards glutathione.

Material and Methods

Chemicals

The 4-hydroxyalkenals were prepared according to Esterbauer *et al.* [34]. Pentenal and hexenal were prepared from their diethylacetals [35] by acidification with 10% citric acid [34]. 4-Keto-pentenoicacid was prepared according to Hurd *et al.* [36]. 4-Ethoxypentenal was synthesized analogus to the procedure given in the literature for the preparation of 4-ethoxybutenal [37]. All other carbonyls were obtained from Schuchardt (München) or Merck (Darmstadt).

The forward reaction was measured as described previously [29].

Measurement of the equilibrium constant K and the rate constant k_2 for the reverse reaction

10 ml of a neutral solution of GSH was mixed with 5 ml of a neutral solution of the carbonyl and 5 ml 266 mM phosphate buffer pH 7.4 and allowed to equilibrate. The final concentration of GSH (a_0) was between 0.1 and 10 mM; the carbonyl concentration (b_0) was usually 2 times higher than GSH, except reaction 5 and 17 where carbonyl was in 20- and 10-fold excess resp. In the experiments 6 − 15 and 20 the reaction mixtures were degased with oxygen free N_2, whereas in the experiments with volatile carbonyls only the stock solutions of GSH and buffer were degased. In appropriate time intervals the GSH content of the reaction mixture was

estimated. From the constant end value (a) the equilibrium constant K was calculated according to: $K = a(b_0 - a_0 + a)/(a_0 - a)$. For GSH determination an aliquot of the reaction mixture was given into a 2 cm cell (for very low GSH-values 5 cm cells were used) and the unspecific absorbance of the sample was measured at 412 nm against phosphate buffer pH 7.4. Then to both cells a solution of 5,5′-dithio-bis-(-2-nitrobenzoic acid) = DTNB was added (0.2 ml 50 mM DTNB in 66 mM phosphate buffer pH 7.4 to 10 ml solution of adduct). The added DTNB reacts immediately with free GSH present in the mixture and then continuously with GSH formed by the reverse reaction. The reaction was allowed to proceed until a small percentage of the adduct was dissociated (depending on the half-live of the reverse reaction 0.5 – 5 hours). The increase of the absorbance was followed at 412 nm. The graph of absorbance *versus* time gives a straight line with an intercept on the ordinate. From the intercept, corrected by the unspecific absorbance, the GSH concentration (a) and the adduct concentration ($a_0 - a$) were calculated. The first order rate constant k_2 was calculated from the reaction rate v given by the slope of the curve: $k_2 = v/(a_0 - a)$. The adduct concentration was assumed to be constant during the reverse reaction; an example for k_2-determination is given in Fig. 2. It should be noticed that on complete equilibration of GSH and carbonyl attention was paid only for K determination, whereas k_2 determinations were carried out whether equilibrium was reached or not.

Results

The forward reaction

The reaction of GSH with α,β-unsaturated carbonyls leads to an equilibrium which may be described by Eqn (1)

$$GSH + R - CH = CH - COR$$
$$\underset{k_2}{\overset{k_1}{\rightleftharpoons}} R - CH(SG) - CH_2 - COR. \quad (1)$$
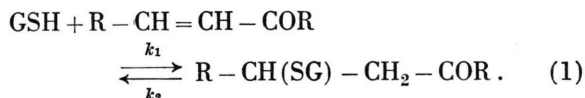
The forward reaction was found to follow second order kinetics. An example illustrating the independence of the second order rate constant k_1 from the concentrations of the reactants is given in Table I. The rate constants for the reactions of various α,β-unsaturated carbonyls with GSH are summarized in Table II and Table III.

The vairous carbonyls differ considerably in their reactivity towards GSH. A major factor governing

Table I. Rate constants for the reaction of GSH with 4-hydroxy-pentenal. Reaction were carried out in 66 mM phosphate buffer pH 7.4 at 20 °C. Concentration of GSH and aldehyde in the reaction mixture as indicated. The first two reactions were followed by measuring the decrease of the ultraviolet absorbance of the aldehyde ($\varepsilon_{222\,nm} = 13600$). The last three reactions were followed by measuring the decrease of GSH with DTNB.

GSH [mM]	Aldehyde [mM]	k_1 [mol^{-1} sec^{-1}]
0.1	0.1	2.08
0.3	0.1	2.30
0.1	0.3	2.19
0.2	0.2	2.17
0.1	2.0	2.20
Average ± standard deviation		2.19 ± 0.08

the reactivity is the polarisation of the double bond by conjugation with the carbonyl group. Due to the decreasing electron-withdrawing effect of the carbonyl group the reaction rate of analogous compounds decreases approx. five orders of magnitude in the sequence: aldehyde > ketone > ester > amide > carboxylate (Table III). Besides electronic also steric effects associated with alkyl substituents play an important role for the reactivity. Aldehydes and ketones with alkyl groups on the α- and/or β-carbon of the double bond are much less reactive than the unsubstituted compounds acrolein and methyl vinyl ketone. The replacement of one ethylen hydrogen by an alkyl group results approx. in a hundred fold decrease of reactivity. Consequently, if two alkyl residues are introduced in the $CH_2 = CH - CO$ grouping (*i. e.* citral, mesityl oxide) the reactivity falls by the factor 10^4 compared to acrolein or methyl vinyl ketone. An additional electron withdrawing group on the β-carbon leads to an increased polarisation of the double bond and therefore to an increased reactivity. This fact explains that 4-hydroxyalkenals react more rapidly than analogous 2-alkenals and that 4-keto pentenoic acid is highly reactive whereas pentenoic acid and also crotonic acid does not react with GSH at all. The slight differences of the reactivity within the series of 4-hydroxy-2-alkenals is difficult to explain, actually one would assume that the reactivity decreases with increasing chain length as it was found for 2-alkenals.

Finally we also investigated the question if GSH adds to the α,β-unsaturated lactonring in cardenolides and we found that strophanthin g (ouabain), strophanthidin g and digitoxin do not react with GSH in a measurable extent.

Table II. Rate- and equilibrium constants for reactions of GSH with conjugated carbonyls. All reactions were carried out in 66 mм phosphate buffer pH 7.4 at 20 ± 1 °C. In reactions 5, 12, 13 and 24 the medium contained 10% ethanol. The forward reaction was followed by measuring the decrease of GSH (with DTNB) or the carbonyl (spectrophotometrically at 222 nm). The initial concentrations of GSH and carbonyl were as follows (in mм): reaction 1 and 16: 0.05+0.05; reaction 2−4, 6−17, 15−20: 0.1+2.0; reaction 5: 0.1+1.0; reaction 14 and 19: 10+10; reaction 17: 5+5; reaction 18: 0.1+40; reaction 21 und 22: 0.2+8.0. The reverse reaction was followed by measuring the increase of absorbance at 412 nm after addition of 0.2 ml 50 mм DTNB to 10 ml adduct prepared by equilibrating GSH and carbonyl in the following initial concentrations (in mм): reaction 1 and 16: 0.1+0.2; reaction 2−4, 6−15, 19 and 20: 2.5+5.0; reaction 18 and 21: 10+20; reaction 5 (for K determination): 0.1+2.0; reaction 17 (for K determination): 1.0+1.0. The equilibrium constant K was estimated by measuring the equilibrium GSH concentration with DTNB after equilibrating GSH and carbonyl in concentrations as given for determinations of reverse reaction. All values are the average of at least three determinations and have a standard deviation of $5-10\%$ for k_1, $3-5\%$ for k_2, and $10-15\%$ for K.
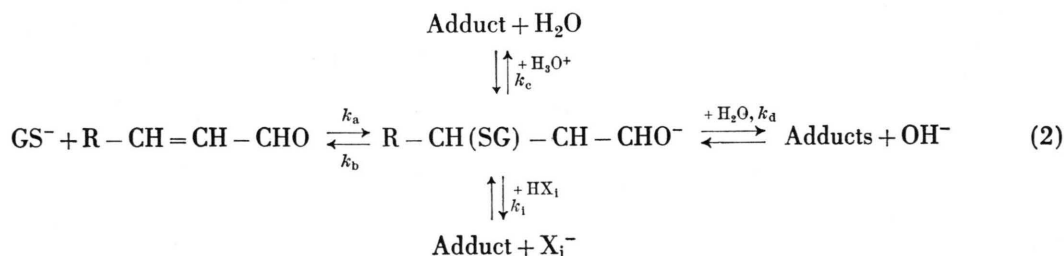
Carbonyl compound	Forward reaction GSH+Carb → Add k_1 [mol^{-1} sec^{-1}]	Reverse reaction Add → GSH+Carb k_2 [sec^{-1}]	Equilibrium (GSH) (Carb)/(Add) K [mol^{-1}]	% free GSH **
1. Acrolein	1.21×10^2	1.76×10^{-6}	1.40×10^{-8}	1.2
2. Crotonaldehyde	7.85×10^{-1}	3.01×10^{-5}	4.16×10^{-5}	46.8
3. 2-Pentenal	4.71×10^{-1}	3.44×10^{-5}	7.38×10^{-5}	56.0
4. 2-Hexenal	3.30×10^{-1}	4.70×10^{-5}	1.42×10^{-4} *	67.0
5. Citral	3.23×10^{-2}	5.00×10^{-6} *	1.63×10^{-4}	70.5
6. 4-Hydroxy-2-pentenal	2.19	5.60×10^{-7}	2.96×10^{-7}	5.2
7. 4-Hydroxy-2-hexenal	1.56	4.10×10^{-7}	2.63×10^{-7} *	5.0
8. 4-Hydroxy-2-heptenal	1.83	4.57×10^{-7}	2.50×10^{-7} *	4.9
9. 4-Hydroxy-2-octenal	1.74	7.49×10^{-7}	4.89×10^{-7}	6.7
10. 4-Hydroxy-2-nonenal	1.09	9.60×10^{-7}	8.85×10^{-7} *	8.5
11. 4-Hydroxy-2-decenal	1.96	9.50×10^{-7}	4.85×10^{-7} *	4.7
12. 4-Hydroxy-2-undecenal	1.47	9.36×10^{-7}	6.38×10^{-7}	7.7
13. 4-Hydroxy-2-dodecenal	2.44	1.26×10^{-6}	5.42×10^{-7} *	7.1
14. 4-Hydroxy-4-isopropyl-2-pentenal	3.57×10^{-2}	7.76×10^{-7}	2.31×10^{-5}	38.0
15. 4-Ethoxy-2-pentenal	1.83	1.75×10^{-5}	9.60×10^{-6}	26.4
16. Methyl vinylketone	3.19×10	1.19×10^{-7}	3.60×10^{-9}	0.6
17. 3-Methyl-3-buten-2-one	6.00×10^{-1}	9.00×10^{-3} *	1.50×10^{-2}	99
18. Mesityl oxide	2.30×10^{-3}	1.12×10^{-5}	4.80×10^{-3}	98
19. 2-Cyclohexen-1-one	3.36×10^{-1}	5.16×10^{-6}	1.53×10^{-5} *	32
20. 4-Keto-2-pentenoic acid	3.42	2.32×10^{-6}	6.07×10^{-7}	7.5
21. Ethyl-crotonate	3.10×10^{-3}	5.75×10^{-5}	1.65×10^{-2}	99
22. Crotonate, 2-Pentenoate	no measurable reaction			

* Calculated from the corresponding other two constants.
** Percentage of GSH in equilibrium if 0.1 mм GSH is allowed to react with 0.1 mм carbonyl, calculated by the K values.

Effect of pH and buffer on the forward reaction

Fig. 1 shows the effect of the H$^+$-concentration on the rate of the reaction of GSH with croton aldehyde. The pH rate profile is very similar to those found for other thiols [29] and confirms the reaction pathway as suggested by Eqn (2):

$$\text{Adduct} + \text{H}_2\text{O}$$

$$\Big\updownarrow \Big| {\scriptstyle + \text{H}_3\text{O}^+ \atop k_c}$$

$$\text{GS}^- + \text{R} - \text{CH} = \text{CH} - \text{CHO} \underset{k_b}{\overset{k_a}{\rightleftarrows}} \text{R} - \text{CH(SG)} - \text{CH} - \text{CHO}^- \overset{+\,\text{H}_2\text{O},\,k_d}{\underset{\longleftarrow}{\longrightarrow}} \text{Adducts} + \text{OH}^- \qquad (2)$$

$$\Big\updownarrow \Big| {\scriptstyle + \text{HX}_i \atop k_i}$$

$$\text{Adduct} + \text{X}_i^-$$

$$k_1 = k_a \cdot \alpha_{\text{SH}} \cdot \beta$$

$$\beta = \frac{k_c (\text{H}^+) + k_d (\text{H}_2\text{O}) + \ldots k_i (\text{HX}_i)}{k_b + k_c (\text{H}^+) + k_d (\text{H}_2\text{O}) + \ldots k_i (\text{HX}_i)} \qquad (3)$$

Table III. Rate constants for the reaction of GSH with acrylyl derivatives. Reactions were carried out in 66 mM phosphate buffer pH 7.4 at 20 °C, concentration of GSH and carbonyl in the reaction mixture as indicated; the reactions were followed by measuring the decrease of GSH with DTNB.

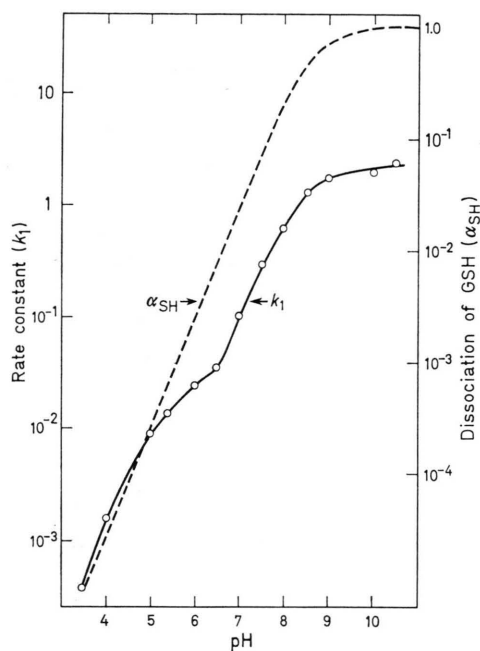|	Carbonyl [mM]	GSH [mM]	k_1 [mol^{-1} sec^{-1}]
Acrolein	0.05	0.05	121
Methyl vinylketone	0.05	0.05	31.9
Acrylic acid ethylester	10.0	0.5	0.243
Acrylic amide	10.0	1.0	0.013
Acrylic acid	200	10.0	0.001

Fig. 1. Effect of pH on the rate constant k_1 of the reaction of GSH with crotonal. The dotted line shows the dissociation degree of GSH (pK$_{SH}$=8.56). 1−5 ml solution of GSH (desired pH adjusted with HCl or NaOH) were added to solutions of crotonal (desired pH adjusted with HCl or NaOH) to give a final volume of 50 ml, final concentrations of GSH were 0.1 mM for reactions at pH 7.0 and above and 1.0 mM for reactions below pH 7.0, crotonal concentrations were 20 times higher than those of GSH. The pH value was maintained constant during the reaction according to the pH-stat principle with HCl or NaOH. The temperature was 20 ± 1 °C. The reactions were followed by measuring the decrease of GSH (with DTNB) on aliquots of the reaction mixture.

Depending on the pH the rate limiting step is either (pH <4.5)) the addition of the sulfhydryl anion ($k_1 = k_a \cdot \alpha_{SH}$, $k_b \ll k_c(H^+)$, $\beta = 1$) or (pH >6.0) the proton transfer reaction by which the intermediate RCH(SG)CHCHO$^-$ is finally stabilized

($k_1 = k_a \cdot \alpha_{SH} \cdot \beta$, $k_b > k_d(H_2O) \gg k_c(H^+)$, $\beta =$ constant < 1). It is evident from Eqn (3) that in the later case the rate of the over all reaction does not only depend on pH but also on concentration and type of buffers used, as the intermediate can than react simultanously with H_2O and all other potent proton donors (HX) present in the medium. In Table IV the catalytic effects of various buffers are

Table IV. Catalytic effects of buffers on the rate of the reaction of thiols with crotonal. All reactions were carried out at pH 7.4, 20 °C, the reaction mixture contained 0.1 mM thiol (GSH, cysteine, thioglycolic acid ethylester = TAE), 2.0 mM crotonal and buffers or salt as indicated. The reactions were followed by measuring the decrease of thiol with DTNB.

Thiol	Addition	k_1 [mol^{-1} sec^{-1}]
CySH	none	16.5
GSH	none	0.27
TAE	none	0.10
CySH	0.1 M phosphate	16.7
GSH	0.1 M phosphate	1.25
TAE	0.1 M phosphate	2.00
GSH	0.066 M Phosphate	0.78
GSH	0.1 M phosphate	1.25
GSH	0.1 M tris	0.83
GSH	0.1 M triethanolamine	0.53
GSH	0.1 M borate	0.66
GSH	0.1 M acetate	0.25
GSH	0.1 M formiate	0.27
GSH	0.1 M ammoniumchloride	0.48
GSH	0.5 M natriumchloride	0.28

summarized. At pH 7.4 the highest effect is shown by phosphate, while Tris, triethanolamine, borate and ammonium chloride are less effective. This is in agreement with Broensted catalysis law that the catalytic activity of a proton donor depends on its dissociation constant. With acetate and formiate only minimal amounts of the potent acid forms are present at pH 7.4, thus these compounds are without any effect. Finally NaCl is also without any effect even at high concentration, indicating that the rate increase caused by buffers is not due to the increased ionic strength.

The reaction rate of cysteine is not increased by buffers, presumably because the resonant intermediate formed is stabilized by a fast intramolecular proton transfer mediated by the NH$_3^+$ group of the cysteine residue. As a result the rate limiting step is the addition of CyS$^-$ and the reaction proceeds

with the maximum rate possible at a given pH ($k_1 = k_a \cdot a_{SH}$; $\beta = 1$). This also makes clear why cysteine (pK_{SH} 8.20) reacts about 100 times faster than GSH (pK_{SH} 8.56) or thioglycolic acid ethylester (pK_{SH} 8.15) although these thiols are quite similar in respect of pK_{SH} and therefore in a_{SH} and the nucleophilic strength (k_a) of the mercaptide ion [35].

The reverse reaction

The reversion of the reaction is forced by addition of excess DTNB to the adduct solution. DTNB has a higher affinity to GSH than the carbonyls and reacts thus with the equilibrium GSH shifting the equilibrium of Eqn (1) to the left until all the adduct is dissociated. Fig. 2 shows a typical
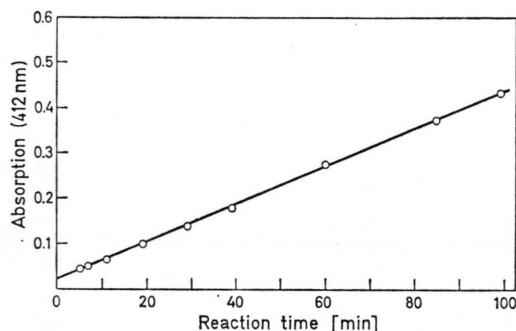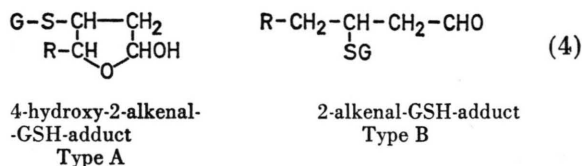

Fig. 2. Rate of decomposition of the adduct of GSH and 4-hydroxynonenal by the reverse reaction. The adduct solution was prepared by reacting (3.5 hours) of 2.5 mM GSH with 5.0 mM 4-hydroxynonenal in 66 mM phosphate buffer pH 7.4. The reaction mixture was degased with N_2. After 3.5 hours 0.2 ml DTNB (50 mM in phosphate pH 7.4) was added to 10 ml reaction mixture (= solution of adduct) and the reverse reaction was followed by measuring the increase of absorbance in a 2 cm cell at 412 nm at 20 °C. Phosphate buffer + DTNB was used as blank. The rate constant k_2 was calculated as follows: concentration of adduct = 2.5×10^{-3} M, rate $v = \Delta A/\Delta t \cdot 13600 \cdot 2 = 2.52 \times 10^{-4}$ mol sec^{-1}; $k_2 = v/$ (adduct) $= 10 \times 10^{-7}$ sec^{-1}.

experiment for measuring the rate of the reverse reaction. For crotonaldehyde adduct the rate constant was also examined as a function of adduct-, DTNB- and crotonaldehyde concentration. The reaction was found to be of first order. The concentration of DTNB has no influence on k_2 values, thus one can assume that the "indicator reaction" does not affect the rate of the reverse reaction. When p-chlormercuribenzoate was used instead of DTNB, we found that the rate increases with the reagent concentration. The α,β-unsaturated carbonyl can interfere by reacting with the DTNB-anion, probably in a Michael type addition. The tolerable limit con-

centration which exerts no disturbing effect varies with the carbonyl and was estimated for each in a preliminary experiment as reported previously [29].

The rate constants for the various reverse reactions are summarized in Table II. The rate of the reverse reaction increases by the factor 10^4 in the order: methyl vinyl-ketone > 4-hydroxyalkenals > acrolein > 4-ketopentenoic acid > citral > cyclohexanone > mesityl oxide > higher 2-alkenales > crotonic acid ethylester > 3-methyl butenone. There seems to be no general predictable relation between k_1 and k_2, nevertheless in many cases a high rate for the forward reaction means a slow reverse reaction. Within the homologous series of 2-alkenal- or 4-hydroxy-2-alkenal-adducts the rate of the reverse reaction increases roughly with the chain length. Hydroxy-alkenal-adducts reverse about 100 times slower than the homologous alkenal-adducts. This remarkable difference is due to the fact that the latter exist in the open chain form B, while the former exist in the cyclic hemi-acetal form A with les than 1% free aldehyde present in the oxo-cyclo-tautomerism-equilibrium [29, 32]. Since the reactive species for the reverse reaction is the aldehydoform (Eqn (2)), adducts of type B decompose much faster than those of type A. This is also clearly manifested by the 4-ethoxy-pentenal-adduct, which can not cyclisize and therefore behaves like 2-pentenal and not like 4-hydroxy-pentenal.

$$
\begin{array}{ccc}
\underset{\substack{\text{4-hydroxy-2-alkenal-}\\\text{-GSH-adduct}\\\text{Type A}}}{
\begin{array}{c}
\text{G-S-CH——CH}_2\\
\;\;\;|\quad\quad\;\;\;|\\
\text{R-CH} \quad \text{CHOH}\\
\quad\;\;\backslash_{\;\;}O_{\;\;}/
\end{array}}
& \quad &
\underset{\substack{\text{2-alkenal-GSH-adduct}\\\text{Type B}}}{
\begin{array}{c}
\text{R-CH}_2\text{-CH-CH}_2\text{-CHO}\\
\qquad\;\;\;|\\
\qquad\;\;\text{SG}
\end{array}} \quad (4)
\end{array}
$$

The equilibrium

The equilibrium constants for the various reactions are summarized in Table II. The experimental constants generally agree well the constants calculated from k_1 and k_2, indicating the validity of the experimental methods. From the physiological and biochemical view it may often be useful to know the equilibrium concentration of GSH in the presence of a distinct concentration of α,β-unsaturated carbonyl. The answer to this question is given for some selected carbonyls in Fig. 3.

From this figure one can predict that for instance at a concentration of 10^{-5} M only acrolein, methyl vinylketone and hydroxyalkenals can inactivate
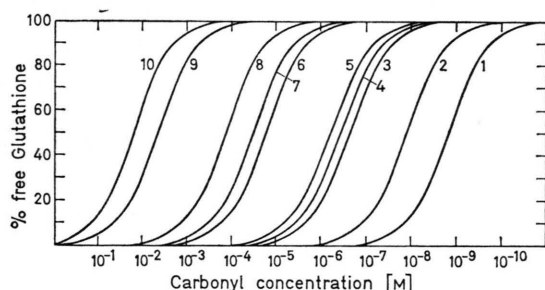
Fig. 3. Percentage of free GSH present in equilibrium as function of the concentration of carbonyls. The curves were calculated on the basis of the equilibrium constants listed in Table II. 1, methyl vinylketone; 2, acrolein; 3, hydroxypentenal; 4, hydroxyoctenal; 5, hydroxyundecenal; 6, cyclohexenone; 7, crotonal; 8, hexenal and citral; 9, mesityl oxide; 10, ethyl crotonate and 3-methylbuten-2-one.

Table V. Classification of conjugated carbonyls according to their reactivity with GSH. The half lives $t_{1/2}$ and concentration data were calculated from the constants in Table II.

Carbonyl compound	Adduct formation $t_{1/2}$ *	mM **	Stability of adducts $t_{1/2}$ ***
Group 1			
Acrolein	1.4 min	0.05	4.6 d, very stable
Methyl vinylketone	5.2 min	0.05	60.7 d, very stable
Group 2			
4-Keto-pentenoic acid	0.8 h	0.15	3.4 d, very stable
4-hydroxy-2-alkenals	1.1—2.5 h	0.2—0.4	6.3—19.2 d, very stable
2-cyclohexen-1-on	8.2 h	1.1	1.5 d, stable
Group 3			
Crotonal, Pentenal, Hexenal	3.6—8.5 d	0.6—1.1	4.0—6.3 h, unstable
Citral	3.6 d	12	1.6 d, stable
3-Methyl-3-buten-2-one	4.6 h	15	1.2 min, unstable
Ethyl crotonate	37 d	122	3.3 h, unstable
Mesityl oxide	50 d	166	17 h, stable
Acrylic acid	115 d	380	—
Crotonic acid	>115 d	>380	—

* Half live for reactions of 0.1 mM GSH with 0.1 mM carbonyl.
** Carbonyl concentration necessary to produce a 50% loss of 0.1 mM GSH within 30 min.
*** Half live for the reverse reaction.

more than 95% of GSH, while the other carbonyl are much less effective.

Discussion

The estimated equilibrium constants and rate constants for reactions of GSH with α,β-unsaturated carbonyls (Table II) demonstrate that the various carbonyls differ markedly in their tendency to react with SH groups. One may distinguish three groups of carbonyls characterized by the following parameters: the stability of the adducts (rate of reverse reaction), the rate of adduct formation, and the concentration necessary to produce the same loss of SH in a given time (Table IV). The latter parameter considers not only the importance of the reaction rate, but also the SH/carbonyl ratio and the equilibrium and appears to be particularly useful for the evaluation of the relative biological and biochemical activity of conjugated carbonyls. For example, the concentration data in Table V reflect well the toxicity of conjugated carbonyls against microorganism reported by Stack [15].

Group 1 includes the most reactive carbonyls acrolein and methyl vinylketone. Most attention was paid to the toxicological, biological and biochemical effcets of acrolein [8, 10, 11, 21, 23, 24, 27, 28, 38, 39]. One may assume that the high cytotoxicity of acrolein towards all living organism results from the fact that even relatively low concentrations lead to a rapid inactivation of functional SH groups and that the inhibition cannot be reversed in considerable time because the dissociation of the acrolein-SH-adduct would take several days. From the dissocia-

tion curve in Fig. 3 it is evident that in the presence of 1.4×10^{-8} M acrolein only 50% of the GSH can be free in the equilibrium. In this respect it is of interest to note that for the atmosphere and drinking water a maximum permissible concentration of approx. 2×10^{-9} M (0.043 – 0.013 ppm) and 1.8×10^{-7} M is recommended [40]. In view of our results the latter concentration appears however too high.

Group 2 includes the series of 4-hydroxyalkenals, 4-keto pentenoic acid and cyclohexenone. Of particular interest are the hydroxyalkenals as they possess anticancer activity [4−7, 9]. These aldehydes react about 100 times slower with GSH than acrolein. The adducts, however, are even more stable than those of acrolein as they nearly entirely exist in the form of cyclic hemiacetals (Eqn (4)) thus the reverse reaction would take several weeks. A number of ketones with one substituent on the β-carbon like 2-cyclohexen-1-one were reported to possess fungistatic activity and it was suggested that their antifungal activity is connected with their ability to combine with SH groups [13, 18]. No consistant rela-

tionship was found [18] between toxicity towards fungi and the chemical reactivity with cysteine, probably because besides the reaction rate the stability of the formed adducts plays also an important role.

Group 3 embraces very different conjugated carbonyls which have in common that they either combine very slowly with SH groups or form very labile adducts. Crotonal and higher 2-alkenals indeed inactivate SH groups with relative high rate, the inactivation, however, will last only as long as sufficient excess of the aldehyde is present. If the aldehyde is removed in some way or other (dialysis, washing of preincubated cells, metabolism etc.) the effect can easily be reversed as the labile adduct may dissociate in a few hours librating the original SH group. All other carbonyls listed in group 3 react only under extreme conditions *i. e.* long reaction time or high concentration with SH groups. In *in vivo* experiments such extreme conditions are hardly to be realized thus *in vivo* effects (*e. g.* anti-tumoral activity of citral [2]) of those compounds are likely not caused by inactivation of SH groups.

The reaction of thiols with conjugated carbonyls involves the addition of the nucleophile RS^- to the β-carbon of the double bond followed by a proton transfer reaction (Eqn (3)). For a given reaction the observed rate constants and the equilibrium constant depend on pH, pK_{SH}, type and concentration of buffer. The quantitative relationship of these

parameters was already discussed in detail in a previous paper and, as far as GSH is concerned, under "results". Only two conclusions of more general significance should be noticed here:

a. The reactive species is RS^-, thus the rate of a given reaction increases with pH (approx. parallel with a_{SH}), approaching a maximum at a pH were the SH group is completely ionized. This explains for example that incubation of the SH-enzyme l-alparaginase with acrolein at pH 8.4 results in a more marked inhibition than at pH 5.0 [39].

b. The reaction rate is accelerated by buffers, if the particular thiol does not contain a proton donating group neighbouring the SH group (*e. g.* GSH, thioglycolic acid ethylester). On the other hand thiols with a proton donor near the SH group (*e. g.* cysteine) react in buffer free medium very much faster than SH compounds of the other type, due to an intramolecular proton transfer. The catalytic effects of buffers must be considered when testing enzymes catalyzing the addition of GSH to conjugated carbonyls [41]. The intramolecular proton transfer may also play a significant role in proteins as in this way some SH groups may be greatly activated by neighbouring NH_3^+-groups.

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