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THE REACTION OF SULFHYDRYL GROUPS WITH CARBONYL COMPOUNDS

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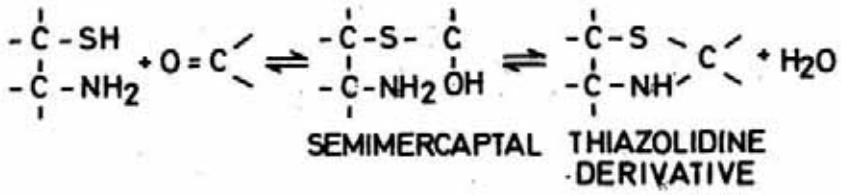
The sulfhydryl groups of L-cysteine and reduced glutathione (GSH) react nonenzymatically with formaldehyde (F), acrolein (Al), acetaldehyde (AA), malondialdehyde (DAM), pyruvate (P), oxoglutarate (oxo-G) and glucose (G) to form thiazolidine derivatives. These reactions show different velocities and the adducts formed show different stabilities.

The equilibrium constants K, as well as the rate constants k_r for the reverse reaction, show considerable variation. The carbonyls reveal higher reactivity with sulfhydryl group of L-Cys than with those of GSH, and the stability of the adducts is higher than that of GSH. Al, F and AA react more rapidly with both thiol compounds than the other carbonyls, but the adducts are less stable.

The sulfhydryl groups level of bovine serum albumin as well as those of high- and low-molecular thiols of human plasma is reduced in the presence of Al, F or DAM.

In our previous study [1] we proved that pyruvate, being a product of cysteine metabolism, reacted with the excess of cysteine and the resulting thiosemiacetal underwent cyclization to a thiazolidine derivative, 2-methyl-2,4--thiazolidinedicarboxylic acid.

This observation focused our attention on nonenzymatic reactions of thiols with carbonyl compounds (cf. Scheme 1).



Scheme 1

Although such reactions have been described by organic chemists long ago [2 - 5], their biological occurrence and significance are still poorly understood. For strongly nucleophilic sulfhydryl groups, carbonyl groups represent important highly reactive electrophils. Hence, the above-mentioned reactions seem to be of considerable interest and significance for the processes in which thiol- and carbonyl-compounds reveal biological activity and toxic effects. Therefore, we have studied nonenzymatic reactions of aldehydes: formaldehyde, acetaldehyde and acrolein, toxic substances most frequently encountered by man, since they represent the most ubiquitous industrial pollutes. Formaldehyde is emitted to the atmosphere which surrounds us by various glues, paints, plywood and resins [6] but it can be also generated as an endogenous metabolite. Acetaldehyde is the main product of ethanol metabolism, responsible for the toxic properties of alcohol [7], and together with acrolein occurs in tobacco smoke [8]. Acrolein can be regarded as an endogenous metabolite of spermine and spermidine [9] and, as suggested by Rees & Tarlow [10], is a product of allyl alcohol conversion, reponsible for its toxicity. Biological action and possible toxic effects of carbonyl compounds are dependent on their affinity for sulfhydryl groups, and on the stability of the adducts formed.

The aim of this study was to characterize the nonenzymatic reactions of cysteine and reduced glutathione [1] with toxic aldehydes and, for comparative purposes, with normal intracellular metabolites such as pyruvate, oxoglutarate, glucose and malondialdehyde.

MATERIAL AND METHODS

Chemicals. L-Cysteine and 5,5′-dithio-bis(2-nitrobenzoic acid) were purchased from Sigma Chemical Company. Oxoglutaric acid and malonylaldehyde bis (diethylacetal) were from E. Merck, Darmstadt, and reduced glutathione from Boehringer, Mannheim. Acetaldehyde was a product of International Enzymes Ltd (Windsor, Berkshire, England). Formaldehyde, acrolein, sodium pyruvate and glucose were obtained from POCh (Gliwice, Poland), and bovine serum albumin from Wytwórnia Surowic i Szczepionek (Kraków, Poland).

Determination of the equilibrium constant K. Thiosemiacetal was synthesized in 0.1 M phosphate buffer, pH 7.3, at the initial concentration of carbonyl compounds (a_0) of 0.1 - 1 mM (0.1; 0.3; 0.5; 0.7; 1.0 mM) and 10 times higher concentration of thiol compounds (b_0), L-Cys or GSH. The reaction mixture was fluxed with N_2, whereas in the experiments with volatile

[1] Abbreviations used: GSH, reduced glutathione; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); Al, acrolein; AA, acetaldehyde; F, formaldehyde; oxo-G, 2-oxoglutarate; P, pyruvate; G, glucose; DAM, malondialdehyde.

carbonyl only the stock solution of L-Cys or GSH and buffer were fluxed. At appropriate time intervals 1 ml samples were withdrawn from the reaction mixture (total volume 20 ml) and the content of carbonyl compounds was determined. The reaction was continued until the carbonyl concentration in the reaction mixture reached constant end values (a). The equilibrium constant was calculated according to the equation:

$$K = \frac{a_0 - a}{a\,(b_0 - a_0 + a)}.$$

The concentration of 2-oxoglutarate was determined by the method of Friedeman & Haugen [11], slightly modified when applied to other carbonyl compounds. In this case the reaction in 1 ml samples of the reaction mixture was terminated by adding 0.5 ml of 3 mM 2,4-dinitrophenylhydrazine in 1.5 M HCl. The condensation reaction product, 2,4-dinitrophenylhydrazone, was extracted to 3 ml of toluene. Then, 2 ml of 2.5% alcoholic KOH was added to 1.5 ml of the toluene extract. After 10 min the absorbance at 520 nm was measured.

The course of the reaction between glucose and L-cysteine or GSH was followed at the initial thiol concentration (a_0) of 0.1 - 1 mM and 10 times higher glucose concentration (b_0) in 0.1 M phosphate buffer, pH 7.3, in a final volume of 20 ml. The reaction mixture was fluxed with N_2 and thiol concentration (a) was estimated according to Ellman [12]. The solution of DTNB (0.1 ml of 5 mM DTNB in 0.1 M phosphate buffer, pH 7.4) was added to 2 ml of the reaction mixture, and the absorbance at 412 nm was measured.

Determination of the rate constant k_r for the reverse reaction. The adduct was prepared from L-cysteine (2.5 mM) or GSH (0.25 mM) and carbonyl compounds (5 mM) in 0.1 M phosphate buffer, pH 7.3, in a final volume of 2.5 ml. The reaction mixture was streamed with N_2. After 3 h incubation at 20°C, 0.1 ml of 25 mM DTNB was added. In the presence of DTNB, due to its high affinity towards thiols, the equilibrium of the reaction was shifted to the left and the carbonyl-thiol adducts were decomposed with a simultaneous increase in absorbance at 412 nm. Thus, the reverse reaction was followed by measuring this increase in absorbance at 412 nm at 20°C, with phosphate buffer + DTNB as a blank. The concentration of thiol (a) and that of the adduct ($a_0 - a$) were calculated. The rate constants k_r were calculated according to Esterbauer *et al.* [13], using the corrected absorption coefficient for DTNB [14]:

$$V = \frac{A}{t \times 14\,150} \times \text{mol} \times \text{s}^{-1}; \qquad k_r = \frac{V}{[\text{adduct}]}.$$

Assay of "non-protein" and "total" sulfhydryl groups in human plasma. Thiols were determined according to Yocelyn [15]. The reaction mixture for non-protein SH estimation contained: 1.5 ml of 0.05 M phosphate buffer, pH 6.8, 0.3 ml of human plasma and 1 ml of aldehyde solution at final concentrations from 2.5 mM to 50 mM. After 60 min incubation at 20°C, 0.5 ml of 1 mM DTNB in 0.2 M phosphate buffer, pH 6.8, was added. The solution was stirred and absorbance at 412 nm was determined within 1 - 3 min. The reaction mixtures for total SH estimation (non-protein + protein SH) contained: 1.5 ml of 0.05 M phosphate buffer, pH 7.6, 0.3 ml of human plasma and 1 ml of aldehyde solution at final concentrations from 2.5 mM to 50 mM. After 60 min incubation at 20°C, 0.5 ml of 10 mM DTNB in 0.2 M phosphate buffer, pH 7.6, was added. The solution was stirred and absorbance at 412 nm was determined after 60 min. The absorbance due to turbidity of the protein solution was subtracted. The value for protein SH was estimated as the difference between "total" and "non-protein" SH. The content of sulfhydryl groups in bovine serum albumin was assayed according to Ghiggeri *et al.* [16].

RESULTS

The nonenzymatic reactions of L-cysteine and reduced glutathione with toxic aldehydes and normal cellular metabolites proceeded at different rates. Figure 1A and B illustrate the diminishing content of carbonyl during the reaction with GSH and L-cysteine, respectively. The effect

Table 1

Equilibrium constants for the reactions of reduced glutathione and L-cysteine with carbonyl compounds

The reactions were carried out in 0.1 M phosphate buffer, pH 7.3, at 20±1 C. Initial concentrations of carbonyls were: 0.1, 0.3, 0.5, 0.7 and 1 mM, with ten times higher concentrations of thiol compounds. For details see Material and Methods. All values are the average of at least five determinations at each of the five concentrations applied. ±S.D. For determination of the K value the concentration of carbonyl was measured, except for the reaction with glucose in which the concentration of thiols was determined

Carbonyl compound	Equilibrium constant K (mol^{-1})	
	L-cysteine	reduced glutathione
Acetaldehyde	0.43 ±0.02	0.02 ±0.002
Acrolein	5.40 ±0.43	0.53 ±0.05
Formaldehyde	0.89 ±0.09	0.36 ±0.03
Malondialdehyde	0.32 ±0.05	0.25 ±0.02
Pyruvate	0.042±0.004	0.011±0.001
2-Oxoglutarate	0.027±0.002	0.008±0.001
Glucose	0.014±0.001	0.005±0.0004

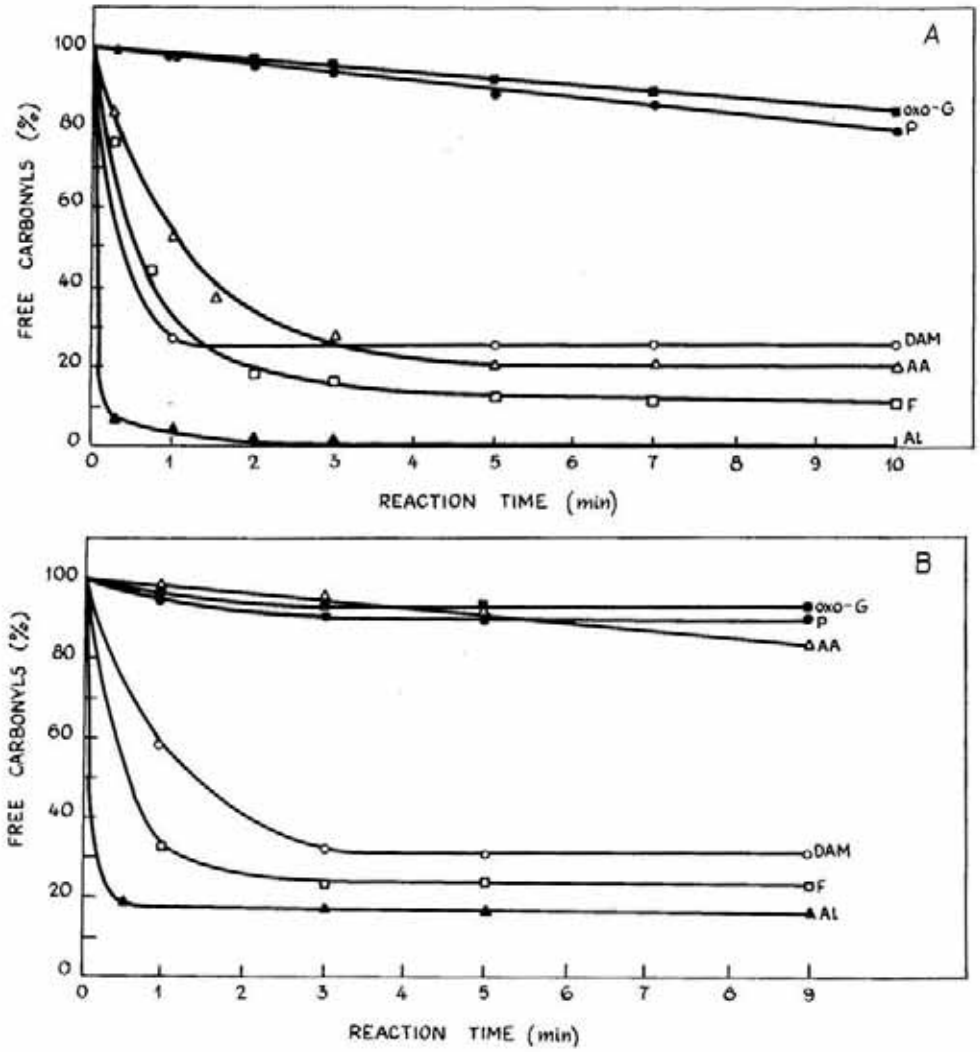


Fig. 1. Time course of binding SH groups of cysteine (A) and reduced glutathione (B) with carbonyl compounds: oxoglutaric acid (■), pyruvate (●), malondialdehyde (○), acetaldehyde (△), formaldehyde (□), and acrolein (▲). Concentration of thiols and carbonyls was 10 and 1 mM, respectively. For details see Material and Methods.

was most pronounced with toxic aldehydes. Consistently, it appears from the values of equilibrium constants (Table 1) that carbonyl compounds show different affinities for thiol groups: the highest values were observed with toxic aldehydes, particularly with unsaturated acrolein. Formaldehyde turned out to be more reactive than acetaldehyde. All the tested carbonyl compounds showed higher reactivity with sulfhydryl group of L-cysteine than with that of glutathione.

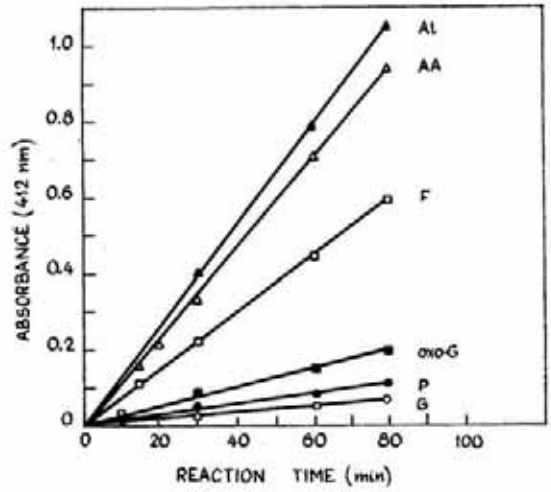


Fig. 2. Rate of decomposition of L-cysteine-carbonyl adducts in the presence of DTNB. Legend as in Fig. 1. For details see Material and Methods.

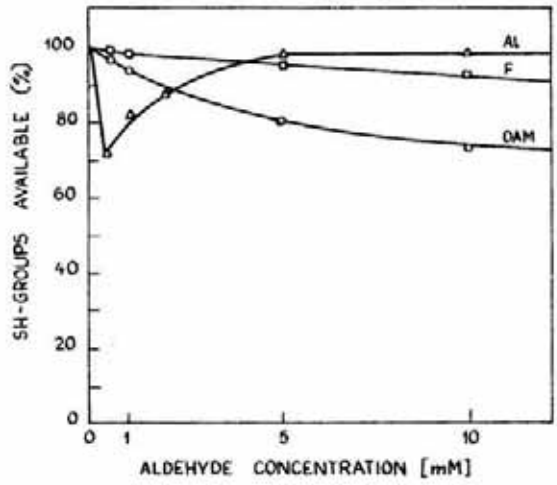


Fig. 3. Effect of concentration of carbonyl compounds on the content of free SH groups of bovine serum albumin. △, Acrolein; □, formaldehyde; ○, malondialdehyde.

In order to determine the degradation rate of individual adducts, the reverse reaction was forced by introducing DTNB, since Ellman's reagent shows higher affinity for sulfhydryl groups than aldehydes [13]. Figure 2 presents the course of degradation of L-cysteine adducts in the presence of DTNB. Determination of the rate constants k_r for the reverse reactions enables a comparison of relative stabilities of these compounds. As can be seen from Table 2, adducts of L-cysteine were decomposed with lower rate constants, i.e. they showed higher stabilities than those

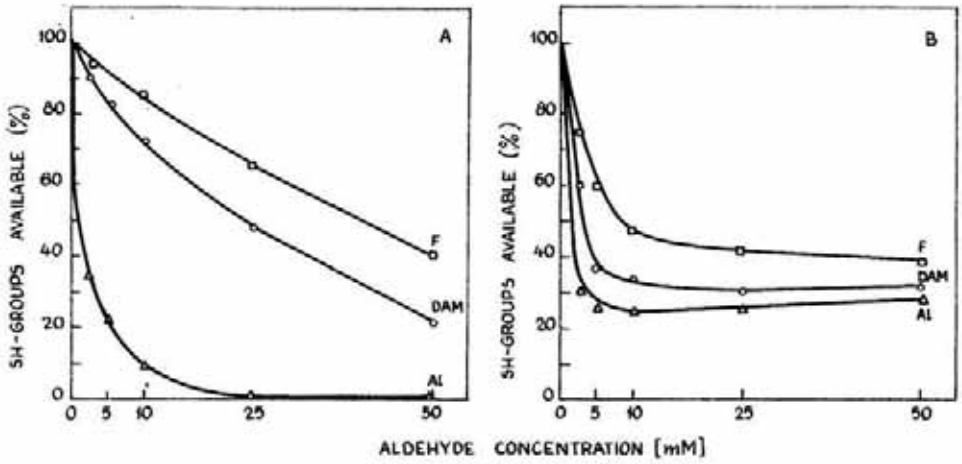


Fig. 4. Effect of concentration of carbonyls on the content of protein (A) and non-protein (B) SH groups of human plasma. □, Formaldehyde; ○, malondialdehyde; △, acrolein.

of GSH. The only exception was acrolein which formed with GSH an extraordinarily stable adduct, and no reverse reaction could be observed under our experimental conditions. The adducts of both thiols with intra-

Table 2

Rate contants for the reverse reaction of L-*cysteine and reduced glutathione adducts with carbonyls*

All values are the average of at least five determinations ±S.D. For details see Material and Methods; n.d., not detectable.

Carbonyl compound	$10^{-6} \times k$, values (s^{-1}) of adduct decomposition of	
	L-cysteine	reduced glutathione
Acetaldehyde	5.0 ±0.45	11.0±0.9
Acrolein	6.5 ±0.58	n.d.
Formaldehyde	3.4 ±0.28	24.0±2.1
Malondialdehyde	0.70±0.065	15.0±1.3
Puryvate	0.77±0.058	5.6±0.39
2-Oxoglutarate	1.40±0.112	7.0±0.49
Glucose	0.45±0.039	4.5±0.41

cellular metabolites: glucose, DAM, pyruvate or oxoglutarate were more stable than those with toxic aldehydes.

Bovine albumin served as a model thiol macromolecule for studies on the aldehyde binding to sulfhydryl groups of protein. As shown in Fig. 3, a decrease in the amount of sulfhydryl groups in albumin was

induced by formaldehyde, DAM and, most markedly, by acrolein, albeit only at low concentration. These aldehydes led also to disappearance of detectable sulfhydryl groups, both of low and high molecular weight compounds present in human plasma (Fig. 4). A coloured product of the reaction of acetaldehyde with protein unabled the use of the DTNB method in the investigation of the acetaldehyde-induced blocking of sulfhydryl groups in proteins.

DISCUSSION

The mechanism of L-cysteine reactions with aldehydes and the kinetics of subsequent formation of both Schiff bases and thiosemiacetals were extensively investigated by Kallen [17, 18], Lienhard & Jencks [19] and Gilbert & Jencks [20]. In our study we attempted to shed light on the biological significance of these reactions. We demonstrated that various carbonyl compounds exhibited at physiological pH a structure-dependent reactivity with sulfhydryl groups. All compounds studied (Table 1) showed higher equilibrium constants of the reactions with the sulfhydryl group of L--cysteine than with that of glutathione. This is probably due to a close vicinity of the amino groups in the former compound and its possible cyclization to a thiazolidine derivative with the equilibrium shift to the right. Toxic aldehydes showed a higher reactivity than the other carbonyl compounds tested. This explains their ability to lower the level of free sulfhydryl groups in tissues [21, 22] and the protective effect of thiol compounds in aldehyde intoxication [23, 24, 25]. The observed very high activity of acrolein towards sulfhydryl groups corresponds with the known cytotoxicity of that aldehyde [26] which blocks biologically important SH groups. As it appears from the data summarized in Table 1, the carbonyl compounds which are normal cellular metabolites, i.e. pyruvate, 2-oxoglutarate, glucose and DAM, are less active towards both glutathione and L-cysteine; their adducts, however, are more stable (Table 2). Higher stability is also characteristic for all adducts of L-cysteine, with the exception of acrolein which forms a particularly stable adduct with glutathione.

As shown in Figs. 3 and 4, aldehydes lower the level of free sulfhydryl groups in albumin and blood serum. This explains the formation of stable bonds between acetaldehyde and albumin, inhibited by L-cysteine and glutathione (Donohue *et al.* [27]).

Interest in aldehydes as potential carcinostatic compounds stems from the hypothesis of Egyiid & Szent-Györgi [28] and Gregg [29] who postulated that glyoxal influenced cell division by reacting with sulfhydryl groups which played a significant role in this process. It seems that this hypothesis could be considerably extended since their reaction with sulfhydryl groups

is no longer attributed only to glyoxal but also to a number of endogenous and exogenous carbonyl compounds.

Formation of thiosemiacetals capable of cyclization to thiazolidine derivatives can constitute a possible mechanism of catabolism and detoxication of both endogenous and exogenous aldehydes. The reactions between thiol and carbonyl groups may control the level of free SH groups in the cell. The lifetime of these products depends mainly on their hydrolytic degradation. If a thiol or a carbonyl of higher reactivity than that of the compound which is in equilibrium with the thiazolidine derivative is introduced the hydrolysis of the latter is accelerated and a new derivative is formed. These reactions are probably of considerable importance as it has recently been found that thiazolidine derivatives are natural cell metabolites [31, 32]. Besides, the adduct of cysteine and formaldehyde, thiazolidine-4-carboxylic acid, has found therapeutic application as a hepatoprotective [33, 34] and cancerostatic [35, 36] drug.

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