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Short Communication

Reactivity of chloronitrobenzenes towards glutathione under physiological conditions: the relationship between structure and reaction rate*

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Introduction

The drug-metabolizing enzyme glutathione transferase exists as a family of about a dozen different isoenzymes. These isoenzymes have different amino-acid sequences and possess differing catalytic abilities [1]. A good general substrate for spectroscopic assay of enzyme activity is 1-chloro-2,4-dinitrobenzene (CDNB) which is conjugated by the enzyme to glutathione (GSH), an endogenous thiol [2]. To discover new selective substrates, i.e. substrates which are catalysed by some isoenzymes but not others, an extensive series of structural analogues of CDNB has been examined in order to obtain their non-enzymic, as well as their enzymic, rates of reaction with GSH. This short communication reports on the non-enzymic reactivity of a representative selection of six chloronitrobenzenes, which have been chosen to cover the full range of chemical reactivity exhibited by the larger series.

The conjugation reaction catalysed by glutathione transferase is shown in Fig. 1. Glutathione (GSH) is an endogenous cysteine-containing tripeptide having a thiol group (—SH) as part of its structure which reacts with CDNB in a nucleophilic aromatic substitution reaction. Under physiological conditions, and in the absence of enzyme, this reaction between CDNB and GSH takes place slowly. As part of our enzyme studies, it was necessary to obtain these corresponding non-enzymic rates of reaction given by the series of analogues of CDNB. This communication reports the general procedures used to obtain these non-enzymic rates. The chloronitrobenzenes were chosen on the basis of their structural relationships to CDNB and not initially on their non-enzymic reactivity. The non-enzymic results obtained from the selected analogues have been analysed to give a very good quantitative structure–activity relationship.

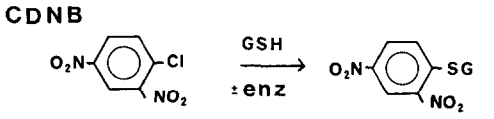

Figure 1
The reaction of CDNB (**4**) with GSH. This reaction occurs in the absence of enzyme, and is catalysed by glutathione transferases.

Experimental

CDNB analogues

1-Chloro-4-nitrobenzene (**1**) and 4-chloro-3-nitrobenzotrifluoride (**3**) were purchased from Aldrich. 1,2-Dichloro-4-nitrobenzene (DCNB; **2**) and 1-chloro-2,4-dinitrobenzene (CDNB; **4**) were obtained from BDH Ltd (UK) and recrystallized from toluene and 95% ethanol, respectively. Ethyl 4-chloro-3,5-dinitrobenzo-

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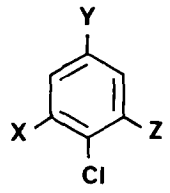
ate (**5**) was the kind gift of Dr A.T. Peters, Chemistry Department, University of Bradford, and 4-chloro-3,5-dinitrobenzonitrile (**6**) originated from PCR Inc. (Gainesville, FL, USA). Figure 2 shows the structures.



cmp.	X	Y	Z
1	H	NO_2	H
2	Cl	NO_2	H
3	NO_2	CF_3	H
4	NO_2	NO_2	H
5	NO_2	CO_2Et	NO_2
6	NO_2	CN	NO_2

Figure 2
The chemical structures of the chloronitrobenzenes.

UV spectrum of glutathione conjugates

For each CDNB analogue it was important to obtain the UV–vis spectrum of the final product, the glutathione conjugate. This product was prepared *in situ* by taking the slow reaction of the CDNB analogue with GSH to completion. This was accomplished by using some or all of the following rate-enhancing procedures: (a) using excess GSH, (b) by addition of glutathione transferase to catalyse the reaction, (c) by working with buffer of pH 7.5 rather than 6.5, which increases the non-enzymic rate 10-fold, (d) by using moderately elevated temperature (37°C instead of 20°C) and extended time of reaction (e.g. overnight).

Choice of analytical wavelength

A comparison of the spectrum of the glutathione conjugate with that of the starting chloronitrobenzene allowed a suitable single wavelength to be chosen for analytical use. This wavelength was usually the wavelength at which maximum absorbance change was seen, was similar to the position of maximum absorbance of the product, and was at longer wavelength than the maximum absorbance of the starting chloronitrobenzene. The GSH conjugate of CDNB is measured at 340 nm. Accurate absorbance measurements of known concentrations of the starting CDNB analogue and of the resulting product allowed the molar absorbance change, ΔE_M, to be obtained for each compound. This value was necessary to convert rates of reaction, obtained experimentally in terms of changes in absorbance with time ($\Delta A \text{min}^{-1}$) into absolute rates of product formation (mol l^{-1} min^{-1}).

Kinetic studies

In previous studies with CDNB, in which the concentrations of CDNB and GSH were varied extensively, the following bimolecular rate equation for the conjugation reaction was established:

$$v = k_2 \text{ [cmpd] [GSH]},$$

where v is the initial rate of reaction, k_2 is the second order rate constant, [cmpd] is the initial concentration of the CDNB analogue, and [GSH] is the initial concentration of GSH.

In this study, initial rates of reaction were obtained in phosphate buffer (0.1 M, pH 6.5 or pH 7.5, depending on the reactivity of the compound) at 25°C. These rates were recorded for 0–15 min, to ensure that linearity of absorbance change was observed. The concentrations of the CDNB analogue and GSH were generally both 1.0 mM, following usual enzyme assay conditions. Values of k_2 (in mol^{-1} l min^{-1}) were calculated for each analogue using the rate equation shown above.

Structure–reactivity correlation

Hammett σ substituent constants, which provide a quantitative measure the electron-withdrawing properties of the various substituents, came from standard tables [3]. The normal *meta-* and *para*-Hammett σ values were used for substituents in these positions; *para*-substituent values were also used for *ortho*-substituents. These values, except for the value of the Cl— or F— group being displaced, were arithmetrically combined to give the overall total value ($\sum\sigma$) for the molecule.

Results

A study of chloronitrobenzene analogues of CDNB was undertaken to establish whether a particular analogue could react with GSH under physiological conditions, and therefore whether it was a potential substrate for glutathione transferase. In most cases standard conditions could be found to give a slow non-enzymic rate of reaction, which then enabled one to observe whether addition of enzyme catalysed the reaction.

For the present purposes the slow non-enzymic rate of reaction (measured as the absorbance change min^{-1}) for each analogue was converted into a bimolecular rate constant (k_2) by taking into account the corresponding

Table 1
Rates of reaction of CDNB analogues with GSH. [All experiments were performed in sodium phosphate buffer, (0.1 M at 25°C, 3 ml) at either pH 6.5 or pH 7.5]

Compound	Compound concentration (mM)	GSH concentration (mM)	pH	Wave-length (nm)	Change in molar absorbance ΔE_M	Non-enzymic rate ΔA/min	Rate constant k_2 (mol^{-1} l min^{-1})	Relative rate (%) CDNB = 100‡	Log_{10} k_2 at pH 7.5§	Σσ‖
1 1-Chloro-4-nitrobenzene*	0.1	10.0	7.5	340	9000*	<0.0001	<0.001	<0.1	<−3.0	0.78
2 1,2-Dichloro-4-nitrobenzene (DCNB)	1.0	5.0	7.5	345	8500	0.0005	0.012	0.32	−1.92	1.01
3 4-Chloro-3-nitrobenzotrifluoride†	1.0	5.0	7.5	370	6700	0.007	0.21	5.5	−0.68	1.32
4 1-Chloro-2,4-dinitrobenzene (CDNB)	1.0	1.0	6.5	340	9600	0.004	0.42‡	100	0.58	1.56
5 Ethyl 4-chloro-3,5-dinitrobenzoate†	0.4	0.5	6.5	335	6000	0.008	6.67	1590	1.82§	2.01
6 4-Chloro-3,5-dinitrobenzonitrile	0.5	0.5	6.5	330	4000	0.305	305	72,600	3.48§	2.22

*Overnight incubation at 37°C gave no detectable reaction, estimated value for molar absorbance change.
†DMSO (150 μl) was added to improve solubility.
‡The rate constant for CDNB at pH 7.5 is 3.8 mol^{-1} l min^{-1}.
§For compounds measured at pH 6.5, the k_2 value of pH 7.5 is taken as 10 times greater than at pH 6.5.
‖Calculated as described in the text: the displaced Cl group is given a zero value.

molar absorbance change for complete reaction and the concentrations of the analogue and GSH used.

Table 1 records the results obtained from the selection of the six CDNB analogues chosen to span the full range of chemical reactivity that has been observed. These non-enzymic conjugation reaction rates are given as the absorbance change min^{-1} for the conditions shown. The more reactive compounds were measured at a pH of 6.5, the less reactive ones at a pH of 7.5. The table also shows the corresponding calculated second-order rate constants, and the relative reactivity of each compound compared with CDNB, expressed as a percentage (i.e. CDNB = 100%). As a particular compound reacts faster at pH 7.5 than at pH 6.5 (because of the greater ionization of GSH to the more reactive GS^- anion at the higher pH), a 10-fold rate enhancement factor was used to convert rate-constants obtained at pH 6.5 into k_2 (and hence $\log_{10} k_2$) values at pH 7.5. The $\sum\sigma$ value is calculated as described in the Experimental section.

The reactivity of the compounds covered a very wide range. The most reactive was 4-chloro-3,5-dinitrobenzonitrile (**6**) which had a relative rate of 72,600% (CDNB = 100%) and the least was the simple 1-chloro-4-nitrobenzene (**1**) with relative rate of <0.1% of CDNB. The reactivity range therefore spanned nearly a 1-million-fold range. Of other compounds studied (not shown in Table 1), most were less reactive than CDNB. Compounds more reactive than CDNB possessed the 1-chloro-4-X-2,6-dinitrobenzene structure (where X is an electron-withdrawing substituent). The table shows two of these, ethyl 4-chloro-3,5-dinitrobenzoate (**5**) and 4-chloro-3,5-dinitrobenzonitrile (**6**).

The reactivity of the CDNB analogues depends on the number, strengths and positions of the electron-withdrawing substituents. Their strengths for various positions are given by Hammett substituent σ values. Their sum ($\sum\sigma$) gives a measure of the total reactivity of the molecule. This analysis of the structure–reactivity relationships [4, 5] has allowed us to obtain a very good positive linear correlation between log k_2 (calculated at pH 7.5) and the sum of the Hammett substituent constants ($\sum\sigma$). This relationship is shown graphically in Fig. 3. The linear regression equation corresponding to Fig. 3, obtained for these six chloronitrobenzene analogues of CDNB is:

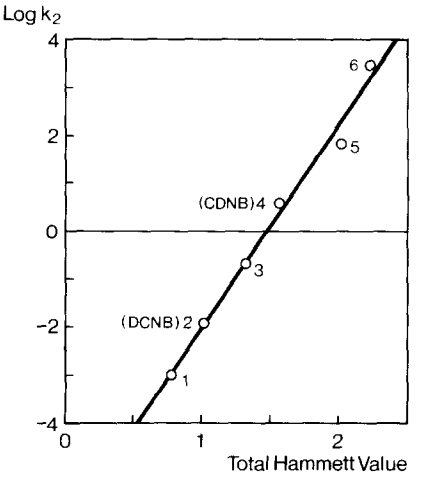


Figure 3
The relationship between chemical reactivity of chloronitrobenzenes and the combined electron-withdrawing power of their substituents. Chemical reactivity is expressed as the logarithm of their bimolecular rate constants with GSH at a pH of 7.5 at 25°C, and the total electron-withdrawing properties of the substituents is given by their combined Hammett σ constants.

$$\log_{10} k_2 = 4.26(\pm 0.24) \sum\sigma - 6.3(\pm 0.4),$$

with $r = 0.994$, $n = 6$, and standard errors as given.

Further analogues reinforce this linear correlation. This very good structure–reactivity relationship, when taken with enzyme studies, makes possible the identification of new enzyme substrates which are selective for individual glutathione transferase isoenzymes.

Summary

An excellent quantitative structure–reactivity correlation between the structural features of CDNB analogues (as expressed by their electronic substituent constants) and the non-enzymic reactivity of these analogues with GSH has been discovered.

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