Enzyme-Catalysed Reactions between some 2-Substituted 5-Nitrofuran Derivatives and Glutathione

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1. A glutathione transferase present in rat and human liver supernatant catalyses the reaction of some 2-substituted 5-nitrofuran derivatives with GSH, with formation of a conjugate and release of the nitro group as inorganic nitrite. Some of the substrates undergo the same reaction at a slower rate in the absence of enzyme. Nitrofuran derivatives commonly used as drugs, and five other drugs containing nitro groups, did not react. 2. Substrate activity in the nitrofuran derivatives showed an approximate correlation with the lability of the nitro group to alkali. 3. Optimum pH values ranging from 6.6 to 9.0 were found for the enzymic reaction with various derivatives, the values being influenced by alkali-lability and pK values of the compounds. 4. Tenfold purification of rat liver glutathione *S*-aryltransferase resulted in an equal purification of the activities that catalyse the reaction of two of the nitrofuran derivatives with GSH.

Certain foreign compounds react with GSH in the mammalian liver as a stage in their conversion into mercapturic acids (derivatives of *N*-acetylcysteine), which are excreted. The enzyme glutathione *S*-aryltransferase, catalysing the reaction of 3,4-dichloronitrobenzene with GSH, with release of chloride and formation of *S*-(2-chloro-4-nitrophenyl)glutathione, was partially purified from rat liver supernatant by Booth, Boyland & Sims (1961). Al-Kassab, Boyland & Williams (1963) reported that rat liver supernatant catalyses the release as nitrite of the nitro group of certain polychloromononitrobenzenes, 4-nitropyridine *N*-oxide and 4-nitroquinoline *N*-oxide with consumption of GSH, and suggested that the enzyme involved was glutathione *S*-aryltransferase. Other studies have indicated that a number of glutathione transferases with different specificities towards foreign compounds are present in the soluble fraction of rat liver homogenate (Boyland & Williams, 1965; Johnson, 1966; Suga, Ohata, Kumaoka & Akagi, 1967; Boyland & Chasseaud, 1967, 1968).

The 2-substituted 5-nitrofuran derivatives that are used as anti-microbial drugs largely depend for their effectiveness on the presence of the nitro group (Dodd, Stillman, Roys & Crosby, 1944). The present paper reports the finding that the soluble fractions of rat and human liver homogenates catalyse the reaction of certain 2-substituted 5-nitrofurans with GSH, with formation of a conjugate and release of the nitro group as inorganic nitrite.

EXPERIMENTAL

Nitrofuran derivatives IV, XII, XIII, XV, nidroxyzone, 5-nitrofurfuryl acetate, furmethoxadone, nifurthazone and nifurdazil were gifts from the Norwich Pharmacal Co. (Norwich, N.Y., U.S.A.). 2-(2-Formylhydrazino)-4-(5-nitro-2-furyl)thiazole, 2-(2-isopropylidenehydrazino)-4-(5-nitro-2-furyl)thiazole, *N*-[4-(5-nitro-2-furyl)thiazol-2-yl]formamide and *N*-[4-(5-nitro-2-furyl)thiazol-2-yl]acetamide were gifts from Abbott Laboratories (N. Chicago, Ill., U.S.A.). Nitrofuran derivative III was obtained from K & K Laboratories Inc. (Plainview, N.Y., U.S.A.), derivative I from Schuchardt (Munich, Germany), and nifuratel from Calmic Ltd. (Crewe, Cheshire, U.K.). All other nitrofuran derivatives were gifts from Smith, Kline and French Research Institute (Welwyn Garden City, Herts., U.K.). Compounds VII and IX were prepared by Dr S. K. Mallick. Metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] was obtained from May and Baker Ltd. (Dagenham, Essex, U.K.), niridazole [1-(5-nitrothiazol-2-yl)imidazolidin-2-one] from Ciba Laboratories Ltd. (Horsham, Sussex, U.K.), nitroxoline (8-hydroxy-5-nitroquinoline) from Dales Pharmaceuticals Ltd. (Steeton, Yorks., U.K.), chloramphenicol from Parke, Davis and Co. (Hounslow, Middx., U.K.), nitraxepam (1,3-dihydro-7-nitro-5-phenyl-1,4-benzodiazepin-2-one) from Roche Products Ltd. (Welwyn Garden City, Herts., U.K.) and 4-nitropyridine *N*-oxide from Koch-Light Laboratories Ltd. (Colnbrook, Bucks., U.K.). Glutathione was from Schwarz Bio-Research Inc. (Orangeburg, N.Y., U.S.A.), and 5,5'-dithiobis-(2-nitrobenzoic acid) was from Aldrich Chemical Co. Inc. (Milwaukee, Wis., U.S.A.). All other chemicals were from BDH Chemicals Ltd. (Poole, Dorset, U.K.).

Phosphate buffers were prepared from KH_2PO_4 and

Na_2HPO_4 , pyrophosphate buffers from $\text{Na}_4\text{P}_2\text{O}_7$ and $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$, and borate buffers from H_3BO_3 and NaOH .

Determination of nitrite. The method of Snell & Snell (1949) was used. A 0.1 ml portion of acetic anhydride was added to 1 ml of incubation mixture to remove GSH that would otherwise interfere (Al-Kassab *et al.* 1963). Then 2 ml of 1% (w/v) sulphanilamide in 20% (v/v) HCl was added followed by 2 ml of 0.02% (w/v) *N*-(1-naphthyl)-ethylenediamine hydrochloride. After the mixture had been centrifuged and left for 20 min, the E_{540} was measured in a 1 cm light-path cell in a Unicam SP.500 spectrophotometer against a water blank. If the E_{540} was greater than 1.0 the solutions were diluted with 10% HCl soon after diazotization. The E_{540} was proportional to nitrite concentration up to 1 mm-nitrite.

Determination of thiol. A 1 ml portion of 6% (w/v) sulphosalicylic acid was added to 1 ml of incubation mixture, and the mixture centrifuged. A 0.5 ml portion of supernatant [diluted if necessary with 3% (w/v) sulphosalicylic acid] was added to 4 ml of 5,5'-dithiobis-2-nitrobenzoic acid reagent (0.5 mm in 1.0 M-phosphate buffer, pH 7.0, containing 1 mM-EDTA) (Harrap, 1967). The E_{412} was measured within 2 min in a 1 cm light-path cell against a blank of 0.5 ml of water and 4 ml of 5,5'-dithiobis-2-nitrobenzoic acid reagent. The E_{412} was proportional to GSH concentration up to 0.25 μmol of GSH in the final coloured solution.

Liver supernatant. Rat liver supernatant was prepared from male rats (Chester Beatty strain) by the method of Booth *et al.* (1961), which includes centrifugation at 95000g. Dialysis against water was omitted unless

otherwise stated. Human liver supernatant was prepared by the same method, from livers removed at post-mortem after 1–2 days of cold storage.

Effect of rat or human liver supernatant on the release of nitrite from nitrofurans derivatives in the presence of glutathione. The procedure is described in Table 1. To decrease autoxidation of GSH, incubations longer than 5 min were carried out in Thunberg tubes that were evacuated on a water pump until bubbling of the solution commenced. Dimethyl sulphoxide (10%, v/v) was used in the reaction mixtures to increase the solubility of the nitrofurans derivatives; it had no effect on the rate of the enzymic reaction. The same concentration of dimethylformamide, dioxan or pyridine decreased the final colour due to nitrite. However, dimethyl sulphoxide reacted slowly with GSH, probably oxidizing it (Yiannios & Karabinos, 1963), and during incubation for 1 h in Thunberg tubes in the absence of nitrofurans derivatives the GSH concentration fell both in the presence and absence of liver supernatant to approx. 60% of the initial value. Endogenous thiol compounds present in the liver supernatant contributed 2% of the total thiol groups present in the test. The recovery of 0.05–0.10 mm-inorganic nitrite incubated for 1 h was 70% in the presence of liver supernatant and 94% in its absence. The lower recovery in the presence of liver supernatant may have been due to reduction of nitrite or to other reactions. This effect would presumably decrease slightly the sensitivity of the test. The values in Table 1 are not corrected for the loss of nitrite.

Glutathione S-aryltransferase activity. This was

Table 1. *Effect of rat liver supernatant on the release of nitrite from nitrofurans derivatives in the presence of GSH*

Reaction mixtures contained 0.1 M-phosphate buffer, pH 7.4, 1 mM (unless otherwise indicated) of the nitrofurans derivative, 10% (v/v) dimethyl sulphoxide as solvent, 10 mM-GSH and, where indicated, 25% (v/v) rat liver supernatant. Incubation was at 37°C. Incubations longer than 5 min were carried out in evacuated Thunberg tubes. The concentration of nitrite in the reaction mixtures at the end of incubation was determined as described in the Experimental section. Values are not corrected for loss of nitrite that occurred during incubation (see the Experimental section). The alkaline hydrolysis was carried out by incubating the same concentration of the nitrofurans derivative and solvent as in the previous test in 0.075 M-borate buffer, pH 9.0, for 30 min at 37°C. Nitrite was then determined as before except that no acetic anhydride was added and extinctions were read against a reagent blank containing the non-incubated nitrofurans derivative.

Compound	R in the formula	Time of incubation (min)	Nitrite released (mm)		Nitrite released by alkaline hydrolysis (mm)
			Liver supernatant present	Liver supernatant absent	
I 5-Nitrofurfural	–CHO	1	0.84	0.26	0.52
II 3-(5-Nitrofurfuryl-ideneamino)-1,2,4-triazole	–CH=N–	1	0.73	0.21	0.37
III 5-Nitrofurfural diacetate	–CH(O·CO·CH ₃) ₂	1	0.34	0.03	0.42
IV Nifurmerone	–CO·CH ₂ Cl	1	0.26	0.08	0.54
V 4-(5-Nitrofurfuryl-ideneamino)-1,2,4-triazole	–CH=N–	1	0.08	0.02	0.32

Table 1 (continued)

Compound	R in the formula	Time of incubation (min)	Nitrite released (mm)		Nitrite released by alkaline hydrolysis (mm)
			Liver supernatant present	Liver supernatant absent	
VI *3-Mercapto-5-methyl-4-(5-nitrofurfurylideneamino)-1,2,4-triazole		1	0.02	0	0.01
VII †3-Methyl-5-(5-nitrofurfurylidene)rhodanine		1	0.02	0.01	0.02
VIII 5-Nitro-N-(2-pyridyl)-2-furoamide		5	0.06	0.01	0.03
IX 5-(5-Nitrofurfurylidene)thiazolidine-2,4-dione		5	0.03	0.01	0
X 5-Nitro-2-furonitrile	-CN	5	0.07	0.05	0
XI 5-Nitro-2-furoic acid	-CO ₂ ⁻	60	0.20	0.01	0
XII Methyl 5-nitro-2-furoate	-CO·O·CH ₃	60	0.20	0.03	0
XIII Ethyl 5-nitro-2-furoate	-CO·O·C ₂ H ₅	60	0.17	0.03	0.01
XIV Ethyl 5-nitro-2-furimide hydrochloride		60	0.13	0.01	0
XV Nifuraldezone	-CH=N·NH·CO·CO·NH ₂	60	0.10	0.05	0.01
XVI †3-Methyl-1-(5-nitrofurfurylideneamino)-5,6-dihydrouracil		60	0.03	0.01	0.01

* Compound tested at 0.1 mm because of its low solubility.

† Compound tested at 0.02 mm because of its low solubility.

measured by the method of Booth *et al.* (1961) with 1 mM-3,4-dichloronitrobenzene as substrate. Protein was measured by a biuret method (Layne, 1957).

Partially purified glutathione S-aryltransferase. Female rats (Chester Beatty strain) aged 6 months were used because they were more readily available in quantity than males. The partial purification was carried out by the following modification of the method of Booth *et al.* (1961) which, when these rats were used, gave higher recoveries of enzyme than the original method. All operations were carried out between -5°C and 8°C. Rat liver supernatant was prepared by the method of Booth *et al.* (1961) except that the livers were perfused *in situ* through the portal vein with ice-cold 0.25 M-sucrose, then

homogenized in 4 vol. of 0.25 M-sucrose instead of phosphate. The undialysed rat liver supernatant was adjusted to pH 5.0 with 50% (v/v) acetic acid, centrifuged at 2000g for 15 min, and the precipitate was discarded. The supernatant was adjusted to pH 7.0 with M-NaOH and dialysed overnight against water. After adjustment of the pH to 8.0 with M-NaOH, (NH₄)₂SO₄ (40g/100ml) was added and the pH readjusted to 8.0 if necessary. After standing for 30 min the precipitate was removed by centrifuging at 10000g for 20 min. The supernatant was adjusted to pH 4.5 with 50% (v/v) acetic acid, centrifuged at 10000g for 20 min and the precipitate discarded. (NH₄)₂SO₄ (15g/100ml of the original pH 8.0 supernatant) was added, the solution centrifuged at 10000g

Table 2. *Partial purification of glutathione S-aryltransferase*

A unit of enzyme is the amount that forms 1 μ mol of *S*-(2-chloro-4-nitrophenyl)glutathione/min per 3 ml of reaction mixture, which corresponds to an increase in E_{440}^{344} of 3.47/min. Corresponding values for activity/ml, total activity and specific activity, reported by Booth *et al.* (1961) are in error by a factor of 10.

Step	Volume (ml)	Activity (units/ml)	Total activity (units)	Protein (mg/ml)	Specific activity (units/mg of protein)	Recovery (%)
1. Rat liver supernatant	350.0	0.96	336	20.5	0.047	100
2. After pptn. at pH 5.0 and dialysis	395.0	0.69	274	13.1	0.053	83
3. After (NH ₄) ₂ SO ₄ pptn. and dialysis	26.5	5.75	153	14.2	0.406	46
4. After acetone pptn.	7.7	13.60	105	30.5	0.446	32

Table 3. *Glutathione conjugates of nitrofurans derivatives*

The nitrofurans derivatives were incubated with GSH and partially purified glutathione *S*-aryltransferase and the products examined by paper chromatography as described in the Experimental section.

Nitrofurans derivative	Composition of incubation mixture		Time of incubation (min)	R_F of conjugate	Whether conjugate apparent when enzyme omitted from incubation mixture
	Dilution of enzyme	Solvent			
I	1 in 133	Ethanol (12.5%, v/v)	2	0.36	+
II	1 in 133	Ethanol (25%, v/v)	2	0.38	+
III	1 in 33	Dimethyl sulphoxide (10%, v/v)	1	0.37	+
IV	1 in 133	Ethanol (12.5%, v/v)	5	0.18	+
V	1 in 133	Dimethyl sulphoxide (10%, v/v)	10	0.36*	+
VI	1 in 133	Ethanol (25%, v/v)	60	0.44	—
VII	1 in 133	Ethanol (25%, v/v)	60	0.59	+
VIII	1 in 10	Dimethyl sulphoxide (10%, v/v)	60	0.47	—
IX	1 in 33	Ethanol (12.5%, v/v)	60	0.48	—
X	1 in 33	Ethanol (12.5%, v/v)	60	0.56	—
XII	1 in 33	Ethanol (12.5%, v/v)	60	0.52	—
XV	1 in 7	Dimethyl sulphoxide (10%, v/v)	60	0.28	—

* This conjugate ran with free GSH in the usual system, but was separated from it by descending chromatography for 18 h on Whatman no. 1 paper (46 cm $\times$ 57 cm) with the same solvent as before.

for 20 min and the supernatant discarded. The precipitate was dissolved in 10 ml of water, adjusted to pH 7.0 with *m*-NaOH, dialysed against water for 2–3 h, and stored overnight at -20°C . The following day it was thawed, cooled to 0°C , and acetone at -10°C (1.125 ml/ml of enzyme solution) was added with stirring. After centrifuging for 5 min at -5°C the precipitate was dissolved in 5 ml of water, dialysed against water for 3 h and stored at -20°C . The enzyme activity was stable for at least 6 months. The partial purification is summarized in Table 2.

Partially purified glutathione *S*-aryltransferase was used in certain of the experiments because of its higher specific activity in releasing nitrite from nitrofurans derivatives in the presence of GSH compared with that of rat liver supernatant (Table 5).

Chromatographic detection of conjugates of nitrofurans derivatives with GSH. Reaction mixtures were incubated at 37°C and contained 20 mM-phosphate buffer, pH 7.4, 5 mM-GSH, 1 mM of the nitrofurans derivative (except for compounds VI and VII, which were 0.5 mM), ethanol or dimethyl sulphoxide to dissolve the nitrofurans derivative and either partially purified glutathione *S*-aryltransferase at dilutions shown in Table 3 or human liver supernatant. After incubation, approx. 20 μ l of each mixture was applied to a 20 cm square of Whatman no. 4 chromatography paper, and ascending chromatography was carried out with a solvent of butan-1-ol-acetic acid-water (12:3:5, by vol.). The dried chromatograms were sprayed with either 0.1 M-K₂Cr₂O₇-acetic acid (1:1, v/v), followed by 0.1 M-AgNO₃ (Knight & Young, 1958) or with 0.2% (w/v) ninhydrin in acetone-pyridine (49:1,

Table 4. Apparent optimum pH values for the enzymic release of nitrite from nitrofurans derivatives in the presence of GSH

Nitrofurans derivative	Composition of incubation mixture				Conditions of incubation		Apparent optimum pH	Spectral change at 0.2 pH unit above optimum (% of peak extinction)	pK of substrate within pH range 4-9
	Buffer	Dilution of enzyme	Concn. of nitrofurans derivative (mm)	Solvent	Temp. (°C)	Time (min)			
III*	Pyrophosphate	1 in 333	1.00	Dimethyl sulphoxide (5%, v/v)	25	1	9.0	1	None
V	Pyrophosphate	1 in 333	1.00	Dimethyl sulphoxide (5%, v/v)	25	3	9.0	4	None
X	Pyrophosphate	1 in 133	1.00	Ethanol (5%, v/v)	37	10	8.2	1	None
VII	Phosphate	1 in 1667	0.02	Dimethyl sulphoxide (10%, v/v)	37	0.5	8.0	4	None
II	Phosphate	1 in 333	0.20	Dimethyl sulphoxide (5%, v/v)	37	1	7.6	1	None
VI	Phosphate	1 in 333	0.10	Dimethyl sulphoxide (10%, v/v)	37	5	6.6	0	7.0
IX	Phosphate	1 in 333	1.00	Dimethyl sulphoxide (10%, v/v)	37	15	6.6	0	5.3

* Concn. of GSH was 5 mM, because the depletion of 1 mM-GSH during incubation was greater than 20%.

v/v) followed by heating in an oven at 105°C for 2-3 min. Glutathione conjugates give yellow and violet colours respectively with the above procedures. Spots with these staining reactions that were reaction products of nitrofurans derivatives and GSH were conjugates. Spots were also sprayed with 0.4% (w/v) 2,4-dinitrophenylhydrazine in 2M-HCl (Bland, 1949) to test for breakage of the furan ring with formation of carbonyl groups.

Optimum pH of enzymic release of nitrite from nitrofurans derivatives in the presence of GSH. Reaction mixtures (Table 4) contained the nitrofurans derivative, 0.1M-phosphate or pyrophosphate buffer, 1 mM-GSH, ethanol or dimethyl sulphoxide to solubilize the nitrofurans derivative and partially purified glutathione S-aryl-transferase. The reaction was started by addition of the nitrofurans derivative and stopped by addition of 0.1 vol. of acetic anhydride. Nitrite was then measured as described above. At the same time the non-enzymic release of nitrite was measured in reaction mixtures lacking only enzyme, and the nitrite release in the presence of enzyme was corrected by this value. Conditions of incubation were chosen such that in the presence of all the reactants the GSH concentration decreased during incubation by less than 20%. The recovery of nitrite added at the start of incubation was 97-100%.

As decomposition of the nitrofurans derivatives in alkaline solution could affect the optimum-pH determination, 0.025 or 0.050 mM solutions of the nitrofurans derivatives in buffer were incubated under the same conditions as employed for the optimum-pH determination. The incubation was carried out in 0.2 cm light-path silica cells in the water-jacketed cell housing of a Unicam SP.800 spectrophotometer. The spectrum was scanned at the beginning and end of incubation, and the maximum change in extinction occurring between 250 nm and 450 nm was expressed as a percentage of the peak extinction in this region to give an approximate indication of the extent of decomposition of the nitrofurans derivatives.

Determination of pK values of nitrofurans derivatives. Small amounts of carbonate-free 0.1M-KOH (prepared by the method of Albert & Serjeant, 1962) were added from a 1 ml burette to 25 ml of a 1 or 2 mM-solution of the nitrofurans derivative in 10% (v/v) dimethyl sulphoxide at 37°C under nitrogen. After each addition the solution was stirred mechanically, then stirring was stopped and the pH was read by means of a glass and a calomel electrode attached to a Radiometer pH meter. pK values were calculated as described by Albert & Serjeant (1962).

RESULTS

Effect of rat liver supernatant on the release of nitrite from nitrofurans derivatives in the presence of GSH. The 2-substituted derivatives of 5-nitrofurans listed in Table 1 released a greater amount of nitrite in the presence of rat liver supernatant than in its absence. With boiled liver supernatant (tested on substrates I, II and III) the same amount of nitrite was released as in the absence of liver supernatant. The nitrite released due to the presence of liver supernatant was less than 0.02 mM/h for the following 36 nitrofurans derivatives (com-

pounds tested at less than 1mM due to their low solubilities are marked <1mM): 5-nitrofuran-2-aldehyde semicarbazone (nitrofurazone); 1-(5-nitrofurfurylideneamino)hydantoin (nitrofurantoin); 3-(5-nitrofurfurylideneamino)oxazolidin-2-one (furazolidone); 5-nitro-2-furaldoxime (nifuroxime); 5-nitrofurfuryl acetate; ethyl 5-nitrofurfurylidene carbazate; 5-nitro-2-furaldehyde 2-(2-hydroxyethyl)semicarbazone (nidroxyzone); 5-nitro-2-furaldehyde 2-(2-dimethylaminoethyl)semicarbazone (nifurethazone); 3-(5-nitrofurfurylidenehydrazino)sulpholan; 1-(5-nitrofurfurylideneamino)-imidazolidine-2-thione; 1-(2-hydroxyethyl)-3-(5-nitrofurfurylideneamino)-2-imidazolidinone (nifurdazil); 3-amino-5-methyl-*N*-(5-nitro-2-furfurylidene)oxazolid-2-one (furmethoxadone); 5-[(methylthio)methyl]-3-(5-nitrofurfurylideneamino)oxazolidin-2-one (nifuratel); 5-morpholino-methyl-3-(5-nitrofurfurylideneamino)oxazolidin-2-one (furaltadone); 5,5-dimethyl-1-(5-nitrofurfurylideneamino)hydantoin; 1-(5-nitrofurfurylideneamino)hydantoin-3-acetic acid; ethyl 1-(5-nitrofurfurylideneamino)hydantoin-3-acetate; 1-(5-nitrofurfurylideneamino)-5-phenylhydantoin; 2-(2-formylhydrazino)-4-5-nitro-2-(furyl)thiazole; 2-(2-isopropylidenehydrazino)-4-(5-nitro-2-furyl)thiazole (<1mM); *N*-[4-(5-nitro-2-furyl)thiazol-2-yl]formamide (<1mM); *N*-[4-(5-nitro-2-furyl)thiazol-2-yl]acetamide (Furium) (<1mM); 4-(5-nitrofurfurylideneamino)-1,2,4-triazolidine-2,4-dione; 2,6-dihydroxy-5-methyl-1-(5-nitrofurfurylideneamino)-1,6-dihydropyrimidine; 6-methyl-4-(5-nitro-2-furyl)-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylic acid methyl ester (nitractin); 5,6-dihydro-5-methyl-1-(5-nitrofurfurylideneamino)uracil; 5,6-dihydro-1-(5-nitrofurfurylideneamino)-5-phenyluracil; 6-(5-nitro-2-furyl)-*as*-triazine-3,5(2*H*,4*H*)-dione; 2-(5-nitro-2-furyl)benzimidazole; 1-(2-dimethylaminoethyl)-2-(5-nitro-2-furyl)benzimidazole; methyl 2-(5-nitro-2-furyl)benzimidazole-5(6)-carboxylate (<1mM); 1-(2-hydroxyethyl)-2-(5-nitro-2-furyl)benzimidazole (<1mM); 5,6-dichloro-2-(5-nitro-2-furyl)benzimidazole (<1mM); 2-(5-nitro-2-furyl)-5(6)-trifluoromethylbenzimidazole (<1mM); 5-trifluoro-1-(2-hydroxyethyl)-2-(5-nitro-2-furyl)benzimidazole (<1mM); 2-(5-nitro-2-furyl)benzoxazole (<1mM).

Under anaerobic conditions undialysed liver supernatant can reduce nitro groups of nitrofuran derivatives (Paul & Harrington, 1967). Such a reaction could interfere with nitrite release by depleting the nitrofuran derivatives. All compounds were therefore tested again under aerobic conditions, with dialysed rat liver supernatant and 1.6mM-GSH at pH6.5. The lower pH was to minimize autoxidation of GSH. Other conditions were as in Table 1, with incubation for 1h. The

recoveries of GSH and added nitrite at the end of incubation were the same as under the conditions of Table 1 (see the Experimental section). Results in general confirmed those in Table 1, and no additional substrates were found with the second set of conditions. The enzymic release of nitrite for substrates XI, XIV and XVI was less than 0.01mM under the second set of conditions, which appeared to be the less sensitive.

Betts, James & Thorpe (1955) found that the extent to which polychloronitrobenzenes were converted into mercapturic acids in the rabbit varied with the lability of the nitro group in alkali (measured by the nitrite released on incubating the compound in ethanolic sodium hydroxide). Nitrofuran derivatives are unstable in alkali, and in addition to loss of the nitro group they may undergo ring cleavage and resinification to brown tars (Olivard & Heotis, 1968). The last column of Table 1 shows the nitrite released on incubation of the nitrofuran derivatives at pH9 for 30min at 37°C. Nitrofurans that were attacked the most rapidly in the enzymic reaction also showed the greatest release of nitrite at pH9. Of the 36 nitrofurans that did not act as substrates for the enzyme only three released more than 0.01mM-nitrite at pH9. These were 2-(2-formylhydrazino)-4-(5-nitro-2-furyl)thiazole (0.12mM-nitrite released), 5-nitrofurfuryl acetate (0.07mM-nitrite) and 1-(2-hydroxyethyl)-2-(5-nitro-2-furyl)benzimidazole (0.02mM-nitrite from a 0.1mM solution of the nitrofuran derivative). After incubation at pH9 the reaction mixtures of nine out of the 52 compounds were brown owing to resinification.

Glutathione conjugates of nitrofuran derivatives. Each of the incubation mixtures of 12 of the compounds listed in Table 1 were subjected to paper chromatography and gave rise to a spot with the characteristics of a conjugate (Table 3). Some substrates also formed a conjugate in the absence of enzyme, but except for compounds IV and VII amounts were smaller than in the presence of enzyme. Compound IV is unstable at pH7.4 and compound VII is sparingly soluble. The conjugate from compound III gave a positive reaction for carbonyl groups with 2,4-dinitrophenylhydrazine, which may have been due to hydrolysis of the diacetate group to an aldehyde group. The conjugate from compound II also gave a positive reaction for carbonyl groups, which suggests that breakage of the furan ring with formation of carbonyl groups had occurred (Olivard & Heotis, 1968). The only other conjugates to give a positive reaction for carbonyl groups were those from compounds I, IV and XV, where it is not necessary to postulate ring breakage, because the parent compounds have carbonyl groups. There was also a delayed reaction from compound V and its conju-

gate, which was probably due to breakdown caused by the acidic conditions of the test.

Optimum pH of enzymic release of nitrite from nitrofuran derivatives in the presence of GSH. The substrates had different stabilities in alkaline solution but all showed a decrease in stability with increase in pH. With some substrates, breakdown was evident at pH values as low as 8. The values for optimum pH obtained could (except for substrates VI and IX) have been affected by breakdown of the substrate, and are therefore given as apparent optima in Table 4. Fig. 1 shows enzymic nitrite release as a function of pH for the various substrates.

When substrates III and V were preincubated with buffer, pH9.2, for 5min before addition of a

mixture of GSH and enzyme 19% and 11% less nitrite was released in the subsequent reaction than without preincubation, indicating that the nitrofuran derivative itself, and not a breakdown product, is the substrate in the enzymic reaction. Thus the true optimum pH of the enzymic reaction with these two substrates appears to be at or above pH9. The pH optima for substrates II, VII and X could well have been lowered by alkaline breakdown of the substrates. The low pH optima for substrates VI and IX are not due to alkaline breakdown, but are probably due to the substrates having pK values in this region (last column of Table 4).

The optimum pH values of substrates I and IV were not determined because of the instability and high GSH consumption of these substrates.

Other drugs containing nitro groups. Five drugs containing nitro groups were tested by the procedure described in Table 1. The drugs (with solvent in parentheses) were nitrazepam (10% ethanol), chloramphenicol (none), nitroxoline (15% ethanol), niridazole (12.5% ethanol-2.5% dimethyl sulphoxide) and metronidazole (none). No nitrite was released in the presence or absence of enzyme. When the incubation was repeated with 1mM-GSH no more thiol was consumed than with controls without the drug.

Activity of partially purified glutathione S-aryltransferase towards various substrates. Table 5 shows the specific activity of partially purified glutathione S-aryltransferase towards three nitrofuran derivatives, 4-nitropyridine N-oxide and 3,4-dichloronitrobenzene, expressed as a multiple of the corresponding specific activity of dialysed rat liver. The ratios of activity are similar for 3,4-dichloronitrobenzene and nitrofuran derivatives I and V, and this is consistent with these reactions being catalysed by the same enzyme in the rat.

Fig. 1. Effect of pH on enzymic release of nitrite from nitrofuran derivatives in the presence of GSH. Experimental details are given in the text and Table 4. The nitrofuran derivatives were as follows: ○, III; △, V; □, X; ●, II; ▲, VI; ■, IX; ●, VII.

Table 5. Activity of partially purified glutathione S-aryltransferase towards various substrates

Activities towards 3,4-dichloronitrobenzene were measured by the method of Booth *et al.* (1961). For the other substrates, 5mM-GSH, 1mM-substrate, 10% (v/v) dimethyl sulphoxide and either 10% (v/v) dialysed rat liver supernatant or 1% (v/v) partially purified glutathione S-aryltransferase were incubated in 0.1M-phosphate buffer, pH7.4. The reaction was started by addition of substrate and stopped by addition of 0.1 vol. of acetic anhydride. Nitrite was then measured as described in the Experimental section. Nitrite release in the absence of enzyme was subtracted to give enzymic release of nitrite.

Substrate	Temp. (°C)	Time (min)	Sp. activity of partially purified enzyme/sp. activity of dialysed rat liver supernatant
Nitrofuran derivative I	25	0.5	10
Nitrofuran derivative III	37	1.0	5
Nitrofuran derivative V	37	1.0	13
4-Nitropyridine N-oxide	25	4.0	16
3,4-Dichloronitrobenzene	As in Booth <i>et al.</i> (1961)		11

Table 6. *Effect of human liver supernatant on the release of nitrite from nitrofuran derivatives in the presence of GSH*

The method and conditions were as described in Table 1, incubation being for 1 min. Glutathione *S*-aryltransferase activities and results on rat liver supernatant are included for comparison.

Liver supernatant	Donor of post-mortem specimen of liver			Nitrite released (mm)			Glutathione <i>S</i> -aryltransferase activity (units/ml)
	Sex	Age	Disease before death	Nitrofuran derivative I	Nitrofuran derivative III	Nitrofuran derivative V	
Human liver supernatant 1	F	60	Carcinoma of ovary	0.76	0.52	0.09	0.02
Human liver supernatant 2	F	69	Carcinoma of bladder	0.76	0.56	0.13	0.02
Human liver supernatant 3	M	61	Chronic myeloid leukaemia	0.68	0.41	0.07	0.02
Rat liver supernatant				0.84	0.34	0.08	1.54
No liver supernatant				0.26	0.03	0.02	—

However, for nitrofuran derivative III and 4-nitro-pyridine *N*-oxide the ratios are somewhat different, and other enzymes or interfering substances may be involved.

Effect of human liver supernatant on the release of nitrite from nitrofuran derivatives in the presence of GSH. Human liver supernatant had approximately the same activity as rat liver supernatant in releasing nitrite from nitrofuran derivatives in the presence of GSH (Table 6). However, the activity of glutathione *S*-aryltransferase was much lower in human than in rat liver supernatant, as previously reported by Grover & Sims (1964). The results may reflect differences either in enzyme concentrations or in specificities. Chromatography showed that the R_F values of conjugates formed from each of the nitrofuran derivatives I, II and V in the presence of human liver supernatant and GSH (0.39, 0.38 and 0.36) were similar to those of the conjugates produced by rat liver enzyme. Amounts of conjugate were greater in the presence than in the absence of liver supernatant.

DISCUSSION

Under the conditions of the test 16 of the 52 2-substituted derivatives of 5-nitrofuran showed enzyme-catalysed conversion to glutathione conjugates accompanied by release of nitrite. The reaction may well occur *in vivo*, and would lead to loss of the antimicrobial activity of the nitrofuran derivative, since this is markedly dependent on the presence of the nitro group. If formed, glutathione conjugates might be expected, by analogy with mercapturic acids, to undergo conversion into the urine (Boyland & Chasseaud, 1969). Paul, Austin, Paul & Ells (1949) and Dodd, Cramer & Ward (1950) found that of a number of 2-substituted 5-nitrofuran derivatives tested nearly all had antibacterial activity *in vitro*. Certain compounds (styled class B nitrofuran derivatives) in which the

R

C-2 substituent had the structure $-C=N-R'$ were

also active against bacterial infections in the mouse. Compounds lacking the $C=N-$ (azomethine) group at C-2 of the furan ring and having instead alkyl, acyl, hydroxyalkyl or carboxyl groups, or certain derivatives of these groups, as the C-2 substituent (class A derivatives) were inactive against bacterial infections *in vivo*. Paul *et al.* (1949) found that certain class A compounds, including 5-nitrofurfural (I), 5-nitrofurfural diacetate (II), methyl 5-nitro-2-furoate (XII) and 5-nitro-2-furoic acid (XI) itself were recovered as 5-nitro-2-furoic acid

in the urine of the rat in amounts equal to 35–75% of the dose administered. This largely explained the lack of activity of compounds I, II and XII *in vivo*, since the antibacterial activity of 5-nitro-2-furoic acid is weak even *in vitro*. Other class A compounds, including 5-nitro-2-furonitrile (X), were metabolized by processes other than conversion into nitrofuroic acid, since neither the original compound nor nitrofuroic acid appeared in the urine. The method employed by Paul *et al.* (1949) relied on the spectra of compounds in the urine and would not detect mercapturic acids that lack the nitro group. Further experiments are necessary to determine whether mercapturic acids are formed *in vivo* from nitrofurans derivatives and whether reaction with GSH is important in the inactivation *in vivo* of those nitrofurans derivatives that form conjugates *in vitro*.

In the present study, eight compounds of class A having aliphatic C-2 substituents were tested, seven of which were substrates for the glutathione transferase. Six of the 36 class B compounds were substrates (included in class B are compounds in which the azomethine linkage forms part of a ring). The remaining eight nitrofurans derivatives have no azomethine linkage but have a ring system in the C-2 substituent, and three of these were substrates. The presence of an azomethine linkage at C-2 thus appears to diminish the likelihood that the nitrofurans derivative will react with GSH either enzymically or non-enzymically. Where the ring attached to the azomethine linkage was triazole, however, reaction with GSH occurred. Thus three out of four azomethine triazole derivatives tested were substrates for the glutathione transferase. These four triazole derivatives all reacted more rapidly with GSH in the absence of enzyme than any of the other azomethine nitrofurans derivatives with secondary ring systems that were tested.

The limited information on antibacterial activities of the compounds *in vivo* indicates that compounds found to be effective as antimicrobials *in vivo* seldom react either enzymically or non-enzymically with GSH. Compounds that react with GSH are usually ineffective as antimicrobials *in vivo*. Nitrofurans derivatives reported to be effective *in vivo* include (Miura & Reckendorf, 1967) nitrofurazone, nidroxyzone, furazolidone, furaltadone, nitrofurantoin, nifuraldezone (Dodd *et al.* 1950), Furium, 2-(2-formylhydrazino)-4-(5-nitro-2-furyl)thiazole (Holper, Otto, Kimura & Bower, 1962), 6-(5-nitro-2-furyl)-*as*-triazine-3,5(2*H*,4*H*)-dione (Hayes, 1964), and 2-(5-nitro-2-furyl)benzimidazole and its derivatives (Bavin, 1966). Of these compounds only nifuraldezone (XV) released more than 0.02mM-nitrite either enzymically or non-enzymically, and with this compound the release was slow.

The reaction of compounds with glutathione generally decreases biological activity of drugs. Compounds that are substrates for the reaction are unlikely to be useful drugs. Measurement of the reaction might therefore be a useful way of screening compounds for indication that they will be rapidly inactivated.

The substrates attacked most rapidly by the glutathione transferase with release of nitrite showed a high lability of the nitro group to alkali, analogous to the finding of Betts *et al.* (1955) on polychloronitrobenzenes. Of the derivatives that did not act as substrates for the glutathione transferase, 2-(2-formylhydrazino)-4-(5-nitro-2-furyl)thiazole released the most nitrite at pH 9. This compound is carcinogenic in the rat (Morris, 1967) but nitrofurazone, which is also carcinogenic, did not release nitrite at pH 9. Three other reported carcinogens tested were too insoluble for measurements to be reliable.

The p*K* values at 7.0 and 5.3 of substrates VI and IX are probably due to the thiol and secondary amino groups present in the C-2 substituents. The low optimum pH of the enzymic reaction with these two substrates suggests that the negatively charged ion is less active as a substrate than the un-ionized molecule. A negative charge on the nitrofurans derivative might decrease the rate of reaction by altering the affinity of the derivative for the enzyme or by hindering the approach of GS[−] if this is the reactive form of the second substrate in the enzymic reaction. The high optimum pH of the enzymic reaction with substrates III and V lends support to the suggestion that GS[−] is the active species in the enzymic reaction, as the p*K* of the thiol group of GSH is 9.2 (Benesch & Benesch, 1955).

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