The Metabolism of Acetamidothiazoles in the Rat

2-ACETAMIDO-, 2-ACETAMIDO-4-METHYL- AND 2-ACETAMIDO-4-PHENYL-THIAZOLE

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The metabolism of some anti-inflammatory acetamidothiazoles was studied in the rat. The main metabolites were the corresponding acetylthiohydantoic acids, produced by fission of the thiazole ring. Minor metabolites arising from oxidation of the methyl or phenyl substituents were also identified. The structures of metabolites were established spectroscopically (u.v., i.r., n.m.r. and mass spectroscopy) and by identification with authentic specimens. The excretion of the original compounds and of metabolites, labelled with ^{14}C is also reported.

Substituted 2-acetamidothiazoles of the general structure (I) have shown anti-inflammatory activity in the rat (Evans, 1972). We have studied the metabolism of some of these compounds (I, R = H, Me and Ph) in the same species. There are few previous reports of the metabolic fate of thiazoles. Several studies have indicated that the thiazole ring was degraded, but the fragments were not identified.

Thus, 2-aminothiazole was degraded to unknown fragments (Perrault & Bovet, 1946) and 2-acetamido-5-nitrothiazole gave six unidentified metabolites on incubation with slices of liver or kidney tissue from turkeys (Bousquet *et al.*, 1965). In the latter study the degradation of the thiazole ring was not reported, so it seemed possible that 5-substituted thiazoles were not so easily degraded as were compounds with a free 5 position.

Materials and Methods

Chemicals

Thio[^{14}C]urea (0.1 mCi; specific radioactivity 0.30 mCi/mg) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. 2,5-Diphenyl-oxazole, 1,4-bis-(5-phenyloxazol-2-yl)benzene and

Hyamin 10X were purchased from Packard Instrument Co., Wembley, Middx., U.K. Bacterial type-1 β -glucuronidase, NADP $^{+}$, NAD $^{+}$ and glucose 6-phosphate were obtained from Sigma (London) Chemical Co., London, S.W.6, U.K.

Unlabelled 2-acetamidothiazole was obtained from Ralph N. Emmanuel Ltd., London S.E.1, U.K. 2-Acetamido-4-methylthiazole and 2-acetamido-4-phenylthiazole were synthesized by known methods (Traumann, 1960).

2-Acetamido-4-hydroxymethylthiazole was prepared by the hydrolysis of 2-acetamido-4-chloromethylthiazole (Bhargava & Singh, 1960) (10g) by hydrolysis in buffer solution, pH 7.3, for 3 h at 60°C. The buffer solution (1 litre) contained NaH_2PO_4 (15.6g) and K_2HPO_4 (8.8g). The solution was cooled, extracted with ethyl acetate (3 $\times$ 80 ml) and the solvent was evaporated. The gummy residue was crystallized from acetone-light petroleum (b.p. 60-80°C) (9:1, v/v) to give crystalline 2-acetamido-4-hydroxymethylthiazole (I, R = CH_2OH), m.p. 133°C, yield 4.4g. (Found: C, 41.8; H, 4.6; N, 16.4; $\text{C}_6\text{H}_8\text{N}_2\text{O}_2\text{S}$ requires C, 41.8; H, 4.7; N, 16.3%).

2-Acetamidothiazole-4-carboxylic acid was prepared by addition of thiourea (3.8g) to a solution of

(II)

(IV)

bromopyruvic acid (3-bromo-2-oxopropanoic acid) (8.4g) in acetone (50ml). The solution was stirred for 30 min at room temperature and heated for 1 h under reflux. The hydrobromide of 2-aminothiazole-4-carboxylic acid was precipitated as a white solid and was removed by filtration (yield 9.0g). The hydrobromide was heated under reflux for 2 h with acetic anhydride (50ml). The acetic anhydride was removed by distillation and the residue was crystallized from ethanol–dimethylformamide (5:1, v/v) to give 2-acetamidothiazole-4-carboxylic acid, m.p. 295–298°C (yield 6g) (Found: C, 38.9; H, 3.3; N, 15.1; $C_6H_6N_2O_3S$ requires C, 38.9; H, 3.2; N, 15.1%).

5-Acetyl-2-methylthiohydantoic acid (II, R = Me) was prepared by mixing ($\pm$)alanine (2.9g) and ethyl dithiocarbamate (4.9g) in aq. 80% ethanol (80ml). NaOH (0.6g) was added and the mixture was left for 16 h at room temperature. The solution was made alkaline with 20ml of 1M-NaOH and the insoluble oil was discarded. The solution, adjusted to pH 5 with HCl, deposited crystalline 5-acetyl-2-methylthiohydantoic acid, m.p. 180°C (yield 4.5g) (Elmore & Toseland, 1957) (Found: C, 37.9; H, 5.4; N, 14.6; $C_6H_{10}N_2O_3S$ requires C, 37.9; H, 5.3; N, 14.7%).

5-Acetylthiohydantoic acid (II, R = H), m.p. 200–202°C (Elmore & Toseland, 1957) was prepared similarly from glycine and ethyl dithiocarbamate (Found: C, 34.1; H, 4.7; N, 16.1; $C_5H_8N_2O_3S$ requires C, 34.1; H, 4.5; N, 15.9%).

5-Acetyl-2-phenylthiohydantoic acid (II, R = Ph), m.p. 190–192°C was prepared similarly from phenylglycine and ethyl dithiocarbamate (Found: C, 52.5; H, 5.0; N, 11.3; $C_{11}H_{12}N_2O_3S$ requires C, 52.4; H, 4.8; N, 11.1%).

Synthesis of 2-acetamido-4-methyl[2- ^{14}C]thiazole (I, R = Me). Thio[^{14}C]urea (0.190g, 0.025mCi) was heated under reflux with redistilled chloroacetone (0.232g) in acetone (100ml) for 2 h. The solution was evaporated *in vacuo* and acetic anhydride (50ml) was added. The solution was refluxed for 2 h then the excess of acetic anhydride was evaporated *in vacuo*. The brown residue was dissolved in ethanol (25ml) and the solution was decolorized with activated charcoal. The solution was filtered and the 2-acetamido-4-methyl[2- ^{14}C]thiazole was recrystallized twice from ethanol–water (9:1, v/v), m.p. 137–138°C (yield 300mg). An i.r. spectrum of the product was identical with that of an authentic sample of compound (I, R = Me). The product was shown to be radiochemically pure by t.l.c. in three different solvent systems and had a specific radioactivity of 0.058mCi/g.

2-Acetamido-4-phenyl[2- ^{14}C]thiazole (I, R = Ph) was prepared similarly from thio[^{14}C]urea (0.190g, 0.025mCi) and phenacyl bromide (0.5g). The product, m.p. 213–216°C (yield 0.4g) had an i.r. spectrum identical with that of an authentic sample. The purity was established by t.l.c. in three different solvent

systems and the product had a specific radioactivity of 0.043mCi/g.

Measurement of radioactivity in biological samples

Radioactive samples were assayed in a Packard Tri-Carb liquid-scintillation counter, model 3320. Samples of urine (5ml) were diluted 1:4 with water and portions (0.5ml) were counted for radioactivity in Bray's (1960) scintillator (10ml). Expired CO_2 was trapped in Hyamine hydroxide (10ml) then it was diluted 1:5 with methanol, and samples (0.5ml) were counted for radioactivity. Total 24 h faeces samples were ground, homogenized in methanol (100ml) and then in water (100ml). The suspensions were centrifuged and a sample (0.5ml) of each supernatant was assayed. Tissues and whole carcasses were dissolved in aq. KOH (30%, w/v) at 20°C for 2 days. Samples (0.5ml) of the resulting suspension were warmed and bleached in counting vials with 100-vol. H_2O_2 (0.25ml). The decolorized solutions were made slightly acidic (pH 5–7) with 10M-HCl and assayed. In all assays, counting efficiencies were determined by using an internal, non-quenching ^{14}C standard and were 65–68%.

Measurement of spectra

U.v. spectra were recorded on a Unicam SP.800 spectrophotometer and i.r. spectra on a Perkin Elmer 21 spectrophotometer. Mass spectra were obtained from either a MS9 or CEC double-focusing mass spectrometer. N.m.r. spectra of metabolite solutions in C^2HCl_3 were determined with a Varian HA 100 spectrometer.

Thin-layer chromatography

T.l.c. was conducted on 20cm $\times$ 20cm plates (E. Merck, Darmstadt, Germany; Kieselgel fluorescent 0.25mm or 2.0mm thick). The solvent systems used for development of chromatograms are shown in Table 1. Plates were developed to a height of 15cm. Spots were located either by being viewed under u.v. light or by spraying the plates with Gibbs reagent (Gibbs, 1927) or with a reagent prepared by mixing equal volumes of 0.1M- $K_2Cr_2O_7$ and acetic acid. Plates were then sprayed with 0.1M- $AgNO_3$. Compounds containing S appeared as yellow spots against a red background (Knight & Young, 1958). The R_f values are shown in Table 1.

Collection and isolation of the metabolites

Male Lodgemoor Wistar rats (200g) were housed in pairs in all-glass metabolism cages with food (Oxoid diet 41B) and water available *ad libitum*. Labelled compounds were administered orally by gavage at 100mg/kg body wt. and urine samples

Table 1. R_F values of metabolites of 2-acetamido-4-methylthiazole, 2-acetamido-4-phenylthiazole and 2-acetamidothiazole on silica gel plates

Spots were located by u.v. light, by Gibb's reagent or by $K_2Cr_2O_7$ -AgNO₃ reagent. The solvent systems were: A, benzene-methanol (9:1, v/v); B, benzene-hexan-1-ol-ethyl acetate (8:1:1, by vol.); C, butan-1-ol-acetic acid water (12:3:5, by vol.); D, propan-1-ol-3% aq. NH₃ (17:3, v/v).

Compound administered	Metabolite	pH of extraction	R_F				Colour with Gibb's reagent
			Solvent A	Solvent B	Solvent C	Solvent D	
2-Acetamido-4-methylthiazole	Acetylthiourea	6.5	0.49-0.55	0.78-0.85	0.82-0.98	0.87-0.90	—
	Unidentified metabolite	6.5	0.31-0.37	0.14-0.23	0.82-0.98	—	—
	2-Acetamido-4-hydroxymethylthiazole	6.5	0.19-0.27	0.08-0.14	0.82-0.98	0.76-0.84	Pale yellow
	5-Acetyl-2-methylthiohydantoic acid	2.0	0 -0.05	0 -0.10	0.68-0.80	0.49-0.62	Orange
	Unidentified metabolite	2.0	—	—	—	0.35-0.41	Yellow
2-Acetamido-4-phenylthiazole	2-Acetamidothiazole-4-carboxylic acid	2.0	0	0	0.30-0.45	0.11-0.19	Pale yellow
	2-Hydroxyacetamido-4-phenylthiazole	6.5	0.47-0.57	0.68-0.75	0.86-0.97	0.77-0.94	Pale yellow
	2-Acetamido-4-(hydroxyphenyl)thiazole	6.5	0.25-0.30	0.62-0.74	0.70-0.79	0.77-0.94	Blue
	Unidentified metabolite	6.5	0.14-0.19	0.19-0.27	0.70-0.79	0.77-0.94	Brown
	Unidentified metabolite	6.5	0.32-0.40	0.79-0.91	0.70-0.79	0.77-0.94	Brown
	Acetylthiourea	6.5	0.49-0.52	0.78-0.85	—	—	—
	5-Acetyl-2-phenylthiohydantoic acid	2.0	0 -0.07	0 -0.10	0.67-0.80	0.63-0.76	Orange
	Glucuronides of compounds I (R = C ₆ H ₄ OH) and III (R = Ph, R' = COCH ₃ OH)	2.0	0 -0.08	0 -0.09	0.37-0.42	0.28-0.36	—
2-Acetamidothiazole	Unidentified metabolite	2.0	0 -0.08	0 -0.09	0.37-0.42	0.38-0.46	—
	Acetylthiourea	6.5	0.49-0.53	0.78-0.85	—	0.86-0.90	—
	5-Acetylthiohydantoic acid	2.0	—	—	0.69-0.75	0.48-0.57	Orange
	Glucuronide of compound III (R = H, R' = COCH ₃ OH) (?)	2.0	0	0	—	—	—

were collected for 24h after administration. Unlabelled compounds were dosed daily at 100mg/kg intraperitoneally for 5 days to groups of six rats. Urine samples were collected daily and stored at -20°C until required.

Samples of urine (10ml) containing radioactive metabolites were extracted at pH6.5 and at pH2.0 with ethyl acetate ($4 \times 50\text{ml}$). The extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness. Residues were dissolved in methanol (0.5ml) and portions ($50\mu\text{l}$) were applied to t.l.c. plates in 8cm bands. The chromatograms were developed in solvent systems A and C (see Table 1). Samples of control urine (10ml) were treated identically. Chromatograms were examined under u.v. light and the total chromatogram was sectioned off into appropriate areas by marking the back of the plate. Radioactive areas were detected with a Panax Radiochromatogram RTLS-1A TLC scanner unit. Each radioactive area of silica gel was scraped off and eluted with methanol (5ml). The suspension was centrifuged and the methanol supernatant was evaporated to small volume, then the eluates were chromatographed again in solvent systems B or D. Single radioactive spots were eluted from the silica gel with water (4ml). The u.v. spectra of the solutions were recorded and portions (0.5ml) were assayed to confirm the presence of ^{14}C radioactivity.

The extraction of metabolites for detailed physico-chemical analysis was conducted by similar methods, with urine (100–150ml) containing unlabelled metabolites. The ethyl acetate extracts were evaporated and the residues dissolved in 0.1M- KH_2PO_4 (5ml). The preliminary separation of unlabelled metabolites was conducted on a column (10cm $\times$ 2cm) packed with Permutit Zeo-Carb 225 cation-exchange resin prepared as follows: the resin (30g) was washed alternately with 1M-NaOH (100ml) and 1M-HCl (100ml). The process was repeated three times and followed by one wash with 0.1M- KH_2PO_4 (pH4.5). The solutions of metabolites, in 0.1M- KH_2PO_4 , were applied to the column and eluted with 0.1M- KH_2PO_4 . Most of the constituents of the urine remained adsorbed on the column. Fractions (3ml) were collected, diluted 1:10 with water and their u.v. spectra determined and compared with those of the corresponding radioactive metabolites.

The quantitative distribution of the radioactive urinary metabolites excreted in a 0–24h sample of urine was examined by t.l.c. of $50\mu\text{l}$ of urine in solvent systems C and D. Chromatograms were examined under u.v. light and appropriate areas of the silica gel were eluted into Bray's (1960) scintillator. The identity of the labelled metabolites was established by comparison of the R_F values of the spots with the R_F values of reference compounds (Table 1).

The metabolites that were extracted at pH2.0, and might therefore be conjugates, were isolated by

evaporation of the ethyl acetate. The presence of metabolites was detected by the t.l.c. spots with R_F values of 0–0.10 in solvent systems A and B and by the detection of ^{14}C radioactivity at these R_F values when labelled compounds had been used. The residues remaining after evaporation of the ethyl acetate were dissolved in 0.1M- Na_2HPO_4 (10ml, pH6.8) and incubated with β -glucuronidase (1mg) at 37°C for 20h. The solutions were extracted with ethyl acetate ($2 \times 50\text{ml}$) and the concentrated extracts were chromatographed in solvent systems A and B.

Metabolism experiments in vitro

Male Wistar rats (150g) received an intraperitoneal injection of phenobarbitone at 100mg/kg 72 and 48 h before the experiments. The animals were killed by cervical fracture and their livers quickly removed, rinsed in ice-cold KCl (1.15%, w/v), blotted and weighed. The livers were homogenized with 2vol. of KCl (1.15%, w/v) in a Waring Blendor and centrifuged at 10000g for 30min at 0°C . The supernatant, containing the microsomal and soluble fractions, was used in the incubation experiments.

Each incubation flask contained 1.0 μmol of NADP^+ , 0.5 μmol of NAD^+ , 20 μmol of glucose 6-phosphate, 60 μmol of MgCl_2 and 6.4 μmol of substrate all dissolved in 0.1M- K_2HPO_4 buffer (50ml, pH7.5), and liver 10000g supernatant (2.0ml). Control flasks contained the above cofactors, from which substrate or liver supernatant was omitted. Incubations were conducted at 37°C for 45min and they were terminated by addition of 2ml of saturated $\text{Ba}(\text{OH})_2$, then the solutions were centrifuged. The supernatants from duplicate incubations were pooled, adjusted to pH8.0 and extracted with ethyl acetate to remove free amines and the unchanged acetamidothiazoles. The solutions were then adjusted to pH3.0 and acidic metabolites were extracted with ethyl acetate for chromatography in solvent systems A and D.

Results

Metabolism of 2-acetamido-4-methylthiazole (I, R = Me)

After an oral dose of 2-acetamido-4-methylthiazole at 100mg/kg, male Lodgemoor Wistar rats excreted 68% of the dose in the urine and 5% in the faeces in 24h. No labelled CO_2 was expired and the remainder of the dose was located in the animal body or intestinal regions (Table 2).

Analysis by t.l.c. of urine extracts containing radioactive metabolites showed three minor radioactive areas, resulting from extractions made at pH6.5, that were resolved in solvent systems A and B (Table 1). In an extract made at pH2.0 one major area and two minor areas were resolved on chromato-

grams developed in solvent systems C and D (Table 1).

The major metabolite was isolated by column chromatography of a pH 2 extract of urine (100 ml). Those fractions with u.v.-absorption maximum at 266 nm (absent from control urine) were pooled and extracted three times at pH 3.0 with diethyl ether. Evaporation of the solvent yielded a brownish solid that crystallized from acetone–water as white needles (m.p. 173–176°C, yield 5 mg).

The i.r. spectrum of this metabolite had an absorption maximum at 1740cm^{-1} , indicative of a carboxylic acid structure. Absorption bands at 1672, 1570 and 1515cm^{-1} indicated that the secondary

amide group was retained. The metabolite gave a negative result in the naphthoresorcinol test for a glucuronide.

The low-resolution mass spectrum of the metabolite showed a molecular ion at m/e 190. After reaction with diazomethane the metabolite gave a molecular ion at m/e 204, indicating that the carboxylic acid function had been converted into the corresponding methyl ester. The loss of keten (42 mass units) in the fragmentation process confirmed the presence of the acetyl amino function (Scheme 1a).

The n.m.r. spectrum contained a doublet peak at $\tau = 8.43$ equivalent to three protons. This signal was

Table 2. Excretion of ^{14}C radioactivity by male Wistar rats

The rats were given 2-acetamido-4-methyl[2- ^{14}C]thiazole and 2-acetamido-4-phenyl[2- ^{14}C]thiazole orally. Values represent 0–24 h recoveries expressed as a percentage of a mean dose of 100 mg/kg body wt. to a group of four rats in each case.

Fraction	Compound administered	...	Recovery	
			2-Acetamido-4-methylthiazole	2-Acetamido-4-phenylthiazole
Urine			68	63
Faeces			5	2
Intestines			18	24
Whole body			2	6
CO_2 expired			0	0
Total recovery			93	95

Scheme 1. Mass-spectral fragmentation of some metabolites

Detection of a metastable ion is represented by m^* . For explanation of parts (a)–(d) see the text. The m/e values are indicated in the Scheme.

assigned to a CH₃ group attached to a C-atom with one proton. A singlet peak equivalent to three protons at $\tau = 7.86$ was attributed to the CH₃ group of the acetyl function. A quintet at $\tau = 6.08$, of relative intensity equivalent to a single proton, slowly collapsed to a quartet after proton exchange in ²H₂O. It was attributed to a proton on a C atom attached to both a CH₃ group and an NH group. At $\tau = 0.41$, a singlet peak equivalent to one proton disappeared on deuteration and was attributed to a CO₂H proton. Poorly resolved signals at $\tau = -1.00$ (doublet) and at $\tau = -1.20$ (singlet) each equivalent to single protons, disappeared after deuteration. The signals were assigned to the imine protons of part structures -CHMe-NH-CO- and -CS-NH-CO- respectively (Table 3). The suggested structure of the metabolite

was 5-acetyl-2-methylthiohydantoic acid (II, R = Me). The m.p., *R_F* values in systems C and D (Table 1) and u.v.-absorption maxima (Table 4) were identical with those of authentic 5-acetyl-2-methylthiohydantoic acid.

Two other metabolites of the compound (I, R = Me) were identified by comparison of their physicochemical characteristics with those of reference compounds. Thus a minor metabolite extractable at pH 6.5 had the same *R_F* values in systems A, B, C and D as had 2-acetamido-4-hydroxymethylthiazole (I, R = CH₂OH). The u.v.-absorption maximum in aqueous solution (269nm) was identical with that of the authentic compound (Table 4). An acidic minor metabolite extractable at pH 2.0 had the same *R_F* values in systems A, B, C and D as 2-

Table 3. *N.m.r. spectrum in C²HCl₃ of 5-acetyl-2-methylthiohydantoic acid*

Chemical shift (τ)	Multiplicity	Exchange with ² H ₂ O	Number of protons	Assignment
8.43	Doublet	None	3	CH ₃ -CH-
7.86	Singlet	None	3	CH ₃ -CO-
6.08	Quintet	Slow, quartet	1	CH ₃ -CH-NH
-0.41	Singlet	Rapid	1	-CO ₂ H
-1.00	Doublet	Slow	1	CH ₃ -CH-NH-
-1.20	Singlet	Rapid	1	CH ₃ -CO-NH-CS-

Table 4. *U.v. spectral data for metabolites of 2-acetamido-4-methylthiazole (I, R = Me), 2-acetamido-4-phenylthiazole (I, R = Ph) and 2-acetamidothiazole (I, R = H)*

Compound administered	Metabolite	pH ...	$\lambda_{\max.}$ in H ₂ O (nm)		
			Neutral	Acid	Alkaline
2-Acetamido-4-methylthiazole	Acetylthiourea (IV)		267	267	236*
	2-Acetamido-4-hydroxymethylthiazole (I, R = CH ₂ OH)		269	272	287
	5-Acetyl-2-methylthiohydantoic acid (II, R = Me)		266	266	237
	2-Acetamidothiazole-4-carboxylic acid (I, R = CO ₂ H)		270	256	268
2-Acetamido-4-phenylthiazole	2-Hydroxyacetamido-4-phenylthiazole (III, R = Ph, R' = COCH ₂ OH)		264	—	276
	2-Acetamido-4-(hydroxyphenyl)thiazole (I, R = C ₆ H ₄ OH)		268	—	285
	2-Amino-4-phenylthiazole (?)		270	—	264
	5-Acetyl-2-phenylthiohydantoic acid (II, R = Ph)		267	—	239
	Unidentified metabolite		265	—	273
2-Acetamidothiazole	5-Acetylthiohydantoic acid (II, R = H)		265	265	237
	2-Hydroxyacetamidothiazole glucuronide (?)		265	—	285

* Rapid hydrolysis to thiourea, $\lambda_{\max.}$ 236nm.

acetamidothiazole-4-carboxylic acid (Table 1) and the same absorption maxima in aqueous solution at pH 7.0 and 10.0 (Table 4).

The structure of a third acidic minor metabolite (R_F 0.35–0.41 in system D) was not determined. Incubation of this metabolite with β -glucuronidase did not release an aglycone.

Metabolism of 2-acetamido-4-phenylthiazole (I, R = Ph)

The pattern of excretion of ^{14}C radioactivity for this compound in the rat was similar to that observed with the 4-methyl compound. After compound I ($R = \text{Ph}$) was administered orally at 100 mg/kg, 63% of the dose was recovered in the 24 h urine sample, 24% remained in the intestinal regions and 2% was excreted in the faeces (Table 2).

Eight radioactive metabolites were resolved by t.l.c. Five of these were extracted at pH 6.5 and three at pH 2.0 (Table 1).

A non-labelled sample of a minor metabolite (III, $R = \text{Ph}$, $R' = \text{COCH}_2\text{OH}$) was purified by preparative t.l.c. in system A and had a u.v.-absorption maximum at 266 nm in aqueous solution. A molecular-ion peak appeared at m/e 234 in a low-resolution mass spectrum of the sample. The molecular-ion peak of the parent compound (I, $R = \text{Ph}$) appeared at m/e 218 and the increase of 16 mass units in the molecular ion indicated addition of an oxygen atom to the molecule (Scheme 1c). A metastable peak at m/e 132.4 in the molecular fragmentation pattern confirmed the loss of 58 mass units from m/e 234 to m/e 176. This fragment of $m/e = 58$ corresponded to the loss of the keten fragment ($m/e = 42$) from all the other acetamido compounds. The increase of 16 mass units indicated that addition of oxygen had occurred in the acetyl portion of compound I ($R = \text{Ph}$) to give a glycollylamide (III, $R = \text{Ph}$, $R' = \text{COCH}_2\text{OH}$). An

ion at m/e 134 corresponding to $\text{C}_6\text{H}_5-\text{C}(\text{S})=\text{CH}^+$ confirmed that oxidation had not occurred on the phenyl group or in the 5 position of the thiazole ring.

Another metabolite, $R_F = 0.25\text{--}0.30$ in solvent system A also had a molecular-ion peak at 234 mass units but loss of 42 mass units in the fragmentation pattern confirmed the integrity of the acetyl function and indicated oxidation on either the 5 position of the thiazole ring or on the phenyl group (Scheme 1d). The fragment ion m/e 134 was absent and instead a fragment ion at m/e 150, corresponding to $\text{C}_6\text{H}_5\text{SO}$, appeared. The reaction of this metabolite with Gibbs spray reagent produced a blue colour, indicating oxidation in the phenyl ring. The indicated structure of this metabolite was therefore 2-acetamido-4-(hydroxyphenyl)thiazole (I, $R = \text{C}_6\text{H}_4\text{OH}$).

A minor metabolite, extracted at pH 6.5, of R_F value 0.14–0.19 in solvent system A (Table 1), was not

identified. Another minor metabolite, R_F 0.32–0.40, gave a colour with Gibbs reagent (brown) that was characteristic of amines. The u.v.-absorption maximum (270 nm) suggested that it might be the deacetylated form of one of the other metabolites.

The acidic major metabolite was isolated from the non-radioactive urine extract by preparative t.l.c. in solvent system D. A mass spectrum of the sample showed the molecular ion (m/e 252) and fragmentation pattern (m/e 252 $\rightarrow$ m/e 234 $\rightarrow$ m/e 192) identical with those obtained from a sample of authentic 5-acetyl-2-phenylthiohydantoic acid. The structure of this metabolite was therefore established as 5-acetyl-2-phenylthiohydantoic acid (II, $R = \text{Ph}$).

Chromatography of the acidic radioactive urine extract in solvent system D resolved two slower-running areas. These were separately eluted from the silica gel into phosphate buffer pH 6.8 and incubated with β -glucuronidase (1 mg) for 20 h. The solutions were extracted with ethyl acetate, then evaporated to small volume and the residue was chromatographed in solvent systems A and B.

The metabolite area R_F 0.28–0.36 in solvent system D produced two radioactive aglycones. The slower-running aglycone in solvent systems A and B co-chromatographed with the metabolite of structure I ($R = -\text{C}_6\text{H}_4\text{OH}$) (R_F 0.25–0.30 and 0.64–0.74 respectively). It also formed a blue spot when sprayed with Gibbs reagent. The faster-running aglycone had the same R_F in system A and B and gave the same yellow colour reaction with Gibbs reagent as did the metabolite of structure III ($R = \text{Ph}$, $R' = \text{COCH}_2\text{OH}$) (Table 1). The radioactive area on the thin-layer chromatograms corresponding to this metabolite was therefore a mixture of two glucuronides, the aglycones of which were 2-hydroxyacetamido-4-phenylthiazole and 2-acetamido-4-(hydroxyphenyl)thiazole.

Acetylthiourea (IV) was also identified as a minor metabolite of the phenyl compound by the u.v. and i.r. spectra and by the occurrence of the molecular ion at m/e 118 in the mass spectrum. The identification was confirmed by comparison with an authentic sample of acetylthiourea.

One minor metabolite, extracted at pH 2.0 was not identified (Table 1).

Metabolism of 2-acetamidothiazole (I, R = H)

An acidic metabolite of 2-acetamidothiazole was isolated from rat urine (100 ml) after intraperitoneal administration of the compound to rats. The metabolite isolated by column chromatography, as for the methyl compound, was obtained as a white solid, m.p. 191–196°C (yield 2 mg) and gave a u.v.-absorption maximum at 264.5 nm in aqueous solution. In 1 M NaOH the maximum appeared at 236 nm. The i.r. spectrum indicated a carboxylic acid function

(1720 cm^{-1}) in addition to the secondary amide group (bands at 1690, 1550, 1420 and 1250 cm^{-1}). The mass spectrum had a molecular ion at m/e 176 and a fragmentation pattern similar to that shown by the major acidic metabolite of the methyl compound. After proton exchange in $^2\text{H}_2\text{O}$ the molecular ion appeared at m/e 179, the increase of 3 mass units indicating that three protons were exchangeable with ^2H . These are the carboxyl and amide protons in the structure, 5-acetylthiohydantoic acid (II, $\text{R} = \text{H}$), suggested for the metabolite. This structure was confirmed by the identical R_F values of the metabolite and the authentic material in systems C and D and by the identical u.v.-absorption maxima in neutral and alkaline aqueous solutions (Table 4). The identification was confirmed by comparison of the i.r. spectra.

Acetylthiourea was detected as a minor metabolite of 2-acetamido-2- ^{14}C thiazole on t.l.c. The formation of this metabolite from the unlabelled compound was subsequently proved by t.l.c. and by u.v. and mass spectroscopy of the material isolated from the t.l.c. plate.

A very small proportion of the ^{14}C radioactivity ($\pm 1\%$) was detected as a base-line spot in solvent systems A and B. The corresponding spot, obtained as a metabolite of the unlabelled compound, was extracted and the material was incubated with β -glucuronidase. The aglycone was extracted with ethyl acetate but the amount of metabolite did not allow determination of its i.r. spectrum. The aglycone, R_F 0.37 in solvent system A, had a u.v. spectrum (Table 4) very similar to that of the original compound, 2-acetamidothiazole, which has $\lambda_{\text{max.}} = 267$ and 287 in water and in 0.1M-NaOH respectively. This similarity suggested that the thiazole ring was still intact and that the aglycone may be 2-hydroxy-acetamidothiazole (III, $\text{R} = \text{H}$, $\text{R}' = \text{COCH}_2\text{OH}$).

Metabolism in vitro

2-Acetamido-4-methylthiazole (I, $\text{R} = \text{Me}$) was metabolized to four compounds (Table 5). The main metabolite accounted for 5% of the ^{14}C radioactivity recovered from the chromatogram. The R_F value in solvent system A (0.45–0.51) and colour reaction with Gibbs reagent (mauve) identified it as 2-amino-4-methylthiazole (III, $\text{R} = \text{Me}$, $\text{R}' = \text{H}$). A second metabolite ($\pm 2\%$), R_F 0.22–0.27 in system A, gave a pale-yellow colour with Gibbs reagent and was identified as 2-acetamido-4-hydroxymethylthiazole (I, $\text{R} = \text{CH}_2\text{OH}$). A small spot running just behind this spot (R_F 0.16–0.21) and giving a mauve colour with Gibbs reagent was the de-acetylated form, 2-amino-4-hydroxymethylthiazole. About 2% of the recovered activity remained at the base-line, and when chromatographed in systems C and D had the same R_F as 5-acetyl-2-methylthiohydantoic acid (II, $\text{R} = \text{Me}$) (0.70–0.80 and 0.50–0.62 respectively). The metabolite produced an orange colour with Gibbs reagent and had u.v.-absorption maxima in aqueous solution at pH 7.0 and pH 14.0 (266 nm and 237 nm respectively) identical with those of the authentic compound (II, $\text{R} = \text{Me}$) (Table 4).

Similar transformations were observed with 2-acetamido-4-phenylthiazole (I, $\text{R} = \text{Ph}$) (Table 5). Five radioactive areas were detected. Unchanged substrate accounted for 43% of the radioactivity recovered. 2-Amino-4-phenylthiazole (III, $\text{R} = \text{Ph}$, $\text{R}' = \text{H}$) (40%), 2-acetamido-4-(hydroxyphenyl)thiazole (I, $\text{R} = \text{C}_6\text{H}_4\text{OH}$) (6%), and the corresponding amine (6%) were identified by their R_F values and colour reactions. About 4% of the recovered radioactivity, R_F values 0.05, 0.73 and 0.68 in systems A, C and D respectively, co-chromatographed with 5-acetyl-2-phenylthiohydantoic acid (II, $\text{R} = \text{Ph}$) in these solvents.

Table 5. *Metabolism of 2-acetamido-4-methyl-2- ^{14}C thiazole and 2-acetamido-4-phenyl-2- ^{14}C thiazole by the 10000g supernatant of rat liver homogenate*

Values represent % of total radioactivity recovered from separation by t.l.c. in solvent system A (Table 1) after removal of most of the unchanged substrate by solvent extraction from protein-free supernatant.

Compound administered	Metabolite	^{14}C recovered (%)	R_F range	Colour with Gibbs reagent
2-Acetamido-4-methyl-2- ^{14}C thiazole	2-Acetamido-4-methylthiazole	90	0.63–0.69	Yellow
	2-Amino-4-methylthiazole	5	0.45–0.51	Mauve
	2-Acetamido-4-hydroxymethylthiazole	1	0.22–0.27	Yellow
	2-Amino-4-hydroxymethylthiazole	1	0.16–0.21	Mauve
	5-Acetyl-2-methylthiohydantoic acid	2	0 –0.08	Orange
2-Acetamido-4-phenyl-2- ^{14}C thiazole	2-Acetamido-4-phenylthiazole	43	0.67–0.73	Yellow
	2-Amino-4-phenylthiazole	40	0.52–0.58	Mauve
	2-Acetamido-4-(hydroxyphenyl)thiazole	6	0.34–0.40	Blue-grey
	2-Amino-4-(hydroxyphenyl)thiazole	6	0.24–0.30	Mauve
	5-Acetyl-2-phenylthiohydantoic acid	4	0 –0.09	Orange

No evidence was found from the experiments *in vitro* for the presence of the hydroxymethyl compound (III, R = Ph, R' = COCH₂OH). Moreover, although deacetylation represented a major pathway for metabolism *in vitro*, no thiohydantoic acid, resulting from deacetylation of the acetylthiohydantoic acid, was found.

Discussion

Identification of the acetylthiohydantoic acids as the major metabolites of the thiazoles was the most important factor in defining the metabolic pathway. I.r. spectra proved useful in confirming some of the identifications but the u.v. spectra of many of the metabolites were so similar that they provided little information about chemical structures. It was apparent from the i.r. spectra that the amide grouping was still intact but the positive identification of the acetylthiohydantoic acids depended on the n.m.r. and mass spectra.

The most important ion peaks in the mass spectrum of the metabolite (II, R = Me) from 2-acetamido-4-methylthiazole, are shown in Scheme 1(a). Replacement of three protons by deuterium, to give a molecular ion at $m/e = M+3$, suggested at first sight a hydroxylated structure, the third proton being in the amide group. The n.m.r. spectrum, however, did not allow the presence of a hydroxylated thiazole ring (Table 3). The occurrence of the double coupled proton in -MeCH-NH- as a quintet was particularly significant because it proved that the aromatic structure (as in compound I) was no longer intact. [The 4-methyl proton signal of compound (I, R = Me)

occurs as a singlet at $\tau = 7.63$.] The total number of protons, ten, was too great to be accounted for by a hydroxylated thiazole structure. The reaction of this metabolite with diazomethane to give a compound with an m/e of 14 mass units greater, finally demonstrated that the metabolite was indeed the carboxylic acid of structure II (R = Me). The phenyl and hydrogen analogues also gave metabolites with n.m.r. and mass spectra that were consistent with the structures (II, R = Ph) and (II, R = H) respectively. In all three cases, most of the administered drug was excreted in the form of the acetylthiohydantoic acids (Table 6). The other metabolites, whether derived directly from the thiazole administered or by degradation of the acetylthiohydantoic acids, represented only minor pathways for metabolism. An unexpected finding was the hydroxylation of the acetamido group of 2-acetamido-4-phenylthiazole. This type of hydroxylation has been reported previously (Kiese & Lenk, 1968) but there is no previous report of glycollylamide formation from an acetamido compound in the rat.

With the exception of the glucuronides, the major metabolites produced *in vivo* were detected when compounds (I, R = Me) and (I, R = Ph) were incubated with liver homogenate, though, as might be expected, hydrolysis of the acetyl group is a more significant route *in vitro*. The processes *in vitro* were not very efficient, as measured by the recovery of unchanged compound, due probably to the low solubility of the substrates in the incubation medium.

The similarity of the metabolic pathway for all three compounds suggests that the ring-scission reaction proceeds by a similar mechanism in each case.

Table 6. Distribution of metabolites of 2-acetamido-4-methylthiazole (I, R = Me), 2-acetamido-4-phenylthiazole (I, R = Ph) and 2-acetamidothiazole (I, R = H) in rat urine, over 24h, after a single dose at 100mg/kg

For details see the text.

Compound administered	Metabolites	% of dose
2-Acetamido-4-methylthiazole	5-Acetyl-2-methylthiohydantoic acid (II, R = Me)	47
	2-Acetamidothiazole-4-carboxylic acid (I, R = CO ₂ H)	10
	2-Acetamido-4-hydroxymethylthiazole (I, R = CH ₂ OH)	5
	Acetylthiourea (IV)	5
	Unidentified metabolites	<5
2-Acetamido-4-phenylthiazole	5-Acetyl-2-phenylthiohydantoic acid (II, R = Ph)	34
	2-Hydroxyacetamido-4-phenylthiazole (III, R = Ph, R' = COCH ₂ OH)	7
	2-Acetamido-4-(hydroxyphenyl)thiazole (I, R = C ₆ H ₄ OH)	7
	Acetylthiourea (IV)	<1
	Unidentified metabolites	6
2-Acetamidothiazole	5-Acetylthiohydantoic acid (II, R = H)	60
	Acetylthiourea (IV)	2
	Unknown glucuronide*	1

* Possibly of 2-hydroxyacetamidothiazole on basis of u.v. spectrum.

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References

- Bhargava, P. N. & Singh, P. R. (1960) *J. Indian Chem. Soc.* **37**, 241–244
- Bousquet, W. F., Rogler, J. C., Knevel, A. M., Spahr, J. & Christian, J. E. (1965) *J. Agr. Food. Chem.* **13**, 571–573
- Bray, C. (1960) *Anal. Biochem.* **1**, 279–285
- Elmore, D. T. & Toseland, P. A. (1957) *J. Chem. Soc. London* 2460–2466
- Evans, D. (1972) *Brit. Patent* 1290841
- Gibbs, H. D. (1927) *J. Biol. Chem.* **72**, 649–664
- Kiese, M. & Lenk, W. (1968) *Biochem. Pharmacol.* **20**, 379–391
- Knight, R. H. & Young, L. (1958) *Biochem. J.* **70**, 111–119
- Perrault, M. & Bovet, D. (1946) *Lancet* **i**, 731–734
- Traumann, K. (1960) *Ann. Chem.* **249**, 38–42