Biochemical Pharmacology, 1965, Vol. 14, pp. 457–466. Pergamon Press Ltd., Printed in Great Britain.

THE BIOTRANSFORMATION OF A SULFONAMIDE TO A MERCAPTAN AND TO MERCAPTURIC ACID AND GLUCURONIDE CONJUGATES*

D. F. COLUCCI and D. A. BUYSKE

Department of Chemical Pharmacology, Lederle Laboratories,
American Cyanamid Company, Pearl River, N.Y., U.S.A.

(*Received* 11 *September* 1964; *accepted* 16 *November* 1964)

Abstract—Benzothiazole-2 sulfonamide undergoes complete metabolism by the rat, rabbit, and dog to form three urinary metabolites conclusively identified by u.v. spectra, radiochemical analysis, and paper chromatography with authentic compounds as benzothiazole-2 mercapturic acid, benzothiazole-2 mercaptan, and benzothiazole-2 thioglucuronic acid. These products result from replacement of the sulfonamide by glutathione to form the intermediate tentatively identified as benzothiazole-2 glutathione. This intermediate then undergoes a multiple step reaction including the loss of two of the amino acids of the tripeptide followed by acetylation of the cysteine to form the mercapturic acid conjugate as a major metabolite. A cleavage of a sulfur-carbon bond of the benzothiazole-2 glutathione conjugate (or cysteine derivative) results in formation of a mercaptan group at a point where the sulfonamide was originally attached. Most of this mercaptan forms a thio ester with glucuronic acid before it is excreted in the urine.

BENZOTHIAZOLE-2 SULFONAMIDE initially emerged as a compound of high interest because it inhibited carbonic anhydrase in an *in-vitro* test procedure at a concentration that was well below that of a series of heterocyclic sulfonamides which were being evaluated as diuretic agents. However, when administered i.v. to dogs the expected diuresis usually caused by carbonic anhydrase inhibition was not produced.

Clapp[1] was able to show that the drug was extensively metabolized by the dog and that only a small fraction of the administered dose appeared in the urine. He was further able to isolate a metabolite which was chemically characterized as a glucuronic acid conjugate of benzothiazole-2 mercaptan. It was suggested that the metabolic sequence of events was the reduction of the sulfonamide to a sulfhydryl which in turn underwent a glucuronidation to form the isolated product.

The metabolic conversion of any sulfonamide group is sufficiently unusual to warrant further interest. In addition, the implied mechanism of a reduction of a sulfonamide to a mercaptan and then glucuronidation of the sulfhydryl required two biochemical reactions which are intriguingly unique and that seemed to warrant detailed investigation. This paper describes experiments designed to clarify the mechanism of these suggested biotransformations.

* Presented in part at the meeting of the Federation of American Societies for Experimental Biology, April 1964.

MATERIALS AND METHODS

Benzothiazole-2 sulfonamide, containing sulfur-35 in the sulfonamide group, was prepared by the usual sulfonamide chemistry methods, starting with ^{35}S-labeled benzothiazole-2 mercaptan obtained from the French Atomic Energy Commission.

Benzothiazole-2 glutathione, containing ^{35}S at the 2-position, was prepared by the reaction of benzothiazole-2 chloride with ^{35}S-labeled glutathione in the presence of metallic sodium in a liquid ammonia solution. The ^{35}S-labeled glutathione was obtained from Schwarz BioResearch, Inc.

Benzothiazole-2 cysteine was prepared by the reaction of benzothiazole-2 chloride with cysteine in the presence of metallic sodium in a liquid ammonia solution.

Benzothiazole-2 N acetyl cysteine (benzothiazole-2 mercapturic acid) was prepared by direct acetylation of benzothiazole-2 cysteine with acetic anhydride.

Benzothiazole-2 mercaptoglucuronide was obtained from J. W. Clapp, American Cyanamid Co., Stamford, Conn.

In-vivo metabolism

Adult male albino rats from the Lederle colony were given i.p. injections of benzothiazole-2 sulfonamide-^{35}S (100 mg/kg); benzothiazole-2 mercaptan-^{35}S (50 mg/kg); benzothiazole-2 glutathione-^{35}S (130 mg/kg); or benzothiazole-2 cysteine (75 mg/kg). Urines from these animals were quantitatively collected for up to 2 weeks and examined by radiometric, spectrophotometric, and paper chromatographic techniques as described below.

Reaction of benzothiazole-2 sulfonamide with glutathione in boiled and unboiled fortified rat liver supernatant

An adult albino rat was decapitated and its liver promptly excised and homogenized with 2·5 volumes of cold 0·2 M KH_2PO_4 buffer (pH 7·4) in a Lourdes homogenizer. The homogenate was centrifuged in a Spinco ultracentrifuge at 9,000 *g* for 30 min.

Fifty ml of a coenzyme solution was prepared by mixing 5 ml 0·04 M magnesium chloride, 15 ml 0·2 M KH_2PO_4 buffer (pH 7·4), 15 ml 0·25 M sucrose and 0·04 M nicotinamide solution, 2 ml 0·3 M glucose-6-phosphate, 2 ml 0·3 M sodium lactate, 2 ml 0·01 M NADP, 2 ml 0·01 M NAD, and 7 ml 0·1 M ATP.

Twenty ml of the coenzyme solution was added to the supernatant and the volume diluted to 60 ml. To a 15-ml portion was added 7·5 mg benzothiazole-2 sulfonamide and 9 mg of reduced glutathione. The same concentrations of benzothiazole-2 sulfonamide and glutathione were added to 15 ml of boiled liver supernatant.

Both samples were incubated at 37° and 5-ml aliquots withdrawn at 2, 30, and 60 min, extracted with chloroform, and the amount of nonreacted benzothiazole-2 sulfonamide quantitated by spectrophotometric and paper chromatographic methods as described below.

Reaction of benzothiazole-2 sulfonamide with glutathione

Thirty mg reduced glutathione was added to 50 ml phosphate buffer (pH 7·4) containing 25 mg benzothiazole-2 sulfonamide. The solution was incubated at 37° and 5-ml aliquots withdrawn at hourly intervals. These aliquots were extracted with

chloroform. The amount of benzothiazole-2 sulfonamide in the chloroform extract was quantitated by u.v. absorption at 268 mμ.

The aqueous layer after extraction was quantitated for its benzothiazole-2 glutathione content by measuring u.v. absorption at 280 mμ.

Metabolism of benzothiazole-2 cysteine and benzothiazole-2 glutathione-^{35}S by rat liver fractions

Whole homogenates. An adult albino rat was decapitated and the liver promptly excised and placed on ice. The liver was homogenized in a Lourdes homogenizer with 2 volumes of cold 0·2 M KH_2PO_4 buffer, (pH 7·4). The incubation mixture contained 2 ml liver homogenate, 5 ml coenzyme solution (pH 7·4), and 2 mg of either of the benzothiazole derivatives. Samples were incubated at 37° for 1 hr, extracted with chloroform, concentrated to dryness, taken up in methanol, and compared in the paper chromatographic systems with authentic compounds. Samples containing everything except the homogenate were treated in an identical manner and served as controls.

Intracellular fractions. Two adult albino rats were decapitated and the livers excised immediately. Each liver was homogenized in a Lourdes homogenizer with 50 ml cold 0·25 M sucrose. The homogenates were combined and diluted with 0·25 M sucrose for a 1 : 9 dilution.

The homogenates were centrifuged at 8,700 *g* in a Spinco ultracentrifuge for 15 min. The supernatant was carefully drawn off and kept cold. The sediment, containing unbroken cells, nuclei, and mitochondria, was shaken up with 0·25 M sucrose and recentrifuged at 8,700 *g* for 20 min. After discarding the washings, the mitochondrial fraction was resuspended in 35 ml of 0·25 M sucrose.

The supernatant, meanwhile, was centrifuged at 105,000 *g* for 60 min. The resultant clear solution was carefully drawn off and termed the soluble fraction. The residue was shaken up with 0·25 M sucrose and centrifuged at 105,000 *g* for 30 min. The wash was discarded and the residue, termed the microsomal fraction, was suspended in 30 ml 0·25 M sucrose.

Two mg each of substrate and benzothiazole-2 cysteine or benzothiazole-2 glutathione-^{35}S, plus 2 ml of coenzyme solution (pH 7·4) were incubated with 4-ml portions of the fractionated liver. Control samples contained no liver fractions. All samples were incubated for 1 hr at 37°. After incubation all samples were extracted with chloroform. The chloroform was evaporated and the residue dissolved in a measured amount of methanol. The u.v. absorption at 325 mμ due to benzothiazole-2 mercaptan was recorded and quantitated. The methanolic solutions were compared in paper chromatographic systems with authentic compounds. The localization of the various benzothiazole derivatives by radiometric and chemical procedures was similar to that described below.

Procedure for the extraction and identification of metabolites from the urine of rat, rabbit, and dog receiving various benzothiazole derivatives

Five-ml portions of the 24-hr predose and postdose urines were extracted with 25 ml chloroform. The chloroform was evaporated and the residue dissolved in methanol. The u.v. spectrum of this methanolic solution was recorded on a Cary model 14

recording spectrophotometer. The same solutions were concentrated and preliminary paper chromatograms run in order to remove extraneous urinary constituents. The eluates from the paper strips were then rerun in various paper chromatographic systems and compared with authentic compounds (Table 1).

TABLE 1. R_f VALUES OF AUTHENTIC COMPOUNDS AND URINARY METABOLITES IN VARIOUS CHROMATOGRAPHIC SYSTEMS*

	System			
	1	2	3	4
Authentic compounds				
Benzothiazole-2 sulfonamide	0·59	0·93	0·40	
Benzothiazole-2 mercaptan	0·66	0·93	0·75	
Benzothiazole-2 N-acetyl cysteine†	0·54	0·88	0·10	
Benzothiazole-2 mercaptoglucuronide	0·20	0·65	0·00	
Benzothiazole-2 cysteine	0·44	0·74	0·00	
Benzothiazole-2 glutathione	0·03	0·50	0·00	
Sulfate (ammonium)	0·00	0·00	0·00	0·90
Metabolites‡ appearing in urine from rat receiving benzothiazole-2 sulfonamide-^{35}S				
Benzothiazole-2 mercaptan	0·66	0·93	0·75	
Benzothiazole-2 N-acetyl cysteine†	0·54	0·88	0·10	
Benzothiazole-2 mercaptoglucuronide	0·20	0·65	0·00	
Sulfate	0·00	0·00	0·00	0·90

* Systems: 1, *n*-butanol : ammonium hydroxide : water, 4 : 1 : 5; 2, *n*-butanol : acetic acid : water, 4 : 1 : 5; 3, benzene : petroleum ether : methanol : water, 12 : 8 : 13 : 7; 4, isopropanol : ammonium hydroxide : water, 1 : 1 : 18.

† Commonly referred to as mercapturic acid.

‡ These urinary metabolites were selectively eluted from preliminary chromatographs of the urine extracts and rechromatographed to minimize streaking due to normal urinary constituents.

The u.v.-absorbing area for benzothiazole-2 mercaptan on the developed chromatogram was eluted with methanol and the u.v. spectrum recorded (Fig. 1). In order to locate metabolites containing a sulfhydryl group, chromatograms were dipped into an alcoholic solution of N-ethyl maleimide, followed by a dip into alcholic potassium hydroxide.[2] Benzothiazole-2 mercaptan, isolated in this urinary fraction, was detected as a pink area on a white background.

The previously extracted urine was acidified to pH 3 with 1 N HCl and extracted again with chloroform. The u.v. spectrum and chromatography of this extract was treated like the first extract. Benzothiazole-2 mercapturic acid isolated in this acidified urinary fraction was detected by its u.v. absorption on paper chromatograms and its characteristic u.v. spectrum (Fig. 1) when eluted with methanol. In addition, the metabolite showed the characteristic yellow color on a brown background of mercapturic acids when the chromatogram was sprayed first with 0·1 M $K_2Cr_2O_7$: acetic acid (1 : 1) and then 0·1 M $AgNO_3$.[3] The urinary metabolite and authentic benzothiazole-2 mercapturic acid were positive to ninhydrin only after acid hydrolysis. These two deacetylated compounds migrated in two chromatographic systems in an identical manner.

The acidified, chloroform-extracted urine was further extracted with *n*-butanol that had been previously saturated with water. This extract was concentrated and

applied to paper chromatograms. The u.v.-absorbing area for benzothiazole-2 mercaptoglucuronide isolated in this urinary fraction was eluted with water and its u.v. spectrum recorded (Fig. 1). This eluate was divided into two portions. One half was treated with β-glucuronidase preparation from beef liver (Ketodase, Warner-Chilcott) at pH 5 and incubated at 37° for 1 hr. The second portion was acidified to 2 N HCl and heated at 100° for 20 min. Both treated portions were extracted with chloroform. Paper chromatography of the extract and subsequent u.v. absorption spectra and treatment of the u.v.-absorbing areas with N-ethyl maleimide reagent gave positive proof for benzothiazole-2 mercaptan as the product of the enzymatic and acid hydrolysis. These experiments conclusively confirm that the glucuronide

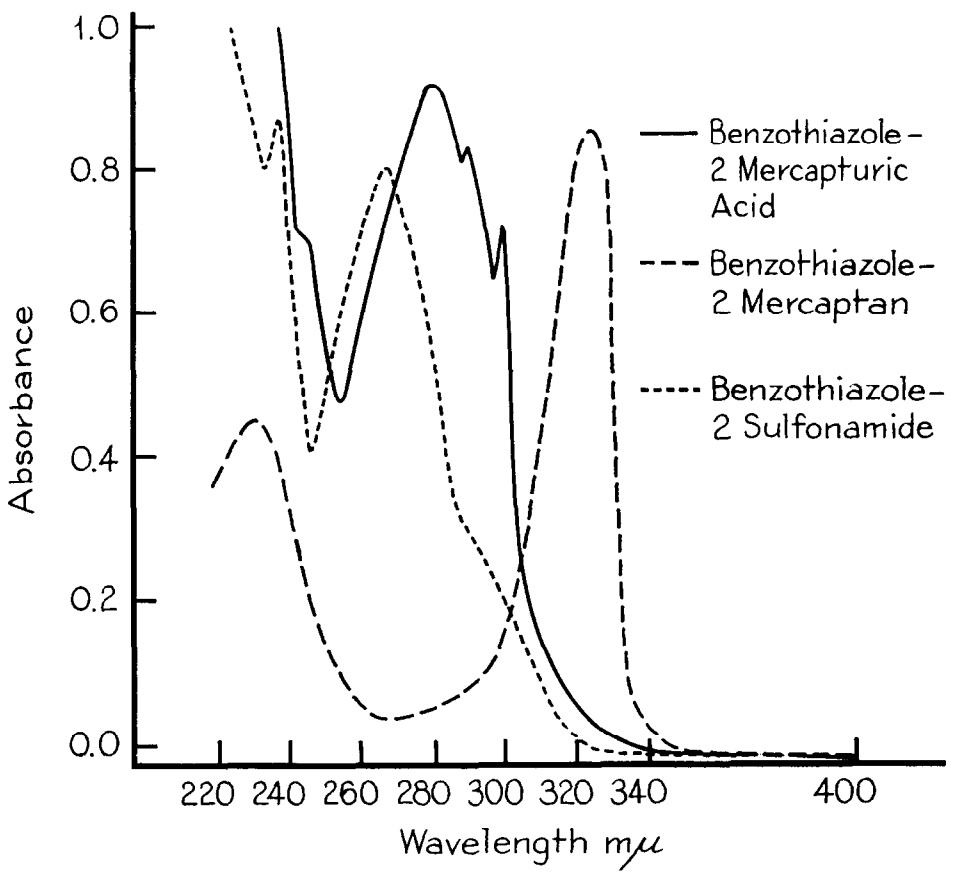

FIG. 1. The spectra of benzothiazole derivatives in methanol. The spectrum for benzothiazole-2 mercapturic acid is also typical for benzothiazole-2 cysteine, benzothiazole-2 glutathione, and benzothiazole-2 mercaptoglucuronide except that the fine structure bands at 290 and 300 mμ are not so pronounced.

of benzothiazole-2 mercaptan is a metabolite formed from benzothiazole-2 sulfonamide.

The urine remaining after all extractions was chromatographed (Table 1). Inorganic sulfate was detected as a yellow color on a red background when sodium rhodizonate was applied as an overspray to an initial barium chloride spray.[4]

Determination of radioactivity in metabolites

Radioactivity was determined by use of a liquid scintillation spectrometer (Packard Instrument Co.). Radioactive areas on paper chromatograms were located, and the amount present was quantitatively determined by appropriate segmentation and dicing of the paper strips. Counting was done either directly or after elution of the activity in a suitable solvent.

Location and recording of u.v. absorption due to metabolites

A major tool in the identification of these metabolites was their characteristically different different absorption spectra. Ultraviolet-absorbing areas were located on chromatograms under a Mineralight short-wave lamp (2,537 Å). The recording of u.v. absorption was done on a Cary model 14 recording spectrophotometer in the 200–400 mμ range. The u.v. spectra of compounds were compared in methanol, chloroform, acid, and base. One distinguishing feature of benzothiazole-2 mercaptan was a hypsochromic shift from 325 mμ in acid to 308 mμ in base.

RESULTS AND DISCUSSION

Metabolism of benzothiazole-2 sulfonamide-^{35}S in the rat

After administration of ^{35}S-labeled benzothiazole-2 sulfonamide to the rat, 59% of the radioactivity was recovered in the urine after the first 24 hr. A total of 83% was recovered in the urine over a period of 2 weeks. After isolation, chromatography and identification of the urinary metabolites as benzothiazole-2 mercaptan, benzothiazole-2 mercapturic acid, and benzothiazole-2 mercaptoglucuronide, it was determined by quantitative radiometric assay that none of these benzothiazole metabolites was radioactive. No parent radioactive sulfonamide was recovered from the urine. The radioactivity in the urine was due to radioactive inorganic sulfate.

The three benzothiazole metabolites (containing no radioactivity) were also found in the urines of a rabbit and a dog that had been dosed with benzothiazole-2 sulfonamide-^{35}S.

From the data of the experiments reported it was apparent that the sulfonamide group was completely cleaved from the benzothiazole moiety. It therefore was of interest to determine the origin of the carbon–sulfur covalent bond at the 2-position in the three benzothiazole metabolites.

Metabolism of benzothiazole-2 glutathione-^{35}S and benzothiazole-2 cysteine in the rat

Mercapturic acid conjugates of drugs are known to occur[3, 5–7] and appear to be formed from an initial conjugation with glutathione. In order to ascertain if a benzothiazole glutathione were the source of the mercapturic acid metabolite, a rat was dosed with benzothiazole-2 glutathione-^{35}S. Careful examination of the first 24-hr urine by paper chromatographic and liquid scintillation counting techniques revealed the same three benzothiazole metabolites produced when the benzothiazole-2 sulfonamide was given. An important difference was that all three metabolites retained their radioactive sulfur at the 2-position.

When benzothiazole-2 cysteine was administered to the rat the acetylated derivative, benzothiazole-2 mercapturic acid, and benzothiazole-2 mercaptoglucuronide were isolated from the first 24-hr urine sample as major metabolites. Benzothiazole-2 mercaptan was also evident but as a minor metabolite.

From these experiments it appeared probable that the thiol group of glutathione or cysteine was the origin of the sulfur atom at the 2-position of the benzothiazole-containing metabolites formed from the administration of benzothiazole-2 sulfonamide.

In addition to the formation of a new sulfur–carbon bond, the presence of benzothiazole-2 mercaptan, as the thioglucuronide, in the urine of the rat that had received benzothiazole-2 cysteine demonstrated that the animal was also capable of cleavage of this conjugate at the sulfur–carbon bond.

The enzymatic system responsible for this reaction was investigated. When 8 μmoles benzothiazole-2 cysteine was incubated for 1 hr with a homogenate of rat liver (equivalent to 1 g liver), 1·08 μmoles benzothiazole-2 mercaptan was recovered as product. When 4·5 μmoles benzothiazole-2 glutathione-^{35}S was added as substrate in an equivalent experiment, 0·047 μmole of the mercaptan of the same specific radioactivity was formed. Homogenates from liver that had been boiled prior to the incubation formed no mercaptan.

Distribution of "thionase" in rat liver fractions

The intracellular localization of the enzyme splitting the sulfur–carbon bond was studied in fractionated rat liver, with benzothiazole-2 cysteine as substrate. The presence of enzyme was ascertained by measuring the amount of benzothiazole-2 mercaptan extracted from incubation mixtures containing 8 μmoles benzothiazole-2 cysteine and coenzyme-fortified rat liver fractions equivalent to 1 g liver.

It was found that the enzymatic activity was distributed between the mitochondrial and soluble fractions. When these two fractions were combined, the amount of benzothiazole-2 mercaptan formed was additive. The microsomal fraction contributed no activity (Table 2).

TABLE 2. ENZYMATIC CLEAVAGE OF THE SULFUR–CARBON BOND OF BENZOTHIAZOLE-2 CYSTEINE (R-CySH) AND BENZOTHIAZOLE-2 GLUTATHIONE-^{35}S (R-GSH)

Fraction	R-CySH (μmoles benzothiazole-2 mercaptan formed/g liver)	R-GSH
Whole homogenate	1·08	0·047
Supernatant	0·90	0·072
Mitochondria and nuclei	0·39	0·046
Microsomes	0·00	0·000
Soluble	1·00	0·013
Soluble + mitochondria and nuclei	1·40	0·146
Soluble + microsomes	0·34	0·007

When benzothiazole-2 glutathione-^{35}S was offered as substrate, the liver fractions formed markedly less mercaptan. This finding could be anticipated if the generation of the benzothiazole-2 cysteine were a prerequisite to the efficient cleavage of the sulfur–carbon bond.

Since the microsomal fraction showed no activity alone and no enhancement of activity when combined with the soluble fraction, it appears that the enzyme system effecting the cleavage of the cysteine or glutathione derivatives from the benzothiazole

linkage at the sulfur bond is different from the S-demethylation system recently reported by Mazel *et al.*[8]

The presence of relatively high endogenous concentration of glutathione[9] as well as the localization of a "thionase" in the supernatant fraction of liver homogenates lends support to the concept that benzothiazole-2 sulfonamide is metabolized by an initial substitution of glutathione for the sulfonamide group. This tripeptide conjugate then undergoes further metabolism to produce the three benzothiazole derivatives found in the urine.

The sulfonamide group is commonly thought of as being unreactive and a particularly stable constituent on an organic molecule. However, Cooper[10] did report a high nonenzymatic-catalyzed lability of the SO_2NH_2 group of 6-uracilsulfonamide. The rapid disappearance of benzothiazole-2 sulfonamide observed in our laboratories in very simple biological systems suggested a high reactivity of this particular sulfonamide.

Experiments were performed to measure the effectiveness of glutathione in the nonenzymatic replacement of the sulfonamide group from the thiazole ring under conditions of pH and concentration simulating those that could occur *in vivo*. Table 3

TABLE 3. NONENZYMATIC REACTION OF GLUTATHIONE WITH BENZOTHIAZOLE-2 SULFONAMIDE UNDER PHYSIOLOGICAL CONDITIONS

Time (min)	Unreacted benzothiazole-2 sulfonamide recovered from solution (mg)	Benzothiazole-2 sulfonamide converted to benzothiazole-2 glutathione (mg)
0	2·50	0·00
1	2·33	0·16
60	1·30	1·30
120	1·03	1·60
180	0·90	1·70
240	0·86	1·71

shows that after 1 min at pH 7·4 and 37°, a co-solution of 117 μmoles of the sulfonamide and 104 μmoles of glutathione reacts to give a measurable amount of the benzothiazole-2 glutathione. In 1 hr, 50% of the benzothiazole is present as the glutathione conjugate. It was found that the sulfhydryl group of cysteine could also displace the sulfonamide from benzothiazole when tested in a similar experiment.

When the glutathione experiment was repeated in the presence of boiled and unboiled supernatant from rat liver, there was a loss of 52% and 66% of sulfonamide in the respective samples after 30 min.

The boiling of the supernatant would be expected to oxidize the endogenous glutathione in the supernatant fraction of liver to the disulfide form. This would result in the unboiled fraction having a higher concentration of glutathione in the chemically more reactive reduced form and thus account for the modest increase in the amount of glutathione addition product formed by this fraction from liver.

On the other hand, the work of several investigators[7, 9] leaves little doubt that glutathione conjugates of drugs are formed under the catalytic influence of the enzyme glutathione kinase.

The data obtained in our laboratory with benzothiazole-2 sulfonamide do not allow an assignment of the relative contributions of the possible enzymatic and the nonenzymatic reactions in the formation of the glutathione conjugate *in vivo*.

Metabolism of benzothiazole-2 mercaptan-^{35}S in the rat

Since benzothiazole-2 mercaptan was a metabolite of the sulfonamide, the details of its metabolic fate were investigated. Urinary metabolites isolated from the urine of rats dosed with benzothiazole-2 mercaptan-^{35}S (50 mg/kg) were identified as follows: benzothiazole-2 mercaptan with the same specific radioactivity as the dosed compound, benzothiazole-2 mercaptoglucuronide with a slightly lower specific

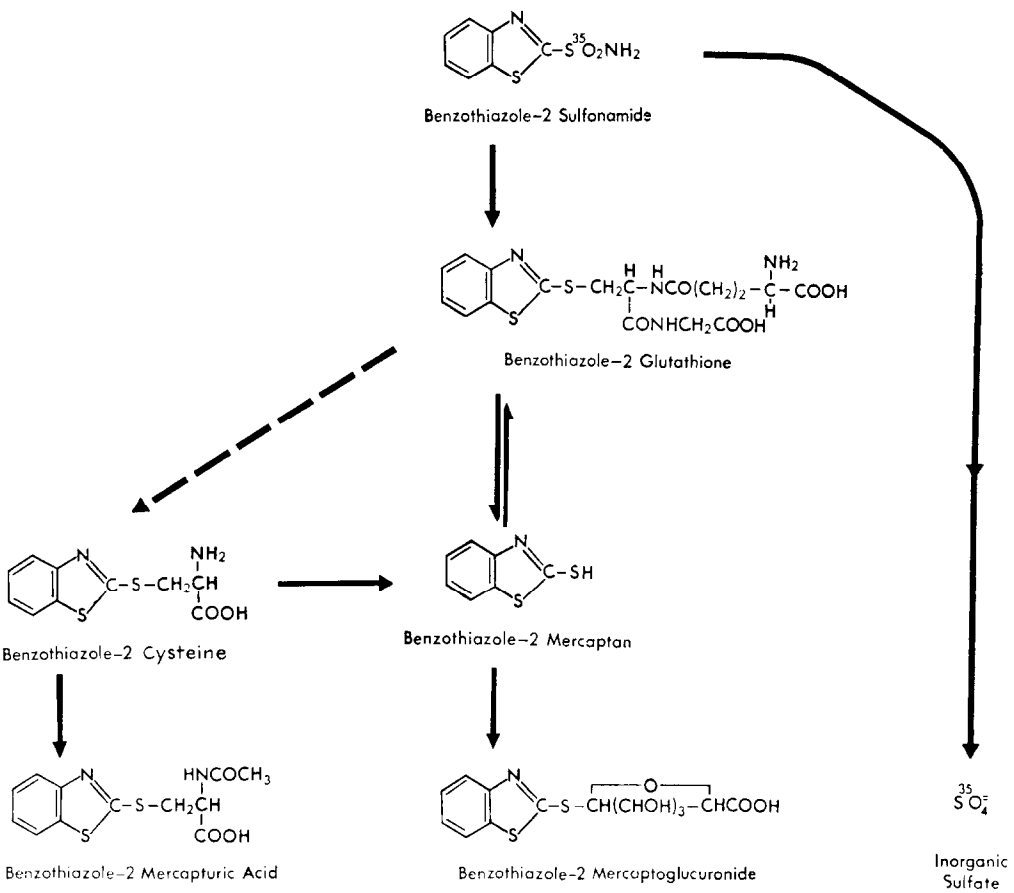

FIG. 2. Pathway for the metabolic transformation of benzothiazole-2 sulfonamide in the rat, rabbit, and dog.

radioactivity than the parent compound, and benzothiazole-2 mercapturic acid which was nonradioactive. In addition, radiolabeled inorganic sulfate was also identified in the urine.

The finding of radiolabeled thioglucuronide of the parent benzothiazole-2 mercaptan was of course anticipated. The excretion of a nonradioactive benzothiazole-2 mercapturic acid metabolite, however, exposed an enzymatic mechanism capable of

causing an efficient detachment of the mercaptan sulfur from benzothiazole-2 mercaptan. The loss of the radioactivity in the organic molecule (verified by the presence of radioactive sulfate in the urine) during the formation of this metabolite strongly suggested that glutathione or cysteine was again serving as the displacing agent and initiating a reaction series similar to that described for the sulfonamide.

The slight reduction of the specific molar radioactivity of the benzothiazole-2 mercaptoglucuronide was added evidence for this reaction. The radioactive mercaptan would be diluted with the nonradioactive compound that is generated via the reaction series:

$$R\text{-}^{35}SH \rightarrow R\text{-S-glutathione} \rightarrow R\text{-SH} \rightarrow R\text{-S-glucuronide}$$

When the *in vivo* rat studies described were performed with the rabbit and the dog, similar results were obtained.

Figure 2 is a diagrammatic summation of the detailed metabolic pathways that occur *in vivo* with benzothiazole-2 sulfonamide in the rat, rabbit, and dog. This scheme is constructed from a consideration of the results of the experiments reported herein. The broken line indicates a recognition that this particular conversion probably results from a multiple-step reaction.

Acknowledgements—The assistance of Drs. George Anderson, Robert Shepherd, and Andrew Kende in the form of helpful discussion, and the participation of Dr. James Florini in early portions of this work, are gratefully acknowledged.

REFERENCES

1. J. W. CLAPP, *J. biol. Chem.* **223**, 207 (1956).
2. R. BLOCK, E. DURRUM and E. ZWEIG, *Manual of Paper Chromatography and Paper Electrophoresis*, 2nd ed., p. 132. Academic Press, New York (1958).
3. R. H. KNIGHT and L. YOUNG, *Biochem. J.* **70**, 111 (1958).
4. R. BLOCK, E. DURRUM and E. ZWEIG, *op. cit.*, p. 428.
5. J. A. STEKOL, *Proc. Soc. exp. Biol. (N.Y.)* **43**, 108 (1940).
6. B. COMBES and G. S. STAKELUM, *J. clin. Invest.* **39**, 1214 (1960).
7. J. BOOTH, E. BOYLAND and P. SIMS, *Biochem. J.* **79**, 516 (1961).
8. P. MAZEL, J. F. HENDERSON and J. AXELROD, *J. Pharmacol. exp. Ther.* **143**, 1 (1964).
9. B. COMBES and G. S. STAKELUM, *J. clin. Invest.* **40**, 981 (1961).
10. J. R. COOPER, *Cancer Res.* **18**, 1084 (1958).