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TITLE The Metabolism of Monobromobenzene, Benzene, Benzyl Chloride, And Related Compounds In The Rabbit

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THE METABOLISM OF MONOBROMOBENZENE, BENZENE, BENZYL CHLORIDE, AND RELATED COMPOUNDS IN THE RABBIT

by

ROBERT F. WITTER

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TABLE OF CONTENTS

Page

I. Introduction - 1

II. Historical - 3

A. The Acetylation of the S-Aryl Derivatives of Cysteine - - - - - - - - - - - - - - - - - 11
B. The Relation of the Phenols to Mercapturic Acid Synthesis - - - - - - - - - - - - - - - - 13
C. The Role of the Endogenous Sulfur-Containing Amino Acids in the Synthesis of Mercapturic Acids - 20
D. The Role of the Exogenous Sulfur-Containing Amino Acids in the Synthesis of Mercapturic Acids - 25
1. Mercapturic Acid Synthesis in Relation to Rat Growth - - - - - - - - - - - - - - - - - 28
a. Monobromobenzene - - - - - - - - - - - - 30
b. Benzyl Chloride - - - - - - - - - - - - - 32
E. The Effect of the Alkalinity or Acidity of the Diet on Mercapturic Acid Synthesis - - - 35
F. Pathways of Excretion of the Metabolic Products of the Monohalogen Substitution Products of Benzene - 36

III. Experimental - 37

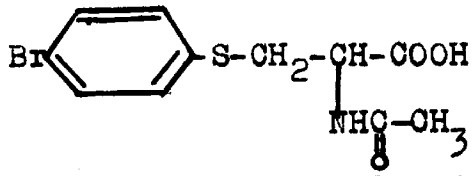
A. Methods - 37
1. Animal Experiments - - - - - - - - - - - - - 37
2. Methods of Analysis - - - - - - - - - - - - 38
B. Results and Discussion - - - - - - - - - - - - 43
1. Metabolism Experiments - - - - - - - - - - - 43
a. Benzyl Chloride - - - - - - - - - - - - - 44
b. S-Benzylcysteine - - - - - - - - - - - - - 66
c. Cinnamyl Chloride - - - - - - - - - - - - 71
d. Monobromobenzene - - - - - - - - - - - - - 71
e. p-Monobromophenol - - - - - - - - - - - - 74
f. Benzene - - - - - - - - - - - - - - - - - 83
g. S-Phenylcysteine - - - - - - - - - - - - - 84
2. The Isolation of Mercapturic Acids - - - - - - 93
a. Benzyl Chloride and S-Benzylcysteine - - 94
b. Benzene - - - - - - - - - - - - - - - - - 107
c. S-Phenylcysteine - - - - - - - - - - - - - 111

TABLE OF CONTENTS (Continued)

		Page
	C. General Discussion	113
	1. The Metabolism of Benzyl Chloride and S-Benzylcysteine	113
	2. The Metabolism of Monobromobenzene, p-Monobromophenol, and Benzene	119
	a. The Relationship between the Changes in the Glucuronic Acid and Phenol Excretions and the Sulfur Distribution	119
	b. Changes in the Partition of Sulfur	123
	D. Summary	126
	E. Conclusions	131
IV.	Bibliography	133
V.	Appendix	139
	A. Methods of Analysis	139
	1. Ether-Soluble Sulfur	139
	2. Hippuric Acid	142
	3. Glucuronic Acid	143
	4. Phenol	146
	5. p-Monobromophenol	151
	B. Synthesis	158
	1. N-Acetyl-S-benzylcysteine	158
	2. S-Phenylcysteine	159
	C. Sources and Purity of the Compounds Used in the Animal Experiments	165
	D. Animal Experiments	166
	1. Numbering of Experiments	167
	2. Protocols	168

INTRODUCTION

The fate of foreign organic compounds in the animal body has been and still is a subject of great interest to the biochemist. In 1879 Baumann and Preusse (1), after the administration of monobromobenzene, discovered p-bromophenylmercapturic acid, representative of a new class of conjugation products, called mercapturic acids. Jaffe' (2) simultaneously made the same discovery. Chemical investigation by the former two authors as well as by Friedmann (3) proved that the mercapturic acid had the following formula:



N-acetyl-p-bromophenylcysteine [1]

Baumann (4) presented evidence that the new sulfur-containing compound was excreted in the urine of the dog as a labile complex with glucuronic acid. He isolated what was claimed to be the potassium salt of the complex. The non-sulfur-containing component of the complex was so

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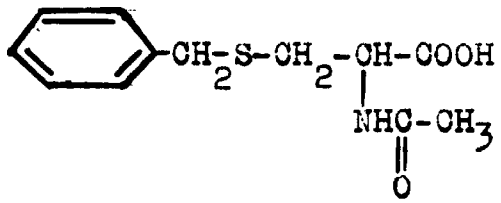
[1]Unless otherwise stated all sulfur-containing amino acids and their N-acetyl derivatives are of the l configuration.

unstable that investigation of its properties was abandoned. Sufficient information was obtained so that Baumann considered the unstable component to be glucuronic acid.

Outside of a few reports of qualitative tests for glucuronic acid in the urine of dogs to which monobromobenzene had been fed, this report has been the only evidence in the literature that p-bromophenylmercapturic acid was excreted in the urine of the dog as a glucuronic acid complex. Since improved methods for the determination of glucuronic acid are now available, it was felt that some evidence on this point might be obtained by the quantitative estimation of the glucuronic acid excretion following the feeding of monobromobenzene and its most probable metabolic product, p-monobromophenol.

The same type of experiment was carried out in the second phase of the problem, a study of the metabolism of benzene. With the exception of a report of a positive test for thiophenol in the hydrolyzed urines of rabbits to which benzene had been fed, no evidence of the formation of phenylmercapturic acid by this species was available (5). Hence, attempts were made to isolate this mercapturic acid.

The third phase of the problem was an investigation of the metabolism of benzyl chloride, reported by Stekol (6) to form a mercapturic acid, N-acetyl-S-benzylcysteine.



This conjugation product was unusual in that the sulfur was attached to an aliphatic side chain rather than directly to the carbon of the benzene nucleus.

Also the metabolism of two possible intermediates in mercapturic acid formation, S-benzylcysteine and S-phenylcysteine, was investigated.

HISTORICAL

In the following section a general review of the subject of mercapturic acids in which will be emphasized the studies of the metabolism of the individual compounds under investigation here will be given. It will be restricted to those phases of the metabolism of the individual compounds in which the sulfur-containing amino acids are involved in some way.

The discussion of the mercapturic acids can be divided into two sections: the nature of the compounds which give rise to the acids, and the mechanism of their biological synthesis. This last problem can be subdivided into two phases: the intermediary products in the formation of the mercapturic acids, and the effect of the diet, particularly

the content of the sulfur-containing amino acids, on their synthesis. The subject will be developed by a consideration of each of these related aspects rather than a strictly chronological discussion of the whole.

Great interest has been shown in the study of mercapturic acid formation in recent years. In 1931 only three conjugation products of this type had been isolated; but since that time five additional mercapturic acids have been discovered. Evidence that an organic compound, when administered to an animal, gives rise to a mercapturic acid has been obtained in three ways: (a) actual isolation of the conjugation product;[2] (b) changes in the sulfur distribution of the urine; (c) inhibition of the growth of young rats, which can be counteracted by supplementing the diet with cystine or methionine. The latter section will be discussed only in so far as it applies to compounds which have been shown to form mercapturic acids by the other methods.

It is now known that benzene and chloro-, bromo-, and iodobenzene give rise to mercapturic acids in certain species. A list of all the organic compounds for which corresponding mercapturic acids have been isolated, the species of animal, the date of isolation, and the observer is presented in Table I.

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[2]Stekol (7) has developed a quantitative method for p-bromophenyl- and α-naphthylmercapturic acids, which does not involve their isolation.

TABLE I

MERCAPTURIC ACIDS WHOSE BIOLOGICAL SYNTHESIS FROM CYCLIC PRECURSORS HAS BEEN PROVEN.

Compound administered	Mercapturic acid isolated	Species	Date	Observers and references
Monobromobenzene	Bromophenylmercapturic	Dog	1879	Baumann and Preusse(1), Jaffé(2)
" "	" "	Cat	1881	" " " (8)
" "	" "	Rabbit	1925	Abderhalden and Wertheimer(9)
" "	" "	Rat	1935	White and Jackson (10)
" "	" "	Mouse	1935	Stekol (11)
" "	" "	Pig	1936	" (12)
Monochlorobenzene	Chlorophenylmercapturic	Dog	1879	Jaffé(2)
" "	" "	Rabbit	1929	Nishimura (13)
Monoiodobenzene	Iodophenylmercapturic	Dog	1895	Baumann and Schmitz (14)
" "	" "	Rabbit	1931	Laurie (15)
" "	" "	Rat	1931	Laurie (15)
Naphthalene	α-Naphthylmercapturic	Rabbit	1934	Inge, Bourne, and Young (16)
"	"	Dog	1935	Stekol (17)
"	"	Pig	1936	Stekol (12)
"	"	Rat	1937	Stekol (18)
Anthracene	1-Anthracenemercapturic	Rat	1936	Boyland and Levi (19)
"	"	Rabbit	1936	Boyland and Levi (19)
Benzyl chloride	Benzylmercapturic	Dog	1938	Stekol (6)
" "	" "	Rat	1939	Stekol (20)
" "	" "	Rabbit	1939	Stekol (20)
p-Bromobenzyl bromide	p-Bromobenzylmercapturic	Rat	1940	Stekol (21)
Benzene	Phenylmercapturic	Rat	1943	Zbarsky and Young (22)

In general the amounts of mercapturic acid isolated from the urine of animals to which have been administered the various compounds listed in Table I have varied with the nature of the compound, the species of animal, and the method of isolation. For example, after the administration of from 7 to 16 gm. of benzene to the rat in daily doses of 0.1 cc., only from 11 to 177 mg. of the corresponding mercapturic acid were isolated (22). Other investigators were not successful in isolating this conjugation product from the urine of rabbits to which benzene had been fed (5). In contrast, the administration of a total of one gm. of benzyl chloride in doses of 0.15 gm. at three-hour intervals to each of three dogs resulted in the isolation of 150 to 200 mg. of benzylmercapturic acid (6). Sherwin (23) isolated mercapturic acid corresponding to 18 per cent of the monobromobenzene which was fed to the dog, yet by the use of the same procedure obtained a yield of only3.5 per cent when the monohalogen derivative of benzene was fed to the rabbit.

The amounts of mercapturic acid isolated by various investigators are probably minimal figures because the methods of isolation are poor. The difficulties of separating the acid from the other constituents of the urine in the absence of any specific precipitant for the mercapturic acid are no doubt responsible for the small amount of the conjugation

product isolated.

In addition to the actual isolation of a mercapturic acid from the urine of animals to which an organic compound has been fed, there is a second type of evidence of the formation of the conjugation product. This is a rise in the level of organic sulfur in the urine after the administration of an organic compound. This method is based on the assumption that the increases in the organic sulfur of the urine after the administration of monobromobenzene and similar compounds are due solely to the presence of mercapturic acids. The validity of the method has been questioned by several workers (24, 7) but, in the absence of any satisfactory method for the determination of the mercapturic acids, has been utilized frequently as presumpting evidence of the presence of these conjugation products. In Table II is given a list of the most important of these compounds for which the only evidence of mercapturic acid synthesis following their administration to a given animal species is an increase in the organic sulfur of the urine.

TABLE II

Precursors of Mercapturic Acids That Have Not Been Isolated but Whose Existence Is Postulated on the Basis of Changes in the Urinary Sulfur Distribution

All of the compounds were fed to the dog with the exception of styrene which was administered also to the rabbit (29) or man (29). The results in the latter case were not conclusive.

Compound	Date	Observers and Reference
o-Dichlorobenzene	1926	Hele and Callow (25)
m-Dichlorobenzene*	1926	Hele and Callow (25)
Monohalogen substitution products of naphthalene**	1881	Baumann (4)
Monofluorobenzene	1927	Coombs (26)
Benzene***	1926	Hele and Callow (25)
Phenanthrene	1935	Stekol (27)
Styrene	1942	Irish et al (28)

*Baumann (4) on the basis of a qualitative test for mercapturic acid found no evidence that m-dichlorobenzene might be converted to a mercapturic acid in the animal body.

**No one has studied the effect of these compounds on the sulfur distribution of the urine, but Baumann (4) reported a qualitative test for mercapturic acid in the urine of a dog that was fed these compounds.

***Phenylmercapturic acid has been isolated from the urine of rats fed benzene (22).

Metabolism of the S-Aryl Derivatives of Cysteine

The results of recent years have shown that mercapturic acid formation is not restricted to the compounds listed in Tables I and II. Some compounds of the type $R\text{-}S\text{-}CH_2\text{-}CH(NH_2)COOH$[3]

are excreted as the corresponding mercapturic acid when they are fed to an animal. These derivatives of cysteine have been postulated to be intermediates in the formation of the mercapturic acids. The aromatic compound, according to this theory, combines with cysteine to form the intermediate product which is then acetylated and excreted as the mercapturic acid (30).

The metabolism of only a few compounds of the type $R\text{-}S\text{-}CH_2\text{-}CH(NH_2)COOH$[3] had been investigated until about six years ago. At that time adequate methods of preparing the d and l enantiomorphs of certain of these amino acids were developed. The revival of Knoop's theory (31) of the deamination and acetylation of amino acids by du Vigneaud and Irish (32) has stimulated the investigation of the biological acetylation of the d and l enantiomorphs of these derivatives of cysteine. As a result of these studies a number of mercapturic acids have been isolated from the urine of animals to which these cysteine derivatives were fed. These investigations are summarized in Table III. In addition, a number of derivatives of homocysteine $R\text{-}S\text{-}CH_2\text{-}CH_2\text{-}CH(NH_2)COOH$ have been shown to be converted to the corresponding acetylated amino acids; and the results of the investigation of these compounds are also presented in Table III.

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[3] R is an aromatic radical such as p-bromophenyl, benzyl, etc.

TABLE III

S- Substituted Amino Acids for Which the Biological Synthesis of the N-Acetyl Derivative Has Been Proven

The amino acid and the corresponding N-Acetyl amino acid are of the l configuration. In all experiments the rat was the experimental animal; but in addition Stekol (20) has demonstrated the conversion of benzylcysteine to benzylmercapturic acid in the rabbit.

Compound	Date	Observer and Reference
Phenylcysteine	1943	Zbarsky and Young (33)
p-Bromophenylcysteine	1943	Stekol (18)
p-Fluorophenylcysteine	1943	Zbarsky and Young (34)
Benzylcysteine	1938	Stekol (6)
p-Bromobenzylcysteine	1941	Stekol (21)
o-Bromobenzylcysteine	1944	du Vigneaud et al (35)
Benzylhomocysteine	1938	Stekol (20)
p-Bromobenzylhomocysteine	1944	du Vigneaud et al (35)

The amount of mercapturic acid isolated after feeding these derivatives of cysteine has varied with the nature of the substituent groups attached to the sulfur. Thus, after the administration of p-bromobenzylcysteine to the rat, an amount of the corresponding mercapturic acid equivalent to 48 per cent of the compound fed was isolated (21); the feeding of phenylcysteine to this same species resulted in the isolation of 14 to 15 per cent of the amount of phenylmercapturic acid theoretically possible (35).

Other investigators have studied the changes in the

sulfur distribution of the urine of animals to which were administered the S-substituted derivatives of the sulfur-containing amino acids. Negative or inconclusive results were obtained when p-iodophenylcysteine was fed to a dog. This compound was very toxic, and the results of iodine determination on the urine indicated that less than 5 per cent was absorbed. Since no analytical data regarding urinary sulfur distribution were reported for this experiment, no conclusion can be drawn regarding its metabolism (36). The results of the partition of urinary sulfur indicated that in the rabbit about 50 to 60 per cent of the sulfur of p-chlorobenzylcysteine or S-benzylcysteine that had been fed was oxidized and excreted as ethereal sulfate sulfur, the rest appearing as organic sulfur (37). The results obtained with the latter compound were not confirmed when S-benzylcysteine was fed to a dog for in this case the main effect was to increase the level of organic sulfur in the urine (38).

The Acetylation of the S-Aryl Derivatives of Cysteine

Several theories have been proposed for the mechanism of the acetylation of the S-aryl derivatives of cysteine. Studies have been made of the acetylation of the d and l enantiomorphs of these compounds, but this short discussion will be confined to the fate of the latter isomers.

Stekol (6) proposed that S-benzylcysteine was first converted to the keto acid and that this acid was acetylated by a reaction with ammonia and pyruvic acid to yield benzylmercapturic acid. He has, however, changed his original theory; and in his most recent publications favors the direct acetylation of S-benzyl-l-cysteine (39, 40).

Another possible mode of formation of benzylmercapturic acid involving a different mechanism of acetylation was suggested by Brand and Harris (41). They pointed out that the amino acids present in glutathione are those used in detoxication. "One can conceive that the substance being detoxified first comes in contact with glutathione, thereby rendering it more unstable as a result of which the detoxified product splits off with part of the glutathione molecule... . The acetyl group in bromophenylmercapturic acid may be a remnant of the glutamic acid radical of glutathione."

The metabolism of S-benzyl- and S-p-bromobenzylglutathione in the rat has been studied by Stekol (42, 21). From the urine of rats fed these glutathione derivatives, the corresponding mercapturic acids were isolated. This investigator postulated that the substituted glutathionine was hydrolyzed *in vivo* to give the cysteine derivative which was then acetylated. He does not believe that glutathione is

involved in the formation of mercapturic acids.

The Relation of the Phenols to Mercapturic Acid Formation

There is ample evidence that phenols are formed in the metabolism of the monohalogen substitution products of benzene. Baumann (1) demonstrated that one of the products of monobromobenzene metabolism was a sulfuric acid ester of p-monobromophenol. In addition, a substance having the properties of pyrocatechol was also detected. In another paper (4) he reported the isolation of small quantities of bromohydroquinone from the urine of dogs to which monobromobenzene had been fed. Likewise Hele (43) obtained evidence of the excretion of phenolic compounds after the administration of monochlorobenzene to this same species.

The fact that phenol is one of the metabolic products of benzene is also well established. Schultzen and Naunyn (44) reported an excretion of phenol in the urine of men or of dogs to which benzene had been given. Munk (45) substantiated this claim and in addition found that not all of the phenol was in the free state. Baumann and Herter (46) found that an increase in the ethereal sulfate sulfur excretion followed the administration of benzene to the dog. Nencki and Giacosa (47) detected pyrocatechol in the urine of dogs to which benzene had been fed. Since only a small portion of the hydrocarbon that was administered could be ac-

counted for as phenols, these investigators postulated that a major portion of the benzene was eliminated by the lungs.

The fact that monohalogen derivatives of benzene are converted in part to the corresponding p substituted phenols in the animal body has suggested to one group of workers that these phenols may be the intermediates in the formation of the mercapturic acids.

Rhode (48) reached the above conclusion as a result of his experiments with rabbits. Following the oral administration of p-bromophenol or monobromobenzene, an increase in the ethereal sulfate sulfur level of the urine took place. If at the same time he injected l-cysteine subcutaneously, the rise in ethereal sulfates was diminished by one-third in both cases. He concluded that the monohalogen substitution product of benzene was oxidized to the p-substituted phenol, which, in turn, was converted to the mercapturic acid.

Sherwin and his coworkers (49) supported this theory as a result of their study of the metabolism of phenol, p-chlorophenol, and monobromobenzene in a pig to which a protein-free diet was fed. The oral administration of each of these compounds was followed by an increase in the level of urinary ethereal sulfates. Then l-cystine was simultaneously fed with each of the three organic compounds. The addition of this amino acid to the diet when phenol was fed increased the excretion of ethereal

sulfate sulfur above the level observed after the feeding of phenol alone. In contrast, when monobromobenzene and p-chlorophenol were fed, the addition of cystine to the diet decreased the ethereal sulfate sulfur level of the urine to a value below the amount of this fraction of the oxidized sulfur observed when either of the two halogen containing compounds were fed alone.

The addition of sodium sulfate to the diet when either phenol, p-chlorophenol, or monobromobenzene was fed did not cause an increase in the level of ethereal sulfate above that value observed when the three organic compounds were fed alone. In other experiments Muldoon, Sheple, and Sherwin (50) found that the administration of sodium sulfate together with monobromobenzene to a dog which was fed on a protein-free diet did not increase the level of ethereal sulfates above that observed when the monohalogen substitution product of benzene was fed without the additional sulfate ion. Sherwin (49, 50) concluded from these results that the sulfur for ethereal sulfate formation must be of endogenous origin. Because of the results of other experiments, he believed that the mercapturic acids were involved in the synthesis of the ethereal sulfates by the animal. Several years before Hopkins (5[illegible]) had suggested that the ethereal sulfates were formed by the combination of a phenol with some sulfur-containing compound to form a sub-

stance which was oxidized to a sulfuric acid ester of the phenol and then excreted by the kidney.

There is some evidence that the sulfur of mercapturic acids may be oxidized to sulfate. Baumann (4) fed a dog eight grams of the ammonium salt of bromophenylmercapturic acid and was able to isolate only traces of the conjugation product from the urine. This author did not determine the distribution of urinary sulfur but relied solely on the isolation procedure to detect the presence of the mercapturic acid. Marriot and Wolf (52) administered one gram of this acid together with 0.5 gram of monobromobenzene to a dog. The changes in the sulfur distribution did not indicate whether any unchanged acid was excreted, or whether any of the mercapturic acid was oxidized. Sherwin (49) obtained more positive evidence that the sulfur of certain mercapturic acids was oxidized in the animal body and excreted as ethereal sulfates.

The sulfur of phenyl-, p-bromophenyl-, and p-chlorophenylmercapturic acids, which were administered to a pig, was oxidized and excreted as ethereal sulfates in amounts of 58, 43, and 23 per cent respectively (49). Furthermore 23 to 30 per cent of the sulfur of the p-bromophenylmercapturic acid that was fed to a rabbit, or 59 per cent of the sulfur of dl-benzylmercapturic acid that was fed to the same animal

were oxidized to ethereal sulfates (37).

From these results it was concluded that not only were the ethereal sulfates of endogenous origin, but also that the mercapturic acids were intermediates in the formation of this fraction of the oxidized sulfur (49). According to this theory, the first step in the metabolism of a monohalogen substitution product of benzene is oxidation to the phenol. In the presence of exogenous cystine, the phenol reacts with cysteine to form a mercapturic acid which is then either eliminated as such or oxidized to ethereal sulfates. Thus, when phenol was fed to the pig which was maintained on a protein-free diet supplemented by cystine, the phenylmercapturic acid that was formed was readily oxidized, and its sulfur was excreted as ethereal sulfates. Hence, the level of ethereal sulfates was higher in the urine of the pig when phenol and cystine were fed together than when phenol was administered alone. In the case of monobromobenzene and p-chlorophenol, the corresponding mercapturic acids which were formed when these organic compounds were fed together with cystine to the pig were not readily oxidized and were excreted unchanged. Hence, a smaller amount of ethereal sulfate sulfur was present in the urine of this animal when either of the halogen containing organic compounds was fed with cystine than when either monobromobenzene or p-chloro-

phenol was fed alone.

The results and the theory of Sherwin (49, 50) were questioned by Hele and coworkers (53), When p-chlorophenol was fed to the dog, they observed no change in the organic sulfur level of the urine and concluded that the phenol did not form a mercapturic acid. When p-bromophenol was administered to the dog (24) or to the rat (54), similar results were obtained.

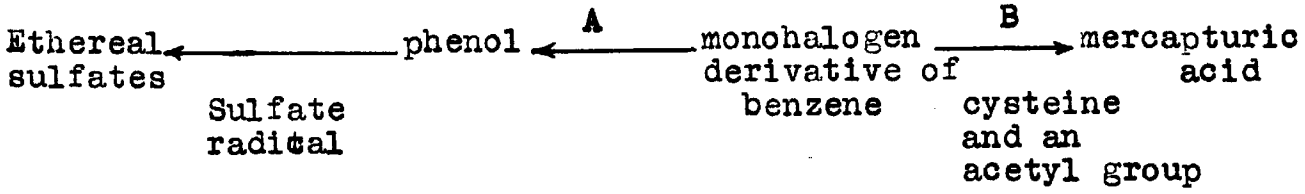
Hele (55) found that the ability of the pig to synthesize either mercapturic acid or ethereal sulfates was limited. The increase in the reducing power of the urine after the administration of p-chlorophenol suggested that the phenol was excreted as a glucuronide. Hence, the administration of sodium sulfate would not affect the synthesis of ethereal sulfate in this species. When p-chlorophenol was administered to the dog, an increase in ethereal sulfates of the urine was observed. But, if p-chlorophenol and sodium sulfate were fed together, the resulting increase in ethereal sulfate sulfur excretion was greater than that observed when the phenol alone was fed (56). This result has been confirmed by other workers (24, 54).

Hele (56) suggested that Rhode (48) did not observe an effect of sodium sulfate on ethereal sulfate excretion because the salt was injected subcutaneously while the monobromophenol,

phenol, or p-bromophenol was given orally, and that it was possible that the sodium sulfate was excreted by the kidney before the orally administered compounds were absorbed from the gastro-intestinal tract.

Lastly, Hele and his coworkers found that mercapturic acids were not oxidized by the pig (56) or the dog (57). The amount of organic sulfur in the urines of both species increased when bromophenylmercapturic acid was fed. The acid had a toxic action in the dog, producing a transient hemoglobinuria. The animals were capable of dealing with an amount of monobromobenzene equivalent to ten times the toxic amount of bromophenylmercapturic acid without the production of hemolysis (57). Laurie (15) found that in the rabbit iodophenylmercapturic acid was not oxidized and was not toxic.

Hele (56) suggested that two paths of metabolism were available for the metabolism of the monohalogen substitution products of benzene: 1) oxidation to the phenol and conjugation with the sulfate radical to form ethereal sulfates; 2) formation of a mercapturic acid by combination with cysteine and subsequent acetylation.



In the presence of adequate cystine, the latter pathway (B) would be favored; in the absence of the sulfur-containing amino acid, the former path (A) would be utilized.

The results of Lewis (24) and Stekol (58) are in accord with this theory rather than that sponsored by Sherwin (49). The simultaneous administration of sodium sulfate and p-monobromophenol was followed by an increased excretion of urinary ethereal sulfate sulfur which exceeded the amount excreted when the phenol alone was fed both in the dog (24) and in the rat (54). Both investigators observed that the level of ethereal sulfates in the urine of the dog which was fed a low protein diet and monobromobenzene was higher than the amount of this fraction of oxidized sulfur in the urine of the same animal when a high protein diet and monobromobenzene was fed (24, 58, 59). This finding was interpreted as evidence that more of the monohalogen substitution product of benzene was oxidized to the phenol in the absence of adequate dietary cystine and methionine than in the presence of the sulfur-containing amino acids (24, 58, 29).

<u>The Role of the Endogenous Sulfur-Containing Amino Acids in the Synthesis of Mercapturic Acids</u>.

Much of the evidence on the origin of the sulfur-containing moiety of the mercapturic acid has been based on the isolation of the conjugation products from the urine by procedures

that are not very reliable. Hence, many conflicting results have been reported.

All the early investigators are agreed as to the importance of dietary cysteine for the synthesis of mercapturic acids. The problem which arises is: can the animal utilize tissue or endogenous cystine to form mercapturic acids? One group of investigators emphasized the importance of the exogenous cystine and denied that the amino acid of endogenous origin could be utilized in the synthesis of these conjugation products.

In 1914 Zeller and Straczewski (60) investigated the formation of bromophenylmercapturic acid in dogs which were fed a low protein diet. The authors not only followed changes in the distribution of urinary sulfur but also attempted to isolate the mercapturic acid from the urine. On the day that the monohalogen substitution product of benzene was fed, the organic sulfur excretion rose markedly, but only traces of p-bromophenylmercapturic acid could be isolated from the urine. They suggested that perhaps the cystine in the tissues did not come in contact with the monobromobenzene; hence, no mercapturic acid was formed. On the basis of further experiments, Thomas and Straczewski (61) discarded this theory. When the monobromobenzene was injected subcutaneously into a dog that was maintained on a

low protein diet, traces only of mercapturic acid were isolated from the urine. But when the experiment was repeated with an animal to which was fed a high protein diet, appreciable amounts of the conjugation product were isolated from the urine. The authors concluded that the endogenous cystine could not be utilized for the synthesis of the mercapturic acid.

Kapfhammer (62) also failed to isolate bromophenylmercapturic acid from the urine of dogs that were maintained on a protein-free or a low protein diet and fed monobromobenzene. He considered this as proof that endogenous or tissue cystine could not be utilized for the synthesis of the mercapturic acid. In further support of this belief, when the sodium salt of cystine was injected subcutaneously at the same time that the monohalogen substitution product of benzene was fed, bromophenylmercapturic acid was isolated from the urine. The effect of the high protein diet previously observed was believed to be due to its cystine content. It should be pointed out that, in both of his experiments, Kapfhammer (62) observed an increase in the excretion of organic sulfur on the experimental day.

Similar results were obtained in 1924 by Muldoon, Shiple, and Sherwin (50), who studied the ability of the dog to furnish cystine for the detoxication of monobromobenzene. Only when cystine was administered with the monohalogen substitution

product of benzene could mercapturic acid be isolated from the urine. Hence, they concluded that the tissues could not furnish cystine for detoxication purposes.

This conclusion has been disproved by the work of many investigators. Mercapturic acid has been isolated from the urine of fasting animals, including the rabbit (13), cat (63), and the growing (11, 64) and adult (11, 65) dog. Marriot and Wolf (52) found that similar changes took place in the sulfur distribution of fasting and well fed dogs upon the administration of monobromobenzene. However, the fasting dogs did not exhibit as great increases in the organic sulfur of the urine as did the well fed animals. Abderhalden and Wertheimer (65) were also able to isolate the mercapturic acid from the urine of fasted phlorhizinized dogs and dogs which were maintained on a protein-free diet. They believed that the extent of synthesis of p-bromophenylmercapturic acid in the fasting animals was limited by cystine requirements more essential than the detoxication of monobromobenzene. Stekol (64) isolated bromophenylmercapturic acid from the urine of young dogs which were subjected to a fast of four to six weeks.

The synthesis of mercapturic acids in the fasting rabbit has been demonstrated. Nishimura (13) was able to isolate small amounts of the corresponding mercapturic acid from the

urine of the fasting rabbits which were fed chloro-, iodo-, or bromobenzene. Conway (66) also proved that the fasting rabbit could form mercapturic acid. He pointed out that the fasting period in Nishimura's experiments was short and that, since the pooled urine sample was taken for the isolation procedure, perhaps the stored circulating protein was not depleted. In his experiments, rabbits were fasted for a period of 37 days, and on the twenty-first and thirty-second day, two grams of monobromobenzene were injected subcutaneously. He was able to isolate bromophenylmercapturic acid from the urine. Previously Lough and Lewis (67) had demonstrated by studies of the sulfur partition of fasted rabbits that a limited synthesis of the mercapturic acid took place.

Virtue (63) observed an increase in the organic sulfur of the urine of either fasting or well fed cats which had received monobromobenzene subcutaneously. Since in the former case the level of urinary nitrogen rose markedly on the experimental day, he concluded that tissue protein was being broken down to yield cysteine for the detoxication of the monohalogen substitution product of benzene.

The generally accepted view is that the animal can use its own tissue cystine or cysteine if necessary for the detoxication of monobromobenzene and similar compounds. One must not conclude that this synthesis is independent of the

diet, since the results of many workers demonstrate that the diet, particularly the content of the sulfur-containing amino acids, plays a very important role.

The Role of the Exogenous Sulfur-Containing Amino Acids in the Synthesis of Mercapturic Acids.

Early evidence of the effect of the content of the exogenous sulfur-containing amino acids on the synthesis of mercapturic acids was available in the first report of Baumann (1), who stated that greater yields of bromophenyl-mercapturic acid could be obtained from the urine of animals that were fed a high meat diet with the monobromobenzene.

The first quantitative study of the influence of the protein of the diet, particularly the sulfur-containing amino acids, on the extent of the synthesis of bromophenyl-mercapturic acid by the dog was carried out by White and Lewis (24) in 1932. Dogs, maintained on diets containing proteins low in cystine content, were fed monobromobenzene together with l-cystine or dl-methionine. The extent of mercapturic acid synthesis was estimated by the changes in the urinary sulfur distribution, in particular, the organic sulfur fraction.

When the bromobenzene was fed, greater increases in the urinary organic sulfur level were obtained when the dietary protein was lactalbumin, rich in cystine, than when casein

or peas were fed. The addition of either l-cystine or dl-methionine to the low cystine diet (peas) resulted in the excretion of a higher level of organic sulfur than was obtained on the pea diet alone. These facts indicated that more bromophenylmercapturic acid was formed as evidenced by the higher level of organic sulfur when the diet was rich in the sulfur-containing amino acids. Further evidence of the specific effect of l-cystine and dl-methionine on the detoxication of monobromobenzene was the unchanged level of total nitrogen in the urine after the sulfur-containing amino acids were fed together with the monohalogen substitution product of benzene to the dog which was maintained on a low cystine diet. The administration of monobromobenzene alone to the dog which was fed the low cystine diet resulted in a marked increase in the excretion of urinary nitrogen. However, the nitrogen level remained unchanged if either of the two sulfur-containing amino acids were fed with the monohalogen substitution product of benzene. Likewise no increase in the excretion of total urinary nitrogen was observed if monobromobenzene was administered to the animal on the lactalbumin diet, which contained adequate cystine. It should be pointed out that the sulfur-containing amino acids were given in two doses; the first at the time of the administration of monobromobenzene, the second three hours later

to take care of any possible difference in the rate of absorption of the halogen compound and the amino acids and thus to assure an adequate supply of cystine or methionine at the time that the halogen derivative of benzene was absorbed.

The authors interpreted their results in the following way. When dietary cystine was not available in adequate amounts, the protein of the tissues was catabolized to furnish cystine for mercapturic acid synthesis; hence, the level of urinary nitrogen was increased. This change did not occur when the diet contained adequate cystine or methionine since the sulfur-containing amino acids of the diet were used in place of those of the tissues.

The authors pointed out that the results with methionine must be interpreted with caution. They suggested that methionine may function as does cystine in the detoxication of the monohalogen derivatives of benzene. "It is possible also that cystine and methionine may have some common product of intermediary metabolism which is essential for the normal function of the organizm and that when methionine is supplied by the diet, the cystine present is thereby made available for the detoxication of the benzene derivatives."[4]

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[4]It should be borne in mind that at the time these experiments were performed, evidence of the conversion of methionine to cystine was not yet available.

Stekol (58) carried out experiments similar to those of White and Lewis (24) with the exception that he also estimated the amount of bromophenylmercapturic acid in the urine of dogs which were maintained on diets of varying cystine and methionine content and fed monobromobenzene.[5] From a consideration of the changes in the amount of total urinary nitrogen, the sulfur distribution, and the mercapturic acid excretion on the days when monobromobenzene was fed, Stekol (58) came to the conclusion that the immediate source of the cystine for detoxication purposes was the tissues regardless of the presence of the sulfur-containing amino acids in the diet. Other evidence that he cited for this hypothesis was that the extent of the synthesis of bromophenylmercapturic acid appeared to be independent of the content of the sulfur-containing amino acids in the diet under conditions in which the animal presumably had adequate stores of tissue protein (59) and also of the time interval between the administration of the food and the monobromobenzene (68). In growing female pups the extent of the synthesis was dependent on the body weight (69).

Mercapturic Acid Formation in Relation to Rat Growth

In the previous discussion the effect of the diet on

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[5]According to Stekol, who developed this method (7), the quantitative determination of this mercapturic acid can be used for comparative purposes only (18).

mercapturic acid formation has been estimated by studying the changes in the amounts of the metabolic products of the monohalogen derivatives of benzene that are excreted in the urine. Another approach to the problem was first employed by White and Jackson (10, 70) in 1933. When bromobenzene was added to the diet of young rats fed a low protein diet, the animals ceased to grow. But if, in addition, either cystine or methionine was added to the diet containing monobromobenzene, growth ensued, and the growth inhibiting action of the monohalogen substitution product of benzene was thus prevented. The authors suggested that the administration of the bromobenzene checked the growth of the young rats since the dietary cystine or methionine was used for the synthesis of bromophenylmercapturic acid rather than for the construction of new tissue. The addition of either cystine or methionine to this diet prevented this deficiency, thereby promoting normal growth.

Since that time this procedure has been commonly used as a method of study of the formation of a mercapturic acid in the animal body. The administration of a number of organic compounds will inhibit the growth of young rats, and this effect can frequently be counteracted by the addition of cystine or methionine to the diet. This is presumptive evidence that the organic compounds are converted to mercapturic acids in the animal body. In this discussion only those substances

which are believed on the basis of other experimental evidence to give rise to a mercapturic acid will be considered.

Monobromobenzene.

The pioneer report of White and Jackson (10, 70) and the interpretations of their results have already been discussed. Their findings were confirmed and extended by Stekol, who found that glutathione (71) and homocystine (72) also were effective in promoting the growth of young rats which were fed a low protein diet containing monobromobenzene. The amount of mercapturic acid excreted in the urine was greater when cystine or methionine were included in the diet than when monobromobenzene was fed in the absence of the extra sulfur-containing amino acids. On the other hand, the addition of glutathione or homocystine to the diet had no effect on the amount of mercapturic acid found in the urine of the animals which were fed monobromobenzene (71, 72). The promotion of growth following the administration of glutathione was attributed to a sparing action on tissue cystine or methionine (71).

In his latest publication Stekol (73) reported the quantitative relationships between the content of cystine and methionine in the diet of rats, which were fed monobromobenzene, and the excretion of mercapturic acid. The results were correlated with growth responses for the purpose of determining

whether the withdrawal of cysteine as mercapturic acid was the sole factor responsible for the cessation of growth. Groups of rats were fed different diets which contained the same amount of bromobenzene but varying amounts of the sulfur-containing amino acids. The total amount of sulfur present in the diet as cystine and methionine was calculated, and the amount of sulfur lost as mercapturic acid in the urine was determined by the use of his method. By subtracting the amount of sulfur lost as mercapturic acid from the amount present in the diet, the amount of the sulfur-containing acids actually available to the animal for the promotion of growth was calculated. Another group of rats was fed a diet which contained an amount of organic sulfur calculated to be the same as that available for the promotion of growth in the group of rats which had received monobromobenzene. The growth rates of the two groups were practically identical. He concluded that these results confirmed the hypothesis of White and Jackson (10); i.e., the monobromobenzene inhibited the growth of rats by creating a cystine deficiency due to the withdrawal of the sulfur-containing amino acids for mercapturic acid synthesis.

In another experiment he studied the effect of the prolonged administration of a low protein diet which contained one per cent monobromobenzene on the excretion of mercapturic acid by

adult rats. After the animals had been fed this diet for about four weeks, the level of bromophenylmercapturic acid in the urine dropped, and at the same time the rats began to lose weight. At this point, when either cystine or methionine was added to the diet, the rats regained their lost weight and in the next six weeks excreted twice as much mercapturic acid as in the previous period. Hence, the extent of the synthesis of the mercapturic acid was dependent on the amount of sulfur-containing amino acids available.

The studies with monobromobenzene have thus demonstrated the importance of the content of the sulfur-containing amino acids in the diet in determining the extent of the synthesis of bromophenylmercapturic acid in the dog and in the rat. There is disagreement, however, as to the immediate source of the cystine which is utilized for detoxication purposes.

Benzyl Chloride

Although the results of experiments in which the growth of rats was inhibited by the incorporation of monobromobenzene into the low casein diet indicate that the effect is caused by a cystine or methionine deficiency arising from a demand for these amino acids for the synthesis of mercapturic acid, the findings with benzyl chloride are difficult to interpret according to

this theory. The administration of benzyl chloride inhibited the growth of young rats which were fed a low casein diet, and this effect was counteracted by the addition of cystine or methionine to the low casein diet (74). The addition of S-benzylcysteine, a possible intermediate in the formation of the mercapturic acid, did not affect the growth of the rats, whereas the incorporation of benzylmercapturic acid, the conjugation product, in the diet caused a 50 per cent drop in the rate of growth of the rats. This effect could be nullified by the addition of cystine or methionine to the diet (75). Hence, Stekol (75) concluded that demand for cystine or methionine for the synthesis of benzylmercapturic acid was not the sole factor responsible for the growth inhibition observed when benzyl chloride was fed to rats that were maintained on a low casein diet.

The other compounds coming within the scope of this review have not been as intensively investigated as monobromobenzene and benzyl chloride. In Table IV is given a list of the more important compounds which are stated to inhibit the growth of rats on a low protein diet and whose growth inhibiting action is prevented by the addition of cystine or methionine to the diet. There is some other experimental evidence for all of these compounds that mercapturic acids are formed

in the course of their metabolism.

TABLE IV

Known Mercapturic Acid Precursors That Inhibit the Growth of Young Rats When Incorporated in a Low Casein Diet

Compound administered	Investigators
Monobromobenzene	White and Jackson (10, 70)
Benzyl chloride	Stekol (74)
p-Bromobenzyl bromide	Stekol (21)
Naphthalene	Stekol (76)
Phenanthrene*	Stekol (77)

*The administration of phenanthrene to the dog causes a rise in the organic sulfur of the urine (27).

The addition of glutathione to a low protein diet prevented the inhibition of the growth of young white rats observed when p-bromobenzyl bromide (21) or naphthalene (71) were added to the diet. The effect of the latter compound on growth was also counteracted by the addition of homocystine (75) to the diet, but the amount of α-naphthylmercapturic acid in the urine was not increased by the presence of this amino acid or glutathione in the diet (71). Therefore, it was considered that the stimulation of growth of the rats by these substances was not due to an increased conjugation of

the naphthalene and that glutathione was not directly utilized for detoxication purposes but was first hydrolyzed. The cysteine thus released was utilized for the synthesis of mercapturic acid (72).

Effect of the Alkalinity or Acidity of the Diet

Thus far one dietary factor, the content of protein or of the sulfur-containing amino acids, has been considered. Abderhalden and Wertheimer (78, 9) asserted that the reaction of the diet influenced the synthesis of bromophenylmercapturic acid by the rabbit. When bromobenzene was injected subcutaneously into rabbits that were fed a diet of oats (acid) or of green vegetables (alkaline), only in the former case were they able to isolate mercapturic acid from the urine. The authors concluded that the synthesis of bromophenylmercapturic acid was favored by a diet that produced an acid urine and inhibited by a diet that produced an alkaline urine.

These results were disproven by Sherwin and coworkers (79). Simple chloroform extraction of the urine of a rabbit that was maintained on a diet of green vegetables (alkaline) and fed monobromobenzene resulted in the isolation of mercapturic acid. Hence, it was concluded that the acidity or alkalinity of the diet had nothing to do with the synthesis of mercapturic acid. One reason that Abderhalden and Wertheimer (78, 9), after the administration of monobromobenzene, were

able to isolate mercapturic acid from the urine of the animal fed a diet of oats and not from the urine of the animal fed a diet of green vegetables might be the high protein content of the former as contrasted to the latter diet. Sherwin (79) writes, "Until the factors of diuresis and method of isolation are fully considered, the retardation of mercapturic acid synthesis on a basic diet must be called into question."

Pathways of Excretion of the Metabolic Products of the Monohalogen Substitution Products of Benzene

Although various investigators have reported that possibly some monobromobenzene (24) or benzene (48) might be eliminated by the lungs, pathways of excretion of the conjugation products of monobromobenzene other than the kidney had not been investigated until 1937 when a paper by Stekol and Mann (80) appeared. They measured the excretion of bromophenylmercapturic acid or α-naphthylmercapturic acid in the bile and in the urine after the administration of monobromobenzene or naphthalene respectively to bile fistula dogs which were fed a standard diet. The authors found that no mercapturic acids were excreted in the bile and that the same amount of the conjugation products were present in the urine as in that of the normal animal. Hence, they concluded that the bile was not a channel of excretion of these mercapturic acids, nor was the extent of the synthesis of the latter changed by the absence of bile from the intestine.

EXPERIMENTAL

Methods

Animal Experiments

Adult male rabbits, approximately 2.5 kg. in weight, were housed in individual cages constructed for the quantitative collection of urine. At the beginning of an experiment the urine in the bladder of the animal was removed by gentle pressure on the abdominal wall. This sample was discarded and the animal was placed in the cage. The urine excreted in a 24 hour period was collected in a small bottle, and the remainder of the specimen was obtained in the manner previously described.

Oats and cabbage or a commercial rabbit chow in weighed amounts sufficient to maintain the body weight of the animal made up the stock diet.

The compounds were fed by stomach tube. Benzene and its halogen derivatives were emulsified in gum tragacanth solution. l-Cystine was administered as a suspension in water, and l-methionine was fed in aqueous solution. The other amino acids were dissolved in the theoretical amount of sodium hydroxide in a total volume of 15 to 20 cc. of solution and fed through a tube.

Data as to the chemical identity and purity of the compounds fed are given in the Appendix.

Methods of Analysis

Sulfur Partitions of the Urine. -- The inorganic and total sulfate sulfur content of the urine was determined by the Folin gravimetric procedure (81) and the total sulfur by the method of Benedict (82) as modified by Denis (83).

A fraction not usually measured in the sulfur partition was the ether-soluble sulfur which was determined in one series of experiments by the following method.

The urine sample was made acid to congo red indicator with hydrochloric acid and extracted continuously in a Griffith (84) extraction apparatus with ether for a period of five hours. The solvent was removed by distillation, and the total sulfur in the residue was determined by the Benedict-Denis method. The details of the procedure are stated in the Appendix.

Hippuric Acid. -- Hippuric acid was determined by the method of Griffith (84). This procedure is not specific for hippuric acid but any nitrogenous compound which is ether-soluble and from which gaseous nitrogen is not liberated by the treatment with sodium hypobromite will interfere. In control experiments which are detailed in the Appendix, it was demonstrated that the nitrogen of benzylmercapturic acid was determined in the estimation of hippuric acid by this procedure.

Total Nitrogen. -- In the first experiments, total nitrogen

was estimated by the macro Kjeldahl-Gunning (85) method. In the remainder of the experiments the method of Ma and Zuazaga (86) was utilized. The ammonia liberated by the alkali from the digested sample of urine was steam distilled into 4 per cent boric acid and titrated with standard hydrochloric acid using a mixture of bromocresol green and methyl red as the indicator. This procedure was more convenient than the usual method involving a back titration.

Creatinine. -- The method for the determination of creatinine was a modification of the Jaffe-Folin (87) procedure adapted to the Evelyn photoelectric colorimeter. The estimation of creatinine by this method was less time consuming and certainly more objective than the original Jaffe-Folin procedure.

Glucuronic Acid. -- The method for glucuronic acid was based on the Tollens' reaction; glucuronic acid and naphtholresorcinol react in the presence of acid and heat to form a deeply colored complex which is extracted by ether, and the depth of the color can be measured in the Evelyn photoelectric colorimeter.

The procedure utilized was devised by Dziewiatkowski (88) and is a modification of the method of Evelyn and Maughan (89). In the former method an aqueous solution of 1, 3-dihydroxy-2-naphthoic acid is substituted for the naphtholresorcinol re-

agent of the latter procedure.

The level of glucuronic acid was estimated in the urine of rabbits fed monobromobenzene, p-monobromophenol, and benzene. When p-monobromophenol or bromophenylmercapturic acids were added to urine in amounts that might be expected in the experimental sample, no additional color was produced. In other words, these two best characterized products of the metabolism of monobromobenzene did not interfere in the determination of glucuronic acid. Likewise an aqueous solution of phenol gave the same reading as the blank.

The details of this procedure and the K value for this method are presented in the Appendix. Glucuronic acid is calculated in terms of its γ-lactone, d-glucurone, since the latter was utilized as the standard in determining the K value as detailed in the Appendix.

Phenol and p-Monobromophenol. -- "Total urinary phenols" were determined by the method of Bernard and McCullagh (90) as modified in this laboratory by Shacter (91). In this procedure the modified Millon reaction is employed. In the experiments in which benzene was fed, the extra total phenol on the experimental day was assumed to be phenol (monohydroxybenzene), and its concentration was calculated from the increase in color intensity on the experimental day with the use of the K value for phenol. In the experiments in which monobromo-

benzene or p-monobromophenol was administered, the extra total phenol on the experimental day was assumed to be p-monobromophenol, and the concentration of this substituted phenol was calculated from the increase in color intensity on the experimental day with the use of the K value for p-monobromophenol.

Since it was found in control experiments that various polyphenols did not react in the modified Millon reaction in concentrations much greater than those which would be expected to be found in the experimental urine, it is probable that the excreted phenol observed in the urine after the administration of benzene was monohydroxybenzene; but in the case of the administration of p-monobromophenol and monobromobenzene, there is no evidence that the extra phenol was p-monobromophenol. It seems, however, more logical to assume that the extra phenol that was present in the urine after the feeding of monobromobenzene or the p-substituted phenol was p-monobromophenol rather than monohydroxybenzene. This question is discussed in the section where the results of the administration of benzene, monobromobenzene, and p-monobromophenol are considered.

Recovery experiments with p-monobromophenol, experiments showing that the various polyphenols do not react in concentrations much greater than those which might be expected to

be present in urine and do not influence the color developed by phenol, and a calibration curve for p-monobromophenol are to be found in the Appendix.

Bromide Ion. -- It seemed possible that in the course of the metabolism of monobromobenzene, bromine might be split off, and the bromide ion excreted in the urine along with the other products of metabolism of the bromobenzene. A test for the bromide ion under such conditions would be complicated by two factors: 1) the slow rate of excretion of the bromide ion, a factor discussed in another section; 2) the presence of phenolic substances in the urine that might react with the free bromine formed in the usual qualitative test so that it could not be extracted with chloroform.

The test for the bromide ion was carried out in this manner. The urine was made acid with dilute sulfuric acid and extracted with chloroform to remove phenols and pigmented materials. The supernatant layer was pipetted into another test tube, a few drops of calcium hypochlorite solution were added, and the solution was again extracted with chloroform.

A sample of urine collected from a rabbit fed monobromobenzene gave no test for the bromide ion. However, if a small crystal of sodium bromide was added to this urine, a yellow color appeared in the second chloroform extract; i.e., a test was obtained thus establishing the validity of the

procedure.

Keto Acids. -- In two experiments the keto acids in the urine were determined by the procedure devised by Shacter (91). The urine sample was treated with the 2, 4-dinitrophenylhydrazine reagent, and the hydrazone of the keto acid was then extracted with benzene. The benzene extract was extracted with a borate buffer, which removed the hydrazone of the keto acid from the organic solvent. Alkali was added to the borate buffer solution of the hydrazone, which produced a color whose intensity was estimated in the Evelyn colorimeter.

Results and Discussion

Metabolism Experiments

The results of the metabolism experiments are presented in Tables V to XVIII of this section and in Tables XXIX to XXXVIII of the Appendix.

Benzyl Chloride

Stekol (6, 20) isolated N-acetyl-S-benzylcysteine from the urine after the subcutaneous administration of benzyl chloride. We have investigated the further fate of this organic compound in the animal body by the determination of the partition of urinary sulfur and by attempts to isolate the metabolic products from the urine. In this section the results of the sulfur partitions will be considered. In two experiments the benzyl chloride was administered by subcutaneous

injection; in the others it was fed orally. The results of the former method will be considered first and are presented in Tables V and VI.

Subcutaneous Injection. -- 260 mg. of benzyl chloride suspended in gum tragacanth solution were injected subcutaneously into a rabbit (Table V, Experiment 1-4) causing a marked rise in nitrogen excretion, a small decrease in the level of total sulfate and inorganic sulfate sulfur compensated for by a small rise in the organic sulfur equivalent to about 10 per cent of the injected compound. The level of total sulfur in the urine remained constant. Injection of gum tragacanth solution alone (Table V, Experiment 1-5) caused no change in the sulfur or nitrogen excretions, indicating that the results of the previous experiment were due to the administration of benzyl chloride. The injection of benzyl chloride was repeated with the same rabbit (Table VI, Experiment 1-7). Again an increase in the level of urinary nitrogen and of organic sulfur was observed. The latter was of doubtful significance for similar changes in the organic sulfur of the urine of the same order of magnitude occurred on the days after the injection of the benzyl chloride. The sulfur fractions had not returned to the normal control level one week after the experimental day.

These results would indicate that either the benzyl chloride

TABLE V

THE EFFECTS OF THE SUBCUTANEOUS INJECTION OF BENZYL CHLORIDE AND THE ORAL ADMINISTRATION OF BENZYL ALCOHOL TO THE RABBIT (EXPERIMENTS 1-3, 1-4, and 1-5 (4-185)).

Rabbit 1, 2.8 kg. Diet; cabbage, 75 gm., oats, 45 gm.

Date	Experiment No.	Nitrogen	Creatinine	Sulfur as					N	N
				Total	Total SO_4	Inorganic SO_4	Ethereal SO_4	Organic	Total S ratio	Total SO_4-S ratio
		gm.	mg.	mg.	mg.	mg.	mg.	mg.		
1942										
11/4		0.628	124	102	80	73	7	22	6.2	7.8
11/5		0.674	120	110	85	80	5	25	6.2	7.9
11/6	1-3	0.649	120	95	71	67	4	24	6.8	9.1
11/7		0.650	120	94	70	66	4	24	6.9	9.3
11/8		0.604	122	102	78	75	3	24	6.9	9.1
11/9		0.689	120	95	76	72	4	19	7.2	9.1
11/10	1-4	0.878	143	94	63	58	5	31	9.4	14
11/11		0.956	147	108	85	79	6	23	8.9	11
11/12		0.890	138	108	85	80	5	23	8.2	10
11/13		0.715	130	96	72	67	5	24	7.4	9.9
11/19		0.779	121	106	82	76	6	24	7.4	9.5
11/20		0.745	118	98	79	72	7	19	7.6	9.4
11/21	1-5	0.797	129	105	79	72	7	26	7.6	10
11/22		0.736	126	106	80	72	8	26	7.0	9.2

Experiment No. Key.

1-3 Administered orally 263 mg. benzyl alcohol.
1-4 Injected subcutaneously 243 mg. benzyl chloride suspended in 10 cc. of tragacanth solution.
1-5 Injected subcutaneously 10 cc. of gum tragacanth solution.

TABLE VI

THE EFFECTS OF THE SUBCUTANEOUS INJECTION OF BENZYL CHLORIDE AND THE ORAL ADMINISTRATION OF BENZYL ALCOHOL TO THE RABBIT (EXPERIMENTS 1-6 AND 1-7 (6-215)).

Rabbit 1, 2.8 kg. Diet; cabbage, 75 gm., oats, 45 gm.

Date	Nitrogen	Creatinine	Sulfur as					Hippuric acid	N	N
			Total	Total SO_4	Inorganic SO_4	Ethereal SO_4	Organic		Total S ratio	Total SO_4-S ratio
	gm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.		
1942										
12/2	0.704	116	101	78	73	5	23	63	7.1	9.0
12/3	0.748	111	102	82	73	9	20	86	7.3	9.1
12/4*	0.731	105	109	86	83	3	23	78	6.7	8.5
12/5	0.719	103	129	107	99	8	22		5.6	7.3
12/6	0.708	108	100	79	74	5	21	68	7.1	9.0
12/7**	0.905	134	110	77	72	5	33	133	8.2	12
12/8	0.886	120	108	87	81	6	21		8.2	10
12/9	1.01	115	127	97	93	4	30	81	8.0	10
12/10	0.867	112	127	97	92	5	30		6.8	8.9
12/11	0.942	112	140	105	100	5	35		6.7	9.0
12/13	0.766	105	115	89	82	7	26		6.7	8.5
12/14	0.822	111	137	102	98	4	33		6.1	8.1
	0.786	111	127	100	95	5	27		6.2	7.9

*242 mg. benzyl alcohol were administered orally.
**241 mg. benzyl chloride suspended in gum tragacanth solution were injected subcutaneously.

was so slowly absorbed that its effect on the sulfur distribution could not be noted, or else that the compound was metabolized in some other way than as a mercapturic acid. The former hypothesis is favored. The high nitrogen levels of the experimental and after days suggest tissue damage due to the irritating properties of the benzyl chloride which may have prevented its absorption. The sites of the injection remained hard for several weeks and later became open sores. In one of the experiments (Table VI, Experiment 1-7), the extra "hippuric acid" of the urine was increased by a small amount, indicating that some mercapturic acid was formed.[6] However, the changes in the "hippuric acid" level of the urine were very small and might have been due to normal variation.

The failure of the organic sulfur of the urine to return to the control value in the second injection experiment (Table VI, Experiment 1-7) might indicate either a slow absorption of the benzyl chloride or an upset in the metabolism caused by the irritating action of this organic compound.[7]

The results obtained by this method of administering the benzyl chloride were unexpected in view of the report of the

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[6] In the Griffith procedure (84) the nitrogenous compounds soluble in ether and not destroyed by sodium hypobromite are determined. In control experiments detailed in the Appendix it was shown that benzylmercapturic acid would interfere in this method.

[7] The failure of benzyl alcohol to cause an increase in the excretion of hippuric acid is discussed in the next section.

isolation of benzylmercapturic acid from the urine of rabbits which were injected subcutaneously with benzyl chloride. Stekol (20) injected 500 mg. of benzyl chloride into each of four rabbits and from the pooled urines was able to isolate benzylmercapturic acid. He did not report the amount isolated. Similar results were obtained with rats (subcutaneously injected)(20) and with dogs (fed) (6). From the urine of the latter 150-200 mg. of benzylmercapturic acid per gram of benzyl chloride fed was isolated (6).

In contrast to effect of the subcutaneous injection the changes produced in our experiments by the oral administration of benzyl chloride were very marked. In none of the experiments was there observed an effect on the ethereal sulfate level of the urine, indicating that conjugation with the sulfate radical is not a pathway of the metabolism of benzyl chloride. This factor will be considered again after the discussion of the effect of the oral administration of benzyl chloride.

The Oral Administration of Benzyl Chloride. -- The oral administration of benzyl chloride to the rabbit resulted in marked changes in the distribution of urinary sulfur. The changes observed can best be illustrated by a typical experiment which is presented in Table VII. The other experiments are summarized in Tables VIII, IX, and X of this section and

TABLE VII

THE EFFECT OF THE ORAL ADMINISTRATION OF BENZYL CHLORIDE TO THE RABBIT (EXPERIMENT 3-1 (3-171)).

Rabbit 3, 2.6 kg. Diet; cabbage, 75 gm., oats, 45 gm.

Date	Nitrogen	Creatinine	Sulfur as					N	N
			Total	Total SO_4	Inorganic SO_4	Ethereal SO_4	Organic	Total S ratio	Total SO_4-S ratio
	gm.	mg.	mg.	mg.	mg.	mg.	mg.		
1942									
10/27	0.660	114	99	76	72	4	23	6.7	8.7
10/28	0.648	115	98	77	71	6	21	6.6	8.5
10/29	0.609	114	93	73	68	5	20	6.6	8.3
10/30*	0.797	120	122	53	40	13	69	6.6	15
10/31	0.715	96	70	50	42	8	20	10.	14
11/1	0.849	126	119	89	82	7	30	7.1	9.6
11/2	0.698	112	90	66	60	6	24	7.8	10
11/3	0.710	111	89	68	64	4	21	8.0	10

*Administered orally 365 mg. of benzyl chloride. The increase in organic sulfur of 48 mg. of sulfur corresponds to 52 per cent of the benzyl chloride administered.

the detailed protocols are presented in Tables XXIX, XXX, XXXI, and XXXVI of the Appendix. The main effect of the feeding of benzyl chloride in the experiment given in Table VII was an increase in the organic sulfur fraction of the experimental urine which accounted for 52 per cent of the compound fed. On the first afterday the level of organic sulfur returned to normal. This fact indicates that the conjugation product was excreted by the kidney within 24 hours. On the experimental day, the rise in organic sulfur of 48 mg. was accompanied by a fall in the level of total sulfate sulfur (22 mg.), associated with a decrease in the amount of inorganic sulfate sulfur in the urine. On the afterday, although the creatinine value was low, the high nitrogen to total sulfur ratio was evidence that there had been a retention of sulfur. On the experimental day the level of total nitrogen remained relatively constant, but the amount of total sulfur increased slightly. The slight increase that took place in the ethereal sulfate sulfur excretion is the greatest that was observed after the feeding of benzyl chloride. In other experiments virtually no change at all took place in this fraction of the oxidized sulfur. (Table IX, Experiments 2A-5 and 2A-6).

TABLE VIII

Summary of the Extra Sulfur Fractions Found in the Urine after the Oral Administration of Benzyl Chloride

Rabbit 1, 2.9 kg. Diet; cabbage, 75 gm., oats, 45 gm.
Rabbit 3, 2.6 kg. Diet; cabbage, 75 gm., oats, 45 gm.
Rabbit 2A, 2.2 kg. Diet; cabbage, 100 gm., oats, 45 gm.
Rabbit 6, 3.2 kg. Diet; chow, 75 gm.

Experiment No.	Amount of benzyl chloride fed	Sulfur: Decrease in total SO_4	Sulfur: Extra total	Sulfur: Extra Organic		Sulfur: Extra ether-soluble	
	mg.	mg.	mg.	mg.	per cent*	mg.	per cent*
1-1	240	16	18	34	57		
1-2	233	0	17	24	40		
3-1	365	25	22	48	52		
2A-5	337	39	9	48	56	44	51
2A-6	357	42	5	47	52	47	52
2A-12	347	36	13	49	56	42	48
2A-13	347	21	21	40	45	40	45
6-6	340	30	20	50	58		

*Per cent equivalent to the benzyl chloride fed.

In other experiments where 230 to 240 mg. of benzyl chloride were fed (Table VIII, Experiments 1-1 and 1-2), similar results were obtained except that no retention of sulfur was observed on the day immediately following the experimental day, and the ethereal sulfate sulfur did not change at all. Likewise, no change was observed in this fraction of the total sulfate sulfur when 340 mg. of benzyl

chloride were fed to a rabbit that was maintained on a diet of chow (Table XXXVI, Experiment 6-6). An examination of Table IX will show that when 350 mg. (150 mg. per kg.) of benzyl chloride were given on four occasions (Experiments 2A-5, 2A-6, 2A-12, and 2A-13) to another rabbit, no change in the level of ethereal sulfate sulfur was observed. A striking difference observed in these experiments was a marked rise in the nitrogen excretion on the day that benzyl chloride was fed. This effect was not as clearly evident in the other experiments and will be discussed later in connection with a consideration of the influence of cystine and methionine on benzylmercapturic acid synthesis.

It seemed possible that in the course of its metabolism benzyl chloride might be converted to benzyl alcohol. When the latter compound in amounts equivalent to the smaller doses of benzyl chloride was administered to a rabbit (Table V, Experiment 1-3; Table VI, Experiment 1-6) no change in sulfur distribution was observed. Hence, benzyl alcohol does not appear to be an intermediate in the metabolism of benzyl chloride. Further evidence of this point is the well-known fact that this compound is converted to hippuric acid by the animal (92, 93). Stekol has shown that benzaldehyde is also converted to hippuric acid (20). It will be noted in one experiment where the hippuric acid excretion

in the urine after the administration of benzyl alcohol was estimated, no change was observed (Table VI, Experiment 1-6). This result is contrary to reports previously published by this laboratory (93) and may be due to the small amount of this compound fed, which was about one-fifth of the amount administered in the previous experiments.

In one experiment (Table XXXVI, Experiment 6-6) the level of urinary phenol and glucuronic acid was determined after the administration of benzyl chloride. No increases in the excretion of either substance were observed, hence, benzyl chloride was presumably not converted to a phenol or to a substance conjugated with glucuronic acid.

Thus the only increase observed in the urinary constituents after benzyl chloride feeding was an increase in the organic sulfur fraction accounting for 40 to 50 per cent of the compound fed (Table VII). The true hippuric acid excretion was not measured. Benzyl chloride is unique among the compounds that have been proven to form mercapturic acids in that no ethereal sulfates are formed in the course of its metabolism. The administration of styrene (28, 29) or phenanthrene (27) is also reported to increase the organic sulfur of the urine without affecting the ethereal sulfate sulfur fraction of the urinary sulfur. However, isolation of the mercapturic acid believed to be formed after the

ingestion of these substances has not yet been achieved, nor are the details of the sulfur partitions available in the reports that have been published.

The Effect of the Administration of l-Cystine and l-Methionine on Benzylmercapturic Acid Synthesis

In one series of experiments summarized in Tables IX and X of this section and given in full detail in Table XXXI of the Appendix, the effect of dietary cystine or methionine on the extent of benzylmercapturic acid synthesis was determined by following the changes in the distribution of urinary sulfur. Similar experiments in which monobromobenzene was fed have been carried out by White and Lewis (24) and by Stekol (58). Since the administration of moderate amounts of cystine or methionine alone to a rabbit results in slight increases in the organic sulfur of the urine (Table IX, Experiments 2A-8 and 2A-15), it is difficult to interpret changes in this fraction of the urinary sulfur when these amino acids are fed together with monobromobenzene, benzyl chloride, or other potential mercapturic acid precursors. It was decided that a more specific method for the estimation of the expected benzylmercapturic acid excretion would be a determination of the ether-soluble sulfur. In control experiments detailed in the Appendix, it was shown that cystine and benzylcysteine were not extracted from acid urine by ether. Methionine is re-

TABLE IX

THE EFFECT OF l-CYSTINE AND l-METHIONINE ON THE SYNTHESIS OF BENZYLMERCAPTURIC ACID.

Periods 1,6, 9, 10, 15, 18 and 22 are the averages of 2 or 3 control days. One week elapsed between Periods 9 and 10. Periods 5,8,14,17, and 21 respectively are the days immediately following the experimental day. The key to this table can be found on the next page.

Rabbit 2A, 2.2 kg. Diet; cabbage, 100 gm., oats, 45 gm.

Period	Experiment No.	Nitrogen	Creatinine	Sulfur						N / Total S ratio	N / Total SO_4-S ratio
				Total	Sulfate			Organic	Ether soluble		
					Total	Inorganic	Ethereal				
		gm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.		
1.		0.698	108	87	62	61	1	25	5	8.0	11
2.	2A-5	0.939	114	126	53	46	7	73	49	7.5	18
3.	2A-6	0.895	108	129	57	52	5	72	52	6.9	16
4.	2A-7	0.740	104	232	146	139	7	86	52	3.2	5.1
5.		0.730	108	118	94	89	5	24	8	6.2	7.8
6.		0.732	108	98	73	71	2	25	8	7.5	9.7
7.	2A-8	0.741	102	289	235	230	5	54	5	2.6	3.1
8.		0.681	102	114	90	86	4	24		6.0	7.6
9.		0.685	101	94	70	67	3	24	8	7.3	9.7
10.		0.632	109	93	65	60	5	28	8	6.9	9.8
11.	2A-12	0.948	115	129	52	47	5	77	50	7.3	18
12.	2A-13	0.912	106	114	46	39	7	68	48	8.0	20
13.	2A-14	0.793	105	158	83	78	5	75	47	5.0	9.3

TABLE IX (Continued)

Period	Experiment No.	Nitrogen	Creatinine	Sulfur						N / Total S ratio	N / Total SO_4-S ratio
				Total	Sulfate			Organic	Ether soluble		
					Total	Inorganic	Ethereal				
		gm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.		
14.		0.765	120	145	112	110	2	33	15	5.3	6.8
15.		0.784	116	84	63	59	4	21	5	9.1	13
16.	2A-15	0.657	103	190	157	152	5	33	7	3.5	4.2
17.		0.728	115	137	110	106	4	27		5.3	6.6
18.		0.744	102	85	67	63	4	18	8	8.8	11
19.	2A-16	0.677	101	131	110	108	2	21	9	5.2	6.2
20.	2A-17	0.734	110	196	122	119	3	74	46	3.8	6.0
21.		0.608	104	128	102	95	7	26	11	4.7	6.0
22.		0.725	107	68	52	47	5	16		11	13

KEY TO TABLES IX AND X.

Experiment No.	Compounds fed and notes
2A-5	337 mg. of benzyl chloride at 8 A.M.
2A-6	357 mg. " " " " 8 A.M.
2A-7	342 mg. " " " " 8 A.M. plus 500 mg. l-cystine at 8 A.M.; 500 mg. of l-cystine at 11 A.M. All of food was not consumed.
2A-8	500 mg. l-cystine at 8 A.M.; 500 mg. of l-cystine at 11 A.M.
2A-12	347 mg. of benzyl chloride at 8 A.M.
2A-13	347 mg. " " " " 8 A.M.
2A-14	350 mg. " " " " 8 A.M.; 600 mg. of l-methionine at 8 A.M.; 600 mg. of l-methionine at 11 A.M. All of food was not consumed.
2A-15	600 mg. of l-methionine at 8 A.M.; 600 mg. of l-methionine at 11 A.M. All of food was not consumed.
2A-16	600 mg. of l-methionine at 8 A.M. All of food was not consumed.
2A-17	354 mg. of benzyl chloride and 600 mg. of l-methionine at 8 A.M.; 600 mg. of l-methionine at 11 A.M. All of food was not consumed on this or following days.

TABLE X

Extra Total Sulfate, Ether-Soluble, and Organic Sulfur Observed after the Administration of Benzyl Chloride Alone or with l-Cystine or l-Methionine

The key to this table is given in the previous paragraph. Control values may be found in the Appendix with Table XXXI. The extra total sulfate sulfur observed on the days when l-cystine or l-methionine was fed is calculated as per cent of the sulfur administered as either of the two amino acids and is given for both the experimental day and the first afterday.

Experiment No.	Extra sulfur					
	As sulfur			As per cent compound fed		
	Organic	Ether-Soluble	Total SO_4	Organic	Ether Soluble	Total SO_4
	mg.	mg.	mg.	per cent	per cent	per cent
2A-5	48	44		56	51	
2A-6	47	47		52	52	
2A-7	61	47	91	70	54	34
			39			15
2A-8	30		162	11		61
			17			6
2A-12	49	42		56	48	
2A-13	40	40		45	45	
2A-14	47	39	34	55	44	13
			63			24
2A-15			94			37
			47			18
2A-16			42			30
2A-17	56	38	54	62	42	21
			34			

ported to be insoluble in ether, and the feeding of this amino acid did not result in a change in the level of the ether-soluble sulfur observed on the control days (Table IX, Experi-

ment 2A-15). Benzylmercapturic acid was added to urine, and under the conditions detailed in the Appendix, the extra ether-soluble sulfur corresponded to from 94 to 98 per cent of the mercapturic acid. Hence, it was decided that evidence of the extent of mercapturic acid synthesis in the type of experiment indicated could be obtained by the determination of ether-soluble sulfur. The procedure would be valid if no sulfur-containing compounds soluble in ether other than benzylmercapturic acid were formed after the ingestion of benzyl chloride. There is a possibility that some of the ether-soluble sulfur may represent an α-keto acid corresponding to S-benzylcysteine which might be formed in the process of the acetylation of benzylcysteine.

In the first series of experiments (see Tables IX and X of this section and Table XXXI of the Appendix) on the effect of the administration of l-cystine or l-methionine on the synthesis of benzylmercapturic acid, approximately 350 mg. of benzyl chloride were fed for two successive days and, on the third day, either l-cystine or l-methionine was administered together with the benzyl chloride. The sulfur-containing amino acids were given in amounts that furnished three mols of sulfur for every mol of benzyl chloride and were fed in two equal doses; one at the time that the benzyl chloride was fed, and the other three hours later in order to be sure

that the cystine or methionine was available to the tissues at the time the benzyl chloride was absorbed.

On the days when benzyl chloride alone was fed (Tables X and XXXI, Experiments 2A-5 and 2A-6), the amounts of extra organic sulfur and ether-soluble sulfur checked each other and accounted for about 50 per cent of the compound administered. On these days an increase in the level of urinary nitrogen and total sulfur was observed. At the same time the level of total sulfate sulfur dropped which resulted in a decrease in the nitrogen to total sulfate sulfur ratio.

On the third day (Tables IX and X, Experiment 2A-7) when l-cystine was given, the extra organic sulfur rose to a level equivalent to 70 per cent of the benzyl chloride fed; but the amount of the extra ether-soluble sulfur did not change significantly from the value obtained by the administration of benzyl chloride alone. However, the level of urinary nitrogen was reduced to the control value on the third day and remained constant for five days after the cystine had been given in addition to the benzyl chloride.

l-Cystine (1.0 gm.) was then fed in two equal doses three hours apart four days after the benzyl chloride had been fed (Tables IX and X, Experiment 2A-8). A rise in

organic sulfur of 30 mg. was observed, but the greatest effect was an increase in the total sulfate sulfur excretion of 162 mg. On the next day the extra total sulfate sulfur excretion was 17 mg. In contrast when the cystine was fed on the previous period together with benzyl chloride, only 94 mg. of extra sulfur appeared in the total sulfate sulfur fraction; and on the day after the experimental period 39 mg. of extra total sulfate sulfur was excreted (Tables IX and X, Experiment 2A-7). The difference in the extent of oxidation of the l-cystine in the presence and in the absence of benzyl chloride plus the marked effect of the l-cystine on the nitrogen excretion of the animal fed benzyl chloride indicate that the dietary amino acid was utilized by the animal in preference to his tissue amino acid. The difference between the level of extra total sulfate sulfur on the day when the cystine was fed alone and when this amino acid was fed in conjunction with benzyl chloride is 68 mg., an amount equivalent to the rise in organic sulfur on the latter day.

Similar experiments were carried out on the same animal with l-methionine (Tables IX and X; Experiments 2A-12, 2A-13, and 2A-14). The administration of two successive doses of benzyl chloride caused the same effects noted in the previous experiments with the exception that a progressive decline in

the level of total sulfate sulfur was observed. On the third day when a total of 1.2 gm. of l-methionine in two equal doses was also given (Experiment 2A-14), the nitrogen excretion returned to the control level, the excretion of organic sulfur was increased to a level higher than that of the two previous days; but the amount of ether-soluble sulfur did not change. The animal was fed the same amount of l-methionine which was administered in two portions three hours apart. (Table IX and X, Experiment 2A-15). This feeding resulted in a very slight rise in organic sulfur, no change in the ether-soluble sulfur, and an increase of 94 mg. in the total sulfate sulfur. On the day after the methionine was fed, the extra total sulfate sulfur was 47 mg. When the l-methionine was fed in conjunction with benzyl chloride, the rise in total sulfate sulfur was only 35 mg. on the experimental day, but 67 mg. on the afterday (Experiment 2A-14). The difference in the amount of oxidation of the l-methionine when fed with benzyl chloride or alone and the decrease in total urinary nitrogen observed in the previous case suggest the same explanation as for the results for cystine and benzyl chloride. That is, in the presence of methionine the tissue protein was spared, and the dietary sulfur-containing amino acids were utilized for detoxication purposes. The interpretation of these experiments is complicated by the

fact that the rabbit did not eat all of his food on the days either when methionine was fed with benzyl chloride or when the amino acid alone was administered. The decrease in urinary nitrogen observed in the former case is all the more significant since the nitrogen level would tend to increase in the fasting animal.

It was thought that perhaps the failure to observe an increase in the synthesis of mercapturic acid (ether-soluble sulfur) after supplementing the diet with l-cystine or l-methionine might be due to a depletion of the stores of sulfur-containing amino acids of the animal by the two previous doses of benzyl chloride. The increased nitrogen and total sulfur excretion and the drop in the level of inorganic sulfate sulfur of these days were evidence for this hypothesis.

Hence, the rabbit was given 600 mg. of l-methionine 24 hours before the next feeding period at which time benzyl chloride and 600 mg. of l-methionine were given, followed three hours later by 600 mg. of the same amino acid (Tables IX and X, Experiments 2A-16 and 2A-17). No definite change in the level of organic or ether-soluble sulfur was observed on the first day, but on the second day the organic sulfur was increased by an amount equal to 62 per cent of the benzyl chloride fed, and the per cent of the benzyl chloride excreted as ether-solutle sulfur was no different than that observed

when benzyl chloride alone was fed. Again the animal did not eat all of his food on either experimental day.

These results can be interpreted in the same manner as the previous ones, that is, the animal used the dietary amino acids in preference to those of his tissue protein for the synthesis of mercapturic acid. It may also be true that the amount of methionine given on the previous day was not sufficient to replenish the animal's stores of the sulfur-containing amino acid.

Other findings in this experiment are worthy of comment. An examination of Table X will reveal that the extra organic sulfur was equal to the extra ether-soluble sulfur of the urine after the feeding of benzyl chloride alone, indicating in this case that the organic sulfur fraction was a true index of mercapturic acid synthesis. This statement involves the assumption that the main component of the ether-soluble sulfur fraction was benzylmercapturic acid. The possibility that an α-keto acid corresponding to the mercapturic acid might be present in this fraction is not ruled out. However, the correspondence between the organic and ether-soluble sulfur fractions does indicate that little or no S-benzylcysteine is excreted in the urine after the feeding of benzyl chloride at the level of these experiments.

The discrepancy between the organic sulfur and the ether-

soluble sulfur on the days when cystine or methionine was fed cannot be explained on the basis of the experimental data. In the former case, the difference is probably not due to unchanged cystine for a negative nitroprusside test was obtained from the urine after the feeding of benzyl chloride and cystine, whereas a positive test was observed with the urine following the feeding of cystine alone. However, it is to be noted that it is not possible at present to explain the similar increases in organic sulfur which are observed after the administration of cystine or methionine alone.

In this experiment the assumption was made that the extra ether-soluble sulfur represented benzylmercapturic acid. This hypothesis was based on the results of other experiments, described in another section, in which the mercapturic acid was isolated from urine by a process involving ether extraction. As a further test of this assumption, benzylmercapturic acid was isolated from the experimental urines which remained after the analyses for ether-soluble sulfur were completed. An amount of mercapturic acid that corresponded to 24 per cent of the benzyl chloride fed and to 47 per cent of the average increase in ether-soluble sulfur was isolated. Since the method of isolation was probably not quantitative, this finding appeared to substantiate the assumption that the extra ether-

soluble sulfur fraction (and hence the extra organic sulfur) of the urine represented, for the most part, benzylmercapturic acid.

S-Benzylcysteine

The metabolism of S-benzylcysteine in the rabbit was investigated by the methods already utilized for benzyl chloride. The results of these experiments are presented in Tables XI and XII of this section and Tables XXI and XXII of the Appendix. The changes that were observed in the sulfur distribution of the urine after the administration of this amino acid to the rabbit are illustrated in a typical experiment presented in Table XI.

The only changes noted in the experimental urine after the administration of 750 mg. of S-benzylcysteine were marked increases in the amounts of organic sulfur and total sulfur. The rise in total sulfur could be accounted for by the increase in the level of organic sulfur since the levels of inorganic and ethereal sulfate sulfur did not change from the control values. Likewise, the total nitrogen of the urine did not change on the experimental day. An examination of Table XII will reveal that similar changes took place in the other experiments after the administration of S-benzylcysteine. The extra organic sulfur in the experimental urines accounted for from 76 to 93 per cent of the amino acid that was fed.

TABLE XI

THE EFFECT OF THE ORAL ADMINISTRATION OF S-BENZYLCYSTEINE TO THE RABBIT (EXPERIMENT 3A-1 (7-277)).

Rabbit 3A, 2.6 kg. Diet; cabbage, 75 gm., oats, 45 gm.

Date	Nitrogen	Creatinine	Sulfur as					N	N
			Total	Total SO_4	Inorganic SO_4	Ethereal SO_4	Organic	Total S ratio	Total SO_4-S ratio
	gm.	mg.	mg.	mg.	mg.	mg.	mg.		
1943									
2/23		132							
2/24	0.922	136	133	89	82	7	33	7.6	10
2/25	0.896	122	121	92	83	9	29	7.4	9.7
2/26	0.897	129	123	97	91	6	26	7.3	9.3
2/27 *	0.873	127	201	85	73	12	116	4.4	10
2/28	0.819	116	108	87	75	12	21	7.5	9.4
3/1	0.856	116	114	89	85	4	25	7.6	9.6
3/3	0.950	129	120	94	85	9	26	7.9	10

*Administered orally 750 mg. S-benzylcysteine dissolved in sodium hydroxide. The increase in organic sulfur of 87 mg. corresponds to 76 per cent of the S-benzylcysteine. From the urine left after analysis an amount of benzylmercapturic acid corresponding to 9 per cent of the S-benzylcysteine was isolated.

TABLE XII

Summary of the Changes in the Sulfur Distribution Observed after the Administration of S-Benzylcysteine

Rabbit 3A, 2.6 kg. Diet; cabbage, 75 gm., oats, 45 gm.
Rabbit 6, 3.2 kg. Diet; chow, 75 gm.
Rabbit 2A, 2.2 kg. Diet; cabbage, 100 gm., oats, 45 gm.

Experiment No.	S-Benzyl-cysteine fed	Extra Sulfur				
		Total	Organic		Ether-Soluble	
	mg.	mg.	mg.	per cent*	mg.	per cent*
3A-1	750	79	87	76		
6-7	560	77	75	88		
2A-9	500	64	64	86	47	63
2A-10	500	63	69	93	57	77

*Per cent of the sulfur administered as S-benzylcysteine.

In one series of experiments, (Tables XI and XII, Experiments 2A-9 and 2A-10) the amount of ether-soluble sulfur in the urine after the administration of S-benzylcysteine was estimated. The procedure and the facts that S-benzylcysteine was insoluble and benzyl mercapturic acid was soluble under the conditions used have already been discussed. It was found that the extra ether-soluble sulfur of the urine after the administration of S-benzylcysteine was equivalent to from 63 to 77 per cent of the sulfur fed as the amino acid or from 77

to 83 per cent of the extra organic sulfur. (Tables XII and XXI, Experiments 2A-9 and 2A-10). The small difference between the amount of extra organic sulfur and extra ether-soluble sulfur indicates that most of the S-benzylcysteine was changed to a substance soluble in ether. If the assumption is made that the differences between the extra organic sulfur and the extra ether-soluble sulfur on the experimental days are due to unchanged benzylcysteine, the amount of the latter excreted unchanged is still very small.

In the experiment which is presented in Table XI, an amount of benzylmercapturic acid that corresponded to 9 per cent of the benzylcysteine given or 12 per cent of the increase in organic sulfur was isolated from the urine. In other experiments double this amount was isolated; and if the fact that the method of isolation is not quantitative is taken into consideration, the results support the hypothesis that most of the increase in organic sulfur on the experimental day was due to benzylmercapturic acid. Further evidence in support of this hypothesis was the fact that the extra ether-soluble sulfur and organic sulfur of the urine in other experiments were almost the same. There is a possibility that some of the ether-soluble sulfur may represent the α-keto acid corresponding to benzylcysteine;[8] but, in the absence of any

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[8] It has been shown in preliminary experiments, discussed elsewhere, that the administration of S-phenylcysteine does increase the level of keto acids in the urine.

quantitative data as to the keto acid excretion after the administration of the amino acid, it is felt that the results are in accord with the theory that benzylcysteine is largely converted to and excreted as benzylmercapturic acid. The data also support the theory that benzylcysteine is an intermediate in the synthesis of the mercapturic acid corresponding to benzyl chloride.

In Experiment 6-7 (Tables XII and XXXII) no change in the levels of phenols or of total glucuronic acid was observed after the feeding of S-benzylcysteine. Similar results were obtained with benzyl chloride. Hence, the mercapturic acid corresponding to benzyl chloride does not appear to be excreted in conjugation with glucuronic acid, as has been suggested for bromophenylmercapturic acid.

The relation of these results to those reported by other investigators will be discussed in the General Discussion.

Cinnamyl Chloride

Cinnamyl chloride ($C_6H_5CH=CH-CH_2Cl$) is a vinylog of benzyl chloride and might thus be expected to behave similarly chemically. When this unsaturated compound was fed to a rabbit (Table XXXIII, Experiment 4-2) no change in the distribu- of sulfur was observed, hence a mercapturic acid was probably not present. The rise in the level of ether-soluble nitro-

genous compounds in the urine as estimated by the Griffith method (84) is presumptive evidence that the cinnamyl chloride was converted to hippuric acid particularly if the results of other investigators, who have demonstrated that cinnamic acid is converted to hippuric acid in the animal body, are considered (94).

Bromobenzene

The metabolism of monobromobenzene was investigated by studying the partition of sulfur and the excretion of phenols and glucuronic acid in the urine. The results are summarized in Table XIII of this section and detailed in Tables XXXIV, XXXV, and XXXVI of the Appendix.

TABLE XIII

The Metabolism of Monobromobenzene in the Rabbit

Rabbit 1, 3.2 kg. Diet; cabbage, 100 gm., oats, 75 gm.
Rabbit 4, 3.0 kg. Diet; cabbage, 100 gm., oats, 75 gm.
Rabbit 3A, 2.5 kg. Diet; cabbage, 75 gm., oats, 45 gm.
Rabbit 6, 3.2 kg. Diet; 75 gm. of chow.

Experiment No.	Amount of bromobenzene fed	Decrease in inorganic SO4-S	Per cent of bromobenzene fed excreted as			
			Ethereal SO4-S	Glucuronic acid	Bromophenol*	Organic S
	gm.	mg.	per cent	per cent	per cent	per cent
1-8	1.0			9		
4-1	1.0			21		
3A-2	1.0			26		
3A-3	1.1	60	25	26		13
6-1	1.0	60	23	21	14	16

*Extra total phenol calculated as bromophenol.

Other workers have indicated (4) that p-bromophenylmercapturic acid may be excreted as a glucuronic acid complex. Consequently in the first experiments (Tables XIII and XXXIV, Experiments 1-8, 4-1, and 3A-2) the excretion of total glucuronic acid following the administration of monobromobenzene to the rabbit was measured. When the halogenated derivative of benzene (one gm.) was fed to each of three rabbits, resulting increases in glucuronic acid excretion took place equivalent to 9, 21, and 26 per cent of the monobromobenzene administered. So far as we are aware, this is the first quantitative evidence of the effect of this monohalogen substitution product of benzene on glucuronic acid excretion. An examination of Table XIII will show that comparable increases in the glucuronic acid level of the urine were obtained in two other experiments. In four out of five experiments, a rise in total glucuronic acid level in the urine equivalent to about 20 to 25 per cent of the monobromobenzene was observed.

The estimation of the glucuronic acid alone after the administration of monobromobenzene did not reveal how the other urinary constituents, the ethereal sulfate sulfur and the organic sulfur, were affected at the same time. Information of this nature might be of value in deciding with which of the two best-known metabolic products of monobromobenzene, p-bromophenol or p-bromophenylmercapturic acid, the glucuronic acid is associated.

In the next experiment (Tables XIII and XXXV, Experiment 3A-3) the partition of sulfur as well as the glucuronic acid excretion was followed. The increases in the glucuronic acid and the ethereal sulfate sulfur were equal, being 25 and 26 per cent respectively of the monobromobenzene administered; whereas the extra organic sulfur accounted for only 13 per cent of the amount fed. The increase in total glucuronic acid was thus about twice the increase in the organic sulfur level of the urine. It is known that phenols are conjugated with glucuronic acid; and since phenols are products of the metabolism of monobromobenzene, in the next experiment the level of "bromophenol" in the urine was estimated[9] (Tables XIII and XXXVI, Experiment 6-1).

The rises in glucuronic acid and ethereal sulfate sulfur on the experimental day accounted for 21 and 23 per cent respectively of the monobromobenzene, whereas the levels of "bromophenol" and extra organic sulfur were 14 and 16 per cent respectively.

These results are very difficult to interpret, and from

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[9] As has been pointed out before in the section of Methods and in the Appendix, the method for monobromophenol is not specific and is based on the assumption that the increased color intensity given in the modified Millon reaction by a sample of the urine of a rabbit fed monobromobenzene or p-monobromophenol is due solely to the presence of this phenol. Its concentration is obtained by the use of a calibration curve prepared from p-monobromophenol after correcting for the color given by control samples of urine. For further details consult the Appendix.

them no definite conclusion can be made regarding the existence of a bromophenylmercapturic acid complex.

If the metabolism of bromobenzene resulted only in the formation of p-bromophenol and bromophenylmercapturic acid, and if the phenol were conjugated with ethereal sulfates and glucuronic acid, the per cent of the compound excreted as bromophenol should be greater than the per cent converted to ethereal sulfate. Actually, the rise in "bromophenol" found in the urine is about half the increase in the ethereal sulfate sulfur observed after the administration of monobromobenzene. One possibility is that the conditions of hydrolysis in the "bromophenol" determination were not drastic enough to hydrolyze all of the ethereal sulfates produced. This hypothesis is improbable because when an equivalent amount of bromophenol was administered (Tables XIV and XXXVII, Experiment 6-8), the level of bromophenol, which was much larger than in the monobromobenzene experiments, was about twice the value for extra ethereal sulfate sulfur.

Comment on possible interpretations of these results and of the changes in the sulfur partition of the urine after the administration of monobromobenzene will be reserved until a discussion of the results of the feeding of p-monobromophenol has been completed.

p-Monobromophenol

This potential product of monobromobenzene metabolism was

investigated by the methods used in the study of the fate of monobromobenzene itself. The partition of urinary sulfur and the excretions of glucuronic acid and "bromophenol" were determined. A summary of the results is presented in Table XIV, and the protocols are listed in Tables XXXVII and XXXVIII of the Appendix.

TABLE XIV

The Metabolism of p-Monobromophenol in the Rabbit

Rabbit 6, 3.2 kg. Diet; chow, 75 gm.
Rabbit 2A, 2.2 kg. Diet; cabbage, 100 gm., oats, 45 gm.

Experiment No.	p-Monobromophenol fed	Sulfur			Extra glucuronic acid		Bromophenol*	
		Decrease in inorganic SO_4	Extra ethereal SO_4					
	gm.	mg.	mg.	per cent+	mg.	per cent+	mg.	per cent+
6-8	1.10	70	45	22	588	52	576	52
2A-1**	0.407	27	17	23	213	52	342	84
2A-2++	0.404	19	16	21	234	57	118	29
2A-3	0.400	16	29	39	273	67	189	47
2A-4	0.403	17	20	27	291	71	116	29

*Total phenols calculated as bromophenol.
+Per cent equivalent to the amount of p-monobromophenol fed.
**Single dose
++Repeated daily administration.

Changes in the Glucuronic Acid, Ethereal Sulfate, and "Bromophenol"

The changes observed in the levels of the three urinary

constituents after the administration of p-monobromophenol will be discussed before the changes in the sulfur distribution are considered.

In Experiment 2A-1 (Tables XIV and XXXVII), 0.4 gm. of p-monobromophenol was fed to another rabbit resulting in an excretion of extra total phenols, calculated as bromophenol, which accounts for 84 per cent of the substituted phenol administered. At the same time the glucuronic acid in the urine and ethereal sulfates increased by amounts that were equivalent to 52 and 23 per cent respectively of the p-monobromophenol fed. Associated with this rise in ethereal sulfates was a compensatory drop in the level of inorganic sulfate sulfur.

The results of this experiment can be interpreted as evidence that the p-monobromophenol was conjugated with ~~ethereal sulfate~~ SULFURIC ACID and glucuronic acid. The extent of excretion as a glucuronide is an indication of the importance of this mechanism in the detoxication of phenols in this species.

Later the same amount of p-monobromophenol was administered to this animal on each of three successive days (Tables XIV and XXXVIII, Experiments 2A-2, 2A-3, and 2A-4). An examination of Tables XIV and XXXVIII will show that the excretion of glucuronic acid, bromophenol, and ethereal sulfate sulfur was increased on the experimental day. Although the level of

bromophenol in the urine was higher than the amount of ethereal sulfate sulfur present, the changes in the two constituents were parallel, rising to a maximum on the second day, and then falling to lower values on the third experimental day. However, the glucuronic acid excretion increased steadily reaching a maximum on the third day. The results obtained for the three successive feedings of p-monobromophenol are similar to those obtained when a larger amount of the substituted phenol was fed to another rabbit (Tables XIII and XXVIII, Experiment 6-8). That is, the sum of the glucuronic acid and ethereal sulfate sulfur levels was much greater than the amount of p-bromophenol in the urine. If the metabolism of p-monobromophenol involved simply a conjugation of the phenol with glucuronic acid and with sulfuric acid, the amount of bromophenol in the urine should have been greater than or equal to the sum of the glucuronic acid and ethereal sulfate levels. The results of Experiment 2A-1 support this hypothesis.

The Relationship between the Phenols, Ethereal Sulfates, and Glucuronic Acid.

The facts that the level of total phenols calculated as monobromophenol was less than the amount of ethereal sulfates in the urine (Tables XIV and XXVI, Experiment 6-1) after the feeding of monobromobenzene and that the calculated bromophenol excretion was much less than the sum of the extra glucuronic

acid and ethereal sulfate sulfur in the urine (Tables XIV, XXXVII, and XXXVIII, Experiments 2A-2, 2A-3, 2A-4, and 6-8) after the administration of p-monobromophenol are difficult to explain.

In control experiments, when amounts of bromophenol similar to those calculated to be present in the experimental sample were added to urine aliquots of the size usually analyzed on the day the phenol or bromobenzene was fed, satisfactory recoveries were obtained. Similarly p-monobromophenol or bromophenylmercapturic acid did not interfere in the determination of glucuronic acid.

A theory that might be offered as an explanation of the low amount of extra total phenol (calculated as bromophenol) after the feeding of monobromobenzene or p-monobromophenol is that, in the course of the metabolism of these two compounds, phenols are produced that either do not react in the modified Millon reaction or give much less color than the p-substituted phenol. Thus the extra phenol, calculated as bromophenol, present in the experimental urine samples would be too low. There is some evidence that bromohydroquinone (4) and pyrocatechol (1) are produced in the course of the metabolism of monobromobenzene. In control experiments it was found that various polyphenols did not give a color in the modified Millon reaction. Diechmann and Thomas (95) found that the

administration of hydroquinone to rabbits increased the excretion of ethereal sulfate but not of glucuronic acid. The administration of pyrogallol or resorcinol increased the level of both constituents. Perhaps, if such polyphenols were produced in the metabolism of bromobenzene or p-monobromophenol, they might be excreted in conjugation with ethereal sulfates and with glucuronic acid. When the urine was hydrolyzed prior to analysis, the level of glucuronic acid and ethereal sulfates would be increased by the fraction of the respective constituent conjugated with the polyphenol; but the amount of "total phenol" which is estimated by means of the modified Millon reaction would not be affected by the presence of the polyphenols since the latter are not determined in the modified Millon reaction. Hence, the total phenol would not represent all of the phenolic compounds in the urine.

Furthermore the other phenols which might give a color in the modified Millon reaction such as phenol itself would probably not give as intense a color as p-monobromophenol. On a molar basis, phenol gives about one half the color that p-bromophenol does. The calculation of "bromophenol" in the experimental urine is based on the assumption that the increase in color intensity after the feeding of monobromobenzene or p-monobromophenol is due solely to the presence of p-monobromophenol. But if, for example, phenol were produced, the calcu-

lation would not be valid because the unsubstituted phenol gives less color than p-monobromophenol. It was also found that the presence of various polyphenols did not weaken the color given by phenol in the modified Millon reaction, and hence this factor is ruled out.

It is possible that polyphenols could have been produced with or without the removal of bromine from the benzene nucleus of bromophenol or bromobenzene. The urines obtained from rabbits fed these substances were examined for the presence of the bromide ion by the procedure described under "Methods," but none would be detected. However, this result does not rule out the possibility that bromine was removed from the monobromobenzene or p-monobromophenol molecules because, as Hele (43) points out, this inorganic ion would be excreted slowly and be present in the urine in amounts that might not be detected. Hastings and coworkers (96) have shown that bromide ion is slowly excreted. Steinauer (97) could not detect the bromide ion in the urine of animals fed monobromobenzene.

Another possibility is that a stable glucuronide was formed with a product or products of the metabolism of monobromobenzene or its corresponding p-substituted phenol, which was not hydrolyzed under the conditions of the phenol determination. This hypothesis is not very probable since in one experiment (Tables XIV and XXXVIII, Experiment 2A-1) the

amount of bromophenol in the urine was greater than the sum of the extra glucuronic acid and extra ethereal sulfates.

Although from these results no conclusion can be made in regard to the existence of a bromophenylmercapturic acid-glucuronic acid conjugate, they do prove the quantitative importance of conjugation with glucuronic acid in the metabolism of monobromobenzene and its corresponding phenol.

Changes in the Sulfur Distribution of the Urine

Monobromobenzene. -- The changes observed in the urinary sulfur distribution after the feeding of monobromobenzene were similar to those reported by Lough and Lewis (67). On the day that the monohalogen substitution product of benzene was fed (Tables XXXV and XXXVI of Appendix) slight rises in the levels of total nitrogen and sulfur took place, which were accompanied by increases in the amounts of organic and ethereal sulfate sulfur, and a very marked drop in inorganic sulfate sulfur. There was a slight decrease in the nitrogen to total sulfur ratio but a slight increase in the nitrogen to total sulfate sulfur ratio. On the first day after the experimental day, the total sulfate and the total sulfur excretions were below the control level, which indicates a retention of the sulfur-containing amino acids to replace that amount utilized in the detoxication of monobromobenzene. These facts are reflected in the increased nitrogen to total sulfur and nitrogen to

total sulfate sulfur ratios which were observed on the first afterday. The decrease in the total sulfur was accompanied by a decrease in the inorganic sulfate sulfur level of the urine. The changes which were observed by Lough and Lewis (67) on the first afterday were more pronounced than the changes that were observed in this experiment, but the former investigators fed two successive doses of the benzene derivative, whereas only one feeding of monobromobenzene was carried out in the present experiments.

p-Monobromophenol. -- The changes in the distribution of urinary sulfur produced by the administration of moderate amounts of p-bromophenol (Tables XIV and XXXVIII, Experiments 2A-1, 2A-2, 2A-3, and 2A-4) are similar to results reported by other workers (24, 54). On the experimental days there was an increase in ethereal sulfate sulfur which was compensated for by a decrease in the inorganic sulfate fraction of the oxidized sulfur. However, the amount of organic sulfur in the experimental urines was not significantly changed. These results are in accord with previous findings with the rat (54) and the dog (24) and are additional evidence that p-monobromophenol is not an intermediate in the synthesis of bromophenylmercapturic acid.

In one experiment, (Tables XIV and XXXVII, Experiment 6-1) the dose (1.1 gm.) of p-monobromophenol given to the rabbit

was somewhat toxic, for he ate only part of the food the experimental day and refused all food for the next three days. It is of interest to point out certain similarities between the experiment and that of Lough and Lewis (67) in which three grams of monobromobenzene were given. In their experiment a marked drop in the level of total sulfur was observed on the experimental day, and in our experiments on this day the total sulfur excretion dropped from a control level of 124 mg. to 88 mg. These results indicate that a large amount of p-monobromophenol like bromobenzene depresses the excretion of total sulfur by the animal. This factor is illustrated in our experiments by the marked rise in the nitrogen/sulfur and nitrogen to total sulfate sulfur ratios on the experimental day. The progressive decline in the level of the urinary constituents after the experimental day can be attributed to the fasting condition of the animal.

Benzene.

The metabolism of benzene was investigated by the methods previously described for monobromobenzene and p-monobromophenol. The results are summarized in Table XVI and the protocols are given in Tables XV and XXXV.

TABLE XV

THE EFFECT OF THE ORAL ADMINISTRATION OF BENZENE TO THE RABBIT (EXPERIMENTS 3A-5, 3A-6, AND 3A-7(3-43B)).

Rabbit 3A, 2.5 kg. Diet; cabbage, 75 gm., oats, 45 gm.

Date	Nitrogen	Creatinine	Sulfur as					Phenol	N Total S ratio	N Total SO_4-S ratio	Extra Ethereal SO_4-S	Extra phenol	Per cent of compound excreted as	
			Total	Total SO_4	Inorganic SO_4	Ethereal SO_4	Organic						Ethereal SO_4-S	Phenol
	gm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.			mg.	mg.	per cent	per cent
1943														
5/13	0.692	120	91	67	65	2	24		7.6	10				
5/14	0.612	124	85	64	57	7	21	48	7.2	9.6				
5/15*	0.720	120	94	70	26	44	24	133	7.7	10	40	95	9.0	7.3
5/16*	0.722	124	76	54	0	54	22	168	9.5	13	50	131	11	10
5/17*	0.681	124	79	54	3	51	25	171	8.6	13	47	134	13	10
5/18	0.714	116	101	80	77	3	21	27	7.1	9				
5/19	0.731	116	105	82	78	4	23		7.0	9				

*Administered 1.08 gm. of benzene orally.

TABLE XVI

Summary of Benzene Metabolism Experiments

Rabbit 3A, 2.5 kg. Diet; cabbage, 75 gm., oats, 45 gm.
Rabbit 6, 3.2 kg. Diet; chow, 75 gm.

Experiment No.	Amount of benzene fed	Decrease in inorganic SO_4-S	Per cent of the benzene excreted as		
			Extra ethereal SO_4-S	Glucuronic acid	Phenol
	gm.	mg.	per cent	per cent	per cent
3A-4*	1.00	29	6.6	4.4	
3A-5**	1.08	35	9.0		7.3
3A-6	1.08	61	11		10
3A-7	1.08	58	10		10
6-2**	1.02	35	9.1	5.3	11
6-3	1.01	57	10	11	13
6-4	1.02	59	11	7.4	13
6-5	1.00	47	8.5	0	11

*Single dose.
**Repeated daily doses.

In two of the three experiments with benzene the effect of repeated doses was noted. In Table XV is presented the first experiment in which a rabbit was fed benzene for three days in succession with a resultant progressive decrease in the inorganic sulfate sulfur fraction of the urine so that by the second day this fraction of the oxidized sulfur had disappeared from the urine, and on the third day only three mg. were present. Increases in the ethereal sulfate sulfur level of the urine that corresponded to 9, 11, and 10 per cent

of the benzene administered occurred on each of the three days. Parallel to the changes in the ethereal sulfate sulfur was an equivalent increased excretion of phenol on each of the three experimental days. The excretions of total sulfur and total sulfate sulfur were depressed on the second and third day that benzene was fed, since the increases in ethereal sulfate sulfur did not entirely compensate for the fall in the level of the inorganic sulfate fraction of the oxidized sulfur. The amount of the organic sulfur fraction did not change from the control value. The latter observation is quite striking and indicates that this hydrocarbon did not form a mercapturic acid in this experiment.

Similar results were obtained in another experiment where four repeated doses of benzene were fed to a rabbit (Tables XVI and XXXVI, Experiments 6-2, 6-3, 6-4, and 6-5). Again the excretion of inorganic sulfate sulfur was depressed and fell to a value of about 40 per cent of the control level on the third day but rose again on the fourth day. The changes in the ethereal sulfates and in the level of phenols present in the urine were similar to those of the previous experiment. In addition, changes in glucuronic acid excretion were measured. The level of this constituent of the urine rose to a maximum on the second day and fell to a value not significantly different

from the control on the fourth day. In view of the variation in the control values of glucuronic acid, it is doubtful if the small differences between the excretion of this constituent of the urine observed on the first and on the third experimental days are significant (Tables XVI and XXXVI).

The administration of a single dose of benzene (Table XXXVI, Experiment 3A-4) caused increases in the excretion of ethereal sulfate sulfur and glucuronic acid (phenols were not estimated) and a fall in the level of the inorganic sulfate sulfur fraction of the oxidized sulfur.

Discussion

These results indicate that one of the main pathways of benzene metabolism in the rabbbit is the conversion of the hydrocarbon to phenols that are excreted in combination with glucuronic and sulfuric acids. These findings are in accord with previous work which has demonstrated that the administration of benzene to an animal gives rise to phenol (44), hydroquinone and pyrocatechol (47), and causes an increase in the excretion of the ethereal sulfates (46, 5). In view of the specificity of the modified Millon reaction, which has already been discussed, it is probable that the extra "phenol" in the experimental urine is actually phenol (monohydroxybenzene).

The relationship between the excretions of glucuronic acid, phenol, and ethereal sulfates in the experiment when

four repeated daily doses of benzene were given (Tables XVI and XXXVI, Experiments 6-2, 6-3, 6-4, and 6-5) was similar to that observed with p-monobromophenol (Tables XXXVII and XXXVIII). In the benzene experiments the total phenol level of the urine was less than the sum of the glucuronic acid and ethereal sulfate excretion except for the last day. The hypothesis that was suggested in the discussion of the monobromobenzene and p-monobromophenol experiments is offered as an explanation of these results. That is, phenols such as hydroquinone and pyrocatechol that do not react in the modified Millon reaction were produced in the animal body from the benzene and excreted as ethereal sulfates and glucuronides. This theory is in accord with the known facts of benzene, pyrocatechol, and hydroquinone metabolism in the rabbit, which have been discussed previously.

The failure to observe an increase in the level of glucuronic acid in the urine on the fourth day that benzene was fed (Table XXXVII, Experiment 6-5) might be due to the fact that the animal's ability to mobilize or synthesize glucuronic acid was depressed by the repeated administration of benzene. This seems unlikely, however, since the same animal excreted much more glucuronic acid than was found in the urine after the feeding of benzene when three repeated doses of p-monobromo-

phenol were administered. (Table XIV, Experiment 6-7). Another interesting observation was the small changes that took place in the organic sulfur fraction of the urine after the feeding of benzene. In the experiments illustrated in Table XV no change took place in the excretion of organic sulfur on the experimental days. In the other experiments (Tables XXXV and XXXVI of the Appendix) the changes of 3 to 4 mg. that were noted are of doubtful significance since the control value of this fraction of the urinary sulfur is not constant and the latter fraction is determined by difference. On the basis of these experiments the synthesis of a mercapturic acid from benzene by the rabbit is questionable. This question will be considered again in the discussion of our attempts to isolate phenylmercapturic acid which are reported in another section of the thesis.

S-Phenylcysteine

The metabolism of S-phenylcysteine had not been investigated at the time of the initiation of these studies. While our experiments were in progress the isolation of phenylmercapturic acid from the urine of rats to which this derivative of cysteine was fed was reported (33).

S-phenylcysteine was not extracted by ether under the conditions described in the Appendix. As a more specific measure of the expected excretion of phenylmercapturic acid

following the administration of the unacetylated amino acid to a rabbit, the ether-soluble sulfur was determined. The results of this experiment are summarized in Table XVII and given in detail in Table XXXI, Experiment 2A-11 of the Appendix.

TABLE XVII

The Metabolism of S-Phenylcysteine (Experiment 2A-11)

Periods 1 and 4 are the averages of the control days. Period 3 is the day after the experimental day and Period 2 is the day when 500 mg. of S-phenylcysteine (Schultz) were given.

Rabbit 2A, 2.2 kg. Diet; cabbage, 100 gm., oats, 45 gm.

Period	Nitrogen	Sulfur						N / Total S ratio	N / Total SO_4-S ratio
		Total	Sulfate			Organic	Ether soluble		
			Total	Inorganic	Ethereal				
	gm.	mg.	mg.	mg.	mg.	mg.	mg.		
1	0.750	97	69	67	2	28	7	7.7	11
2	0.661	152	75	70	5	77	50	4.2	8.8
3	0.687	109	75	72	3	34		6.3	9.2
4	0.633	92	65	60	5	27	8	6.9	9.8

It was found that the changes in the sulfur distribution of the urine following the feeding of S-phenylcysteine were similar to those produced by S-benzylcysteine. The organic sulfur and ether-soluble sulfur increased by amounts equivalent

to 60 and 53 per cent respectively of the compound fed. The amount of total nitrogen in the urine remained constant but a slight decrease took place in the nitrogen to total sulfate sulfur ration, indicating that a very small portion of the S-phenylcysteine may have been oxidized to the sulfate. The change is so small, however, that it may not be significant.

The correlation between the extra organic and ether-soluble sulfur values on the experimental day demonstrates that little if any of the S-phenylcysteine was excreted unchanged but that it was converted to a form soluble in ether. In view of the similarity of the behavior of the compound of S-benzylcysteine it was thought probable that the ether-soluble sulfur fraction was composed mainly of phenylmercapturic acid. However, from an aliquot of this urine calculated to contain 50 mg. of phenylmercapturic acid, none could be isolated by the procedure successful in the case of S-benzylcysteine. The details of this experiment are reported in another section. It is probable that either the mercapturic acid was destroyed in process of isolation (Zbarsky and Young (33) report the acid to be very labile to alkali), or else the method of isolation was not quantitative enough to isolate this small amount.

The ether-soluble sulfur fraction may also contain another component, the keto acid corresponding to S-phenylcysteine. The only evidence for this was obtained in two preliminary experiments carried out with the cooperation of Dr. C. W. Shen of this laboratory. Two rabbits were each given 750 mg. of S-phenylcysteine (Witter). Analyses of the urines by the method of Shacter (91) carried out by Dr. Shen demonstrated increases in total keto acids over the amounts present on the control days equivalent to 14 and 7.5 per cent respectively of the compound fed. The data obtained in these experiments are presented in Table XVIII.

TABLE XVIII

The Formation of a "Keto Acid" from S-Phenylcysteine

Date	Rabbit	Keto acid	Extra keto acid	
		mg.	mg.	per cent*
1944				
4/15	8	4.5		
4/16*		93.7	89	14
4/15	S	2.8	47	7.5
4/16**		50.5		

*Per cent of S-phenylcysteine excreted as the keto acid.
**Calculated as mg. of phenylpyruvic acid.

In control experiments in which dl-benzylmercapturic acid was used, it was shown by Dr. C. W. Shen that this mercapturic acid did not interfere in the determination of keto acids by the method of Shacter (91).

The substitution of a benzyl or a phenyl group on the sulfhydryl group of cysteine appears to protect the sulfur of cysteine from oxidation. The compound, instead of being oxidized, is acetylated (proven in our experiments with S-benzylcysteine) and excreted as the mercapturic acid. The significance of the rise in keto acids is not known, but one might expect on the basis of analogous compounds that oxidative deamination could occur even if the sulfur could not be oxidized normally. Also the increase in keto acid may be evidence of acetylation by the mechanism proposed by du Vigneaud and Irish (32). These hypotheses are mere speculation and have no supporting experimental evidence.

The Isolation of Mercapturic Acids.

The isolation of mercapturic acids from the urine of animals fed the compounds listed in Table I has been accomplished by many workers using various techniques. In the present study the major part of the isolation experiments was carried out after the feeding of benzyl chloride and benzyl chloride and benzylcysteine, and the procedure worked out successfully for the isolation of benzylmercapturic acid

was utilized in attempts to isolate a mercapturic acid after the administration of benzene or phenylcysteine to the rabbit. Two other methods were tried out in endeavors to isolate a mercapturic acid after the administration of benzene to the rabbit.

Benzyl Chloride and S-Benzylcysteine

The General Method. -- This method was a modification of that of du Vigneaud and his coworkers (98). The procedure was changed slightly from time to time in the hope of improving the yield, but all of the modifications were based on the following preliminary procedure. The filtered urine was concentrated in vacuo to a small volume.[10] The concentrate was acidified (congo red paper) and extracted with ether in a Kutscher and Steudel type of extractor for a period of from 10 to 24 hours. After most of the ether had been removed by distillation on the steam bath, the remainder was removed by distillation in vacuo. The residue was dissolved at a temperature of 50 to 60° in the minimum amount of 5 per cent ammonium hydroxide required to make the solution alkaline. The ~~ammoniacal~~ ALKALINE solution was filtered, warmed to 70-80°, and decolorized with norit. After the norit was removed by

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[10] In the benzylcysteine experiments the urine was not concentrated but was acidified with hydrochloric acid and extracted continuously with ether.

filtration, the warm solution was carefully acidified (congo red paper) with 2.5 N hydrochloric acid. The oily material that formed could be solidified by alternately heating and cooling the solution and scratching the sides of the vessel with a stirring rod. The mixture was cooled in the refrigerator,[11] and the impure product could be removed by filtration.

In all of the experiments with benzylcysteine and benzyl chloride except two, the method was the same up to this point. In two of the benzyl chloride experiments (Experiments 2A-9 and 9-171B, Table XIX) the modification introduced was a treatment of the concentrated urine with solid basic lead acetate and filtration before ether extraction of the filtrate. The lead acetate procedure will be discussed after a consideration of the other methods.

The Purification of the Acid Precipitate. -- Consideration of the chemical behavior of benzyl chloride suggested that, in vivo, it might well be converted to benzyl alcohol, which in turn would be oxidized to benzoic acid and excreted in conjugation with glycine as hippuric acid. It was therefore necessary to be able to separate benzylmercapturic acid from hippuric acid. The method was based on the assumption that there might be enough difference in the solubilities of the two acids in

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[11] In the discussion which follows this precipitate will be designed as the "acid precipitate." The modifications in the procedure were concerned with the further purification of this precipitate.

ether so that hippuric acid could be dissolved in this solvent and that most of the mercapturic acid would be left in the residue insoluble in ether. Hence, in the first three experiments (Table XIX, Experiments 1-229, 2-257, and 2-245) the acid precipitate was washed with a few cc. of ice-cold ether; and the supernatant fluid was decanted. The solvent was removed from the ether-soluble fraction by evaporation, and the residue was subjected to the same general procedure that will be described for the portion of the acid precipitate that was insoluble in ether. From the ether-soluble fraction of the acid precipitate, small amounts of benzoic and benzylmercapturic acids but no hippuric acid were isolated.

When the acid precipitate was washed with ether, this solvent removed some deeply pigmented impurities; but the residue insoluble in ether was still colored. It was therefore dissolved in 30 to 40 cc. of alcohol; the solution was warmed to 50-60° and decolorized with norit. This treatment with norit was repeated again two or three times, and in this way a light yellow colored solution was obtained. After the solvent was removed by distillation in vacuo, the light pink residue was recrystallized from aqueous alcohol and from hot water. The resulting product was practically colorless, had the correct melting point, and crystallized in the form

beautiful, glistening needles.

Laurie (15) isolated iodophenylmercapturic acid by absorption on charcoal; hence, the norit employed in decolorizing the solution was allowed to stand in contact for a day with each of several portions of alcohol. After the norit was removed from the combined extracts by filtration, the filtrate was concentrated in vacuo to dryness, and the residue was recrystallized from hot water. Varying amounts of the benzylmercapturic acid isolated was recovered in this way; but in one experiment in which benzylcysteine was fed, no benzylmercapturic acid was obtained except from the fraction recovered from the norit precipitate.

The results obtained in the isolation experiments with benzyl chloride are presented in Table XIX. The method of preserving the urine, the doses of the compounds that were fed, and other pertinent information are given with the table.

TABLE XIX

The Isolation of Mercapturic Acids After the Administration of Benzyl Chloride and S-Benzylcysteine

In Experiments 1-229, 1-257, and 2-245, the benzyl chloride was fed in doses of 0.3 to 0.4 gm. at 48 hour intervals to each of two rabbits until the total amount listed in the second column was fed. The urine was collected for 48 hours after the last feeding and preserved with sodium fluoride and toluene. In Experiment 2A-9 the amount of benzylmercapturic acid listed in the third column was isolated from a urine aliquot that was equivalent to the amount of benzyl chloride given in the second column. In the experiments in which S-benzylcysteine was fed, this amino acid was administered in one dose, and the urine was collected for 24 hours after the feeding. In Experiment 3A-1 the mercapturic acid was isolated from an aliquot of a 24 hour urine sample, and the amount of the mercapturic acid given in the third column is calculated as the amount isolated from the complete urine sample.

Experiment No.	Compound fed and amount		Mercapturic acid isolated: Total amount	Yield	Melting point
		gm.	gm.	per cent	
1-229	Benzyl chloride	1.9	0.30	8.0	142.5-3°*
2-257	" "	1.7	0.45	13.0	142 -3°
2-245	" "	2.1**	0.49	12.0	142.5-3°
2A-9	" "	0.36	0.17	24.0	143 -4°
9-171B	" "	0.34	0.10	16.0	143.5°
3-193	S-Benzylcysteine	0.75***	0.16	18.0	142.5-3.5°
2-193	" "	0.75	0.07	8.0	142 -3°
4-13B	" "	0.75	0.14	16.0	142.5-3.5°
3A-1	" "	0.75	0.08	9.0	142 -3

* $[\alpha]_D^{23°} = -39°$ (0.5 per cent in alcohol).

** A total of 3.0 gm. of l-cysteine was fed with the benzyl chloride and was administered in 0.5 gm. doses 3 hours after each feeding of the benzyl chloride.

*** Anhydrous sodium acetate (750 mg.) was given in three equal portions, one at the time the amino acid was fed, the other two 3 and 6 hours later respectively.

In the first experiment with benzyl chloride (Table XIX, Experiment 1-229) a total of 1.9 gm. of benzyl chloride was given to two rabbits. From the residue which remained after the acid precipitate had been washed with a small amount of ice-cold ether 0.29 gm. of a white crystalline product with the following properties was obtained:

	Melting point	Rotation
Found	142.5-3°	$[\alpha]_D^{23^\circ} = -39^\circ$ (0.5 per cent in alcohol)
du Vigneaud[12]	143 -4° (Corrected)	$[\alpha]_D^{23^\circ} = -40.5^\circ$ (1.0 per cent in alcohol)

Thus the physical properties of the purified product agreed with those found by du Vigneaud for synthetic N-acetyl-S-benzyl-l-cysteine. The amount isolated corresponded to 8 per cent of the compound fed.

No benzylmercapturic acid could be isolated from the charcoal or from the ether-soluble fractions. From the latter 0.05 gm. of benzoic acid which melted at 121° was isolated, and when mixed with a sample of pure benzoic acid, which melted at 121-2°, a melting point of 120-1° was obtained.

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[12] Stekol (6) reported a melting point of 147-148°.

In the second experiment in which this method of purification of the acid precipitate was employed, two rabbits were fed a total of 1.7 gm. of benzyl chloride in doses of 0.3 to 0.4 gm. every 48 hours. From the portion of the acid precipitate insoluble in ether, 0.34 gm. of benzylmercapturic acid was obtained; from the ether-soluble fraction, 0.08 gm., and from the charcoal, 0.03 gm. Each fraction had the same melting point of 142-3°. The combined yield was 0.45 gm. or 13 per cent of the theoretical.

In a third isolation experiment each animal was fed 0.3 to 0.4 gm. of benzyl chloride at 48-hour intervals, and three hours after each dose of benzyl chloride, 0.5 gm. of l-cysteine were fed until a total of 2.1 gm. of benzyl chloride and 3.0 gm. of l-cysteine had been administered. This amount of the sulfur-containing amino acid was equivalent to about 1.5 mols of cysteine per mol of benzyl chloride. The purpose of the experiment was to discover whether significantly larger amounts of benzylmercapturic acid could be isolated after supplying the animal with extra dietary l-cysteine since the reports of other workers (24) suggested such a possibility. A total of 0.49 gm. of benzylmercapturic acid (melting point 142.5-3°) was isolated in this experiment, an amount which is equivalent to 12 per cent of the theory. Of this total amount 0.46 gm. was isolated from the residue insoluble in ether, a few crystals from the ether

washings, and 0.03 gm. from the charcoal.

Thus the simultaneous administration of l-cystine with benzyl chloride did not increase the amount of benzylmercapturic acid that could be isolated. The variation observed in the yield in the previous two experiments shows that the method of isolation is not quantitative and any difference in the extent of synthesis would have to be very marked to detectable in an isolation experiment with the use of this method.

Basic Lead Acetate Procedure. -- In the other two isolation experiments with urines obtained after benzyl chloride was fed, a modification of the procedure was introduced. The neutralized urine was concentrated, and solid basic lead acetate was added to the concentrate. After the removal of the precipitate by filtration, the filtrate was subjected to a procedure to remove excess lead.[13] After filtration the acid solution was continuously extracted with ether as in the previous experiments. This procedure was utilized in two isolation experiments (Experiments 2A-9 and 9-171B). It was found that the acid precipitate obtained by this method was a light brown crystalline solid which melted at 138-40 ° in Experiment 3A-9 and at 141° in Experiment

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[13] The solution was made alkaline and filtered and then acidified (congo red paper) with hydrochloric acid.

9-171B. Hence, the only step necessary for its purification was to dissolve the acid precipitate in hot water, decolorize with norit, and, after the charcoal was removed by filtration, separate the purified product when the solution cooled. The fact that the acid precipitate was pure enough so that only one recrystallization from water and one decolorization with norit were necessary to obtain a purified mercapturic acid was an advantage over the previous procedure, for the use of the lead acetate method reduced the dangers of absorption of the product on charcoal and of mechanical loss. Emulsification of the urine in the ether extraction was prevented by the use of the lead acetate procedure--another distinct advantage of this method.

In Experiment 2A-9, the urine sample was the combined residual urines remaining after the sulfur partition had been carried out. The volume of urine was equivalent to 0.86 gm. of benzyl chloride and 0.17 gm. of benzylmercapturic acid; 24 per cent of the total amount theoretically possible, or 47 per cent of the average increase in ether-soluble sulfur was obtained. In Experiment 9-171B, 0.34 gm. of benzyl chloride was fed to a rabbit in one dose, and the amount isolated corresponded to 16 per cent of the theoretical amount. In this experiment, a second crop was obtained by diluting the mother liquor from which the acid precipitate

was obtained. After the second crop was recrystallized from hot water, 30 mg. of a product with a melting point of 186.5° was separated. When it was mixed with a known sample of hippuric acid, no depression of the melting point of 186.5° was observed.

All of the preparations of benzylmercapturic acid from benzyl chloride were combined, recrystallized from hot water, and analyzed for sulfur by the bomb method.

	Found	Theory
Sulfur	12.71 per cent	12.66 per cent

The analysis is a close check of the theoretical, further proof that the substance isolated was benzylmercapturic acid.

Stekol (6) has isolated benzylmercapturic acid from the urine of rabbits that were subcutaneously injected with benzyl chloride but did not report the amount isolated. After the oral administration of a total of 1.0 gm. of benzyl chloride to two dogs in 0.15 gm. doses at intervals of 3 hours, this investigator isolated about 8 to 10 per cent of the theoretical amount. In our experiments in which the rabbit was fed benzyl chloride, the benzylmercapturic acid isolated was from 8 to 24 per cent of the theoretical amount.

S-Benzylcysteine. -- The method of seperation of the acid precipitate in the experiments in which benzylcysteine was fed has been described already. The acid precipitate was recrystallized from hot water after the hot solution had been decolorized with norit. An examination of Table XIX will show that, in four experiments after the feeding of 750 mg. of S-benzylcysteine, mercapturic acid equivalent to from 8 to 18 per cent of the compound fed was isolated. In Experiment 3A-1, the mercapturic acid was isolated from an aliquot of a urine in which the sulfur distribution had been determined. The amount isolated corresponded to 12 per cent of the increase in organic sulfur observed on the experimental day.

In Experiment 3-193, a total of 0.75 gm. of anhydrous sodium acetate was given in addition to the S-benzylcysteine in three equal doses, one at the time of the administration of the amino acid, and two more 3 and 6 hours later respectively. The purpose of this experiment was to determine whether more benzylmercapturic acid could be isolated if dietary sodium acetate were available for the acetylation of the S-benzylcysteine. The amount of benzylmercapturic acid isolated corresponded to 18 per cent of the compound fed, and most of the product isolated in this experiment was extracted with alcohol from the charcoal that was used in decolorizing the solutions.

This finding illustrates the importance of this step in the isolation of benzylmercapturic acid. In still another experiment (No. 4-138), after the same amount of S-benzylcysteine was fed, the equivalent of 16 per cent was isolated as the mercapturic acid. From these results it is evident that there was little difference in the amount of mercapturic acid isolated in the presence and in the absence of acetate in the diet.

It is of interest that, in the two experiments in which the ether-soluble sulfur was determined, only a small difference was noted between this fraction of the urinary sulfur and the organic sulfur. This result may be interpreted to mean that very little S-benzylcysteine was excreted unchanged.

The preparations of benzylmercapturic acid from the feeding of S-benzylcysteine were combined and recrystallized from water. When a sample of this recrystallized acid was mixed with benzylmercapturic acid[14] isolated from the urine of animals that were fed benzyl chloride, the melting point was not depressed, proving that the mercapturic acids formed by the animal from the different precursors were identical.

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[14] This product was analyzed as previously described.

	Found	Theory
Sulfur	12.71 per cent	12.66 per cent

The urine of the animals that were fed either benzyl chloride or S-benzylcysteine had a very peculiar and offensive odor that was noted in urines voided as short a time as three hours after the administration of either compound. A positive cyanide-nitroprusside test was obtained on the 24 hour urine after the administration of S-benzylcysteine. The odor suggests the possible presence of benzyl mercaptan, but the failure to obtain uniformly a positive nitroprusside test does not support this hypothesis.

Discussion of Benzyl Chloride and S-Benzylcysteine Isolation Experiments. -- The isolation experiments have demonstrated that benzyl chloride and S-benzylcysteine are converted in part, at least, to the same metabolic product, benzylmercapturic acid, which was isolated in about the same yield in both series of experiments. These results together with the chemical relationship of S-benzylcysteine to the mercapturic acid support the hypothesis that this amino acid is an intermediate in the transformation of benzyl chloride into the mercapturic acid.

In some of the isolation experiments small amounts of benzoic and hippuric acid were also isolated. The conversion of benzyl chloride to benzoic or hippuric acid, both normal constituents of urine, is probable from the chemical viewpoint, but, as yet, the experiments reported are not convincing evidence for it.

Although the amount of mercapturic acid isolated in our experiments is the same as or more than other investigators have reported, the method is undoubtedly not quantitative. In view of that fact, the isolation of benzyl mercapturic acid in amounts equivalent to about 50 per cent of the extra-ether-soluble sulfur found in the urine after the feeding of benzyl chloride (Experiment 2A-9) demonstrates that a large portion of this fraction of the organic sulfur of the urine is made up of mercapturic acid.

Benzene

Although it is generally stated in review articles that benzene forms a mercapturic acid in the animal body as evidenced by the rise in the organic sulfur of the urine after feeding the hydrocarbon, this latter fact has been shown only in the case of the dog (25). The other investigators who have reported changes in the sulfur distribution in the urine of the rabbit following the administration of benzene have never measured the organic sulfur fraction of the total urinary sulfur. Likewise the isolation of phenylmercapturic acid from the urine of animals fed benzene had never been acomplished at the time these experiments were carried out. Drummond and Finar (5) in their work on the isolation of muconic acid from the urine of rabbits that were fed benzene present the only hint available in the

literature that this species forms mercapturic acid. In samples of the acid-hydrolyzed urine of rabbits that were fed benzene, they were able to obtain qualitative tests for thiophenol. Hence, an attempt was made to isolate this mercapturic acid from the urine of rabbits fed benzene by two isolation procedures: (a) the ether extraction method without previous treatment of the urine with lead acetate; (b) the chloroform extraction proceeding of McQuinn and Sherwin (23). By neither method was it possible to isolate a product although large amounts of benzene, 9 and 10 gms. respectively, were fed in 1 gm. daily doses to rabbits. All that could be isolated were black gums and oils that could not be crystallized.

In the first experiment a rabbit that was maintained on a diet of cabbage and oats was fed a total of 9 gm.. of benzene, 1 gm. a day for a 9-day period. The animal exhibited no toxic symptoms at any time. The urine was neutralized and concentrated <u>in vacuo</u> to about 50 to 100 cc., acidified to congo red paper with hydrochloric acid, and then continuously extracted with ether for 24 hours. The ether extract so obtained was subjected to the procedure previously described. The acid precipitate so obtained was a black oil. The pH of the acid solution containing the black oil was readjusted by adding ammonium hydroxide until the solution was barely acid to

congo red paper. After the solution had stood in the refrigerator for a few days, crystals appeared on top of the oil. The crystals, melting at 118-20°, were separated mechanically. Not enough material was available for further recrystallization. The oil which did not crystallize on standing in contact with the acid mother liquor was separated and extracted with hot water in which phenylmercapturic acid is reported to be soluble (99, 100). The hot aqueous layer was decanted from the oil, treated with norit, and filtered. On cooling and upon concentration of the filtrate to a small volume *in vacuo*, no product could be obtained. Likewise, when the residue insoluble in water was recrystallized from aqueous alcohol, a small amount of a brown powder was obtained that did not melt on being heated to 150°.

In the second experiment after two rabbits were fed a total of 10 gm. of benzene over a period of 5 days in 1 gm. doses, an attempt was made to isolate phenylmercapturic acid by the method of McQuinn and Sherwin (23). The combined urines were neutralized, and concentrated *in vacuo* to 100 cc., acidified to congo red with hydrochloric acid, and extracted four times with equal volumes of chloroform. The resulting emulsions were broken by centrifugation, filtration, and dilution with chloroform. After the combined chloroform extracts were washed with water, the chloroform layer was separated.

The solvent was removed by distillation in vacuo, and the residue was dissolved in hot 5 per cent ammonium hydroxide. The hot alkaline solution was treated with norit and filtered. The filtrate was made acid to congo red which resulted in the separation of an oil that did not crystallize on standing in contact with the mother liquor or when separated mechanically and redissolved in hot aqueous alcohol.

The charcoal used in attempts to decolorize these alkaline solutions was extracted with several portions of alcohol by allowing the solvent to stand in contact with it for several days, but no product was obtained from the alcohol extract.

The results of subsequent sulfur partitions of the urine of rabbits that were fed benzene indicated that little or no mercapturic acid was formed, which might be an explanation of the failure to isolate phenylmercapturic acid after the administration of benzene. About 8 months after these isolation experiments were carried out, Zbarsky and Young (22) reported the isolation of phenylmercapturic acid from the urine of rats to which benzene was fed. The amount isolated was very small and varied from 11 to 177 mg. after the feeding of from 7 to 16 gm. of benzene. The small yield reported may be due to the fact that this mercapturic acid is formed in very small amounts or that the difficulties of purification and separation of the acid from the other products of benzene metabolism are so

great that the per cent of the actual amount isolated from the urine is very small. Probably both factors are to be considered. Zbarsky and Young (22) conclude that the formation of a mercapturic acid from benzene is a minor pathway of metabolism of this hydrocarbon in the rat. They were unable to isolate the acid from control samples of rat urine and therefore concluded that this compound was not a normal product of metabolism which was formed from some precursor normally present in the diet. This question will again be discussed under the "General Discussion."

Phenylcysteine

Attempts were made to isolate phenylmercapturic acid from the urine of rabbits that were fed phenylcysteine by the methods that had been successful with benzylcysteine and benzyl chloride. No success was attained in these experiments; hence, the rabbit either does not form the mercapturic acid from phenylcysteine, or the isolation procedure was not suitable for the purpose of separating the detoxication products. In experiments previously described, S-phenylcysteine was found to be insoluble in ether; and since an increase in ether-soluble sulfur was obtained upon feeding S-phenylcysteine to the rabbit, it was assumed that the extra sulfur in this fraction represented mainly phenylmercapturic acid.

Consequently, an aliquot of the urine in which the increase in sulfur soluble in ether had been observed was subjected to the ether extraction-lead acetate procedure described for benzyl chloride. The excess lead was removed as the sulfide, and the urine was made just acid to litmus paper and concentrated to about 5 cc. The concentrate was then made acid to congo red with hydrochloric acid and continuously extracted with ether for six hours. After removing the ether *in vacuo*, the residue was dissolved in warm 5 per cent ammonium hydrochloride. The alkaline solution was warmed with norit at 60-70° for about 5 minutes, filtered, and acidified to congo red with hydrochloric acid. No precipitate was obtained on cooling the solution.

It was possible that the amount of mercapturic acid calculated to be present in the aliquot from the increase in ether-soluble sulfur was too small (40 to 50 mg.) to be detected. In the next experiment 500 mg. of S-phenylcysteine were fed. Using the identical procedure employed in the previous experiment, 55 mg. of a product melting at 127-8° were obtained upon acidifying and concentrating the ammonium hydroxide solution to a small volume *in vacuo*.

The precipitate obtained after adding solid basic lead acetate to the urine was suspended in alcohol and decomposed with H_2S in hope of obtaining a product from this fraction.

After the lead sulfide was removed by filtration, the alcohol filtrate was concentrated to a thick gum *in vacuo*. This residue would not dissolve in hot water, but dissolved in aqueous alcohol, reappearing as a gum on cooling. The impure material was redissolved in warm 5 per cent ammonium hydroxide, the solution was warmed with norit, filtered, and acidified with hydrochloric acid. The gum reappeared on cooling. An identical procedure was employed on a third experiment in which 400 mg. of S-phenylcysteine was fed. The acid precipitate was a solid melting at 130-1°, and weighing 24 mg., and after recrystallization from hot water it still melted at 130-1°. Phenylmercapturic acid is reported to melt at 141-2°.

Zbarsky and Young (33) reported the isolation of phenylmercapturic acid from the urine of rats fed benzene or S-phenylcysteine a few months after these experiments were completed. They stated that the mercapturic acid was labile to alkali and in isolation experiments with benzene always kept the urine slightly acid to litmus paper (22). It is possible that the mercapturic acid may have been destroyed in the process of decolorizing the hot alkaline solution.

General Discussion

The Metabolism of Benzyl Chloride and S-Benzylcysteine

The experiments have proven that benzyl chloride and

S-benzylcysteine yield the same end product in their metabolism in the rabbit. The results support the theory that S-benzylcysteine is an intermediate in the metabolism of benzyl chloride.

Benzyl chloride + cysteine ⟶ benzylcysteine ⟶ (acetylation) benzylmercapturic acid

Benzyl alcohol cannot be an intermediate product in the formation of the mercapturic acid as evidenced by the results reported in this thesis and those of others (20). Another possibility, benzaldehyde, is ruled out by the fact that it is converted to hippuric and not benzylmercapturic acid (20). It is probable that the benzyl chloride unites with the sulfhydryl group of cysteine or some intermediate in its metabolism. The results of du Vigneaud and coworkers (99) and Stekol (20) indicate that the benzyl group is not combined first with homocysteine to form benzylhomocysteine, which is then converted to benzylcysteine. If the current theory that only the sulfhydryl group of homocysteine contributes to the synthesis <u>in vivo</u> of cystine, the carbon chain being supplied by serine (101, 102), is correct, then S-benzylhomocysteine would hardly be expected to be converted to benzyl-

mercapturic acid.

If benzylcysteine is an intermediate product in the metabolism of benzyl chloride, the correspondence between the organic and ether-soluble fractions of the urine after the administration of the latter compound indicates that little, if any, of the unacetylated amino acid is excreted. Similarly, very little of the S-benzylcysteine that is fed to a rabbit appears to be excreted unchanged but is converted to a form soluble in ether. Since it is believed that the main component of the ether-soluble sulfur fraction is benzylmercapturic acid, the rabbit appears to have the ability to acetylate the amounts of S-benzylcysteine that were administered as such or formed from benzyl chloride when this substance was fed.

The main path of the metabolism of benzyl chloride and benzylcysteine in the rabbit appears to be conversion to the mercapturic acid. Neither compound, at the level fed, was oxidized to a phenol or other substance that was conjugated with ethereal sulfate or glucuronic acid. Hence, benzylmercapturic acid is not associated with a glucuronic acid complex as has been suggested for the bromophenylmercapturic acid (4).

Other precursors of the mercapturic acids such as bromo-, iodo-, or chlorobenzene are believed to have two pathways of

metabolism: oxidation to phenols that are excreted as conjugates with ethereal sulfates and glucuronic acid, and conversion to mercapturic acids. Only one of these paths of metabolism, formation of mercapturic acid, appears to be open to benzyl chloride, at least at the level at which it was fed in our experiments. Conversion of benzyl chloride to hippuric acid is a logical alternate pathway but the results of our experiments and those of others (20) do not support this hypothesis. If an alternate pathway of benzyl chloride metabolism exists, conversion to the mercapturic acid is probably the main pathway. It is not known whether all of the benzyl chloride was absorbed from the gastrointestinal tract. If one assumes that the benzyl chloride was completely absorbed, about 50 per cent cannot be accounted for. Our data have eliminated certain possible alternate pathways of benzyl chloride metabolism but have given no indication of what the alternate pathway, if any exists, may be. Failure of absorption of ~~the~~ benzyl chloride may, however, explain the low recovery as the mercapturic acid.

The administration of cystine or methionine to a rabbit that was fed benzyl chloride did not increase the excretion of ether-soluble sulfur, which is considered to be a measure of the benzylmercapturic acid, but did augment the organic

sulfur of the urine to values higher than those observed when benzyl chloride alone was fed. However, the level of nitrogen, which was elevated when the benzyl chloride was fed alone, remained at the control level when the sulfur-containing amino acids were added to the diet. Also less cystine or methionine was oxidized in the presence of benzyl chloride than in its absence, as far as can be judged by the excretion of oxidized (sulfate) sulfur. These experiments are interpreted as evidence that the animal utilized the dietary sulfur-containing amino acids to form the mercapturic acid and to replace the cystine and methionine depleted from the tissues on the previous days when benzyl chloride was administered or when the animal did not eat. The increased nitrogen excretion on the days that benzyl chloride alone was fed appears to be evidence that the content of the sulfur-containing amino acids in the diet was not sufficient to conjugate the benzyl chloride, and therefore that the tissue protein was broken down to furnish cystine or methionine.

The results obtained with S-benzylcysteine and with S-phenylcysteine indicate that the substitution of a benzyl or a phenyl group on the sulfhydryl group of cysteine protects the sulfur from oxidation. However, oxidative deamination of the amino acid is not ruled out, and our preliminary experiments with S-phenylcysteine suggest that this process does

take place to some extent for an increase in keto acids of the urine was observed after this compound was fed. In the case of benzylcysteine it was proven that the amino acid was acetylated to form the mercapturic acid. It is possible that some of the extra ether-soluble sulfur in the urine after the feeding of S-benzylcysteine may be due to a keto acid, but there is no direct experimental evidence for this hypothesis.

Sherwin and coworkers (37) found evidence of extensive oxidation of S-benzylcysteine in the rabbit. This amino acid was fed at a much higher level than in the present experiments. It has been suggested that the results obtained by Sherwin (37) might be due to the action of intestinal flora (103). Pirie (38) found that the sulfur of S-benzylcysteine was not oxidized in the dog; results which are similar to those reported in this thesis for the rabbit.

In contrast to the effects of the oral administration of benzyl chloride which have been discussed, the subcutaneous injection of benzyl chloride did not affect the distribution of urinary sulfur to any great extent. Probably this compound was not well absorbed by the animal, and hence no effect could be observed.

The Metabolism of Monobromobenzene, p-Monobromophenol, and Benzene.

The Relationship between the Changes in the Glucuronic Acid and Phenol[15] Excretions and the Sulfur Distribution.

The relationship between the changes in the excretion of glucuronic acid and phenol,[15] and the distribution of urinary sulfur after the administration of these three compounds will be discussed together since a common hypothesis is offered as an explanation of the results.

From the changes in the excretion of glucuronic acid, ethereal sulfate, organic sulfur, and "bromophenol" after the administration of monobromobenzene, it is impossible to decide whether or not the mercapturic acid was excreted as a glucuronic acid complex since the glucuronic acid might also be associated with phenolic products of the metabolism of monobromobenzene. The "bromophenol" found in the urine was less than the ethereal sulfate sulfur excretion. This finding is impossible to explain with the simple theory that the only phenolic product of the metabolism of monobromobenzene associated with ethereal sulfate is p-monobromophenol.

Likewise the observation that the sum of the glucuronic acid and the ethereal sulfate sulfur excretions was much greater

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[15] Or "bromophenol."

than the phenol or "bromophenol" found in the urine after the administration of p-monobromophenol and benzene respectively cannot be explained by the hypothesis that the only path of metabolism of the former compound is conjugation with glucuronic and sulfuric acids, and that the only phenolic compound produced from the latter compound is phenol (monohydroxybenzene), which is conjugated with glucuronic and sulfuric acids.

It is suggested as a working hypothesis that the phenol or "bromophenol" estimated in the urine by the use of the modified Millon reaction is actually only a part of the total phenolic compounds that are present in the urine. This theory is based on the observation that the various polyphenols did not react in the modified Millon reaction. Thus, if polyphenols were produced that did not react with the reagent, the total phenol concentration when estimated by this method would be lower than the actual amount of total phenols (mono- and polyphenols) present in the urine. There is some evidence that pyrocatechol and hydroquinone are products of the metabolism of benzene (47), and that pyrocatechol and bromohydroquinone are formed in the animal body from monobromobenzene (1, 4).

In the case of monobromobenzene and p-monobromophenol, phenols which gave less color than p-monobromophenol or no color at all in the modified Millon reaction might be pro-

duced with or without the removal of bromine from the molecule. In the case of benzene, the phenol estimated by means of the modified Millon reaction is probably the compound,phenol (monohydroxybenzene), since the polyphenols did not react.

The formation of a conjugate with glucuronic acid appears to be an important method, from the quantitative viewpoint, of the elimination of certain products of the metabolism of p-monobromophenol or monobromobenzene.

If it is assumed that one mol of glucuronic acid is associated with one mol of bromophenol or its metabolic products, the extra glucuronic acid observed after feeding the substituted phenol accounts for from 50 to 70 per cent of the p-monobromophenol fed. Certainly conjugation with gulcuronic acid is an important step in the metabolism of p-monobromophenol.

If a similar assumption is made regarding the conjugation of glucuronic acid with monobromobenzene, about 20 to 25 per cent of the monohalogen substitution product of benzene that was fed are associated in some way with glucuronic acid. Hence, conjugation with glucuronic acid is a most important path in the metabolism of monobromobenzene and its most probable metabolic product, p-monobromophenol. Is this also true of the other monohalogen substitution product of phenol and benzene? The results of Diechmann (95) indicated that only

the administration of o-chlorophenol caused a rise in the level of glucuronic acid when the substituted phenol was fed to a rabbit. The administration of the m- and p-substituted isomers did not change the level of glucuronic acid in the urine. A study of the effect of the other isomeric monohalogen substitution products of phenol and of benzene will answer this question.

The glucuronic acid excretion following the administration of benzene was variable ranging from 0 to 11 per cent of the benzene fed. Although a smaller per cent of the hydrocarbon was converted to substances conjugated with glucuronic acid than with monobromobenzene or p-monobromophenol, on a molar basis the amount of the former compound fed was about 2 times the amount of the monohalogen substitution product of benzene that was administered. It may be that some of the difference observed in the per cent of monobromobenzene and benzene that was conjugated with glucuronic acid is due to the fact that levels of the two compounds fed were not the same on a molar basis. Other investigators have reported qualitative tests for glucuronic acid in the urine of animals that were fed benzene (104), but the only other quantitative evidence of the effect of the administration of this hydrocarbon on glucuronic acid excretion was published by Diechmann (95) a few months after our experiments

had been completed. In contrast to the results reported here, he observed no change in the excretion of glucuronic acid after the administration of four cc. of benzene to a rabbit.

The Changes in the Partition of Sulfur

Monobromobenzene and p-Monobromophenol. -- The effects of the administration of monobromobenzene and p-monobromophenol were the same as those observed in previous reports from this laboratory (24, 67). The chief difference in the effect of the two compounds was that the feeding of the former resulted in a marked rise in organic sulfur whereas no significant change took place in this fraction after the administration of p-monobromophenol, which suggests that p-monobromophenol is probably not an intermediary product in the formation of bromophenylmercapturic acid in the rabbit. These results are similar to those obtained with the dog (24) and the rat (54).

The administration of either compound caused a rise in the excretion of ethereal sulfates, which was accompanied by a fall in the level of the inorganic sulfate fraction of the oxidized sulfur, indicating that the inorganic sulfate or its precursors were being utilized for the synthesis of ethereal sulfate.

Benzene. -- The effect of the administration of benzene

on the partition of urinary sulfur was an increase in the ethereal sulfate sulfur excretion, which was accompanied by a fall in the level of inorganic sulfate sulfur that was so marked in one experiment in which two successive doses of benzene were given that the latter fraction of the urinary sulfur disappeared from the urine. Similar results were obtained by White and Lewis (24) after the continued administration of p-monobromophenol and monobromobenzene to the dog. As in the experiments in which monobromobenzene and p-monobromophenol were fed to the dog (24) or the rat (54) or to the rabbit in our experiments or those of Lough and Lewis (67), the ethereal sulfates were formed at the expense of the inorganic sulfate sulfur during the period of the administration of benzene. The observation that the administration of benzene causes a rise in the excretion of ethereal sulfates by the rabbit is a confirmation of other reports (5).

The administration of benzene did not significantly affect the level of organic sulfur. The changes observed in this fraction of the urinary sulfur in two experiments are probably not significant since the organic sulfur fraction is determined by difference and varies by small amounts on the control days.

These results indicate that phenylmercapturic acid is not a product of the metabolism of benzene in the rabbit.

The level of benzene fed (0.3 to 0.4 gm. per kg.) was much lower than other investigators have administered. For example, Diechmann (95) fed 3 gm. per kilo. The work of Nishimura (13) indicated that better yields of mercapturic acid were obtained when smaller doses of the monohalogen derivative of benzene were fed to the rabbit. Thus it is believed that the small amounts of benzene that were fed in our experiments should result in a change in the organic sulfur fraction if a mercapturic acid was formed in the course of the metabolism of this hydrocarbon. The failure to observe a change in this fraction of the organic sulfur is thus more significant.

Likewise in isolation experiments no phenylmercapturic acid was isolated. This evidence together with the results of sulfur partitions would lead to the conclusion that no phenylmercapturic acid was formed. However, Zbarsky and Young (22) have reported the isolation of small amounts of phenylmercapturic acid from the urine of rats to which benzene had been fed. If the rabbit converted benzene to phenylmercapturic acid in amounts comparable to those isolated by these authors, the level of organic sulfur in the urine would not be changed. In view of the variation in yield reported by Zbarsky and Young (22), it is probable that the rat forms more phenylmercapturic acid than the

rabbit. The difficulty of separating the phenolic conjugation products of the metabolism of benzene is no doubt responsible for the low yield. Hele (25) reported increases in the level of the organic sulfur in the urine of dogs that corresponded to from 5 to 16 per cent of the benzene fed. It is thus apparent that a species difference in the metabolism of benzene probably exists between the dog and the rabbit, and hence, might exist between the rat and the rabbit. In the light of these considerations, the failure to observe significant increases in the level of organic sulfur in the urine of rabbits to which benzene has been fed can be accepted as proof that this species does not form phenylmercapturic acid.

Summary

1. In confirmation of previous investigations, when benzyl chloride or S-benzylcysteine was fed to a rabbit, benzylmercapturic acid was isolated from the urine.

2. The isolation procedure involved ether extraction of the urine with or without previous treatment with lead acetate. By the use of these methods from 8 to 24 per cent of the benzyl chloride and from 8 to 18 per cent of the S-benzylcysteine that was fed to a rabbit was isolated as benzylmercapturic acid.

3. The oral administration of benzyl chloride to the

rabbit in amounts of from 0.25 to 0.35 gm. resulted in increases in the organic sulfur of the urine which corresponded to from 40 to 58 per cent of the benzyl chloride fed. No change was observed in the excretion of ethereal sulfate sulfur, nor was there an increase in the level of glucuronic acid or phenols in the urine after the administration of benzyl chloride. In one series of experiments in which the ether-soluble sulfur level of the urine was determined, this fraction of the extra urinary sulfur was equal to the extra organic sulfur of the urine which was observed after the administration of benzyl chloride. Since S-benzylcysteine was insoluble in ether and, from an aliquot of the urine of the animals to which benzyl chloride had been fed, an amount of benzylmercapturic acid which corresponded to 50 per cent of the average increase in ether-soluble sulfur was isolated, it was concluded that most of the increase in ether-soluble (hence organic sulfur) was probably due to benzylmercapturic acid, and that this mercapturic acid was probably not excreted in association with glucuronic acid. The only pathway of benzyl chloride metabolism that could be proven in our experiments was conversion to the mercapturic acid.

4. The simultaneous administration of l-cystine or l-methionine with benzyl chloride did not increase the excretion of

ether-soluble sulfur but did increase the level of organic sulfur in the urine. However, the increase in urinary nitrogen observed when benzyl chloride was fed alone was prevented by the simultaneous administration of either of the sulfur-containing amino acids. Likewise less cystine or methionine was oxidized in the presence of than in the absence of benzyl chloride, at least as far as can be judged by the excretion of oxidized sulfur fraction. It was concluded that the animal utilized the sulfur-containing amino acids of the diet in preference to those of his tissues.

5. The administration of S-benzylcysteine (500 to 750 mg.) to the rabbit resulted in an increased excretion of organic sulfur corresponding to from 76 to 93 per cent of the amino acid fed. In one series of experiments the extra ether-soluble sulfur was measured, and this fraction of the urinary sulfur corresponded to about 80 per cent of the increase in organic sulfur observed after the administration of S-benzylcysteine. Apparently most of the S-benzylcysteine was converted to a form soluble in ether, which fraction of the total urinary sulfur is probably mainly mercapturic acid. There was no increase in ethereal sulfate sulfur, total phenols, or glucuronic acid after the administration of S-benzylcysteine. Hence the sulfur of this amino acid is not oxidized to ethereal sulfates, nor does the mercapturic acid

appear to be excreted in combination with glucuronic acid. It is postulated that S-benzylcysteine is an intermediate in the metabolism of benzyl chloride.

6. The administration of S-phenylcysteine resulted in an increase in the excretion of ether-soluble and organic sulfur in the urines, but the sulfur of this amino acid was not oxidized to sulfate. Apparently the substitution of a phenyl or a benzyl group on the sulfhydryl group of cysteine protects the sulfur from oxidation. In two preliminary experiments it was demonstrated that an increase of keto acid equivalent to from 7 to 14 per cent of the amino acid fed resulted from the administration of S-phenylcysteine.

7. The administration of monobromobenzene (1.0 gm.) caused a rise in the excretion of glucuronic acid which was equivalent to from 20 to 25 per cent of the monohalogen substitution product of benzene fed in 4 out of 5 experiments. Since the administration of p-monobromophenol, which is considered to be a product of the metabolism of monobromobenzene, resulted in the excretion of extra glucuronic acid that corresponded to from 50 to 70 per cent of the substituted phenol fed, it is not possible to decide whether the extra glucuronic acid observed in the urine after the feeding of monobromobenzene was associated with bromophenylmercapturic acid.

The administration of monobromobenzene caused an increase in the level of organic sulfur of the urine, whereas the feeding of the corresponding p-substituted phenol did not significantly affect this fraction of the total urinary sulfur. Thus p-monobromophenol does not appear to be an intermediate in the formation of bromophenylmercapturic acid.

8. The administration of benzene (1.0 gm) resulted in the excretion of extra ethereal sulfate sulfur, extra total phenol, and extra glucuronic acid. The amounts of the first two constituents accounted for about 10 per cent, whereas the amounts of the third constituent were variable, accounting for from 0 to 10 per cent of the benzene fed. The rise in ethereal sulfate sulfur was accompanied by a fall in inorganic sulfate sulfur.

9. The extra phenol or extra total phenol calculated as p-monobromophenol was much less than the sum of the ethereal sulfate sulfur and/the extra glucuronic acid after the administration of benzene and monobromobenzene or p-monobromophenol respectively. A possible explanation for these results is discussed.

10. The organic sulfur fraction of the urinary sulfur was not significantly affected by the administration of benzene. In experiments employing two different procedures no phenylmercapturic acid could be isolated from the urine. It is

probable that the formation of phenylmercapturic acid is not a pathway of the metabolism of benzene in the rabbit.

Conclusions.

1. Benzyl chloride and S-benzylcysteine were both converted to benzylmercapturic acid by the rabbit. Hence, S-benzylcysteine may be an intermediate in the formation of benzylmercapturic acid from benzyl chloride. When either l-cystine or l-methionine was fed together with the benzyl chloride, the sulfur-containing amino acids of the diet appeared to be used for the synthesis of the mercapturic acid in preference to those of the tissues.

2. The administration of either monobromobenzene or p-monobromophenol caused an increase in the excretion of glucuronic acid. Since both bromophenylmercapturic acid and p-monobromophenol are products of the metabolism of monobromobenzene, it is not possible to decide whether glucuronic acid was excreted in combination with the mercapturic acid. The experiments demonstrate the quantitative importance of conjugation with glucuronic acid in the metabolism of monobromobenzene and p-monobromophenol.

3. Benzene appeared to be converted to phenols which were excreted in conjugation with glucuronic acid and ethereal sulfates. Formation of a mercapturic acid did not appear to be a pathway of the metabolism of benzene in the rabbit for

no significant increase was observed in the level of organic sulfur nor could any phenylmercapturic acid be isolated after the administration of this hydrocarbon.

BIBLIOGRAPHY

1. Baumann, E., and Preusse, C., Ber., 12, 806 (1879).
2. Jaffe', M. Ber., 12, 1092 (1879).
3. Friedmann, E. E. Beit. chem. Physiol. Path., 4, 486 (1904).
4. Baumann, E., Z. physiol. Chem., 8, 190 (1883-4).
5. Drummond, J.C., and Finar, I.L., Biochem. J., 32, 79 (1938).
6. Stekol, J.A., J. Biol. Chem., 124, 129 (1938).
7. Stekol, J.A., J. Biol. Chem., 113, 279 (1936).
8. Baumann, E., and Preusse, C., Z. physiol. Chem., 5, 309 (1881).
9. Abderhalden, E., and Wertheimer, E., Arch. ges. Physiol., 207, 215 (1925).
10. White, A., and Jackson, R.W., J. Biol. Chem., 111, 507 (1925).
11. Stekol, J.A., Proc. Soc. Exp. Biol. Med., 33, 115 (1935).
12. Stekol, J.A., J. Biol. Chem., 113, 675 (1936).
13. Nishimura, K., Acta Schol. Med. Univ. Imp. Kyoto, 12, 73 (1929).
14. Baumann, E., and Schmitz, P., Z. physiol. Chem., 20, 586 (1895).
15. Laurie, N.R., Biochem. J., 25, 1037 (1931).
16. Inge, H.R., Bourne, M.C., and Young, L., Biochem. J., 28, 809 (1934).
17. Stekol, J.A., J. Biol. Chem., 110, 463 (1935).
18. Stekol, J.A., J. Biol. Chem., 122, 333 (1937).
19. Boyland, E., and Levi, A.A., Biochem. J., 30, 1225 (1936).
20. Stekol, J.A., J. Biol. Chem., 128, 199 (1939).
21. Stekol, J.A., J. Biol. Chem., 138, 225 (1941).

22. Zbarsky, S.H., and Young, L., J. Biol. Chem., 151, 487 (1943).

23. McQuinn, A.F., and Sherwin, C.P., Proc. Soc. Exp. Biol. Med., 30, 1115 (1933).

24. White, A., and Lewis, H.B., J. Biol. Chem., 98, 607 (1932).

25. Hele, T.S., and Callow, E.H., Biochem. J., 20, 588 (1926).

26. Coombs, H.I., Biochem. J., 21, 623 (1927).

27. Stekol, J.A., Proc. Soc. Exp. Biol. Med., 33, 170 (1935).

28. Spencer, H.C., Irish, D.D., Adams, E.M., and Rowe, V.K., J. Ind. Hyg. Tox., 24, 295 (1942).

29. Carpenter, C.P., Shaffer, C.B., Weil, C.S., Symthe, H.F., Jr., J. Ind. Hyg. Tox., 26, 69 (1944).

30. White, A., Ph.D. Thesis, University of Michigan (1930).

31. Knoop, F., and Blanco, J.G., Z. physiol. Chem., 146, 267 (1925).

32. du Vigneaud, V., Cohn, M., Brown, G.B., Irish, C.J., Schonheimer, R., and Rittenberg, D., J. Biol. Chem., 131, 273 (1939).

33. Zbarsky, S.H., and Young, L., J. Biol. Chem., 151, 217 (1943).

34. Zbarsky, S.H., and Young, L., J. Biol. Chem., 152, 599 (1944).

35. Binkley, F., Wood, J.C., and du Vigneaud, V., J. Biol. Chem., 153, 495 (1944).

36. Hele, T.S., and Pirie, N.W., Biochem. J., 25, 1095 (1931).

37. Rose, A.R., Shiple, G.P., and Sherwin, C.P., Am. J. Physiol., 69, 518 (1924).

38. Pirie, N.W., Biochem. J., 26, 2041 (1932).

39. Stekol, J.A., Proc. Soc. Exp. Biol. Med., 45, 693 (1940).

40. Stekol, J.A., Proc. Soc. Exp. Biol. Med., 47, 292 (1941).

41. Brand, E., and Harris, M.M., Science, 77, 589 (1933).

42. Stekol, J.A., Proc. Soc. Exp. Biol. Med., 43, 108 (1940).

43. Hele, T.S., Biochem. J., 18, 586 (1924).

44. Schultzen, O., and Naunyn, B., Arch. Anat. Physiol., 349 (1867).

45. Munk, I., Arch. ges. Physiol., 12, 142 (1876).

46. Baumann, E., and Herter, E., Z. physiol. Chem., 1, 142 (1876).

47. Nencki, M., and Giacosa, P., Z.physiol. Chem., 4, 325 (1880).

48. Rhode, H., Z. physiol. Chem., 124, 15 (1922-3).

49. Muldoon, J.A., Shiple, G.J., and Sherwin, C.P., J. Biol. Chem., 60, 59 (1924).

50. Muldoon, J.A., Shiple, G.J., and Sherwin, C.P., J. Biol. Chem., 59, 675 (1924).

51. Hopkins, G.F., Guy's Hosp. Gazette, 21, 426 (1907).

52. Marriot, W.M.K., and Wolf, C.G.L., Biochem. Z., 7, 213 (1907-8).

53. Hele, T.S., and Coombs, T.S., Biochem. J., 20, 606 (1926).

54. Stekol, J.A., and Dash, H.H., Proc. Soc. Exp. Biol. Med., 43, 261 (1939).

55. Hele, T.S., and Coombs, H.I., Biochem. J., 21, 611 (1927).

56. Hele, T.S., Biochem. J., 18, 110 (1924).

57. Callow, E.H., and Hele, T.S., Biochem. J., 21, 606 (1927).

58. Stekol, J.A., J. Biol. Chem., 117, 147 (1937).

59. Stekol, J.A., Proc. Soc. Exp. Biol. Med., 35, 623 (1937).

60. Zeller, H., and Straczewski, H., Arch. physiol, 585 (1914).

61. Thomas, K., and Straczewski, H., Arch. physiol., 249 (1919).

62. Kapfhammer, J., Z. physiol. Chem., 116, 302 (1921).

63. Virtue, R.L., Proc. Soc. Exp. Biol. Med., 34, 425 (1936).

64. Stekol, J.A., Proc. Soc. Exp. Biol. Med., 33, 119 (1935).

65. Abderhalden, E., and Wertheimer, E., Z. physiol. Chem., 201, 267 (1931).

66. Conway, W.J., J. Biol. Chem., 121, 27 (1937).

67. Lough, S.A., and Lewis, H.B., J. Biol. Chem., 94, 739 (1931).

68. Stekol, J.A., J. Biol. Chem., 118, 155 (1937).

69. Stekol, J.A., J. Biol. Chem., 121, 93 (1937).

70. White, A., and Jackson, R.W., J. Biol. Chem., 100, CIII (1933).

71. Stekol, J.A., J. Biol. Chem., 127, 131 (1940).

72. Stekol, J.A., J. Biol. Chem., 133, XCVIII (1940).

73. Stekol, J.A., Arch. Biochem., 2, 151 (1943).

74. Stekol, J.A., Proc. Am. Chem. Soc., Detroit Sect., Sept., 1940, Biol. Chem., p. 140.

75. Stekol, J.A., Fed. Proc. Am. Soc. Exp. Biol., 1, Part II, 136 (1942).

76. Stekol, J.A., J. Biol. Chem., 121, 87 (1937).

77. Stekol, J.A., Proc. Soc. Exp. Biol. Med., 42, 328 (1940).

78. Abderhalden, E., and Wertheimer, E., Arch. ges. Physiol., 209, 611 (1925).

79. Sherwin, C.P., Ann. Rev. Biochem., 2, 386 (1933).

80. Stekol, J.A., and Mann, F.C., J. Biol. Chem., 117, 619 (1937).

81. Folin, O., J. Biol. Chem., 1, 131 (1906).

82. Benedict, S.R., J. Biol. Chem., 6, 363 (1909).

83. Denis, W., J. Biol. Chem., 8, 401 (1910).

84. Griffith, W.H., J. Biol. Chem., 69, 197 (1926).

85. Hawk, P.B., and Bergheim, O.B., Practical Physiological Chemistry, 11th Ed., p. 701. P. Blakiston's Son and Co., Philadelphia, Pa.

86. Ma, T.S., and Zuazaga, G., Ind. Eng. Chem., Anal. Ed., 14, 280 (1942).

87. Folin, O., J. Biol. Chem., 17, 469 (1914).

88. Dziewiatkowski, D., Ph.D. Thesis, University of Michigan (1944).

89. Laughan, G.B., Evelyn, K.A., and Browne, J.S.L., J. Biol. Chem., 126, 567 (1938).

90. Bernard, F.W., and McCullagh, D.R., Federation Proc., 1, 101 (1942).

91. Shacter, B., Ph.D. Thesis, University of Michigan (1943).

92. Macht, D.I., J. Pharmacol. and Exper. Therap., 11, 263 (1918).

93. Diack, S.L., and Lewis, H.B., J. Biol. Chem., 77, 89 (1923).

94. Erdmann and Marchand, Annalen, 44, 344 (1842).

95. Diechmann, W., and Thomas, G., J. Ind. Hyg. Tox., 25, 286 (1943).

96. Hastings, A.B., Harkins, H.N., and Leu, S.K., J. Biol. Chem., 94, 681 (1931).

97. Steinauer, O., Arch. Anat. Path., 59, 65 (1874).

98. du Vigneaud, V., Wood, J.L., and Irish, O.J., J. Biol. Chem., 131, 273 (1939).

99. Zbarsky, S.H., and Young, L., J. Biol. Chem., 151, 211 (1943).

100. Baumann, E., and Preusse, C., Z. physiol. Chem., 5, 335 (1879).

101. Binkley, F., and du Vigneaud, V., J. Biol. Chem., 144, 507 (1942).

102. Stetten, D., Jr., J. Biol. Chem., 144, 501 (1942).

103. Lewis, H.B., Physiol. Rev., 4, 394 (1924).

104. Schmiedeberg, O., Arch. Exp. Path. Pharm., 14, 288 (1881).

105. Shiple, G.J., and Sherwin, C.P., J. Biol. Chem., 55, 671 (1923).

106. Clarke, H.T., and Inoyue, J.M., J. Biol. Chem., 94, 571 (1932).

APPENDIX

Methods of Analysis

Ether-Soluble Sulfur

In those experiments in which the excretion of ether-soluble mercapturic acids was anticipated, the amount of sulfur that could be extracted from the acidified urine by ether was determined. When a Kutscher and Steudel type of extractor of about 50 cc./capacity was employed, about 70 per cent of benzylmercapturic acid added to the urine could be recovered. With a Griffith type of extractor under the conditions outlined in the next paragraph, 94 to 98 per cent of the benzylmercapturic acid added to urine could be recovered as ether-soluble sulfur. S-phenylcysteine, S-benzylcysteine, and cystine were not soluble in ether. Hence the mercapturic acid should be separated from these amino acids by this procedure. If the keto acid corresponding to the mercapturic acid was excreted in the urine together with benzylmercapturic acid, it would probably be soluble in ether. Thus the mercapturic acid would not be separated from the keto acid by extraction with ether.

Procedure. -- To a 20 cc. sample of urine in a Griffith extraction tube were added 2 drops of congo red indicator and 0.5 to 1.0 cc. of concentrated hydrochloric acid. 100 cc. of

ether were placed in the Kjeldahl flask, and the acidified sample was extracted 5 hours with ether at 60-70°. The solvent was removed by distillation, and the residue was transferred quantitatively to an evaporating dish by dissolving in 3 to 5 cc. of 0.1 N. alkali and rinsing the Kjeldahl flask 5 times with water. The total sulfur was then determined by the Benedict method (82) as modified by Denis (83).

Recovery Experiments. -- In Table XX it is shown that l-cystine, S-benzylcysteine, and S-phenylcysteine are not extracted by ether under the conditions used. This finding is to be expected since the amino acids are present as salts in the strongly acid solution and are also insoluble in ether. With benzylmercapturic acid (Table XXI) in extraction of 94 to 98 per cent was obtained. This compound is slightly soluble in ether and, because of the N-acetyl group, would not be expected to be as readily soluble in the strongly acid solution as the S-benzylcysteine.

TABLE XX

Extraction of Benzylmercapturic Acid by Ether from Urine

Duplicate samples (20 cc. volume) were prepared which contained synthetic benzylmercapturic acid equivalent to the amount of barium sulfate given in the second column. The value (expressed as mg. of barium sulfate), which is listed in the third column, has been corrected for the urine blank. The procedure was as

previously described except that in Experiment 1 the time of extraction was 4 hours.

Experiment No.	Benzylmercapturic Acid*		
	Added	Recovered	
	mg.**	mg.**	per cent
1	16.8	15.7	93.5
	16.8	16.4	98.0
2	27.6	26.8	97.2
	27.6	26.6	96.5
3	43.8	42.7	97.5
	43.8	42.9	98.0

*Preparation 1-140, melting point 153-4°, S = 12.71 per cent.
**Expressed as mg. of barium sulfate.

TABLE XXI

Extraction by Ether of the Sulfur-Containing Amino Acids Added to Urine

The procedure was the same as that described for Table XX, with the exception that the time of extraction was 4 hours. The value listed in the third column has not been corrected for the urine blank.

Compound	Added	Recovered
	mg.*	mg.*
1. Urine (control)	0	2.7
2. Cystine	33.2	2.2
3. S-Benzylcystine	18.7	2.8
f. S-Phenylcystine	21.9	3.3

*Expressed as mg. of barium sulfate.

An examination of Table XXI will show that the differances between the values for the urine blank (2.7 mg. of barium sulfate) and the amounts of barium sulfate recovered from the experimental urines (these values are not corrected for the urine blank) are within the limits of error of the method.

Hippuric Acid

Hippuric acid was determined by the method of Griffith (84). In this procedure the urine was made acid to congo red and extracted with ether. After this solvent was removed by distillation, the residue was treated with a solution of sodium hypobromite, which liberates the nitrogen from urea, ammonia, and the amino group of the amino acids. The remaining nitrogen was estimated by the usual Kjeldahl-Gunning method. The procedure of Griffith (84) is not specific for hippuric acid but will be affected by the presence in the urine of ether-soluble nitrogenous compounds whose nitrogen is not liberated by the action of sodium hypobromite. It was found that benzylmercapturic acid would interfere in this determination. When duplicate 10 cc. samples of urine to each of which had been added 9.4 mg. of benzylmercapturic acid [16] were subjected to the procedure of Griffith (84), the extra ether-soluble nitrogen in the samples corresponded to 8.8 mg. and 9.2 mg. respectively of the mercapturic acid added.

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[16] Preparation 1-140.

Procedure. -- The procedure was that described by Griffith (84). A 10 cc. sample of urine that had been pipetted into a Griffith extraction tube was acidified (congo red) with 0.1 cc. of concentrated hydrochloric acid. The sample was extracted continuously with 100 cc. of ether for 1 hour with the extraction apparatus immersed in a water bath at 60 - 70°. The ether was distilled off, and to the residue 2 cc. of sodium hypobromite[17] solution was added. The flask was shaken for 1 minute, and 2 cc. of dilute sulfuric acid (1:5) was added. The contents of the flask were mixed thoroughly, and 2 cc. of 25 per cent sodium hydroxide and 2 cc. of the hypobromite solution were added. After the flask was shaken for 1 minute, the remaining nitrogen was determined by the Kjeldahl method in which approximately 0.02 N acid was used for the titrations.

Glucuronic Acid

The level of total glucuronic acid in the urine was estimated by the procedure of Dziewiatkowski (88).

The desired aliquot of urine was made acid to congo red with concentrated hydrochloric acid and diluted to 50 cc. The diluted sample was then hydrolyzed for 1½ hours on the steam

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[17] Prepared by mixing equal volumes of 25 per cent sodium hydroxide and a solution containing 12.5 gm. of bromine and 12.5 gm. of sodium bromide dissolved in 100 cc. of water.

bath, transferred to a 100 cc. volumetric flask, and diluted to volume. To 2 cc. samples of the diluted hydrolyzed urine in large pyrex test tubes were added 2 cc. of freshly prepared 1,3-dihycroxy-β-napthoic acid and 2 cc. of concentrated hydrochloric acid. After the contents were mixed, the tubes were placed in a boiling water bath for exactly 30 minutes and subsequently were cooled in running water for 5 to 10 minutes, and 2 cc. of alcohol were added to each tube. 25 cc. of peroxide-free ether were measured from a burette into each tube, which was immediately stoppered with a cork that had been extracted with boiling water and with ether.

The samples were extracted by gentle agitation for 1 minute or more. The corks were loosened to allow the acid to drain from the sides of the tube, and after 15 minutes the ether was pipetted into colorimeter tubes which were then stoppered with corks that had been cleaned as previously described. After 30 minutes the intensity of the color was estimated by the use of the Evelyn photoelectric colorimeter (Filter 540).

Preparation of the Reagent. -- A suspension of 100 mg. of 1, 3-dihydroxy-β-napthoic acid in 20 to 30 cc. of water was heated on the steam bath in a large stoppered pyrex test tube with frequent gentle shaking. After 5 minutes the solution was cooled under running water, filtered into a 50 cc.

volumetric flask, and the contents of the flask were diluted to the mark.

Preparation of Peroxide — Free Ether. -- 500 cc. of ether were shaken for 5 minutes with 100 cc. of a 1 per cent ferrous sulfate solution. After the ether layer had separated, it was washed twice with distilled water and dried over anhydrous sodium sulfate. The ether thus purified should be used within an hour.

K Value. -- The K value was determined with 2 cc. samples which contained amounts of pure glucurone ranging from 30 to 100 γ.

TABLE XXII

K Value for Glucurone*

Concentration γ	G	L	K x 10^{-3}
Blank	74		
Blank	74		
30	78 3	0.1037	3.45
30	79 1	0.1010	3.37
40	74 2	0.1264	3.16
40	74 3	0.1264	3.16
50	69 3	0.1565	3.12
50	70 2	0.1518	3.04
75	60	0.2218	2.95
75	60 2	0.2182	2.91
80	59 1	0.2273	2.84
80	58 3	0.2310	2.89
100	51 3	0.2861	2.86
100	51 3	0.2861	2.86

*Obtained from D. Dziewiatkowski and designated by him as preparation No. 1

The K value which corresponded to the galvanometer reading obtained with the sample was utilized in the calculation of the glucurone in the urine.

Phenol

Phenol was determined by the procedure of Shacter (91). A urine sample containing 0.01 to 0.2 mg. was pipetted into a 10 cc. volumetric flask. After diluting the sample to a volume of 7 cc. with distilled water, 0.5 cc. of 7 N. sulfuric acid was added, followed by 1.5 cc. of 15 per cent mercuric sulfate in 6 N. sulfuric acid solution; both solutions were measured from burettes. After loosely stoppering the flasks, they were placed in a boiling water bath for 6 minutes. The samples were cooled in running water to room temperature and diluted to a volume of 10 cc. with distilled water. The solution was transferred to a 10 cc. centrifuge tube, and after centrifugation, the supernatant layer was transferred to an Evelyn colorimeter tube. The center setting of each tube was checked, and 1 drop of a 5 per cent solution of sodium nitrite was added and mixed with the contents of the tube. After 6 minutes (4 to 8 minutes), the galvonmeter reading was taken using filter 520 and a K value of 3.13. Shacter's reagents were used in the benzene experiments, and the K value was that established by him.

Specificity of the Modified Millon Reaction. -- The effect

of the presence of polyphenols on the modified Millon reaction was investigated. Solutions containing 1 mg. per 2 cc. of the more common and readily available polyphenols were prepared. The 2 cc. samples were subjected to the procedure outlined in the previous section, and the results are presented in Table XXIII.

TABLE XXIII

The Effect of Certain Polyphenols on the Modified Millon Reaction

2 cc. samples containing 1 mg. were used except as noted.

Phenol	G
Pyrocatechol (1, 2-dihyroxy benzene)	99^{2} 99^{1}
Resorcinol (1, 3-dihydroxy benzene)	99^{2} 99^{2}
Hydroquinone (1, 4-dihydroxy benzene)	100 99^{3}
Pyrogallol (1, 2, 3-trihydroxy benzene)	99^{2} 99^{1}
Phloroglucinol (1, 3, 5-trihydroxy benzene)*	97 98

*This solution contained 2 mg. per 2 cc.

It is obvious that in the concentration used none of the polyphenols gave a measurable color in the modified Millon reaction.

The Effect of the Presence of Polyphenols on the Color Developed by Phenol. -- The effect of the presence of various polyphenols on the color due to phenol was found to be negligible since the same galvonmeter reading was obtained in the presence and in the absence of the various polyphenols tested.

TABLE XXIV

The Effect of Polyphenols on the Color Due to Phenol

By suitable dilutions 2 cc. samples were prepared that contained from 90 to 100 γ of phenol and the same amount of phenol plus 50 γ of the respective polyphenol. The procedure was that previously described.

Polyphenol present	G
None	$52^{2}_{52^{1}}$
Pyrocatechol	$53_{52^{2}}$
Resorcinol	53_{523}
Hydroquinone	52^{2}_{523}
Pyrogallol	53_{523}
Phloroglucinol	53_{523}

None of the polyphenols reacted in the modified Millon reaction in concentration of 1 mg. per 2 cc., a concentration much greater than the usual amount of phenol present in the experimental urine sample (0.05 to 0.1 mg.). Furthermore, when the various polyphenols, which by themselves did not react, were added individually to a phenol solution, they did not affect the color intensity in any way. Hence, these substances could not be responsible for the increase in color intensity observed on the experimental day after benzene feeding and, if present, would not affect the color development due to phenol.

With solutions containing 1 mg. per 2 cc. of the polyphenol it was observed that, when the acid mercuric sulfate reagent was added, a precipitate appeared. It appeared immediately in the solutions of phloroglucinol or resorcinol; with the solutions of the other three phenols, a precipitate appeared after the addition of the mercuric sulfate upon heating in the boiling water bath. When the acid mercuric sulfate reagent was added to solutions containing 50γ of the above polyphenols, a precipitate appeared immediately in the solutions containing phloroglucinol or resorcinol. No precipitate appeared in the other solutions when they were boiled in a water bath, but they assumed a very faint yellow-green color. The solutions were centrifuged; and when to the supernatant layer a 5 per cent solution of sodium nitrite was added, no

color developed. The faint yellow color observed in three solutions did not change the galvonmeter reading to a value different than that of the control phenol solution.

It is postulated that the polyphenols, which are powerful reducing agents, were oxidized to the corresponding quinones by the mercuric sulfate and hence did not yield a color when the solution of sodium nitrite was added.

p-Monobromophenol

The tests of p-monobromophenol were carried out in exactly the same way as for phenol except that 10 minutes was allowed for maximum development of color after the addition of the sodium nitrite solution. Detailed directions for this determination are given in the last paragraph in this section.

A calibration curve was prepared using 2 cc. samples of p-monobromophenol solutions. This compound was an Eastman Kodak product with a melting point of 63.5° and a bromine content of 45.59 per cent. The galvonmeter reading was taken 6 and 14 minutes after the addition of the sodium nitrite solution.

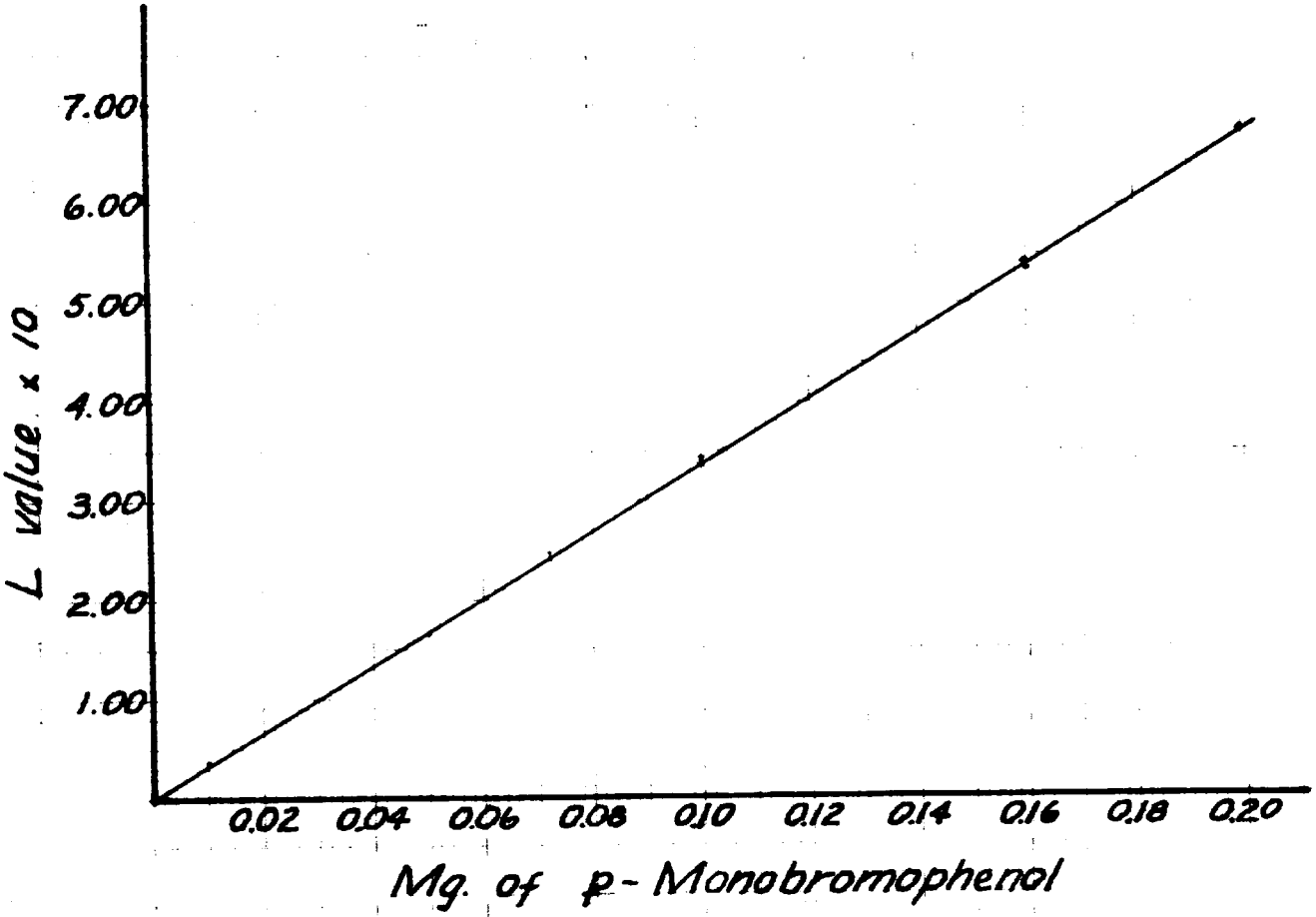
TABLE XXV

K Value for p-Monobromophenol in the Modified Millon Reaction

p-Monobromophenol	Time for Color Development: 6 minutes			14 minutes		
	G	L(corrected)	K	G	L(corrected)	K
mg.						
0.01	92^{1}	0.0362	3.62	92_{2}	0.0374	3.74
0.01	91^{3}	0.0386	3.86	$91_{}$	0.0398	3.98
0.05	$69_{}$	0.1627	3.26	68^{1}	0.1675	3.35
0.05	68^{3}	0.1643	3.29	68	0.1691	3.39
0.072	57^{2}	0.2403	3.33	$57_{}$	0.2441	3.39
0.072	$57_{}$	0.2441	3.39	56^{2}	0.2480	3.45
0.10	46^{2}	0.340	3.32	45^{2}	0.342	3.42
0.10	46^{3}	0.332	3.40	$46_{}$	0.337	3.37
0.16	29^{2}	0.534	3.34	28^{3}	0.542	3.39
0.16	30	0.523	3.27	29	0.538	3.36
0.20	22	0.658	3.29	21	0.678	3.39
0.20	22	0.658	3.29	21	0.678	3.39

A calibration curve prepared from these data may be found on the next page. The average K value, if the reading of the concentration of 0.01 mg. is excluded, was 3.32 for 6 minutes of color development and 3.39 for 14 minutes of color development. The time for the development of the maximum color intensity was found to be 9 minutes, and the galvonmeter reading remained constant for as long as it was observed, that is, for 11 additional minutes. Hence, in the urine samples the color was allowed to develop 10 minutes before the galvonmeter reading was taken.

p - Monobromophenol Determination
Filter 520



The experiment with bromobenzene in which the total phenols were determined was carried out before p-monobromophenol was available in this laboratory. Since in the procedure as applied to phenols (monohydroxybenzene) the color is allowed to develop 6 minutes after adding the solution of sodium nitrite, the experimental and control samples of urine were allowed to stand 6 minutes after the addition of a drop of sodium nitrite solution. In the calculation of the results of that experiment (Table XXXVI, Experiment 6-1) the K value of 3.32 for 6 minutes of color development was used. In the experiments in which p-monobromophenol was fed, the time of color development of the control and experimental urine samples was 10 minutes; and a K value of 3.39 was used in the calculations.

Choice of Color Filter. -- An examination of Table XXVI will show that apparently any Evelyn filter between 470 and 520 would be satisfactory, and Evelyn filter 520 was chosen.

TABLE XXVI

Maximum Color Absorption by p-Monobromophenol

In this series of readings the center setting was 50. All filters available were tested but only the four that gave the lowest galvonmeter readings are reported. The color did not fade during the course of the experiment because the same galvonmeter reading was obtained with the p-monobromophenol solution (2 cc. containing 0.05 mg.) at the beginning and at the end of the experiment, when readings were taken with the Evelyn filter 520.

Filter	G	Filter	G
520	46	470	45
520	45	470	46
490	45	420	53
490	45	420	53

Recovery Experiments. -- Experiments in which p-monobromophenol was added to a urine sample comparable to that of the experimental period are presented in Table XXVII.

TABLE XXVII

Recovery of p-Monobromophenol Added to Urine

The amounts of p-monobromophenol indicated in Column 2 were added to 2 cc. of normal urine. Tubes 1 and 2 contained the control urine, that is, 0.08 cc. of urine diluted to a volume of 2 cc. The other tubes contained the respective amounts of p-monobromophenol given in the second column plus 0.08 cc. of urine in a total volume of 2 cc. Duplicate analyses were run, and the galvanometer readings for the duplicates are given in column 4. The average of the corresponding duplicate values is given in Columns 4, 5, and 6.

Tube No.	p-Monobromophenol added to urine *	G	L Urine	L Urine minus L of the control urine	p-Monobromophenol Recovered***
	γ				γ
1 and 2	0	94, 94	0.0263**	0	0
3 and 4	20	79, 80	0.0976	0.0713	21.1
5 and 6	40	68, 68	0.1635	0.1372	40.4
7 and 8	60	59 , 59	0.2282	0.2019	59.5
9 and 10	80	50 , 50	0.300	0.274	80.8

*Two control tubes each of which contained 50γ of p-monobromophenol in water gave galvanometer readings of 67 and 67, which are equivalent to L values of 0.1723 and 0.1691 or

recoveries of 50.8 and 49.9γ respectively.
**This is the L value of the control urine.
***This value was calculated by the method illustrated in the next section with the exception that the quotient was multiplied by 1000 to convert mg. to γ. For example, for tubes 7 and 8,

$$\gamma \text{ of } \underline{p}\text{-monobromophenol recovered} = \frac{(\text{L urine} - (\text{control urine})1000}{3.39}$$

$$40.4 = \frac{(0.1635 - 0.0263)(1000)}{3.39}$$

Calculation. -- The extra total phenols found by the use of the modified Millon reaction in the urine of the animals that were fed monobromobenzene or p-monobromophenol were assumed to be p-monobromophenol. Thus instead of calculating the extra total phenols as monohydroxybenzene on the experimental day by subtracting the average L value of the control urine from the L value for the experimental day and dividing by the K value for bromophenol 3.39. This process yielded as a quotient mg. of p-monobromophenol. A formula will illustrate this point.

$$\frac{\text{L experimental urine} - \text{L control urine}}{\text{K for bromophenol}} = \text{mg. of bromophenol}$$

This method of calculation is equivalent to expressing the color intensity of urinary constituents that react in the modified Millon reaction in terms of p-monobromophenol. Since the latter is not found in normal urine, the control values are reported as mg. of phenol. This calculation is based on the assumption that the increase in total phenols that reacted in the modified Millon reaction on the days when p-monobromophenol or monobromobenzene was fed is due solely to the p-substituted phenol. This is, it seems, a more logical assumption than to assume that the extra phenol is monohydroxybenzene. However, if the latter were produced in the course of the metabolism of the monobromobenzene or p-monobromophenol, the calculation could not be valid for on a molar basis phenol gives about one-half the color that p-monobromophenol does. Similar considerations would apply if an isomeric bromophenol were produced from monobromobenzene or p-bromophenol, which might give less color than the p-substituted isomer. In addition, if any of the polyphenols that did not react in the modified Millon reaction were produced in the course of the metabolism of monobromobenzene or p-monobromophenol, the extra total phenol on the experimental day, which is measured by this procedure, would not include some of the phenolic compounds present in the urine.

Procedure for the Determination of p-Monobromophenol.[18] -- The procedure was the same as that for phenol with the exception that after the addition of a drop of a 5 per cent solution of sodium nitrite, the galvanometer deflection was not read for 10 minutes. The K values were 3.32 and 3.39 for 6 and 10 minutes of color development respectively.

Synthesis

N-Acetyl-S-benzylcysteine

This compound was prepared according to the method of Shiple and Sherwin (105). All melting and boiling points reported are uncorrected unless otherwise stated.

To 2.5 gm. of finely divided S-benzylcysteine, suspended in 25 cc. of redistilled pyridine[19] and cooled in an ice bath, 1.3 cc. of acetyle chloride were added drop by drop. The resulting solution was allowed to stand for 2 hours at room temperature. Then 27.9 cc. of concentrated hydrochloric acid were added from a burette to give a reaction acid to congo red. The acid pyridine solution was placed in the refrigerator for 4 hours, but no precipitate appeared.

At the end of this time the cold solution was made alkaline by the slow addition of 70 cc. of 20 per cent sodium hydroxide and extracted 4 times with 25 cc. portions of ether.

After separation of the ether phase, the aqueous layer was acidified to congo red paper with 12 cc. of concentrated

[18] Or total extra phenols calculated as p-monobromophenol.

[19] The pyridine was stored over barium oxide and freshly distilled in the presence of this solid.

hydrochloric acid. Upon cooling, a cream colored precipitate appeared.

After filtration the precipitate was washed 3 times with 10 cc. portions of cold water (5 to 10°). After drying for 48 hours over phosphorous pentoxide in a vacuum dessicator, the precipitate weighed 2.35 gm. (melting point, 149-154°) or a yield of 78 per cent of the theoretical.

The crude benzylmercapturic acid was dissolved in the minimum amount of hot 95 per cent alcohol and decolorized with norit. The norit was removed by filtration, and to the hot filtrate, water at 70° was added until the solution became turbid. Upon cooling the turbid solution in the refrigerator, 1.48 gm. of a white crystalline product, which was designated as preparation 1-140 and melted at 153.5° to 154°, was obtained. This amount is 50 per cent of the theoretical. By concentrating the mother liquor <u>in vacuo</u> a second crop which melted at 152-4° and weighed 0.3 gm. was obtained, increasing the overall yield to 59 per cent.

A satisfactory analysis for sulfur by the Parr bomb method was obtained for preparation 1-140. (1.48 gm.).

	Found	Theory
Sulfur	12.65 per cent	12.66 per cent

Shiple and Sherwin (105) reported a melting point of 156-7°

and a yield of about 50 per cent. du Vigneaud (98) reported a melting point of 157-8° (corrected) and considered that this preparation was the dl-mercapturic acid. The l-mercapturic acid melted at 143-4° (corrected).

S-Phenylcysteine

S-phenylcysteine was prepared according to the method of Zbarsky and Young (99).

10 gm. of l-cystine were dissolved in 200 cc. of 1.5 N sulfuric acid. Six gm. of granular zinc (Mallinckrodt, Mesh No. 20) were added, and the mixture was heated on the steam bath for four hours with the occasional addition of mossy zinc.

At the end of this time the solution was filtered into a 3-necked liter flask equipped with a mechanical stirrer. A suspension of 6 gm. of cuprous oxide[20] in water was poured in small portions into the cysteine solution, maintained at about 50°, over a period of 45 minutes with vigorous mechanical stirring. When all of the oxide had been added, the reaction mixture contained a voluminous red precipitate.

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[20]The suspension was prepared by grinding 6 gm. of cuprous oxide (Baker's) with water in a mortar, allowing the cuprous oxide to settle, and decanting the supernatant layer until a test for chloride ion was no longer positive. Then water was added and the oxide was ground until a suspension was obtained.

The mixture containing the cuprous mercaptide of cysteine was cooled in a salt ice bath to 0° and stirred mechanically. An ice cold solution of benzene diazonium sulfate[21] was added dropwise through a separatory funnel the top of which extended below the surface of the reaction mixture. During the addition, the temperature was maintained between 0 and 5°. Gas was evolved during the coupling and as the reaction mixture was allowed to come to room temperature with vigorous mechanical stirring. Then the mixture was heated at 60 to 70° for 20 minutes; the precipitate was removed by filtration; and the filtrate, which had become dark green in color, was extracted with two 25 cc. portions of ether. The aqueous phase was saturated with hydrogen sulfide until the precipitation was complete. After the copper sulfide was removed by filtration, the filtrate was distilled <u>in vacuo</u> for 30 minutes. At the end of this time the solution, which no longer had the odor of hydrogen sulfide, was immersed in a salt ice bath and carefully neutralized to congo red paper with concentrated ammonium hydrox-

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[21] Ten gm. of aniline (redistilled from zinc dust) were mixed with 100 gm. of cracked ice made from distilled water, and 14 cc. of 1 to 1 sulfuric acid were added with vigorous stirring. The resulting suspension of aniline sulfate was immersed in a salt-ice bath and stirred mechanically. A 50 per cent solution of 6 gm. of sodium nitrite was added dropwise through a separatory funnel the tip of which was extended below the surface of the reaction mixture. The temperature was maintained between 0° and 5). Completeness of diazotization was ascertained when a drop of the solution diluted with 2 or 3 drops of water on a spot plate turned starch-iodide paper blue.

ide added from a burette. A precipitate which appeared at this point was removed by filtration. After the crude product was dried in a vacuum dessicator over phosphorus pentoxide, it weighed 4.8 gm., melted at 172-5° (initial temperature 168°), and gave a strong nitroprusside test upon reduction with sodium cyanide (due presumably to cystine as an impurity).

The crude S-phenylcysteine was dissolved in 100 cc. of 2 N hydrochloric acid, and 6 gm. of granular tin were added. After the mixture was heated on the steam bath for 3 hours, it was filtered to remove excess tin, and the filtrate was saturated with hydrogen sulfide until precipitation was complete. The tin sulfide was removed by filtration, and the filtrate was freed of hydrogen sulfide by distillation <u>in vacuo</u> for 30 minutes. The solution was cooled in a salt ice bath and neutralized to congo red by the careful dropwise addition of concentrated ammonium hydroxide from a burette. After the resulting precipitate was washed with ice-cold water, it was repeatedly extracted with water at 90°, until no more went into solution. The aqueous extracts were combined and placed in the refrigerator. 1.5 gm. of S-phenylcysteine melting with decomposition at 176-8° (initial temperature 170°) were obtained.

A second run was made using a 5 gm. sample of l-cystine. The conditions were as described for the first synthesis except for the separation of l-cystine and S-phenylcysteine. Instead of dissolving the crude product obtained from the reaction mixture in 2 N hydrochloric acid and reducing the l-cystine to l-cysteine, which is soluble at the isoelectric point of S-phenylcysteine, the mixture was extracted with several portions of boiling 50 per cent alcohol until no precipitate appeared on cooling the extract. According to Clark and Inoyue (106), l-cystine is insoluble in hot 50 per cent alcohol, whereas S-phenylcysteine is sparingly soluble. The alcohol extracts were combined and upon cooling 1.2 gm. of a product decomposing at 183° (initial temperature 168°) was obtained. It was recrystallized from 300 cc. of boiling water. The product separated on cooling the aqueous solution. Concentration *in vacuo* yielded a second crop which was combined with the first. The total yield was 0.8 gm. of S-phenylcysteine which decomposed at 193° (initial temperature 25°).

The preparations of S-phenylcysteine obtained in the two runs were combined and analyzed for sulfur by the Parr bomb method.

	Found	Theory
Sulfur	15.98 per cent	16.26 per cent

Zbarsky and Young (99) do not report a sulfur analysis of their product, but Clark and Inoyue (106) found 16.1 S. The combined

analyzed preparation decomposed at 181-2° when the initial temperature of the bath was 165°. Sintering and charring took place at 176°.

The melting point varied with the rate of heating and the temperature of the bath. Other workers (99, 100, 106) have reported different melting points of their preparations.

	Melting Point	Rotation
Zbarsky and Young (99)	170-2°	$[\alpha]_{546}^{23^\circ} = +13.4^\circ$ (2% in 1.0 N alkali)
Clark and Inoyue (106)	201-2°	$[\alpha]_{D}^{25^\circ} = +11^\circ$ (1% in 0.1 N alkali)
Baumann and Preusse (100)	Above 160°	
Present preparation	181-2°	Not tested

The preparation analyzed gave a much fainter nitroprusside test than the crude product. 0.1 cc. of 0.001 M cystine in 5 cc. of solution gave a stronger test than 5 cc. of a 0.01 M solution of this product. Hence, only traces of cystine (or cysteine) were present in the analyzed preparation.

The Sources and Purity of the Compounds Used in the Animal Experiments

Benzene Derivatives

a. The benzene was a preparation obtained from the General Chemical Company (Thiophene Free Grade, B.P. = 80 - 81°)

b. The cinnamyl chloride was obtained from the Eastman Kodak Company (Reagent Grade, M. P. = 8°)

c. The monobromobenzene was freshly redistilled at 153-4°.

d. The benzyl alcohol was freshly redistilled at 203-4°.

e. The benzyl chloride was redistilled at 78° and 27 mm.

f. The p-monobromophenol, which was obtained from the Eastman Kodak Company, melted at 63.5°.

Sulfur-Containing Compounds

The analyses of the sulfur content (Parr bomb method) of the amino acids and derivatives used are presented in Table XXVIII.

TABLE XXVIII

Sulfur Content of Amino Acids Fed

Compound	Source	Melting Point	Sulfur Found	Sulfur Theory
l-Cystine*	This lab.	--	26.29	26.68
l-Methionine	Staley	--	20.85	21.49
S-Phenylcysteine**	Schultz	195°	16.45	16.26
S-Phenylcysteine***	Witter	181-2°	15.98	16.26
S-Benzylcysteine	Blood	217°	15.16	15.18

* $[\alpha]_D^{20^\circ} = -205^\circ$

** Prepared by the method of Clark and Inoyue (106).

*** Prepared by the method of Zbarsky and Young (99).

Animal Experiments

Number of Experiments. -- The protocols are numbered according to the animal and the sequence of the experiment. The first number refers to the number of the rabbit, the second to the experiment. Thus 2A-1 would be Experiment No. 1 on rabbit 2A. The numbers in parenthesis in the headings serve to identify the data in the original notes.

TABLE XXIX

THE EFFECT OF THE ORAL ADMINISTRATION OF BENZYL CHLORIDE TO THE RABBIT (EXPERIMENT 1-1 (1-108)).

Rabbit 1, 3.05 kg. Diet; cabbage, 75 gm., oats, 45 gm.

Date	Nitrogen	Creatinine	Sulfur as					N / Total S ratio	N / Total SO_4-S ratio
			Total	Total SO_4	Inorganic SO_4	Ethereal SO_4	Organic		
	gm.	mg.	mg.	mg.	mg.	mg.	mg.		
1942									
5/29	0.97	166	103	78	75	3	25	9.3	12
5/30	0.97	163	108	83	79	4	25	8.9	12
5/31	1.04	168	114	89	83	6	25	9.1	12
6/1	1.04	162	118	91	86	5	27	8.8	12
6/2*	1.06	166	126	67	61	6	59	8.4	16
6/3	0.91	161	97	70	65	5	27	9.4	13
6/4	0.88	163	102	80	72	6	22	8.6	11

*240 mg. of benzyl chloride were administered orally. The increase in organic sulfur of 34 mg. corresponds to 57 per cent of the benzyl chloride administered.

TABLE XXX

THE EFFECT OF THE ORAL ADMINISTRATION OF BENZYL CHLORIDE TO THE RABBIT (EXPERIMENT 1-2 (2-155)).

Rabbit 1, 2.85 kg. Diet; cabbage, 75 gm., oats, 45 gm.

Date	Nitrogen	Creatinine	Sulfur as					N / Total S ratio	N / Total SO_4-S ratio
			Total	Total SO_4	Inorganic SO_4	Ethereal SO_4	Organic		
	gm.	mg.	mg.	mg.	mg.	mg.	mg.		
1942									
10/9	0.625	155	90	69	66	3	21	6.9	9.0
10/10	0.597	155	90	68	64	4	22	6.6	8.8
10/11	0.614	142	88	67	62	5	21	7.0	9.2
10/12*	0.625	142	96	51	47	4	45	6.5	12
10/13	0.685	141	99	78	73	5	21	6.8	8.8
10/14	0.696	141	93	73	69	4	20	7.4	9.5
10/15	0.632	138	85	67	62	5	18	7.4	9.5

*Administered orally 233 mg. of benzyl chloride. The increase in organic sulfur of 24 mg. corresponds to 40 per cent of the benzyl chloride fed.

TABLE XXXI

THE EFFECT OF l-CYSTINE AND l-METHIONINE ON THE SYNTHESIS OF BENZYLMERCAPTURIC ACID AND THE METABOLISM OF S-BENZYLCYSTEINE AND S-PHENYLCYSTEINE IN THE RABBIT.

For an explanation of this table, see the key and the explanatory notes at the end.

Rabbit 2A, 2.2 kg. Diet; cabbage, 100 gm., oats, 45 gm.

Date	Experiment No.	Nitrogen	Creatinine	Sulfur						N / Total S ratio	N / Total SO_4-S ratio
				Total	Sulfate			Organic	Ether-soluble		
					Total	Inorganic	Ethereal				
		gm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.		
1943											
9/31		0.669	104	83	60	59	1	23	4	8.1	11
10/1		0.727	112	91	65	63	2	26	5	7.9	11
10/2	2A-5	0.939	114	126	53	46	7	73	49	7.5	18
10/3	2A-6	0.895	108	129	57	52	5	72	52	6.9	16
10/4	2A-7	0.740	104	232	146	139	7	86	52	3.2	5.1
10/5		0.730	108	118	94	89	5	24	8	6.2	7.8
10/6		0.734	112	101	77	74	3	24		7.3	9.5
10/7		0.731	103	96	70	69	1	26	8	7.6	10
10/8	2A-8	0.741	102	289	235	230	5	54	5	2.6	3.1
10/9		0.681	102	114	90	86	4	24		6.0	7.6
10/10		0.648	100	91	70	67	3	21		7.1	9.3
10/11		0.723	102	98	70	68	2	28	8	7.4	10
10/12	2A-9	0.792	108	158	70	67	3	88	55	5.0	11
10/13	2A-10	0.680	96	157	64	61	3	93	65	4.3	11
10/14		0.750	117	97	69	67	2	28	7	7.7	11
10/15	2A-11	0.661	108	152	75	70	5	77	50	4.2	8.8

TABLE XXXI (Continued)

Date	Experiment No.	Nitrogen	Creatinine	Sulfur						N / Total S ratio	N / Total SO_4-S ratio
				Total	Sulfate			Organic	Ether-soluble		
					Total	Inorganic	Ethereal				
		gm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.		
1943											
10/16		0.687	116	109	75	72	3	34		6.3	9.2
10/17		0.584	103	83	61	56	5	22	9	7.0	9.6
10/18		0.681	115	102	69	64	5	33	7	6.7	9.9
10/19	2A-12	0.948	115	129	52	47	5	77	50	7.3	18
10/20	2A-13	0.912	106	114	46	39	7	68	48	8.0	20
10/21	2A-14	0.793	105	158	83	78	5	75	47	5.0	9.3
10/22		0.765	120	145	112	110	2	33	15	5.3	6.8
10/23		0.802	122	77	55	52	3	22		10	15
10/24		0.766	110	92	72	67	5	20	5	8.3	11
10/25	2A-15	0.657	103	190	157	152	5	33	7	3.5	4.2
10/26		0.728	115	137	110	106	4	27		5.3	6.6
10/27		0.608	87	70	54	51	3	16		8.7	11
10/28		0.932	120	106	84	80	4	22		8.8	11
10/29		0.691	100	78	62	57	5	16	8	8.9	11
10/30	2A-16	0.677	101	131	110	108	2	21	9	5.2	6.2
11/1	2A-17	0.734	110	196	122	119	3	74	46	3.8	6.0
11/2		0.608	104	128	102	95	7	26	11	4.7	6.0
11/3		0.736	108	69	52	45	7	17		11	14
11/4		0.742	107	74	57	52	5	17		10	13
11/5		0.697	106	60	46	41	5	14		12	15

KEY TO TABLE XXXI.

The compounds were fed according to the method listed under Animal Experiments.

Experiment No.	Compounds fed and notes
2A-5	337 mg. of benzyl chloride at 8 A.M.
2A-6	357 mg. " " " " 8 A.M.
2A-7	342 mg. " " " 8 A.M. plus 500 mg. of l-cystine at 8 A.M., 500 mg. of l-cystine at 11 A.M. All of food not consumed.
2A-8	500 mg. of l-cystine at 8 A.M., 500 mg. of l-cystine at 11 A.M.
2A-9	500 mg. of benzylcysteine at 8 A.M. Feeding not quantitative.
2A-10	500 mg. of S-benzylcysteine at 8 A.M.
2A-11	500 mg. of S-phenylcysteine (Schultz) at 8 A.M.
2A-12	347 mg. of benzyl chloride at 8 A.M.
2A-13	347 mg. " " " at 8 A.M.
2A-14	350 mg. of benzyl chloride at 8 A.M. plus 500 mg. of l-methionine at 8 A.M., 600 mg. of l-methionine at 11 A.M. All of food not consumed on this day and succeeding ones except 10/24 and 10/28.
2A-15	600 mg. of l-methionine at 8 A.M.; 600 mg. of l-methionine at 11 A.M. All of food not consumed.
2A-16	600 mg. of l-methionine at 8 A.M. All of food not consumed.
2A-17	354 mg. of benzyl chloride plus 600 mg. of l-methionine at 8 A.M. 600 mg. l-methionine at 11 A.M. All of food not consumed on this or succeeding days.

Notes for Table XXXI

All of the urines with the exception of the urine excreted by the rabbit after the administration of cystine gave negative nitroprusside tests.

From an aliquot of the combined urines obtained after the administration of benzyl chloride, an amount of benzyl-

mercapturic acid corresponding to 24 per cent of the benzyl chloride fed or 47 per cent of the average increase in ether-soluble sulfur was isolated.

NORMAL VALUES FOR TABLE XXXI.

The normal levels of the sulfur fractions were calculated by averaging the values of the two or three preceding days.

Experiment No.	Sulfur		
	Total SO_4-S	Organic	Ether-soluble
	mg.	mg.	mg.
2A-5		25	5
2A-6		25	5
2A-7	55	25	5
2A-8	73	24	
2A-9		24	8
2A-10		24	8
2A-11		28	7
2A-12		28	8
2A-13		28	8
2A-14	49	28	8
2A-15	63		
2A-16	68		
2A-17	68	18	8

EXTRA SULFUR FRACTIONS OF THE EXPERIMENTAL DAYS FOR TABLE XXXI

Experiment No.	Compounds fed	Extra sulfur: As sulfur: Organic	Ether-soluble	Total SO_4-S	Extra sulfur: As per cent of compound fed: Organic	Ether-soluble	Total SO_4-S
		mg.	mg.	mg.	mg.	per cent	per cent
2A-5	Benzyl chloride	48	44		56	51	
2A-6	Benzyl chloride	47	47		52	52	
2A-7*	Benzyl chloride and l-cystine	61	47	91 39	70	54	34 15
2A-8*	l-cystine	30		162 17	11		61 6
2A-9	S-Benzylcysteine	64	47		86	63	
2A-10	S-Benzylcysteine	69	57		93	77	
2A-11	S-Phenylcysteine	49	43		60	53	
2A-12	Benzyl chloride	49	42		56	48	
2A-13	Benzyl chloride	40	40		45	45	
2A-14*	Benzyl chloride and l-methionine	47	39	34 63	55	44	13 24
2A-15*	l-Methionine			94 47			37 18
2A-16*	l-Methionine			42			30
2A-17*	Benzyl chloride and l-Methionine	56	38	54 34	62	42	21 13

*The extra total sulfate sulfur is calculated as per cent of the sulfur administered as l-cystine or l-methionine and is given for the experimental day and the day afterwards.

TABLE XXXII

THE EFFECT OF THE ORAL ADMINISTRATION OF S-BENZYLCYSTEINE TO THE RABBIT
(EXPERIMENT 6-7(5-69B))

Rabbit 6, 3.2 kg. Diet; chow, 75 gm.

Date	Nitrogen	Creatinine	Sulfur as					Phenol	Glucuronic acid	N / Total S ratio	N / Total SO_4-S ratio
			Total	Total SO_4-S	Inorganic SO_4	Ethereal SO_4	Organic				
	gm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.		
1943											
6/20	1.39	151	113	89	82	7	24			12	16
6/21	1.40	158	109	86	74	12	23	84	112**	14	16
6/22*	1.66	154	188	89	86	3	99	105	83	8.8	19
6/23	1.51	151	114	91	81	10	23	90	92**	13	17
6/24	1.43	158	115	88	81	7	27			12	16

*Administered orally 560 mg. of benzylcysteine dissolved in sodium hydroxide. The increase in organic sulfur of 75 mg. corresponds to 88 per cent of the S-benzylcysteine.

**Average values for 6/20 and 6/21 and for 6/23 and 6/24 obtained by analyses of a mixture of equal parts of the respective hydrolyzed urine samples.

TABLE XXXIII

THE EFFECT OF THE ORAL ADMINISTRATION OF CINNAMYL CHLORIDE (EXPERIMENT 4-2 (9-163B))

Rabbit 4, 2.7 kg. Diet; cabbage, 100 gm., oats, 45 gm.

Date	Nitrogen	Creatinine	Sulfur as					Hippuric Acid	N / Total S ratio	N / Total SO_4-S ratio
			Total	Total SO_4	Inorganic SO_4	Ethereal SO_4	Organic			
	gm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.		
1943										
10/7	0.823	92	129	93	86	7	36		6.4	8.8
10/8	0.845	91	128	90	82	8	38	140	6.6	9.4
10/9*	0.906	81	125	88	79	11	37	300	7.3	10
10/10	0.922	95	145	107	96	11	38	110	6.4	8.5

*Administered orally 360 mg. of cinnamyl chloride ($C_6H_5CH = CH - CH_2Cl$). The increase in hippuric acid of 160 mg. corresponds to 42 per cent of the cinnamyl chloride fed.

TABLE XXXIV

GLUCURONIC ACID EXCRETION BY THE RABBIT AFTER THE ORAL ADMINISTRATION OF MONOBROMOBENZENE

Rabbit 4, 3.0 kg. Diet; cabbage, 100 gm., oats, 7.5 gm.
Rabbit 3A, 2.5 kg. Diet; cabbage, 75 gm., oats, 45 gm.
Rabbit 1, 3.2 kg. Diet; cabbage, 100 gm., oats, 45 gm.

Date	Experiment No.	Creatinine	Glucuronic acid	Extra glucuronic acid	
		mg.	mg.	mg.	per cent*
1943					
4/17		157	133		
4/18		171	143		
4/19	4-1	157	375	237	21
4/20		157	163		
4/21		166	112		
4/17		120	101		
4/18		122	96		
4/19	3A-1	128	385	287	26
4/20		122	140		
4/21		128	59		
3/22		152	153		
3/23		162	147		
3/24	1-8	172	241	91	9

*Extra glucuronic acid calculated as per cent equivalent to the one gm. of monobromobenzene that was fed.

TABLE XXXV

THE EFFECT OF THE ORAL ADMINISTRATION OF MONOBROMOBENZENE AND BENZENE TO THE RABBIT (EXPERIMENTS 3A-3 AND 3A-4 (1-19B)).

Rabbit 3A, Diet; cabbage, 75 gm., oats, 45 gm.

Date	Experiment No.	Nitrogen	Creatinine	Sulfur					Glucuronic acid	N / Total S ratio	N / Total SO_4-S ratio	Extra Organic S	Extra Ethereal SO_4-S	Extra Glucuronic acid	Per cent compound excreted as		
				Total	Total SO_4	Inorganic SO_4	Ethereal SO_4	Organic							Organic S	Ethereal SO_4-S	Glucuronic acid
		gm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.			mg.	mg.	mg.	per cent	per cent	per cent
1943																	
5/29		0.693	124	90	69	65	4	21	47	7.6	10						
5/30		0.749	123	100	75	70	5	25	47	7.5	10						
6/1	3A-3*	0.803	129	120	68	7	61	52	367	6.7	12	29	55	320	13	25	26
6/2		0.828	127	89	60	52	8	29	72	9.3	14						
6/3		0.668	120	88	67	63	4	21	59	7.5	10						
6/4	3A-4**	0.714	124	96	74	72	2	22	42	7.4	9.6						
6/5		0.758	129	99	71	38	33	28	150	7.7	10	--	27	100	--	6.6	4.4
6/6		0.734	124	90	67	75	2	23	40	8.2	11						
		0.769	116	101	80	70	10	21	25	7.6	11						

*Administered 1.08 gm. of monobromobenzene.
**Administered 1.00 gm. of benzene.

TABLE XXXVI

THE EFFECT OF THE ORAL ADMINISTRATION OF BENZENE, MONOBROMOBENZENE, AND BENZYL CHLORIDE TO THE RABBIT (EXPERIMENTS 6-1, 6-2, 6-3, 6-4, 6-5, AND 6-6(4-53B))

For an explanation of this table, see the key on the next page.

Rabbit 6, 3.2 kg. Diet; chow, 75 gm.

Date	Experiment No.	Nitrogen	Creatinine	Sulfur					N / Total S ratio	N / Total SO_4-S ratio	Glucuronic acid	Phenol
				Total	Total SO_4	Inorganic SO_4	Ethereal SO_4	Organic				
1943		gm.	mg.	mg.	mg.	mg.	mg.	mg.			mg.	mg.
5/23		1.63	144	128	101	93	8	27	13	16	107	
5/24		1.61	171	154	126	111	15	28	10	13	137	113
5/25	6-1	1.77	168	159	98	37	61	61	11	18	378	164*
5/26		1.54	156	99	73	63	10	26	16	21	141	
5/27		1.64	158	133	109	101	8	24	12	15	144	
5/28		1.67	156	139	112	102	10	27	12	15	148	
5/29		1.52	150	132	106	93	13	26	12	14	133	93
5/30	6-2	1.82	159	142	112	62	50	30	13	16	254	232
5/31	6-3	1.57	156	130	97	40	57	33	12	16	386	258
6/1	6-4	1.67	150	126	97	38	59	29	13	17	303	266
6/2	6-5	1.69	158	131	98	50	48	33	13	17	166	236
6/3		1.77	166	133	112	97	15	21	13	16	171	104
6/4		1.79	169	155	127	114	13	28	11	14	125	90
6/5	6-6	1.79	155	176	99	86	13	77	10	18	70	99
6/6		1.76	150	132	104	91	13	28	13	17	91	
6/7		1.65	151	135	108	96	12	27	12	15	130	

*Total extra phenol calculated as mg. of p-monobromophenol

CHANGES IN CERTAIN URINARY CONSTITUENTS ON THE EXPERIMENTAL DAYS FOR TABLE XXXVI.

Experiment No.	Glucuronic acid		Extra Ethereal SO4-S		Extra phenol	
	mg.	per cent +	mg.	per cent+	mg.	per cent+
6-1	246	21	49 34**	23 16**	164*	15
6-2	122	5.3	38	9.1	133	11
6-3	254	11	45	11	159	13
6-4	171	7.4	47	11	167	13
6-5			36	8.8	137	11
6-6			50**	58**		

+Per cent of compound fed excreted as....
*Total extra phenol calculated as bromophenol.
**Extra organic S.

KEY TO TABLE XXXVI

Experiment No.	Compound fed and notes
6-1	1.04 gm. of monobromobenzene. A test for bromide ion on the experimental urine was negative.
6-2	1.02 gm. of benzene.
6-3	1.01 gm. " "
6-4	1.02 gm. " "
6-5	1.01 gm. " "
6-6	0.34 gm. of benzyl chloride

Average Control Day Values

By averaging the values found on the different control days, the following average figures were obtained:

Organic S	Ethereal SO_4-S	Glucuronic acid	Phenol
mg.	mg.	mg.	mg.
27	12	132	99

TABLE XXXVII

THE EFFECT OF THE ORAL ADMINISTRATION OF p-MONOBROMOPHENOL TO THE RABBIT
(EXPERIMENT 6-8(6-93B))

Rabbit 6, 3.2 kg. Diet; chow, 75 gm.

Date	Nitrogen	Creatinine	Sulfur					Glucuronic acid	Phenol	N	N
			Total	Total SO_4	Inorganic SO_4	Ethereal SO_4	Organic			Total S ratio	Total SO_4-S ratio
1943	gm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.		
7/18	1.63	151	132	104	90	14	28		100	12	16
7/19	1.58	145	116	93	80	13	23	77***	104	14	17
7/20**	1.48	172	88	73	15	58	15	665	576*	17	20
7/21	1.37	160	74	60	42	18	14	100	125	18	23
7/22	1.26	183	65	54	34	20	11		67	19	25
7/23	1.07	145	54	43	29	14	11		48	20	

*This value is extra total phenol calculated as mg. of p-monobromophenol.

**Administered 1-10 gm. of p-monobromophenol. On this day the animal ate only 50 gm. of the chow and nothing on the succeeding days. The extra glucuronic acid, ethereal sulfate sulfur and the bromophenol excreted in the urine on the experimental day were 588, 45, and 576 mg., respectively, and were equivalent to 52, 22, and 52 per cent, respectively, of the p-monobromophenol fed. The experimental urine gave a negative sulfosalicylic acid test for protein.

***This value is the analysis of a mixture of equal parts of urine hydrolysates of 7/18 and 7/19.

TABLE XXXVIII

THE EFFECT OF THE ORAL ADMINISTRATION OF P-MONOBROMOPHENOL TO THE RABBIT. (EXPERIMENTS 2A-1, 2A-2, 2A-3, and 2A-4 (7-115E))

For an explanation of this table see the key on the next page.

Rabbit 2A, 2.2 kg. Diet; cabbage, 100 gm., oats, 45 gm.

Date	Experiment No.	Nitrogen	Creatinine	Sulfur					N / Total S ratio	N / Total SO_4-S ratio	Glucuronic acid	Phenol
				Total	Total SO_4	Inorganic SO_4	Ethereal SO_4	Organic				
1943		gm.	mg.	mg.	mg.	mg.	mg.	mg.			mg.	mg.
8/10		0.878	132	96	78	73	5	18	9.2	11		20
8/11		0.759	112	86	72	66	6	14	8.8	11	53*	18
8/12	2A-1	0.737	120	81	64	42	22	17	9.1	11	266	342**
8/13		0.718	114	82	65	60	5	17	8.8	11	73	33
8/14		0.711	105	73	58	54	4	15	9.8	12		22
8/15		0.705	109	74	57	55	2	17	9.6	12	96*	26
8/16	2A-2	0.724	98	73	55	35	20	18	9.9	13	330	118**
8/17	2A-3	0.810	115	95	71	38	33	24	8.5	11	369	189**
8/18	2A-4	0.787	103	80	61	37	24	19	9.8	13	387	116**
8/19		0.753	110	77	61	55	6	16	9.8	12	108	
8/20		0.667	95	70	49	42	7	21	9.5	14		

*These values (averages for 8/10 and for 8/14 and 8/15 respectively) were obtained by analyses of a mixture of equal parts of the urine hydrolysates of 8/10 and 8/11 and of 8/14 and 8/15 respectively.

**Total extra phenol calculated as p-monobromophenol. No test for bromide ion was obtained with the experimental urine.

CHANGES IN CERTAIN URINARY CONSTITUENTS ON THE EXPERIMENTAL DAYS FOR TABLE XXXVIII.

Experiment No.	Extra glucuronic acid		Extra Ethereal SO_4-S		Bromophenol*	
	mg.	per cent**	mg.	per cent**	mg.	per cent**
2A-1°	213	52	17	23	342	84
2A-2°°	234	57	16	21	118	29
2A-3	273	67	29	39	189	47
2A-4	291	71	20	27	116	29

*Total extra phenol calculated as p-monobromophenol.
**Per cent of the p-monobromophenol excreted as.....
°Single dose.
°°Repeated dose.

KEY TO TABLE XXXVIII.

Experiment No.	Amount of p-monobromophenol fed mg.
2A-1	407
2A-2	404
2A-3	400
2A-4	403

CHANGES IN CERTAIN URINARY CONSTITUENTS ON THE EXPERIMENTAL DAYS FOR TABLE XXXVIII.

Experiment No.	Extra glucuronic acid		Extra Ethereal SO_4-S		Bromophenol*	
	mg.	per cent**	mg.	per cent**	mg.	per cent**
2A-1°	213	52	17	23	342	84
2A-2°°	234	57	16	21	118	29
2A-3	273	67	29	39	189	47
2A-4	291	71	20	27	116	29

*Total extra phenol calculated as p-monobromophenol.
**Per cent of the p-monobromophenol excreted as.....
°Single dose.
°°Repeated dose.

KEY TO TABLE XXXVIII.

Experiment No.	Amount of p-monobromophenol fed mg.
2A-1	407
2A-2	404
2A-3	400
2A-4	403

THE METABOLISM OF MONOBROMOBENZENE, BENZENE, BENZYL CHLORIDE AND RELATED COMPOUNDS IN THE RABBIT

by Robert F. Witter

1. The metabolism of monobromobenzene, benzene, benzyl chloride, S-benzylcysteine, S-phenylcysteine, p-monobromophenol, and cinnamyl chloride in the rabbit has been investigated by following the changes in the distribution of urinary sulfur and the excretions of total phenols and total glucuronic acid, and by attempts to isolate the corresponding mercapturic acids.

2. A procedure in which the mercapturic acid was isolated by means of ether extraction of the acid urine with or without previous treatment with basic lead acetate is described.

3. In confirmation of earlier reports, from 8 to 24 per cent of the benzyl chloride (0.3 to 0.4 gm. fed) or from 8 to 18 per cent of the S-benzylcysteine (0.75 gm. fed) administered was isolated from the urine as a mercapturic acid.

4. The oral administration of either benzyl chloride (0.25 to 0.35 gm.) or S-benzylcysteine (0.5 to 0.75 gm.) resulted in an increased excretion of organic sulfur corresponding to from 40 to 58 per cent or from 76 to 93 per cent, respectively, of the amount fed. However, the feeding of either compound did not alter the excretion of ethereal sulfates, total phenols, or glucuronic acid. It was concluded that S-benzylcysteine may be an intermediate in the conversion of benzyl chloride to benzylmercapturic acid. Evidence has been presented that the rabbit utilizes the cystine or methionine of the diet in preference to the sulfur-containing amino acids of the tissues for the synthesis of benzylmercapturic acid from

benzyl chloride.

5. The administration of either monobromobenzene (1.0 gm) or p-monobromophenol (0.4 gm.) was followed by increases in the excretion of glucuronic acid of from 20 to 25 per cent or from 50 to 70 per cent, respectively, and in the excretion of total phenols (calculated as p-monobromophenol and estimated by means of the modified Millon reaction) of 14 per cent and from 29 to 84 per cent, respectively, of the compound fed. The experiments have demonstrated the quantitative importance of conjugation with glucuronic acid in the metabolism of these two compounds. The changes observed in the distribution of urinary sulfur after the administration of either monobromobenzene or p-monobromophenol were similar to those which have been previously reported in the literature.

6. Benzene, when fed in doses of 1.0 gm., appeared to be converted in part to phenols which were excreted in conjugation with glucuronic and sulfuric acids. The increases in total phenols, as measured by the modified Millon reaction, and in ethereal sulfates were each equivalent to about 10 per cent, and the increases in glucuronic acid to from 0 to 10 per cent of the benzene that was fed. No significant change was observed in the excretion of organic sulfur, nor could any phenylmercapturic acid be isolated from the urine of rabbits to which benzene was fed; hence, conversion to a mercapturic acid is probably not an important pathway in the metabolism of this hydrocarbon in this species.