4, and 6 atmospheres (absolute) has been found directly proportional to the nitrogen pressure. These results agree with those of Van Slyke, Dillon, and Margaria (1934) who found that nitrogen was dissolved in blood and hemoglobin solutions under nitrogen pressures, varying from atmospheric downward, in accordance with Henry's law.

SUMMARY

The solubility coefficient of nitrogen in whole blood of normal dogs, equilibrated at atmospheric pressure, was found to vary from 0.0138 to 0.0148, and in ox bloods from 0.0135 to 0.0140.

The amount of nitrogen dissolved by whole blood under nitrogen pressures varying from 1 to 6 atmospheres (absolute) has been found directly proportional to the nitrogen pressure, according to Henry's law.

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THE DETERMINATION OF *p*-BROMOPHENYLMERCAPTURIC ACID IN THE URINE OF THE DOG

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The extent of the synthesis of mercapturic acids in animals is usually estimated by studying the partition of urinary sulfur or by isolating mercapturic acids from the urine of animals which were fed halogenated benzenes. The former procedure is based on the assumption that the rise in the output of neutral sulfur in the urine of animals on the day following the administration of benzene derivatives is due entirely to the excreted mercapturic acids. This assumption lacks experimental justification. The isolation of mercapturic acid, when used as a sole criterion of its presence in the urine, has led to contradictory interpretations of the results obtained, since an isolation procedure such as that of Baumann and Schmitz (1), most commonly used, is not only far from being quantitative, but also fails to detect small amounts of mercapturic acid in the urine (*cf.* 2–5). The need for a quantitative method for estimating mercapturic acids in the urine has been emphasized on several occasions (6). An attempt to devise such a method was made by McGuinn (7), but no quantitative procedure was worked out.

We desired a comparatively simple, reasonably accurate method, requiring small volumes of urine, in order that the determination of urinary sulfur partitions, mercapturic acid, and isolation of the mercapturic acid could be made on the same sample of urine. The method presented here is believed to fulfil these requirements and offer a means of comparison between the rise in the output of neutral sulfur and the amount of mercapturic acid present in the urine after the administration of bromobenzene.

Reagents

1. Iodine in potassium iodide (0.01 N). 1.2692 gm. of resub-

limed iodine and 2 gm. of potassium iodide in 1000 cc. of distilled water. The solution is standardized against 0.02 N sodium thiosulfate.

2. Sodium thiosulfate (0.02 N). Standardized by the use of either potassium dichromate or potassium iodate.
3. Sodium hydroxide (2.5 N).
4. Sulfuric acid (2.5 N).
5. Zinc sulfate (10 per cent).
6. Sodium hydroxide (0.5 N).
7. Starch indicator (2 per cent solution of soluble starch in a saturated solution of sodium chloride).
8. Mercuric chloride (5 per cent).
9. Ethyl alcohol (95 per cent).
10. Ethyl ether.

Removal of Interfering Substances from Urine—The 24 hour sample of dog or pup urine is filtered and made up to 500 cc. with distilled water. To 50 cc. of the diluted urine, 10 cc. of 10 per cent $ZnSO_4$ are added with continuous shaking, followed by 10 cc. of 0.5 N NaOH, added dropwise during shaking. The mixture is allowed to stand for a few minutes and then filtered through a dry filter.[1]

Determination of the Mercapturic Acid by Use of $HgCl_2$—25 cc. of the clear filtrate are transferred to a 250 cc. Erlenmeyer flask. 4 cc. of 2.5 N NaOH are added, the flask is covered with a small watch-glass, and placed on a hot-plate at "low" heat for 25 to 30 minutes. After this time, the flask and its contents are thoroughly cooled, first under the tap and then in an ice bath, and 10 cc. of 95 per cent alcohol are added and the liquids mixed. To the mixture, 5.6 cc. of 2.5 N H_2SO_4 are added, followed, after the flask has been whirled, by 1 to 2 cc. of 5 per cent $HgCl_2$. The precipitate, which forms at once, is immediately filtered through a weighed Gooch crucible, washed with water, 95 per cent alcohol, and finally with ether, dried *in vacuo*, and weighed.

[1] Highly pigmented urines, collected from dogs fed mixed and high protein diets, show rather high absorption of iodine. In this case, it is advisable to increase the amounts of $ZnSO_4$-NaOH used for the removal of interfering substances. The proportions given here were used by us on our dogs. It is probable that individual variations in dogs would demand an increase in the amounts of $ZnSO_4$ and NaOH to remove most of the interfering substances. It is essential that the amounts of $ZnSO_4$ and NaOH used be such that the filtrates of normal urines show the same iodine blank before and after the alkaline hydrolysis of the filtrates. In any case, the amount of 10 per cent $ZnSO_4$ added to the urine must be exactly the same as that of 0.5 N NaOH. Of course, proper corrections for the increased dilutions must also be applied in the final calculation.

Calculation—1 gm. of the mercury complex of *p*-bromophenylmercaptan is equivalent to 1.1 gm. of *p*-bromophenylmercapturic acid. If 25 cc. of the $ZnSO_4$ = NaOH filtrate of the urine, prepared as described above, are used, the calculation is as follows: $a \times (500/17.86) \times 1.1$ = *p*-bromophenylmercapturic acid in gm. per 24 hour sample of urine, where a is the weight of $Hg(SC_6H_4Br)_2$ obtained from 25 cc. of $ZnSO_4$-NaOH filtrate.

Determination of Mercapturic Acid by Use of Iodine

Blank Titration—10 cc. of the $ZnSO_4$-NaOH filtrate are placed in a 250 cc. Erlenmeyer flask and cooled in an ice-salt bath; 10 cc. of 95 per cent alcohol are added, followed by 15 cc. of distilled water and 1.6 cc. of 2.5 N H_2SO_4 and 10 drops of starch solution. The mixture is cooled in an ice-salt bath, then titrated directly with a standard iodine solution added from a microburette until the blue color persists for at least 30 seconds. If desired, a definite volume of iodine solution may be added and the excess iodine titrated with standard thiosulfate solution. Either procedure was found satisfactory, yielding identical values. If direct titration with iodine is preferred, the standard solution must be checked from time to time by titration with standard thiosulfate and proper corrections applied, if necessary. As 0.02 N thiosulfate solution does not keep well, this solution was made up daily before use from 0.1 N thiosulfate by proper dilution.

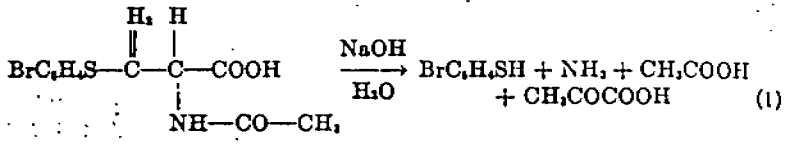
Final Titration—10 cc. of the $ZnSO_4$-NaOH filtrate are placed in a 250 cc. Erlenmeyer flask, 4 cc. of 2.5 N NaOH and 15 cc. of distilled water are added, and the flask is covered with a watch-glass and placed on a hot-plate, at "low" heat, for 25 to 30 minutes. The flask is then thoroughly cooled under the tap and then in an ice-salt bath. 10 cc. of 95 per cent alcohol are now added, followed by 5.6 cc. of 2.5 N H_2SO_4 and 10 drops of starch solution. The contents are kept in an ice-salt bath throughout the manipulation. The mixture is then titrated by either of the procedures described in the blank determination.

Calculation—1 cc. of 0.01 N iodine solution is equivalent to 3.18

mg. of *p*-bromophenylmercapturic acid. If 10 cc. of the $ZnSO_4$-NaOH filtrate, prepared as described above, are used, the calculation is as follows: $(a - b) \times (500/7.14) \times 3.18 = p$-bromophenylmercapturic acid in mg. per 24 hour sample of urine, where a represents cc. of 0.01 N iodine used in the final titration and b is the cc. of 0.01 N iodine used in the blank titration.

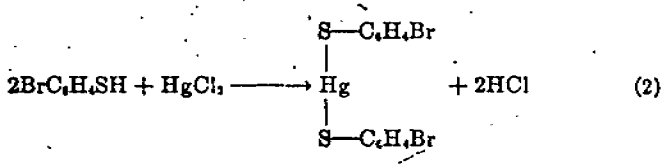
DISCUSSION

The determination of *p*-bromophenylmercapturic acid by the use of $HgCl_2$ is based on the following reactions:



$$BrC_6H_4S{-}C(H_2){=}C(H){-}COOH \xrightarrow[H_2O]{NaOH} BrC_6H_4SH + NH_3 + CH_3COOH + CH_3COCOOH \quad (1)$$

$$\text{NH—CO—CH}_3$$



$$2BrC_6H_4SH + HgCl_2 \longrightarrow Hg(S{-}C_6H_4Br)_2 + 2HCl \quad (2)$$

Reaction 1 was first described by Baumann (8). Reaction 2 was investigated in the following experiment performed on several occasions, yielding almost identical results. 0.10 gm. of *p*-bromophenylmercapturic acid, prepared from dog urine, was dissolved in 100 cc. of distilled water and 16 cc. of 2.5 N NaOH, and heated on a hot-plate at "low" heat for 25 to 30 minutes. The solution was then cooled in an ice-salt bath, and 40 cc. of 95 per cent alcohol were added, followed by 22.4 cc. of 2.5 N H_2SO_4. The flask was whirled, and 3 cc. of 5 per cent $HgCl_2$ were added. The precipitate was centrifuged off, washed successively with water, 95 per cent alcohol, and ether, and finally dried *in vacuo*. The yield was 0.110 gm., or 100 per cent of the theoretical (calculated on the basis of Reactions 1 and 2). Analysis of the compound gave 35 per cent Hg and 11.3 per cent S. Calculated for $Hg(S{-}C_6H_4Br)_2$, 34.7 per cent Hg and 11.15 per cent S. The substance was analyzed for Hg by dissolving the mercury compound in fuming nitric acid under a reflux with gentle heat, evaporating the solution nearly to dryness on a water bath, followed by the addition of water, and precipitation of the Hg as iodate as described by Spacu and Spacu (9). The mercury complex obtained by the precipitation of *p*-bromophenylmercaptan (synthesized according to Hübner and Alsberg (10)) with $HgCl_2$ from acidified alcoholic solution gave nearly identical analytical results; *i.e.*, 34.3 per cent Hg and 11.15 per cent S. Thus it seems that the Reaction 2 is essentially correct.

Titration of the *p*-bromophenylmercaptan by iodine was suggested to us by the work of Klason and Carlson (11), who successfully determined *p*-thiocresol and *p*-thionaphthol by iodine titration of alcoholic solutions of these mercaptans, on the basis of Reaction 3.

$$2R{-}SH + I_2 \rightarrow R{-}S{-}S{-}R + 2HI \quad (3)$$

When precipitation with $HgCl_2$ and iodine titration were applied to dog urine, it was found that the urines gave small but consistent precipitates with $HgCl_2$ in acid medium and iodine absorption by normal urines was also rather high. The absorption of iodine by normal urines was previously observed by Fürth (12) and Lewis (13) and their coworkers. The use of charcoal for the removal of the interfering reducing substances, as suggested by Virtue and Lewis (13), was found to be unsuitable, since *p*-bromophenylmercapturic acid, similar to other mercapturic acids (14), is adsorbed by charcoal. Since $HgCl_2$ reacts with creatinine, uric acid, pyruvic acid, SH— compounds, etc., by combining with them or by being reduced, the use of $ZnSO_4$-NaOH, as used by Somogyi (15) for the removal of interfering reducing substances for glucose determinations in blood, seemed promising. The use of $ZnSO_4$-NaOH proved to be satisfactory for our purpose. $HgCl_2$ produced no precipitate with the acidified $ZnSO_4$-NaOH filtrate of normal dog urine within the time allowed to carry out the filtration and washing of the mercury precipitate, as described in the foregoing procedure. Faint turbidity, however, appears on prolonged standing, even in $ZnSO_4$-NaOH filtrates, when $HgCl_2$ is added as directed in the procedure. The interference with the determination by this extraneous matter which appears on prolonged standing is, however, improbable, since the determination is usually completed before any turbidity due to interfering matter can possibly develop. It seems unlikely that the turbidity which

develops on prolonged standing can be due to a slower secondary reaction between *p*-bromophenylmercaptan and $HgCl_2$.

Absorption of iodine by the acidified $ZnSO_4$-NaOH filtrate is reduced to one-third or less of the value obtained with normal acidified urines. We were unable to eliminate completely all the reducing substances which titrate with iodine. This necessitated making a blank determination on each $ZnSO_4$-NaOH filtrate before subjecting it to alkaline hydrolysis, and subtracting the value found from the titer obtained after the alkaline hydrolysis. Blanks on $ZnSO_4$-NaOH filtrates of normal urines containing no mercapturic acid were found to be the same before or after alkaline hydrolysis.[1]

As has been pointed out by Lucas and King (16) and Virtue and Lewis (13), in the iodine titration of cysteine, it is important to keep the temperature of the reacting mixture low and to use a rather high concentration of acid. They suggest an approximately 2 per cent acid concentration. In our method, the concentration of the acid is about 1.0 per cent. Lucas and King (16) have pointed out that in the case of aromatic mercaptans, such as thiophenol or thiocresol, the iodine absorption was constant between pH 0 and 7. We have also found that exact control of the acidity is not essential for the accuracy of the method, using either procedure. Although Lucas and King (16) have found that aromatic mercaptans can be safely titrated at room temperature, we preferred to carry out all determinations at 0°.

The procedure with $HgCl_2$ yields slightly higher results than those given by the iodine titration method. The consistency of the two procedures, however, in spite of these differences, serves as a satisfactory check on the procedure for the determination of mercapturic acid as a whole. As will be discussed in greater detail in our next report, under certain dietary conditions the rise in the output of neutral sulfur of the urine after feeding bromobenzene corresponds almost exactly to the output of mercapturic acid as determined by either of the procedures outlined above. Such a relationship, however, was not always found to hold true, especially in those cases where *l*-cystine, cysteine, *dl*-methionine, and taurine were administered with the bromobenzene over a period of several days. It was found that the rise in the output of neutral sulfur of the urine, under these conditions, was invariably con-

TABLE I

Recovery of p-Bromophenylmercapturic Acid from Water and Urine

Medium	Mercapturic acid added	0.01 *N* I_2 absorbed			$Hg(SC_6H_3Br)_2$	Mercapturic acid found by methods		Recovery
		Blank (*b*)	Final (*a*)	(*a* − *b*)		I_2	$HgCl_2$	
	mg.	cc.	cc.	cc.	mg.	mg.	mg.	per cent
Water.....	10.0				8.7		9.6	96
"	10.0				9.0		9.9	99
"	6.0				5.5		6.1	101
"	10.3				10.1		11.1	107
"	12.0	0	3.64	3.64		11.60		97
"	10.3	0	3.13	3.13		9.96		97
"	10.3	0	3.26	3.26		10.37		101
"	12.0	0	3.71	3.71		11.80		98
Pup urine...	10.0				8.9		9.8	98
" " ...	10.0				9.6		10.6	106
" " ...	10.0	0.70	3.88	3.18		10.10		101
" " ...	10.0	0.70	3.84	3.14		10.00		100
Dog " ...	10.0				9.4		10.3	103
" " ...	10.0				9.3		10.2	102
" " ...	10.0				9.1		10.0	100
" " ...	10.0				9.6		10.6	106
" " ...	4.1	0.60	1.83	1.23		3.91		95
" " ...	4.1	0.60	1.93	1.33		4.20		102
" " ...	4.1	0.60	1.84	1.24		3.97		97
" " ...	4.1	0.60	1.89	1.29		4.11		100
" " ...	10.0*				9.4		10.3	103
" " ...	10.0†				9.5		10.4	104
" " ...	10.0‡				8.8		9.7	97
" " ...	10.0§				9.3		10.2	102
" " ...	10.0*	0.63	3.71	3.08		9.80		98
" " ...	10.0†	0.60	3.71	3.11		9.90		99
" " ...	10.0‡	0.60	3.77	3.17		10.10		101
" " ...	10.0§	0.61	3.69	3.08		9.80		98

The recoveries were made on 25 cc. of water and $ZnSO_4$-NaOH filtrate of urine with the $HgCl_2$ procedure, and on 10 cc. of water and $ZnSO_4$-NaOH filtrate of urine with the I_2 procedure. The mercapturic acid was dissolved in urine before the addition of $ZnSO_4$-NaOH.

* Urine collected after repeatedly feeding *l*-cystine (1.0 gm. per day).

† Urine collected after repeatedly feeding cysteine hydrochloride (1.54 gm. per day).

‡ Urine collected after repeatedly feeding *dl*-methionine (1.26 gm. per day).

§ Urine collected after repeatedly feeding taurine (1.08 gm. per day).

TABLE I—*Concluded*

Medium	Mercapturic acid added	0.01 N I_2 absorbed			$Hg(SC_6H_4Br)_2$	Mercapturic acid found by methods		Recovery
		Blank (b)	Final (a)	(a − b)		I_2	$HgCl_2$	
	mg.	*cc.*	*cc.*	*cc.*	*mg.*	*mg.*	*mg.*	*per cent*
Pup urine...	10.0*				9.2		10.1	101
" " ...	10.0†				9.1		10.0	100
" " ...	10.0‡				9.4		10.3	103
" " ...	10.0§				9.6		10.6	106
" " ...	10.0*	0.73	3.81	3.08		9.80		98
" " ...	10.0†	0.71	3.85	3.14		10.00		100
" " ...	10.0‡	0.70	3.87	3.17		10.10		101
" " ...	10.0§	0.70	3.75	3.05		9.70		97
" " ...	‖	39.00	224.2	185.2	540	589	594	
" " ...	‖	40.00	216.1	176.1	510	560	561	
Dog " ...	‖	36.00	259.9	223.9	724	712	797	
" " ...	‖	36.60	269.3	232.7	706	740	777	

‖ Urine collected after a single feeding of 1.0 gm. of bromobenzene. These results are expressed per 24 hour sample of urine.

siderably higher than the output of mercapturic acid sulfur. *l*-Cystine, cysteine, taurine, and *dl*-methionine, when *fed alone repeatedly* to the same dog, increased the output of neutral sulfur of the urine. The recovery of *p*-bromophenylmercapturic acid added to such urines, as is shown in Table I, was, however, satisfactory, thus indicating that the presence of unchanged taurine, or of partially oxidized *l*-cystine, cysteine, or methionine does not interfere with the determination of *p*-bromophenylmercapturic acid in the urine.

Table I summarizes the experiments in which the recovery of *p*-bromophenylmercapturic acid from water and the urines of several dogs maintained on diets of various sulfur contents was determined by both of the procedures presented here. The recoveries seem satisfactory enough to warrant the application of the method for metabolic studies on dogs. Several determinations of *p*-bromophenylmercapturic acid in the urine of dogs which were fed bromobenzene are illustrated in Table I.

Pending the application of the procedure to urines of other animals, we suggest that the method as presented here be reserved for the urines of dogs and pups only. Inasmuch as other halogen-mercapturic acids and *l*-α-naphthalenemercapturic acid are essentially similar to *p*-bromophenylmercapturic acid, in so far as they all yield aromatic mercaptans on alkaline hydrolysis, it seems probable that the method as outlined here is also applicable for the determination of these mercapturic acids in dog urine. However, further work is necessary in order to check such a possibility.

We realize that the reactions on which our method is based are not specific in nature and therefore limit the value of the method. However, under the conditions under which the extent of the synthesis of mercapturic acid in dogs is generally studied, the method outlined seems more reliable in its nature than quantitative interpretation of the fluctuation of the neutral sulfur in the urine after feeding bromobenzene under varying dietary conditions.

SUMMARY

A method for the determination of *p*-bromophenylmercapturic acid in dog urine by two procedures is presented.

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