Analysis of PHENOLIC COMPOUNDS of Interest in Metabolism

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I. INTRODUCTION

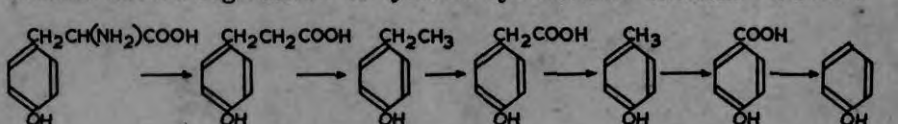
The total number of phenolic compounds which have been identified as constituents or metabolites of living organisms is very large. In an article of this scope a comprehensive survey of these compounds would scarcely be possible. Such a survey, indeed, would be of only partial interest to the average reader whose studies tend to be confined either to the animal or to the plant kingdom. The authors have, therefore, restricted their field to methods of analysis of the simpler phenolic compounds which are involved in the metabolic processes of the higher animals and which are ex-

creted in urine. In most cases methods used for the detection and estimation of phenols are not highly specific and are, in principle, applicable to phenolic compounds in general; only the preliminary preparation of the material in which they are contained requires modification. Only those methods with which the authors have had personal experience have been described in detail. The fact that these methods have been chosen must not be taken as implying that methods not so selected are necessarily inferior. The methods described were originally chosen for their convenience for the authors' purpose and have proved satisfactory in their hands.

Phenols may be formed in the animal body either as a result of normal metabolic processes or as a result of some abnormal treatment of the animal which may cause either some quantitative interference in the normal metabolism of the animal or the appearance of some new phenolic metabolite. The commonest abnormal treatment of the animal in this sense is by the administration of drugs or foreign organic compounds. These compounds are often modified in various ways in their passage through the body. Since the formation of phenolic metabolites and their conjugation with glucuronic and sulfuric acids are common reactions which foreign compounds undergo, the analysis of phenols in urine has, perforce, been extensively used by workers in the field of what was formerly called "detoxication" but is now more fittingly described as "the metabolism of foreign compounds" or "conjugation reactions."

1. Phenols of Normal Occurrence

The naturally occurring phenol which has most metabolic significance is tyrosine, an amino acid widely distributed in proteins. Many of the simpler phenols which are normally found in urine are believed to be derived from absorption of phenols formed by the bacterial decomposition of tyrosine and other aromatic amino acids in unabsorbed protein in the intestine. Between 1879 and 1886 Baumann (7,8) postulated the accompanying scheme for the degradation of tyrosine by intestinal bacteria. Full evi-



dence for intermediate steps in this degradation was not provided at the time but since then all these compounds have been identified by various workers as products of the bacterial degradation of either tyrosine or phenylalanine. The bulk of the *p*-cresol found in most urines is believed to be formed in the gut in this way from tyrosine or phenylalanine. Indoxyl is

believed to originate in a similar manner from tryptophan. Early workers claimed that phenol, *o*- and *p*-cresols, catechol, *p*-hydroxybenzoic acid, *p*-hydroxyphenylacetic acid, *p*-hydroxyphenylpyruvic acid, and indoxyl were constituents of the urines of various species, although not all these observations have been confirmed by later investigators. It is, for example, very doubtful whether phenol itself is excreted by the normal animal. One early worker, Mooser (64), was of the opinion that *p*-cresol was the only phenol present in urines. Some phenols other than those mentioned above are now known to be present in urine, e. g., *m*-hydroxybenzoic acid. As will be seen later the presence of some phenols in urine depends upon the phenols or phenol precursors which are present in the diet. In the pathological condition alkaptonuria another phenol, homogentisic (2,5-dihydroxyphenylacetic) acid, is excreted; this is believed to be the result of abnormal tyrosine metabolism. Tyrosine itself is sometimes excreted in pathological conditions, e. g., in acute yellow atrophy of the liver. Since the liver is the organ mainly concerned in reactions resulting in the formation of phenol and in conjugation reactions, the excretion of phenols is liable to be abnormal in conditions of liver disease.

In recent years interest in the phenols of the tissues and urine has been revived and our knowledge of them has been advanced by the development of modern techniques. In 1941 to 1943 Lederer (56,57), extending earlier work of Walbaum and Rosenthal (92), isolated from the scent glands of the beaver *m*- and *p*-hydroxybenzoic acids, gentisic (2,5-dihydroxybenzoic) acid, and 2-hydroxy-5-methoxybenzoic acid, which were present unconjugated in the free form, and salicylic acid in the form of a conjugate; other phenols found were *p*-ethylphenol, *p*-propylphenol, quinol, *p*-ethoxyphenol, chavicol (*p*-allylphenol), 5-ethyl-2-hydroxyanisole, catechol, 4-methylcatechol, 4-ethylcatechol, betuligenol (1-(*p*-hydroxyphenyl)butan-3-ol), 2,4′-dihydroxydiphenylmethane, 2′,3″-dihydroxydibenz-2-pyrone, 4,4′-dihydroxydiphenyl-2,2′-dicarboxylic acid dilactone, salicylaldehyde, and *p*-hydroxyacetophenone. The presence of many of these phenols in the scent glands could be attributed to phenols or closely related compounds in the bark of trees and other items of the beaver's diet; it is of interest that in this secretion the phenols are mainly unconjugated whereas in urine conjugated phenols predominate. In a later investigation Lederer and Polonsky (57,58) isolated from the urine of a pregnant mare *p*-ethylphenol, *p*-cresol, *o*-, *m*-, and *p*-hydroxybenzoic acids, vanillic acid, *p*-cumaric(*p*-hydroxycinnamic) acid, dihydroferulic (4-hydroxy-3-methoxyphenylpropionic) acid, and ferulic (4-hydroxy-3-methoxycinnamic) acid. These investigations are the only extensive studies which have been reported up to the present time. Grant (49) isolated the ethereal sulfate of *p*-ethylphenol from goat urine. *m*-Hydroxybenzoic acid was isolated by Bielig and Haya-

sida (9) from the urine of rabbits dosed with β-ionone and by Bray, Hybs, James, and Thorpe (18) from the urine of rabbits which had received 2,3,5,6-tetrachloronitrobenzene. In neither case could *m*-hydroxybenzoic acid reasonably be regarded as a metabolite of the compound administered. In 1953, Boyland, Manson, Solomon, and Wiltshire (10) isolated this acid from the urine of normal rabbits as well as from that of rabbits injected with naphthalene.

One of the most useful techniques for the investigation of phenolic constituents of biological material is paper chromatography (see Section II,3) which may be used for identification or estimation. A preliminary investigation (21,26) of normal rabbit urine showed the presence of *m*- and *p*-hydroxybenzoic acids, *p*-hydroxyphenylacetic acid, catechol, 2,3-, 3,4-, and 2,5-dihydroxybenzoic acids, vanillic acid, and *p*-cumaric acid as well as several unidentified substances reacting like phenolic compounds. *p*-Cresol and *p*-ethylphenol are phenols which cannot be easily detected by paper chromatography.

The excretion of phenols in urine, both qualitatively and quantitatively, will depend to a large extent upon the nature of the diet. The amounts of phenols derived from bacterial breakdown of aromatic amino acids in the gut will be related to the amounts of these amino acids in the food and the extent to which they are absorbed. Phenols which are excreted by some animals but not by others, or phenols the excretion of which appears to be a species characteristic, may well be traceable to peculiarities of diet. It is, therefore, important when comparing the findings of different workers that differences in the diets of the subjects of the experiments should be taken into consideration.

Little is known of the nature of the phenols present in the tissues. With the exception of those derived from tyrosine, phenylalanine, and tryptophan, the phenols of animal tissues are probably derived from aromatic precursors present in items of the diet, especially those of plant origin (cf. Lederer (57)), since the benzene ring does not appear to be synthesized by the animal. Phenols in urine are either free or conjugated with sulfuric or glucuronic acid. Since, in general, free phenols are not readily excreted, while ethereal sulfates and glucuronides are (16,17), it is likely that there will be more free than conjugated phenol present in the tissues but more conjugated than free phenol in urine. Various investigators in the past, e. g., Pelkan and Whipple (67), have reported the rapid "fixing" of ingested phenol by the dog, probably by binding to protein. It is possible that some phenols may be present normally bound in tissues in this way.

From the foregoing it can be seen not only that there may be many different phenols in normal urine but also that these phenols may vary in amount depending upon the nature of the diet. This is of considerable im-

portance, as will be seen, when the excretion of abnormal phenols is measured.

2. Phenols Arising from Administration of Foreign Aromatic Compounds

The administration of a foreign aromatic compound to an animal frequently results in the excretion in the urine of phenolic metabolites of the compound even if the administered compound is not itself a phenol. There are several ways in which a phenol may be formed from nonphenolic precursors. Many aromatic compounds are oxidized in the body by the introduction of one or more phenolic hydroxyl groups (hydroxylation); as examples may be quoted the hydroxylation of benzene to phenol, catechol, quinol, and hydroxyquinol (69) and the oxidation of salicylic acid to dihydroxybenzoic acids (25,26). While the introduction of one hydroxyl group may take place to a considerable extent, di- or trihydric phenols are usually formed only in very small amounts. If the administered compound is not of such a nature as to be rapidly excreted it is likely to be hydroxylated to some extent; phenolic compounds may be further hydroxylated, and even those aromatic acids which are readily excreted may be hydroxylated to a small extent (see Thorpe (87), Smith (84), and Young (94)). Hydroxylation is not, however, the only metabolic process by which phenols can be formed; they may be formed by hydrolysis of precursors such as esters, glycosides, and ethers. The first two can usually be readily hydrolyzed (65,71) but the latter appear to be hydrolyzed to a significant extent only when other substituents are present in the ring (19). The kinetics of the formation of phenols from precursors has been studied by Bray, Humphris, Thorpe, White, and Wood (15).

It will be appreciated that a phenol derived from an administered compound may be the same as one which is normally excreted and that in such a case it is important to have accurate knowledge of the extent of the normal excretion of the phenol by the animal on the given diet before the extent of the formation of the phenol from the administered compound can be assessed.

3. Form in Which Phenols Are Excreted

Before discussing the methods of analysis of phenolic compounds, it is pertinent to consider the forms in which these compounds are actually excreted. The proportion of a phenol excreted in the free state is usually small since the healthy animal can readily conjugate phenols with glucuronic and sulfuric acids. (In cases of liver dysfunction the facility of conjugation may be impaired.) The relative amounts of the two conjugates formed depend upon the nature of the phenol. The conditions governing

the formation of glucuronides and ethereal sulfates have been studied by the authors and co-workers (16,17,28). Phenylglucuronides are usually somewhat resistant to hydrolysis whereas ethereal sulfates are rapidly hydrolyzed by low concentrations of acid. The relative amounts of a phenol conjugated with glucuronic and sulfuric acids can, in fact, be determined on this basis (Section III,1). In the quantitative analysis of phenols in urine it is, therefore, necessary to make certain that the method is such as to ensure the complete hydrolysis of the conjugated phenols, and it is often of value for assessment of the results to determine the free as well as the total phenols (Section IV,1,A(1)), since this can be done with little additional expenditure of time and labor.

It is often practicable, particularly where there is no specific method for the phenol, to measure the excretion of conjugated phenol by determination of the increase in excretion of the conjugating acids. Alternatively, the estimation of these conjugating acids may provide a useful check upon the values obtained by determination of phenol liberated after hydrolysis. A significant discrepancy between the results obtained by the two methods may even reveal the existence of a hitherto unsuspected metabolite. To take a hypothetical example, hydroxylation of an administered compound might have produced two isomeric phenols, only one of which was detected by isolation. If the identified phenol gave a relatively feeble color with the reagent used for colorimetric estimation compared with that given by the unidentified phenol, the value obtained by direct estimation of conjugated phenol would be much greater than that obtained by estimation of the conjugating acids. Such a result would provide a strong reason for a more searching examination of the nature of the metabolites formed.

II. QUALITATIVE EXAMINATION

1. Introduction

Before a reliable quantitative assessment of the excretion of phenols formed as metabolites can be obtained, it is essential that the nature of these phenols be discovered. This ultimately should involve the isolation and characterization of the compounds or derivatives by the standard methods of organic chemistry. The first step toward the detection and identification of phenols in urine is the separation of the phenols from the bulk of the accompanying material. Although some qualitative tests may give apparently satisfactory results when applied directly to urine, it is advisable to extract the phenols from the urine if maximum sensitivity and minimum interference are required. Since phenols are excreted mainly in conjugated form it is usually necessary to hydrolyze these conjugates be-

fore proceeding to identify the phenols. It has already been mentioned that some phenylglucuronides may be somewhat resistant to hydrolysis and require heating with relatively concentrated acid to effect complete hydrolysis. In such circumstances it may be necessary to ascertain whether the hydrolytic process causes decomposition of any phenol, e. g., decarboxylation of a phenolic acid.

The most convenient way of separating phenols from the bulk of the material excreted in urine is steam distillation. Comparatively few compounds, however, are volatile in steam and a more general method which can be applied to almost all simple phenols is to extract the hydrolyzed urine with ether in a continuous extractor for several hours. By adjusting the pH of the urine, it may be possible to separate phenols from phenolic acids; a pH of 7.8 usually serves to prevent extraction of the acids while it permits extraction of other phenols. The phenolic acids can be subsequently extracted if the residual urine is adjusted to pH 1 (cf. Schmidt (80)). Tests may then be applied to the residue left after evaporation of the ether. If the phenols are liable to oxidation by atmospheric oxygen, e. g., aminophenols, the ether should be removed *in vacuo* or in a stream of nitrogen. The ether extract can be fractionated by conventional methods. Chromatographic separation of an ether extract on columns of powdered cellulose often provides a convenient method for the separation of mixtures of phenolic compounds (Section II,4).

In the course of the isolation of phenols from urine it is frequently helpful to use certain qualitative tests as a guide to the progress of a separation. Such tests as those described in Section II,2,A to H are rarely specific and, moreover, are often given by normal constituents of urine, so that the application is usually of greater value as a guide in the later stages of a separation or in the final characterization of a phenol. These tests rarely yield useful or reliable information when applied directly to urine.

Undoubtedly the most rewarding technique for the qualitative examination of phenols is that of paper chromatography (Section II,3). It can be carried out easily, quickly, and without elaborate apparatus and permits identification of the phenols with some measure of certainty if the appropriate reference compounds are available; it can give an indication of the total number of phenols present in a mixture and often a rough estimate of the relative amounts present.

2. Color Reactions

The reactions commonly used for detection of phenols are not specific for particular phenols or even for phenols in general but may serve as a guide in narrowing down the identification. With color reactions shades of color

are difficult to describe precisely and, whenever possible, final interpretation should be by direct comparison with a similar test performed upon an appropriate amount of an authentic sample.

A. FERRIC CHLORIDE TEST

This test is not highly sensitive but ferric chloride has the advantage of forming complexes of different colors, from violet with phenol itself, bluer shades with cresols, deep blue with gentisic acid, and red with *p*-hydroxybenzoic acid, to green with catechols. The colors are discharged by acid. The color with catechols is particularly dependent upon pH and yellow, green, blue, purple, and red colors are given as the pH increases. The test may not be given readily by phenolic compounds which are sparingly soluble in water. For such compounds the addition of ethanol up to about 50% often dissolves sufficient phenol to give a positive reaction. Similar colors are also given by certain β-keto acids, e. g., acetoacetic acid.

Reagent. Ferric chloride, 0.5 *N* in water.

Test. A few drops of reagent are added to 1 ml. of the neutral test solution. The color is usually destroyed on acidification. (Ferric hydroxide separates in alkaline solution.)

B. MILLON'S TEST

This test is given by a number of phenols. In general monohydric phenols tend to give a red color but with di- and trihydric phenols the colors tend to be yellow-orange or brown. With vanillin and vanillic acid a characteristic purple color is obtained which appears to be specific for these two compounds (88).

Reagent. Mercury (30 ml.) is dissolved in nitric acid, sp. gr. 1.42 (570 ml.) and the resulting solution is diluted with two volumes of water.

Test. A drop of reagent is added to 1 ml. of test solution, which is then boiled. The red color may not be seen if an excess of reagent is used, e. g., with tyrosine an excess of reagent gives a yellow color due to nitration. The test should not be used in the presence of inorganic salts (e. g., in urine), which precipitate mercury complexes.

C. FOLIN AND CIOCALTEU TEST

This is a very sensitive test given by a large number of phenols. It depends upon the reduction of compounds containing sexivalent tungsten and molybdenum to colored compounds in which the metals have lower valence. It is thus liable to give colors with nonphenolic reducing substances, e. g., uric acid and thiophenols. Some phenols (e. g., *p*-hydroxybenzoic acid) only give feeble colors.

Reagents. As for quantitative estimation (Section IV,1,A).

Test. About 1.0 ml. of reagent is added to 1 ml. of test solution. Sodium carbonate solution (2.0 ml.) is then added. The blue color may appear on standing in the cold but comes up quickly on warming.

D. INDOPHENOL TEST WITH 2,6-DICHLOROQUINONE CHLOROIMIDE (10,48)

Many phenolic compounds which are unsubstituted in the *para* position combine with 2,6-dibromo- or 2,6-dichloroquinone chloroimide to give a blue indophenol dye. There are, however, a large number of phenols which give only a very feeble or no color with this reagent. The test must be carried out at or about pH 9–10. Oxidizing and reducing substances interfere and some amines may form indamine dyes with this reagent.

Reagents.

(a) The powdered imide or a 1% solution in aldehyde-free ethanol may be used.
(b) Powdered sodium bicarbonate or borate buffer, pH 9.6.

Test. After the addition of a trace of solid 2,6-dichloroquinone chloroimide (or a few drops of the solution) to the test solution, an excess of powdered sodium bicarbonate or an equal volume of borate buffer is added. A blue color usually develops on standing. With nonacidic phenols, the color can usually be extracted with *n*-butanol.

E. *p*-NITROANILINE TEST

This test depends on the coupling of the phenol with a diazonium salt formed from *p*-nitroaniline. Colors of various shades, usually red or orange, are given by most phenols but it should be remembered that the diazonium salt will also couple with bases. The test is usually very sensitive.

Reagents. The reagents for paper chromatography can be used (Section II,3,B(1)).

Test. A few drops of the phenol solution are added to 1 ml. of the diazotized *p*-nitroaniline solution. The sodium carbonate solution is then added. The color usually develops quickly.

Diazotized sulfanilic acid may be used in the same way. The colors are not always the same as with diazotized *p*-nitroaniline.

F. AMMONIACAL SILVER NITRATE TEST

This reagent is reduced with the separation of metallic silver by a number of dihydric and aminophenols. The test is, of course, liable to be given by a number of reducing substances.

Reagent. Ammonia (2 *N*) is added to silver nitrate (0.1 *N*) until the precipitate first formed just redissolves.

Test. Phenol solution is added to the reagent. Metallic silver separates either as a dark brown precipitate or is deposited on the walls of the tube as a mirror. The test usually works on standing at room temperature.

G. INDOPHENOL TEST FOR AMINOPHENOLS

Phenols with an amino group in the *para* position usually form indophenol dyes on treatment with phenol and sodium hypobromite. These dyes are deep blue in alkaline and red in acid solution. The test is more specific with hypobromite than with bleaching powder or calcium hypochlorite (Thorpe, Williams, and Shelswell (89)), which give a color with ammonia.

Reagents.

(a) Sodium hypobromite prepared by adding bromine to 1 *N* sodium carbonate solution until the color is just yellow.

(b) Phenol solution, 5% w/v in water.

Test. A few drops of 5% phenol solution are added to 1 ml. of neutral or faintly acid test solution. On addition of sodium hypobromite a deep blue color appears, becoming more intense on standing. Under these conditions ammonia only gives a very feeble color.

H. BROMINE WATER TEST

Many phenols are readily brominated in aqueous solution by bromine to give sparingly soluble polyhalogenophenols. Thus, phenol and *o*- and *p*-bromophenol yield 2,4,6-tribromophenol, m. p. 94°, and 2,4-dichlorophenol yields 6-bromo-2,4-dichlorophenol, m. p. 68°.

Reagent. Water saturated with bromine.

Test. Bromine water is added to the phenol solution with frequent mixing until no more bromine is taken up.

Great care must be taken over the interpretation of this test. In particular, the isolation of 2,4,6-tribromophenol must not be taken as proof of the presence of phenol. The importance of this is illustrated by the early claim of Baumann and Herter (8a) that phenol was formed as a metabolite of *p*-hydroxybenzoic acid by the dog because after distilling the acidified urine with steam they obtained a distillate from which 2,4,6-tribromophenol was isolated after addition of bromine water. This is not valid proof of the presence of phenol in the urine since when *p*-hydroxybenzoic acid is treated with bromine water decarboxylation occurs and 2,4,6-tribromophenol is formed. (Some other phenolic acids are also decarboxylated in this way.) Subsequent work has, in fact, made it almost certain that the bulk, if not all, of Baumann and Herter's tribromophenol arose from *p*-hydroxybenzoic acid or its conjugates.

3. Paper Chromatography

This technique was first developed by Consden, Gordon, and Martin (31) for the identification of amino acids and peptides in protein hydrolyzates. Since that time it has been extended, with various modifications, to virtually any compounds which can be made to give a color reaction or can otherwise be detected on paper. The original method employed a "descending" technique but the simpler "ascending" method, suggested by Williams and Kirby (93), has much to recommend it and, as far as phenols are concerned, is suitable for the separation of most of the mixtures encountered. The following is a brief description of the ascending procedure used in this laboratory (26) which can be carried out with very simple and inexpensive apparatus.

Solvent Mixtures. A large number of solvent mixtures of varying composition have been used by different workers. Useful mixtures for the separation of phenols are varying proportions of benzene:acetic acid:water (e. g., 2:2:1 by volume), chloroform:acetic acid:water (e. g., 2:1:1), or *n*-butanol:acetic acid:water (e. g., 10:1:9), an increasing proportion of acid tending to increase the R_f. Formic acid can replace acetic acid. Variations in the proportions of these mixtures causes changes in R_f values and so permits adjustment to suit the separation of a particular mixture. Benzene saturated with 98% formic acid often gives good separation. Light petroleum saturated with formic acid is of value for fast-moving compounds and a mixture of *n*-butanol:pyridine:saturated aqueous sodium chloride:ammonia, sp. gr. 0.880 (4:8:5:3 by volume) gives good separation of *o*-, *m*-, and *p*-hydroxybenzoic acids (cf. Evans, Parr, and Evans (37)).

Detecting Reagents. Although the reactions used are the same as those for qualitative tests the colors obtained on paper are not always the same as those obtained in the test tube.

(*1*) *Diazotized p-Nitroaniline.* *p*-Nitroaniline 0.3% in 8% (w/v) hydrochloric acid (25 ml.) is mixed with 5% (w/v) sodium nitrite (1.5 ml.) mixed immediately before spraying. The application is followed by one of sodium carbonate (20% w/v).

(*2*) *Diazotized Sulfanilic Acid.* As above, sulfanilic acid replacing *p*-nitroaniline.

(*3*) *2,6-Dichloroquinone Chloroimide.* A freshly prepared solution (0.1% w/v in ethanol) is sprayed, followed by veronal or phosphate buffer, pH 10.

(*4*) *Ammoniacal Silver Nitrate.* Silver nitrate (25 ml. 0.1 *N*) and ammonia, sp. gr. 0.880 (8 ml.), made up to 50 ml. with water.

(*5*) *Ferric Chloride.* 0.2% (w/v) in water.

(*6*) *Potassium Carbonate.* 20% (w/v) in water.

The diazo reagents have the most general application for phenolic compounds. The quinone reagent tends to be hypersensitive. Ammoniacal silver nitrate is convenient for dihydric and aminophenols, since most monohydric phenols do not reduce this reagent. The ferric chloride reaction is not very sensitive. Potassium carbonate is useful for nitrophenols which give a yellow color with this reagent.

satisfactory for the separation and identification of a large number of phenolic compounds. Exceptions are the fast-moving phenols such as phenol, *p*-ethylphenol, the cresols, and chlorophenols which move with the solvent front in all solvents examined. These can, however, be studied if they are first coupled with diazotized *p*-nitraniline or sulfanilic acid and the azo dye formed is applied to the paper as an alkaline solution (see Hossfeld (51), and Johnson, Stein, and Weiss (53)). A modification of this procedure which has been found useful for *p*-chlorophenol consists in applying the solution of the azo compound to paper which has previously been soaked in 4% aqueous sodium carbonate and dried (51).

The information about solvents suitable for the chromatography of phenols and their R_f values is too extensive to be given here in full, and in any case would not be of great value since a worker wishing to use the methods would have to discover for himself the exact conditions which apply to his particular investigation. Table I records references to a number of papers describing the paper chromatography of phenols which should serve as a guide for finding appropriate solvent mixtures.

Quantitative Estimation. Paper chromatograms prepared as above may be used for rough quantitative estimation of phenols by comparing the size and intensity of spots with those obtained from suitably graded standards run on the same paper, standard solutions of graded strength and the unknown being applied alternately along the starting line. Measurement of the area of spots with a planimeter (39,40) has also given satisfactory results.

4. Separation and Characterization of Phenols

The method by which a given phenol is separated and characterized must obviously depend upon the properties of the particular phenol. Most phenolic substances are sufficiently soluble in ether to permit their extraction in a continuous extractor (e. g., Kutscher-Steudel type). Ether extraction, therefore, usually provides an effective means of separating phenolic material from many of the other constituents of urine. Since the phenols are mainly present in the conjugated state the urine must first be hydrolyzed. The conditions will depend upon the stability of the phenylglucuronide but usually hydrolysis can be achieved by boiling the urine under reflux for 1 hour after addition of an equal volume of 10 N H_2SO_4. An alternative to ether extraction which can be applied for a limited number of phenols is steam distillation. This gives a purer product than ether extraction. The crude phenolic material obtained by either of these methods can be fractionated or purified by conventional methods such as fractional crystallization, differential extraction, or conversion into appropriate derivatives. For mixtures difficult to separate in these ways chromatographic separation on columns of paper powder, hydrocellulose, or Hyflo-Supercel (cf. Hough, Jones, and Wadman (52)) can be used. The solvents used are those found to give satisfactory results on paper strips. The resulting fractions after identification can be grouped and the phenols thus separated identified or estimated in conventional ways.

Apparatus. *Cylinders of paper 33 cm. long are made from sheets of paper* 33 x 28 cm. by stapling so that the edges do not overlap. Whatman No. 4 paper is suitable for most purposes. (Since results may vary somewhat with different grades of paper, the grade used should always be specified when reporting results.) The paper cylinder stands in a petri dish which is placed in a tall cylindrical jar with a ground top or a filtrate jar. The jar is covered with a sheet of plate glass if the top is ground or with a glass food cover if a filtrate jar is used. The petri dish contains the nonaqueous phase of the solvent mixture and the bottom of the jar is covered by the aqueous phase.

Procedure. Spots of the required solution containing 1 to 5 γ of phenol are placed on the starting line about 2.5 cm. from the bottom of the paper cylinder and 3 cm. apart, and are dried. Reference compounds should always be run alongside the unknown on each paper. The cylinder is placed in the petri dish and the jar is covered. A run of 3–5 hours is usually sufficient, although with some slow-moving compounds 15 hours may be necessary, when evaporation at the solvent front takes place. After the run the sheet is unfolded and dried in a convenient drier at about 50° and the positions occupied by the compounds are revealed by spraying with the selected detecting reagent.

Provided that the jars are covered and protected from drafts, there is usually little difficulty in attaining a level solvent front during a run. Temperature changes and changes in composition of the solvent mixtures on exposure in the jars may cause slight changes in R_f values but if reference compounds are run on the paper alongside the unknowns these differences are of little account.

The procedure outlined above with slight modifications of solvents has proved

TABLE I

Examples of Application of Paper Chromatography for Identification of Phenols

Type of phenol	References
Acyl amidophenols	13
Adrenaline derivatives	32
Aminohydroxybenzoic acids	29
Aminohydroxypyridines	24
Aminophenols	13,78
Anthocyanins	4,5
Catechins of tea	11,75,76
Coumarin derivatives	85
Flavonoid compounds	5,46
Hydroxy-4,6-dimethylpyrimidines	20
Hydroxyphenyl ethers	19
Hydroxysulfanilic acids and amides	20
Lignin derivatives	3
Phenolic acids	21,26,29,38,85
Phenols of pine wood	61
Phenols, run as azo dyes	1,51,53
Phenols, various mono and dihydric, acids and amides	5,6,21,26,36,37,74
Nitrophenols	77

There are a large number of reagents which form derivatives with phenols which can be readily crystallized and separated. Those commonly used include *p*-nitrobenzyl bromide, chloroacetic acid, *p*-toluenesulfonyl chloride, phenyl isocyanate, and benzoyl chloride. An extensive list of the melting points of these and other derivatives suitable for the characterization of phenols is given by Johnson, Shennan, and Reed (54) who also describe the methods of preparation of the compounds.

III. QUANTITATIVE EXAMINATION

1. Introduction

As may be seen from the experimental section different phenols give different responses to the various reagents. Consequently, unless the nature of the phenols being estimated is known, the experimental values obtained by the quantitative application of color reactions cannot be correctly interpreted, since it will be uncertain whether a reliable standard for colorimetry has been selected. Similar conditions apply in metabolic studies in which changes in concentrations of phenols are followed; in such cases it must be ascertained whether or not the nature and proportions of the phenols present change during the experiment. Here again, paper chromatography is a most useful tool since rough quantitative estimates can usually be readily made.

Quantitative methods for the estimation of conjugated phenols depend upon the estimation either of phenols themselves or of the conjugating moieties. In methods in which phenol is estimated, "free" or unconjugated phenol may be estimated directly and "total" (i. e., free + conjugated) phenol after hydrolysis. In actual practice it is usually advantageous to extract phenols from the material under examination in order to minimize interference by other substances. Extraction (usually with ether) from solutions at various pH values (cf. Schmidt (80)) gives an opportunity for the fractionation of phenols into type groups, e. g., phenols and phenolic acids. Fractionation of conjugated phenols into those conjugated with sulfuric acid and those conjugated with glucuronic acid can also be achieved by using two degrees of hydrolysis, e. g., mild (in 1 *N* hydrochloric acid for 15 minutes at 100°) for liberating phenols from ethereal sulfates, and strong (in 5.0 *N* sulfuric acid for 1 hour at 100°) for hydrolyzing ether glucuronides (cf. Garton, Robinson, and Williams (47)). It cannot be too strongly emphasized that ether glucuronides are not readily hydrolyzed and for the estimation of total phenols vigorous hydrolysis is necessary. In some methods described in the literature, especially those like that of Volterra (90), in which acidified material such as urine is simulta-

neously hydrolyzed and distilled, the conditions are often not adequate for complete hydrolysis of many glucuronides and low results may be obtained. It is advisable whenever possible to confirm that the conditions of hydrolysis are adequate by control experiments with the pure glucuronide. Failing this it should be ascertained that no further glucuronic acid can be liberated by applying more drastic conditions for the hydrolysis.

2. Direct Estimation of Phenols

The most useful colorimetric method for the estimation of phenols is, in our experience, that based on the Folin and Denis method (45) using the reagent of Folin and Ciocalteu (44). It is very sensitive and widely applicable provided nonphenolic reducing substances are absent. (The Gibbs method (48,68) using 2,6-dibromoquinone chloroimide can also be used for many phenols but is more troublesome to apply.) A deep blue color is not given with the Folin and Ciocalteu reagent by all phenols, notable exceptions being nitrophenols. Other phenols, e. g., *p*-hydroxybenzoic acid, give relatively feeble colors. It is thus important that the standard for comparison should be made up from the same phenol as that being estimated. Where phenols cannot be estimated by the Folin and Ciocalteu method, a procedure based on coupling of the phenol with diazotized *p*-nitraniline (86) can often be used. Again it should be emphasized that the colors given by this reagent with different phenols differ greatly and, therefore, the standard for comparison must be appropriate. Furthermore, it should be remembered that bases as well as phenols can couple with diazotized amines and may have to be removed or allowed for after separate estimation.

Methods which are applicable to relatively few phenols, include the bromine method (82) for phenol, the indophenol method (77) for *p*-aminophenol, the phloroglucinol method (68) for quinol, and the cobalt method for catechols (2).

3. Estimation of Ethereal Sulfate

The methods for estimation of ethereal sulfate are based upon hydrolysis of the ester and precipitation of the resultant inorganic sulfate either as barium or benzidine sulfate. Many modifications of these methods are described in the literature. Other methods, e. g., the rhodizonic acid (73) method, are usually unsuitable owing to interference by urinary constituents. In all methods it is necessary to distinguish between inorganic and ethereal sulfate, the usual procedure being to estimate inorganic sulfate before and after hydrolysis, it being assumed that the difference is all due to ethereal sulfate.

Precipitated barium sulfate may be estimated either gravimetrically or

turbidimetrically. The pioneer method for the gravimetric estimation of sulfate in urine is that of Folin (43), a method the reliability of which remains unchallenged to the present day. The gravimetric method is the most accurate method available for ethereal sulfate estimations but in using it with urine, i. e., in the presence of inorganic sulfate, it becomes unreliable if the concentration of ethereal sulfate is less than about 2 mg. (SO_3) per 100 milliliters. The small amount of sulfate in blood makes it difficult to estimate unless large samples of blood are used. To obviate this Denis (34) and Denis and Reed (35) developed a method in which the barium sulfate was suspended in the blood filtrate and measured nephelometrically. This led to the development of turbidimetric methods and Sperber (83) avoided the disadvantage of the "difference" method for urine by first removing the inorganic sulfate and then estimating the ethereal sulfate turbidimetrically after hydrolysis of the filtrate. Turbidimetric methods have the advantage that they can be carried out much more rapidly than gravimetric methods, and are thus more convenient when a large number of samples have to be analyzed in a limited time.

Benzidine methods make use of the very low solubility of benzidine sulfate which can be isolated and titrated as an acid as in the method of Rosenheim and Drummond (79), or determined by means of a color reaction such as that with hydrogen peroxide and ferric chloride (91), or sodium β-naphthoquinone-4-sulfonate (59), or by diazotization and coupling (33,55,72). The colorimetric methods are claimed to be very sensitive. The benzidine method provides a rapid method for estimating ethereal sulfate but since, like the Folin (43) method, it is a "difference" method it becomes less reliable as the inorganic:ethereal sulfate ratio increases. The precipitation of benzidine sulfate by the original method, especially from hydrolyzed urines, may occasionally be capricious, even to the extent that less benzidine sulfate is precipitated from hydrolyzed urine than from unhydrolyzed urine. This difficulty can be largely overcome by preliminary removal of phosphates and precipitation under conditions recommended by Fiske (42). Under experimental conditions dietary control may serve to ensure that the excretion of phosphate is so low as not to interfere with precipitation of benzidine sulfate (e. g., Maw (63)).

4. Estimation of Glucuronic Acid

Most of the methods in use for the estimation of glucuronides are based on the reaction of hexuronic acids with naphthoresorcinol to form a colored complex (Tollens reaction). Several procedures have been described, e. g., Maughan, Evelyn, and Browne (62), Hanson, Mills, and Williams (50), and Fishman, Smith, Thompson, Bonner, Kasdon, and Homberger (41).

The authors (15) have modified the method of Hanson, Mills, and Williams so as to avoid separation of the colored complex in a separate phase. This is more convenient when a large number of samples have to be handled.

Analysis by a naphthoresorcinol method gives the total hexuronic acids. Glucuronic acid is the most common hexuronic acid found in nature and appears to be the only one found in animal material. Thus, results obtained from urines by the naphthoresorcinol method may be reasonably assumed to refer to glucuronic acid. It is desirable, however, to confirm the identity of the acid, using paper chromatography (66), or by the isolation and preparation of a derivative, e. g., Levvy (60).

Glucuronic acid derivatives other than phenylglucuronides may be present in urine, the chief of these being ester glucuronides (conjugates of carboxylic acids). When both types of conjugate are present it may be necessary to differentiate between the two. This can often be done by determining reducing values. Ester glucuronides reduce alkaline cupric reagents directly, while ether glucuronides, being true glycosides, must first be hydrolyzed before cupric reagents can be reduced (70). Thus, by determining the reducing power of the material in question before and after hydrolysis, an estimate of the relative amounts of glucuronic acid combined with carboxylic acids and with phenols (or alcohols) may be obtained provided that the glucuronide can be completely hydrolyzed without loss of glucuronic acid (see Section IV3,B(2)). This procedure is not applicable without correction factors when other glycosides are present or when non-carbohydrate reducing material is liberated on hydrolysis. Glucuronides can also be formed from aliphatic alcohols. Ether glucuronides of this type cannot be distinguished from phenylglucuronides by copper reduction methods. The formation of diglucuronides is possible although only one has been identified (see Bray (12)). These could be of several types; for example, the diglucuronide of a phenol could theoretically be formed by attachment to two phenolic hydroxyl groups, one phenolic and one carboxylic group, or one alcoholic and one phenolic group. The formation of such diglucuronides would obviously complicate the interpretation of glucuronic acid estimation. The naphthoresorcinol and reducing methods may be applied directly to urine without further purification. In special cases other methods may be used. Although most phenylglucuronides cannot be extracted from aqueous solutions by ether, some are soluble in ether, e. g., the six xylylglucuronides (Bray, Humphris, and Thorpe (14)). Such glucuronides may be extracted from urine by means of ether and determined in the extract: (*a*) by a naphthoresorcinol method; (*b*) by titration—if no other abnormal ether-soluble acids are present (or if these can be estimated independently); or (*c*) after hydrolysis, by estimation of reducing power.

5. Normal Excretion of Phenolic Compounds

If the excretion of phenols after the administration of drugs or foreign compounds is being studied quantitatively, it is essential to determine the normal excretion of phenolic metabolites since the excretion of the metabolites of the administered compound will be superimposed upon these. The normal "base-line" excretion is usually reasonably steady provided that the animal is kept on a constant diet for a considerable period before dosage, and maintained on this diet until the excretion of phenols has returned to the base-line value. Ideally, determinations should be made for several days before and after dosage (e. g., see Maw (63)), but, with animals whose excretion characteristics have been determined in previous experiments, the predose period may often be reduced to two days. The period required after dosage depends upon the rate of excretion of the metabolites of the substance administered. Many substances are completely excreted in 24 hours but some may be slowly excreted over three or four days.

There may be considerable variation in the excretion of normal metabolites even in animals of the same species and strain, and reliable results for administered compounds can only be obtained if normal excretions are determined for each individual animal used. Table II gives some typical

TABLE II

Typical Values for Normal Excretion
of Phenolic Metabolites in 24 Hours by Rabbits on Diet No. 18

Value determined	Standard used and results expressed as mg. of	Range	Av. individual variation (±%)	Ref.
Phenol				
Free	*p*-Cresol	6–24	20	27
Total		40–87	15	
Phenol				
Free	Xylenol	12–18	18	14
Total		90–99	7	
Phenol				
Free	*p*-Methoxyphenol	135–173	15	19
Conjugated		31–83	14	
Phenol				
Free	*p*-Hydroxyphenyl phenyl ether	224–279	7	19
Conjugated		116–196	24	
Ethereal sulfate	SO_3	28–55	9	19
Glucuronic acid (naphthoresorcinol method)	Glucuronic acid	130–301	8	19
Reducing value	" "	175–210	10	13
After hydrolysis	" "	305–480	10	13

values for the normal excretion of phenolic metabolites by rabbits; these values were obtained in the authors' laboratory on animals which had become accustomed to the standard laboratory diet (Diet No. 18 of Bruce and Parkes (30)). The extent of the variation in individual animals is expressed as the average percentage by which individual daily values differed from the corresponding weekly averages. It will be appreciated that the amount excreted corresponding to this percentage is very small in comparison with the amount of the increased excretion resulting from the administration of a dose of a foreign compound of the magnitude usually given. The values for phenols given in Table II show clearly the importance of the selection of an appropriate standard for the determination. Tables giving values for normal and pathological human urines are given by Volterra (90).

IV. METHODS

1. Phenols

A. USING FOLIN AND CIOCALTEU REAGENT

Phenols can be estimated directly on urine if interfering reducing substances such as uric acid are first removed (44, 45). The authors find it more convenient to separate the phenols by ether extraction or by steam distillation and then to apply the Folin-Ciocalteu reagent (27).

Reagents.

Folin-Ciocalteu Reagent. A mixture of 100 g. of sodium tungstate ($Na_2WO_4.2H_2O$), 25 g. of sodium molybdate ($Na_2MoO_4.2H_2O$), 700 ml. of water, 50 ml. of 85% phosphoric acid, and 100 ml. of concentrated hydrochloric acid is boiled under reflux for 10 hours. Then 150 g. of lithium sulfate, 50 ml. of water, and a few drops of bromine are added. The mixture is boiled for 15 minutes without a condenser to remove excess of bromine, cooled, diluted to 1 liter, and filtered. The reagent should have no greenish tint, and should be kept in a stoppered bottle. Before removing the stopper it should be carefully cleaned since organic material in dust is liable to cause reduction of the reagent.

Sodium Carbonate Solution. 20% w/v, Na_2CO_3 in water.

Phenol Standard. A solution of the selected phenol (50 mg./100 ml.) is appropriately diluted before use, e. g., for *p*-cresol so as to give 2 mg./100 ml.

Procedure.

(1) Free Phenol. Urine (20 ml.), at pH 7.8–8.0, is continuously extracted with ether for 6 hours. After the addition of 10 ml. of water, the extract is carefully heated (particular care is necessary with steam-volatile phenols) to remove the ether and the aqueous residue is diluted to 25 ml. A suitable portion of this, de-

pending on the phenol content, is transferred to a boiling tube and made up to 1(ml. Folin-Ciocalteu reagent (1 ml.) and sodium carbonate solution (2 ml.) ar added. The tube is heated in the boiling water bath for 1 minute and is ther cooled in water. The blue solution is diluted to 25 ml. and compared with s standard prepared by using 1 ml. of standard solution in place of the portion o urine extract. If a photoelectric absorptiometer is used, a Chance OR2 orang filter is suitable.

(2) *Total Phenol.* The above estimation is repeated with urine which has beer hydrolyzed by being boiled under reflux for 1 hour with an equal volume of 10 N sulfuric acid and then adjusted to pH 7.8–8.0.

The method described is suitable for the estimation of nonacidic phenols. Fo phenolic acids which give a suitable reaction with the reagent the pH of the urin would have to be adjusted, e.g., by the addition of 2 ml. of 2 *N* sulfuric acid be fore ether extraction.

B. USING *p*-NITROANILINE

This method (16) is suitable for some phenols which give only a feebl color with the Folin-Ciocalteu reagent, e. g., *p*-hydroxybenzoic acid. The procedure for the estimation of this acid in the unconjugated state will b described.

Reagents.

p-Nitroaniline. 3 g./l. in 0.8 *N* hydrochloric acid.
Sodium Nitrite. 5% w/v in water.
Ethanol. 96%.
Sodium Carbonate. 5% Na_2CO_3 (w/v) in water.
Diazotized p-Nitroaniline. 25 ml. of *p*-nitroaniline and 1.5 ml. of sodium nitrit mixed immediately before use.

Procedure. Urine (20 ml.) acidified with 2 *N* sulfuric acid (2 ml.) is con tinuously extracted with ether. The extract, after removal of the ether, i neutralized with 0.02 *N* sodium hydroxide using phenol red as the indicator and i diluted so that concentration of *p*-hydroxybenzoic acid is between 0.2 and 2 mg./10 ml. This solution (5 ml.) is mixed with ethanol (5 ml.) and diazotized *p*-nitro aniline (1 ml.). After 2 minutes sodium carbonate (3 ml.) is added and the solutio is made up to 25 ml. with water. The absorption is measured immediately on photoelectric absorptiometer using a Chance OB1 blue filter. The calibratio curve is constructed using solutions containing 0.01 to 0.10 mg. of *p*-hydroxy benzoic acid in 5 ml.

For estimation to include conjugated acid, urine must be hydrolyzed as de scribed under method A(2).

2. Ethereal Sulfate

A. FOLIN'S GRAVIMETRIC METHOD (43)

Reagents.

Barium Chloride. 5% w/v in water.

Dilute Hydrochloric Acid. Concentrated hydrochloric acid diluted with 3 volumes of water.

Procedure.

Inorganic Sulfate. Urine (25 ml.), filtered if necessary, dilute hydrochloric acid (10 ml.), and water (100 ml.) are mixed in a conical beaker and barium chloride (10 ml.) is added slowly drop by drop from a special dropper which delivers 10 ml. in approximately five minutes. The mixture must not be stirred during the addition and is left undisturbed for 1 hour afterward. The precipitate is then collected by filtration through a prepared Gooch crucible having an asbestos pad and is washed well with water (200 ml.). The crucible is dried at 100° and placed inside a silica crucible to which the flame of a Meker burner is applied at first gently but finally with full force for 1 hour or until constant weight is attained.

Total Sulfate. Urine (25 ml.) is treated with dilute hydrochloric acid (20 ml.) in a conical beaker, covered with a clock glass, and boiled gently for 30 minutes. After the solution has cooled, water (100 ml.) is added and barium sulfate is precipitated and weighed as described above.

B. TURBIDIMETRIC METHOD

The authors (15) have found that the following methods are reliable and convenient when a rapid method for a large number of samples is required. The barium sulfate suspension is not stabilized with gelatin and absorption readings should be taken within ten minutes.

Reagents.

Hydrochloric Acid. About 7.1 *N*.

Sodium Hydroxide-Potassium Sulfate Solution. Sodium hydroxide (2.5 *N*) containing 0.128 g./l. of potassium sulfate. The sulfate is added to ensure that the calibration curve for absorption is linear over the required range.

Barium Chloride. Analar grade powdered $BaCl_2.2H_2O$.

Potassium Sulfate. Analar grade K_2SO_4, 0.44 g./l.

Procedure.

(*1*) *Inorganic and Total Sulfate.* To achieve uniformity of conditions both estimations are carried out simultaneously. Urine is diluted if necessary so as to contain 0.1 to 1.0 mg. of SO_3. For inorganic sulfate, sodium hydroxide-potassium

sulfate solution (5 ml.) and hydrochloric acid (2 ml.) are mixed in a 16 x 150 mm. test tube and cooled. Urine (5 ml.) is then added. For total sulfate, urine (5 ml.) is heated with hydrochloric acid (2 ml.) in a 16 x 150 mm. test tube for 1 hour in a boiling water bath, using a "cold finger" condenser in the top of the tube. Tube and contents are cooled and sodium hydroxide-potassium sulfate solution (5 ml.) is added. The tubes for both inorganic and total sulfate are prepared in duplicate, one of each being required for blanks. To one of each, powdered barium chloride (200 mg.) is added and the tubes are vigorously shaken for 30 seconds. The absorption values are then determined between 7 and 10 minutes after mixing using a photoelectric absorptiometer with a Chance neutral filter H 508. (The barium sulfate suspension is stable for up to 20 minutes.) The calibration curve made by using appropriate dilutions of potassium sulfate solution is linear from 0 to 1 mg. of SO_3 per tube.

This method is reliable if the ratio of inorganic:ethereal sulfate does not exceed 5. Sulfite does not interfere with the determination. If the inorganic:ethereal sulfate ratio exceeds 5, inorganic sulfate can be determined by this method and ethereal sulfate as described below.

(2) *Ethereal Sulfate When Only Small Amounts Are Present* (cf. Sperber, 83).

Reagents.

Barium Chloride-Hydrochloric Acid. 1% $BaCl_2.2H_2O$ w/v in 0.2 *N* hydrochloric acid.

Sodium Carbonate. 10% w/v in water.

Procedure. Unfiltered urine is diluted five times. Diluted urine (20 ml.) is mixed with barium chloride-hydrochloric acid (3 ml.). After the addition of 2 ml. of sodium carbonate the solution is well mixed and filtered through Whatman No. 1 paper (or centrifuged) until clear. (This usually requires only one passage through the paper.) Portions (5 ml.) of the filtrate (or supernatant) are hydrolyzed with 7.1 *N* hydrochloric acid (2 ml.) as described in method B(1), and from then the same procedure is followed. There appears to be a constant loss of about 0.01 mg. of SO_3 per sample, probably due to absorption on the precipitate of barium salts.

3. Glucuronic Acid

A. NAPHTHORESORCINOL METHOD (15)

In most of the naphthoresorcinol methods (41,50,62) the colored complex formed by boiling the reagent with glucuronic acid is extracted with an organic solvent and the color intensity of that phase is measured. If *n*-butanol is used in place of the amyl alcohol used by Hanson, Mills, and Williams (50), a monophasic system is obtained which obviates the separation of the two phases.

Reagents.

Naphthoresorcinol Solution. 0.375% w/v in water; it is "aged" by being kept at 0° for 1 week before use. The "aged" solution can be kept for another week.

Hydrochloric Acid. Concentrated technical grade. If pure hydrochloric acid is used the blue color may not develop.

n-Butanol.

D-*Glucurone.* A solution (0.1 mg./ml.) in water is stable for one week if kept at 0°.

Procedure. Urine is diluted so as to contain not more than 0.04 mg./ml. of glucuronic acid. Diluted urine (2 ml.), hydrochloric acid (4 ml.), and naphthoresorcinol (2 ml) in a test tube (13 x 150 mm.) are heated in boiling water for 2 hours. Evaporation is reduced by a "cold finger" condenser placed in the top of the tube. The tube and contents are then cooled in cold water. *n*-Butanol (5 ml.) is added, the tube is corked, and is shaken well and left to stand for 3 minutes for air bubbles to disperse. The absorption value is then read within 5 minutes in a photoelectric absorptiometer using an Ilford Spectrum Orange 607 filter. The calibration curve can be constructed using 0.2 to 2 ml. of glucurone solution made up to 2 ml. with water.

B. COPPER REDUCTION METHOD

Of the numerous copper reduction methods for the estimation of sugar in urine that of Shaffer and Hartmann (81) is particularly satisfactory when adapted for the estimation of glucuronic acid (22,23). The method cannot be satisfactorily used if sugar is present and must be used with caution for ether glucuronides (see end of section).

Reagents.

Alkaline Copper Iodide Reagent. In 600 ml. of warm water are dissolved 81 g. of potassium citrate ($K_3C_6H_5O_7.H_2O$), 70 g. of potassium bicarbonate, and 92 g. of potassium oxalate ($K_2C_2O_4.H_2O$). A solution of 25 g. of copper sulfate ($CuSO_4.5H_2O$) is run into the bottom of the main solution with constant stirring. To the mixture a solution of 3.57 g. of potassium iodate and 50 g. of potassium iodide in 200 ml. of water is added. The whole is cooled and made up to 1 liter.

Sulfuric Acid. Approximately 5 *N* (140 ml. of concentrated sulfuric acid diluted to 1 liter).

Sodium Thiosulfate. 0.1 *N*.

Starch Indicator. Soluble starch (1 g.) is dissolved in water and made up to 100 ml.

Procedure.

(*1*) *Ester Glucuronide.* Urine (10 ml.) and reagent (10 ml.) in a boiling tube are heated in a boiling water bath for 15 minutes. The mixture is then cooled and acidified with 5 *N* sulfuric acid (5 ml.), and the iodine liberated is titrated with 0.1 *N* sodium thiosulfate. A blank determination using 10 ml. of water in place of urine

is carried out simultaneously. The difference between the blank and urine titrations represents the glucuronic acid, which should correspond to 3.154 mg. for each milliliter of sodium thiosulfate. It is, however, advisable to calibrate the reagent with pure glucuronic acid or a pure ester glucuronide, e. g., veratroyl glucuronide.

(*2*) *Total Glucuronide.* Urine is hydrolyzed by adding one-tenth volume of concentrated hydrochloric acid and heating in a boiling water bath for 90 minutes. The mixture is then cooled, neutralized with solid sodium carbonate, and made up to known volume, and the glucuronic acid is estimated as in (1). The difference between the values obtained in (2) and (1) gives the ether glucuronide.

It is advisable to confirm that the hydrolysis conditions are sufficient to give maximal reducing values. The use of an unnecessarily high concentration of acid should be avoided since glucuronic acid is liable to be decomposed. If the glucuronides are very resistant to hydrolysis it may not be possible to achieve complete hydrolysis without loss of glucuronic acid. In such cases total glucuronide would have to be determined by the naphthoresorcinol method.

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