PHENOLIC SUBSTANCES

IN

FEEDS, FECES, URINE, AND BLOOD

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TABLE OF CONTENTS

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INTRODUCTION

Projects for the purpose of studying the problems connected with salt water contamination of streams have been conducted at this laboratory for several years. The results from these projects show detailed effects of various kinds of salts on the metabolic systems of different animals, and the results obtained when saline waters in various concentrations were used as a sole source of drinking supply.

Phenol and phenolic substances are present in varying amounts in the waste products of many industrial plants. This is particularly true of many of the processes connected with the petroleum industry. When these waste products are turned into the streams they present the additional problem of water contaminated with phenol.

When the problem of phenol contamination of streams became paramount, this laboratory was called upon to arbitrate the controversies. The need for information whereby the above mentioned controversies may be settled, as well as the desire for more complete knowledge concerning the physiological effect of phenol upon the body prompted this investigation.

REVIEW OF LITERATURE

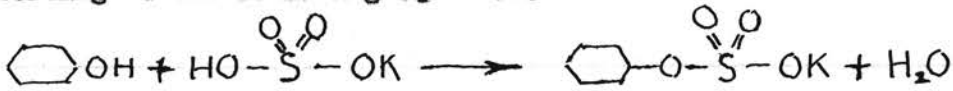
A detailed review of the literature upon which is founded our knowledge of the physiology of phenols is given by Tisdall (22) and also by Pelkan and Whipple (18). Consequently, only a few of the more important facts will be repeated here.

Formerly the word "phenols" included only the volatile phenols, phenol and the cresols. Since the advent of Folin and Denis' method

for determining phenols, this terms includes not only the volatile phenols, but also the non-volatile phenols and the aromatic hydroxi-acids. This is due to the fact that the Folin and Denis phenol reagent will react with all compounds containing a benzene ring that has a free hydroxi group substituted for a hydrogen atom. In this paper the words phenols, phenol bodies, and phenolic substances will be given the more inclusive meaning.

The phenolic substances normally found in the urine and sometimes in the feces are: volatile phenols, consisting of phenol and ortho and para cresol, the non-volatile phenol pyrocatechin, the aromatic hydroxi-acids p-hydroxi-benzoic acid, p-hydroxi-phenylacetic acid, and p-hydroxi-phenylpropionic acid. All of the above named compounds are considered as decomposition products of the amino acid tyrosine. Thus, the amounts of these phenolic substances excreted have been taken as an index of the extent to which putrefaction is taking place in the intestine.

The body uses conjugation as a method for detoxifying phenols. One conjugation reaction takes place when phenol combines with $KHSO_4$ according to the following equation:



This reaction takes place in the liver. It has been suggested that other organs have the ability to conjugate phenols, while Masuda (17) points out the possibility of this reaction taking place in the lymph. The products formed as a result of this reaction are called conjugated phenols and they form a part of the compounds referred to as ethreal sulfates. Also, the product formed when phenol combines with glucuronic acid constitutes a part of the conjugated phenols. These

conjugated phenols are then excreted by way of the kidneys. Folin and Denis (11) claim that no conjugated phenols exist in the feces. Also, Theis and Benedict (21) have made the claim that only some of the bloods contain conjugated phenols. This claim has been confirmed by Castex and Arnaudo (2).

A survey of the literature shows five independent color reactions that have been used in determining phenolic substances in biological materials. There are any number of modifications of these basic methods in use. The modifications consist in changing the treatment of the sample before the final color formation is brought about. Each of these basic methods will be taken up and discussed briefly.

Theis and Benedict (21) employ a color reaction between phenols and diazotized p-nitroaniline as a basis for the quantitative estimation of phenol bodies. The reagent is made by diazotizing a water solution of p-nitroaniline with a solution of sodium nitrite. The diazotized reagent does not keep so this diazotizing must be done at the beginning of each analysis. The final reaction yields an orange to red color. A solution of gum acacia is added during the procedure to prevent turbidity in the case of blood filtrates. Theis and Benedict did their original work on blood. Morenzi (16) has successfully applied this procedure to urine. The results obtained by this method are quite satisfactory as a whole except that they fail to show the presence of conjugated phenols in the blood.

Weis (24) has utilized the Millon reaction in a colorimetric determination of phenols in urine. This is the characteristic reaction used to detect the presence of protein. This reaction shows

the presence of a monohydroxi-benzene nucleus, so the determination will include all phenolic substances. The proteins are precipitated by means of barium hydroxide. A mercuric sulfate-sulfuric acid solution is added and the sample is refluxed for one-fourth hour. After cooling, sodium nitrite is added and the Millon reaction is allowed to take place. The resulting turbidity is filtered out before the color comparison is made. It is seen at once that this procedure will not permit the estimation of free and conjugated phenols as all conjugated phenols will be broken down during the refluxing.

Gonzales (14) determined phenols in sera by bringing about the formation of brominated indophenol which gives a blue color. The proteins are coagulated by means of ethyl alcohol, it being claimed that some phenols are lost when the precipitation is accomplished by means of acid. Heating is also involved in this method, so it does not permit the determination of conjugated phenols.

Blood phenols have also been determined by causing a yellow color to be formed. Blanco and Comesana (1) accomplished this by boiling a hydrochloric acid-sodium nitrite solution of the sample and adding ammonium hydroxide after cooling. The determination is run on a filtrate obtained by precipitating the proteins from oxalated plasma, using the Folin and Wu tungstic acid method. Blanco and Comesana's method falls into the class with the last two named. That is, the heating eliminates the possibility of estimating conjugated phenols.

In 1912, Folin and Denis (8) announced the development of a phosphotungstic-phosphomolybdic acid reagent that gave a deep blue color when it was reduced by phenolic compounds. They called it the "phenol" reagent and immediately used it in determining tyrosine and

other substances containing the mono-hydroxi-benzene nucleus.

Since that time this reagent has been used in any number of instances to investigate the phenolic content of various biological substances. As explained by Folin, the reagent is destroyed by alkali, but the blue color forms only in alkaline solutions, it being necessary to add the alkali last. A large excess of alkali will destroy the blue color so this alkali is added with care. Sodium carbonate has proved to be the alkali best suited for use in this determination. As pointed out by Gortner and Holm (13) and others, this reagent is not specific for phenolic substances alone. Tyrosine, tryptophane, and even cuprous oxide, among various other substances, will reduce the phenol reagent to give the characteristic blue color. This has always been a serious draw back to the use of the reagent for determining phenolic substances in biological samples as the samples almost always contain materials other than phenol bodies that give this blue color with the phenol reagent. However, when the interfering substances are controlled the color reaction is very sensitive and this method has been successfully applied to the determination of phenols in urine and blood. Its successful application to the determination of phenols in feeds and feces will be described in this paper.

EXPERIMENTAL

As previously stated, the object of this investigation was to study the complete physiological action of phenol on the body as well as to determine to what extent phenol is toxic when it is ingested in the drinking water. To accomplish this it was necessary to analyze

the feeds, feces, urine, and blood by the same method as all of these results were to be correlated and compared in the same tables. A careful investigation revealed that Folin and Denis' method was the only one that had been applied to the urine, feces, and blood. As far as it was able to ascertain, no attempt has been made to quantitatively determine the phenolic substances in feeds.

For the reasons stated above, and also because it permitted the estimation of both free and conjugated phenols, the phosphotungstic-phosphomolybdic acid reagent of Folin and Denis was chosen as the method for running all of these analyses. In the remaining part of this paper this reagent will be referred to as the "phenol" reagent. Of the various modifications of this method that have been used, Tisdall's (22) ether extraction method proved to give the best control of the interfering substances when urine was analyzed. The procedure will be explained in detail later. In Folin and Denis' (11) original application of the phenol reagent to the determination of phenols in feces no attempt was made to remove tyrosine and tryptophane before the color determination was made. Consequently, the procedure of the ether extraction method was modified so that it could be applied to feces. This method for feces was chosen in preference to the modification used by Debergh and Goiffon (5), as the results obtained can be more justly compared with those obtained on the urine when the ether extraction method is used. The method used on the feces was applied to feeds, the only changes being that different dilutions and a different strength standard were used.

For determining phenols in the blood, Rakestraw's (19) method was used. This method also makes use of the phenol reagent. About

the only objection to this method is that it includes the tyrosine that is present, which results in values that are too high. It will be explained later how this objection may be overcome.

From the list of substances given by Gortner and Holm (13) that interfere with the determination of phenols by the phenol reagent, tyrosine, tryptophane, and uric acid are the ones most likely to be present in normal urine and feces. All three of these substances are insoluble in ether. Thus, they are removed from the determination. The final results include all substances that are soluble in ether, the sodium salts of which are insoluble in ether, and that finally give the characteristic blue color with the phenol reagent. It is seen that this includes all volatile and non-volatile phenols present in urine and feces as well as the aromatic hydroxi-acids.

Only one complete set of reagents is required for the performance of the four methods of analyses chosen. This simplifies the procedure to a great extent. The preparation of these reagents will be described in detail.

The phenol reagent is prepared according to the new method given by Folin and Ciocalteau (12). 100 gm. of sodium tungstate ($Na_2WO_4 \cdot 2H_2O$), 25 gm. of sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$), and 700 ml. of water are transferred to a 1500 ml. Florence flask. 50 ml. of 85% phosphoric acid and 100 ml. of concentrated hydrochloric acid are then added. The flask is connected to a reflux condenser by means of a cork or rubber stopper wrapped in tin-foil and the solution is boiled gently for 10 hours. After the boiling is completed 150 gm. of lithium sulfate, 50 ml. of water and a few drops of liquid bromine are added. The mixture is boiled, without the condenser, for about 15

minutes to remove the excess bromine. It is then cooled, diluted to one liter, and filtered. The finished reagent should be deep yellow in color and should have no greenish tint. This green color indicates the presence of partially reduced reagent and limits the range of true proportionality obtained between different concentrations of the substances analyzed for. The reagent should be protected against dust, such as organic materials, that will gradually produce slight reductions. These reductions may be detected by the presence of the green color. If this green color appears it may be removed by the addition of more bromine and subsequent boiling. Phenol reagent that had been allowed to stand for almost a year and had taken on a considerable green tint was treated in this way and was found to give results identical with those obtained when freshly prepared reagent was used.

The working standard used consisted of a solution of phenol in 0.1 N hydrochloric acid that contained 0.1 milligram of phenol per milliliter of solution. The phenol stock solution was standardized by the method of Messinger and Vortmann which was later described by Tisdall (22). As some difficulty was encountered in getting this titration to proceed accurately, the method will be repeated in detail. 10 ml. of the phenol stock solution, containing approximately one milligram of phenol per milliliter, are taken for titrating. The solution is made slightly alkaline by means of sodium hydroxide, using a capillary tube and brom-phenyl-blue paper as indicator. It is then heated to 65°. 15 ml. of 0.1 N iodine solution are added and the mixture is allowed to stand in the dark, at room temperature, for one half hour. No attempt is made to keep the temperature up during or after the addition of the iodine solution. After the one half

has elapsed, 5 ml. of concentrated hydrochloric acid are added and the excess iodine is titrated with 0.1 N sodium thiosulfate, using starch as indicator. The number of milliliters of 0.1 N iodine taken up by the phenol multiplied by 1.567 equals the total number of milligrams of phenol present in the 10 ml. aliquot taken. The working standard is then prepared by making the necessary dilution with 0.1 N hydrochloric acid.

The standard, prepared as directed above, was found to be permanent over a period of at least one year if it had been stored in a bottle that had been previously sterilized by boiling in water for about one half hour. The standard seemed to deteriorate if it was not made up in 0.1 N acid or if it was stored in a non-sterile bottle.

The remaining solutions are very simple to prepare; the mere naming of them will tell how they are made. 20% sodium carbonate, 10% sodium carbonate, 2.5% zinc chloride, 10% sodium hydroxide, 5% lead acetate, 10% aluminum sodium sulfate, and 5% sodium cyanide are all made by taking the weight of the solute in grams equal to that figure arrived at by multiplying the desired final volume by the given per cent and adding water to make the required dilution.

Analysis of Urine: A detailed procedure for the determination of phenols in urine by means of ether extraction will be given at this time. This method was first worked out by Tisdall (22). It is repeated here because it is modifications of this method that have been applied to the analysis of feces and feeds. Also, the convenience of having the methods for determining phenolic substances in feeds, feces, urine and blood all collected in a single paper will be realized, as Rakestraw's procedure for blood will be discussed later.

In analyzing for phenolic substances, urine may be extracted with the ether directly, as tyrosine, tryptophane, and uric acid are insoluble in ether. The pH of a portion of the urine sample is adjusted to a value between 5 and 6, using a capillary tube and alizarine red paper as indicator. For free phenols, the extraction is made on 10 ml. of this treated portion. For total phenols, a 10 ml. aliquot is transferred to a large test tube. Eight drops of concentrated hydrochloric acid are added and the mouth of the test tube is closed with a small funnel. The sample is then heated in boiling water for 10 minutes. This heated sample is cooled in running water before the extraction is made.

The sample for the free phenols and the one for the total phenols are now transferred to separatory funnels. Each is extracted three times with ether, each extraction being continued for a period of five minutes. The first extraction is made with 100 ml. of ether and each of the other two is made with 50 ml. of ether. At this point the desired phenol bodies are in the ether layer. They are removed from this ether by extracting with 25 ml. of 10% sodium hydroxide solution.

The above extractions may best be performed in the following manner. Deposit the sample to be extracted in a 300 ml. separatory funnel. Add 100 ml. of ether and shake for five minutes. Allow the water and ether layers to separate. This requires about 10 minutes. Drain the water layer into a 150 ml. separatory funnel, leaving the ether layer in the original funnel. Add 50 ml. of ether to the sample in the smaller funnel and extract again for a period of five minutes. Again allow 10 minutes for the two layers to separate. Drain

the water layer into a second 150 ml. separatory funnel and then deposit this second ether layer in the 300 ml. funnel. Now make the third extraction on the water layer. After the separation of the layers as described above, add the third ether layer to the other two in the large funnel.

Remove any traces of water that have collected in the bottom of the large separatory funnel. After removing this water add 25 ml. of a 10% sodium hydroxide solution and shake for a period of 10 minutes. After the layers separate, drain the sodium hydroxide layer into a 50 ml. Erlenmeyer flask and run the final color determination on it. Place the ether layer in a waste-ether bottle. This ether may be redistilled and used again.

To form the blue color, the sodium hydroxide layer is neutralized with concentrated hydrochloric acid, three or four drops in excess being added. The sample is then transferred to a 100 ml. volumetric flask.

At this point the standard is made up. 5 ml. of the standard solution containing a total of 0.5 milligram of phenol, are placed in another 100 ml. volumetric flask. Both the standard and the unknown are given the same treatment from this point on. They are diluted to about 75 ml. with boiling water. From 3 to 5 ml. of the phenol reagent are added to each, depending upon the amount of phenols expected. 20% sodium carbonate is then added until all of the acid present has been neutralized. This is shown when no more carbon dioxide is given off. 5 ml. excess sodium carbonate are added. The samples are allowed to stand for one half hour or more. During this time the color forms and the samples cool. They are then diluted to

volume and compared in a colorimeter.

With the standard set at 20 the following equation represents the calculation:

$$0.5 \times \frac{20}{x} \times \frac{1}{10} = \frac{1}{x} = \text{mg. phenols/ml. of urine.}$$

It will be well to analyze the above procedure in detail. Since the removal of the phenolic substances from ether depends upon converting them into their sodium salts which are insoluble in ether, these substances will not be extracted from the urine if they are present in the form of a salt. Hence, the sample is made acid before extracting it. This converts all the phenolic substances of the urine into their free form so that they may be extracted.

When the ether layer is extracted with sodium hydroxide all the phenolic substances are converted back into their sodium salts. As has been said, this puts them into forms that are insoluble in ether, so they all collect in the sodium hydroxide layer. If this ether layer is extracted with sodium carbonate insteam of sodium hydroxide, only the aromatic hydroxi-acids are removed. Thus, it is seen that this procedure offers a method whereby the volatile phenols may be separated from the aromatic hydroxi-acids, if this separation is desired. This may be done by extracting the ether layer first with sodium carbonate and then with sodium hydroxide. The first extraction will give the aromatic hydroxi-acids and the second will give the volatile phenols. The sum of the two extractions will give the total phenolic substances present.

All of the sodium hydroxide must be neutralized before the color is formed. The reduction of the phenol reagent depends upon the

presence of a free hydroxyl group, and this reduction takes place only in slightly acid solutions. As a result, any of the phenols that are present as sodium salts will not be included in the final determination. However, a large excess of acid at this point seems to interfere with the color formation.

All bothersome precipitates are avoided by diluting the sample with boiling water after it (the sample) has been neutralized. If the phenol reagent and the sodium carbonate are added while the sample is still hot, no turbidity will appear. The added heat seems to intensify the blue color. This hot water also drives off the ether that has remained in the sample.

The sodium carbonate is added for two reasons. First, it it necessary to make the solution alkaline before the blue color will appear, even though the actual reduction of the reagent takes place in an acid solution. It seems that the compound as formed by the reduction is colorless, but when this compound is placed in an alkaline solution its chromophore group changes so that a blue color is produced. The second reason is that the sodium carbonate destroys the excess phenol reagent, thereby removing its yellow color from the determination. The complete destruction of the reagent is shown when the color developed is deep blue and contains no green tinge.

In the case of rats drinking considerable quantities of phenol water, the total phenolic content of the urine was so high that only 5 ml. of urine were taken for analysis and the final dilution of the sample was 200 ml. This was done to bring the intensity of the color into the range of that of the standard that was used for the determination of free phenols.

Analysis of Feces: The method as applied to feces, consisted simply of the combination of Tisdall's (22) ether extraction method with the original method applied to feces by Folin and Denis (11). About the only objection to the original method of Folin and Denis is that no attempt was made to remove tyrosine and tryptophane. The primary object of extracting urine with ether was to remove these two compounds and also to remove uric acid. Now, if the water filtrate from the feces in Folin and Denis' procedure be put through this ether extraction process, these interfering substances will be removed, the results obtained will be a truer index of the amount of phenolic substances in the feces, and a better comparison may be made between these results and the ones obtained on the urine. The detailed procedure follows.

10 grams of well mixed, undried feces are weighed into a 250 ml. beaker. 75 ml. of water are added and the sample is allowed to stand for from 30 to 45 minutes, with occasional stirring. The mixture is then transferred quantitatively to a 200 ml. volumetric flask. 20 ml. of 10% sodium aluminum sulfate and 1 ml. of 5% lead acetate are added and the sample is diluted to the mark. It is allowed to stand for 15 to 20 minutes with occasional shaking. The sample is then filtered and 10 ml. aliquots of the filtrate are taken for analysis.

For free phenols, the 10 ml. aliquot is made to pH 5 to 6, and then it is transferred to a 300 ml. separatory funnel and extracted directly with the ether. For total phenols, a 10 ml. aliquot is transferred to a large test tube. Eight drops of concentrated hydrochloric acid are added and the test tube is closed with a small funnel. The sample is then heated in boiling water for 10 minutes.

After cooling, the sample is transferred to a 300 ml. separatory funnel and extracted with ether.

From this point on the procedure is exactly the same as that given for the urine. The same strength standard is used also. When the colorimeter reading for the standard is 20, the following equation represents the calculation:

$$\frac{0.5 \times 20}{x} \times \frac{1}{10} \times \frac{200}{1} \times \frac{1}{10} = \frac{20}{x} = \text{mg. phenols/gram feces.}$$

Analysis of feeds: Feeds were analyzed by almost the same procedure as was applied to the feces. However, as the results obtained on the feeds were much lower than those obtained on the feces, different dilutions and a different strength standard were necessary.

For free phenols, 10 grams of the feed are weighed into a 250 ml. beaker. 50 ml. of water are added and the mixture is allowed to stand for one half hour or more. At the end of this time the sample is transferred to a 100 ml. volumetric flask. 20 ml. of 10% sodium aluminum sulfate and 1 ml. of 5% lead acetate are added and the sample is diluted to the mark. It is allowed to stand for 15 to 20 minutes with occasional shaking, then it is filtered. 10 ml. of the filtrate are taken for extraction.

For total phenols, 10 grams of the feed are weighed into a 250 ml. beaker. 50 ml. of water and 25 drops of concentrated hydrochloric acid are added. The mixture is brought to boiling quickly by placing it over a free flame. It is then transferred to a hot water bath and heated for one half hour. Longer heating may or may not give higher results. After cooling, the sample for total phenols is treated the same as the sample for free phenols.

From this point on the procedure is the same as that for the urine and feces.

The standard used for the feeds should contain a total of 0.3 milligram of phenol. It is necessary to add only 3 ml. of the phenol reagent. In all determinations on feeds, the final volume is 100 ml. With the standard set at 20 the following equation shows the calculation:

$$\frac{0.3 \times 20}{X} \times \frac{1}{10} \times \frac{100}{1} \times \frac{1}{10} = \frac{6.0}{X} = \text{mg. phenols/gram of feed.}$$

Analysis of blood: The phenolic substances in the blood were determined by Rakestraw's (19) method. This method is described in the article cited but it will be repeated here so that it may be discussed with the view of pointing out possible errors.

5 ml. of blood are drawn and placed in 25 ml. of water. The proteins are precipitated by the familiar tungstic acid method of Folin and Wu, enough additional water being added to give a 1 to 10 dilution.

25 ml. of this tungstic acid filtrate are transferred to a 50 ml. centrifuge tube. Uric acid is precipitated by adding 1 ml. of 2.5% zinc chloride and 1 ml. of 10% sodium carbonate. The sample is allowed to stand for one hour, with occasional shaking. The uric acid precipitate is then thrown down by means of centrifuging. 10 ml. aliquots are pipetted off the top and placed in a large test tube graduated at 20 ml., one aliquot being used for the total phenols and one for the free phenols.

For total phenols, add four drops of concentrated hydrochloric acid and close the test tube with a small funnel. Heat in hot water

for 10 minutes. The water is never allowed to boil during this heating but is held at a temperature of from 90 to 95 degrees. After heating the sample is cooled immediately in running water. 5 drops of 20% sodium carbonate are added to partially neutralize the acid.

At this point the standard is made up. A quantity of the standard solution containing a total of 0.05 mg. of phenol is placed in a similar test tube. Sufficient water is added to bring the volume of the standard to 10 ml.

The standard and both the free and total determinations receive the same treatment from here on. 0.5 ml. of diluted phenol reagent (1-4) is added with shaking. After one minute 2 ml. of 20% sodium carbonate are added dropwise. The sample is then allowed to stand for at least 5 minutes, or until a definite blue color has formed. 1 ml. of 5% sodium cyanide is added from a burette and the sample is heated in boiling water for one minute. It is then cooled in running water, diluted to the mark, and compared in a colorimeter. With the standard set at 20 the following equation represents the calculation:

$$\frac{0.05 \times 20}{X} \times \frac{1}{10} \times \frac{27}{25} \times \frac{10}{1} \times 100 = \frac{108}{X} = \text{mg. phenols/100 ml. blood.}$$

This method has always shown the presence of conjugated phenols in the blood, provided that the tungstic acid filtrate is analyzed at once. However, it was found that the conjugated phenols broke down if the filtrate was allowed to stand before it was analyzed, even though it was stored at low temperatures. This decomposition was quite slow. It required at least a week for all the conjugated phenols to disappear. Analyses after standing showed the filtrate to contain practically no conjugated phenols while the total phenolic content approached the value determined when the blood sample was

first drawn. Also, it is believed that the mild form of heating in the determination of total phenols tends to increase the value obtained for conjugated phenols. It seems that vigorous boiling of the water will drive off some of the cresols, thereby reducing the increase obtained by the deconjugation of phenolic substances.

The formation of the blue color, particularly in the case of the micro determination on blood, is quite sensitive to pH change. The amounts of acid and alkali should be used strictly as directed. Sodium hydroxide cannot be substituted for sodium carbonate as an alkali.

As previously stated, about the only objection to this method for blood analysis is that it includes the tyrosine that may be present. According to Folin and Ciocalteau (12), the phenol reagent will not be reduced by tyrosine except in solutions that are alkaline enough to destroy the reagent. As a result, when the phenol reagent is used to estimate tyrosine a large excess is used so that the reduction by tyrosine will take place before all of the reagent has been destroyed by the sodium carbonate. In the analysis of blood for phenols, if the sodium carbonate is added slowly the excess reagent will be destroyed before the solution becomes alkaline enough for the reagent to be reduced by tyrosine. This will eliminate tyrosine from the determination.

There is another reason why the excess phenol reagent should be destroyed as quickly as possible. The sodium cyanide, while being used to intensify the color already present, will also reduce the phenol reagent. As long as the sample shows a green tinge the sodium cyanide should not be added as this green color indicates the pres-

ence of unreduced, undestroyed phenol reagent which will be reduced by the cyanide and result in high phenolic values for blood.

TABULATION AND DISCUSSION OF RESULTS

The animals used in connection with the study of the physiological action of phenol were grown male rats, selected from the healthy stock of the rat colony maintained in connection with this laboratory. Four rats were placed in each cage, with from six to twelve cages being under observation at a single time. The rations received by these rats were exact duplicates of the ones received by the stock rats of the colony except that the drinking water contained phenol in varying amounts. Of course one cage of rats were maintained as control rats and they received no phenol in their water.

The first set of rats was fed phenol in the water in amounts varying from 50 to 250 P. P. M. As the animals showed no ill effect from this phenol, the next set was given phenol in the water in amounts varying from 500 to 5000 P. P. M. The phenol content of the drinking water was finally raised to the point where some rats were receiving 12,000 P. P. M. phenol and not a single rat died from phenol poisoning.

The rats could be started on this high phenol water at once. That is, it was not necessary to start the rats on low phenol water and gradually increase the amount of phenol given until the rats were drinking the desired quantities. This shows that the body of the rat is able to detoxify large quantities of phenol at all times and that it was not necessary to build up a resistance to phenol poisoning.

Careful observation of these rats revealed that the animals drinking up to 4000 or 5000 P. P. M. phenol water were, if anything, livelier, healthier, and more vigorous than the control rats. Also, female rats drinking phenol water at these lower concentrations were able to give birth to and suckle their young without any difficulty. However, when the phenolic content of the drinking water was increased above 5000 P. P. M. the rats showed a definite detrimental effect, although none of them really died. The mature rats seemed to be more nervous and lacked the large, healthy appearance that the lower phenol rats had. Also, the females on this high phenol water did not make such good mothers.

To make metabolism studies, the phenol rats were placed in special cages. These cages were equipped with large dishes covered with screens so that the feces could be separated from the urine. The feed was given in a special feeding device so that the amount consumed could be measured accurately. The amount of drinking water consumed was measured, also.

The special test periods lasted for four days. To run these tests, four rats were placed in each metabolism cage, three cages being used. One of these cages contained the control rats. The rats in each of the other two cages were given phenol water of different concentration. The rats were fed and watered once daily, the amount of feed and water given being recorded each time. The feces and urine were collected twice daily and were stored in an ice box throughout the test period.

The total urine collected during the four day period was diluted to 500 ml. before it was analyzed. The feces were weighed and ana-

lyzed wet as drying seemed to drive off some of the volatile phenols. This was indicated by the fact that the phenolic content of dried feces was always low.

The phenolic content of the urine and feces was not appreciably changed by allowing the samples to stand at reduced temperatures for some time before they were analyzed. This was true for the conjugated phenols as well as for the total phenols. Also, the moisture content of the feces was found to be practically constant during this period. Any difference between both the moisture and the phenolic determinations before and after standing may well be attributed to technical errors in analysis. These tests did not extend over a period longer than one week, however.

In spite of the above statements, it is well to complete the analysis of the urine and feces as quickly as possible after the test period is completed. Although the tests do not show any deterioration of the phenolic substances on standing, no reason can be found why they should not deteriorate, much the same as they do in the case of the blood filtrates, unless it is because the urine and feces are more alkaline.

Table No. 1
PHENOLIC SUBSTANCES EXCRETED IN NORMAL RAT URINE, IN MG./RAT/DAY

Value	Total Phenols Mg.	Free Phenols Mg.	Conjugated Phenols Mg.
Maximum	5.844	2.844	2.750
Minimum	3.656	1.547	1.522
Average	4.487	2.186	2.302

Table No. 2
PHENOLIC SUBSTANCES EXCRETED IN NORMAL RAT FECES, IN MG./RAT DAY

Value	Total Phenols Mg.	Free Phenols Mg.	Conjugated Phenols Mg.
Maximum	5.344	4.242	1.102
Minimum	2.118	2.241	0.027
Average	3.200	2.859	0.342

Tables No. 1 and 2 show the amounts of phenolic substances excreted in the urine and feces of normal rats. In each instance, the results show the number of milligrams excreted by one rat during a 24 hour period and represent an average of the values obtained on ten different samples. The important thing shown by these two tables is that about 55% of the free phenols excreted, and about 40% of the total phenols excreted are excreted in the feces. The amount of conjugated phenols excreted in the feces is comparatively small. Also, of the total amount of phenols excreted in the urine, about 50% of them are excreted as conjugated phenols, while only about 10% of the phenols excreted in the feces are conjugated.

Tables No. 3, 4, and 5, when considered in the order named, constitute a complete balance of phenolic substances as obtained from the four day metabolism tests described above. The results are all expressed on the basis of one rat day. As was shown by previous investigators, the total amount of ingested phenol could not be recovered. The per cent of the ingested phenol that was recovered decreased considerably as the amount of the phenol ingested increased. This tends to verify the fact that the body has means other than conjugation for detoxifying phenols.

Table No. 3

SOURCE OF PHENOLIC INTAKE

Phenol Content P.P.M.	Water Intake ml.	Water Phenol Intake mg.	Feed Intake gm.	Feed Phenolic Compounds mg. Total	Feed Phenolic Compounds mg. Free	Feed Phenolic Compounds mg. Conj.	Total Phenolic Intake, mg. Total	Total Phenolic Intake, mg. Free	Total Phenolic Intake, mg. Conj.
0	11.75	0	13.14	5.08	1.97	3.11	5.08	1.97	3.11
500	14.75	7.37	13.28	5.14	1.99	3.15	12.51	9.36	3.15
1000	15.00	15.00	15.41	5.96	2.31	3.65	20.96	17.31	3.65
2000	10.00	20.00	12.60	4.88	1.89	2.99	24.88	21.89	2.99
3000	13.25	39.75	11.88	4.59	1.78	2.81	44.34	41.53	2.81
4000	12.25	49.00	11.72	4.54	1.76	2.78	53.54	50.76	2.78
5000	8.75	43.75	12.92	5.00	1.94	3.06	48.75	45.69	3.06
6000	13.12	78.72	8.69	3.36	1.30	2.06	82.08	80.02	2.06
8000	12.75	102.00	9.79	3.79	1.47	2.32	105.79	103.47	2.32

Table No. 4

AMOUNTS OF PHENOLIC COMPOUNDS EXCRETED

Phenol Content of Water P.P.M.	Excreted in Urine mg.			Excreted in Feces mg.			Total Phenolic Compounds Excreted mg.		
	Total	Free	Conj.	Total	Free	Conj.	Total	Free	Conj.
0	4.49	2.18	2.30	3.20	2.86	0.34	7.69	5.04	2.64
500	5.69	2.09	3.60	3.26	2.66	0.60	8.95	4.75	4.20
1000	7.59	2.47	5.12	3.99	3.61	0.38	11.58	6.08	5.50
2000	6.19	2.56	3.63	5.35	3.99	1.36	11.54	6.55	4.99
3000	10.59	2.50	8.09	4.22	3.59	0.63	14.81	6.09	8.72
4000	12.63	2.94	9.69	3.74	3.17	0.57	16.37	6.11	10.26
5000	10.16	3.31	6.85	4.25	3.43	0.82	14.41	6.74	7.67
6000	12.10	4.59	7.51	5.53	4.40	1.13	17.63	8.99	8.64
8000	7.28	4.13	3.15	4.21	3.80	0.41	11.49	7.93	3.56

Table No. 5

RELATIVE PATHS OF EXCRETION AND THE BALANCE FOR PHENOLIC COMPOUNDS

Phenol Content of Water P.P.M.	Per Cent Excreted in Feces			Per Cent Excreted in Urine			Balance Phenols Ingested Minus Phenols Excreted, mg.		
	Total	Free	Conj.	Total	Free	Conj.	Total	Free	Conj.
0	41.6	56.7	12.87	58.4	43.3	87.1	-2.61	-3.07	0.47
500	36.5	56.0	14.30	63.5	44.0	85.7	3.56	4.61	-1.05
1000	34.5	59.4	6.92	65.5	40.6	93.1	9.38	11.23	-1.86
2000	46.4	60.9	27.20	53.6	39.1	72.8	13.40	15.33	-1.99
3000	28.5	58.9	7.23	71.5	41.1	92.8	29.53	34.82	-5.91
4000	22.8	51.9	5.50	77.2	48.1	94.5	37.18	44.64	-7.47
5000	29.5	50.8	10.70	70.5	49.2	89.3	34.34	38.95	-4.61
6000	31.4	48.9	13.10	68.6	51.1	87.0	64.48	71.06	-6.57
8000	36.6	47.9	5.12	63.4	52.1	88.6	94.30	95.54	-1.24

Table No. 6

FREE AND CONJUGATED PHENOLS EXCRETED
IN THE URINE AND FECES EXPRESSED AS PER CENT
OF THE SUM OF THE TOTAL PHENOLIC SUBSTANCES EXCRETED

Phenol Content of Water P.P.M.	Urine		Feces	
	Free	Conj.	Free	Conj.
0	28.3	29.9	37.2	4.42
500	23.4	40.1	29.7	6.70
1000	21.3	44.2	31.1	3.30
2000	22.2	31.4	34.6	11.70
3000	16.9	54.6	24.2	4.30
4000	18.0	59.2	19.4	3.40
5000	23.0	47.6	23.7	5.70
6000	26.0	42.5	24.9	6.40
8000	35.9	27.5	33.1	3.54

It has been pointed out by Folin and Denis (9), and by Pelkan and Whipple (18) that the body apparently has the ability to oxidize benzene to phenol. The hexa-hydroxi derivative of cyclo-hexane, inosite, has been found in normal urines. These two facts suggest the possibility that either one, or both of two means may be used by the body to detoxify phenols. It may be that the body forms inosite from the excess phenol. This inosite may be considered as an addition derivative of benzene as well as a substitution derivative of cyclo-hexane. Inosite is insoluble in ether and will not react with the phenol reagent. Consequently it will not appear in the phenol determinations.

The other possible means of detoxifying phenol is that of more complete oxidation. The body may attempt to further oxidize the pyrocatechin that has already been formed from phenol. Further substitution of hydroxyl groups in the ring of pyrocatechin will produce an unstable molecule that breaks down, allowing complete oxidation of the benzene ring. This is contrary to the general belief that the body cannot oxidize the benzene ring, but the possibility has been mentioned by Macleod in the seventh edition of his "Physiology in Modern Medicine." It may be that hippuric acid is formed from this unstable compound produced by the oxidation of phenol, as is the case for benzoic acid, benzyl alcohol, and phenylpropionic acid. Also, it seems entirely possible that large quantities of the volatile phenols may be thrown off by means of the lungs.

The phenol in the drinking water seems to have little effect on the amount of water consumed by the rats. The amount of feed consumed by the rats remained practically constant until the phenol

content of the water passed 5000 P. P. M.; after this point the amount decreased. The amount of free phenols excreted in the urine increased only slightly until the drinking water contained as much as 5000 P. P. M. phenol. The increase in the total phenols excreted in the urine is accounted for by the fact that the amount of conjugated phenols in the urine increases as the phenol content of the water increases. The amounts of total, free, and conjugated phenols in the feces all have a tendency to remain constant. However, the figures show that excessive amounts of ingested phenol will force up the amounts of these three substances that are excreted in the feces.

Table No. 6 shows the free and conjugated phenols excreted in the urine and in the feces expressed as per cent of the sum of the total phenolic substances excreted. This table shows that of the increase in the phenols excreted, due to increasing the phenol content of the drinking water, the greater part of this increase is accounted for by the increase in the conjugated phenols in the urine. Another important point brought out by this table is the fact that as the phenol content of the drinking water passes 5000 P. P. M., the per cent of the total phenols excreted as free phenols increases, while the per cent excreted as conjugated phenols decreases. This is true for both feces and urine although it is not so pronounced in the feces. It tends to show that when the rat is drinking phenol water at a concentration of 5000 P. P. M. or more, the capacity of its body to conjugate and detoxify phenolic compounds is overtaxed. This fact verifies observations already stated.

During the completion of the phenolic balance just described,

the idea presented itself that a true balance could not be arrived at without analyzing the feeds for any possible substances that might react with the phenol reagent. As a result, the method as used on the feces was applied to the feed, and it was quite surprising to find a definite blue color developed. Since the feed used was made up of a mixture of various ingredients, the next task was to determine which of these materials supplied the substances that reduced the phenol reagent. The results of these individual tests are shown in table No. 7. The results are calculated in mg. per gram of feed and are expressed in terms of phenol, as a phenol standard was used.

Table No. 7
RESULTS OBTAINED WHEN FEEDS ARE
ANALYZED FOR PHENOLIC SUBSTANCES

Substance Analyzed	Total Phenols mg./gram	Free Phenols mg./gram	Conjugated Phenols mg/gram
Mixed feed	0.387	0.150	0.237
Wheat Bran	0.600	0.130	0.470
Cotton Seed Meal	0.313	0.174	0.139
Alfalfa Hay	0.500	0.351	0.149
Butter Milk (dried)	0.392	0.392	0.000
Corn Meal	0.445	0.191	0.254
Tankage	0.088	0.108	-0.020

According to table No. 7, the feeds of animal origin do not contain the greater amount of phenolic substances. Also, the plant feeds of high protein content do not contain greater amounts of phenolic compounds. In fact, the exact opposite is true. Tankage proved to be the lowest in phenolic content, and cotton seed meal was next to

low. These two substances are the highest in protein content. These facts are exactly opposite to what was expected.

In passing, it might be well to add that the mixed feed also contained cod liver oil. Extreme difficulties were encountered when an attempt was made to analyze this oil. Consequently, no results are listed for this ingredient of the feed.

Throughout this paper the results obtained when the feeds were analyzed have been called phenols. This was done so that the tabulation and correlation of data could be simplified. This naming may be incorrect as it is not logical that the substances that reduce the phenol reagent are present in the feeds as phenolic compounds. Certainly the difference between the free and the total determinations cannot be properly called conjugated phenols.

It requires eight hours boiling in 20% hydrochloric acid to break down protein so that tyrosine and tryptophane will be present in forms that will reduce the phenol reagent. This shows that the so called conjugated phenols as obtained in the analysis of feed are not due to the hydrolysis of protein, as the acid concentration during the heating of the feed was only about 2% and the heating lasts for only 30 minutes, without boiling. Inspection of the data obtained on the feeds will verify this statement. Certainly any compound liberated by this mild acid treatment will be made available to the body by any ordinary digestion system. Consequently it was necessary to take these compounds into account when the phenol balance was being made. Also, there was the possibility that any one, or all of these so called phenolic substances present in the feed might pass through the body unabsorbed, or unchanged, or both. If this happened,

their presence would affect the results obtained on the urine and feces. The conjugated phenols of the feces may be due to these conjugated phenols of the feeds passing through the body unchanged. This again justifies the use of the phenolic content of the feed in striking the balance for phenolic substances.

As yet, no attempt has been made to determine the exact nature of the substances present in the feed that reduce the phenol reagent. However, after considering the method of analysis used, they may be confined to a rather narrow group of compounds. Some of them are soluble in water in the state in which they normally exist. Some of them may be hydrolyzed into water soluble compounds by heating over a water bath in the presence of 2% hydrochloric acid. They are not precipitated by sodium aluminum sulfate and lead acetate. All are more soluble in ether than they are in water, their sodium salts are insoluble in ether, and they reduce the phenol reagent to give the characteristic blue color.

The phenolic content of the blood of rats drinking phenol water was determined. Figure No. 1 shows a family of curves representing these results. The blood was drawn by means of heart puncture, and

Table No. 8
URIC ACID AND PHENOLIC CONTENT OF THE BLOOD
OF NORMAL RATS EXPRESSED IN MG. PER 100 ML. OF BLOOD

Value	Total Phenols	Free Phenols	Conjugated Phenols	Uric Acid
Maximum	6.47	4.48	2.32	1.78
Minimum	4.32	3.93	0.38	1.64
Average	5.494	4.167	1.327	1.74

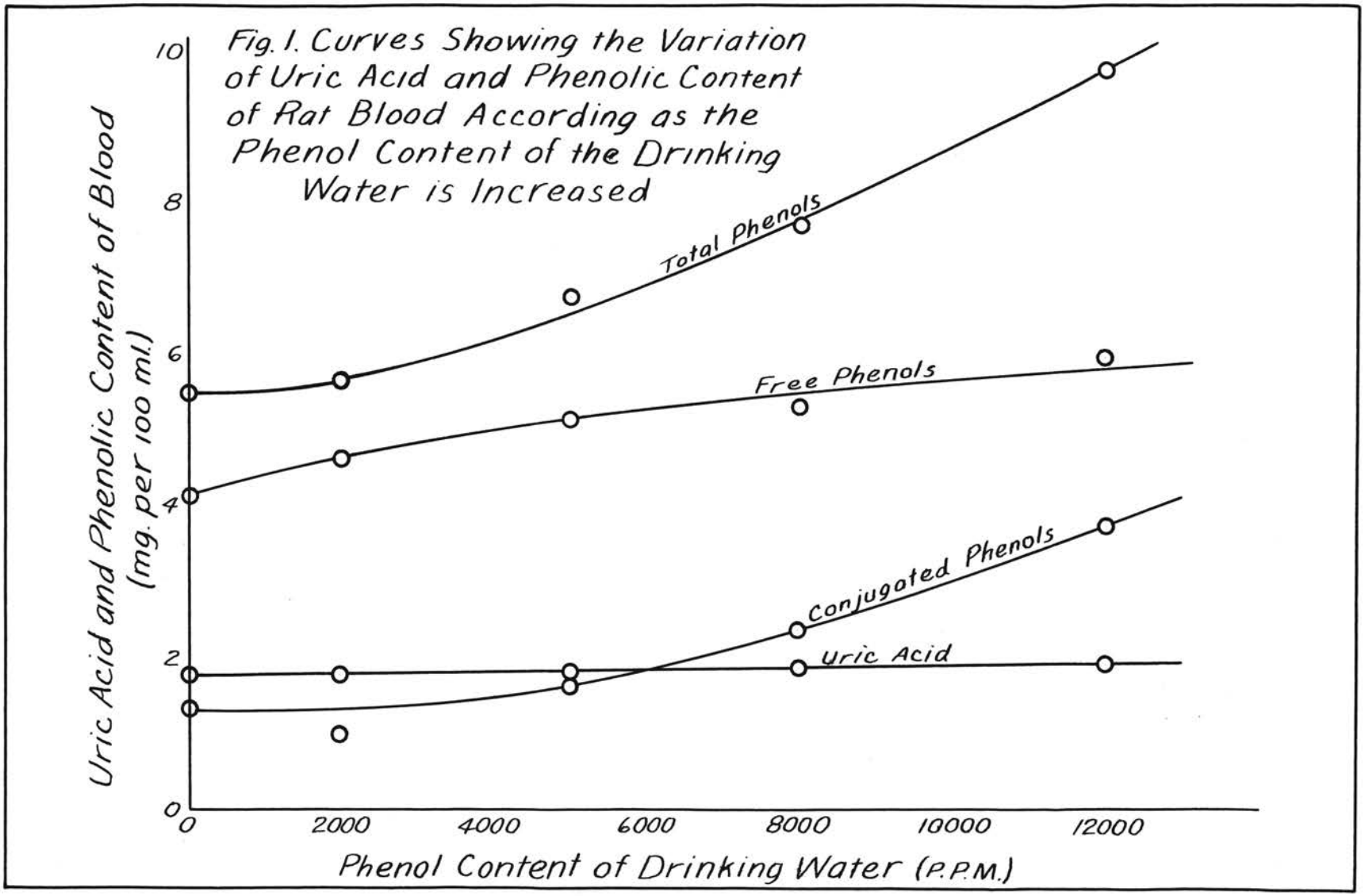


Fig. 1. Curves Showing the Variation of Uric Acid and Phenolic Content of Rat Blood According as the Phenol Content of the Drinking Water is Increased
Uric Acid and Phenolic Content of Blood (mg. per 100 ml.)
Total Phenols
Free Phenols
Conjugated Phenols
Uric Acid
0
2
4
6
8
10
0
2000
4000
6000
8000
10000
12000
Phenol Content of Drinking Water (P.P.M.)

was analyzed immediately, according to the instructions already given. The sample was analyzed for uric acid at the same time. These uric acid results are also shown in figure No. 1.

Table No. 8 gives the values obtained for phenols and uric acid when normal rat blood was analyzed. These results are given in mg. per 100 ml. of blood and were arrived at by analyzing ten different blood samples. They were used as a standard for comparison.

The phenolic content of the blood was fairly constant for rats on a given concentration of phenol water. However, as the phenol content of the water increased the total, free, and conjugated phenols of the blood increased. The total and conjugated phenols show a slight increase as phenol was first added to the drinking water; as the phenol in the water was increased in amount, the rate of increase of the total and conjugated phenols increased. The exact opposite is true for the free phenols. The rate of increase of the free phenols was greater when the smaller amounts of phenol were added to the water, while the rate of increase decreased as the amount of phenol in the water was increased. This shows that although the free phenols of the blood increased as the phenol content of the drinking water increased, they tended to approach a constant value.

Early in the progress of this investigation, evidence seemed to indicate that high uric acid accompanied high phenolic values. It was impossible to check the urine and feces for uric acid at the same time that they were analyzed for phenols, due to the length of time required for the phenol analysis. However, a uric acid determination was run on every blood sample that was analyzed for phenols, Folin's

direct method being used. In every case, the blood with the higher phenolic content also showed the higher uric acid content. The phenols do not interfere with the uric acid determination as they do not react with the uric acid reagent. Also, it can be shown that the uric acid was quantitatively removed from the phenolic determination by estimating the uric acid in the precipitate from the phenolic procedure. The results obtained will check within 99% the ones obtained by Folin's direct method on the same samples.

As far as could be ascertained, the time of day at which the blood was drawn had no effect on the amount of phenolic substances present.

The blood work was extended so as to include analyses made on the blood of chickens that had been drinking phenol water. Two lots, each containing four chickens, were provided. One lot received tap water while the other lot received 5000 P. P. M. phenol in the water. The chickens were allowed to remain on the phenol water for 30 days before the first blood samples were taken.

The blood samples were taken by heart puncture, deposited in a known volume of water, and then analyzed for phenols and uric acid immediately. The results obtained were recorded according to the age of the chickens and are shown in Table No. 9.

In chicken blood, as well as in rat blood, high uric acid always accompanied high phenolic values. There is one exception to this statement shown in Table No. 9, and that is in the results obtained at 17 weeks of age. However, at 17 weeks the blood of the control chickens contained the larger amounts of phenols and uric acid, but the uric acid determinations seem unusually low in this instance.

Table No. 9

URIC ACID AND PHENOLIC CONTENT OF THE BLOOD
OF CHICKENS DRINKING PHENOL WATER
ALL RESULTS IN MG. PER 100 ML. OF BLOOD

Age Weeks	Control Chickens				Chickens on 5000 P.P.M. Phenol Water			
	Phenolic Substances			Uric Acid	Phenolic Substances			Uric Acid
	Total	Free	Conj.		Total	Free	Conj.	
14	11.86	9.31	2.55	5.84	10.00	7.88	2.12	3.92
15	9.27	7.10	2.17	5.92	10.83	7.83	3.00	6.15
17	10.30	7.87	2.43	3.75	9.16	7.20	1.96	2.89
18	7.20	5.74	1.46	3.07	9.47	7.40	2.07	4.36
20	8.12	6.71	1.41	4.47	10.20	8.06	2.14	5.67
21	11.02	9.27	1.75	6.09	13.50	9.82	3.68	6.67

Even in the two cases where, for some unaccountable reason, the phenolic content of the blood of the control chickens exceeded that of the blood of phenol chickens, the uric acid was also higher in the blood of the controls.

While there is no regular variation with age of the phenolic content of chicken blood, the general trend is for the blood phenols to first decrease with age and then increase. This particular fact will be studied further.

SUMMARY AND CONCLUSIONS

A. The technique for analyzing feed, feces, urine and blood for phenolic substances has been developed in detail.

B. The ether extraction method may be used to analyze feeds and feces, thereby making it possible to apply the Folin and Denis phenol reagent to these substances as well as to urine and blood.

C. The amount of phenolic substances in the blood, urine and feces of normal rats has been established.

D. The amount of phenolic compounds excreted, when determined by Folin and Denis' method, is not a true index of the extent of putrefaction in the intestine due to the presence of the compounds in the feeds that give a blue color with this phenol reagent.

E. It has been proved that both the blood and the feces of rats contain appreciable amounts of conjugated phenols.

F. It has been proved that the uric acid content of the blood of rats and chickens increases as the phenolic content increases.

G. The fact that the body has means other than conjugation for

detoxifying phenol has been verified.

H. Several new means whereby the body may detoxify phenol have been suggested.

I. A method whereby a phenol standard may be made permanent has been developed.

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