DETERMINATION OF CITRULLINE AND ALLANTOIN AND DEMONSTRATION OF CITRULLINE IN BLOOD PLASMA

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I. CITRULLINE

Fearon (1) introduced the use of diacetylmonoxime, \( \text{CH}_3\cdot\text{C}:\text{NOH}\cdot\text{CO}:\text{CH}_3 \), in strongly acid solution (trichloroacetic) as a reagent for the colorimetric determination of citrulline. The color produced is a reddish yellow peach shade. Fearon recognized that allantoin and urea, when present, vitiated the results by producing colors which are somewhat more yellowish.

Gornall and Hunter (2) used Fearon's reaction to measure citrulline after removal of urea with urease. They realized that the urease contributed a rather appreciable blank. We have found that allantoin or allantoic acid and canavanine present in commercial preparations of urease are responsible for most, if not all, of this blank and have removed these materials by dialysis (3). When dialyzed urease is used to destroy urea, no urease blank need be run.

Interference by allantoin is prevented by adsorbing the citrulline with Amberlite at pH 6 to 7. Allantoin is not adsorbed. The difference in chromogenic material before and after adsorption is a measure of the citrulline. Interference by other plasma extractives besides allantoin is also eliminated by this procedure.

It is desirable to keep at a minimum the color formed by allantoin in the mixture analyzed before adsorption of the citrulline. This object has been attained by forming the color in a solution acidified with a 1:3 mixture of sulfuric and phosphoric acids, and heating for 10 minutes. Under these conditions color development from citrulline is almost maximal, while the amount of color formed by allantoin is only about one-fiftieth as much as that from equinolar amounts of citrulline.

The nature of the carbamido-diacetyl reaction and the structure of the products formed remain unknown. Formation of the colored product is apparently favored by the presence of an oxidizing agent and to some extent by light. The product, however, is at the same time photolabile and is destroyed by excess oxidant. Color formation takes place slowly at room temperature.

The following observations on the nature of the alternative reagents and the subsequent discussion of the specificity of the reaction relate to
the mechanism of the reaction only in so far as they characterize the structure of the reactants.

Diacetyl, $\text{CH}_2\cdot\text{CO} \cdot \text{CO} \cdot \text{CH}_3$, or diacetyl-\textit{dioxide}, $\text{CH}_3\cdot\text{C} : \text{NOH} \cdot \text{C} : \text{NOH} \cdot \text{CH}_3$ (dimethylglyoxime), and diacetyl-\textit{monoxide}, each in equimolecular concentrations, when heated in acid solution with a given carbamido compound give colors with absorption curves of the same shape and with maxima at the same wave-length. However, the optical density is slightly greater when diacetyl-\textit{monoxide} is used. Benzoylacetetyl, $\text{C}_6\text{H}_5\cdot\text{CO} \cdot \text{CO} \cdot \text{CH}_3$, and its \textit{monoxide}, $\alpha$-\textit{isonitrosopropiophenone}, $\text{C}_6\text{H}_5\cdot\text{CO} \cdot \text{C} : \text{NOH} \cdot \text{CH}_3$, also react with carbamido compounds, and the use of $\alpha$-\textit{nitosopropiophenone} has proved of value in the determination of urea, as will be shown in a subsequent publication. It offers, however, no advantage for the determination of citrulline.1

Lang observed (4) that the diacetyl color reaction with guanidine derivatives in alkaline solution takes place only when an alkyl group is present on at least one end of the diketo group. We have found the same to be true of the diacetyl color reaction with carbamido derivatives in acid solution. Neither benzil, $\text{C}_6\text{H}_5\cdot\text{CO} \cdot \text{CO} \cdot \text{C}_6\text{H}_5$, nor $p$-benzoquinone (which may be considered to be a vinylogue of diacetyl) gives color when heated in acid with citrulline, urea, or allantoin.

A number of the commercially available zeolites and cation exchange resins adsorb substances from solution more or less selectively. The specificity of the adsorption depends largely on the basicity of the substances presented for adsorption and the affinity and to some extent the capacity of the adsorbent for basic groups. Of those substances which give a strong carbamido-diacetyl reaction, citrulline is the most basic one likely to be present in biological material. (Stronger bases such as arginine would be adsorbed but do not give the carbamido-diacetyl reaction.) The following procedures for the determination of citrulline and allantoin utilize these cation exchange materials and thereby increase the specificity of the methods. Conditions for pretreatment of adsorbents have been worked out which permit adsorption of citrulline but not of the less basic substances (such as allantoin).

When urease is used to remove urea, it is advantageous to have the enzyme act in the presence of KCN. Commercial preparations of urease contain a number of other enzymes, some of which alter the specificity of the method unless their action is inhibited by KCN. Although the use of crude crystalline urease (5) might largely overcome the need for using KCN, the convenience and economy of dissolving the dialyzed urease in cyanide solution have led us to adopt this alternative. Dried

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1 The color produced by reaction of citrulline with benzoylacetetyl or its monoxide is a gray-purple.
dialyzed urease (3) has been stored in an ice box for 2 years without loss of more than 10 per cent of its activity.

**Method for Citrulline**

*Apparatus—*
*Dialysis units* as described by Hamilton and Archibald (6).

*Adsorption columns.* Columns similar to the one illustrated in Fig. 1 are prepared. Amberlite IR-100 (analytical grade⁴) fills the bottom 2 inches of the column and the rate of filtration should not be faster than 3 drops per 2 seconds. Before use, each column is flushed in succession with 10 cc. of 10 per cent NaCl solution, 5 cc. of H₂O, 10 cc. of 12 N HCl, and 25 cc. of H₂O. It is then dried with 5 cc. of alcohol followed by 5 cc. of ether and a current of air. Acetone may be used in place of alcohol and ether. The columns may be used at least ten times before being recharged with fresh adsorbent if they are flushed out before each use with salt, acid, water, alcohol, ether, and air.

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⁴ Amberlite was obtained from the Resinous Products and Chemical Company, 225 West Washington Square, Philadelphia.
Zeo-Karb H may be used in place of Amberlite, and for some purposes may be preferred. In this case the newly charged columns are washed with 12 N HCl until the washings are no longer yellow and allowed to stand for 3 hours or overnight in contact with the HCl before being washed again with salt, acid, water, alcohol, and ether. This treatment with concentrated acid alters somewhat the properties of the adsorbent but renders it better suited to the needs of this analysis.

Reagents—

Sulfuric-phosphoric acid mixture, 1 volume of concentrated sulfuric acid and 3 volumes of syrupy phosphoric acid.

Diacetylmonoxime, 3 per cent solution in water.

0.02 N H₂SO₄ (approximate), 1.0 cc. of concentrated H₂SO₄ diluted to 1800 cc.

0.4 M KCN, pH 7.2. Dissolve 1 gm. of KCN in 18 cc. of H₂O. Add cautiously in a hood, with stirring, 20 cc. of M NaH₂PO₄. Store in an ice box.

Dialyzed urease prepared according to the directions of Archibald and Hamilton (3); 2 per cent solution in 0.4 M KCN buffered with NaH₂PO₄ to pH 7.2.

Stock standard. 10.00 mg. of citrulline per 100 cc.

Working standard. 0.01 mg. per cc.; 1 cc. of stock standard is diluted to 10 cc. with water.

Procedure

0.56 cc. of the 2 per cent urease is added to 4 cc. of plasma and the mixture allowed to stand at room temperature for 20 minutes to hydrolyze urea. (Plasma from uremic patients is allowed to incubate 1 hour.) 4 cc. of the plasma thus digested are then dialyzed for 2 hours against 10 cc. of 0.02 N H₂SO₄ in the apparatus described by Hamilton and Archibald (6). 4 cc. of the dialysate, 2 cc. of the sulfuric-phosphoric acid mixture, and 0.25 cc. of diacetylmonoxime reagent are pipetted into test-tubes and mixed. The remaining dialysate is adjusted with the help of indicator paper to a pH between 6.0 and 7.0 by addition of 18 N NaOH.

³ Zeo-Karb H is obtained from The Permutit Company, 330 West 42nd Street, New York.

⁴ Squibb’s double strength urease was employed for the preparation of the dialyzed urease.

⁵ Dilute sulfuric acid instead of water is used for dialysis in order to stop the action of enzymes present in the jack bean urease preparation before appreciable amounts of allantoin or uric acid have been converted to allantoic acid or to other compounds which give a positive carbamido-diacetyl reaction. When 4.0 cc. of the 4.56 cc. of urease digest are dialyzed against 10 cc., the protein-free solution, at equilibrium, is exactly a 1:4 dilution of the plasma concentration.
solution, and passed through an adsorption column to remove citrulline. 4 cc. of the filtrate are mixed with monoxime reagent and acid in the same manner as is the untreated dialysate. Standards and reagent blanks are prepared with 4, 2, 1, 0.5, or 0 cc. of standard solution and 0, 2, 3, 3.5, or 4.0 cc. respectively of water, and the same amount of acid and monoxime as was used for the samples. After mixing the solution, the tubes are capped with glass marbles or bulbs and heated in a boiling water bath, from which light is excluded. After exactly 10 minutes, they are set in a covered container to cool in water. Once the tubes are heated, it is essential that they be protected from light until readings are made. Approximately 10 minutes after the end of the heating period, the optical densities are read in a photometer with light of wave-length of 490 m. The photometer scale is set at zero optical density with the reagent blank.

Calculation for Citrulline—The optical density of each standard is plotted against the mg. of citrulline, and the citrulline equivalents of the samples are read from this curve. The citrulline equivalent of the Amberlite or Zeo-Karb filtrate is subtracted from the citrulline equivalent of the untreated dialysate to give the true weight of citrulline. Since 4 cc. of dialysate are equivalent to 1.00 cc. of plasma, the mg. of citrulline found in 4 cc. of the dialysate is multiplied by 100 to obtain the concentration in mg. per 100 cc. of plasma.

\[ \text{Mg. citrulline per 100 cc. plasma} = 100 (D - F) \]

\[ D = \text{citrulline equivalent (in mg.) of color obtained on heating 4 cc. of dialysate of urease-treated plasma} \]

\[ F = \text{citrulline equivalent (in mg.) of color obtained on heating 4 cc. of dialysate which has been treated with Amberlite or Zeo-Karb} \]

Discussion of Method

As pointed out by Gornall and Hunter (2), to obtain consistent results it is necessary to adhere rigidly to a given set of conditions. They noted in their method, as we did in ours, that a calibration curve is necessary, because optical density is proportional to concentration only over a narrow range and there is relatively too little color with the lower concentrations. If the solutions (after being heated) are exposed for a few minutes to light of ordinary laboratory intensity, appreciable fading of color results. The per cent of color lost in a given interval is greater the lower the concentration of citrulline. This light sensitivity is decreased by the presence of some unrecognized constituent (not glycine, arginine, ammonium carbonate, or glucose) of plasma. Hence unless tubes are protected from light, color development appears to obey Beer's law better when it takes place in plasma dialysates than when solutions of pure citrulline are used. For some purposes it has proved convenient to construct calibra-
tion curves by use of solutions of stock citrulline diluted with Amberlite or Zeo-Karb filtrates of dialysates of urease-treated plasma. If, however, the heated standards are protected from light, dilution of citrulline with distilled water has proved adequate.

The advantages of this method over those previously published are as follows: A high yield of a concentrated protein-free filtrate of plasma or its equivalent is available by use of the dialysis technique. By use of dialyzed urease, the high blanks obtained with commercial preparations are avoided. By use of a high concentration of KCN during urease action, formation of chromogenic by-products from allantoin and uric acid is avoided. By use of the high concentration of phosphoric acid the color formed by action of the reagent on allantoin in 10 minutes is minimized. This same acid mixture is more satisfactory for development of color with citrulline than is either acid alone. Except for Abelin (7), who described a method for the rapid approximate determination of serum urea, previous investigators, using the carbamido-diacetyl reaction, have employed persulfate to achieve maximum color intensity. The use of this oxidant is attended subsequently by a rapid fading of color. When the heating takes place in a mixture of sulfuric and phosphoric acids, addition of persulfate becomes unnecessary and the color obtained fades much less rapidly than when persulfate is used.

Adsorption of the chromogen on Amberlite is an addition which increases enormously the specificity of the determination.

The method has been applied successfully to the determination of citrulline in dialysates of enzymatic hydrolysates of protein.

Attempts were made to simplify the determination of citrulline by passing plasma through Zeo-Karb, washing the columns with 0.3 per cent saline, then with water, then measuring citrulline in an acid eluate of the Zeo-Karb. However, under conditions which were adequate to achieve quantitative adsorption of citrulline from plasma, elution was incomplete.

Specificity of Citrulline Method

Fearon (1) and Gornall and Hunter (2) have considered in detail the specificity of the color formation with diacetylmonoxime in acid. It should be noted that to interfere with the specificity of the present citrulline method a substance after treatment with urease in KCN must (a) dialyze through cellophane, (b) cause appreciable absorption of light of wave-length 490 m\(\mu\) after being heated for 10 minutes under the con-

6 A small fraction of the urea present is adsorbed on Zeo-Karb H and is eluted with the citrulline by acid. The peach color of citrulline was therefore partly masked by the yellow from the urea. Nevertheless the absorption curve indicated that the citrulline-like compound was present in the eluate.
ditions specified for the analysis, and (c) be partly or completely adsorbed by Amberlite or Zeo-Karb. The peach color formed when citrulline is present is visibly different from the more yellow color formed with a number of other substances which give a positive carbamido-diacetyl reaction. The product formed with citrulline has an absorption maximum at wave-length 490 m\(\mu\). Citrulline is not attacked by urease and is completely adsorbed by the Amberlite or Zeo-Karb; these properties serve to separate citrulline from other plasma constituents that give color with diacetylimonoxime.

Urea is removed completely from normal plasma by 20 minutes incubation with the specified amount of urease. The ammonium carbonate formed from blood urea does not interfere with either the adsorption of citrulline or the diacetyl color reaction. The large amount of ammonia formed on incubating buffered urine with urease must be removed prior to adsorption of citrulline or the large excess of ammonium ion would interfere with the adsorption of the less basic citrulline. The removal of ammonia may be accomplished either by vacuum distillation at pH (8) or by passing the solution through a column of Decalso (Folin permutit) arranged as illustrated in Fig. 1. The Decalso does not adsorb citrulline in the presence of as much salt as is present in plasma dialysates and the amount used in the column for removal of ammonia removes insignificant amounts of chromogen from dialysates, and may be freed from ammonia for repeated use by treating it with 10 cc. of 10 per cent solution of NaCl followed by water.\(^7\)

Allantoin, \(\text{NH}_2\cdot\text{CO}\cdot\text{NH}\cdot\text{CH}\cdot\text{CO}\cdot\text{NH}\cdot\text{CO}\cdot\text{NH}\), like urea, yields a product having a yellow color with an absorption maximum at 480 m\(\mu\). However, because of the high concentration of phosphoric acid in the acid mixture used, the amount of color formed during 10 minutes heating is inappreciable unless relatively large quantities of allantoin are present. Further, allantoin is not adsorbed by Amberlite and only 5 to 8 per cent of that present is adsorbed by Zeo-Karb.

In the absence of KCN, Squibb's preparation of urease, even when partially purified by dialysis, forms from allantoin a substance yielding with diacetylimonoxime a product with an absorption curve indistinguishable from that obtained with citrulline. In the presence of 0.04 M KCN no chromogen was formed by the action of the urease preparation. Allantoin, therefore, does not interfere with the specificity of the citrulline determination.

\(^7\) As shown by Dubnoff (9), 3 per cent NaCl will elute arginine from Decalso. Ammonia, however, is but slightly eluted by 3 per cent NaCl, although almost completely by 10 per cent solution.
Uric acid does not form a colored product under the conditions of the analysis. In the absence of KCN the dialyzed preparation of urease used in the present analysis acting on uric acid forms a product which, on being heated with diacetylmonoxime, yields a colored product with an absorption curve like that given by urea or allantoic acid. In the presence of 0.04 M KCN, however, uric acid, like allantoin, yields no chromogens on treatment with urease. In this connection it may be noted that uricase and allantoinase have been said to occur in soy bean preparations (10, 11). Ling (12), however, says that soy bean contains no allantoinase. There appear to be no data in the literature indicating whether these enzymes accompany the urease preparation made from jack beans.

Allantoic acid, \((\text{NH}_2\cdot\text{CO}\cdot\text{NH})_2\text{CH}\cdot\text{COOH}\), does not appear to have been considered by previous workers who investigated the specificity of the carbamido-diacetyl reaction. Allantoic acid was prepared according to the directions of Young and Conway (13). On being heated for 10 minutes in the presence of phosphoric acid, it gives (unlike allantoin) appreciable color with diacetylmonoxime. The absorption curve of the product is similar to that obtained with urea, or with allantoin after prolonged heating. Allantoic acid is not adsorbed by Amberlite or Zeo-Karb, and hence does not interfere in the present citrulline analysis.

Alloxanic acid, \(\text{NH}_2\cdot\text{CO}\cdot\text{NH}\cdot\text{CO}\cdot\text{COOH}\), the first product of action of alkali on alloxan, also yields a positive carbamido-diacetyl reaction and a color similar to that given by allantoic acid. Alloxanic acid is not adsorbed by Zeo-Karb or by Amberlite.

Alloxan, \(\text{CO}\cdot\text{CO}\cdot\text{NH}\cdot\text{CO}\cdot\text{NH}\), heated under the conditions outlined for citrulline determination, yields a product with a golden yellow color, the shade of which is indistinguishable from that of the products from allantoic acid or urea. However, the intensity of the color is only 1/200 that of an equal weight of citrulline and the chromogen is not adsorbed by Zeo-Karb or Amberlite.

Alloxan reacts with KCN slowly at pH 6, rapidly at higher pH to yield a product which reacts with diacetyl yielding 70 times as much color in 10 minutes heating as is given by the alloxan from which it is derived. The chromogenic product is not urea. The maximal amount of the product formed by action of KCN on a given weight of alloxan yields with diacetyl one-third the amount of color given by an equal weight of citrulline. The absorption curve given by the product is similar to that given by urea and allantoic acid. This reaction with KCN is accompanied by liberation of 0.5 mole of CO\(_2\). In a concentration of 0.05 M KCN at pH 7 the reaction is complete within 2 minutes. The structure of the product is unknown. These findings are the bases of two of four methods for the determination of alloxan to be published shortly.
When treated with urease, alloxan forms a chromogen which yields a faint color that has an absorption curve like that given by citrulline, with its maximum at 490 m\(\mu\). The reaction proceeds slowly at room temperature and is not attended by a liberation of \(\text{CO}_2\). Neither alloxan nor the chromogen formed on treatment with urease or KCN is adsorbed by Zeo-Karb.

\textit{Alloxantin}, \((\text{CO} \cdot \text{NH} \cdot \text{CO} \cdot \text{NH} \cdot \text{CO} \cdot \text{C} \cdot \text{OH})_2\), is not adsorbed by Amberlite. Heating 1.652 mg. of it under the specified conditions for 10 minutes gives an optical density of 0.164 at wave-length 480 m\(\mu\). The color produced is a yellow like that given with urea.

\textit{Parahanic acid}, \((\text{NH} \cdot \text{CO} \cdot \text{NH} \cdot \text{CO} \cdot \text{CO})\), is not adsorbed by Amberlite. Heating 1.384 mg. under the specified conditions for 10 minutes gives an optical density of 0.940 at the wave-length of the absorption maximum, \textit{viz.} 480 m\(\mu\).

As pointed out by Gornall and Hunter (2) both \textit{methylurea} and \textit{phenylurea} yield with the diacetyl reagent colors which are almost indistinguishable from that obtained with citrulline. These compounds are partly removed from solution by the adsorbents and are the only compounds known to interfere appreciably with the specificity of the citrulline test. Zeo-Karb, even after overnight treatment with 12 N HCl, adsorbs 80 to 85 per cent of phenylurea from dilute aqueous solutions. Amberlite, on the other hand, adsorbs only 40 per cent under the same conditions. Amberlite also removes 50 per cent of the methylurea, whereas treated Zeo-Karb removes only 20 to 30 per cent.

As pointed out by Barker (14), \textit{thymol} interferes and gives with diacetylmonoxime a color resembling somewhat that obtained with citrulline. Unlike citrulline, thymol is only partially adsorbed by Zeo-Karb. \textit{Cresol} interferes less than does thymol and \textit{phenol} is almost without effect on the reaction.

Traces of \textit{protein} yield a pink color easily distinguished from that given by citrulline. It is a wise precaution to add a drop of 30 per cent sulfosalicylic acid to a 1 cc. aliquot of each dialysate to make sure that no protein has escaped from the dialysis cell.

\textit{Biuret}, both before and after treatment with urease, yields a positive carbamido-diacetyl reaction with a color similar to that given by urea.

\textit{Thiourea} yields no color with the diacetyl reagent during 10 minutes heating and almost inappreciable color during a 60 minute heating period.

Neither \textit{glutathione}, \textit{ergothioneine} (thioneine), \textit{glutamyltyrosine}, \textit{glycylglycine}, \textit{proline}, \textit{histidine}, \textit{carnosine},\textsuperscript{8} \textit{anserine},\textsuperscript{8} \textit{glutamine}, \textit{hippuric}

\textsuperscript{8} Carnosine and anserine were made available through the kindness of Dr. D. W. Wilson, University of Pennsylvania.
acid, p-aminohippuric acid, uric acid, benzimidazole, nor urethane gives color on being heated in the prescribed acid mixture with diacetyl reagent. Neither barbituric acid, phenobarbital, nembutal, caffeine, nor hydantoin, \( \text{NH} \cdot \text{CO} \cdot \text{NH} \cdot \text{CH}_2 \cdot \text{CO} \), gives appreciable color under the 10 minute heating conditions outlined for citrulline. When the heating period is prolonged or when the 2 cc. of phosphoric-sulfuric acid mixture are replaced by 18 \( \text{N} \) \( \text{H}_2\text{SO}_4 \), barbituric acid yields a light yellow color and hydantoin yields a color similar to that obtained with citrulline.

*Phenobarbital* and *nembutal* (sodium ethylmethylbutyl barbiturate) yield on 1 hour’s heating in the phosphoric-sulfuric acid mixture only 3 per cent of the color given by an equal weight of barbituric acid.

**Presence of Citrulline in Plasma**—Gornall and Hunter (2) were aware of the fact that blood filtrates contained small amounts of chromogenic material which was different from urea and citrulline. Ormsby (15) noted that the color obtained on heating blood filtrates in acid with diacetylmonoxime was not exactly the same as if urea alone were present and he mentioned “citrulline or other material” as the cause. Barker (14) noted that Decalso (Folin permufrit) removed from blood filtrates some of the chromogenic material which was not urea. He concluded, however, that the material removed was probably not citrulline. This conclusion was based on the shape of the absorption curve obtained with filtrates containing all of the urea. Had urea been removed, the presence of citrulline and (in the case of dog blood filtrates) allantoin would probably have been more apparent.

The conclusion that the chromogen measured in urease-treated plasmas as citrulline is indeed citrulline rests on the following evidence. (1) The color yielded by citrulline urea and phenylurea or high concentrations of hydantoin in the diacetyl reaction is distinctive. The difference in color obtained with urea or allantoic acid (golden yellow) on the one hand and citrulline (peach) on the other is more readily appreciated by the eye than by photoelectrometric readings. For the difference in color apparent to the eye the adsorption curves are remarkably close. (2) Of those few substances which yield a color similar to that obtained with citrulline, only citrulline is known to be removed completely by Amberlite or Zeo-Karb. Hydantoin, methylurea, and phenylurea are partly removed.

When fresh Zeo-Karb is pretreated with dilute sulfuric acid, instead of 12 \( \text{N} \) \( \text{HCl} \) as recommended in the above procedure, it removes all of the phenylurea and nearly all of the methylurea from solution, whereas Amberlite removes only 40 to 50 per cent of the phenyl- and methylurea. Since the amount of chromogen (10 minutes heating), left in the filtrate from urease-treated plasma dialysates is almost the same after treatment with Amberlite as it is after passage through sulfuric acid-treated Zeo-
Karb (which removes all of the phenylurea and nearly all of the methylurea), one may conclude that not more than an insignificant portion of the material measured as citrulline can be either phenylurea or methylurea.

Although 5 to 8 per cent of the allantoin present is adsorbed when Zeo-Karb is used, the optical density attained in 10 minutes heating of the analyzed solution is not thereby appreciably reduced unless relatively enormous quantities of allantoin are present. The color given by urease-treated dialysates of most human plasmas is indistinguishable from that obtained with citrulline. Almost all of the chromogen in dialysates of human plasma and all of the chromogen yielding the characteristic color in dog plasma dialysates are removed by Amberlite or Zeo-Karb.

Determinations of citrulline content in aliquots of dog and human plasma started within 30 minutes after the blood was drawn gave the same results as determinations on other aliquots of the same plasma after storage in the ice box for 1 and 2 weeks. This would suggest that the citrulline found was not the result of proteolysis or autolysis of the plasma protein. Nevertheless all the plasma values recorded in Table I were obtained on plasma from freshly drawn blood.

The possibility that the citrulline found in plasma arises from plasma protein or any other constituent of plasma as a result of the action of proteolytic enzymes in the urease preparation has been eliminated by the following two observations. (1) Plasma which had not been treated with urease was passed through Zeo-Karb and the column was washed with 0.3 per cent NaCl solution, then with water. The chromogen was eluted with a mixture of 1 part of the phosphoric-sulfuric acid mixture and 2 parts of water. The eluted material gave the peach color characteristic of citrulline when heated in acid with diacetylmonoxime.6 (2) Plasma was dialyzed against a large volume of water to remove preformed citrulline. The protein was then treated with urease in KCN for 1 hour and redialyzed. The amount of citrulline found in the second dialysate was inappreciable.

There is present, in the protein-free dialysates of some human and dog plasmas, a small amount of material which gives a color in the carbamido-diacetyl reaction that has an absorption maximum at a wave-length higher than that of citrulline (490 μ) and near that for plasma protein (500 to 510 μ). The dialysate of dog plasma represented in Fig. 2 is an example. The color may perhaps be due to a polypeptide or a di- or tripeptide. This component is partly adsorbed by Zeo-Karb and Amberlite. As a result some plasmas will yield an apparent citrulline value which is too high. The maximal positive error from this source is about 15 per cent; usually the error is negligible.

The fact that dialysates of urease-treated normal human plasma lose
by far the greater part of their chromogen (10 minutes heating) on passage through the adsorption columns indicates that (1) the total of the concentration of alloxanic acid, allantoic acid, biuret, methylurea, or phenylurea present in normal human plasma must be small, and (2) the concentration of KCN employed during the urease incubation is adequate to prevent formation of chromogen from allantoin and uric acid.

II. DETERMINATION OF ALLANTOIN IN PRESENCE OF CITRULLINE

When both allantoin and citrulline are present, the amounts of each may be determined without using more of the sample than is needed to determine citrulline alone.

Method

The apparatus and reagents are those described above for citrulline. Allantoin standard. A stock solution containing 0.10 mg. per cc. keeps at least a week if stored in the ice box.

Procedure

Standards containing allantoin instead of citrulline are set up as outlined above under the citrulline method. These are heated for 10 minutes, together with the citrulline standards, blank, and dialysates of samples (both with and without Amberlite treatment). Readings are taken at λ = 490 µm, and the citrulline is calculated as outlined above.

The tubes containing the blank, the allantoin standards, and the portions of the dialysates treated with Amberlite are then heated a second time, this time for 50 minutes. After being cooled in the absence of light, the transmittance or optical density is read at 470 µm. The standard curve for allantoin is constructed by plotting the mg. of allantoin in each known sample against the corresponding increase in optical density resulting from the additional 50 minutes heating. The allantoin equivalents of the samples after 60 minutes heating and after 10 minutes heating are read from the standard curve. The difference gives the figure for allantoin. The amount of allantoin found in 4 cc. of filtrate is the amount present in 1 cc. of plasma.

When allantoin determinations are to be made, the use of Amberlite as an adsorbent is preferable to the use of Zeo-Karb. Amberlite after being pretreated as specified does not adsorb allantoin. Pretreated Zeo-Karb, on the other hand, absorbs 5 to 8 per cent of the allantoin. Zeo-Karb nevertheless may be used if the allantoin found is multiplied by a factor which corrects for the allantoin lost in the column. If the columns are not pretreated with 12 N HCl and are washed instead with dilute H₂SO₄, nearly all of the allantoin as well as the phenylurea, methylurea, and hydantoin is adsorbed.
where

\[ \text{Mg. allantoin per 100 cc. plasma} = 100 \left( A_{60} - A_{10} \right) \]

\[ A_{60} = \text{the allantoin equivalent of the optical density read after 60 minutes heating,} \ \text{\(\lambda\) 470 m\(\mu\)} \]

\[ A_{10} = \text{the allantoin equivalent of the optical density read after 10 minutes heating,} \ \text{\(\lambda\) 490 m\(\mu\)} \]

The use of readings taken at wave-length 490 m\(\mu\), on the one hand, and 470 m\(\mu\), on the other, leads to inappreciable error, since both standards and samples are measured the same way and the absorption curve of allantoin over this range is not steep.

**Specificity of Allantoin Method**

The method is not absolutely specific for allantoin. By it any compound can be measured which after treatment with urease in cyanide is not adsorbed by Amberlite or Zeo-Karb H and which gives more yellow to pink color on being heated in acid with diacetyl reagent for 1 hour than is given in 10 minutes heating.

The molecular rings of cyclic derivatives of urea such as alloxan, hydantoin, barbiturates, and to a small extent purines such as caffeine open slowly during prolonged heating in acid and give relatively much more color after being heated 1 hour than after 10 minutes heating. Compounds such as allantoic acid, biuret, and the product resulting from the action of alloxan with cyanide give relatively much color after 10 minutes heating and therefore can contribute in a relatively small way to the lack of specificity of the allantoin method which depends upon the increase in color obtained between the end of the 10 and 60 minute heating intervals.

Methods for the determination of alloxan and evidence of absence of alloxan from normal dog and human plasma are to be given in a later publication. In any case any alloxan present in the sample would have been destroyed during the removal of urea by urease, by the action of the cyanide in which the urease was dissolved. The concentration of barbiturates resulting from medication will seldom if ever, even during a barbiturate anesthesia, reach a level which would interfere with the specificity of the allantoin determination. Uric acid even in the concentrations present in human plasma causes insignificant error. Caffeine yields very little color even after 1 hour's heating but could be a small source of error in plasmas of non-fasting patients.

The fact that slight though appreciable color is formed during the 10 minutes heating of dialysates of urease-treated plasma after treatment with Amberlite or Zeo-Karb leads one to the conclusion that in human and dog plasma there is present some material (other than urea, citrulline,
or allantoin) which gives the carbamido-diacetyl reaction on 10 minutes heating in the phosphoric-sulfuric acid mixture. The intensity of color from this material varies considerably from one plasma to another and is equivalent in the case of human plasma to 15 to 40 per cent of the citrulline present, and to 30 to 80 per cent of the citrulline in the case of dog plasma. The shade of color in both cases is similar to that obtained in the presence of allantoin after more prolonged heating. The presence of this material does not interfere with the specificity of the allantoin (or citrulline) measurement, since allantoin concentration is determined from the difference between the optical densities after 60 minutes and after 10 minutes heating.

It may be noted that the allantoin method of Young et al. (16) can be used to measure, in addition to allantoin, any allantoic acid present as well as any compound which during the course of the procedure employed by Young would give rise to glyoxylic acid. As pointed out by Christman et al. (17) the reaction employed in Young's method (16) gives positive results with uric acid and ergothioneine. Since neither of these compounds interferes in the diacetyl method, determination of their concentrations is unnecessary when this method is employed.

**Results**

The concentration of citrulline and "allantoin" in a number of blood plasmas from fasting human subjects is indicated in Table I. Corresponding values on normal fasting dogs under nembutal anesthesia appear in Table II. The 2- to 5-fold rise in citrulline content, the simultaneous marked depletion of circulating arginine, and the consequent depression of urea formation which occur in dogs in severe hemorrhagic shock when p-amino hippurate clearance is depressed until it is less than 0.5 per cent of its normal level is to be the subject of a subsequent communication.10

The absorption curves of a standard solution of citrulline and of dialysates of dog and human blood plasma are given in Fig. 2. The similarity in shape of the curves for citrulline and for plasma dialysates favors the conclusion that citrulline is present in blood. Amberlite filtrates of plasma dialysates yield essentially the same absorption curve as is given by Zeo-Karb filtrates. The curve given for the dog plasma dialysate is representative of a few in which maximal absorption occurred near λ 500 μm.

Ormsby (15) has already given the absorption curves of the products formed with urea, allantoin, and citrulline. For comparison, curves given by these and a number of other substances are shown in Fig. 3.

The rates of formation of color on heating dialysates of urease-treated plasmas and solutions of several pure substances under the conditions outlined above are indicated in Figs. 4 and 5. For convenience of comparison all readings were made at a wave-length setting of 470 mp.

**Table I**

*Citrulline and Allantoin of Fasting Human Plasma*

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Urea clearance per cent of normal</th>
<th>Citrulline mg. per 100 cc.</th>
<th>Allantoin mg. per 100 cc.</th>
<th>α-Amino nitrogen by ninhydrin mg. per 100 cc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>W. M.</td>
<td>12M.</td>
<td></td>
<td>Normal</td>
<td></td>
<td>0.48</td>
<td>0.3</td>
<td>3.78</td>
</tr>
<tr>
<td>S. R.</td>
<td>14</td>
<td></td>
<td>&quot;</td>
<td>130</td>
<td>0.55</td>
<td>0.5</td>
<td>3.27</td>
</tr>
<tr>
<td>C. S.</td>
<td>32</td>
<td></td>
<td>&quot;</td>
<td>114</td>
<td>0.48</td>
<td>4.17</td>
<td></td>
</tr>
<tr>
<td>R. A.</td>
<td>34</td>
<td></td>
<td>&quot;</td>
<td></td>
<td>0.48</td>
<td>3.96</td>
<td></td>
</tr>
<tr>
<td>W. G.</td>
<td>45</td>
<td></td>
<td>&quot;</td>
<td></td>
<td>0.57</td>
<td>0.6</td>
<td>3.80</td>
</tr>
<tr>
<td>J. D.</td>
<td>22</td>
<td></td>
<td>&quot;</td>
<td></td>
<td>0.38</td>
<td>3.72</td>
<td></td>
</tr>
<tr>
<td>J. A.</td>
<td>7F.</td>
<td></td>
<td>Healed nephritis</td>
<td>121</td>
<td>0.50</td>
<td>0.62</td>
<td>3.29</td>
</tr>
<tr>
<td>M. R.</td>
<td>5</td>
<td></td>
<td>Convalescent nephrotic</td>
<td>113</td>
<td>0.43</td>
<td>0.94</td>
<td>3.02</td>
</tr>
<tr>
<td>B. W.</td>
<td>15</td>
<td></td>
<td>Convalescent nephrotic</td>
<td>102</td>
<td>0.73</td>
<td>1.13</td>
<td>3.49</td>
</tr>
<tr>
<td>P. P.</td>
<td>43</td>
<td></td>
<td>Essential hypertension</td>
<td>150</td>
<td>0.55</td>
<td>0.91</td>
<td>3.34</td>
</tr>
<tr>
<td>L. W.</td>
<td>5</td>
<td></td>
<td>Nephrosis</td>
<td>120</td>
<td>0.35</td>
<td>0.02</td>
<td>3.38</td>
</tr>
<tr>
<td>J. H.</td>
<td>3M.</td>
<td></td>
<td>Acute nephritis</td>
<td>81</td>
<td>0.92</td>
<td>1.6</td>
<td>3.54</td>
</tr>
<tr>
<td>A. C.</td>
<td>37</td>
<td></td>
<td>Chronic nephritis</td>
<td>37</td>
<td>0.95</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>S. S.</td>
<td>37</td>
<td></td>
<td>&quot;</td>
<td>79</td>
<td>0.57</td>
<td>0.78</td>
<td>2.99</td>
</tr>
<tr>
<td>N. O.</td>
<td>19</td>
<td></td>
<td>&quot;</td>
<td>6</td>
<td>1.51</td>
<td>3.2</td>
<td>5.70</td>
</tr>
<tr>
<td>S. G.</td>
<td>14</td>
<td></td>
<td>Chronic nephritis</td>
<td>(Blood urea N 121 mg. per 100 cc.)</td>
<td>2.02</td>
<td>2.12</td>
<td>3.82</td>
</tr>
<tr>
<td>J. Mo.</td>
<td>19</td>
<td></td>
<td>Malaria</td>
<td>(chill)</td>
<td>0.82</td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td>J. Me.</td>
<td>19</td>
<td></td>
<td>&quot;</td>
<td></td>
<td>0.47</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td>M. R.</td>
<td>21</td>
<td></td>
<td>&quot;</td>
<td></td>
<td>0.60</td>
<td>0.80</td>
<td></td>
</tr>
</tbody>
</table>

* These values multiplied by 0.240 give the total citrulline N by 0.080 α-amino N.
† These values multiplied by 0.3544 give total allantoin N.

The influence of the concentration and character of the acid used for the diacetyl reaction with citrulline is indicated in Fig. 6. In this case all readings were made at 490 mp after 10 minutes heating. The abscissae indicate the concentration of acid in 4 cc. to which were added, in all
PLASMA CITRULLINE AND ALLANTOIN

**Table II**

*Citrulline and Allantoin of Fasting Dog Plasma*

<table>
<thead>
<tr>
<th>Dog</th>
<th>Citrulline mg. per 100 cc.</th>
<th>&quot;Allantoin&quot; mg. per 100 cc.</th>
<th>α-Amino N by ninhydrin mg. per 100 cc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.20</td>
<td></td>
<td>3.45</td>
</tr>
<tr>
<td>B</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>1.28</td>
<td>2.11</td>
<td></td>
</tr>
<tr>
<td>K-67</td>
<td>1.00</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>K-68</td>
<td>1.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K-69</td>
<td>0.92</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>K-70</td>
<td>1.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K-71</td>
<td>1.14</td>
<td>3.0</td>
<td>3.66</td>
</tr>
</tbody>
</table>

**Fig. 2.** Absorption curve of urea-free dog and human plasma dialysates before and after treatment with Zeo-Karb. 4 cc. of dialysate or Zeo-Karb filtrate (equivalent to 1 cc. of plasma) heated 10 minutes at 100° with 2 cc. of sulfuric-phosphoric acid mixture and 0.25 cc. of 3 per cent diacetylmonoxime reagent. Initials of patients are indicated on the curves to enable comparison with the data entered in Table I. In cases, 0.1627 mg. of citrulline in 2 cc. of water and 0.25 cc. of the diacetylmonoxime reagent. Since, in the procedure outlined above, only 2 cc. of phosphoric-sulfuric acid mixture are used, the point corresponding
FIG. 3. Absorption obtained with substances giving the carboximido-diaceetyl reaction. The weight indicates the amount present in the 6.25 cc. of mixture heated 10 minutes at 100°.

FIG. 4. Rate of formation of color at 100° in the carboximido-diaceetyl reaction with pure substances. Volume 6.25 cc.

to the conditions specified for the citrulline procedure is at X. The lighter curves indicate the intensity of color obtained when 0.25 cc. of 1 per cent potassium persulphate was added after the heating. After addition of persul-
FIG. 5. Rate of formation of color at 100° in the carbamido-diacetyl reaction, before and after removal of citrulline with Zeo-Karb, from a dialysate of urease-treated plasma of a normal fasting dog. 4 cc. of dialysate or Zeo-Karb filtrate in a total volume of 6.25 cc.

FIG. 6. Effect of amount and kind of acid used in the carbamido-diacetyl reaction with citrulline. Heated 10 minutes at 100°.
### Table III

*Effect of Light (Diffuse Daylight) on Development and on Stability of Color Obtained with Citrulline*

<table>
<thead>
<tr>
<th>Citrulline in 6.25 cc. solution</th>
<th>Optical density at $\lambda = 490$ mp</th>
<th>Time standing at room temperature after 10 min. heating</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$5$ min.</td>
<td>$15$ min.</td>
</tr>
<tr>
<td>$mg.$</td>
<td>Dark</td>
<td>Dark</td>
</tr>
<tr>
<td>0.1627</td>
<td>0.975</td>
<td>0.980</td>
</tr>
<tr>
<td>0.0813</td>
<td>0.575</td>
<td>0.585</td>
</tr>
<tr>
<td>0.03254</td>
<td>0.215</td>
<td>0.222</td>
</tr>
<tr>
<td>0.01627</td>
<td>0.089</td>
<td>0.094</td>
</tr>
<tr>
<td>0.00814</td>
<td>0.039</td>
<td>0.042</td>
</tr>
</tbody>
</table>
fate the color fades rapidly, especially in the absence of sulfuric acid. The effect of light on the fading of the color obtained in the carbamido-diacetyl reaction in the absence of persulfate and the stability of this color in the dark are indicated in Table III.

Fig. 7 indicates the range of concentration over which Beer's law is approximated. Above a concentration of about 0.0005 mm per 6.25 cc. of mixture, additional amounts of the urea derivative cause relatively little increase in color. Curves obtained with methylurea when double and half the usual amounts of diacetylmmonoxime reagent in the same vol-

![Graph showing the relationship between concentration and optical density](image)

**Fig. 7.** Relation between concentration of the carbamido compound and amount of color formed in the reaction with diacetylmmonoxime. Conditions were as prescribed in the procedure for analysis.

Both citrulline and allantoin when added to plasma have been "recovered" by the above procedure with an error of less than ±5 per cent. This by itself does not mean, of course, that the material measured in either fraction is entirely citrulline or entirely allantoin. Christman et al. (17) have recently applied the principle of Young’s method to the
measurement of allantoin in human blood. After correcting for the uric acid and ergothioneine present, they obtained a negative value for allantoin. Similar negative values were obtained with whole horse blood, although results obtained with horse plasma indicated the presence of allantoin. At present there is insufficient evidence to indicate whether an overcorrection by Christman has led him to miss allantoin present in human plasma or whether the positive allantoin figures given by the method here described include "allantoin-like" substance. In any case the present method, unlike that of Christman, does not give positive results with either uric acid or thioneine. It is probable that the values given by this method for plasma allantoin are somewhat too high.

The curves presented were obtained with a Coleman junior spectrophotometer. The location of each absorption maximum was verified by using the Coleman spectrophotometer No. 10S. For convenience of reference and comparison of results a constant for the cuvette used is given.

A solution of CuSO₄·5H₂O m/80 in 2 N NH₄OH read at a wave-length of 620 mp against 2 N NH₄OH had an optical density of 1.15. According to Drabkin and Austin (18) the extinction coefficient of m/80 copper ammonium sulfate under these conditions is 58.

The author wishes to acknowledge the helpful suggestions of Dr. D. D. Van Slyke in whose laboratory the work was conducted, the help of Dr. R. A. Phillips and Dr. P. B. Hamilton in obtaining specimens of dog blood, and the technical assistance of Miss E. Stroh and Miss P. Ortiz.

SUMMARY

1. A method for the determination of citrulline in plasma is outlined. The method has been applied also to the determination of citrulline in dialysates of enzymatic digests of proteins.

2. The specificity of the carbamido-diacetyl reaction has been investigated further and its usefulness extended by including a fractionation with Amberlite or Zeo-Karb H.

3. Evidence is presented which indicates that the normal fasting plasma level is between 0.3 and 1.0 mg. of citrulline per 100 cc. for man and 0.8 to 1.5 mg. per 100 cc. for the dog.

4. A simple method for the determination of allantoin in plasma is included. This method is uninfluenced by the presence of uric acid or thioneine, but is not entirely specific for allantoin.

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