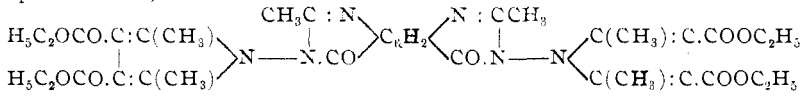
2,8-Dimethyl-3,7-di(2,5-dimethyl-3,4-dicarbethoxypyrrole)-4,6-diketotetrahydro-1,3,7,-9-naphthotetrazine,

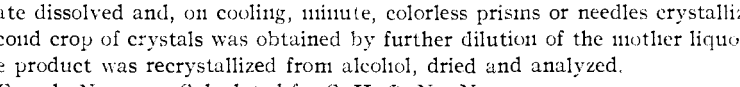


—The diaminonaphthotetrazine was boiled for an hour with a glacial acetic acid solution of the calculated amount of ethyl diacetosuccinate. On concentrating, some unchanged naphthotetrazine separated. This was removed and the mother liquor diluted with water. A white, amorphous precipitate appeared. On boiling the solution, the precipitate dissolved and, on cooling, minute, colorless prisms or needles crystallized out. A second crop of crystals was obtained by further dilution of the mother liquor. The crude product was recrystallized from alcohol, dried and analyzed.

Found: N, 12.1. Calculated for $C_{36}H_{40}O_{10}N_6$: N, 11.75.

The pure compound melts at 268.2° (corr.).

3,7-Diphenyl-2,4,6,8-tetraketo-octahydro-1,3,7,9-naphthotetrazine (*3,7-Diphenyl-2,8-dihydroxy-4,6-diketotetrahydro-1,3,7,9-naphthotetrazine*).



—When diethyl 4,6-diphenyluramino-*m*-phthalate was boiled with aniline, no change was evident. But when the two were heated together in a sealed tube for six hours at 225°, a reaction occurred accompanied by considerable decomposition. The crude product was boiled with glacial acetic acid, the solution filtered, and on cooling large, brownish-green crystals were obtained. These were washed with glacial acetic acid, then with water, dissolved in dilute potassium hydroxide solution and reprecipitated by carbon dioxide.

Found: N, 14.07. Calculated for $C_{22}H_{14}O_4N_4$: N, 14.07.

The pure compound is colorless and amorphous, melts above 300°, is apparently insoluble in water, alcohol or benzene, but dissolves in glacial acetic acid.

ORGANIC LABORATORY, June, 1909.

[FROM THE LABORATORY OF PHYSIOLOGICAL CHEMISTRY, DEPARTMENT OF ANIMAL HUSBANDRY, UNIVERSITY OF ILLINOIS.]

THE DETERMINATION OF UREA IN URINE.

NUTRITION INVESTIGATIONS, PUBLICATION NO. 26.

BY F. W. GILL, F. G. ALLISON, AND H. S. GRINDLEY.

Received July 6, 1909.

The method of Folin[1] for the determination of urea in urine has been very generally used since it was first described. It has been conclusively proven repeatedly that Folin's method gives quite uniform results when properly carried out with due regard to all details. On the other hand, the method requires very careful work, and it is long and tedious, requiring close attention continuously. The difficulties attending the accurate determination of urea in urines by the Folin method have been espe-

[1] *Z. physiol. Chem.*, **32**, 504 (1901); **36**, 333 (1902); **37**, 548 (1903); *Amer. Jour. Physiol.*, **13**, 45 (1905).

cially pointed out by Kober,[1] by Benedict and Gephart,[2] by Ronchese[3] and C. G. L. Wolf and E. Osterberg.[4]

The method for the estimation of urea described by Benedict and Gephart,[3] which consisted in heating the urea-containing solutions to 140–160° in an autoclave and then distilling the resulting solution after dilution, with 20 cc. of a 10 per cent. solution of sodium hydroxide, apparently had a number of advantages as compared with the Folin method. Benedict and Gephart noted that their method gave higher urea values than did the method of Folin, and called attention to the fact that the higher results obtained must indicate either that the autoclave procedure decomposes other nitrogenous urinary constituents than urea, or that Folin's process fails to obtain all of the urea nitrogen as ammonia. They found as a result of the examination of six samples of human urine that the urea as determined by the Benedict and Gephart method formed upon an average 103.86 per cent. of the urea as determined by the Folin method. They did not prove experimentally the reason for the higher results which were obtained by their method as compared with Folin's method. However, they did demonstrate experimentally that their method, if carried out in detail in the presence of magnesium chloride, gave a urea value as a result of the average of six examinations of human urine, which amounted to 100.62 per cent. of the Folin's values upon the same samples. This value is within the limits of experimental error accompanying the determination of urea in urine by the Folin method.

Later Wolf and Osterberg in comparing the Folin and the autoclave method of Benedict and Gephart confirmed the work of the latter investigators, namely, that the autoclave method invariably gives higher results than the magnesuim chloride method of Folin. They found as a result of the examination of six samples of human urine that the urea as determined by the Benedict and Gephart method formed upon an average 101.74 per cent. of the urea as determined by the Folin method. These same investigators proved that pure creatinine and uric acid are decomposed in part, yielding as one product ammonia, under the conditions attending the application of the Benedict and Gephart method. Indirectly their experiments indicated that this decomposition of the creatinine occurred in the hydrolysis in the autoclave and not in the distillation process with sodium hydroxide. As a result of their work, Wolf and Osterberg drew the following conclusions: "Benedict and Gephart's method cannot be used for the accurate determination of urea in urine. Urea is quantitatively decomposed, but at the same time uric

[1] THIS JOURNAL, 30, 1279 (1908).
[2] *Ibid.*, **30**, 1760 (1908).
[3] *Bull. Soc. Chim.* [4], **3**, 1138 (1908).
[4] THIS JOURNAL, 31, 421 (1909).

acid and creatinine yield ammonia by this method. To the decomposition of these substances is due the high results which these authors obtained when their method has been compared with that of Folin."

Still later Levene and Meyer[1] confirmed the work of Wolf and Osterberg in that they also found that under the conditions of hydrolysis required by the autoclave treatment of Benedict and Gephart, not only urea, but also uric acid and creatinine suffer hydrolysis. They maintained that results obtained by the method of Benedict and Gephart were satisfactory for urines with a minimal content of uric acid and creatinine, such as dog's urine. On urines of normal man and of dogs in pathological conditions, the method could not be applied without further modification. In this same connection these authors suggested a modification of the Benedict and Gephart method which consists in the precipitation of the creatinine, uric acid and ammonia with phosphotungstic acid before hydrolysis in the autoclave and distillation with sodium hydroxide according to the Benedict and Gephart method. In connection with their paper they report six distinct analyses of the same samples of human urine by this modified method, three analyses of the same urine by Folin's method, and three analyses of the same urine by the Benedict and Gephart method. It is of interest to note, that the urea as determined by the Benedict and Gephart method formed 99.66 per cent. of the urea as determined by the Folin method, and further the urea as determined by the method which they suggest formed only 93.9 per cent. of the urea as determined by the Folin method.

A close study of the work of Wolf and Osterberg, and Benedict and Gephart makes it difficult to understand how the amount of decompositions which the former investigators found in creatinine and uric acid could account for more than 15 to 20 per cent. of the actual difference between the urea nitrogen values as determined by the two methods. Judging from the results of Wolf and Osterberg, and Benedict and Gephart, together with those obtained in this laboratory, it seems more than probable that the substances giving rise to the so-called undetermined nitrogen are also in part, at least, decomposed by the methods of hydrolysis and distillation suggested by Benedict and Gephart. It was with a view of getting a better understanding of these questions and with the hope that thereby a simpler, less tedious, and a more accurate method, than is now available, for the determination of urea in urines, might be devised, that these investigations were undertaken.

In a recent article[2] from this laboratory, during the proof-reading, we inserted the following conclusions as to the results which we had obtained at that time, in studying these methods for the determination of

[1] THIS JOURNAL, 31, 717 (1909).

[2] *Ibid*, 31, 695 (1909).

urea in urine. First, that creatinine is not at all decomposed by heating in the autoclave with 1:4 hydrochloric acid, according to the Benedict and Gephart method. Second, that creatinine, either before or after treatment with the hydrochloric acid in the autoclave, is partially decomposed into ammonia upon distillation with 20 cc. of 10 per cent. sodium hydroxide solution as in the Benedict and Gephart procedure. Third, that uric acid is decomposed in part by the autoclave treatment with 1:4 hydrochloric acid and, moreover, it is still further decomposed into ammonia by the distillation with 20 cc. of 10 per cent. sodium hydroxide solution. Fourth, that the so-called undetermined nitrogenous substances are also broken down, wholly or in greater part, into ammonia by the distillation with the sodium hydroxide and not during the autoclave hydrolysis with 1:4 hydrochloric acid, because by the application of the sodium carbonate aeration method to the autoclave-hydrolyzed urines, we obtained practically the same values for urea per 24 hours as were obtained by the Folin method. Fifth, we find that unhydrolyzed uric acid and also creatinine which has or has not been through the autoclave treatment do not by the sodium carbonate aeration process decompose in the least into ammonia.

It is the object of this paper to describe the experiments and to give the data upon which the above conclusions were based and also to describe a modification of the Benedict and Gephart method for the determination of urea in urines.

A Study of Creatinine and Creatine.—To begin with it seemed desirable to test thoroughly the influence of the Benedict and Gephart autoclave treatment and distillation process as used in the determination of urea upon solutions of creatinine and creatine. For this purpose 25 milligrams or more of either creatinine or creatine (it was found that they both behaved in a similar manner) were dissolved in 5 cc. of water. There were added to the solution 5 cc. of dilute hydrochloric acid (made by adding four volumes of distilled water to one volume of concentrated acid). The solution was then placed in the autoclave and kept at a temperature of 142–145° for a period of 90 minutes. After the autoclave had cooled, the contents of the tube were washed into a 500 cc. Kjeldahl flask, diluted to about 350 cc., treated with 15 or 20 cc. of 10 per cent. sodium hydroxide solution, and distilled into an excess of standard acid until the distillate measured about 275 cc. As a result of forty individual tests, the extent of the decomposition varied from 10.1 to 28.8 per cent., the average value being 16.6 per cent. The following figures represent the results obtained in ten of the individual tests: 17.4, 18.1, 13.0, 19.1, 13.4, 14.8, 16.5, 19.5, 12.9, and 17.0 per cent.

These results fully confirm those of Wolf and Osterberg which showed that creatinine when hydrolyzed in the autoclave and distilled with 20 cc. of 10 per cent. sodium hydroxide yields a considerable amount of ammonia. However, our results show a somewhat greater decomposition of this substance than do those of Wolf and Osterberg. This difference in the degree of the decomposition is undoubtedly due to the differences in the methods of procedure followed, in the hydrolysis, the dilution and the distillation. It has been proven in this laboratory, as will be shown below, that this decomposition of the creatinine is a gradual and continuous process and the amount

of the distillate collected and the time required for the distillation decidedly modify the extent of the decomposition resulting.

From the above results, it seemed desirable to test thoroughly the influence of the process of distillation in the presence of the fixed alkali as used in the Benedict and Gephart method, upon the creatinine and creatine contained in the solution. For this purpose 25 milligrams of either creatinine or creatine (it was found that they both behaved in a similar manner) were dissolved in 350 cc. of nitrogen-free water. Fifteen or 20 cc. of 10 per cent. sodium hydroxide solution were added and the distillation was made exactly as is usually done in the case of the Benedict and Gephart method. As a result of ten individual tests, the following figures which represent the percentage of creatinine or creatine decomposed, were obtained: 18.4, 23.8, 18.4, 16.9, 19.9, 13.1, 16.2, 13.8, 12.3, and 13.0 per cent. The average of these results is 16.8 per cent.

These results do not correspond to those of Wolf and Osterberg, who found in one experiment (No. 68), that creatinine when distilled with 500 cc. of water and 3 cc. of 10 per cent. sodium hydroxide solution underwent no decomposition. Unfortunately, Wolf and Osterberg report only one experiment in duplicate and it is impossible to make out clearly and definitely from their paper, the quantities of creatinine which were used in their test, since the only information available in their paper, as to this point, states that 0.010 cc. of creatinine was used. We have construed this to mean that 10 milligrams of creatinine were used for each test. Again, while it is not evident from their communication, we deemed it probable, that they dissolved this quantity of creatinine in water, diluted the solution to a volume of about 400 cc. and distilled with 20 cc. of 10 per cent. sodium hydroxide for about forty minutes as called for by the Benedict and Gephart method. The 10 milligrams of creatinine which it appears these investigators took for their work would contain all told 0.003717 gram of nitrogen. One cc. of their 0.2 N sulphuric acid would be equivalent to 0.0028 gram of nitrogen. They found that solutions of creatinine yielded by hydrolysis and distillation as in the Benedict and Gephart method 8.4 to 4.2 per cent. of ammonia nitrogen. It is thus evident that if we consider their maximum result the amount of ammonia nitrogen represented by the 8.4 percentage decomposition of the 10 milligrams of creatinine would be equivalent to 0.08 cc. of their 0.2 N acid while their minimum result, namely, 4.2 per cent., would be equivalent to only 0.04 cc. of their standard acid. These volumes 0.08 and 0.04 cc. of standard acid are no greater than would be obtained between duplicate determinations in such work, especially if the subjective element was completely eliminated.

In our work we used 0.1 N sulphuric acid. One cc. of our acid was equivalent to 0.0014 gram of nitrogen. The 25 milligrams of creatinine, which we used, would contain 0.00929 gram of nitrogen. It is thus evident, that if we consider our maximum result, in the experiment reported immediately above, the amount of ammonia nitrogen represented by the 20.6 per cent. decomposition of the 25 milligrams of creatinine would be equivalent to 1.37 cc. of our 0.1 N acid while our minimum result, 17.6 per cent., would be equivalent to 1.17 cc. of our 0.1 N acid. These volumes of our standard acids are much greater than the differences obtained between duplicate determinations, even if the subjective element is entirely eliminated. The object of the above discussion is to point out the fact that the small amount of creatinine taken and the strength of the standard acid used, by Wolf and Osterberg, would make it quite difficult to accurately detect a decomposition of 5 to 10 per cent. of the quantity of creatinine used in testing the action of the alkali during the distillation process.

It is thus quite evident from these results that creatinine and creatine when heated in boiling water in the presence of fixed alkalies are decomposed in part into ammonia.

That these compounds are thus decomposed was demonstrated, or at least made quite probable, by the researches of Neubauer.[1] It also seems to be clearly indicated by the results given above that the decomposition of creatinine when subjected to the hydrolysis with hydrochloric acid and the distillation with alkali involved in the Benedict and Gephart method, is due entirely to the distillation with the alkali and not to the hydrolysis with the strong acid in the autoclave.

That this supposition is true is proven by the following experiments: Twelve and one-half to 25 milligrams of creatine were hydrolyzed as usual by the Benedict and Gephart autoclave method and the resulting solution subjected to aeration for the determination of ammonia as in the Folin method with slight modifications to be mentioned later. In seven different individual tests, all that were made, there was no decomposition in either test.

Incidentally, the results thus obtained are of interest in connection with the autoclave method of F. G. Benedict and V. C. Myers,[2] for the conversion of creatine into creatinine for the purpose of the quantitative determination of the former compound. Indirectly, it would appear from the results obtained by Wolf and Osterberg that the above method may produce a partial decomposition of the creatine or creatinine and therefore result in giving a low creatine value in products containing this constituent. The experimental data here given demonstrate that the Benedict and Myers autoclave method of converting creatine into creatinine does not decompose either creatine or creatinine into ammonia and that it may therefore still be used with impunity in the quantitative estimation of creatine.

These results demonstrate clearly and beyond doubt that when creatine or creatinine are hydrolyzed by the autoclave method with hydrochloric acid, no decomposition into ammonia occurs. It therefore follows that the decomposition of the creatine and creatinine into ammonia which does occur when these urinary constituents are subjected to the Benedict and Gephart treatment for the determination of urea in urine occurs exclusively in the distillation with the 20 cc. of sodium or potassium hydroxide.

The following experiment is of interest in this connection. Four portions of 25 milligrams of creatine were weighed into Kjeldahl flasks. There was then added to each flask 400 cc. of ammonia-free water and 15 cc. of 10 per cent. sodium hydroxide. These solutions were distilled into an excess of standard 0.1 *N* acid until the volume of the liquid in the Kjeldahl flasks amounted to about 75 cc. The excess of standard acid in the receiving vessels was determined by titration with a standard alkali. As a result of the four individual tests, the following figures which represent the percentage of creatine decomposed, were obtained: 28.4, 21.2, 26.3, and 28.4 per cent. A new set of 0.1 *N* acids was at this time placed under the condensers and the distillation of the liquids in the flask measuring only about 75 cc. was renewed and continued without the addition of any more alkali, until the volume of the remaining liquid measured only 10 to 15 cc. The receiving flasks were removed and the excess of acid determined by titration. As a result of the four individual tests, the following figures, representing the percentage of the original creatine decomposed, were obtained: 5.6, 3.6, 7.2, and 7.6 per cent.

To the liquids remaining in the Kjeldahl flasks measuring only about 10–15 cc., there were added 400 cc. of ammonia-free water, but no more alkali was added. A new set of 0.1 *N* acids was again placed under the condensers and the distillation renewed and continued until the volumes of liquids in the Kjeldahl flasks equaled about 75 cc. The excess of standard acid in the receiving flasks was determined by titra-

[1] *Ann.*, **137**, 289 (1866).

[2] *Amer. J. Physiol.*, **18**, 397 (1907).

tion. As a result of the four individual tests, the following figures which represent the percentage of the original creatine decomposed were obtained: 13.5, 12.1, 13.0 and 9.4 per cent. A new set of 0.1 *N* acids was for the fourth time placed under the condensers and the distillation of the liquids in the flasks, measuring only about 75 cc., was renewed and continued without the addition of any more alkali, until the volume of the remaining liquids measured only 10–15 cc. The receiving flasks were removed and the excess of acid determined by titration. As a result of three individual tests the following figures representing the percentage of the original creatine decomposed were obtained: 3.12, 3.80, and 3.80 per cent.

It is evident from these results that the decomposition of creatine, when continuously heated in water to the boiling point in the presence of 15 cc. of 10 per cent. sodium hydroxide, is a gradual and continuous process and the amount of the distillate collected and the time taken for the distillation modifies the extent of the decomposition resulting.

A Study of Uric Acid.—In the next place it was deemed advisable to study the influence of the Benedict and Gephart autoclave treatment and distillation process as used in the determination of urea upon solutions of uric acid. For this work, 12.5 to 25 milligrams of uric acid were dissolved in the smallest possible excess of a solution of sodium hydroxide and the solution diluted to a volume of about 5 cc. There was added to this solution 5 cc. of dilute hydrochloric acid (1:4), and the acid liquid subjected to the autoclave treatment and the distillation process as described above for creatinine and creatine, page 1081. As a result of two individual tests, the following figures which represent the percentage of uric acid decomposed were obtained: 15.7 and 24.1 per cent.

These results upon uric acid fully confirm those obtained by Wolf and Osterberg which demonstrated the fact that uric acid when heated in the autoclave and distilled with 20 cc. of 10 per cent. sodium hydroxide as in the Benedict and Gephart procedure for the determination of urea, yields a considerable amount of ammonia. The results of Wolf and Osterberg show that 14.10 to 20.44 per cent. of the uric acid were decomposed when different amounts of hydrochloric acid were used.

In view of the results obtained above in connection with the work upon creatine and creatinine, it was deemed desirable to test the influence of the process of distillation in the presence of the fixed alkali as used in the Benedict and Gephart method, upon the uric acid contained in the solution. To do this 25 milligrams of uric acid were treated with 350 cc. of nitrogen-free water. Fifteen or 20 cc. of 10 per cent. sodium hydroxide solution were added and the distillation was made exactly as is customary in the case of the Benedict and Gephart method. As a result of seven individual tests, the following figures which represent the percentage of uric acid decomposed were obtained: 10.7, 9.9, 10.8, 8.0, 8.3, 7.2, and 7.6 per cent.

These results do not correspond to those of Wolf and Osterberg who found in one experiment (No. 52) that uric acid when distilled with 300 cc. of water and 3 cc. of 10 per cent. sodium hydroxide solution underwent no decomposition. They report only one experiment in duplicate and the same objections as were noted above as to their work on creatinine in this connection is also applicable to their work upon uric acid. However, it is true that the conditions of our tests are different both in this experiment upon uric acid and also in the corresponding experiment upon creatinine and creatine, from those of Wolf and Osterberg, in that we used 15–20 cc. of the 10 per cent. sodium hydroxide solution while they used only 3 cc. of the alkaline solution. The original Benedict and Gephart method calls for 20 cc. of the 10 per cent. solution of sodium hydroxide, and we have repeatedly found that after hydrolysis in the autoclave, as in the Benedict and Gephart method, there is only required 2 1/2

to 3 cc. of a 10 per cent. solution of sodium hydroxide to neutralize the hydrochloric acid remaining. This being true, in order to make these tests upon uric acid and creatinine under the conditions actually attending the distillation of the urea nitrogen according to the Benedict and Gephart method, at least 17 cc. of the 10 per cent. solution of sodium hydroxide should be used.

It is quite evident from the results given immediately above that uric acid when heated in boiling water in the presence of fixed alkalies is decomposed in part into ammonia. It also seems to be indicated by the results here presented, that the decomposition of uric acid when subjected to the action of hydrochloric acid at increased temperature and pressure in the autoclave and to the distillation with alkali as in the Benedict and Gephart method is due, in part, to each of the two actions namely, first the influence of the hydrochloric acid at the increased temperature and pressure in the autoclave, and, second, to the distillation with the alkali. In other words, the extent of the decomposition of the uric acid into ammonia is greater as a result of the combined action taking place in the autoclave and in the distillation, than is the decomposition resulting from the distillation process itself. In this respect the uric acid differs from the creatinine and creatine. However, the above data, while indicative, are not conclusive. On the other hand, the following experiments prove that the above supposition is true as regards the behavior of uric acid under the above-mentioned conditions.

Twelve and one-half to 25 milligram portions of uric acid were subjected to the Benedict and Gephart method of hydrolysis for urea in the autoclave and the resulting solutions were aerated for ammonia, according to the Folin method. As a result of five individual tests, the following data which represent the percentage decomposition of the uric acid were obtained: 9.0, 10.9, 8.8, 8.7, and 10.9 per cent.

These results prove conclusively that uric acid when simply heated in the autoclave with hydrochloric acid as in the Benedict and Gephart method for urea is decomposed in part into ammonia. The extent of this decomposition amounts approximately to 10 per cent. It therefore follows that the decomposition of uric acid into ammonia which occurs when this urinary constituent is subjected to the Benedict and Gephart method for the determination of urea in urine, occurs both in the autoclave treatment and in the distillation process in the presence of the alkali.

That the uric acid itself was entirely free from ammonia and further that it was not affected by the aeration process in the presence of the 2–3 grams of sodium carbonate is shown by the following experiment: Twelve and one-half to 25 milligram portions of the uric acid were suspended in 10 cc. of water and the mixtures thus obtained were subjected to the Folin aeration method for the determination of ammonia. As a result of four different tests, each in duplicate, no ammonia was found to be given off from the uric acid by this treatment.

It follows from the investigations of Wolf and Osterberg and the results here reported that uric acid is decomposed to the extent of 15–20 per cent. or even more in some cases by the treatment involved in the Benedict and Gephart method for the determination of urea in urine. Further, it is evident from the results presented in this paper that this decomposition of uric acid is about equally divided between the autoclave treatment and the distillation process with 20 cc. of a 10 per cent. solution of fixed alkali. In other words, if uric acid is subjected to the autoclave treatment with hydrochloric acid and the ammonia determined in the resulting liquid by the aeration method and without distillation with fixed alkali, the decomposition amounts to about 10 per cent. On the other hand, if uric acid is subjected to the distillation process in the presence of 15–20 cc. of fixed alkali but not to the autoclave treatment the decomposition amounts to approximately 10 per cent. Further, if the uric acid is

subjected to both the autoclave treatment and the distillation process the decomposition amounts to about 20 per cent.

The gradual and continuous decomposition of creatine when heated in aqueous solution with sodium hydroxide to the boiling point of water made it desirable to test the behavior of uric acid under similar conditions. Four portions of 25 milligrams of uric acid were weighed into Kjeldahl flasks. There were then added to each flask 400 cc. of ammonia-free water and 15 cc. of 10 per cent. sodium hydroxide. These solutions were distilled into an excess of standard acid until the volume of the liquid in the Kjeldahl flasks amounted to about 75 cc. The excess of standard acid in the receiving vessels was determined by titration with a standard alkali solution. As a result of the four individual tests, the following figures which represent the percentage of uric acid decomposed were obtained: 7.2, 8.0, 8.3, and 7.6 per cent. A new set of 0.1 *N* acids was at this time placed under the condensers and the distillation of the liquids in the flask measuring only about 75 cc. was renewed and continued without the addition of any more alkali until the volume of the remaining liquid measured only 10–15 cc. The receiving flasks were removed and the excess of acid determined by titration. As a result of the four individual tests, the following figures representing the percentage of the original uric acid decomposed were obtained: 3.0, 3.4, 3.6, and 8.7 per cent.

To the liquids remaining in the Kjeldahl flasks, measuring only 10–15 cc., there were added 400 cc. of ammonia-free water but no additional sodium hydroxide solution was added. A new set of 0.1 *N* acids was again placed under the condensers and the distillation renewed and continued until the volumes of the liquid in the Kjeldahl flasks equaled about 75 cc. The excess of standard acid in the receiving flasks was determined by titration. As a result of the four individual tests, the following figures which represent the percentage of the original uric acid decomposed were obtained: 9.4, 11.8, 12.1, and 11.8 per cent. A new set of 0.1 *N* acids was for the fourth time placed under the condensers and the distillation of the liquids in the flasks measuring only about 75 cc. was renewed and continued without the addition of any more alkali, until the volume of the remaining liquids measured only 10–15 cc. The receiving flasks were removed and the excess of standard acid determined by titration. As a result of three individual tests, the following data, representing the percentage of the original uric acid decomposed, were obtained: 14.6, 3.8, 4.5, and 3.0.

It is clearly apparent from these results, that the decomposition of uric acid, when continuously heated in water to the boiling point, in the presence of 15 cc. of 10 per cent. sodium hydroxide, is a gradual and continuous reaction, and the amount of the distillate collected and the time taken for the distillation modifies the extent of the decomposition resulting.

A Study of Hippuric Acid.—In connection with this investigation it seemed desirable to test the influence of the Benedict and Gephart autoclave treatment and distillation process as used in the determination of urea upon solutions of hippuric acid, which probably forms one of the most abundant known nitrogenous constituents composing the so-called undetermined nitrogenous substances of urines. For this purpose 25 milligram portions of hippuric acid were dissolved in 5 cc. of water and 5 cc. of dilute hydrochloric acid (made by adding four volumes of distilled water to one volume of concentrated acid). The solutions were then placed in the autoclave and kept at a temperature of 142–145° for a period of 90 minutes. After the autoclave had cooled, the contents of the tube were washed into a 500 cc. Kjeldahl flask, diluted to about 350 cc., treated with 20 cc. of 10 per cent. sodium hydroxide solution, and distilled into an excess of standard acid until the distillate measured about 275 cc. As a result of five individual tests, the extent of the decomposition varied

from 21.2 to 24.5 per cent., the average value being 23.7 per cent. The following figures represent the results obtained: 24.5, 21.2, 24.5, 24.5, and 23.6 per cent. These results demonstrate the fact that the Benedict and Gephart method for the determination of urea causes simultaneously the partial decomposition of the hippuric acid of the urine into ammonia. The average excretion of hippuric acid of an adult man for twenty-four hours is said to be about 0.7 gram, but since the percentage of nitrogen in this compound is small, being only 7.82, the amount of nitrogen represented by a decomposition of 23.7 per cent. of the hippuric acid by the Benedict and Gephart method would amount to only about 15 milligrams in the urine for twenty-four hours.

While this is true, it was nevertheless deemed desirable to determine whether or not this decomposition of the hippuric acid resulted in the process of hydrolysis with hydrochloric acid in the autoclave or in the process of distillation with the fixed alkali. In order to accomplish this end, several tests were made to determine the influence of the process of distillation in the presence of the fixed alkali as used in the Benedict and Gephart method, upon solutions of hippuric acid. For this purpose 25 milligram portions of pure hippuric acid were dissolved in 350 cc. of nitrogen-free water. Fifteen cc. of 10 per cent. sodium hydroxide solution were added and the distillation was made exactly as is usually done in the case of the Benedict and Gephart method. As a result of four individual tests, the following figures, which represent the percentage of hippuric acid decomposed, were obtained: 17.7, 18.5, 16.9, and 15.3 per cent. The average of these results is 17.1 per cent. It is thus quite apparent from these results that hippuric acid when heated in boiling water in the presence of fixed alkalies is decomposed in part into ammonia.

It seems to be clearly indicated by the results given above that the decomposition of hippuric acid, when subjected to the hydrolysis with hydrochloric acid and the distillation with fixed alkali involved in the Benedict and Gephart method, is due entirely to the distillation with the alkali and not to the hydrolysis with the acid in the autoclave. That this supposition is true is proven by the following experiment: Twenty-five milligram portions of hippuric acid were hydrolyzed as usual by the Benedict and Gephart autoclave method and the resulting solution subjected to aeration for the determination of ammonia as per the Folin method. In three individual tests, all that were made, there was no decomposition in either test. It is thus clearly evident that when hippuric acid is hydrolyzed by the autoclave method with hydrochloric acid, no decomposition into ammonia occurs. It therefore follows that the decomposition of the hippuric acid into ammonia, which occurs when this urinary constituent is subjected to the Benedict and Gephart treatment for the determination of urea in urine, occurs exclusively in the distillation with the 20 cc. of 10 per cent. sodium or potassium hydroxide.

The gradual and continuous decomposition of creatine and uric acid when distilled in aqueous solution in the presence of sodium hydroxide led us to test the behavior of hippuric acid under similar conditions. Four portions of 25 milligrams of hippuric acid were weighed into Kjeldahl flasks. There were then added to each flask 400 cc. of ammonia-free water and 15 cc. of 10 per cent. sodium hydroxide. These solutions were distilled into an excess of standard acid until the volume of the liquid in the Kjeldahl flask amounted to approximately 75 cc. The excess of standard acid in the receiving vessels was determined by titration with a standard alkali solution. As a result of the four individual tests, the following figures which represent the percentage of hippuric acid decomposed were obtained: 17.7, 18.5, 16.9, and 15.3 per cent. A new set of 0.1 *N* acids was at this time placed under the condensers and the distillation of the liquids in the flasks measuring only about 75 cc. was renewed and continued without the addition of any more alkali until the volume of the re-

maining liquid measured only about 10–15 cc. The receiving flasks were removed and the excess of acid determined by titration. As a result of four individual tests, the following data, representing the percentage of hippuric acid decomposed, were obtained: 9.6, 7.2, 9.6, and 8.0 per cent.

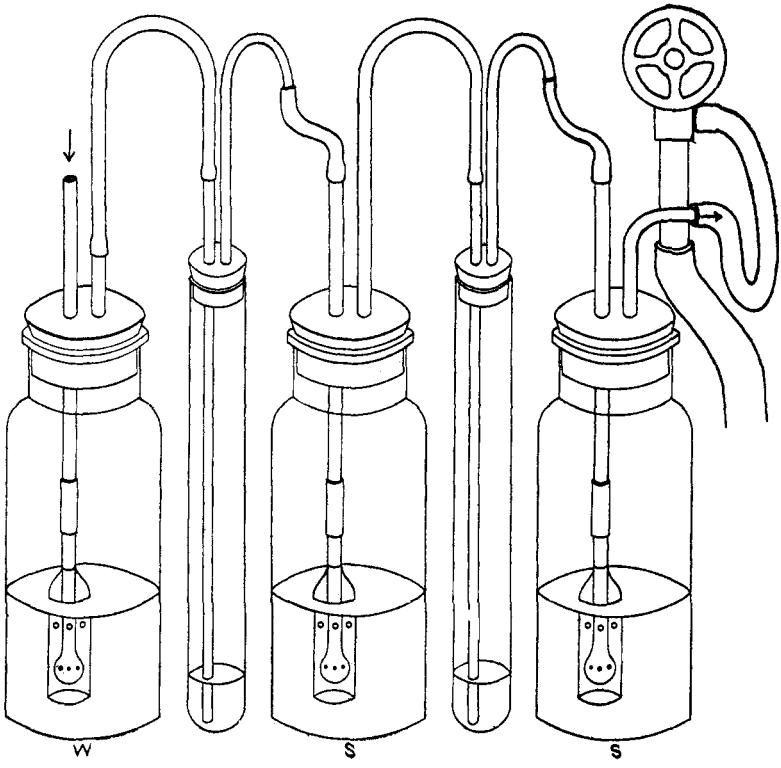
To the liquids remaining in the Kjeldahl flasks, measuring only 10–15 cc., there were added 400 cc. of ammonia-free water but no additional sodium hydroxide was added. A new set of 0.1 N acids was again placed under the condensers and the distillation renewed and continued until the volumes of the liquid in the Kjeldahl flasks equaled about 75 cc. The excess of standard acid in the receiving flasks was determined by titration. As a result of the three individual tests, the following figures which represent the percentage of hippuric acid decomposed were obtained: 12.9, 17.7 and 33.8 per cent. A new set of 0.1 N acids was for the fourth time placed under the condensers and the distillation of the liquids in the flasks measuring only about 75 cc. was renewed and continued without the addition of any more alkali, until the volume of the remaining liquids measured only 10–15 cc. The receiving flasks were removed and the excess of acid determined by titration; as a result of four individual tests the following data representing the percentage of hippuric acid decomposed were obtained: 5.6, 4.7, and 6.4 per cent.

It is clearly evident from the data here presented that the decomposition of hippuric acid, when continuously heated in water to the boiling point in the presence of 15 cc. of 10 per cent. sodium hydroxide, is similar in character to the decompositions which take place with creatine and uric acid under like conditions, namely, a gradual and continuous reaction involving the evolution of nitrogen in the form of ammonia. The time taken to effect the distillation and the quantity of the distillate collected modifies the extent of the decomposition resulting.

Since the above results show that creatine, creatinine and hippuric acid are not decomposed by the autoclave treatment itself, as described by Benedict and Gephart, and since uric acid under the above treatment is only decomposed to the extent of about 10 per cent., it is quite apparent that the Benedict and Gephart method for the hydrolysis of the urea may be still used with accuracy in so far as the errors arising from these urinary constituents are concerned, providing the ammonia resulting from the hydrolysis of the urea may be determined in the resulting solution without involving in the process a decomposition of the other nitrogenous urinary constituents into ammonia. The average normal uric acid nitrogen content of urine is approximately 0.20 gram for twenty-four hours. The error of 10 per cent. upon this quantity occurring in the autoclave treatment would amount to only 0.020 gram which would be equivalent to only 0.15 per cent. of a total urinary nitrogen value of 13.00 grams per 24 hours. It has been our experience that it is very seldom possible to get differences in duplicates in urea nitrogen by the Folin method to fall below this value of 0.020 gram per day.

Method for the Determination of Urea.—It is apparent from the data presented above, that one of the most promising methods of accomplishing this end, namely, the removal of the ammonia resulting from the hydrolysis of the urea without bringing about the decomposition of the creatinine, the uric acid and hippuric acid, is the Folin aeration method in the presence of sodium carbonate. The details of the method of thus determining urea in urines as finally perfected are as follows: The 5 cc. portions of the urine are measured into 8 × 1 or 10 × 1 inch hard glass test tubes, to which are added the 5 cc. portions of the 1 : 4

solution of hydrochloric acid. The urine and acid are thoroughly mixed and the tubes and their contents are placed in the autoclave at a temperature of 142–145° for a period of 90 minutes. The autoclave is allowed to cool for 30 minutes after the hydrolysis is complete before the tubes are removed. The contents of the tubes are allowed to become stone-cold and then 3 drops of 1 per cent. solution of alizarin red and 3 to 4 drops of pure cottonseed oil are added. The aeration tube and stopper are inserted as shown in the accompanying figure and con-



nected with a 16 ounce wide-mouthed bottle, containing 25 cc. of approximately 0.2 *N* sulphuric acid solution plus 130 cc. of ammonia-free water and a Folin absorption tube. The apparatus, in sets of four if necessary, are connected to a Chapman water pump for the aeration process. A block of sodium carbonate weighing from 2 to 3 grams is dropped into each tube, beginning, in case a series of tubes are attached to one pump, at the end furthest from the pump. The blocks of sodium carbonate are prepared by moistening by a suitable means, for example, by the use of a spatula and a porcelain plate, anhydrous sodium carbonate with ammonia-free water, to a soft consistency and quickly shaping the moistened carbonate into a mass from which blocks weighing 2 to

3 grams each can be cut. If too much water is not used the carbonate will harden in a very few minutes to a compact mass.

We have found that the proportion of 10 to 15 drops of water to 2 to 3 grams of the anhydrous sodium carbonate gives the proper consistency to work well. Upon the addition of the stick of sodium carbonate, a stormy effervescence at first results, but do not slip in the rubber stopper, carrying the glass tubes, until most of the effervescence has ceased. Then while the solution is yet acid as shown by the alizarin indicator, insert the stopper carrying the tubes, beginning at the end of the set or train furthest from the pump. The contents of the tubes should be thoroughly shaken until they have become alkaline, that is, turn red. When all the solutions in one set attached to a single pump are alkaline then operate the water pump so as to produce a rapid but not violent aeration. Continue the aeration for 5 hours. From a series of carefully conducted tests we have demonstrated without doubt, that all the ammonia is removed in five hours with a rapid though not violent aeration. At the end of the above-mentioned time disconnect the apparatus and titrate the excess of standard sulphuric acid, using 0.1 *N* sodium hydroxide as the standard alkali and Congo red as the indicator.

The results of the comparative tests of the Folin method, the Benedict and Gephart method, and the hydrolysis-aeration method described above, which is a combination of the autoclave hydrolysis of the urea suggested by Benedict and Gephart and the Folin method for the determination of the ammonia in urines are given in the following table.

Discussion of the Results.—The results recorded in the above table indicate that the hydrolysis-aeration method as here purposed gives practically the same urea nitrogen values as does the Folin method. It is also evident that the Benedict-Gephart method gives higher results than does the Folin method. The average of the analysis of the twenty-five samples of urine in triplicate by the Folin method gives a value of ten grams of urea nitrogen, while the hydrolysis-aeration method gives a value of 10.05 grams and the Benedict-Gephart method gives a value of 10.25 grams of urea nitrogen per 24 hours.

The average result of the hydrolysis-aeration method exceeds the average result of the Folin method by only 0.05 gram of urea nitrogen per day of 24 hours, while the average results of the Benedict-Gephart method exceeds the average of the Folin method by 0.25 gram of urea nitrogen for 24-hour period.

Viewing the data in still another form it is evident that the results of the hydrolysis-aeration method expressed as percentage of the Folin results vary from 99.45 to 101.94 per cent., the average being 100.43 per cent. The results of the Benedict-Gephart method expressed as

percentage of the Folin results vary from 100 88 to 103.86 per cent., the average being 102.52 per cent.

UREA NITROGEN EXPRESSED AS GRAMS PER 24 HOURS.

Lab. No.	Folin method. Grams.	Hydrolysis-aeration method. Grams.	Benedict-Gephart method. Grams.	Difference between Folin and hydrolysis-aeration. Grams.	Difference between Folin and Benedict-Gephart. Grams	Hydrolysis-aeration as per cent. of the Folin. Per cent.	Benedict-Gephart as per cent. of the Folin Per cent
19	7.34	7.34	7.61	0.00	0.27	100.00	103.68
20	11.36	11.39	11.46	0.03	0.10	100.26	100.88
21	9.61	9.62	9.82	0.01	0.21	100.10	102.18
22	7.94	7.91	8.06	—0.03	0.12	99.62	101.51
23	6.79	6.78	6.97	—0.01	0.18	99.85	102.65
24	11.14	11.10	11.30	—0.04	0.16	99.64	101.40
25	7.71	7.86	7.97	0.15	0.26	101.94	103.37
26	11.44	11.55	11.74	0.11	0.30	100.96	102.62
27	9.85	9.87	10.13	0.02	0.28	100.20	102.84
28	11.88	11.90	12.11	0.02	0.23	100.17	101.94
29	9.89	10.07	10.25	0.18	0.36	101.82	103.64
30	6.31	6.36	6.55	0.05	0.24	100.79	103.80
31	8.95	9.04	9.24	0.09	0.29	101.01	103.24
32	13.40	13.47	13.64	0.07	0.24	100.52	101.79
33	9.11	9.19	9.37	0.08	0.26	100.88	102.85
34	15.49	15.61	15.79	0.12	0.30	100.77	101.93
35	7.73	7.72	7.91	—0.01	0.18	99.87	102.33
36	9.17	9.15	9.30	—0.02	0.13	99.78	101.42
37	11.75	11.78	11.91	0.03	0.16	100.25	101.36
38	7.77	7.77	8.07	0.00	0.30	100.00	103.86
39	18.76	18.92	19.45	0.16	0.69	100.85	103.68
40	7.50	7.54	7.68	0.04	0.18	100.53	102.40
41	7.31	7.27	7.52	—0.04	0.21	99.45	102.87
42	11.00	11.02	11.19	0.02	0.19	100.18	101.73
43	10.80	10.94	11.14	0.14	0.34	101.30	103.15
Av.	10.00	10.05	10.25	0.04	0.25	100.43	102.52

It is thus evident that the hydrolysis-aeration results are much closer the Folin results than are the Benedict-Gephart results. The differences between the results of the hydrolysis-aeration method and the results of the Folin method which vary from —0.04 to 0.18 gram per day, the average being 0.04 gram, are no greater than different determinations by the Folin method even when made by the same analyst.

The results here given clearly indicate that the hydrolysis-aeration method as here proposed gives practically the same results as the Folin method and at the same time obviates the long and tedious operations attending the hydrolysis and distillation processes involved in the Folin method. The hydrolysis-aeration method requires much less time and attention than does the Folin method and it does not require the expert manipulation and training necessary to get concordant results required by the Folin method. In fact the details of the hydrolysis-aeration

method are so simple and easy of operation and they require so little attention and time that it may even be used successfully in clinical work. It should be mentioned that it is probable from the results of Steel and Gies[1] that in urines containing ammonium magnesium phosphate the hydrolysis-aeration method may fail to yield all of the ammonia nitrogen resulting from the hydrolysis of the urea.

This investigation suggests a number of important studies to be made before we know definitely the best conditions necessary to obtain accurate and reliable urea determinations with the least expenditure of labor and time. Among the questions to be studied are the following: The influence of the distillation of aqueous solutions of creatinine, uric acid and hippuric acid, and also of urines which have been hydrolyzed in the autoclave with hydrochloric acid, in the presence of magnesium hydroxide and sodium carbonate. The influence of hydrolysis in the autoclave by the Benedict and Gephart method upon the so-called undetermined nitrogen compounds of urine. The influence of the Folin methods of hydrolysis and distillation upon solutions of creatinine, uric acid and hippuric acid. A comparative study of the three methods—Folin, Benedict and Gephart and the hydrolysis-aeration—upon the same samples of urines by different analysts. The influence of ammonium magnesium phosphate and other interfering substances upon the hydrolysis-aeration method. The influence of the presence of magnesium salts upon the complete removal of ammonia from solutions upon distillation with only a slight excess of magnesium hydroxide. Some of these problems have been studied but they need further careful investigations for the purpose of confirming and extending the previous work.

Conclusions.

First, creatinine and hippuric acid are not at all decomposed by heating in the autoclave with hydrochloric acid but they are partially decomposed either before or after treatment with hydrochloric acid in the autoclave with 20 cc. of 10 per cent. sodium hydroxide solution.

Second, uric acid is decomposed in part by the autoclave treatment with hydrochloric acid and, moreover, it is still further decomposed into ammonia by distillation with 20 cc. of 10 per cent. sodium hydroxide solution.

Third, the hydrolysis-aeration method gives practically the same urea nitrogen values as does the Folin method but the Benedict-Gephart method gives higher results than does the Folin method. The average of the analysis of twenty-five samples of urine by the Folin method gave a value of 10.00 grams of urea nitrogen, while the hydrolysis-aeration method gave a value of 10.05 grams, and the Benedict-Gephart method

[1] *J. Biol. Chem.*, **5**, 71 (1908).

gave a value of 10.25 grams of urea nitrogen per 24 hours. The results of the hydrolysis-aeration method expressed as percentage of the Folin results varied from 99.45 to 101.94 per cent., the average being 100.43 per cent. The results of the Benedict-Gephart method expressed as percentage of the Folin results varied from 100.88 to 103.86 per cent., the average being 102.52 per cent.

Fourth, the hydrolysis-aeration method requires much less time and attention than does the Folin method and it does not require the expert manipulation and training necessary to get concordant results which the Folin method requires.

URBANA, ILL.

THE DEVELOPMENT OF FAT IN THE BLACK WALNUT (JUGLANS NIGRA).

BY F. M. M'CLENAHAN.

Received July 26, 1909.

There exists a recognized uncertainty as to the genesis of vegetable fats and their necessary progenitors. Especially is this true of the fats that occur in the seeds of plants.[1] With a view to shedding some light on this very interesting problem the following observations were made on the chemical development of the kernel of the black walnut during the summer of 1908. On account of the evenness of its development this nut is an ideal one for study. Different positions on the trees and even different trees in the locality did not show an appreciable difference of kernel development. By June 15th the nuts in the neighborhood of the laboratory had reached their full development of size. Thereafter the most significant changes were internal.

Studies were made on crops gathered June 15th, July 15th, July 29th, August 12th, and August 26th. At this last date the nut gave evidence of apparent ripeness, and college duties prevented further analytic study.

Crop of June 15th.—The hull of the nut seemed nearly mature in its structure. The shell had not yet developed its stony consistency, but could be cut easily with a knife. The kernel cavity and kernel capsule were fully developed in volume and form. The kernel, however, was a colorless limpid liquid, having a saline taste and an acid reaction with litmus. There was much tannin in the hull, in the canals of the undeveloped shell, and in the tissue of the kernel capsule, but none whatever in the liquid kernel.

The stem of a fresh nut was cut off close to the hull and the nut was soaked in fuchsine for three days. The fuchsine penetrated the hull and the canals of the shell progenitor, but in no degree was the kernel liquid tinted by it.

[1] See especially Jost's "Plant Physiology," Gibson, 1907, p. 176.