# *The Alleged Excretion of Creatine in Carbohydrate Starvation.*

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CONTENTS.



## *Introduction.*

It was stated by Cathcart (4) and Benedict and Myers (2) independently, in 1907, that creatine was excreted in the urine during inanition. Cathcart (5) has further stated that the output of creatine, caused by fasting for 36 hours, is diminished as soon as a diet consisting of carbohydrates is taken, whereas it is increased by a fat diet.

Rose and Mendel (19) confirm these results, laying great stress on the fact that carbohydrates play a very important *rôle* in preventing the excretion of creatine in the urine.

In the course of a 10 days' experiment on one of us (G. G., 10), where the diet was restricted to protein and fat and was of insufficient calorie value, we found that no creatine was excreted in the urine. The explanation of this discrepancy was not fully investigated at that time, but recent work by Greenwald (12) has suggested a possible explanation.

Folin's (8) method for the estimation of creatinine in urine depends on the orange colour produced by the addition of picric acid and soda (Jaffé, 15). This colour has been shown to be due to a reducing action of the creatinine on the picric acid (Chapman, 6).

Among the reducing substances which also give a similar colour are acetone, aceto-acetic acid, and $\beta$-oxybutyric acid, all of which may be present in urine under different conditions.

Van Hoogenhuyze and Verploegh (13) and Krause (16) stated that urine to which acetone had been added produced, with picric acid and soda, a

slightly darker colour than was obtained with the urine alone, but that the colour soon faded and caused no error in the determination of creatinine. Krause (16), Wolf and Osterberg (22), and Rose (20) found that the addition of the ethyl ester of aceto-acetic acid to urine did not produce any error in the estimation of creatinine, unless large amounts (*i.e.* over 1 per cent.) were added. They seem to have assumed that the action of aceto-acetic acid would be the same as that of the ester.

Recently, however, Greenwald (12), working on diabetic urines, has shown that if aceto-acetic acid is added to urine directly a considerable error is introduced into the estimation of creatinine.

This observation may possibly explain why we did not find any creatine in our experiment on the fat diet. Folin (8) originally stated that acetone and aceto-acetic acid gave the orange colour with picric acid and soda, but remarked that they could easily be removed from the urine. In our experiment we removed the aceto-acetic acid as far as possible from the urine before making the estimation, in order to get rid of any disturbing effect that the acetone bodies might have on the creatinine figures.

In the experiments described in this paper we have studied this question in greater detail and also the means of overcoming the difficulty.

I. *The Effect of Aceto-acetic Acid on the Estimation of Creatinine.*

The different intensities of colour produced by $\beta$-oxybutyric acid, acetone, aceto-acetic ester and aceto-acetic acid when treated with picric acid and soda were first investigated.

The importance of aceto-acetic acid is emphasised by the experiments of Arnold (1), Emden (7), and Hurtley (14). These investigators have, independently, pointed out that in cases of acidosis the fresh urine contains only small amounts of acetone, while aceto-acetic acid may be present in large amounts.

Throughout our experiments the estimation of the creatinine was performed in the usual way; 15 c.c. of a saturated solution of picric acid and 5 c.c. of 10-per-cent. caustic soda were added to 10 c.c. of urine, the mixture was allowed to stand for seven instead of five minutes, and then diluted to 500 c.c. with water. Folin (8) stated that the maximum intensity of colour occurs in five to nine minutes after mixing. We have always waited seven minutes because we found that five minutes was not always sufficient if the urine was slightly diluted, as occurs in the estimation of the creatinine+creatine by this method. The matching was done with a Duboscq colorimeter against an N/2 potassium bichromate solution. All the matching was done by E. P. P. while the scale was read by G. G., six

to eight readings were made and the mean was taken. The total nitrogen was estimated by Kjeldahl's method. The aceto-acetic acid + acetone was estimated by the Messinger-Huppert method.

The substances tested were prepared in the following manner, and we wish to thank Dr. Hurtley for very kindly supplying us with them. The $\beta$-oxybutyric acid was extracted from urine and the strength of the solution was accurately known; the solution was nearly colourless. The acetone was chemically pure. The ethyl ester of aceto-acetic acid was obtained by distilling the pure commercial ester under reduced pressure, and the product boiled constantly at the correct boiling point for the pure substance.

The aceto-acetic acid was obtained from the ester, which was hydrolysed by adding the theoretical amount of normal caustic soda, and allowing it to stand at the room temperature for 36 hours, when the hydrolysis was complete. It will be seen that the solution used consisted of the sodium salt of aceto-acetic acid and an equivalent amount of ethyl alcohol. The mixture was diluted and the amount of acetone present was determined by Folin's method (9). The amount of aceto-acetic acid corresponding to the acetone present was deducted from the theoretical amount of aceto-acetic acid in order to get the correct value for the aceto-acetic acid. The aceto-acetic acid was kept in an ice chest in order to prevent its decomposition, and was tested from time to time by means of the Folin and Messinger-Huppert methods.

$\beta$-oxybutyric acid, acetone, the ethyl ester and the sodium salt of aceto-acetic acid all give an orange colour when treated alone with picric acid and soda, but on dilution the solution is much paler than the usual colour obtained with urine under these conditions. These substances were then added to urine in varying concentrations, and the colour produced by the addition of picric acid and soda was compared with the colour produced by the urine alone with the picric acid and soda, without the addition of these substances. The addition of $\beta$-oxybutyric acid produces practically no alteration in the colour obtained by adding picric acid and soda to urine.

Thus when added to the urine (Table I) in amounts corresponding to 0·036 grm. per 100 c.c. and 1 grm. per 100 c.c. it caused no error at all in the creatinine determination. When present in amounts corresponding to a 2·16-per-cent. solution it made the colour slightly lighter, causing a difference of 0·27 mm. scale reading, which is almost within the limit of accuracy of the method. The amounts of the $\beta$-oxybutyric acid added are quite comparable with those found in the urine in diabetes, and as the error caused even by large amounts is so small its effect may be safely neglected.

Table I shows the Effect of Increasing Amounts of β-oxybutyric Acid, Acetone and Aceto-acetic Ethyl Ester on the Determination of Creatinine in Urine.

| Concentration in urine, per cent. | Grammes per day in 1500 c.c. urine. | Scale reading in mm. | Creatinine, grm. per 100 c.c. | Error in the determination of creatinine, grm. per 100 c.c. |
|---|---|---|---|---|
| β-oxybutyric acid added to urine. | | | | |
| 0 | 0 | 7 | 0·116 | |
| 0·036 | 0·54 | 7 | 0·116 | 0 |
| 1 | 15·0 | 6·88 | 0·118 | +0·002 |
| 2·16 | 32·4 | 7·27 | 0·111 | −0·005 |
| Acetone added to urine. | | | | |
| 0 | 0 | 7 | 0·116 | |
| 0·04 | 0·6 | 7·1 | 0·114 | +0·002 |
| 0·17 | 2·5 | 7 | 0·116 | 0 |
| 1 | 15 | 7·6 | 0·106 | −0·01 |
| 1·6 | 24 | 8·24 | 0·098 | −0·018 |
| 7·6 | | 12·34 | 0·066 | −0·05 |
| Aceto-acetic ethyl ester added to urine. | | | | |
| 0 | 0 | 7 | 0·116 | |
| 0·1 | 1·5 | 7·2 | 0·113 | −0·003 |
| 0·5 | 7·5 | 7·5 | 0·108 | −0·008 |
| 0·75 | 10·7 | 8·17 | 0·099 | −0·017 |
| 1 | 15 | 6·9 | 0·117 | +0·001 |
| 2 | 30 | 6·3 | 0·128 | +0·012 |

Acetone if added to the urine in amounts less than 0·2 per cent. (Table I) does not introduce any error at all. A 1-per-cent. solution makes the colour lighter than usual, while if the acetone is present in larger amounts the colour becomes much lighter and it fades very rapidly on standing. As acetone is excreted in urine in very small amounts the creatinine determinations will not be affected, as a 0·17-per-cent. solution caused no error. These results do not agree with those of van Hoogenhuyze and Verploegh (13) and Krause (16), who found that a 1-per-cent. acetone solution made the colour darker, but that the error disappeared on standing.

Aceto-acetic ethyl ester when present in small quantities produces a slight lightening of the colour. Thus a 0·1- and 0·75-per-cent. solution causes an error in the scale reading of 0·2 and 1·1 mm. Larger amounts, on the other hand, cause a darkening effect, but the colour becomes much redder than usual, which makes it really impossible to match it with the N/2 bichromate solution. These results agree with those obtained by Krause (16), Wolf and Osterberg (22), and Rose (20). This experiment is not of much practical importance, as the ethyl ester is never excreted in urine, but we have made

it because other observers have added this substance to urine instead of aceto-acetic acid.

The sodium salt of aceto-acetic acid produces a much more marked effect than the other acetone bodies. Even when added to urine in small amounts the colour obtained with picric acid and soda is not darker, as stated by Krause (16) and others, but is actually lighter, and when present in large amounts the colour is very much lighter (Table II and fig. 1). The error is not eliminated on standing but increases.

Table II shows the Effect of Increasing Amounts of Aceto-acetic Acid on the Estimation of Creatinine in Urine.

| Aceto-acetic acid added to urine. | | Scale reading. | Creatinine. | Error in the creatinine determination. | |
|---|---|---|---|---|---|
| grm. per 100 c.c. | grm. per 24 hrs. in 1500 c.c. | | grm. per 100 c.c. | grm. per 100 c.c. | per cent. |
| 0 | 0 | 7 | 0·116 | 0 | |
| 0·0234 | 0·35 | 7·33 | 0·111 | 0·005 | 4·3 |
| 0·0468 | 0·702 | 8 | 0·101 | 0·015 | 12·9 |
| 0·093 | 1·4 | 9 | 0·09 | 0·026 | 22·2 |
| 0·187 | 2·8 | 11·3 | 0·072 | 0·044 | 38 |
| 0·374 | 5·6 | 14·66 | 0·055 | 0·061 | 52·6 |

Thus if the concentration of the sodium aceto-acetate is only 0·0234 or 0·0468 per cent. the creatinine estimation is too low, the actual errors being 0·005 and 0·015 grm. respectively. The error produced by larger amounts is very striking, for if the concentration is increased to 0·374 per cent. the error is as great as 0·061 grm., and the percentage error in this case is 52·6 per cent. As amounts of aceto-acetic acid up to a concentration of 0·4 per cent. may be excreted in diabetes, the error caused in such cases must be very great. If the error in the creatinine determination be plotted against the concentration of the aceto-acetic acid the resulting curve is almost a straight line (fig. 1).

The chemistry of this action is at present engaging our attention.

It was not possible to isolate the pure acid and add it to urine, but this does not matter, as the aceto-acetic acid is excreted in the urine partly as the free acid and partly as a salt. Moreover, in the process of estimating the creatinine an excess of caustic soda is added, and this must convert all the free acid into the sodium salt.

The solution of sodium aceto-acetate used in our experiments also contained 0·37 grm. ethyl alcohol to 1 grm. of aceto-acetic acid.

The presence of ethyl alcohol in the sodium aceto-acetate solution (due to its mode of preparation from aceto-acetic ethyl ester) is a possible disturbing

factor, as it might be the cause of the colour change. However, the addition of alcohol to urine in amounts which correspond to those added with the sodium aceto-acetate did not produce any alteration at all in the colour. The possibility still remained that it was the mixture of the alcohol with the sodium aceto-acetate which was responsible for the change in colour. There is no means of directly disproving this hypothesis, as the alcohol cannot be removed from the sodium aceto-acetate solution without destroying the salt. There is, however, indirect proof that this is not the case, as will be shown in the following paragraphs.

Aceto-acetic acid is excreted in the urine during carbohydrate starvation. As will be shown later on, the aceto-acetic acid can easily be removed from the urine without breaking down creatinine or creatine, and this procedure was followed in three diet experiments which will be described in detail later on. The urine which contained aceto-acetic acid had apparently less creatinine in it than the urine from which the aceto-acetic acid had been removed. Thus on the 2nd day Experiment I, a concentration of aceto-acetic acid of 0·065 per cent., caused an error in the creatinine figure of 0·017 grm. per 100 c.c., and a concentration of aceto-acetic acid of 0·081 per cent. on the third day caused an error in the creatine of 0·028 grm. per 100 c.c. On the second and third days of Experiment II the concentration of aceto-acetic acid of 0·085 and 0·112 per cent. produced errors of 0·018 and 0·027 grm. per 100 c.c. respectively (Table III).

Table III shows the Error caused in the Estimation of Creatinine caused by the Excretion of Aceto-acetic Acid in the Urine consequent on Carbohydrate Starvation. (Extracted from Tables VII–IX, pp. 217 and 218, of this paper.)

| Day. | Concentration of aceto-acetic acid per 100 c.c. | Error caused by aceto-acetic acid in determination of creatinine. |
|---|---|---|
| | | grm. per 100 c.c. |
| Diet, Experiment I— | | |
| 2 | 0·065 | 0·017 |
| 3 | 0·081 | 0·028 |
| Diet, Experiment II— | | |
| 1 | 0·029 | 0·005 |
| 2 | 0·085 | 0·018 |
| 3 | 0·112 | 0·027 |
| Diet, Experiment III— | | |
| 1 | 0·036 | 0·01 |
| 2 | 0·072 | 0·008 |

These figures have been plotted on the fig. 1 previously referred to and they lie fairly close to the curve. The figures for the second day of Experiment I and for both days of Experiment II lie somewhat below the curve, while the figure for the third day of Experiment I lies a little above the curve, but the difference is in no case great.

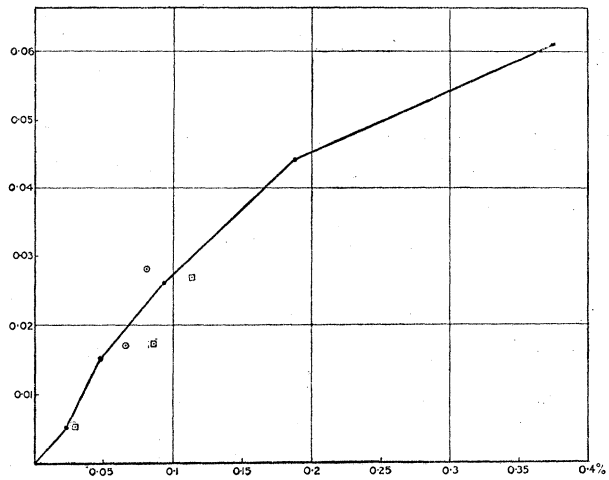


FIG. 1.—The curve shows the error in grammes per 100 c.c. in the estimation of creatinine caused by increasing amounts of aceto-acetic acid. Abscissæ: percentage concentrations of aceto-acetic acid. Ordinates: the error in the creatinine determinations expressed in grammes per 100 c.c. ⊙ = the error in the creatinine determinations on second and third days of Expt. I (Table VII). ⊡ = the error on first, second, and third days of Expt. II (Table VIII).

It must be remembered that the figure for the concentration of the aceto-acetic acid in the diet experiments was obtained by the Messinger-Huppert method, which makes no distinction between acetone and aceto-acetic acid. It is true that acetone is only present in urine in small amounts, but in considering the effect of the aceto-acetic acid some allowance should be made for the amount of acetone present. A diminution in the concentration of the aceto-acetic acid would make the points below the curve more nearly approximate to the curve but would displace the point above the curve away from the curve.

The general agreement between the errors produced by aceto-acetic acid in these experiments, and the errors produced when sodium aceto-acetate containing ethyl alcohol is added to urine, point to the conclusion that it is the aceto-acetic acid itself and not the alcohol that causes the errors, in the latter case.

Our experiments agree with those of Greenwald and show that the aceto-

acetic acid produces a considerable error in the estimation of creatinine, so that the result obtained is too low.

II. *A Method for Removing Aceto-acetic Acid from Urine Preliminary to the Estimation of Creatinine.*

In Folin's method for estimating the creatinine + creatine, the creatine is converted into creatinine by heating on a water-bath for three hours with normal hydrochloric acid. This procedure removes all the aceto-acetic acid from the urine by converting it into acetone, which is distilled away. Thus the aceto-acetic acid could not be detected by Rothera's (21)* nitroprusside test after one hour's heating. Consequently the estimation of the creatinine + creatine will not be disturbed by the presence of any aceto-acetic acid and will be accurate, but the result of the creatinine estimation which is carried out in the presence of aceto-acetic acid will be too low. Consequently the result obtained for the creatinine + creatine will be higher than that for the creatinine and will lead to the conclusion that creatine is present in the urine whether this is actually the case or not.

The aceto-acetic acid must, therefore, be removed from the urine before the determinations are made. Greenwald (12) extracted the urine with ether for two hours and found that the aceto-acetic acid was all removed by that process; the ether was subsequently removed by aëration for one hour. This process involved some dilution of the urine and in order to get over this difficulty Greenwald added twice the amount of picric acid and soda. This method takes some time to carry out and in our experience it is better if possible to avoid all dilution of the urine, especially when the urine is dilute to begin with, in order to get correct results.

The method which we employed (10) is very much simpler and we have now tested it carefully and modified it slightly (11).

1 c.c. of 10-per-cent. phosphoric acid is added to 10 c.c. of urine in a boiling tube 200 mm. long and 30 mm. wide. The mixture is then heated in a water-bath of which the temperature is between 65° and 70° C. and at a pressure of about 210 mm. of mercury produced by means of a filter pump. Bumping is prevented by allowing air to bubble slowly through the liquid by means of a capillary tube dipping into it. The temperature must not rise above 70° C. nor the pressure fall below 210 mm. or else concentration of the urine takes place. If the above directions are followed only a few drops of liquid are distilled over into the receiver, as the result of three-quarters of an hour's distillation. At the end of this time the process is stopped and the solution

* Rothera's test has been shown by Hurtley (14) to be a test for aceto-acetic acid as well as for acetone.

cooled. The mixture in the boiling tube is neutralised with 1·5 c.c. of 10-per-cent. soda* and then 15 c.c. of saturated picric acid and 5 c.c. of the soda are added. The mixture is allowed to stand seven minutes and then the contents of the boiling tube are washed into a 500 c.c. flask and diluted up to 500 c.c. with water. By heating for three-quarters of an hour, the aceto-acetic acid can be completely removed even if it is present in a concentration of 0·2 per cent.

If the concentration of aceto-acetic acid is greater than 0·2 per cent., the distillation must be continued for a longer time, and the complete removal of the aceto-acetic acid must be ascertained by testing a control with Rothera's nitroprusside test. We have continued the distillation for one and a half hours, and find that no error in the creatine estimation occurred.

We have tested the method in the following manner. The amount of creatinine in a normal urine was determined, and aceto-acetic acid was then added in varying amounts to the urine, and the creatinine again estimated. The aceto-acetic acid was then removed by the distillation method, and the creatinine again estimated. The results obtained show that the distillation did not break up any of the creatinine, and that the aceto-acetic acid was completely removed (Table IV).

Table IV shows that the Error caused by Varying Concentrations of Aceto-acetic Acid in the Urine is completely removed by the Distillation Method.

| Amount of aceto-acetic acid added to urine. | Creatinine by Folin's method. | | Creatinine after removal of the aceto-acetic acid by the distillation method. |
|---|---|---|---|
| | Urine alone. | Urine + aceto-acetic acid. | |
| grm. per 100 c.c. | grm. per 100 c.c. | grm. per 100 c.c. | grm. per 100 c.c. |
| 0·088 approx. | 0·145 | 0·128 | 0·145 |
| 0·044 ,, | 0·108 | 0·097 | 0·105 |
| 0·18 | 0·096 | Not estimated | 0·096 |
| 0·27 | 0·096 | 0·053 | 0·094 |

One of the most important questions to decide was whether creatine was converted into creatinine in this process of distillation.

Pure crystalline creatine† was added to normal urine in varying amounts,

* The phosphoric acid must be adjusted against the 10-per-cent. caustic soda, and the correct amount of caustic soda necessary to neutralise 1 c.c. phosphoric acid must be added.

† We wish to thank Dr. F. G. Hopkins and Mr. Mackenzie Wallis for kindly supplying us with the pure creatine.

and the creatinine was then determined by Folin's method and by the distillation method. The creatine was then converted into creatinine by heating on the water-bath for three and a half hours or longer with 5 c.c. normal hydrochloric acid, in order to get the creatinine + creatine figure. These experiments show that in no case was any creatine converted into creatinine by the distillation, even when (Experiment 5) there was actually more creatine (0·152 grm.) than creatinine (0·119 grm.) in the urine (Table V). In the first three experiments, when creatine was present in small amounts, practically all the creatine added to the urine was converted into creatinine by heating for three and a half hours on the water-bath. In the fourth experiment, when 0·076 grm. of creatine was present in the urine, only 48 per cent. of the creatine was converted into creatinine after three and a half hours, and even after five and a half hours only 89 per cent. could be recovered. In the fifth experiment, with a very large amount of creatine (0·152 grm. per 100 c.c.), only 28 per cent. was converted after heating for three and a half hours.

Table V shows that Creatine is not broken down into Creatinine by Heating with Phosphoric Acid in the Distillation Method.

| No. of expt. | Amount of creatine calculated as creatinine added to urine. | Creatinine. | | Creatinine + creatine calculated as creatinine. | Creatine recovered in the estimation as creatinine. | Creatine added to urine as creatinine. | Per-centage recovered. |
|---|---|---|---|---|---|---|---|
| | | Folin's method. | Distillation method. | | | | |
| | grm. per 100 c.c. | grm. per 100 c.c. | grm. per 100 c.c. | grm. per 100 c.c. | grm. per 100 c.c. | grm. per 100 c.c. | |
| 1 | 0·01 | 0·071 | 0·071 | 0·081† | 0·01 | 0·01 | 100 |
| 2 | 0·016 | 0·095 | 0·096 | 0·110† | 0·015 | 0·016 | 95 |
| 3 | 0·034 | 0·112 | 0·113* | 0·145† | 0·033 | 0·034 | 97 |
| 4 | 0·076 | 0·117 | 0·117 | 0·154†<br>0·185‡<br>0·183§ | 0·037†<br>0·068‡<br>0·063§ | 0·076 | 47·5<br>89·5<br>83 |
| 5 | 0·152 | 0·119 | 0·120 | 0·163† | 0·044 | 0·152 | 24 |

* Distilled 1½ hours.
† Heated for 3½ hours on the water-bath.
‡ „ 5½ „ „
§ By the autoclave method.

Benedict and Myers (3) have also noticed that the water-bath method gave an incomplete result. when urine containing 0·066 grm. creatine per 100 c.c. was used. Mellanby (18) has pointed out that five hours' heating on the water-bath is usually necessary to get a complete conversion. However, our observations show that three and a half hours on the water-

bath is sufficient to estimate accurately small amounts of creatine up to 0·034 grm. per 100 c.c., but that a greater quantity than this cannot be converted quantitatively into creatinine in three and a half hours. Larger amounts than 0·034 grm. per 100 c.c. must be heated for more than three and a half hours, and it is very difficult, even so, to convert all the creatine into creatinine. As the greatest amount of creatine found by Cathcart (5) in a case of carbohydrate starvation was 0·38 grm. per day, three and a half hours' heating on a water-bath would be quite sufficient to convert practically all the creatine, that might be present in the urine, into creatinine.

Finally, to test the accuracy of the distillation method, we have employed it for urines containing both creatine and aceto-acetic acid in solution together (Table VI). In one experiment creatine was added to normal urine, and the estimations were carried out with and without the addition of aceto-acetic acid. In the second experiment the creatine was added to part of the urine of the third day of the diet experiment (Table VIII). In this case the urine already contained aceto-acetic acid. The results (Table VI) show that, even when both aceto-acetic acid and creatine are present at the same time, the aceto-acetic acid can be removed from the urine without breaking up the creatine, and that the creatine, if present in small amount, can be converted almost quantitatively into creatinine after three and a half hours' heating. The series of control experiments shows that the distillation method gives satisfactory results.

Table VI shows the Accuracy of the Distillation Method for Urines containing both Creatine and Aceto-acetic Acid.

| | | Creatinine. | | Creatinine + creatine (as creatinine), Folin's method. | Creatine recovered by experiment (as creatinine). | Percentage recovered. |
|---|---|---|---|---|---|---|
| | | Folin's method. | Distillation method. | | | |
| | | grm. per 100 c.c. | grm per 100 c.c. | grm. per 100 c.c. | grm. per 100 c.c. | |
| A | Normal urine + creatine 0·01 grm. per 100 c.c. | 0·071 | 0·070 | 0·080 | 0·01 | 100 |
| | Same urine + creatine 0·01 grm. per 100 c.c. + 0·06 grm. of aceto-acetic acid per 100 c.c. | 0·047 | 0·070 | 0·079 | 0·009 | 90 |
| B | Urine of Day 3, Experiment II, containing 0·112 grm. of aceto-acetic acid per 100 c.c. | 0·10 | 0·119 | 0·119 | 0 | |
| | Same urine + creatine 0·04 grm. per 100 c.c. | 0·10 | 0·120 | 0·156 | 0·036 | 90 |

In the examination of any urine which contains aceto-acetic acid, and which is thought to contain creatine, two estimations are required, namely, that of the total creatinine + creatine by the original Folin method, and that of the creatinine alone by the method given in this paper.

III. *The Alleged Excretion of Creatine on a Carbohydrate-free Diet.*

It is well known that the consumption of a diet containing no carbohydrates produces acidosis, with the excretion of β-oxybutyric acid, aceto-acetic acid, and acetone. The aceto-acetic acid will cause an error in estimating creatinine and creatine, and must be removed to get accurate results.

We have performed three diet experiments on three separate individuals, and have investigated the creatinine and creatine excretion, taking this precaution. The experiments were begun about 12 hours after the last ordinary meal in Experiments I and III, and six hours after, in Experiment II. Tables VII, VIII, and IX show the various determinations made. The creatinine was first of all estimated directly by Folin's method without removing the aceto-acetic acid, and the results are referred to as "apparent creatinine." The true creatinine was then obtained after removing the aceto-acetic acid by the distillation method. The creatinine + creatine was determined by heating the urine for three and a half hours on the water-bath with hydrochloric acid. By subtracting the apparent creatinine values from the creatinine + creatine output, the apparent creatine was obtained, and, by subtracting the true creatinine from the creatinine + creatine, the true creatine output was obtained. Duplicate determinations were performed in each case, and in each determination the mean of six to eight readings of the scale was taken.

In Experiment I (E. P. P.), cream alone was eaten on the first two days; on the third day protein was added to the diet. The calorie value of the diet was low. The effect of the withdrawal of carbohydrates was shown by the prompt appearance of aceto-acetic acid in the urine. On the first day the nitroprusside reaction (Rothera's) was faint, but on the second and third days it was well marked, and 0·872 and 0·874 grm. of aceto-acetic acid were excreted. On the first day the apparent creatinine was 1·82 grm., while the true creatinine and creatinine + creatine was 1·78 and 1·80 respectively, so that no creatine was excreted in the urine, as the difference is within the limits of experimental error. On the 2nd day the apparent creatinine was diminished to 1·58 grm., while the true creatinine and creatinine + creatine were practically the same as on the previous day, *i.e.*, 1·81 and 1·82 grm. The apparent creatine was, therefore, 0·24 grm., while no true creatine was excreted. On the third day the apparent creatinine had fallen to 1·42 grm., while the

true creatinine and creatinine + creatine was still 1·72 grm. The apparent creatine had, therefore, increased to 0·31 grm., while as a matter of fact no true creatine was excreted.

In Experiment II (G. G.) ¾ pint of cream and two eggs were eaten on each day. The amount of aceto-acetic acid excreted was greater than in Experiment I, and the nitroprusside reaction was quite strong on the first day, 0·3 grm. being excreted. On the second and third days the aceto-acetic acid amounted to 1·06 and 1·46 grm. The true creatinine output was slightly lower than in the case of E. P. P., but it remained equally constant

Table VII.—Experiment I. Subject, E. P. P. Date, June 18–20, 1913.

| Day. | Volume. | Total nitrogen. | By the Folin method. | | | | After removal of aceto-acetic acid. | | Aceto-acetic acid. | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Apparent creatinine. | Creatinine + creatine. | Apparent creatine. | | True creatinine. | True creatine. | Grm. per day. | Concentration in 100 c.c. |
| | | | | | Grm. per day. | Grm. per 100 c.c. | Grm. per day. | Grm. per day. | | |
| | c.c. | grm. | grm. | grm. | | | | | | |
| 1 | 740 | 12 | 1·82 | 1·80 | 0 | 0 | 1·78 | 0 | — | — |
| 2 | 670 | 13·5 | 1·58 | 1·82 | 0·24 | 0·018* | 1·81 | 0 | 0·872 | 0·065* |
| 3 | 1070 | 16·25 | 1·42 | 1·72 | 0·30 | 0·028 | 1·72 | 0 | 0·874 | 0·081 |

Diet eaten.—Day 1 and 2: Cream, 300 c.c. Calorie value (approximate), 1060.
Day 3: Cream, 300 c.c.; plasmon, 50 grm.; eggs, 2. Calorie value (approximate), 1640.

* On this day the volume of urine was small and an equal volume of water was added to it before the creatinine determinations were made in order to get a reading on the colorimeter scale within the limits advised by Folin. This dilution will halve the concentration of the aceto-acetic acid in the solution used for the Folin estimation, which becomes actually less than that of the succeeding day.

Table VIII.—Experiment II. Subject, G. G. Date, June 22–25, 1913.

| Day. | Volume. | Total nitrogen. | By the Folin method. | | | | After removal of aceto-acetic acid. | | Aceto-acetic acid. | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Apparent creatinine. | Creatinine + creatine. | Apparent creatine. | | True creatinine. | True creatine. | Grm. per day. | Concentration per 100 c.c. |
| | | | | | Grm. per day. | Grm. per 100 c.c. | | | | |
| | c.c. | grm. | grm. | grm. | | | grm. | grm. | | |
| 1 | 1310 | — | 1·46 | 1·53 | 0·07 | 0·0053 | 1·52 | 0 | 0·3 | 0·029 |
| 2 | 1235 | 12·4 | 1·21 | 1·43 | 0·22 | 0·0175 | 1·43 | 0 | 1·06 | 0·085 |
| 3 | 1310 | 11·86 | 1·17 | 1·53 | 0·36 | 0·0274 | 1·52 | 0 | 1·46 | 0·112 |

Diet eaten.—Cream, 400 c.c.; eggs, 2. Calorie value (approximate), 1400.

Table IX.—Experiment III. Subject, M. D. Date, June 25–28, 1913.

| Day. | Volume. | Total nitrogen. | By the Folin method. | | | | After removal of aceto-acetic acid. | | Aceto-acetic acid. | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Apparent creatinine. | Creatinine + creatine. | Apparent creatine. | | True creatinine. | True creatine. | Grm. per day. | Concentration per 100 c.c. |
| | | | | | Grm. per day. | Grm. per 100 c.c. | | | | |
| | c.c. | grm. | grm. | grm. | | | | | | |
| 1 | 600 | 11·99 | 1·93 | 2·05 | 0·12 | 0·010 | 2·03 | 0 | 0·43 | 0·036* |
| 2 | 750 | 14·43 | 2·02 | 2·14 | 0·12 | 0·008 | 2·15 | 0 | 0·99 | 0·066* |
| 3 | 1220 | 14·82 | 2·09 | 2·25 | 0·16 | — | 2·27 | 0 | — | — |

Diet eaten.—Cream, 500 c.c.; eggs, 3. Calorie value (approximate), 1600 calories.

* On these days the volume of urine was small and an equal volume of water was added to it before the creatinine determinations were made in order to get a reading on the colorimeter scale within the limits advised by Folin. This dilution will halve the concentration of the aceto-acetic acid in the urine.

throughout the experiment, viz., 1·5 grm. On the first day the apparent creatine was already 0·07 grm., and on the second and third days it had risen to 0·22 and 0·36 grm. respectively, but no true creatine was excreted at all. The difference in the scale reading between the apparent and true creatinine was 2 mm. on the third day of this experiment. It has been previously shown (p. 211, fig. 1) that the error in the estimation of the creatinine caused by the aceto-acetic acid in these two experiments agrees fairly closely with the error caused by adding the same concentration of a sodium aceto-acetate solution to normal urine.

As it was necessary to be absolutely certain that if any creatine was present in the urine it would be converted into creatinine by the methods we have used, some pure creatine was added to a part of the urine of the third day in Experiment II. The estimation of the creatinine + creatine in the plain urine and in the urine to which creatine had been added was carried out under precisely similar conditions on the same water-bath. The result (Table VI) showed that in the plain urine no creatine was converted into creatinine, but that the creatine was almost quantitatively converted into creatinine in the sample of urine to which creatine had been added. This control experiment shows that creatine if present in the urine is detected and estimated by the methods employed.

As we wished to confirm the results of these experiments on ourselves, Dr. M. Donaldson very kindly took the following diet for three days, viz., ¾ pint of cream and three eggs each day. We wish to express our thanks to him. The urine gave Rothera's nitroprusside test on the first day, and this reaction was well marked on the second and third days. The true

creatinine in the urine was again very constant for the three days, lying between 2 and 2·3 grm. On the first and second days the apparent creatine was 0·12 grm., and on the third day it was 0·16, while no true creatine was excreted. The apparent creatinine was not so low as in Experiments I and II, but its difference from the true creatinine was quite definite enough to be measured on the colorimeter.

*Discussion of Results.*

These three experiments show that the removal of carbohydrate from the diet causes an excretion of aceto-acetic acid in sufficient amount to cause an error in the estimation of creatinine, so that the results are too low and creatine is apparently excreted.

Cathcart (5), Benedict (2), Mendel and Rose (19) state that creatine occurs in the urine under somewhat similar conditions to those under which we have worked. The amounts of creatine they obtained were about the same as those apparently obtained by us, before we removed the aceto-acetic acid, *e.g.* the largest amount that Cathcart (5) found on a fat diet was 0·38 grm., which is slightly more than the apparent creatine we found on the third day of Experiment II.

Cathcart remarks that the creatinine excretion diminishes to a certain extent, as the result of a fat diet. We have found that it remains constant throughout, and in the case of G. G. agreed very closely with the amount of creatinine excreted 15 months before on a pure fat and carbohydrate diet (10). However, the error caused by the presence of aceto-acetic acid in the urine results in less creatinine being found than is actually present.

Our experiments extended over about the same time as those of Cathcart, but there was a slight difference, viz., that we had no preliminary starvation day. However in Experiment I the condition of semi-starvation was really very similar to that of one day's complete starvation, as only half a pint of cream was taken and the calorie value was 1060.

We have also published a case of carbohydrate starvation (10) lasting for 10 days in which the diet had a calorie value of only 1969 per diem. No creatine was excreted at any time, and the creatinine excretion remained constant throughout.

From these results we draw the conclusion that mere carbohydrate starvation itself does not cause an excretion of creatine in the urine.

Naturally, no conclusion can be drawn from these experiments as to whether creatine is excreted during prolonged periods of total starvation, but we maintain that in all those many physiological and pathological conditions in which acetone bodies are excreted in the urine, the estimations of creatinine

and creatine must be inaccurate, unless the precaution is taken of removing the aceto-acetic acid from the urine.

*Conclusions.*

1. The presence of aceto-acetic acid always causes an error in the estimation of creatinine and the error increases with increasing amounts of aceto-acetic acid. As the result of this error the estimation of creatinine will be too low. This error is not eliminated if the diluted urine is allowed to stand for varying lengths of time before making the readings.

2. The aceto-acetic acid is removed in the estimation of creatinine + creatine and does not cause any error.

3. As the creatinine figure is too low and the creatinine + creatine figure is correct, it will appear that creatine has been excreted.

4. Acetone and $\beta$-oxybutyric acid, if present in amounts comparable to those which usually occur in urine, produce practically no error in the estimation of creatinine.

5. A simple and reliable method has been devised for removing aceto-acetic acid, preliminary to the estimation of creatinine.

6. In our experiments a carbohydrate-free diet did not cause the excretion of any creatine.

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