XLV. OBSERVATIONS ON THE USE OF THE FOLIN METHOD FOR THE ESTIMATION OF CREATINE AND CREATININE.

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During a continuous use, for over three years, of the Folin colorimetric method of estimating creatine and creatinine we have made observations by way of testing its accuracy and limitations which we think may be useful to others if published. Some of these have meanwhile been pointed out by workers in other laboratories, but we include them for the sake of corroboration.

Procedure.

The colorimeter we mostly used was that of Jobin—a modification of the Du Boscq instrument. We also compared its readings with those of two similar instruments made by Pellin[2] and with one of the Kagenaar pattern as described by Hoogenhuyze and Verploegh [1905]. We found that consistent and reliable readings were obtainable with all, after a little experience in their use had been acquired.

For making the standard bichromate solution we used Kahlbaum's purest bichromate of potassium certified to contain 99·97 °/₀ of the pure salt. This was fused, then cooled in a desiccator and a semi-normal solution made from it containing 25·54 g. in the litre.

The readings of the colorimeter were made by one of us (W. H. T.), but

[1] Immediately before sending this paper for publication we learned, to our great sorrow, of the death of our co-worker, Thomas Arthur Wallace, at Agra in India, whither he had gone, a short while before, to take up a post in the Missionary College there. W. H. T., H. R. S. C.

[2] One of these was very kindly lent to us by Dr Ritchie of the Royal College of Physicians Laboratories, Edinburgh, to whom we express our best thanks.

were checked in all important cases by comparative readings made mostly by a second person in the laboratory familiar with the use of the instrument[1].

To avoid unconscious bias, the solutions in all the critical readings were made up by a second person and given to the reader under a number which gave no indication of which solution it was, this being disclosed only when the series was finished. We found this a very useful safeguard.

Great care was taken to obtain equal illumination of the two halves of the colorimeter. One important cause of inequality was found to be uneven blackening of the interior of the metal box in which the glass parallelopipeds are contained. The possibility of shifting of the zero on one or other side of the instrument was constantly examined and allowed for.

Another routine precaution, taken at the beginning of every set of readings and repeated as a rule after every four readings (if the series exceeded six in number), was to read the standard solution against itself. This we regard as of the utmost importance.

Few will believe, without actual experience, how much the reading of the standard by an individual may vary and how necessary is this control. The procedure also furnishes a ready test of fatigue, whether retinal or cerebral. When fresh, there is no difficulty in obtaining similar readings on the two sides using the standard solution in both tubes. When fatigued it was found that the readings were too high or too low, oftenest the latter and frequently to the extent of 0·5 mm.

The degree of illumination unquestionably affects the result. This has been pointed out by several workers. On dull days the readings tended to be lower than on bright days. Our instrument was placed opposite a large window, with northern aspect, and the readings made, for the most part, in the middle hours of the day. The practice of reading the standard against itself reduces the error for variation in the illumination.

Control of the Standard Solution.

Folin [1904], in fixing his bichromate standard, tested it against pure creatinine prepared from urine, also against creatinine solutions made by the conversion of creatine, and likewise against the double picrate of creatinine and potassium obtained from urine[2].

Of subsequent workers, there appear to be only three who controlled

[1] We are grateful to Professor Noel Paton and Dr Rosenheim for assisting us in this way on more than one occasion.

[2] This last was overlooked by one of us (W. H. T.) in a recent note on the subject [1913].

their standard solutions by the first of these procedures, namely: Hoogenhuyze and Verploegh [1905], Koch [1905] and Klercker [1907].

The creatinine used by Hoogenhuyze and Verploegh was prepared from urine and gave almost theoretical readings (8·14 mm. instead of 8·1 mm.). That of Koch was obtained from Merck and required 11·46 mgms. to give the standard reading of 8·1 mm. in the colorimeter, this being normally given by 10 mgms. of creatinine treated in the way described by Folin. Klercker used a solution of creatinine prepared from urine. The content of this was determined by estimating the total nitrogen and found to be 96·6 % of the theoretical, assuming all the nitrogen to be present as creatinine.

We endeavoured to control our standards by this method, but early attempts to prepare pure creatinine did not prove satisfactory. Later we prepared a small sample of pure creatinine free from creatine. With this we obtained theoretical readings. The difficulty we encountered of preparing pure creatinine is admitted by Folin. This has been largely reduced by his newer methods.

Of these latter, that of heating pure crystalline creatine in the autoclave at a pressure of 4·5 kilos per sq. cm. for 3 hours was tried by one of us. The yield was not so high as claimed for it by Folin, nor the creatinine so pure as anticipated, but the method proved valuable in giving an easy means of preparing pure creatinine picrate which we used later as a control.

Most workers have tested their bichromate standard solution against solutions of creatinine prepared by the conversion of pure creatine. This was the method followed by Dorner [1907], Gottlieb and Stangassinger [1907], Hoogenhuyze and Verploegh [1908], Weber [1908], Benedict and Myers [1907]. We gave it an exhaustive trial. For this purpose a large number of samples of pure creatine, obtained from different kinds of flesh (horse, rabbit, dog), were prepared from time to time, and purified by recrystallising six to eight times till absolutely pure. Several Dumas analyses of these were made, the results of which are here shown.

TABLE I. *Showing results of Dumas analyses of samples of creatine.*

	Creatine taken	N. evolved	Temp.	Pressure	N. per cent.	Theory
1	0·1056	29·4 c.c.	16° C.	740 mm.	32·21	32·06
2	0·2220	61·8	18	740·8	32·03	,,
3	0·1625	44·0	17	755·5	31·92	,,
4	0·2020	54·8	16·5	756	32·22	,,
5	0·1260	35·4	22	753·4	32·10	,,
6	0·2160	58·0	18·8	754·6	32·30	,,

(Two of these estimates, Nos. 5 and 6, were kindly made for us by W. Caldwell, M.A., Senior Demonstrator of Physiology in this school, who

also checked readings for us at various times. For this assistance we express to him our best thanks.)

There can be no doubt from the above analyses that our preparations of creatine were absolutely pure. This is borne out also by the fact that the crystals in every case, after being fully air dried at room temperature, lost the calculated amount of water of crystallisation when further dried in the oven at 110° C. In this latter drying—notwithstanding statements to the contrary—creatine does not decompose and we found it unsafe to trust for complete drying to a temperature lower than this. In weighing the anhydrous creatine, which is very hygroscopic, great care was taken to guard against the absorption of water.

In making the test solutions of creatinine we usually took the quantity of creatine requisite to give when fully converted a 0·1 % solution of creatinine, that is to say, 0·116 grm. of anhydrous creatine or 0·132 grm. of crystalline creatine for 100 c.c. In a few cases, solutions of anhydrous creatine of other strengths were used.

From a large number of estimates made with these solutions we found, as is shown in detail in Tables XIII and XIV, that the average conversion by the water bath method amounts to 96·5 % of the theoretical yield—varying from 93·1 % to 100·2 %. The readings on the colorimeter scale corresponding to these were 8·39 mm. for the average, 8·7 mm. for the lower, and 8·08 mm. for the higher, instead of 8·1 mm. for the theoretical yield.

On more carefully examining the results published by others we found that our experiences were not singular. Folin gives no data on this point in his first article, but speaks of the difficulty of complete conversion of creatine in the article in *Hammarsten's Festschrift* [1906]. Dorner [1907] with 0·1 % solutions of creatine obtained results varying from 85 to 100 % of the theoretical yield. Gottlieb and Stangassinger [1907] 83·19 to 99·98 (with 4·56 % HCl) and 91·55 % to 92·98 % (with 2·28 % HCl). Jaffé's [1906] best result with 2–2·5 % HCl was 94·3 %. It did not seem to us therefore that this method was sufficiently accurate to be used as a control for the standard bichromate solution and another test was substituted by one of us the results of which have been published [Thompson 1913]. It was found that solutions of creatinine picrate and also of the double picrate of creatinine and potassium, when taken in quantities equivalent to 0·1 grm. creatinine and treated in the usual way, fulfilled the required conditions, giving readings of 8·1 mm. in the Du Boscq colorimeter. This result settled some doubts which had previously existed in our minds with regard to the accuracy of the Folin standard.

Conditions which affect the Readings.

Our object was not to test all the possible conditions which might affect the results. We confined ourselves to those which came under our notice in the application of the method to our work.

These were (1) in the estimation of creatine, the best amount of acid (HCl) to use for the conversion into creatinine; (2) the optimum time and temperature for the development of the colour in the estimation of creatinine and creatine; (3) the influence of the quantity of alkali added for the same purpose; (4) the relative values of readings at different parts of the scale; (5) the influence of urinary pigment on the estimation of creatine in urine; (6) the estimation of creatine in the presence of dextrose and the use of phosphoric acid; (7) the recovery of creatine from diabetic urine; and (8) the reliability of the autoclave method.

1. *Estimation of Creatine. The amount of acid to be used in the conversion.*

For the conversion of creatine into creatinine, Folin in 1904 recommended 5 c.c. of normal HCl to be added to 10 c.c. of a dilute solution of creatine. Later [1906] he increased this amount to 10 c.c., a quantity which has been adopted by most subsequent workers. A few however, namely Dorner [1907], Hoogenhuyze and Verploegh [1905], have used double that quantity, namely 20 c.c. of N. HCl.

We have tested all three quantities and are convinced that the best results, both with the water bath and autoclave methods, are got by using 10 c.c. N. HCl with 10 c.c. of weak creatine solution. The conversion with 5 c.c. is liable to be less complete, particularly if glucose be present.

From several observations the following two consecutive sets of readings are taken:

N. HCl	Method	Reading
20 c.c.	water bath	8·59 mm.
20	autoclave	8·69
10	,,	8·46
5	,,	8·6

N. HCl	Method	Reading
20 c.c.	autoclave	8·52 mm.
10	,,	8·26
5	,,	8·32

The readings with 20 c.c. and 5 c.c. are uniformly higher, that is, the yield is uniformly less, than with 10 c.c. It would seem as if a destruction of creatinine took place when 20 c.c. of acid were used.

Except at the very outset we have always used in these investigations 10 c.c. of N. HCl for the conversion of creatine.

2. *The Time and Temperature for Development of the Colour.*

Folin originally gave a time limit of 5 to 10 mins. for the development of the Jaffé reaction. Later [1906] he restricts the time to 5 mins. and this period has been adopted by most workers. Some however have departed from it, thus Dorner [1907] gave 5–15 mins., Benedict and Myers [1907] only 3·5 mins., Mellanby [1908] 5 mins. with some latitude, Mendel and Rose [1911] 10 mins.

Comparatively few observers however have recorded the temperature which they adopted. Amongst these are Hoogenhuyze and Verploegh [1905], who diluted with water at 15° C., Dorner [1907], who cooled the solution after adding the picric acid and Klercker [1907], who used water for dilution at different seasons of the year of about the same temperature. Mellanby [1908] also states that it is necessary for the temperature to be constant but does not give the degree.

We found with temperatures of 10° C. to 15° C. that the maximum colour was not developed under eight minutes. Between 15° C. and 17° C. the time required was seven minutes, above 17° C. and below 20° C. five minutes is sufficient.

The results are shown in the following table:

TABLE II. *Creatinine estimation: influence of time and temperature on the development of the Jaffé reaction.*

Readings Time 5 mins.	Readings Time 7 mins.	Temp.	Method of conversion
8·75 mm.	8·52 mm.	10	Water bath 5 hours.
9·30	8·85	10	Autoclave HCl 25 mins.
8·55	8·45	15	Water bath.
8·76	8·50	15	Autocl. HCl 25 mins.
8·80	8·70	15	Autocl. H_3PO_4 30 mins.
8·30	8·11	15	Water bath HCl.
8·48	8·35	17	Water bath HCl 3 hours.
8·29	8·30	20	Water bath HCl 3 hours.

Nearly all our observations were made at a temperature lying between 15° C.–17° C. The flasks in which the reaction was carried out were weighted with leaden rings and kept in a basin of water at this temperature.

3. *Effect of the amount of alkali used in the Folin method.*

We found that the quantity of alkali added to develop the colour of the Jaffé reaction distinctly affected the results. For creatinine Folin recommended 5 c.c. of 10 % NaOH solution; for creatine, the same amount over and above

that necessary to neutralise the acid used in the conversion. These quantities have been adhered to generally by others with few exceptions. Thus Benedict and Myers [1907] used 10 c.c. for 3·5 mins.; Mellanby [1908] concluded that no great accuracy was necessary; he got the same results with 10 c.c. and 3 c.c. His observations were chiefly concerned with meat extracts. Grindley and Woods [1906], also working with meat extracts, used 5 c.c. to 10 c.c. 10 % NaOH. Hehner [1907] soon afterwards stated, in a short publication giving no data, that an excessive quantity of alkali diminished the colour in the case of meat extracts, and that to obtain the best results the amount of alkali added must be quite small. He also considered that more picric acid than the quantity prescribed by Folin should be used when dealing with these extracts. In reply to this Emmett and Grindley [1907] repeated the work of Grindley and Woods, finding that in the determination of preformed creatinine the use of a small or large amount of alkali made almost no difference, but that slightly better results were obtained with 10 c.c. or 15 c.c. than with 5 c.c. For transformed creatine 10 c.c. and 15 c.c. gave the same results, and both were better than 5 c.c. It is not clear, however, that the acid used in the conversion was neutralised before adding the picric acid solution, nor was their method strictly comparable to that followed by others—the quantities of creatinine containing solution were much larger.

Cook [1909] also found in estimating the creatine content of meat extracts, that 5 c.c. of alkali did not give the maximum colour, while 10 c.c. and 15 c.c. gave identical results. With solutions of creatinine he found that 5 c.c. and 10 c.c. gave similar results. Accordingly, he recommended the use of 10 c.c. all round. For a considerable time we followed this recommendation without question, but later discovered that 10 c.c. of 10 % NaOH destroyed some of the creatinine and gave too low results. This applies chiefly to pure solutions of creatinine but also, though to a less extent, to urine. We have not tested the point in the case of meat extracts. Our results are shown in the following readings.

TABLE III. *Influence of the quantity of alkali on the colour developed.*

	Readings with 5 c.c. NaOH of 10 % strength		Readings with 10 c.c. NaOH of 10 % strength
	(a) Creatinine in aqueous solution		
	8·29		8·75
	8·25		8·70
	8·58		8·75
	8·78		9·05
	8·20		8·58
Mean	8·42	Mean	8·77

TABLE III (*continued*)

(*b*) Creatinine in urine

	9·54		9·66
	9·05		9·45
	9·55		9·75
	6·30		6·50
	7·51		7·9
	8·25		8·27
	6·26		6·41
	5·73		5·85
Mean	7·77	Mean	7·97

It will be seen that with pure solutions of creatinine, the average reading of the series, when 5 c.c. of alkali were used, was 8·42 mm. (= 0·0966 °/₀ of creatinine): when 10 c.c. of alkali were used the average reading was 0·35 mm. higher, that is 8·77 (= 0·0924 °/₀ of creatinine). There was also a difference with urine though less marked. The mean of the series of readings with 5 c.c. of alkali is 7·77 mm. (= 0·1042 °/₀), that with 10 c.c. alkali is 7·97 mm. (= 0·1016 °/₀ creatinine). So far as these solutions are concerned we are unable to confirm Cook: on the contrary, we find that better results are given when Folin's original directions are strictly followed.

4. *Relative values of readings at different parts of the scale.*

It has been generally accepted that within a lower limit of 5 mm. and an upper one of 12 mm., as stated by Folin, the values of the readings are proportional. We have not found this to be so, nor is it generally true in colorimetry that the dilution ratio of a solution is inversely proportional to the height of the column of coloured solution. Folin naturally refers to this, and explains the exceptional result in his method as follows. He concludes that dilution produces a diminution of the total colour of the solution—not an increase such as would arise if the colour were due to a red ion—but that the diminution is hidden by the increased relative depth of tint which occurs with increase of the height of column seen through. It has, however, since been shown by Chapman [1909] that the colour in the Jaffé reaction is due not alone to the formation of picramic acid, as had long been held, but to a mixture of picramic acid and a still redder reduction product of picric acid, namely diaminonitrophenol. It is doubtful therefore if Folin's explanation will hold good, nor are the facts quite in accordance with his view.

On this point we made a large number of observations, comparing in each case the reading given at a certain dilution with that given by the same solution diluted to one and a quarter, one and a half, or double the volume.

In the following table we give some of our results confining these to a comparison of readings with dilutions to 250 c.c. and 500 c.c., but making the selection so as to show the results with different solvents. These latter were water, dog's urine, normal human urine, and diabetic human urine.

TABLE IV. *Relative value of readings at different parts of colorimeter scale.*

	Solvent	Readings Dilution 10–250	Readings Dilution 10–500	Ratio	
1.	Water (HCl)	6·3 mm.	11·2 mm.	1 : 1·75	Mean = 1 : 1·76
2.	,,	6·5	11·36	1 : 1·72	
3.	,,	6·55	11·60	1 : 1·77	
4.	,,	5·50	10·10	1 : 1·80	
5.	,,	5·45	9·80	1 : 1·77	
6.	,,	6·15	11·00	1 : 1·79	
7.	,,	6·28	11·00	1 : 1·75	
8.	,,	6·15	10·90	1 : 1·77	
9.	,,	6·15	10·70	1 : 1·74	
10.	,,	6·30	10·90	1 : 1·73	
11.	,,	6·24	11·15	1 : 1·78	
12.	Dog's urine (HCl)	4·80	8·90	1 : 1·85	Mean = 1 : 1·91
13.	,, ,,	4·43	8·50	1 : 1·92	
14.	,, ,,	5·90	11·30	1 : 1·91	
15.	,, ,,	4·85	9·30	1 : 1·92	
16.	,, ,,	4·63	9·00	1 : 1·94	
17.	,, ,,	4·60	8·96	1 : 1·95	
18.	,, ,,	4·87	9·24	1 : 1·90	
19.	Human urine (HCl)	6·30	12·20	1 : 1·94	Mean = 1 : 1·91
20.	,, ,,	4·30	8·10	1 : 1·88	
21.	,, ,,	5·55	10·30	1 : 1·87	
22.	,, ,,	5·50	10·67	1 : 1·94	
23.	Diabetic urine	5·22	10·10	1 : 1·93	Mean = 1 : 1·87
24.	,, ,,	6·60	12·80	1 : 1·94	
25.	,, ,,	6·60	12·00	1 : 1·82	
26.	,, ,,	6·25	11·30	1 : 1·81	

On examining the list it will be seen that the higher reading is never double the lower and that the ratio differs considerably when urine is compared with water as the solvent, also that there is a difference, though not marked, between normal and diabetic human urine. On the other hand, the dilution ratio is identical for human and dog's urine. The mean ratio instead of being 1 to 2, is, for solutions in water 1 to 1·76, in dog's urine and human urine 1 to 1·91, in diabetic urine 1 to 1·87. These results were borne out by dilutions to other degrees than one to two.

Our general conclusions are that for accurate work readings can only be regarded as strictly proportional if they lie between a lower limit of 7 mm. and an upper one of 9 mm. If separated by a wider interval a correction factor has to be applied, and this varies for different solvents, but for all

practical physiological purposes may be based on the mean of all the above ratios for a difference of column of 5 mm., viz. 1:1·8 instead of 1:2. In general therefore the upper readings of the scale give relatively too high results, the lower readings on the contrary too low results, as compared with the normal reading of 8·1 mm.

5. *The influence of urinary pigment: recovery of creatine from normal urine.*

Several observers have called attention to the influence of urinary pigment. Weber [1908] found that the darkening which occurs on boiling urine with HCl lowers the reading and increases the estimation of creatine by fully 5 %. Benedict [1912] also found a similar effect and recommended the use of granulated zinc in the boiling to remedy it. Rose [1912] did not find this procedure efficacious with diabetic urines and recommended the use of phosphoric acid instead of hydrochloric. Dreibholz [1908], with diabetic urine, found a difficulty in matching the colour with the standard bichromate solution after boiling the urine with hydrochloric acid, but failed to find a useful remedy. With normal urine when the pigment was removed by filtration the result was not affected.

Early in these investigations it was clear to us that in the case of dog's urine the darkening on boiling with hydrochloric acid lowered the colorimeter readings. We therefore proceeded to investigate the point, and did so in two ways, both of which were applied to normal human and dog's urine.

In the first method the ordinary creatine readings of a series of urines were compared with those given by similar samples of the same urines after boiling with an equal quantity of normal HCl. In the boiling we used not alone the water bath for three hours, but also the autoclave at 117°–120° C. for two different periods, namely, 15 mins. and 25–30 mins. The results are shown in the following tables:

TABLE V. *Showing colorimeter readings of human urine before and after boiling with normal HCl.*

	Unboiled	Water bath 3 hours	Autoclave 15 mins.	Autoclave 25–30 mins.
1.	7·8	7·53	7·5	7·6
2.	6·5	6·3	6·5	6·51
3.	8·1	7·9	8·15	8·1
4.	7·24	7·15	7·16	7·26
5.	7·6	7·3	—	7·5
6.	6·25	6·16	—	6·1
7.	7·51	7·54	—	7·5
Mean	7·29	7·13	7·19	7·22

The effect on human urine is therefore very slight. Taking the mean yield of the unboiled urines as 100 %, that of the water bath series amounts to 102·25 %, and of the autoclave, 25–30 mins., to 101 %. The yield of those boiled in the autoclave for 15 mins., when compared with the same group of urines unboiled, works out at 101·1 %.

TABLE VI. *Showing similar readings to the above for dog's urine.*

	Unboiled	Water bath 3 hours	Autoclave 15 mins.	Autoclave 25–30 mins.
1.	6·2	5·7	6·35	6·06
2.	7·4	6·08	6·46	6·45
3.	7·1	6·4	6·84	6·73
4.	7·74	7·3	7·54	7·47
5.	6·62	6·2	—	6·67
6.	8·5	7·87	—	7·9
7.	9·3	8·03	—	8·13
Mean	7·55	6·8	6·8	7·06

The results for dog's urine, taking the mean of the unboiled series as 100 %, work out as 111 % for urine treated by the water bath method, and 106·9 % for the same boiled in the autoclave for 25–30 mins. The mean yield of the smaller autoclave series boiled for 15 mins. gives 104·6 % when compared with that of the same group unboiled. It is interesting to note that in both series the effect on the pigment is more pronounced in the water bath than in the autoclave method.

The second method consisted in comparing the recovery of creatine when dissolved in urine with that of the same substance dissolved in water. The procedure adopted in testing the recovery of creatine from urine was as follows. Normal urines were taken and known quantities of the pure samples of creatine we used throughout this research were dissolved in them—for the most part 0·116 g. in 100 c.c. (= 0·1 % of creatinine).

Determinations were then made, (1) of the preformed creatinine in the urines unboiled, (2) of the readings of the same urines without any addition of creatine but treated by the water bath and autoclave methods, (3) of the total creatinine in the urines after creatine was added, these being treated as in (2). Two sets of subtractions were then made; (*a*) the creatinine of the unboiled urine was subtracted from the total creatinine of the urine to which creatine was added; (*b*) the creatinine of the boiled urine was subtracted from that of the same urine plus creatine.

The values for the first set of deductions should give too high results for recovered creatine, at all events in the dog's urine, if the darkening of the pigment affect the recovery. Those of the second set, presumably the

true creatine values, should correspond to the results of recovery of creatine from solution in water provided no other disturbing factor, than the effect of the pigment, entered into the reaction.

Observations were made both with human urine and with that of the dog. The following tables show the results:

TABLE VII. *Showing the recovery of creatine from dog's urine: expressed as volume percentages of the urine taken.*

	Creatine added expressed as creatinine	Creatine recovered expressed as creatinine water bath 3 hrs.		Creatine recovered expressed as creatinine autoclave 25 mins.	
		(a)	(b)	(a)	(b)
1.	0·05	0·0627	0·0598	0·0575	0·0567
2.	0·05	0·0594	0·0475	0·0613	0·0533
3.	0·10	0·1003	0·1003	0·0916	0·0910
4.	0·10	0·1029	0·0935	0·0939	0·0892
5.	0·10	0·0892	0·0809	0·0925	0·0930
6.	0·10	0·1297	0·1221	0·1022	0·0950
7.	0·05	0·0598	0·0461	0·0605	0·0478
Totals	0·5500	0·6040 =109·8 %	0·5502 =100 %	0·5595 =101·7 %	0·5260 =95·4 %

The total yield of the first set of values by the water bath method, column (*a*), works out as 109·8 % of the amount added. That obtained by the second method of deducting, column (*b*), gives a yield of 100 %. The corresponding values furnished by the autoclave method (25 mins. at 117–120° C.) are 101·7 % and 95·4 % respectively. Those therefore in column (*a*) show an increase due to pigment of 10 % by the water bath method and of 6–7 % by the autoclave method, figures which correspond very closely with the effects of darkening of the pigment of dog's urine seen in Table VI. Moreover, the mean of the recovery by the two methods, water bath and autoclave, obtained by the second method of deduction is 97·7 % which is very close to the average recovery from solution in water mentioned in the earlier part of this paper, namely, 96·5 %.

The results of recovery from human urine are seen in Table VIII.

On examining these figures it will be seen that the results of column (*a*) in the water bath method, which include the pigment effect, are just over 2 % higher than those of column (*b*). Similarly, those of the autoclave method show a difference of 1·2 % due to the pigment, and these differences correspond almost exactly with those given earlier for the effect of the pigment in human urine alone. The mean result for the two methods (water bath and autoclave), taking column (*b*) in each case, which excludes

the pigment effect, is 98·26 %, that is slightly higher than the general average for aqueous solutions. It may be concluded therefore that the darkening of the pigment is the only disturbing factor in normal urine in the recovery of creatine, and that its augmenting effect in round numbers is 2 % for human urine and 10 % for dog's urine. The effect, moreover, may be practically eliminated by the procedure of boiling the urine for the normal period (in any given research) and deducting the results from those given by the urine of subsequent periods treated in identically the same way—that is assuming the pigment has remained unaltered throughout.

TABLE VIII. *Showing the recovery of creatine from human urine: expressed as volume percentages of the urine taken.*

	Creatine added expressed as creatinine	Creatine recovered expressed as creatinine water bath 3 hrs.		Creatine recovered expressed as creatinine autoclave 25 mins.	
		(a)	(b)	(a)	(b)
1.	0·05	0·0521	0·0496	0·0563	0·0563
2.	0·10	0·0980	0·0970	0·0970	0·0974
3.	0·10	0·0980	0·0937	0·0958	0·0944
4.	0·10	0·1111	0·1092	0·1104	0·1072
5.	0·10	0·0881	0·0885	0·0922	0·0920
Totals	0·45	0·4473 =99·4 %	0·4380 =97·33 %	0·4517 =100·4 %	0·4473 =99·2 %

6. *Estimation of creatine in the presence of dextrose.*

Different statements had been made with regard to the influence of dextrose on the Jaffé reaction in its application to the estimation of creatinine and creatine. Klercker [1907] found that on long standing, when glucose was present, the colour became deeper; Hoogenhuyze and Verploegh [1905] that glucose produces no effect in the time necessary for the Folin estimation; Dreibholz [1908] that the caramel produced on boiling diabetic urine with HCl adversely affected the estimation of creatine; Taylor [1911] that glucose introduces no effect on the estimation of creatine unless present to above 5 %; while Rose [1912] strongly supports the view of Dreibholz.

We felt it necessary from the outset of our investigations to ascertain for our own satisfaction which statement was to be accepted. Our first observations dealt with aqueous solutions of creatine and dextrose, later we extended them to diabetic urine in which creatine was dissolved.

TABLE IX. *Estimation of creatine in presence of dextrose.*

	Water bath 3 hours HCl	Autoclave 25 mins. HCl	Autoclave 30 mins. H_3PO_4 3 %	Autoclave 30 mins. H_3PO_4 2 %	Dextrose present	Theoretical amt. expressed as creatinine
1.	0·1012	0·1020	—	—	5 %	0·1000
2.	0·1004	0·1002	—	—	10 %	,,
3.	0·0937	0·0936	0·0886	—	10 %	,,
4.	0·0910	0 0907	0·0814	—	10 %	,,
5.	0·0895	0·0900	0·0815	0·0900	10 %	0·0900
6.	0·0975	0·0968	0·0917	0·0960	10 %	0·1000
7.	0·0324	0·0366	0·0323	0·0326	10 %	0·0342
8.	0·0960	0·0964	0·0850	0·0889	10 %	0·1000
Av. % of recovery	96·6	96·6	86·7	92·5		0·1000

The foregoing table gives the first series of observations. It includes also a comparison of the results of the water bath method with those of the autoclave. Further, the efficacy of phosphoric acid in strengths of 3 % and 2 % for the recovery of creatine is compared with that of normal hydrochloric acid.

On examining the table it will be seen that with HCl—both water bath and autoclave methods—the average percentage of recovery (96·6 %) is practically the same as when dextrose is not present in the solution. This, as previously mentioned, was 96·5 %. Our conclusion, therefore, is that dextrose *per se* has very little, if any, influence on the results within the time necessary for estimating creatine.

In the above observations we used 10 c.c. of normal HCl for 10 c.c. of creatine solution, but in some of our early work the quantity of acid added was only 5 c.c. In these we found, when the quantity of sugar was 5 % or over, that the creatine results were much too low. This has also been the experience of M. Ross Taylor [1911]. It is essential therefore to use the larger quantity of acid.

It has been stated by Rose [1912] that the disturbing influence of dextrose is due to the action of hydrochloric acid in causing a transformation of some of the sugar into caramel. It occurred to him therefore to employ phosphoric acid in the conversion of creatine when dextrose is present, since it does not caramelise sugar. Rose claims to have obtained better results in this way than with hydrochloric acid. We tested the effects of the two acids, using solutions of creatine both in the presence and absence of sugar. In these tests varying strengths of phosphoric acid were used, 5 %, 4 %, 3 % and 2 %. The results are shown in Table IX. In all cases the best results were obtained by us with 2 %, by Rose, however, with 3 % phosphoric acid, the

volume of acid added in both sets of observations being 20 c.c. The results with 5 % and 4 % phosphoric acid were much inferior—these strengths causing a considerable destruction of creatinine.

We then felt it necessary to make a comparison of these same acids, using creatine in aqueous solution. The following table gives the results:

TABLE X. *Estimation of creatine in aqueous solution: comparison of hydrochloric and phosphoric acid in transforming creatine into creatinine.*

	Water bath N. HCl	Autoclave 30 mins. H_3PO_4 3 %	Autoclave 30 mins. H_3PO_4 2 %	Theoretical amt. expressed as creatinine
1.	0·0942	0·0910	0·0940	0·1000
2.	0·0935	0·0885	0·0920	,,
3.	0·0931	0·0915	0·0921	,,
4.	0·0928	0·0910	0·0931	,,
5.	0·0924	—	0·0925	,,
6.	0·0951	0·0932	0·0944	,,
7.	0·0976	0·0931	0·0976	,,
8.	0·0972	0·0944	0·0980	,,
9.	0·0960	0·0935	0·0942	,,
Mean recovery %	94·6	81·8	94·6	,,

On examining the table it will be seen that the results with normal HCl and 2 % H_3PO_4 closely correspond, while those with 3 % H_3PO_4 are considerably lower. The mean result with HCl is 94·6 % of the theoretical yield; with 2 % phosphoric acid the same; with 3 % phosphoric acid 81·8 %. It still remained to test the recovery of creatine from diabetic urine—the results are shown in the following section.

7. *Recovery of creatine from diabetic urine.*

In these observations we made use of a series of diabetic urines obtained from time to time from clinical hospitals in Dublin through the kindness of members of the staff. We applied the same four methods for recovery of creatine from diabetic urine which were used when the substance was added to aqueous solutions of dextrose (see Table IX). The results are necessarily somewhat more complicated than with simple solutions, since the preformed creatinine and creatine had to be determined separately.

The full data are given in Table XI. Pure creatine was added to eight samples of diabetic urine obtained from different patients. Three of the samples contained both acetone and aceto-acetic acid, four were free from both these bodies, and one contained a trace of aceto-acetic acid but no acetone.

TABLE XI.

Showing the recovery of creatine when added to diabetic urines.

Urine contained	Preformed creatinine per cent.	Creatine added, expressed as creatinine	Preformed creatine of urine as found by				Creatine recovered as found by			
			(*a*) HCl W. B. method	(*b*) HCl autocl. 25 mins.	(*c*) H_3PO_4 3 % autocl. 30 mins.	(*d*) H_3PO_4 2 % autocl. 30 mins.	(*a*) HCl W. B.	(*b*) HCl autocl. 25 mins.	(*c*) H_3PO_4 3 %	(*d*) H_3PO_4 2 %
1. Dextrose 5·2 %. No acetone or aceto-acetic acid	0·0570	0·1000	0·0355	0·0346	0·0320	—	0·0948 =94·8 %	0·0950 =95 %	0·0945 =94·5 %	—
2. Dextrose 10 %. Both acetone and aceto-acet. ac.	0·0389	0·1000	0·0311	0·0311	0·0310	—	0·0720 =72 %	0·0720 =72 %	0·0611 =61·1 %	—
3. Dextrose 9·7 %. Both acetone and aceto-acet. ac.	0·0365	0·1000	0·0249	0·0319	0·0051	—	0·0859 =85·9 %	0·8660 =86·6 %	0·0812 =81·2 %	—
4. Dextrose 4·6 %. No acetone or aceto-acetic acid	0·0467	0·1000	0·0033	0·0039	0·0015	0·0040	0·0946 =94·6 %	0·0923 =92·3 %	0·0931 =93·1 %	0·0964 =96·4 %
5. Dextrose 3·8 %. No acetone or aceto-acetic acid	0·0675	0·1000	0·0128	0·0129	0·0104	0·0044	0·1065 =106·5 %	0·1053 =105·3 %	0·1001 =100·1 %	0·9250 =92·5 %
6. Dextrose 7·7 %. Both acetone and aceto-acet. ac.	0·0960	0·1000	0·0445	0·0441	0·0213	0·0267	0·0889 =88·9 %	0·0913 =91·3 %	0·0872 =87·2 %	0·0864 =86·4 %
7. Dextrose 2·8 %. No acetone, trace of aceto-acetic acid	0·0475	0·1000	0·0021	0·0022	0·0010	0·0000	0·0968 =96·8 %	0·0928 =92·8 %	0·0860 =86·0 %	0·0942 =94·2 %
8. Dextrose 1·7 %. No acetone or aceto-acetic acid	0·0374	0·0843	0·0268	0·0269	0·0196	0·0249	0·0804 =95·4 %	0·0786 =93·24 %	0·0798 =94·6 %	0·0749 =88·8 %

On examining the results it will be seen that the percentage recoveries with the different methods were as follows: (1) water bath HCl, 72 % to 106·5 %, the mean of the series being 91·8 %; (2) autoclave HCl, 72 % to 105·3 %, the mean being 91·2 %; (3) autoclave H_3PO_4 3 %, from 61 % to 100 %, the mean being 87·1 %; (4) autoclave H_3PO_4 2 %, from 86·4 % to 96·4 %, the mean being 91·76 %.

The results where HCl has been used are about 5 % inferior to the corresponding recoveries from aqueous and dextrose solutions. Those with 2 % H_3PO_4 are less variable but the mean (taken however from a smaller series) is identical with that of HCl. With 3 % H_3PO_4 the results are less favourable. We are forced therefore to conclude that while no special advantage is gained by using phosphoric acid, there is a liability to destruction of creatine by this reagent even with a strength of 3 %. It will be remembered that the same effect was found in the recovery of creatine from aqueous solutions both in the presence and absence of glucose.

The remarkable feature, however, of the results is the variability in the degree of recovery from different diabetic urines. This was not seen in the recovery from normal urine nor from aqueous or dextrose solutions, and points to a disturbing factor other than pigment or sugar. It is also remarkable that the lowest results were obtained from urines containing acetone and aceto-acetic acid. We have not studied the effects of either of these bodies, but they have been called attention to by Jaffé (acetone), Folin (acetone, aceto-acetic acid, and aceto-acetic ester), Klercker (acetone), Hoogenhuyze and Verploegh (acetone), Krause [1910] (acetone and aceto-acetic acid), Wolf and Österberg [1911] (aceto-acetic ester), Rose [1912] (aceto-acetic acid), Greenwald [1913] (acetone and aceto-acetic acid). Krause and Greenwald find the disturbing effect of aceto-acetic acid more serious than that of acetone. Its presence increased the values for preformed creatinine, and thereby lowered those of creatine. This is in harmony with our observations, but we think the matter deserves a more thorough investigation, starting with solutions of pure creatine to which dextrose, acetone, and aceto-acetic acid are added in succession.

Since the foregoing was written Graham and Poulton [1913] have published a note in which they show that aceto-acetic acid and its sodium salt diminish the colour of the Jaffé reaction and thus lower the estimate of creatinine. Aceto-acetic ester, used by Wolf and Osterberg, also by Folin, has no appreciable effect.

8. *Comparison of the autoclave and water bath methods.*

It may seem superfluous to have undertaken this part of the work, but at the period when we began it, the reliability of the autoclave method was not universally accepted. We therefore decided to test it for ourselves, and also to determine the optimum time in the autoclave for the transformation of pure creatine into creatinine at the temperature (117° C. to 120° C.) recommended by Benedict and Myers.

Two series of observations were made. In the first the results of heating on the water bath for three hours were compared with those of the autoclave, heated for 15 mins., 25 mins., and 35 mins. respectively. They are given in Table XIII:

TABLE XIII. *Creatine estimation: comparison of water bath and autoclave methods.*

No.	Water bath 3 hrs.	Autoclave 15 mins.	Autoclave 25 mins.	Autoclave 35 mins.	Theoretical amt. expressed as creatinine
1.	0·1002	0·0994	0·1004	0·0997	0·1000
2.	0·0981	0·0944	0·0982	—	,,
3.	0·0976	0·0935	0·0982	0·0942	,,
4.	0·0997	0·0942	0·0995	—	,,
5.	0·1000	0·0947	0·1000	0·1006	,,
6.	0·0944	0·0880	0·0962	0·0953	,,
Total	0·5900 =98·35 %	0·5642 =94·03 %	0·5925 =98·78 %	0·3898 =97·45 %	0·6000

It will be seen that the results of heating in the autoclave for 15 mins. are too low, while those for 25 mins. are identical, or almost so, with the water bath results. Consequently we are of opinion that the optimum time in the autoclave is 25 mins. at a temperature of 117° C. The results of heating for 35 mins. are slightly lower than those for 25 mins., indicating that the optimum period has been exceeded.

The second series gives a number of comparisons between the water bath results and those of the autoclave heated for one period only, namely, 25 mins.

On examining the mean results of the foregoing a remarkably close correspondence—indeed identity—will be seen, though the degree of conversion 95·7 % is a little lower than the average as given at the beginning of this paper, namely 96·5 %, which was obtained by taking the gross mean of the determinations in Tables XIII and XIV. When we compare this

with the gross mean of the autoclave determinations (25 mins.) the correspondence also works out very close, namely 96·6 % as against 96·5 %. Observations were also made for periods of one hour and two hours in the autoclave. In both cases there was a considerable destruction of creatinine, the results at one hour giving a yield of 92·4 % and at two hours of 88·6 %.

TABLE XIV. *Creatine estimation: comparison of water bath and autoclave methods.*

No.	Water bath 3 hours	Autoclave 25 mins.	Theoretical amount expressed as creatinine
7.	0·0975	0·0969	0·1000
8.	0·0964	0·0961	,,
9.	0·0964	0·0961	,,
10.	0·0994	0·0999	,,
11.	0·0994	0·0993	,,
12.	0·0942	0·0948	,,
13.	0·0935	0·0932	,,
14.	0·0931	0·0927	,,
15.	0·0931	0·0925	,,
16.	0·0944	0·0971	,,
17.	0·0959	0·0953	,,
18.	0·0953	0·0942	,,
19.	0·0960	0·0958	,,
20.	0·0955	0·0959	,,
Total	1·3401 =95·7 %	1·3398 =95·7 %	1·4000

It may be stated that we found no difficulty in obtaining consistent and concordant results with the autoclave, provided that care was taken to see that the time and temperature were kept constant and that the autoclave was not opened till the pressure had fallen to zero. We allowed at least ten minutes for this and then opened the valve slowly. Having set the autoclave for 117° C. it is desirable if possible not to use it for other purposes necessitating different temperatures, during the time it is in use for estimating creatine. It must be borne in mind that the velocity of reaction is greatly accelerated as compared with that at 100° C. and therefore slight differences of time and temperature produce much greater effects.

We may perhaps add also in regard to the water bath that our ordinary rule was to immerse the flask in the water and use a reflux condenser. Latterly we have returned to the use of the funnel and watch glass for condensation and find it more convenient, but the flask should be immersed in the water. If placed on the top of the water bath the results are less consistent. Dr Rosenheim informed one of us (W. H. T.) that he had had the same experience.

SUMMARY OF RESULTS.

1. For control of the bichromate standard solution used in the Folin method of estimating creatinine and creatine we did not find the degree of conversion of creatine into creatinine by boiling with normal HCl sufficiently constant. We recommend for this purpose creatinine picrate as described by Thompson.

2. For the estimation of creatine in weak solutions the best results were got by using an equal quantity of N. HCl and boiling either on the water bath for three hours or in the autoclave for 25 minutes at 117° C.

3. For the development of the colour in the Folin method the optimum time and temperature in our hands proved to be 7 mins. at 15°–17° C.

4. The addition of too much alkali reduces the colour. For aqueous solutions and urine the best results are given by the quantity recommended by Folin, namely 5 c.c. of 10 % NaOH over and above what may be necessary to neutralise the solution.

5. The range of proportional readings on the colorimeter scale should not go lower than 7 mm. nor higher than 9 mm. If it is necessary to compare readings separated by a wider interval a correction factor should be used as explained in the body of this paper.

6. The darkening of the pigments of urine which occurs on boiling with normal HCl adds to the estimate of creatine-creatinine contained in it. With human urine the increase is slight (1–2½ %), with dog's urine as much as 10 %.

7. The presence of dextrose to the extent of 10 % did not affect the estimation of creatine. Phosphoric acid was not found to be superior to hydrochloric in estimating creatine when dextrose is present.

8. The recovery of creatine from diabetic urine gave results lower by 5 % than from aqueous or sugar solutions. Phosphoric acid did not prove more useful for this purpose than hydrochloric. The smaller recovery is probably due to the effect of aceto-acetic acid.

9. The autoclave method gave results identical with those of the water bath. The optimum time for urine and weak solutions of creatine was 25 mins. at 117°–120° C.

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