CL. THE METABOLISM OF CREATINE

I. A MICRO-METHOD FOR THE DETERMINATION OF CREATINE AND CREATININE

BY REGINALD BRETtauER FISHER
AND ALFRED ELLIS WILHELMi

From the Department of Biochemistry, Oxford

(Received 15 May 1937)

WE have been occupied with the problem of estimating accurately the creatinine present in samples of a Ringer-Locke perfusate in which the creatinine content is 1–16 γ /ml. Not more than 10 ml. of perfusate are usually available for duplicate estimation, so that each sample contains 5–80 γ of creatinine. Simple concentration of the perfusates, which contain relatively large amounts of calcium, makes direct estimation of creatinine impossible, owing to interference with the development of the colour in the Jaffé reaction by the formation of a precipitate of calcium hydroxide when the alkali is added. The observation of Gaebler & Kelch [1928] that creatinine is adsorbed selectively on to fuller's earth in solutions containing other chromogenic substances led us to think that an adsorption method might eliminate difficulties due to the presence of substances interfering with the Jaffé reaction and might also be sensitive enough to make possible the accurate estimation of very small amounts of creatinine. We were encouraged by the brief description by Borsook [1935] of a method of this character to adopt his technique, which consists in adsorbing the creatinine on to fuller's earth in acid solution and eluting it with the alkaline picrate in which the colour is developed. The method as we first used it had considerable disadvantages, which are described below. We believe that we have succeeded in overcoming them, and we feel that it may be useful to other workers to describe our technique, which makes possible the estimation of creatinine in 5 ml. of solution containing 5–80 γ of creatinine with an error of the order of $\pm 1\gamma$.

Methods

(1) *Preparation of adsorbent.* An efficient adsorbent can be prepared in the following manner: 200 g. B.D.H. "fuller's earth: levigated powder" are washed twice for 3 hr. in 1 l. 1.2 *N* HCl, and thrice in 1 l. distilled water, the supernatant liquid being siphoned off. The moist fuller's earth is drained as completely as possible on a Büchner funnel, transferred to large watch-glasses and dried slowly over a boiling water-bath, being broken up into small pieces during the process. It is not necessary to remove the last traces of HCl. After drying, the fuller's earth is pulverized in a mortar and sieved (90-mesh). The final preparation is a fine grey-brown powder, at least five times as efficient as an adsorbent of creatinine as the starting material. (210 mg. of the starting material adsorb creatinine quantitatively from 6 ml. solution only when the amount of creatinine is less than 25 γ , whereas 130 mg. of the final product adsorb at least 80 γ quantitatively in the same circumstances.)

(2) *Preparation of samples for colorimetric estimation.* As the method has been applied almost exclusively to the determination of total creatinine in trichloroacetic acid filtrates the technique most used has been as follows:

(a) Aliquots of the filtrate are pipetted into tared 200 ml. flat-bottomed flasks, 5 ml. 2*N* HCl and 25 ml. distilled water are added, and the flasks are heated on a boiling water-bath for 2 hr. and then more vigorously over a Bunsen burner until the weight of the contents is 2–3 g.

(b) The samples are transferred from the flasks to 15 ml. centrifuge tubes. The flasks are washed twice with 2 ml. 2*N* HCl, the washings being added to the samples, making the final volume 6–7 ml. To each tube about 130 mg. prepared fuller's earth are added, and the tubes are shaken in a machine for 15 min. The tubes are centrifuged for 3 min. and the supernatant liquid is poured off, the last drops being removed with filter-paper. 1 ml. *N*/100 HCl is blown into each tube from an Ostwald pipette, and the adsorbent is suspended by a few brisk rotations of the tube. 1 ml. *N*/100 HCl is used to wash down the tube. The tubes are again centrifuged for 3 min. and the supernatant liquid is poured off, the last drops being removed with filter-paper. The adsorbent is dispersed by the brisk addition of 2 ml. alcoholic alkaline picrate (25 ml. purified saturated picric acid, 25 ml. 97 % alcohol and 7.5 ml. 10 % NaOH) from a syringe fitted with a stop and a sawed-off needle. The tubes are shaken for 10 min., during which the elution of the creatinine and the development of the colour take place, and then 1 ml. *N*/20 BaCl₂ is blown into each tube from an Ostwald pipette. The solutions are shaken for 5 min. more and are finally centrifuged for 5 min. The final solutions are perfectly clear, and the adsorbent is well packed down in the bottom of the tubes. In order to prevent as much as possible the inclusion of particles of fuller's earth in the samples, they are withdrawn into a pipette fitted with a syringe and gently expelled into 6 ml. test-tubes. A little of the sample is used to wash the pipette.

Alcoholic alkaline picrate for use in the colorimetry is prepared by diluting 20 ml. of the picrate solution described above with 10 ml. distilled water and filtering the solution into a small Erlenmeyer flask which is then stoppered to keep the solution free of dust. The alcoholic solution thus prepared is indistinguishable in the colorimeter from aqueous alkaline picrate and does not introduce any errors due to differences in depth of colour.

(3) *Colorimetry.* A Leitz three-stage colorimeter is used. The standard consists of the "neutral grey" solution and the appropriate filter for creatinine estimation is Leitz S.F. 6 which passes light around 531 m μ . "Compensation" is introduced to eliminate the absorption of light due to the large excess of alkaline picrate. Although this absorption is small when the appropriate colour filter is used, we have found it in the interests of accuracy to balance it out in the manner indicated in Fig. 1.

Fig. 1. Compensation array for absolute colorimetry. The topmost stage is set to the same depth of solution as the coupled middle and lower stages. The light must therefore pass through the same depth of alkaline picrate on both sides, whatever the position of the coupled stages.

In each series of determinations fresh alkaline picrate is placed in the proper cups of the top and bottom stages. Before the estimations are begun, the right- and left-hand micro-cups of the middle stage are filled with alkaline picrate and distilled water respectively and set to 25 mm. depth of solution. There are thus columns of equal depths of distilled water and alkaline picrate on each side. The two halves of the field are made to match by adjusting the centring device in the base of the machine. Although the light has already been centred with the stages empty, an additional adjustment is often necessary because there is a small but definite absorption of light by the cups and plungers, and this is not the same on both sides.

When the machine is balanced, the distilled water is removed from the left-hand micro-cup, the cup and plunger are washed with a little neutral grey solution, and the cup is filled with neutral grey and replaced in the colorimeter. The absorption of the 25 mm. column of alkaline picrate is then measured by adjusting the depth of neutral grey until the fields match in intensity. This gives the "working zero" of the apparatus, which should be zero reading of neutral grey. If it is not, the machine must be balanced again before the estimations are begun. In this way the accuracy of adjustment of the colorimeter is finally checked.

The alkaline picrate is then removed from the right-hand micro-cup, the cup and plunger are washed with a little of the unknown solution, and the cup is filled, replaced and set to 25 mm. depth of solution. The depth of neutral grey is then adjusted until the fields match. Ten readings are taken, five from above and five from below the position of equality, and the mean is calculated. The creatinine content of the unknown solution is then given by the expression

$$Y = 3.60x - 1.55 \quad \dots\dots(1),$$

where Y = creatinine in γ , and x = mean neutral grey reading in mm.

This relation is derived from 90 observations (78 with one batch of adsorbent and 12 with another) on solutions of creatinine zinc chloride of known concentration which were subjected to the whole of the procedure described above, with the exception of the preliminary heating. These 90 observations are collected in Table I, and it follows that relation (1) affords an accurate estimate of the creatinine content of a sample. The probable error of an individual estimation is $\pm 0.98\gamma$ creatinine.

Table I. *Relation between creatinine content of sample and neutral grey reading*

Amount of creatinine	Neutral grey reading (mm.)									
	Adsorbent 4					Adsorbent 5				
γ										
5	1.51	1.54	1.61	1.71	1.81					
10	3.06	3.07	3.08	3.09	3.70			3.19	3.31	
15	4.55	4.62	4.67	4.69	4.76					
20	5.56	5.86	5.95	6.00	6.14			5.91	6.00	
25	7.54	7.55	7.59	7.59	7.80					
30	8.80	8.84	8.87	9.36	9.39			8.78	9.00	
35	10.10	10.11	10.23	10.39	10.60					
40	11.36	11.44	11.51	12.09				11.22	11.86	
45	12.59	12.60	12.95	13.01	13.05	13.18	13.26	13.42		
50	13.67	13.82	14.06	14.28	14.66			14.26	14.66	
55	15.48	15.60	15.64	15.72						
60	16.54	17.07	17.26	17.60	17.70					
65	18.16	18.29	18.36	18.56						
70	19.40	19.75	19.78	19.90	19.91	20.24				
75	21.09	21.25	21.38							
80	22.32	22.42	22.60	22.62						

Notes on procedure

(1) *Conversion of creatine into creatinine.* This conversion is accomplished by a variant of the Folin open-flask method, and we have satisfied ourselves that the conversion is complete under our conditions. The concentration of acid in the final 2–3 ml. of solution after boiling down is approximately 2.5 *N*.

(2) *Adsorption.* The adsorption of creatinine on to fuller's earth occurs only from acid solution, but we have been able to show that the extent of the adsorption from solutions acidified with HCl is not affected by variation of the normality of the acid between 0.4 and 2.5. There is therefore no necessity to adjust the acid concentration in the samples after the conversion procedure.

Originally 50 mg. of fuller's earth were used in each tube as recommended by Borsook [1935]. We found, however, that considerably more than 200 mg. of the untreated fuller's earth, or 130 mg. of prepared adsorbent, was necessary to obtain a reasonably linear relation between creatinine content and colour development; we have therefore continued to use 130 mg. of prepared adsorbent.

In Borsook's method the shaking is done by hand and special tubes with glass stoppers are used. We have found it more convenient to use ordinary 15 ml. centrifuge tubes and to shake them in a special machine which we have devised and which we propose to describe separately. The tubes are held vertically in the machine and a rapid alternating circular motion in a horizontal plane is imparted to them. Efficient shaking can be obtained by this means without stoppering the tubes, and eight tubes can be dealt with at once.

(3) *Elution.* The major difficulty encountered in connexion with the elution of the adsorbed creatinine was in avoiding the dispersal of fuller's earth by the alkali. B.D.H. "fuller's earth for adsorption purposes" always yielded a definite blank in the colorimeter, due to scattering of light by dispersed particles of the adsorbent, and this blank was extremely variable. Our prepared adsorbents dispersed in alkali to a much greater extent than the original material. It was impossible, in fact, to clear the final solution by centrifuging. We attempted to flocculate the adsorbent by adding dilute BaCl₂ during the elution, but we were only partially successful; the solution was not entirely cleared, and the adsorbent itself was bulky and obviously still considerably hydrated. We then substituted the alcoholic picrate already described for aqueous picrate and retained the BaCl₂ treatment. When this procedure was used the adsorbent did not increase in volume during the elution and the supernatant liquid was perfectly clear after 5 min. centrifuging at 3000 r.p.m.

(4) *Colorimetry.* We chose to estimate the creatinine by absolute colorimetry because whilst we were using untreated adsorbent we saw no reason to suppose that we should obtain a linear relation between the amount of creatinine taken and the colour developed in the eluate. We realized that unless this relation was in fact linear the relation between the colour developed in a standard solution which had been subjected to the same procedure and that developed in an unknown would be of a complex character and peculiar to the particular standard chosen. Fig. 2 illustrates this point. A series of samples of creatinine solution in which the creatinine content increased by steps of 5γ from 5γ to 50γ was submitted to the adsorption and elution procedures with B.D.H. "fuller's earth for adsorption purposes" as the adsorbent. Each sample was then used as a standard for the estimation of the colour developed in the next higher member of the series. Then, on the assumption that the whole of the creatinine had been recovered in the first member of the series, it was possible to calculate the

creatinine recovered in each later tube of the series. Correction curves for particular standards could then be calculated in the following manner:

Let S be the amount of creatinine in the standard, and s be the amount recovered when S is adsorbed and eluted. Let U and u be the corresponding quantities for the unknown. Then the apparent creatinine content of the unknown is

$$\frac{u}{s}(S),$$

and the difference between the true and estimated creatinine is

$$\frac{Us - uS}{s}.$$

Fig. 2 illustrates the nature of one such correction curve. It is obvious that the method of deriving this curve is susceptible to large errors, and that if a more direct procedure is available it should be substituted for simple colorimetry. Absolute colorimetry provides such a substitute, and it has the further advantage that the standard is stable and reproducible. Its advantages are such that we have retained it even though we have since succeeded in obtaining a linear relation between the colour developed in the eluate and the amount of creatinine taken initially.

Since the utility of the method in connexion with a particular problem can be tested only by showing that it will recover creatinine quantitatively from solutions or extracts of the character appropriate to the problem, we do not propose to discuss the matter of recoveries here. We have used the method extensively during the past 2 years and we shall show in subsequent papers of this series that it does permit quantitative recovery of creatinine from tissue extracts and perfusates, and that it is not affected by large excess of such substances as glucose, arginine, glycine and trichloroacetic acid.

Fig. 2. Correction curve for 30γ standard.

SUMMARY

A colorimetric method for the estimation of 5–80γ of creatinine is described, which is based on the adsorption of creatinine from acid solution by prepared fuller's earth and elution by alkaline picrate, followed by "compensated absolute" colorimetry. The standard error of an individual estimation is $\pm 0.98\gamma$.

REFERENCES

- Borsook (1935). *J. biol. Chem.* **110**, 481.
 Gaebler & Kelch (1928). *J. biol. Chem.* **76**, 337.