The Application of the Diacetyl Reaction to the Estimation of Creatine in Urine

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It has been shown (Ennor & Stocken, 1948) that sulphhydryl or disulphide compounds interfere with the Barritt (1936) reaction as applied by Eggleton, Elsdon & Gough (1943) for the determination of creatine in tissue extracts and that this interference can be overcome by the addition of *p*-chloromercuribenzoic acid to the reaction mixture. It thus became possible to estimate directly the creatine content of tissues regardless of their glutathione content. More recently this method has been applied to the determination of the phospho-creatine content of various organs (Ennor & Rosenberg, 1952). In the former publication (Ennor & Stocken, 1948) some advantage was claimed for the method in determining the creatine content of urine, but we subsequently found that the reaction was inhibited by compounds other than those of the sulphhydryl type and that this inhibition could not be removed by the addition of *p*-chloromercuribenzoic acid. Raaflaub & Abelin (1950) have reported similar findings and have detailed a method for the estimation of creatine in urine in which the effect of inhibitors has been reduced by a dilution technique. The present report gives details of experiments designed to determine the nature of the inhibitors, the specificity of the reaction, and details of a method whereby creatine may be determined in urine. A preliminary report of this work has been given (Ennor & Stocken, 1949).

EXPERIMENTAL

Reagents

Creatine hydrate and creatinine. A.R. specimens were recrystallized according to Hunter (1928) and their purity checked by N analysis. All solutions were made up immediately prior to use.

Guanidine and guanidine derivatives. Most samples used were of A.R. quality and were used without further purification.

Diacetyl. A stock solution of approx. 1% was prepared from dimethylglyoxime as described by Walpole (1911) and diluted 1:20 for use.

1-Naphthol. Freshly prepared solutions were used throughout. A.R. quality was satisfactory provided that the 10% (w/v) solution in the stock alkali showed but a faint colour.

Stock alkali. NaOH (60 g.) and Na₂CO₃ (128 g.) were dissolved and made up to 1 l. in water.

***p*-Chloromercuribenzoic acid.** This was synthesized as described by Whitmore & Woodward (1932). A 4×10^{-3} M solution was prepared by dissolving the requisite amount of the compound in the minimum volume of N-NaOH before making up to volume.

Development of method

In the earlier part of this work on model systems the conditions suggested by Eggleton *et al.* (1943) were observed but in experiments with urine it became desirable to increase

Fig. 1. Relationship between 1-naphthol concentration and *E* of 10 µg. of creatine as measured 20 min. after the addition of the reagents.

the sensitivity of the method. This was accomplished by the use of higher 1-naphthol concentrations, the effect of which, on the extinction (*E*) using a constant amount of creatine, is shown in Fig. 1. In these experiments varying amounts of 1-naphthol were dissolved in 2.5 ml. of stock alkali and added to 10 µg. of creatine dissolved in water. 0.5 ml. of the diacetyl dilution was then added, the volume adjusted to 10 ml. by the addition of water and the solution thoroughly mixed. After standing for 20 min. for colour development the optical density was measured in a Hilger Spekker absorptiometer using an Ilford spectrum-green filter (no. 604).

A plot of the extinction against the reciprocal of the 1-naphthol concentration (Fig. 1) indicates a limiting E greater than 0.2. A convenient practical concentration of 160 mg./10 ml. gives an E of about 0.2.

The estimation of creatine in the presence of creatinine

Model experiments. The estimation of creatine in urine, quite apart from the effect of accelerators or inhibitors which may be present, is complicated by the fact that small amounts of creatine are being determined in the presence of relatively large amounts of creatinine. This latter compound hydrolyses in the alkaline reaction mixture and yields creatine at a rate which is dependent upon temperature. The rate of this hydrolysis is such, at ordinary laboratory temperatures and at the pH of the reaction mixture, that the concentration of creatinine 20 min. after the preparation of the mixture is not significantly reduced and it follows therefore that creatine is formed at a constant rate. Since, however, the ratio of creatine to creatinine in urine is of the order $\mu\text{g.}/\text{mg.}$ it is clear that whilst the creatinine loss by hydrolysis may be insignificant the creatine gain is such that a serious error will be introduced unless this is taken into account. The colour density of a solution containing only creatine reaches a maximum at 20° in about 20 min. after which time there is a gradual decrease. In the case of a mixture of creatine and creatinine the colour density continues to increase. The density at 20 min. does not represent the simple sum of the colour due to the creatine initially present plus that due to the creatine produced by hydrolysis of the creatinine, because of the time lag in colour development and because of the continuing hydrolysis of creatinine. However, since the hydrolysis and colour development rates, though different, are constant, it should be possible by a simple extrapolation procedure to determine the initial creatine content. Such a method has in fact been proposed by Raaflaub & Abelin (1950), who have shown that an extrapolated curve obtained with creatinine passes through the origin. In experiments carried out in 1948 in Melbourne and in Oxford, although there was agreement on the principle of extrapolation and on the fact that an extrapolated curve did not pass through the origin, the point on the time axis (extrapolation time) at which this was cut by the extrapolated curve was the subject of controversy. It subsequently became clear that the disagreement was due to differences in temperature and the purity of the reagents, and we therefore undertook a careful purification of the reagents and a rigid control of the experimental conditions. The temperature was controlled either by carrying out the entire procedure in a constant temperature room, by withdrawing samples from a large bulk of reaction mixture maintained at constant temperature or by means of a jacketed absorption cell through which water at the requisite temperature was circulated.

The conversion of creatinine to creatine was followed by measurement of the colour density at various time intervals after the addition of the reagents. For this purpose three concentrations were employed, namely 200, 400 and 800 $\mu\text{g.}$ of creatinine with and without 10 $\mu\text{g.}$ creatine in a final volume of 10 ml.

It will be observed (Fig. 2) that with creatinine alone at 19° the extrapolation time is 5 min. and is independent of concentration. Curves (3–6) were obtained from a similar measurement of colour development but in the presence of added creatine (10 $\mu\text{g.}$). These also intersect at the 5 min.

point but at a distance above the abscissa which is equal to the E of 10 $\mu\text{g.}$ creatine alone. Additional experiments carried out at varying temperatures have led us to the conclusion that the extrapolation time is a function of temperature and is completely independent of creatinine concentration over the range 200–5600 $\mu\text{g.}/10$ ml. The results obtained in these experiments are best shown by plotting the extrapolation time against the reaction temperature.

Fig. 2. Extrapolated curves of the colour development of creatinine and creatinine plus creatine. Curve 1: 200 $\mu\text{g.}$ creatinine; curve 2: 400 $\mu\text{g.}$ creatinine; curve 3: 200 $\mu\text{g.}$ creatinine + 10 $\mu\text{g.}$ creatine; curve 4: 400 $\mu\text{g.}$ creatinine + 10 $\mu\text{g.}$ creatine; curve 5: 600 $\mu\text{g.}$ creatinine + 10 $\mu\text{g.}$ creatine; curve 6: 800 $\mu\text{g.}$ creatinine + 10 $\mu\text{g.}$ creatine.

Fig. 3. Variation of the extrapolation time with temperature.

It will be seen from this curve (Fig. 3) that the slope is comparatively steep over the range of temperature normally experienced in the laboratory.

Comparison of the colour densities, as measured at various time intervals after mixing of solutions containing varying amounts of creatine (0, 10, 20, 30 and 40 $\mu\text{g.}$) in the presence of a constant amount of creatinine (800 $\mu\text{g.}$), shows (Fig. 4) that the curves so obtained are parallel and that the vertical distance between each succeeding curve corresponds to an E which is equal to that given by the creatine increment alone. Provided then that the temperature is kept constant, and the point at which the time axis is cut by the extrapolated pure creatinine curve is known, it is possible to determine creatine in a mixture of creatine and creatinine. That it is necessary to allow colour development to take

place at constant temperature is apparent from Fig. 3, which shows that a small difference in temperature can produce a marked difference in the extrapolation time and thus lead to a significant error in the estimation.

Urine experiments. Although it was possible to determine creatine in the presence of creatinine in model systems the application of the method to urine analysis, for example, remained unsatisfactory due to the effect of inhibitors other than disulphide or sulphydryl compounds and perhaps to other guanidino compounds which yield coloured products. Although a considerable effort was spent in an attempt to identify the interfering compound(s) we were unable to attribute the interference to a single entity. It was found, however, that substances containing an NH group were, in

Fig. 4. Extrapolated curves (1-5) given by varying amounts of creatine (0, 10, 20, 30 and 40 $\mu\text{g.}$) in the presence of a constant amount of creatinine (800 $\mu\text{g.}$).

general, inhibitors of the reaction. A diverse selection of biological and non-biological compounds were tested, and among these piperidine (but not pyridine), glycylglycine, iminodiacetic acid, diketopiperazine, sarcosine, proline and hydroxyproline were effective inhibitors, whereas other common amino acids were comparatively feeble. One of these inhibitors was examined in detail and it will be seen (Fig. 5) that 10 molecular proportions of proline reduce the colour intensity of 13.1 $\mu\text{g.}$ creatine by 45%. It is possible, therefore, that the inhibition in urine is due to the cumulative effect of trivial amounts of a variety of peptides and amino acids as well as to disulphide or sulphydryl compounds.

We were unable to devise a method which could cope successfully with inhibitors in all specimens of urine. On occasion ion-exchange resins, absorptive or complexing reagents proved useful but no single method was either adequate or convenient for routine analysis. The difficulty was overcome by another approach and we reinvestigated the experimental conditions used by Eggleton *et al.* (1943) with the object of increasing the sensitivity of the method. This was achieved as mentioned above by an increase in the concentration of 1-naphthol in the reaction mixture.

With the resultant increased sensitivity it is important to be certain that the coloured complex arises only from creatine since it is known that other guanidino derivatives

may also be excreted. In this connexion Eggleton *et al.* (1943) have reported that under the conditions of this test arginine, glycoeyamine and guanidine produce about one-ninth of the colour of an equivalent amount of creatine. We have tested several guanidino compounds, and the results obtained are compared with creatine in Fig. 6.

Fig. 5. Inhibition produced by varying molecular proportions (0-100) of proline on the colour given by 1 μmole of creatine. The E was measured 20 min. after the addition of the reagents.

Fig. 6. Comparison of the rates of colour development of substituted guanidines. Curve 1: methylguanidine, 2.5 μmoles ; curve 2: glycoeyamine, 2.5 μmoles ; curve 3: arginine, 2.5 μmoles ; curve 4: guanidine, 2.5 μmoles ; curve 5: *as.*-dimethylguanidine, 0.25 μmole ; curve 6: creatine, 0.25 μmole .

It will be noted (Fig. 6) that of the compounds tested only *as.*-dimethylguanidine ($\text{HN}:\text{C}(\text{NH}_2)\cdot\text{NMe}_2$) reacts in the same way as creatine. On the other hand, the reaction in the case of guanidine, arginine, glycoeyamine and methylguanidine shows no sign of reaching completion even in 50 min. At 30 min. these latter compounds require a tenfold concentration to produce the same E as creatine; this finding is in agreement with those of Eggleton *et al.* (1943). Aminoguanidine ($5 \times 10^{-3}\text{M}$) gave no colour.

As discussed in the model experiments it is possible to determine creatine in the presence of creatinine provided that the extrapolation point is known. In urine analysis there are three ways of obtaining the position of this point. (1) Extrapolation to the time axis of the curve obtained by measuring the colour development of a solution of pure creatinine. (2) Intersection of the curves obtained by measuring the colour development of a sample of urine with and without added creatinine (see Fig. 2). (3) Intersection of the curves obtained by measuring the colour development of a sample of urine before and after removal of the creatine present.

The first method has the theoretical disadvantage that the rate of colour development of pure creatinine is not necessarily the same as the rate in urine because of the presence of inhibitors and/or accelerators.

The second method has the obvious advantage of presenting an internally controlled system but will estimate other guanidino compounds if present.

The third method requires for absolute specificity the use of an enzyme system which will attack only creatine. Such a system is found in a pseudomonad described by Nimmo-Smith (1949). This system differs from the bacterial enzyme of Dubos & Miller (1937) in that it has no action on creatinine.

Recommended method

The technique adopted for the enzymic removal of the creatine present in urine was to incubate at 37° for 30 min., with occasional shaking, 0.5 ml. of urine, 0.5 ml. of 0.2M-phosphate buffer (pH 6.5) and 0.5 ml. of the stock bacterial suspension which contained about 6 mg. dry wt. of organisms/ml. This mixture after incubation was made up to 10 ml., centrifuged and the clear supernatant used for experiments which were arranged in the following fashion:

- (1) 2.0 ml. of supernatant (enzyme-treated urine).
- (2) 2.0 ml. of supernatant + 5 μ g. of creatine.
- (3) 2.0 ml. of supernatant + 200 μ g. of creatinine.
- (4) 0.1 ml. of untreated urine.
- (5) 0.1 ml. of untreated urine + 5 μ g. of creatine.
- (6) 0.1 ml. of untreated urine + 200 μ g. of creatinine.

The above arrangement was carried out in duplicate, and to each tube was added 1.0 ml. of *p*-chloromercuribenzoic acid (142 mg./100 ml.) in order to eliminate the inhibitory effect of disulphide or sulphydryl compounds and also to poison small amounts of the enzyme system which remained and which otherwise would rapidly decompose the added creatine. The following reagents were then added at zero time: 2.5 ml. of 1-naphthol solution (640 mg. in 10 ml. of stock alkali) and 1.0 ml. of diacetyl dilution. The mixture was then made up to 10 ml., filtered, and the rate of colour development determined over the period 18–36 min. from zero time. The results of a typical experiment are plotted in Fig. 7.

It will be appreciated that these experiments include both the second and third methods, and moreover that the results are in agreement with each other. Thus the addition of creatinine to either the enzyme-treated urine or the untreated urine (Expts. 1 and 3 and 4 and 6 respectively) leads to results which give an identical extrapolation point. It will also be noted that the same *E* is obtained for the 5 μ g. of creatine which was added to both the treated and the untreated urine (represented by vertical lines marked A and B). The amount of creatine present in the urine is given by C.

Since the curves given by the enzyme-treated urine with and without the addition of creatinine intersect on the time axis it follows that the amount of other guanidino compounds present is insignificant. In other specimens of urine which we have examined the curves intersect above the time axis thus indicating the presence of one or more guanidino compounds, most probably glycoxyamine, which is a normal excretory product (cf. Weber, 1935). It will be appreciated, however, that its presence does not interfere with the determination of creatine (provided that the third method of determining the extrapolation point is used) and that the

Fig. 7. The estimation of the creatine content of urine.

Curve 1: enzyme-treated urine; curve 2: enzyme-treated urine + 5 μ g. creatine; curve 3: enzyme-treated urine + 200 μ g. creatinine; curve 4: untreated urine; curve 5: untreated urine + 5 μ g. creatine; curve 6: untreated urine + 200 μ g. creatinine.

method is capable of determining the creatine content in a direct manner. In order to discover the error at different concentrations, analyses were made of normal urine and of urine which had been freed from creatine and subsequently used to make a solution containing 5 μ g./ml. In the first experiment the results of duplicate runs on urines of four males and two females were: 19, 23; 22, 21; 25, 26; 24, 28; 8, 5; 24, 21 μ g. creatine/ml. At the known lower concentration of 5 μ g./ml., 0.25 ml. urine was taken instead of the usual 0.1 ml. and four trial runs gave 4.5, 6.0, 3.3 and 5.3 μ g./ml. The error of the method is therefore $\pm 10\%$ at a concentration of 20 μ g./ml. and about twice this amount at 5 μ g./ml. The lower values were, however, seldom observed; typical values for males were 11, 19, 14, 15, 35, 7, 40, 38, 25, 27, 22, 8 and 12 μ g. creatine/ml. urine.

DISCUSSION

There are two essential differences between the method described in this communication and that described by Raaflaub & Abelin (1950). The sensi-

tivity of the method has been increased by rather more than 50% and an accurate assessment has been made of the point at which the extrapolated creatinine curve cuts the time axis. Such an assessment gives the distance along the time axis at which the *E* for creatinine must be read and eliminates the error (about 20%) which may arise because of the fact that at ordinary laboratory temperatures the *E*-time curve for pure creatinine does not intersect the time axis at its origin. The point at which the *E* for a solution of pure creatinine cuts the time axis is a function of temperature and the fact that Raaflaub & Abelin (1950) obtained curves passing through the origin could be explained by a slight contamination of creatinine with creatine. The best commercially available specimens of creatinine are not sufficiently pure to be used without further purification; the plots of some commercial samples tested by us have in fact cut the time axis to the left of the origin.

Among those guanidino derivatives which occur naturally and which may reach levels which would lead to interference with the present method only glycocyamine need be considered. If the greatest accuracy is desired, the specific enzymic method must be used since this will lead to a correct assessment of the extrapolation point.

Since an equilibrium exists between creatine and creatinine a solution of the latter will become contaminated with creatine at a rate which is dependent on the temperature and the pH at which it is stored. For this reason it is essential to prepare

all solutions of creatinine immediately prior to use. Similar considerations apply to urine specimens which, because of their high creatinine content, will develop increasing amounts of creatine. Thus a specimen of urine, when analysed immediately after voiding, contained 22 $\mu\text{g.}$ of creatine/ml.; after storage at room temperature for 24 hr. it contained 32 $\mu\text{g.}/\text{ml.}$ In view of this hydrolysis of creatinine it is perhaps pertinent to cast some doubt on the results recorded in the literature for creatine excretion and indeed to query whether or not the creatine which is actually found in small amounts in urine of normal adult males is excreted as such or arises as a result of the hydrolysis of creatinine during the course of the accumulation of urine in the bladder.

SUMMARY

1. The specificity of the Barritt (1936) reaction for the determination of creatine has been investigated.

2. A method is described for the direct determination of creatine in urine.

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