The Estimation of Creatine and of Diacetyl

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Voges & Proskauer [1898] observed that when strong alkali was added to broth cultures of certain species of bacteria there developed, after an interval of time, a pink colour. Subsequently the work of Harden [1906] and of Harden & Norris [1911] established that the chemical reaction responsible was one between diacetyl and creatine (or certain similar substances). Attempts to make the reaction quantitative [Walpole, 1911; Eggleton & Eggleton, 1928] were only partially successful. The reaction was further studied by Dulière [1929], Lang [1932] and Müller [1935], but it was the discovery by Barritt [1936], that α-naphthol greatly intensifies the colour, which made it possible for the first time to use this reaction as a means of estimating either creatine or diacetyl. The present paper presents some additional facts about the reaction and methods of estimation based on it.

EXPERIMENTAL

Preliminary experiments

Preliminary experiments confirmed Barritt's observations and also demonstrated that the reaction took place rapidly at room temp. Early in the research it was noticed that colour development invariably began at the surface of the liquid, suggesting that oxygen was involved in the reaction. This was proved by studying the reaction in the absence of O_2, and by measuring the amount of O_2 used. When the reagents were mixed under an atmosphere of O_2-free hydrogen, no colour formation occurred even after a period of 4 hr., whereas the control in air showed maximum colour development within 30 min. After 4 hr. exposure to O_2-free H_2, with no pigment production, air was admitted and immediately colour formation began and reached approximately the same intensity as the control.

The Warburg manometric apparatus was employed to measure the O_2 used during the reaction. Two ml. of the stock alkali (see later) or 2 ml. of 1% α-naphthol in the stock alkali, were placed in the main chamber of the manometer cup, and the creatine and the diacetyl solutions were placed in the side bulb. By this means the following points were established (for a temp. of 26°): (1) Creatine in alkaline solution does not absorb O_2. (2) α-Naphthol in alkaline solution consumes O_2 at a slow but steady rate over a period of many hours. (3) Diacetyl in alkaline solution shows a rapid initial O_2 uptake which falls off, sometimes completely, after 30 min. It amounts to about 1 mol. O_2/mol. diacetyl. (4) On mixing diacetyl with α-naphthol in alkali there is a consumption of O_2, the rate of which is approximately equal to the sum of the rates of O_2 consumption of diacetyl and α-naphthol separately. (5) The addition of diacetyl and creatine to the alkali causes a rapid consumption of O_2 for the first 30 min., which falls off to a low steady rate. The presence of excess of creatine increases by 2- or 3-fold the amount of O_2 consumed by diacetyl during this 30 min. In other words, O_2 is involved in the Voges-Proskauer reaction, though it is not possible to say how much, since under the conditions of these experiments some of the diacetyl must have oxidized without reacting with creatine. (6) When diacetyl and creatine are added to α-naphthol in alkaline solution, there is an initial rapid phase of O_2 consumption lasting about 30 min., followed by a slow steady O_2 uptake lasting over a period of hours. The amount of O_2 used by this mixture is greater than when creatine is omitted, indicating that the modified Voges-Proskauer reaction also involves an oxidation by molecular O_2. The results of a typical experiment are expressed graphically in Fig. 1. In this experiment, the amount of

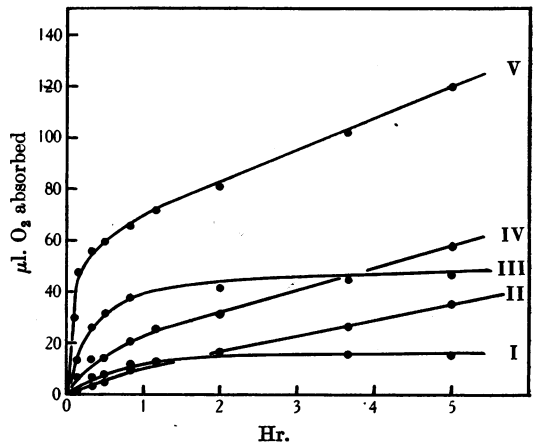

Fig. 1. O_2 uptake at 26° (measured in Warburg manometers) of the following mixtures dissolved in 2 ml. of stock alkali.

I.	α-naphthol	0	mg.,	diacetyl	0·2	mg.,	creatine	0	mg.
II.	„	10	„	„	0·0	„	„	0	„
III.	„	0	„	„	0·2	„	„	7·5	„
IV.	„	10	„	„	0·2	„	„	0	„
V.	„	10	„	„	0·2	„	„	7·5	„

diacetyl (0·2 mg.) was the limiting factor, creatine and α-naphthol being present in excess, 7·5 and 10 mg. respectively. (7) The rate of O_2 consumption is approximately equal to the rate of colour development. The rate of O_2 consumption was obtained as the difference between the rate of O_2 consumption of a mixture of diacetyl, creatine and

α-naphthol and the rate of O_2 consumption of a similar mixture of diacetyl and α-naphthol. Colour development was measured by means of the Zeiss Pulfrich photometer. Within the limits of experimental error, which in this case are large, owing to the speed of the reaction and the presence of side reactions, colour development and O_2 consumption ran parallel.

These experiments show that the Voges-Proskauer reaction and the Barritt reaction both involve an oxidation by molecular O_2, and in consequence special attention must be paid to this fact when using the reaction for the estimation of either creatine or diacetyl.

Nature of the pigment formed in the Voges-Proskauer and Barritt reactions

The isolation of the pigment formed in the modified reaction has been attempted, but so far it has proved impossible to obtain it in the crystalline state. The impure product has some interesting properties. In the solid state it has been stable for 4 years. In alkaline solutions of pH 10 it is coloured red; at pH 8 it is purple, and at pH 6 it is brown; it is least soluble at pH 4. These facts suggest that it is a compound with three ionizing groups and an isoelectric point at pH 4. In concentrated HCl or H_2SO_4 the pigment is quite stable and coloured purple. In solution at pH 12, 40 mg. impart the same intensity of colour as 7 mg. of creatine in excess of diacetyl and α-naphthol, or 5 mg. of diacetyl in excess of creatine and α-naphthol.

The pigmented solution produced in the presence of α-naphthol was examined spectroscopically (our thanks are due to Dr L. E. Bayliss for valuable advice and help) and the absorption spectrum was found to have a single peak centred on wave-length 5250 A., the extinction coefficient (E) having half the peak value at 4650 and 5660 A.

The Zeiss S50 and S53 filters proved to be suitable for the photometric measurement of the pigment. The question of the optimum conditions for the development of maximum colour from small amounts of creatine and small amounts of diacetyl was then investigated.

Investigation of reaction conditions

Alkali. A solution of NaOH was used both in the original Voges-Proskauer test and in the Barritt modification. Investigation of the degree of alkalinity necessary for maximal colour development in the Barritt reaction showed that (*a*) no colour developed when the solution was made alkaline with $NaHCO_3$, (*b*) pigment formation occurred when either Na_2CO_3 or NaOH was used, and (*c*) maximum colour development occurred when a mixture of NaOH and Na_2CO_3 was used. The concentration of these compounds was not critical. A stock alkali solution was made up containing 30 g. NaOH and 80 g. Na_2CO_3 (equimolar amounts) in distilled H_2O and made up to 500 ml. Two ml. of this solution were used for the estimation.

α-Naphthol. Barritt used a solution of α-naphthol in ethanol, but as this rapidly becomes pigmented on standing, a solution in the stock alkali was used instead. The amounts of α-naphthol required for maximal colour development proved to be surprisingly large, about 500 mol./mol. of creatine, and at first the activity of the α-naphthol was thought to be due to the presence of some impurity. However, after thrice distilling in steam from an acid solution, the α-naphthol was neither more nor less active than before. Steam distillation yielded a perfectly white, crystalline product, which, if kept in a dark bottle, did not become discoloured. All subsequent samples of α-naphthol were purified in this manner. The solution of α-naphthol in alkali does not keep more than 2 or 3 hr. and in consequence it was always made up just before it was required. A 1% solution was used.

Diacetyl. A stock solution is prepared from dimethyl glyoxime (British Drug Houses Ltd., 'Analytical Reagent') by heating 1·6 g. with 200 ml. of 5 *N* H_2SO_4 in an all-glass distilling apparatus and collecting the first 50 ml. of distillate; this is made up to 100 ml. with H_2O. The amount of dimethylglyoxime taken yields about 1 g. diacetyl (as assayed by the van Niel [1927] method). This yield (85% of theoretical) is fairly constant, and the solution is stable for at least a month if kept in the ice-box.

Excess diacetyl causes production of a yellow pigment which tends to obscure the colour produced by the creatine; a deficiency causes subnormal colour production. For amounts of creatine up to 60 μg. in a final volume of 10 ml., 1 ml. of 1 : 20 dilution of the stock 1% solution was found to be optimal.

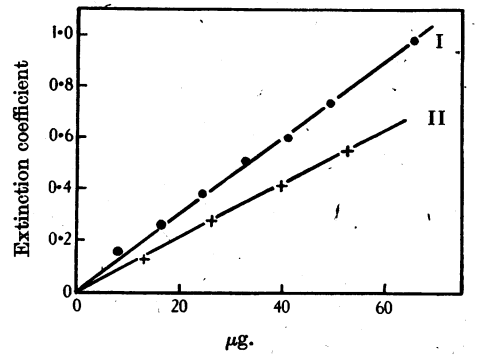

Fig. 2. Colour intensity (measured as extinction coefficient, S53 filter, 10 mm. cuvettes) produced by different amounts of creatine (II) and diacetyl (I) when treated in the manner described in the text.

Creatine. The sample used (British Drug Houses Ltd.) was recrystallized from distilled H_2O. With 0–50 μg. of creatine the colour was proportional to the amount of creatine present. Similarly, it was shown that when creatine was in excess the colour was proportional to the amount of diacetyl present. The results (Fig. 2) show that there is a linear relationship between intensity of colour and amount of diacetyl, and that the chromogenic powers/μ.mol. of diacetyl and creatine are about equal. This may indicate that the pigment contains diacetyl and creatine in equimolar proportions.

METHODS OF ESTIMATION

(*a*) *Creatine*

Reagents. (1) Stock alkali. (2) Stock 1% diacetyl diluted 1 : 20 before use. (3) α-Naphthol purified by steam distillation. A 1% solution in the stock alkali is made up just before use. (4) Saturated solution of creatine.

Procedure. To a neutral solution of creatine (containing not more than 60 μg.) in a standard

10 ml. flask are added 2 ml. of the 1% α-naphthol in alkali followed by 1 ml. of the diluted diacetyl. The solution is briskly shaken and made up to the mark with distilled H_2O and left for 30 min. The colour may be measured with the Zeiss Pulfrich photometer (S 53 filter and a reagent blank in the control cuvette), or by direct colorimetry with standard creatine solutions prepared in the same way. There is no interfering colour from the reagents alone and the intensity of the colour is stable for at least 2 hr. The extinction coefficient for creatine under these conditions is 0·0105/μg. (10 mm. cuvette, and S 53 filter).

Many guanido compounds react under these conditions [cf. Barritt, 1936]. Of those that we examined quantitatively, arginine, guanidine and glycocyamine produce about one-ninth of the colour given by an equivalent amount of creatine. Creatinine and freshly prepared creatine phosphate do not react under these conditions.

(*b*) *Diacetyl*

Reagents. As for the estimation of creatine (see p. 527).

Procedure. To a solution of diacetyl (containing not more than 100 μg.), in a standard 15 ml. flask, are added 4 ml. of a solution made up from 1 part saturated aqueous creatine and 3 parts 1% α-naphthol in stock alkali. The flask is briskly shaken, made up to the mark with distilled H_2O and allowed to stand for 30 min. It is then estimated in the photometer with a reagent blank in the control cuvette, or by direct vision colorimetry. Of the two the latter method is to be preferred owing to the presence of a slight variable blank error which it has proved impossible to eradicate.

Diacetyl does not appear to be formed by cells to any large extent; its reduction product, acetyl methyl carbinol, is far more common. This substance is readily oxidized to diacetyl by refluxing with ferric chloride [van Niel, 1927] and the diacetyl so formed may be removed by distillation, e.g. in the micro-Kjeldahl apparatus provided by Quickfit and Quartz Ltd. The following experiment illustrates the speed with which this occurs. 1 ml. of diacetyl solution containing 163 μg. diacetyl was pipetted into each of four distillation flasks and distilled in turn for varying periods of time. The distillate and washings were collected in calibrated measuring cylinders, the volume noted and 5 ml. taken in each case for analysis (Table 1).

Table 1. *Recovery of diacetyl by distillation*

Exp. no.	1	2	3	4
Diacetyl added (μg.)	163	163	163	163
Time of distillation (min.)	5	10	15	20
Vol. of distillate washings (ml.)	8·8	11·4	15·4	22
Diacetyl recovered (μg.)	164	162	154	159

In Exp. 1, 5 ml. of distillate were collected, the remainder coming from washings. It appears therefore that by collecting 10 ml. of distillate a quantitative recovery is assured.

Sources of interference

(1) The use of $CCl_3.COOH$ as a protein precipitant can cause some interference, since it reacts with alkaline α-naphthol solutions, producing an intense blue pigment which entirely masks the colour given by creatine. This reaction occurs only in ultra-violet light. If the colour development is carried out in the dark room or in the light of a tungsten filament lamp, $CCl_3.COOH$ does not interfere.

(2) Urine, and $CCl_3.COOH$ extracts of liver, contain substances which inhibit the reaction between diacetyl, creatine and α-naphthol. (The reaction between creatine and diacetyl is similarly inhibited, and Walpole believed that NH_3 was responsible for the inhibition in the case of urine.) For example, the colour production from 30 μg. creatine in excess diacetyl was reduced to 74% by the presence of 1 ml. of a normal urine, the effect being proportional to the amount of urine added. Similarly the colour intensity due to diacetyl in the presence of excess creatine was reduced to 68% of normal by 1 ml. of the same urine. This marked inhibitory action was not shown by urea, NH_3, uric acid or creatinine when added in amounts likely to be present in 2 ml. of urine. Glycine, however, proved to be an inhibitor. The colour intensity with 30 μg. of creatine was reduced by one-half in the presence of 4 mg. of glycine. Alanine and glutamic acid did not show this property, but β-alanine and β-alanyl histidine (carnosine) were inhibitory, being about 60% as active, on a molar basis, as glycine.

These substances also inhibited the colour production by diacetyl in the presence of excess creatine, indicating that the mechanism of the interference is not a destruction of either diacetyl or creatine, but a reaction with some compound formed intermediately in the Barritt reaction.

Discussion of methods

It appears from the above experiments that omega amino-acids may be responsible for the inhibitory action of liver extracts and urine. No ornithine or lysine was available so it was impossible to test the hypothesis that all omega amino-acids inhibit the reaction, nor was the effect of primary amines tried, though it may well be that the inhibition is due to the terminal amino group $—CH_2.NH_2$. In the light of these experiments, the claim of Lang [1932] to be able to estimate arginine in protein digests by a diacetyl method seems to require confirmation. Indeed, we observed no colour in a neutralized acid hydrolyzate of gelatine which had been treated with excess diacetyl and α-naphthol in alkaline solution, despite the presence of large quantities of arginine.

The use of the method for the estimation of arginine or creatine is therefore limited by these inhibitors. However, most animal tissues, with the

exception of liver, appear to contain insignificant quantities of interfering substances. In the case of skeletal muscle with its relatively high content of β-alanine in the form of carnosine and anserine, the situation is mitigated by the fact that the creatine content is also very high. Thus, only 0·1 ml. of a 1 : 10 CCl_3.COOH extract of skeletal muscle is required for the estimation of free creatine, and in this small volume the concentration of β-alanine-containing dipeptides is negligible. None the less, if the method is used in connection with muscle creatine, the possibility of interference from these sources should be borne in mind. It is possible that a preliminary separation of the creatine by selective adsorption would overcome the difficulties. In the estimation of diacetyl the preliminary distillation removes these sources of interference.

RESULTS

Except in the case of liver, no noticeable abnormalities have been encountered with animal tissues. Table 2 gives figures obtained with neutralized 1 : 10 CCl_3.COOH extracts of certain tissues of the rat. The animals were killed by stunning. A portion of the tissue was removed, freed from blood by washing with distilled H_2O, dried on filter paper, weighed, and ground with 10 times its weight of 4% CCl_3.COOH and a little sand. The insoluble material was filtered off and portions of the clear filtrate neutralized before development of the colour. It is assumed that the pigment formed was entirely derived from creatine. For comparison are given figures taken from Hunter [1928]; where no figures for the creatine content of rat tissues were available, those for corresponding tissues of other species are quoted. Hunter's results were all obtained by converting the extracted creatine into creatinine and determining the creatinine by the Jaffé reaction.

Table 2. *Creatine content of animal tissues*

Tissue	Present method—rat (mg./100 g.*)	Picrate method [Hunter, 1928] (mg./100 g.*)
Skeletal muscle	524	470
Cardiac muscle (ventricle)	192	168–223 (rabbit)
Smooth muscle (small intestine)	30·4	27·1 (rabbit)
Whole brain	132	114 (rabbit)
Lung	8·1	No figures
Spleen	6·5	19·1
Kidney	19·9	15·8–29 (dog)
Testis	267	212 (sheep)
Liver	0	23·3 (rabbit)

* Wet weight.

SUMMARY

1. Barritt's observation, that the Voges-Proskauer colour test for diacetyl is greatly increased in sensitivity in the presence of α-naphthol, is confirmed.

2. Barritt's reaction can also be used as a sensitive test for creatine.

3. Both the Voges-Proskauer and the Barritt reactions involve an oxidation by molecular oxygen.

4. Certain amino-acids interfere with the colour production in the Barritt reaction when the latter is used to estimate either creatine or diacetyl.

5. A similar interference is noted when urine and extracts of liver are present in the reaction mixture.

6. Arginine, guanidine, and glycocyamine react in a similar manner to creatine, but produce less colour. Creatinine and creatinephosphoric acid produce no colour.

7. Methods are described for the estimation of creatine and of diacetyl, in quantities up to 50 μg.

8. A reaction between α-naphthol and trichloroacetic acid is described, which occurs only under ultra-violet excitation.

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