residual colour is almost wholly accounted for by its tyrosine and tryptophan content. Thus the major part of the colour yielded by proteins must arise from chromogenic amino acid sequences. It had seemed possible, at the outset of this investigation, that the large colour increments of proteins might be ascribed to a rather restricted set of sequences. Had that proved to be the case, the Lowry determination might conceivably have been used specifically to detect such sequences in polypeptides. However, the results with chain B of insulin, as well as those with simple dipeptides, point to the existence of a considerable number of different chromogenic sequences in proteins.

SUMMARY

1. In the Lowry protein determination the phenol reagent is reduced to yield a blue colour. The colour yield of a completely hydrolysed protein is due to its content of tyrosine, tryptophan and cysteine, the only amino acids that react significantly.

2. Preliminary treatment with alkaline copper solution leads to a great increase in the colour yield of proteins. We have shown with simple peptides as well as with chain B of insulin that this large colour increment is largely attributable to sequences of amino acids containing functional side chains. Particularly chromogenic are dipeptides containing a histidine, arginine or glutamic acid residue, the nature of the second residue being relatively unimportant.

3. The behaviour of a compound obtained in small yield and presumed to be the oxidized product of His.His suggests that electron removal from the dipeptide to the phenol reagent involves the amino nitrogen and imidazole ring of the *N*-terminal residue.

4. Efficient copper catalysis of electron transfer from a chromogenic grouping in protein to the phenol reagent is believed to require formation of a coplanar quadridentate chelate. The colour yield depends critically upon the rate of electron transfer since the phenol reagent is rapidly destroyed in the alkaline solution.

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Biochem. J. (1960) **75**, 115

A Titrimetric Method for the Determination of Creatine Phosphokinase

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Creatine phosphokinase is usually assayed by measuring the amount of creatine or creatine phosphate formed in a standard time period or the initial rate of formation of one of these products (Askonas, 1951; Banga, 1943; Chappell & Perry, 1954; Ennor & Rosenberg, 1954; Kuby, Noda & Lardy, 1954; Narayanaswami, 1952; Rosenberg & Ennor, 1955). Oliver (1955) has described a method based on the coupling of adenosine triphosphate formation with reduction of triphosphopyridine

nucleotide; he points out that methods based on estimation of creatine or creatine phosphate are subject to errors arising from the introduction of the reactants with tissue extracts. Oliver's method has obvious disadvantages when inhibitor studies are contemplated, since two other enzymes are used in the system.

The method described below depends upon the fact that a hydrogen ion is quantitatively liberated at pH 9·0 in the reaction between adenosine triphosphate^{4-} and creatine^{-} to give adenosine diphosphate^{3-} and creatine phosphate^{3-} (Fiske & Subbarow 1929; Kumler & Eiler, 1943; Meyerhof & Lohmann, 1928; Noda, Kuby & Lardy, 1954). A constant pH titration can therefore be conducted, the rate of addition of base being equal to the rate of creatine ~ P formation. The sensitivity is such that extreme dilutions of enzyme can be assayed with great accuracy in a few minutes. In the absence of substrates other than creatine and adenosine triphosphate a significant pH shift is produced only by hydrolysis of adenosine triphosphate or transphosphorylation of creatine; the former can be allowed for by a blank conducted without creatine and has proved to be very small in practice. The method is simple, rapid and easy to carry out; the results have proved to be reliable and reproducible.

EXPERIMENTAL

Creatine phosphokinase was prepared by procedure A of Kuby *et al.* (1954), but was not crystallized. Crystalline disodium adenosine triphosphate (ATP) was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. All other reagents used were of analytical grade.

A Beckman model GS pH meter was employed, the high-sensitivity range being used. A sleeve-type reference electrode was found to give greater stability and was used throughout the study. The temperature was maintained at 30° by means of a water-jacketed reaction vessel. The contents were stirred by a Teflon (polytetrafluoroethylene)-covered stirring bar and magnetic stirrer. A Gilmont Ultramicroburette of 0·1 ml. total capacity (Emil Greiner Co., New York, U.S.A.) and calibrated accuracy of $\pm 10^{-5}$ ml. was used, and the titration was conducted with carbonate-free 0·1387 N-NaOH. Since the total reaction volume was 20 ml., it remained essentially constant throughout the titration.

Procedure

The reaction mixture consisted of 10 ml. of 0·06 M-creatine (final concn. 0·03 M), 4 ml. of 0·02 M-ATP (final concn. 4 mM), 4 ml. of 0·01 M-manganous acetate (final concn. 2 mM), 1 ml. of 0·02 M-mercaptoacetic acid (final concn. 1 mM) and 1 ml. of creatine phosphokinase solution with an enzymic activity of less than 1·5 μmoles min.$^{-1}$ of creatine ~ P. In experiments involving other concentrations and activators, the final volume was always made up to 20 ml. All components except the enzyme were placed in the reaction vessel and the pH was adjusted to 9·00 with 1 N-NaOH. The enzyme (at pH 9·0) was then introduced and base was added continuously from the burette to maintain a constant pH. Burette readings were noted at intervals of 1 min. For purposes of assay, the titration was continued for 5–10 min., depending upon the accuracy required. In the kinetic studies which were conducted to evaluate the optimum conditions for the assay procedure, the titration was continued for 25 min. or more. The stability of the pH meter was such that no significant error ($< 0{\cdot}1\%$) was introduced from this source.

Analysis of data

The slope of the titration curve (μmoles of base/min.) may be conveniently estimated by fitting a straight line by the method of least squares. When the interval on the abscissa is constant, as in this case, the slope of the line per unit interval is given by the following expressions (cf. Fisher & Yates, 1953), which may be rapidly evaluated without the necessity of graphical analysis.

$$\frac{2(y_5 - y_1) + (y_4 - y_2)}{10} \text{ (5 min. titration)},$$

$$\frac{3(y_7 - y_1) + 2(y_6 - y_2) + (y_5 - y_3)}{28} \text{ (7 min. titration)},$$

$$\frac{4(y_9 - y_1) + 3(y_8 - y_2) + 2(y_7 - y_3) + (y_6 - y_4)}{60} \text{ (9 min. titration)},$$

where y_i is the burette reading at i min.

RESULTS

Some initial experiments were carried out in which no thiol was incorporated into the reaction mixture. It was found that the reaction rate fell quite rapidly with time, closely approximating the apparent second-order kinetics with respect to ATP described under similar conditions by Kuby *et al.* (1954) (Fig. 1). After about 15 min., the titration

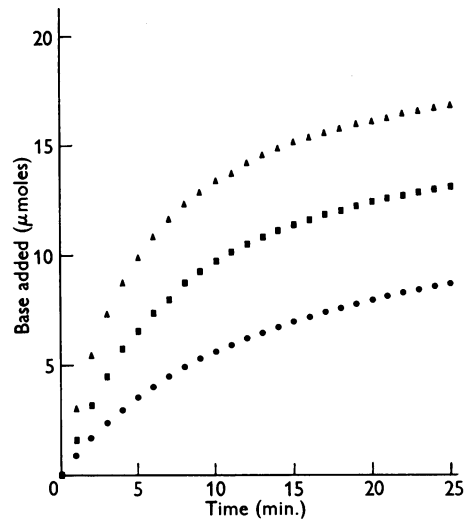

Fig. 1. Titration curves with 1 (●), 2 (■) and 3 (▲) ml. of enzyme preparation to system containing 1 mM-ATP, 6 mM-Mg^{2+} ion, 30 mM-creatine. Final volume, 20 ml.; pH 9·00.

curves obtained with different amounts of enzyme became almost parallel; the horizontal asymptote was evidently a function of the amount of enzyme added, implying that the enzyme was inactivated during the titration period. This possibility was confirmed by allowing the enzyme to stand in the reaction vessel with Mn^{2+} ions and ATP for 20 min. before addition of the creatine, whereupon no reaction occurred. Spontaneous inactivation of highly diluted creatine phosphokinase has previously been shown to be prevented by cysteine (Chappell & Perry, 1954; Oliver, 1955), and several thiols and other agents have therefore been examined for a similar protecting effect on this system. Fig. 2 shows representative titration curves obtained with the most effective of these. The rate falls much more slowly than in the control, and for the first 5–10 min. it is essentially constant. Larger concentrations (up to 10 mM) of the agents tested gave no better protection except with cysteine; in most cases some inhibition was observed. On the other hand, 2-mercaptoethanol or mercaptoacetate was equally effective at mM or 0·1 mM. Mercaptoacetate at 0·01 mM was only partially effective, but the reaction rate was better maintained than in its absence. A concentration of 0·5 mM-KCN was required for maximum effect. Sulphide, 2:3-dimercaptopropanol, ethylenediaminetetra-acetic acid and ascorbate were not effective as protecting agents at 1 mM; the first two inhibited the enzyme. Gelatin showed incomplete protection at a concentration of 1 mg./ml. The loss of activity which occurs in the absence of a protecting agent is reversible; the introduction of mercaptoacetate after the rate had decayed caused an immediate increase in rate to the value observed under standard conditions (Fig. 3).

The influence of varying the metal ion used to activate the system was examined. Mn^{2+} ion was found to give the maximum rate, followed by Mg^{2+} and Ca^{2+} ions (Fig. 4). The titration curves shown

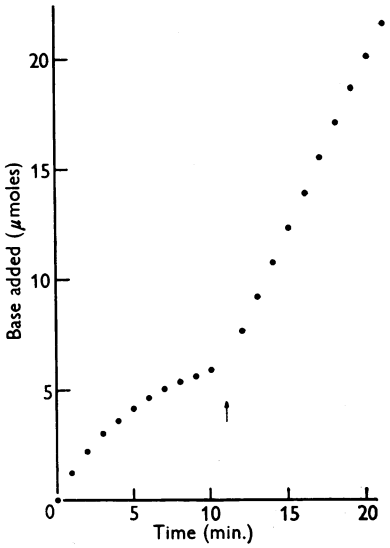

Fig. 3. Titration curve showing recovery of reaction rate on addition of sodium mercaptoacetate at arrow (final concn., 1 mM). Initial reactants were 4 mM-ATP, 2 mM-Mn^{2+} ion, 30 mM-creatine. Enzyme concentration was 0·30 μg. of N/ml. Final volume, 20 ml.; pH 9·00.

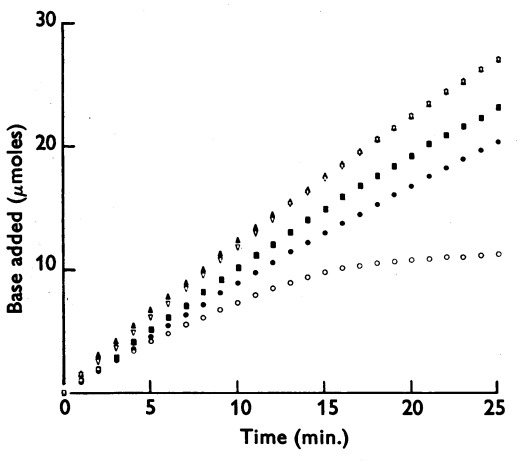

Fig. 2. Titration curves obtained with various protecting agents. 4 mM-ATP, 2 mM-Mn^{2+} ion, 30 mM-creatine. Enzyme concentration was 0·23 μg. of N/ml. Final volume, 20 ml.; pH 9·00. ○, Control; ■, 1 mM-cysteine; ▲, 1 mM-mercaptoethanol; ▽, 1 mM-mercaptoacetic acid; ●, 1 mM-KCN.

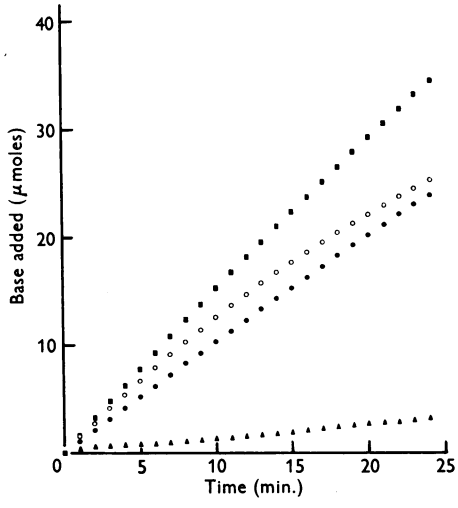

Fig. 4. Titration curves showing ▲, system without metal ions and activation by ●, Ca^{2+} (2 mM), ○, Mg^{2+} (4 mM) and ■, Mn^{2+} (2 mM) ions; 4 mM-ATP, 30 mM-creatine, 1 mM-mercaptoacetate. Enzyme concentration was 0·23 μg. of N/ml. Final volume, 20 ml.; pH 9·00.

in Fig. 4 were obtained with optimum concentrations of metal ions; Mn^{2+} and Ca^{2+} ions produced marked inhibition in higher concentrations, whereas Mg^{2+} ion was less effective in this respect. Fe^{2+} and Co^{2+} ions inhibited the enzyme. The optimum concentration was to some extent dependent on the concentration of mercaptoacetate used; when the latter was increased from 1 to 10 mM, the optimum concentration of Mn^{2+} ion increased from 2 to 4 mM. If the Mn^{2+} ion concentration exceeded the sum of the ATP and mercaptoacetate concentrations, the latter lost its protecting effect and a precipitate formed (presumably manganese hydroxide).

The optimum concentration of ATP was 4 mM, larger concentrations producing some inhibition of the enzyme. Smaller concentrations gave a satisfactory Lineweaver–Burk plot when $[Mn^{2+}]/[ATP]$ was kept at 0·5, with a Michaelis constant of 0·2 mM. Although the initial rate is relatively insensitive to ATP concentration in the range 1–4 mM, it is desirable to use the optimum concentration in the assay procedure to minimize the slight fall in rate which occurs as the reaction proceeds.

The titrimetric method is not suitable for studying the influence of pH on the reaction rate below pH 9·0, since the stoicheiometry of H^+-ion liberation fails as the pK_a values of the reactants are approached. However, there is no evidence of a sharp pH optimum, the rates at pH 8·5 and 9·5 being within 5 % of the rate at pH 9·0. Since the rate of spontaneous hydrolysis of ATP increases quite rapidly above pH 9·0, this pH is a suitable compromise for assay purposes.

With the standard conditions described in the Experimental section, the rate of H^+-ion liberation was linearly related to the amount of enzyme added (Fig. 5). The standard deviation of each estimation was $2·6 \times 10^{-3}$ μmoles/min., which is well below 1 % of the range.

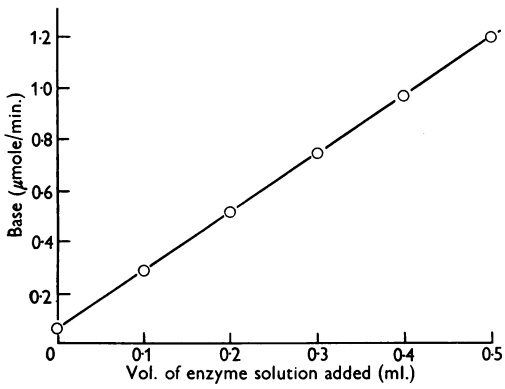

Fig. 5. Relation between reaction rate and amount of enzyme solution (containing 9·3 μg. of N/ml.) added. Duration of titration, 9 min.; 4 mM-ATP, 2 mM-Mn^{2+} ion, 1 mM-mercaptoacetate, 30 mM-creatine. Final volume, 20 ml.; pH 9·00.

Direct comparison with the initial velocity of creatine phosphate production by the method of Kuby *et al.* (1954) was made. The rate was only 55 % of that found by the titrimetric method described here. The addition of 1 mM-mercaptoacetate raised this to 65 %, and substitution of 2 mM-Mn^{2+} ion for 4 mM-Mg^{2+} ion further increased it to 70 %. The remaining difference is perhaps attributable to the glycine buffer used by Kuby *et al.* (1954); a direct comparison of the rate under identical conditions was impossible, since whereas the use of a strong buffer is mandatory with their technique it makes a constant-pH titration too insensitive.

The titrimetric method was found suitable for the estimation of creatine phosphokinase activity in a crude extract of rabbit muscle (10 mM-KCl) in an amount equivalent to 0·2–2·0 mg. of muscle (final dilution 1:10 000–1:100 000). The effect of the reactants present in the extract is negligible at these dilutions. Addition of purified creatine phosphokinase to the crude muscle extract resulted in precise summation of activities. The method will, however, be less accurate in conditions where the acid liberated in the absence of creatine is large compared with that in the assay procedure, i.e. when other enzymes liberating acid are in excess of the creatine phosphokinase.

DISCUSSION

The method described enables creatine phosphokinase activity to be determined in high dilution with great accuracy in about 10 min. The procedure is simple and does not depend upon the use of other enzyme systems, thus making it suitable for inhibitor studies. Provided that the standard conditions are adhered to, the method is specific for creatine phosphokinase. Adenosine triphosphatase activity in the sample to be tested may be accurately estimated by omitting creatine from the reaction system, and can then be subtracted from the total rate to give creatine phosphokinase activity. The main limitation is imposed by the necessity of working at pH 9·0, since at a lower pH the stoicheiometry of hydrogen-ion liberation fails.

It seems likely that the protection afforded by thiols and cyanide against the spontaneous decay in reaction rate is due to maintenance of the thiol groups of the enzyme in the reduced form. However, it is possible that these agents exert their effect by formation of a complex with trace amounts of heavy metals; silver is known to inhibit creatine phosphokinase in very high dilutions

(Martell & Calvin, 1952) and does not form a complex well with ethylenediaminetetra-acetic acid (Solvonuk, McRea & Collier, 1956).

The literature on the relative activating effect of Mn^{2+} and Mg^{2+} ions is not consistent, but Ca^{2+} ion has generally been found to be less effective (Ennor & Morrison, 1958). Part of this disagreement probably arises from the use of glycine as a buffer, since this forms a complex with Mn^{2+} ion more powerfully than with Mg^{2+} ion (Albert, 1950). In the present work Mn^{2+} ion was not only more effective than Mg^{2+} ion, but the rate of the reaction was more nearly constant with both Ca^{2+} and Mn^{2+} ions than with Mg^{2+} ion. The effectiveness of these ions as activators therefore parallels the stability of their complexes with ATP, adenosine diphosphate and creatine phosphate (Smith & Alberty, 1956). The better linearity achieved with Ca^{2+} and Mn^{2+} ions may be related to the severe substrate inhibition of the back-reaction by adenosine diphosphate which occurs with Ca^{2+} ion as the activator (Rosenberg & Ennor, 1955) but not with Mg^{2+} ion (Kuby *et al.* 1954). If this were the case, the rate of the back-reaction would not increase so rapidly as the phosphorylation of creatine proceeds, and the net rate would be more constant.

SUMMARY

1. A titrimetric method for assay of creatine phosphokinase is described.

2. The effects of various protective agents have been investigated and 2-mercaptoethanol and mercaptoacetic acid were found to afford the best protection.

3. The activating effects of various metals have also been studied and Mn^{2+} ion was found to be the most effective.

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Biochem. J. (1960) **75**, 119

Determination of Riboflavin and its Coenzymes in Tissues

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The determination of riboflavin and its coenzymes in tissues is usually done by the fluorimetric method of Bessey, Lowry & Love (1949). Although this method represents an advance on previous procedures it has some disadvantages, such as the burden of separate determinations of flavine mononucleotide and riboflavin, the high salt concentration after hydrolysis which quenches fluorescence to a considerable extent, and the rather long time needed for a complete determination.

Recently we described methods permitting separation and analysis of flavin mixtures in microgram quantities (Cerletti & Siliprandi, 1955) and on a preparative scale (Cerletti, Montesi & Siliprandi, 1957), and have studied the properties of pure flavin nucleotides (Cerletti & Siliprandi, 1958; Cerletti, 1959).