Micro Method for Determination of Reactive Carbonyl Groups in Proteins and Peptides, using 2,4-Dinitrophenylhydrazine

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A method is described for determining carbonyl groups that is especially suitable for use with proteins and peptides. It involves the determination of the extinction at 370nm of a sample solution after adding 2,4-dinitrophenylhydrazine. The reaction of 2,4-dinitrophenylhydrazine with pyruvoylglycine and with transaminated ribonuclease T₁ is presented; the isolation of protein hydrazones is discussed.

Selective modification of proteins or peptides by techniques such as transamination (Dixon, 1964; van Heyningen, Tipton & Dixon, 1968) or periodate treatment (Dixon & Weitkamp, 1962; Fields & Dixon, 1968) or procedures such as that of selective cleavage of peptide chains at cysteinyl residues (Patchornik & Sokolovsky, 1964) lead to derivatives that possess a reactive carbonyl group. Measurement of these groups may be useful for detecting the new derivative in the effluent of an ion-exchange column (Dixon, 1964), for determining the extent of modification or, indeed, may facilitate the detection of such groups in naturally occurring proteins (cf. Havir & Hanson, 1968; Riley & Snell, 1968).

The method of Friedemann & Haugen (1943) as modified by Dixon (1964) necessitated extraction of the oxo acid into an organic solvent, after reaction with 2,4-dinitrophenylhydrazine. The method could not be satisfactorily applied to peptides possessing charged groups at acid pH, or to proteins. The technique reported here makes use of the difference in extinction at 370nm between 2,4-dinitrophenylhydrazine and the hydrazone that is formed and involves measuring the extinction of a sample in the presence of excess of reagent after reaction has occurred.

MATERIALS

A stock solution of approx. 5mm-2,4-dinitrophenylhydrazine in 2M-HCl was prepared by the procedure of Friedemann & Haugen (1943). The 10M- and M-HCl solutions were prepared by diluting AnalaR HCl (sp.gr. 1.18) obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Pyruvoylglycine was prepared from toluene-*p*-sulphonyl chloride and pyruvic acid by the method of Dixon (1964).

Ribonuclease T₁, prepared by chromatography on

DEAE-cellulose in 0.12M-Na₂HPO₄–0.08M-NaH₂PO₄ (Fields, Dixon, Law & Yui, 1971), was transaminated by incubation for 30min at room temperature in a solution of 2M-sodium acetate in 0.4M-acetic acid that contained 0.1M-sodium glyoxylate and 10mm-CuSO₄ (Dixon, 1967). Ribonuclease T₁, so treated, showed the loss of one amino group, as determined by 2,4,6-trinitrobenzenesulphonic acid, the loss of one residue of alanine by automatic amino acid analysis after acid hydrolysis, and the derivative was retarded to 3.5 column volumes when chromatographed on DEAE-cellulose in 0.22M-sodium phosphate buffer, pH6.9. Under the same conditions native ribonuclease T₁ emerged at 2.5 column volumes.

DETERMINATION OF CARBONYL GROUPS

Rapid method (5min). This method is most useful as a semi-quantitative test. A 0.2ml volume (or less) of sample (containing 25–500nmol of carbonyl groups) is allowed to react with 0.2ml of reagent (5mm-2,4-dinitrophenylhydrazine in 2M-HCl) for 5min. The mixture is then diluted to 5ml with M-HCl and the extinction at 370nm determined. A blank is also prepared and may be used in the spectrophotometer to give a difference reading directly. As the blank extinction is near unity accurate dilution is required for determining small concentrations of carbonyl group.

Second method (60min). This method is indicated when many determinations are to be made, as in the assay of fractions of effluent from a chromatographic column, or when the concentration of carbonyl group is high enough so that a small volume of sample may be taken. The reagent is diluted 25-fold with M-HCl before reaction with sample, thus eliminating errors arising from individual dilutions. Samples (10–100μl) are mixed with 2.5ml portions of the diluted reagent solution. After 1h the extinction at 370nm is determined.

Method for reaction with proteins (60min). The sample solution is adjusted with 10M-HCl to give an HCl concentration of 1M. Then 2.4ml of this solution is pipetted into a cuvette. Reagent solution (0.09ml) is mixed with

2.4 ml of *m*-HCl for use as a blank, and with it the spectrophotometer is adjusted to give a null reading at 370 nm. The reaction is initiated by adding 0.10 ml of the reagent to the sample and mixing the contents of the cuvette by inversion. A stopwatch is started and several readings are taken during the first few minutes. These readings are extrapolated to give an accurate initial reading, which is subtracted from the final extinction reached after 1 h.

Standardization. If possible, a standard curve should be prepared for the particular hydrazone that is being studied. If not (as in the case of a protein), a model compound is used.

RESULTS AND DISCUSSION

Fig. 1 shows that a solution that was 10 μ M with respect to pyruvoylglycine reacted with 2,4-dinitrophenylhydrazine in *m*-hydrochloric acid to give a difference in extinction of 0.120 at 370 nm. Fig. 2 shows the progress curve of the reaction of reagent with transaminated ribonuclease T_1 . Since this protein has an *N*-terminal pyruvoyl group, the extinction coefficient of pyruvoylglycine was used to determine its concentration. Fig. 3 shows the absorption spectrum of the 2,4-dinitrophenylated enzyme after gel filtration into 0.1 *m*-glycine-NaOH buffer, pH 9. The hydrazone at this pH has an extinction of 17 500 $\text{M}^{-1} \text{cm}^{-1}$.

The reaction of 2,4-dinitrophenylhydrazine with

carbonyl groups to form a hydrazone is freely reversible at pH 0, and samples of protein hydrazone that were gel-filtered into *m*-hydrochloric acid rapidly dissociated. When gel-filtration was into buffer at alkaline pH no dissociation was observed in 2 days, as judged by lack of spectral change. No investigation of the lower limit of pH as regards stability of the hydrazone was made.

Fig. 2. Reaction of 2,4-dinitrophenylhydrazine with transaminated ribonuclease T_1 . A 0.3 ml portion of 10 *m*-HCl was added to 2.1 ml of transaminated ribonuclease T_1 in 0.2 *m*-phosphate buffer, pH 6.9, before mixing with 0.10 ml of reagent. Readings at 370 nm were made at the times shown against a blank that contained 0.09 ml of reagent in 2.4 ml of *m*-HCl. The graph shows the extrapolation back to zero time which gave a value of 0.025 for the initial extinction. The arrow at 0.92 on the vertical axis shows the value expected for the combination of 1 mol of reagent/mol of protein.

Fig. 1. Standard curve for reaction of 2,4-dinitrophenylhydrazine with pyruvoylglycine in *m*-HCl, determined by the second method. Samples (2–10 μ l) of solutions of pyruvoylglycine were added to 2.5 ml of 0.2 *m*-2,4-dinitrophenylhydrazine in *m*-HCl to give the final concentrations shown. After 1 h the extinction at 370 nm was determined against a blank of reagent.

Fig. 3. Spectrum of ribonuclease T_1 and of the 2,4-dinitrophenylhydrazone of transaminated ribonuclease T_1 . Visible and u.v. spectra of ribonuclease T_1 and of its hydrazone in 0.1 *m*-glycine-NaOH buffer, pH 9, were recorded on a Beckman model DK-2 ratio recording spectrophotometer. A sample of native enzyme was dissolved directly into the buffer and was read against a blank of buffer. The hydrazone was formed in *m*-HCl and gel-filtered on a column (1 *cm* $\times$ 25 *cm*) packed with Sephadex G-25, equilibrated with 0.1 *m*-glycine-NaOH buffer, pH 9, before the spectrum was read against a blank of buffer. ----, Ribonuclease T_1 ; —, hydrazone of transaminated ribonuclease T_1 .

Other spectrophotometric methods have been described for determining carbonyl compounds (see Jordan & Veatch, 1964; Lappin & Clark, 1951) but they are not as suitable for use with proteins, nor as convenient as the one described here. They often involve addition of strong alkali in alcohol to the solution of hydrazone to shift the absorption maximum into the visible region before its extinction is determined.

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