The Use of Maleic Anhydride for the Reversible Blocking of Amino Groups in Polypeptide Chains

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1. Maleic anhydride was shown to react rapidly and specifically with amino groups of proteins and peptides. Complete substitution of chymotrypsigen was achieved under mild conditions and the extent of reaction could be readily determined from the spectrum of the maleyl-protein. Maleyl-proteins are generally soluble and disaggregated at neutral pH. Trypsin splits the blocked proteins only at arginine residues and there is frequent selectivity in this cleavage, e.g. in yeast alcohol dehydrogenase and pig glyceraldehyde 3-phosphate dehydrogenase.

3. The group is removed by intramolecular catalysis at acid pH. The half-time was 11–12 hr. at 37° at pH 3.5 in ε-maleyl-lysine or in maleyl-chymotrypsigen.

4. The unblocking reaction can be used as the basis for a 'diagonal'-electrophoretic separation of lysine peptides and N-terminal peptides, as shown by studies with β-melanocyte-stimulating hormone.

The cleavage of proteins with trypsin frequently gives rise to highly complex mixtures of peptide fragments, and the problem of obtaining all the tryptic peptides in a pure form and in sufficient yield has become rate-limiting in the elucidation of the primary structure of large protein molecules. Much effort has therefore been directed towards decreasing the complexity of tryptic digests by limiting the action of the enzyme, so that it splits only bonds involving arginine residues. These efforts have led to the use of chemical reagents such as S-ethyltrifluoroacetate (Goldberger & Anfinsen, 1962; Goldberger, 1967; Taniuchi, Anfinsen & Sodja, 1967) and substituted imido esters (Hunter & Ludwig, 1962; Ludwig & Byrne, 1962; Ludwig & Hunter, 1967), which are capable of reacting specifically with the amino groups of lysine residues in proteins. Peptide bonds C-terminal to the trifluoroacetyl- or amidino-lysine residues were found to be resistant to cleavage by trypsin, and the simpler mixtures of peptides that were produced by selective cleavage at the arginine residues were found to be amenable to fractionation in their blocked-lysine form. The purified peptide could then be unblocked to regenerate the lysine residues and further digested separately with trypsin to obtain the lysine-containing peptides that occur between any two consecutive arginine residues in the protein chain.

The full complement of tryptic peptides from a given protein could thus be obtained from the individual blocked-lysine fragments by a series of separate fractionation steps more simply than from a total tryptic digest of the whole protein. By this strategy, Davidson, Sajgö, Noller & Harris (1967) used S-ethyltrifluoroacetate to determine the complete sequence of 333 residues (including 28 lysine and 10 arginine residues) in the protein chain of lobster glyceraldehyde 3-phosphate dehydrogenase. Perham & Jones (1967) applied the same reagent to develop a 'diagonal'-separation technique (Brown & Hartley, 1966) for lysine-containing peptides in a protein chain.

As a general method for blocking the lysine residues of proteins or peptides, S-ethyltrifluoroacetate suffers from two major disadvantages. First, the trifluoroacetylamino group is not completely stable under the conditions (pH 9.5–10.0) used for the blocking reaction. Consequently to achieve complete blocking it is necessary to use an enormous excess of the very noxious reagent. Similar difficulties in achieving complete blocking of lysine residues have been encountered with imido ester reagents (Wofsy & Singer, 1963). Secondly, although the peptides produced by tryptic digestion of trifluoroacetylated proteins are generally soluble, they are liable to form aggregates, so that the peptides can be difficult to obtain pure and in good yield even when disaggregating solvents such as 8M-urea are used for their chromatographic separations (Harris & Perham, 1968).

The reagent of choice would overcome these difficulties if it were to combine the reversible properties of trifluoroacetylation and amidination...
with the solubilizing and disaggregating properties of reagents such as succinic anhydride (Habeeb, Cassidy & Singer, 1958; Klotz & Keresztes-Nagy, 1963; Friet, Bendit, Smith & Lauffer, 1965). It should also react specifically and quantitatively with amino groups to form a derivative that is completely stable at the optimum pH for the blocking reaction.

Maleic anhydride appears to be such a reagent and we have investigated its potential use for reversible blocking of amino groups in polypeptide chains. A preliminary account of this work has been given (Butler, Harris, Hartley & Leberman, 1967).

MATERIALS AND METHODS

Reagents. All reagents were of A.R. grade or the highest quality commercially available. Maleic anhydride was purified further by sublimation under reduced pressure. 

\[ 1,4\textsuperscript{14}C_2 \] Maleic anhydride (3-14 mc/m-mole) was obtained from The Radiochemical Centre, Amersham, Bucks.

Enzymes and proteins. Trypsin, chymotrypsin, bovine chymotrypsinogen A and soya-bean trypsin inhibitor were obtained from the Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Samples of MSH* were kindly given by Dr H. B. F. Dixon of the University of Cambridge and by Dr A. B. Lerner of Yale University, New Haven, Conn., U.S.A.

YADH was prepared by a modification of the procedure of Krebs (1955) for the preparation of yeast glyceraldehyde 3-phosphate dehydrogenase. In the preparation of YADH the protein precipitated between 0% and 60% saturation rather than between 55% and 70% saturation with (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} for was taken for crystallization (Butler, 1967).

Paper electrophoresis. High-voltage paper electrophoresis was carried out on Whatman 3MM paper with apparatus similar to that described by Michl (1961) as modified by Ryle, Sanger, Smith & Kitai (1955), with pyridine (10%), \( v/w \) acetic acid (0-5%, \( v/v \)), pH 6-5, as buffer and toluene containing 8% of pyridine as coolant.

Peptides were detected by staining with the ninhydrin-cadmium reagent of Heilman, Barrolier & Watzke (1957), or by using the chlorine-o-tolidine reagent of ReinDEL & Hoppe (1954).

Specific amino acids in peptides were detected as follows: (i) arginine by the Sakaguchi colour reaction on paper (Acher & Crocker, 1953) as modified by Irreverre (1965); (ii) histidine by the Pauly reaction as described by Dalgleish (1952), modified by dipping the paper in a mixed solution of the reagents rather than by spraying it; (iii) tryptophan by the Ehrlich reaction as described by Dalgleish (1952); (iv) tyrosine as described by Acher & Crocker (1952).

Peptide mobilities at pH 8-5 were calculated with respect to that of aspartic acid (\( = +1 \)) and from this and the molecular weight the net charge was calculated (Offord, 1966).

Other techniques. (i) N-Terminal residues of peptides were determined by using the 1-dimethylaminonaphthalene-5-sulphonyl chloride reagent of Gray & Hartley (1963) and Gray (1967). (ii) Amino acid analyses were carried out with a Beckman Spinco 120 amino acid analyser. (iii) Radioactivity counting was carried out by using a Nuclear-Chicago liquid-scintillation counter. (iv) Spectrophotometry was carried out with a Gilford model 2000 recording spectrophotometer with a Unicam SP.500 monochromator. (v) pH-stat digestions were carried out by using a Radiometer pH-stat with an Ole Dich recorder and syringe drive.

Preparation of \( e \text{-maleyl-lysine} \). \( e \text{-Maleyl-lysine} \) was synthesized by the copper-complex technique of Porter & Sanger (1948), but with the soluble CuCl\textsubscript{2} rather than the insoluble carbonate.

L-Lysine hydrochloride (500 mg.) was dissolved in 0-5 ml. of water and 1-5 ml. of 1-0 M-CuCl\textsubscript{2} solution was added, followed by 10 g. of NaHCO\textsubscript{3}. When the effervescence had ceased, the pH was found to be 7-1. Maleic anhydride (400 mg.) was added in small batches and, after the reaction and effervescence had ceased, the pH was again found to be 7-1. The solution was acidified to pH 3-5 with acetic acid and applied to a column (50 ml. bed volume) of Zeo-Karb 225 (H\textsuperscript{+} form) in 0-1 M-acetic acid. The column was eluted with 0-1 M-acetic acid to remove any free maleic acid, and then with 1-0 M-NH\textsubscript{3} to elute the lysine derivative and free lysine. The eluate containing these was freeze-dried.

On submission of a sample to electrophoresis at pH 6-5, it was found to contain a single derivative and a small amount of free lysine (about 5%). The derivative gave \( \alpha-1 \)-dimethylaminonaphthalene-5-sulphonyl-lysine on reaction with 1-dimethylaminonaphthalene-5-sulphonyl chloride followed by hydrolysis with 5-7 M-HCl at 105\textdegree C for 18 hr., and was therefore presumed to be \( e \text{-maleyl-lysine} \).

This material was further purified by chromatography on a column (6 cm. x 4 cm.) of DEAE-cellulose (OH\textsuperscript{-} form). The material was dissolved in water and applied to the column, which was then eluted with water, followed by pyridine-acetate buffer, pH 5-8, 1-0 M with respect to acetate. The unchanged lysine and some of the \( e \)-maleyllysine were not bound to the column and emerged in the breakthrough volume, but most of the \( e \)-maleyl-lysine was eluted by the pyridine-acetate buffer as a separate, symmetrical, peak, and was freeze-dried to give a white powder.

Preparation of maleyl-MSH. MSH (0-4 mg.) was dissolved in 0-8 ml. of 0-2 M-sodium borate, pH 9-0, and a solution of 1-2 mg. of maleic anhydride in dioxan was added. The solutions were mixed and kept at room temperature for several hours before the resulting maleyl-MSH was desalted by passing it down a column (100 cm. x 2-5 cm.) of Sephadex G-25 in 0-1 M-NH\textsubscript{3}. The fractions containing the peptide were pooled.

Reaction of chymotrypsinogen with [\textsuperscript{14}C]-maleic anhydride. [\textsuperscript{14}C]-Maleic anhydride was diluted with carrier maleic anhydride in dry benzene to give a 0-2 M solution of specific radioactivity 11-9 \mu c/m-mole. Samples of this solution were added to solutions of chymotrypsinogen (2-5 mg./ml.) in 1-0 ml. of each of the following buffers: 0-2 M-sodium maleate, pH 6-0; 0-2 M-pyridine-acetate, pH 6-5; 0-2 M-sodium phosphate, pH 7-0 and pH 5-0; 0-2 M-sodium borate, pH 8-5; pH 9-0 and pH 9-5; 0-2 M-sodium phosphate, pH 7-0,
containing 0.02 M-pyridine; 0.2 M-sodium phosphate, pH 7.0, containing 20% (v/v) or redistilled dioxan. The reaction was very rapid and in all cases appeared to be complete after 5 min. After 75 min at 20°C, the samples were dialysed against several changes of 0.5% NH₄HCO₃ until a constant specific radioactivity of protein was obtained. The protein concentration was calculated from the extinction at 280 nm. (ε 50000), corrected for 280 nm.

The determination of the solution specific radioactivity was determined by counting duplicate 50 µl. samples in 4 ml. of scintillation fluid (solvent 6; Baxter, Fanning & Schwartz, 1964) twice for 2 min., with controls of water and of the final diffusate. Triplicate samples of suitable dilutions of the original [14C]maleic anhydride that had been hydrolysed with 0.1 M-NaOH acted as standards. Similar experiments were carried out to investigate the effect of various changes in the reaction conditions on the degree of substitution (Table 1).

Preparation of maleyl-chymotrypsinogen. Bovine chymotrypsinogen A (20 mg.) was dissolved in 2.0 ml. of 0.1 M-sodium pyrophosphate buffer, pH 9.0, and treated at 2°C with 300 µl. of 1.0 M-maleic anhydride in redistilled dioxan. The maleic anhydride solution was added in six additions and the pH of the mixture was maintained at 9.0 by the addition of 0.1 M-NaOH. When the reaction was complete, the maleyl-chymotrypsinogen was desalted by passing it down a column (40 cm × 3 cm.) of Sephadex G-25 in 0.01 M-NH₄H, and the fractions that contained the protein were pooled. These gave a solution with a protein concentration of about 0.6 mg./ml.

Unblocking of maleyl-chymotrypsinogen. Two samples of the solution of maleyl-chymotrypsinogen A were taken and adjusted to pH 3.5 with formic acid and 0.1 M-NH₄H and to a final volume of twice the volume of the sample, one sample also being made 5 M with respect to guanidine hydrochloride. These solutions were incubated for 30 hr. at 37°C and then for 60 hr. at 60°C, and samples were taken at zero and later times. The reaction was stopped in each sample by mixing it with twice its volume of 0.2 M-NaOH, and the extinction was measured at 260 and 280 nm.

Preparation of maleyl-carboxymethyl-YADH. The crystals of YADH were spun down from suspension in (NH₄)₂SO₄ solution and dissolved in 0.1 M-NH₄H (about 10 mg./ml.), and the solution was dialysed against 0.1 M-NH₄H to remove the residual (NH₄)₂SO₄. The solution was then freeze-dried to give a white powder of YADH. This powder was dissolved (at about 10 mg./ml.) in 0.1 M-sodium phosphate-0.6 M-EDTA-0.6 M-guanidine hydrochloride, apparent pH (glass electrode) 8.1, and the solution was oxygenated by bubbling N₂ through it for 10 min. Dithiothreitol (2.5 µmoles/ml. of protein solution) was added and the mixture incubated at room temperature for 2 hr. Iodoacetic acid (10 µmoles/ml. of protein solution) was dissolved in 1.0 ml. of 0.1 M-sodium phosphate-0.6 M-EDTA-0.6 M-guanidine hydrochloride, and the pH was adjusted to 8.1. This solution was oxygenated by bubbling N₂ through it for 10 min. and then mixed under N₂ with the reduced solution of YADH. The resulting solution was incubated at room temperature (20°C) for 3 hr. and then the reaction stopped by the addition of β-mercaptoethanol (5 µmoles/ml. of protein solution).

The side products of the reaction were removed by dialysis against 0.1 M-sodium pyrophosphate-0.6 M-guanidine hydrochloride, pH 9.0, and the carboxymethyl-YADH was treated with maleic anhydride (12.5 mg./ml. of protein solution) added as the solid, a little at a time. During the reaction the pH was maintained between 8.5 and 9.0 by the addition of 1.0 M-NaOH. The solution was dialysed against five changes of 0.5% NH₄HCO₃ and then freeze-dried to give a white powder of maleyl-carboxymethyl-YADH.

RESULTS

Reaction of maleic anhydride with chymotrypsinogen. The blocking reaction was studied by using bovine chymotrypsinogen A as a model. This protein contains 14 lysine residues, which together with the N-terminal residue give a total of 15 free amino groups/molecule.

The effect of pH on the extent of reaction of maleic anhydride (5-0 mM) with chymotrypsinogen A (0.1 mM) is shown in Fig. 1. At the optimum pH (about 9) the reaction proceeds to 90% of the theoretical extent with only a threefold excess of reagent over all amino groups. Fig. 1 also shows that the reaction is relatively unaffected by pyridine or dioxan. The effects of varying the protein concentration, the excess of reagent or the temperature, and of 2 M-guanidine hydrochloride or 8 M-urea, on the extent of reaction were also investigated, and the results are shown in Table 1.

The reaction depends on competition between protein and water for the reagent, so the time for

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**Fig. 1.** Effect of pH on the reaction of maleic anhydride (5-0 mM) with bovine chymotrypsinogen A (0.1 mM) at 2°C in the following 0.2 M buffers: ○, sodium maleate; ■, pyridine-acetate; Δ, sodium phosphate; ▲, sodium phosphate-0.02 M-pyridine; ◆, sodium phosphate-dioxan (20%); V, sodium borate. For other details, see the text.
Table 1. *Effect of conditions on the reaction of maleic anhydride with \(\alpha\)-chymotrypsinogen at pH 9.0 in 0.1 M-sodium pyrophosphate*

The theoretical maximum extent of reaction is 15 moles of maleyl group/mole of chymotrypsinogen. For experimental details, see the text.

<table>
<thead>
<tr>
<th>Concentration of chymotrypsinogen ((\mu)M)</th>
<th>Concentration of maleic anhydride ((\mu)M)</th>
<th>Temperature (°C)</th>
<th>Conditions</th>
<th>Extent of reaction (moles of maleyl group/mole of chymotrypsinogen)</th>
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<td>12-6</td>
</tr>
<tr>
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<td>5</td>
<td>20</td>
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<td>13-3</td>
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<td>2</td>
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<td>2</td>
<td></td>
<td>15-5</td>
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<tr>
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<td>5</td>
<td>2</td>
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</tr>
<tr>
<td>0.1</td>
<td>5</td>
<td>20</td>
<td>8M-Urea</td>
<td>13-3</td>
</tr>
</tbody>
</table>

**Table 2. Hydrolysis of \(\varepsilon\)-maleyl-lysine at 37°**

Samples were incubated at 37° for the times indicated; the lysine released was determined by amino acid analysis. The buffers used were as follows: pH 2.1, acetic acid (8%, v/v)-formic acid (2%, v/v); pH 2.5-6.0, sodium citrate, 0.2M with respect to citrate; pH 7.0 and 8.0, sodium phosphate, 0.2M with respect to phosphate; pH 10.0, sodium borate, 0.2M with respect to borate.

<table>
<thead>
<tr>
<th>pH</th>
<th>Time (hr.)</th>
<th>Lysine released (% of total)</th>
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</thead>
<tbody>
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<td>46.4</td>
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<tr>
<td>2.5</td>
<td>6</td>
<td>46.5</td>
</tr>
<tr>
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<td>6</td>
<td>39.8</td>
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<td>6</td>
<td>18.1</td>
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<td>4.5</td>
<td>6</td>
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<tr>
<td>10.0</td>
<td>72</td>
<td>0.0</td>
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</tbody>
</table>

Fig. 2. Effect of pH on the hydrolysis of \(\varepsilon\)-maleyl-lysine at 37°. Hydrolysis times were: ●, 6 hr.; ■, 18 hr.; ▲, 72 hr. For experimental details, see the text.

The reaction is governed by the rate of hydrolysis of the reagent. This was measured by titrating 2M-maleic anhydride at 3° at various pH values with 0.1 M-sodium hydroxide in a pH-stat. The half-life of maleic anhydride, determined from first-order plots, was 3.6 min. at pH 4.0, 2.5 min. at pH 5.0 and 6.0, 1.2 min. at pH 7.0 and 6.0 min. at pH 8.0. At higher pH values the reaction was too rapid to follow, but it is obvious that a few minutes at pH 9.0 would represent several half-lives.

**Properties of \(\varepsilon\)-maleyl-lysine.** The stability of the product of the reaction of maleic anhydride with amino groups in proteins or peptides was studied by using \(\varepsilon\)-maleyl-lysine as a model compound. The effect of pH was studied by incubating \(\varepsilon\)-maleyl-lysine (approx. 1.5 mM) dissolved in buffers of appropriate pH values at 37° for 6, 18 and 72 hr. The following buffers were used: pH 2.1, acetic acid (8%, v/v)-formic acid (2%, v/v); pH 2.5-6.0, sodium citrate, 0.2M with respect to citrate; pH 7.0 and 8.0, sodium phosphate, 0.2M with respect to phosphate; pH 10.0, sodium borate, 0.2M with respect to borate. The reactions were stopped by freezing the solutions and the lysine released was determined by applying a freshly thawed sample to the 10 cm. column of an amino acid analyser. The total \(\varepsilon\)-maleyl-lysine was determined as lysine after hydrolysis with 5M-hydrochloric acid at 105° for 18 hr., and a control sample of \(\varepsilon\)-maleyllysine was also analysed to determine the contamination with free lysine. Table 2 shows the
degree of unblocking at various pH values, and Fig. 2 shows the half-life of \( \varepsilon \)-maleyl-lysine at 37\(^\circ\)C, assuming first-order kinetics.

The spectrum of \( \varepsilon \)-maleyl-lysine in 0.1 M sodium hydroxide was determined and is shown in Fig. 3, together with that of lysine plus maleic acid, also in 0.1 M sodium hydroxide.

**Unblocking of maleyl-chymotrypsinogen.** To see whether the rate of unblocking would be the same for the amino groups in a protein as for the model compound, the rate of unblocking was studied for maleyl-chymotrypsinogen in both the presence and the absence of guanidine hydrochloride. The protein was insoluble at pH 3.5 in the absence of this reagent.

The extinction of a protein solution at 280 nm. changes very little on unblocking of the lysine residues (see Fig. 3), whereas that at 260 nm. changes markedly. Hence the \( E_{280} \) for each sample could be used to correct the \( E_{260} \) to a constant protein concentration, and in this way to correct for the different amounts of protein taken during sampling of the suspension of the protein, which was insoluble in the absence of the guanidine hydrochloride. Although this correction is an approximation, the magnitude of the errors involved in it are small compared with the other errors in the estimation.

The samples that had been incubated for 30 hr. at 37\(^\circ\)C and then for 60 hr. at 60\(^\circ\)C were assumed to be completely unblocked, and from these and the initial samples the difference in the \( E_{260} \) for complete unblocking of the lysine residues was estimated. Hence the percentage of unblocking in each of the other samples was found.

These percentages are shown in Fig. 4, plotted on a semi-logarithmic graph against hydrolysis time. Also shown are the predicted rate for the hydrolysis of \( \varepsilon \)-maleyl-lysine at pH 3.5 and 37\(^\circ\)C and, from this rate, the range within which the experimental points would lie (±15\%) for an error in sampling and measurement of extinctions of ±2.5\%.

**Specificity of reaction.** The specificity of the reaction of maleic anhydride with the amino groups in proteins or peptides was tested by examining the product of its reaction with MSH. This octadecapeptide contains both tyrosine and tryptophan (Harris & Roos, 1959), and any reaction with these residues should be detected by the spectral change that would be produced on the introduction of the conjugated double bonds of the maleyl group into the aromatic structures of these residues.

The spectra of MSH and maleyl-MSH in 0.1 M sodium hydroxide are shown in Fig. 5. Also shown (broken line) is the spectrum of maleyl-MSH minus the contribution due to the three maleyl groups that would result from the reaction of the two \( \varepsilon \)-amino groups of the lysine residues and of the \( \alpha \)-amino group of the N-terminal aspartic acid in MSH.

**Tryptic digestion of maleyl-MSH.** The sequence of MSH is known (Harris & Roos, 1959) and contains one arginine residue in addition to the two lysine residues. It thus seemed a good model compound

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![Fig. 3. Spectra of \( \varepsilon \)-maleyl-lysine and of lysine plus maleic acid in 0.1 M NaOH. The estimated error was ±3%. For other details, see the text.](image)

![Fig. 4. Hydrolysis of maleyl-chymotrypsinogen at 37\(^\circ\)C, pH 3.5, in water (■) and in 5M-guanidine hydrochloride (□). The broken line (----) shows the rate for \( \varepsilon \)-maleyl-lysine. The vertical lines show the error (±15\%) for an error in sampling and measurement of extinctions of ±2.5\%. For other details, see the text.](image)
with trypsin A protein. on which to try the tryptic digestion of a maleyl-protein. A sample of maleyl-MSH was digested with trypsin in 0.5% ammonium hydrogen carbonate at 37° for 4 hr., and the digest was submitted to electrophoresis at pH 6.5, giving two peptides, T1 and T2. Peptide T2 gave no colour with ninhydrin-cadmium, suggesting that it had a blocked N-terminal residue. Both peptides were eluted and then unblocked with acetic acid (5%, v/v)-pyridine (1%, v/v), pH about 3.5, at 60° for 6 hr. After subsequent redigestion with trypsin, followed by resubmission to electrophoresis at pH 6.5, peptide T1 yielded a single peptide, T1a, whereas peptide T2 gave two peptides, T2a and T2b. When guide strips from the paper electrophoretograms were stained, peptides T1 and T1a gave a reaction for tryptophan, peptides T2 and T2a gave reactions for arginine and histidine, and peptides T2 and T2b gave reactions for tyrosine. The peptides T1a, T2a and T2b were eluted and their analyses and N-terminal residues determined. These are shown in Table 3, together with their mobilities at pH 6.5.

**Chymotryptic digestion of maleyl-MSH.** A further sample of maleyl-MSH was digested with chymotrypsin in 0.5% ammonium hydrogen carbonate at 37° for 4 hr., and the digest was submitted to electrophoresis at pH 6.5. Four peptides, C1–C4, were obtained. Peptides C3 and C4 did not react with ninhydrin-cadmium, suggesting that their N-terminal residues were blocked. When guide strips from the paper electrophoretograms were stained for individual amino acids, peptide C1 gave reactions for arginine and tryptophan, peptides C2 and C3 gave reactions for histidine, and peptides C3 and C4 gave reactions for tyrosine. The peptides were eluted from part of the electrophoretogram and their amino acid analyses and N-terminal residues were determined. These are shown in Table 3, together with their mobilities at pH 6.5.

*Diagonal* electrophoresis for lysine and N-terminal peptides. The mild conditions required for unblocking the lysine residues suggested that maleylation might be used as a technique for a

![Graph](image)

Fig. 5. Spectra of maleyl-MSH and of MSH in 0.1 M-NaOH. The broken line (-----) shows the spectrum of maleyl-MSH corrected for a content of three maleyl groups (see the text). For experimental details, see the text.

<table>
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<th>Peptide ...</th>
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<th>T2b</th>
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<th>C2</th>
<th>C3</th>
<th>C4</th>
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<td>—</td>
<td>—</td>
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<tr>
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<td>1.2</td>
<td>1.1</td>
<td>—</td>
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<tr>
<td>Met</td>
<td>—</td>
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<td>—</td>
<td>—</td>
<td>0.8</td>
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<td>Tyr</td>
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<td>0.7</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Phe</td>
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<td>—</td>
<td>—</td>
<td>0.9</td>
<td>0.6</td>
<td>—</td>
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<tr>
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<td>+</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>—</td>
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<tr>
<td>N-Terminal residue</td>
<td>—</td>
<td>Met</td>
<td>Asp</td>
<td>Arg</td>
<td>Lys</td>
<td>—</td>
<td>—</td>
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<td>Mobility at pH 6.5</td>
<td>0.00</td>
<td>—0.23</td>
<td>+0.34</td>
<td>+0.23</td>
<td>+0.35</td>
<td>+0.88</td>
<td>+1.10</td>
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lysine 'diagonal'-electrophoretic separation, and this was tested on the chymotryptic digest of maleyl-MSH. The peptides on a strip of paper from the electrophoretogram at pH 6.5 were unblocked by incubation in a desiccator, which had been evacuated on a water pump, in the vapour of acetic acid (5%, v/v)-pyridine (1%, v/v) at 60° for 6 hr. The strip was then dried and sewn on to a fresh sheet of paper, and the peptides were re-submitted to electrophoresis at pH 6.5, at right angles to the original direction of electrophoresis. The resulting electrophoretogram was stained with ninhydrin-cadmium, and a drawing of it is shown in Fig. 6. The positions of the peptides are outlined, with a broken line for the weakly reacting ones, and the positions that peptides C3 and C4 would occupy are shown by crosses. The peptide derived by unblocking from a given peptide is indicated by a prime (e.g. peptide C2 gives rise to peptide C2').

Samples of asparagine, glutamine and tryptophan that had been treated on paper under the conditions of demaleylation were submitted to electrophoresis. No deamination of the former could be detected and tryptophan showed no change in mobility, fluorescence or reaction with Ehrlich's reagent.

**Tryptic digestion of maleyl-carboxymethyl-YADH.** A sample of maleyl-carboxymethyl-YADH was dissolved (about 2.5 mg./ml.) in 0.05 M-sodium citrate, pH 7.5, and then digested with trypsin (enzyme/substrate ratio 1:100) at pH 8.5 at 20° in a pH-stat, under carbon dioxide-free nitrogen. The pH was maintained at 8.5 by the addition of 0.05 M-sodium hydroxide. On the addition of trypsin there was an extremely rapid uptake of base, which slowed within the first 3 min., when an amount equivalent to the cleavage of three or four bonds/monomer molecule of maleyl-carboxymethyl-YADH had been added. Since this was equivalent to the cleavage of almost half the susceptible bonds in the maleyl-carboxymethyl-YADH (a total of eight bonds/molecule were expected to be cleaved) the effect of the addition of a further sample of trypsin was investigated. As this did not produce any significant further uptake of base, a sample of the trypsin digest 5 min. after the addition of the trypsin was taken and the reaction was stopped by the addition of soya-bean trypsin inhibitor in a slight excess over the trypsin in the sample.

The remainder of the digest of maleyl-carboxymethyl-YADH was allowed to react with the trypsin for 20 hr. During this time there was a slow, continuing, uptake of base. After 18 hr. this uptake had become even slower, and the rate was not increased by the addition of a further sample of trypsin. The digestion was stopped after 20 hr. by freezing the solution. At this point the total base added was approximately equivalent to the cleavage of eight bonds/molecule of maleyl-carboxymethyl-YADH.

The products of the trypsic digestions for 5 min. and 20 hr. (both at 20°) were compared by chromatography on a column (120 cm. x 2 cm.) of Sephadex G-50, in 0.1 M-ammonia. The extinctions of the fractions at 225 nm. were measured and the resulting profiles were distinctly different.

**DISCUSSION**

The reaction of maleic anhydride with the amino groups of proteins and peptides is that of a typical acid anhydride. The pH profile for the reaction (Fig. 1) suggests that the reaction occurs with the un-ionized amino group and hence the rate of reaction increases with increasing pH. However, since the OH- catalysed hydrolysis of maleic anhydride also increases with increasing pH, the optimum percentage of blocking is found to occur at pH 8.5-9.0. Temperature, 5 M-guanidine or 8 M-urea have no significant effect on the ultimate percentage of blocking, whereas increasing the protein concentration favours the acylation rather than the hydrolytic reaction (Table 1). To obtain the highest possible concentration of protein the reaction may be carried out in the presence of a denaturing solvent such as 8 M-urea. The nature of the buffer does not appear to be important,
although care must be taken to select a buffer that does not contain groups (e.g. amino or thiol) which would react with the anhydride. Complete reaction can be expected to occur with a 20-fold excess of reagent over total amino groups, and the extent of the reaction can be estimated spectrophotometrically from the relative extinction coefficients of the maleylamino group at 250 nm. \((\varepsilon_{250} 3360)\) and 280 nm. \((\varepsilon_{280} 308)\) respectively.

The maleylamino group is completely stable at neutral or alkaline pH, but is readily hydrolysed at acid pH. Although maleyl-proteins are usually insoluble at acid pH the hydrolysis of the maleyl groups occurs with equal facility in aqueous suspension. Alternatively, the unblocking reaction may be carried out in solution in the presence of 5\(\text{m}\)-guanidine hydrochloride. The semi-logarithmic graph in which half-life is plotted against pH (Fig. 2) shows that the rate of hydrolysis of the maleyl group is directly proportional to the \(\text{H}^+\) concentration between pH 6 and pH 4.5, the pH-dependence of the reaction decreases below pH 4.5, and it finally becomes independent of pH between pH 2.5 and pH 2.0, suggesting that the reaction is catalysed by the un-ionized carboxyl group. Possible catalytic mechanisms for this hydrolytic reaction, as well as for the hydrolysis of maleyl ester bonds, are shown in Scheme 1. For the maleyl amide bond it is suggested that the reaction involves an intramolecular attack of the un-ionized carboxyl group, which is locked in the appropriate stereochemical configuration by the double bond, on the carbonyl group of the neighbouring amide bond. At acid pH the maleyl group is thus eliminated as the anhydride and is subsequently hydrolysed to maleic acid. For the maleyl ester bond, on the other hand, the superiority of \(-\text{O}^-\) over \(-\text{NH}^-\) as a leaving group enables the hydrolytic reaction to be catalysed by the ionized carboxyl group, rather than by the un-ionized group as with the maleyl amide bond. Maleyl ester bonds would therefore be unstable at neutral or alkaline pH, in contrast with maleyl amide bonds.

The mechanism that is proposed for the hydrolysis of the maleyl amide bond is essentially the same as that proposed earlier (Bender, 1957; Bender, Chow & Chloupek, 1958) to account for the similar pH-dependence that was observed for the acid-catalysed hydrolysis of phthalamic acid (the monoamide of phthalic acid). In this case hydrolysis of the phthalylamino bond is also catalysed by a neighbouring sterically fixed un-ionized carboxyl group. Bender, Chloupek & Neveu (1958) showed further that methyl hydrogen phthalate was hydrolysed at a much faster rate than methyl benzoate. Moreover, the rate of hydrolysis of the ester bond was independent of pH above pH 4 and decreased as the pH was lowered from pH 4 to pH 2.5. These observations show that the hydrolysis of the phthalyl ester bond is catalysed by the ionized carboxyl group (rather than by the un-ionized carboxyl group as with the phthalylamino bond), and by analogy it is likely that maleyl ester bonds would behave in the same manner. Maleyl derivatives of the hydroxy amino acids and of tyrosine, cysteine and histidine, if formed, would likewise be expected to be labile at neutral or alkaline pH.

The reaction of maleic anhydride with proteins at alkaline pH thus appears to be uniquely specific for amino groups, and this specificity has been clearly demonstrated with MSH. The u.v. spectrum of maleyl-MSH indicates reaction only with the three amino groups (Fig. 5). Digestion with trypsin occurred exclusively at the single arginyl bond and the peptide fragments that were obtained after
digested with trypsin, as well as with chymotrypsin, possessed electrophoretic mobilities and specific staining properties that were entirely consistent with the known amino acid sequence of the molecule (Harris & Roos, 1959) (Fig. 7). Further, the amino groups in these peptides were readily regenerated from their α- and ε-maleylamino derivatives, under conditions (pH 3-5 at 60° for 6 hr.) that did not cause any other detectable change in structure. The tryptic peptides containing regenerated lysine residues were again susceptible to digestion with trypsin.

The maleylamino groups could also be unblocked on paper by exposure to the vapour of pyridine acetate buffer, pH 3-5, and this procedure may thus be used for the purification by ‘diagonal’ paper electrophoresis of N-terminal peptides and of those containing lysine. This is essentially the same as other ‘diagonal’ separations (see e.g. Brown & Hartley, 1966), but in this case removal of a maleyl group results in a net gain of two positive charges and in a decrease of approx. 100 in the molecular weight of the peptide for each lysine residue. It is therefore possible to predict the probable positions in which peptides of different charges and numbers of lysine residues would be found in the ‘diagonal’ electrophoretic ‘map’. Fig. 6 shows that the peptides in the lysine ‘diagonal’ of the chymotrypsin digest of maleyl-MSH do occupy the predicted positions despite the fact that peptides C2 and C3 contain histidine. It should thus be possible to predict the number of lysine residues, the net charge and the approximate molecular weight of any peptide in the ‘diagonal map’.

The effect of maleylation of the lysine residues on the course of the trypsin digestion of carboxymethylated YADH is of particular interest owing to the ‘burst’ reaction that occurs during the first few minutes of digestion. Comparison by gel filtration on Sephadex G-50 of samples removed after 5 min. and after 20 hr. digestion indicated that the rapid digestion occurring in the initial ‘burst’ reaction was due to a highly specific hydrolysis of three or four arginyl bonds (out of a total of eight susceptible arginyl bonds) in the protein chain. A similar effect has been observed with other maleylated proteins, and with glyceraldehyde 3-phosphate dehydrogenase, from pig and lobster muscle, large partially digested fragments containing intact arginyl bonds have been isolated from limited trypptic digests (W. Kenney, M. Sajgô, J. O. Thomas & J. I. Harris, unpublished work). The possibility of isolating intermediate fragments of this type from partial trypptic digests of large proteins aids considerably in determining the order in which arginine residues occur in the primary structure of a protein chain.

This influence of maleylation of the lysine residues on the course of the trypsin hydrolysis of a protein appears to be through a combination of two separate effects. Maleyl-proteins are considerably unfolded in solution and, in spite of the increase in the overall negative charges, a proportion of the trypsin-sensitive arginyl bonds may be more favourably placed for hydrolysis than in the unmaleylated protein. Conversely, other arginyl bonds may find themselves in less favourable environments, possibly due to localized ‘pockets’ of inhibitory negative charges.

Since the preliminary communication of this work (Butler et al. 1967) a number of related acid anhydrides such as tetrafluorosuccinic anhydride (Braunitzer, Beyreuther, Fujiki & Schrank, 1968), 2,3-dimethylmaleic anhydride and 2-methylmaleic
anhidride (citraconic anhydride) (Dixon & Perham, 1968) have also been used for the reversible acylation of amino groups in proteins. The tetrafluorosuccinylaminol bond was shown to be formed at pH 7.0 and to be cleaved at pH 9.5 at 0°, and is stable at low pH, providing another useful addition to the range of acid-stable lysine-protecting groups. The 2,3-dimethylmaleylaminol bond was found to be labile below pH 8 and does not therefore possess the necessary range of stability for general application. The 2-methylmaleylaminol bond has a stability intermediate between that of the dimethylmaleyl-amino bond and the maleylaminol bond. Although it forms two isomeric derivatives with each reacting amino group, these products have a similar stability with an average half-life of 1–2 hr. in 40 mM-hydrochloric acid at 25°. The above authors point out that the conditions for the removal of maleyl groups may be too drastic for some proteins. Although some extremely labile amide groups might undergo hydrolysis under these acid conditions, the greater stability of the maleyl group may be valuable for many chemical studies. Freedman, Grossberg & Pressman (1968) reported that completely maleylated antibodies were unblocked with essentially complete recovery of the number of binding sites and of their affinity for antigen. Further comparison of these various reagents must await the availability of fuller details on the reaction products.

Maleyl anhydride therefore has a variety of different applications in protein chemistry. Maleyl-proteins have an increased negative charge at neutral pH, so that protein–protein interactions are minimized and protein–water interactions are favoured. Insoluble aggregates can therefore be brought into solution in simple salt media, and polymeric proteins can be dissociated into soluble sub-units without the use of urea or guanidine. This greatly facilitates the accurate determination of molecular weights of protein sub-units, as demonstrated with YADH (Butler, 1967) and methionyl-transfer-RNA synthetase from Escherichia coli (Bruton & Hartley, 1968). Since the reaction is specific for amino groups a large excess of the reagent can be used to ensure complete substitution of the protein without any accompanying side reactions. Maleyl-proteins are completely stable under the conditions of tryptic digestion, and the resulting maleyl-peptides may be fractionated without the use of disaggregating solvents (such as 8 M-urea), provided that the pH is not allowed to fall below pH 5. Digestion with trypsin is specific for arginyl bonds and an additional specificity among the susceptible arginyl bonds may be achieved by partial tryptic digestion of the blocked-lysine protein. The protected lysine residues in maleyl-proteins and -peptides may be unblocked under conditions that have proved to be specific for the hydrolysis of maleylamino bonds, other potentially labile residues such as asparagine, glutamine and tryptophan not being affected, and some maleyl-proteins have been shown to regain biological activity after the removal of the blocking group (e.g. see Freedman et al. 1968). Unblocked peptides may be redigested with trypsin to obtain the lysine-containing tryptic peptides that occur between any two consecutive arginine residues in the protein chain, or submitted to an alternative type of digestion to yield peptides with internal lysine residues that can be purified by ‘diagonal’ paper electrophoresis.

This is an impressive list of qualities, but we believe that the principle of intramolecular catalysis is capable of much wider exploitation in the design of other reagents for protein chemistry.

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