Hydrolysis of *p*-*NN'*-Phenylenebismaleimide and its Adducts with Cysteine

IMPLICATIONS FOR CROSS-LINKING OF PROTEINS

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To understand the extent of the cross-linking of proteins by the bifunctional reagent *p*-*NN'*-phenylenebismaleimide, a quantitative study of competing reactions has been undertaken. The two reactive maleimide rings of the bismaleimide are hydrolysed in mildly alkaline aqueous solutions much more rapidly than is the single maleimide ring of the monofunctional analogue *N*-ethylmaleimide. The kinetics of hydrolysis are second-order, depending on both imide and hydroxyl ion concentration in the pH range 8–10. The hydrolysis of the first imide ring of the bismaleimide is more rapid than the second, with second-order rate constants of $1600\text{ M}^{-1}\cdot\text{s}^{-1}$ and $500\text{ M}^{-1}\cdot\text{s}^{-1}$ respectively, at 25°C . The half-times for hydrolysis of the first and second imide rings at pH 9.0 are therefore only 43 s and 140 s. Because it renders the maleimide ring unreactive towards cysteine, this rapid hydrolysis can limit the extent of cross-linking of proteins by the bismaleimide.

N-Ethylmaleimide has been widely used as an alkylating agent for cysteine residues in proteins. More recently a bifunctional analogue of *N*-ethylmaleimide, *p*-*NN'*-phenylenebismaleimide, has been used as a specific cross-linking reagent for cysteine residues. For example, it has been used to cross-link cysteine residues of adjacent proteins in ribosomal subunits (Lutter *et al.*, 1972; Chang & Flaks, 1972) and to show the proximity of two cysteine residues within the myosin molecule (Reisler *et al.*, 1974).

Gregory (1955) showed that *N*-ethylmaleimide (I) underwent alkaline hydrolysis in aqueous solutions, and that the product, *N*-ethylmaleamic acid (II), did not react with cysteine (Scheme 1). At neutral pH the half-time for hydrolysis of *N*-ethylmaleimide (about 45 h) is much longer than typical reaction times with cysteine residues, so that hydrolysis can be neglected. Hydrolysis might, however, be a much more significant effect in understanding the reaction of *p*-*NN'*-phenylenebismaleimide with proteins, since hydrolysis of the reagent after it is monofunctionally bound to protein might compete with cross-linking, which can be a slow process. In the

present paper the rate of hydrolysis of *p*-*NN'*-phenylenebismaleimide both free and after reaction with cysteine has been measured. It is found that *p*-*NN'*-phenylenebismaleimide is hydrolysed at a much greater rate than *N*-ethylmaleimide and the rate is unaffected by prior reaction with cysteine. Hydrolysis is therefore significant in the analysis of the cross-linking reaction of *p*-*NN'*-phenylenebismaleimide with proteins.

Methods

The kinetics of hydroxyl ion uptake by *p*-*NN'*-phenylenebismaleimide at various pH values were followed by using a Radiometer pH-stat at 25°C , with 10 mM-NaOH as titrant in an N_2 atmosphere. The pH of 5 ml of 20 mM-KCl was adjusted by using the titrator and the reaction was started by the addition of $50\text{ }\mu\text{l}$ of 7.5 mM-*p*-*NN'*-phenylenebismaleimide in *NN*-dimethylformamide from a Hamilton syringe.

Spectra of *p*-*NN'*-phenylenebismaleimide and its derivatives were recorded on a Cary 15 spectrophotometer with 1 cm-path-length stoppered cells. The time course of production of absorbing species was observed at 25°C with the wavelength set at the λ_{max} of the expected spectrum, and with 0.1 M-sodium borate buffers to maintain the pH. *p*-*NN'*-Phenylenebismaleimide in dimethylformamide, and cysteine, were kept as concentrated stock solutions on ice. Cysteine solutions were freshly prepared from the hydrochloride, dissolved with EDTA in water and adjusted to pH 6.0. Stock solutions nominally 10 mM in both cysteine and EDTA were standardized by

Scheme 1.

reaction with dithiobis-(2-nitrobenzoate) (Ellman, 1959) before use.

p-*NN'*-Phenylenebismaleimide (Aldrich, Milwaukee, WI, U.S.A.) was recrystallized from dimethylformamide/ethanol (4:1, v/v) before use. *p*-*NN'*-Phenylenebismaleamic acid was synthesized by the method of Ried & Keller (1956). *p*-Phenylenediamine (10g) and 20g of maleic anhydride, both dissolved in acetic acid, were mixed at 23°C. After 2h the orange precipitate was collected by filtration, washed with acetic acid, and then ethanol and dried under vacuum. Dimethylformamide (Uvasol grade) was from Merck A.G., Darmstadt, W. Germany. Dimethylformamide (special-for-spectroscopy grade) from BDH Chemicals, Poole, Dorset, U.K., was found to convert *p*-*NN'*-phenylenebismaleimide into a species unreactive towards cysteine. Other chemicals were reagent grade or the best available.

The kinetic data were analysed on the basis of an irreversible two-step consecutive reaction scheme of the type $A \rightarrow B \rightarrow C$, since this was the simplest scheme that could be expected to fit the hydrolysis of a bisimide. In this scheme only three species are considered: unmodified, singly hydrolysed and doubly hydrolysed *p*-*NN'*-phenylenebismaleimide (Scheme 2). They are designated A, B and C respectively, and their respective molar concentrations at any time, t , are a , b and c . At constant pH, the rate of the hydrolytic step depends only on the concentrations of A or B and hence the two rate constants

k_1 and k_2 are pseudo-first-order constants. If the molar concentration of A at zero time is a_0 , then the concentrations of A, B and C at time t are given by:

$$a = a_0 \cdot \exp(-k_1 t) \quad (1)$$

$$b = \frac{a_0 k_1}{(k_2 - k_1)} \cdot [\exp(-k_1 t) - \exp(-k_2 t)] \quad (2)$$

$$c = a_0 - \frac{a_0 k_2}{(k_2 - k_1)} \cdot \exp(-k_1 t) + \frac{a_0 k_1}{(k_2 - k_1)} \cdot \exp(-k_2 t) \quad (3)$$

Since each hydrolysis reaction consumes a hydroxyl ion, the total uptake of hydroxyl ions measured in the pH-stat at any time during the reaction is $(b+2c)$, which from eqns. (2) and (3) yields:

$$\text{OH}^- \text{ uptake at time } t = 2a_0 + a_0 \frac{(k_1 - 2k_2)}{(k_2 - k_1)} \cdot \exp(-k_1 t) + a_0 \frac{k_1}{(k_2 - k_1)} \cdot \exp(-k_2 t) \quad (4)$$

In the case where there is no interaction between the two imide rings of *p*-*NN'*-phenylenebismaleimide, $k_1 = 2k_2$, and eqn. (4) reduces to:

$$\text{OH}^- \text{ uptake at time } t = 2a_0[1 - \exp(-k_2 t)] \quad (5)$$

If, however, $k_1 > 2k_2$, then both terms of eqn. (4) contribute, but the first exponential term is significant only in the early phase of the reaction, so that for the latter part of the reaction:

$$\ln(\text{Total OH}^- \text{ uptake} - \text{OH}^- \text{ uptake at time } t) = -k_2 t + \ln \left(\frac{a_0 k_1}{k_1 - k_2} \right) \quad (6)$$

This permits the evaluation of both k_1 and k_2 from the ordinate intercept and gradient of a graph of $\ln(\text{total OH}^- \text{ uptake} - \text{OH}^- \text{ uptake at time } t)$ versus time.

Although k_1 and k_2 are pseudo-first-order rate constants, the ratio of k_1 and k_2 should be invariant with pH and hence the value of the intercept of the graph should not vary with pH.

An essentially similar treatment can be given to the spectrophotometric data. So long as Beer's law is obeyed:

$$\text{Absorbance per cm at a time } t = \epsilon_A a + \epsilon_B b + \epsilon_C c \quad (7)$$

where ϵ_A , ϵ_B and ϵ_C are the molar extinction coefficients of species A, B and C respectively, at a given wavelength. In the range of wavelengths studied here, ϵ_A was found to be essentially zero. Designating $Q = \epsilon_C/\epsilon_B$, then from eqns. (2) and (3) it follows that:

$$\text{Absorbance at time } t = a_0 \epsilon_B \left[Q - \frac{(Qk_2 - k_1)}{(k_2 - k_1)} \cdot \exp(-k_1 t) - \frac{k_1(1 - Q)}{(k_2 - k_1)} \cdot \exp(-k_2 t) \right] \quad (8)$$

Scheme 2.

As discussed above, if $k_1 > 2k_2$ a linear relation will be observed for:

$$\ln(\text{Final absorbance} - \text{absorbance at time } t) = -k_2 t + \ln \left[\frac{\text{final absorbance} \times k_1(1 - Q)}{(k_2 - k_1)Q} \right]$$

Thus k_2 can be evaluated from the gradient of the graph of $\ln(\text{final absorbance} - \text{absorbance at time } t)$ versus time. k_1 cannot be directly determined, since Q is unknown.

Results

pH-stat studies of the hydrolysis of *p*-*NN'*-phenylenebismaleimide

A hydroxyl ion uptake with a stoichiometry close to 2mol of hydroxyl ion/mol of *p*-*NN'*-phenylenebismaleimide was observed when *p*-*NN'*-phenylenebismaleimide was mixed with water. At pH9.0 and 25°C the reaction was nearly complete after 10min. Fig. 1 shows that the time course of hydroxyl ion uptake conforms to the expectations of the two-step hydrolysis scheme described in the Methods section. From the gradient and intercept on the ordinate and by using eqn. (6), the pseudo-first-order rate constants

Fig. 1. Hydroxyl ion uptake during hydrolysis of *p*-*NN'*-phenylenebismaleimide

p-*NN'*-Phenylenebismaleimide (380nmol) was added to 5ml of 20mM-KCl at 25°C and maintained at pH 9.0 with a pH-stat. Hydroxyl ion uptake was analysed by eqn. (6).

for the hydrolysis of the first and second imide rings, k_1 and k_2 , were estimated to be 0.018 s^{-1} and 0.0057 s^{-1} respectively.

Earlier studies on the hydrolysis of imides have shown that the reaction rate depends on the concentration of hydroxyl ion (see Hargreaves *et al.*, 1970). The second-order rate constants, k'_1 and k'_2 , are given by $k_1/[\text{OH}^-]$ and $k_2/[\text{OH}^-]$.

Data were gathered in the pH-stat over the range pH8.8–9.5 and k'_2 was obtained by the graphical method. Values of k'_2 calculated at each pH showed no trend with pH, confirming the dependence of hydrolysis on hydroxyl ion concentration. The mean value of k'_2 was $530\text{ M}^{-1}\cdot\text{s}^{-1}$ (range $440\text{--}550\text{ M}^{-1}\cdot\text{s}^{-1}$) at 25°C. As expected, the ratio of pseudo-first-order rate constants k_1 and k_2 did not vary with pH and gave a mean value of 3.04 (range 2.84–3.18). Thus the second-order rate constant, k'_1 , for the first step was $1600\text{ M}^{-1}\cdot\text{s}^{-1}$.

The departure of the data early in the time course from the line extrapolated from the data of the latter part of Fig. 1 confirmed that k_1 is significantly greater than $2k_2$. Therefore the hydrolysis of the two imide groups of each *p*-*NN'*-phenylenebismaleimide molecule does not occur independently, hydrolysis of the first imide group slightly retarding hydrolysis of the second.

Spectrophotometric studies of the hydrolysis of *p*-*NN'*-phenylenebismaleimide

At pH7.0 an aqueous solution of $50\mu\text{M}$ -*p*-*NN'*-phenylenebismaleimide at 25°C did not absorb appreciably in the range 240–450nm, and no absorbance developed after 15min. However, after incubation at pH9.0 a species exhibiting strong absorbance centered at 298nm was formed. This observation suggested that alkaline hydrolysis of *p*-*NN'*-phenylenebismaleimide, which is nearly complete after 10min at pH9.0, but 100-fold slower at pH7.0, had produced a strong chromophore. At pH8.0 the development of absorbance was slower than at pH9.0 and allowed a series of spectra to be recorded during the course of the reaction (Fig. 2). The spectra at increasing time intervals showed that the wavelength of the absorption maximum increased with time, suggesting the existence of an intermediate species with a different absorption spectrum from the final product. These observations are readily correlated with the two-step hydrolysis of *p*-*NN'*-phenylenebismaleimide, the intermediate absorbing species being the half-hydrolysed form of the reagent.

To confirm these correlations, the expected product of the hydrolysis of *p*-*NN'*-phenylenebismaleimide, *p*-*NN'*-phenylenebismaleamic acid (Scheme 2, C), was synthesized. The absorption spectrum of this compound in 0.1 M-sodium borate, pH9.0, was indeed indistinguishable from that of the chromophore finally present after incubation of *p*-*NN'*-phenylene-

Fig. 2. Absorption spectra recorded during hydrolysis of *p*-*NN'*-phenylenebismaleimide ($42\text{ }\mu\text{M}$) was incubated in 0.1 M -sodium borate/HCl, pH 8.0, at 25°C . The number beside each line is the time of incubation in minutes. The zero-time spectrum was recorded at pH 7.0 (see the text).

bismaleimide in the same medium. Values for the absorption coefficient of *p*-*NN'*-phenylenebismaleamic acid are given in Table 1.

The time course of the development of absorbance at 25°C was followed to provide a second estimate of the rate constant for hydrolysis of *p*-*NN'*-phenylenebismaleimide. The variation of the time course over the pH range 8–10 was also examined. When plotted according to eqn. (9) the time courses gave a curve very similar to that obtained by using the pH-stat to follow the hydrolysis. k_2 , estimated from the gradient of the linear latter part of the plot, was $4.3 \times 10^{-3}\text{ s}^{-1}$ at pH 9.0, which compares well with the value of $5.7 \times 10^{-3}\text{ s}^{-1}$ obtained at the same pH by measurement of hydroxyl ion uptake. In addition, the second-order rate constant, $k'_2 (=k_2/[\text{OH}^-])$ did not vary with pH yielding a mean value of $460\text{ M}^{-1}\text{ s}^{-1}$ (range $430\text{--}570\text{ M}^{-1}\text{ s}^{-1}$), in good agreement with the value ($530\text{ M}^{-1}\text{ s}^{-1}$) obtained by hydroxyl ion-uptake measurements.

Scheme 3.

pH-stat studies of the hydrolysis of the biscysteine adduct of p-NN'-phenylenebismaleimide

When *p*-*NN'*-phenylenebismaleimide was added to excess cysteine in the pH-stat at pH 9.0, there was a rapid rise in pH due to proton consumption during the addition reaction of the cysteine anion and the maleimide moieties of *p*-*NN'*-phenylenebismaleimide (Scheme 3). This was followed by proton liberation, thus providing evidence for hydrolysis of the imide rings of the biscysteine adduct of the bismaleimide. However, part of the proton liberation merely neutralized the initial proton consumption, so that the pH-stat could not be used to follow the kinetics of the hydrolysis of the adduct.

Spectrophotometric studies of the hydrolysis of the biscysteine adduct of p-NN'-phenylenebismaleimide

When *p*-*NN'*-phenylenebismaleimide was added to an excess of cysteine at pH 8–10, the biscysteine adduct that rapidly formed did not show appreciable absorption in the range 240–450 nm, but on standing a strong absorbance centered about 260 nm developed. By analogy with *p*-*NN'*-phenylenebismaleimide it is reasonable to suppose that the development of absorbance was caused by breakage of the imide rings.

Breakage of the imide ring of the cysteine adduct of *N*-ethylmaleimide, a monofunctional analogue of

Scheme 4.

p-*NN'*-phenylenebismaleimide, has been shown to occur by two distinct reactions (Smyth *et al.*, 1960). At pH9.0 there is a nearly quantitative conversion into 2-(*N*-ethylcarbamoylmethyl)-3-oxo-2*H*,4*H*,5*H*,6*H*-1,4-thiazine-5-carboxylic acid (IV) by intramolecular aminolysis (Scheme 4). At higher pH, the product of partial hydrolysis, *S*-[(1-carboxy-2-*N*-ethylcarbamoyl)ethyl]cysteine (III), was produced. By analogy, incubation of the biscysteine adduct of *p*-*NN'*-phenylenebismaleimide at mildly alkaline pH might produce a mixture of hydrolytic and aminolytic products.

For the analysis of the spectrophotometric data it was important to establish whether or not both the aminolytic and hydrolytic reactions occurred, and whether both products had the same absorption spectrum. The analysis which follows shows that both reactions do occur and that aminolysis is the cause of the majority of the ring breakage at pH8.0. We found, however, that the absorption spectrum of the mixture of hydrolytic and aminolytic products formed at pH8.0 was indistinguishable from that produced by the exclusively hydrolytic product of the bis-*N*-acetylcysteine adduct of *p*-*NN'*-phenylenebismaleimide (see Table 1). Thus the absorption spectra of hydrolytic and aminolytic products are very similar.

The time course of production of absorbance by the biscysteine adduct was followed at 25°C at the wavelength of the peak of the final absorption spectrum, 263nm, at pH8.0, 9.0 and 10.0. When analysed on the basis of the two-step hydrolytic scheme (eqn. 9), the form of the plots was very similar to Fig. 1. The rate constants, k_2 , measured at each pH were respectively 0.0043, 0.021 and 0.076 s⁻¹. These values do not differ by as much as the factor of 10 per pH unit that is expected for the hydrolytic reaction (see under 'pH-stat studies of the hydrolysis of *p*-*NN'*-phenylenebismaleimide'), suggesting that the aminolytic reaction, which has a lesser dependence on pH, is a significant source of imide ring breakage.

The reactive nucleophile in the aminolytic reaction is the free base form of the α -amino group. Hence the first-order rate constant for aminolysis at any pH is proportional to the fraction of the α -amino group

Table 1. Spectral data for the hydrolytic products of *p*-*NN'*-phenylenebismaleimide and its bis-*N*-acetylcysteine adduct at pH8.0

λ (nm)	Absorption coefficient (ϵ) (mm ⁻¹ ·cm ⁻¹)	
	<i>p</i> - <i>NN'</i> -Phenylenebismaleamic acid	Hydrolysed bis- <i>N</i> -acetylcysteine adduct of <i>p</i> - <i>NN'</i> -phenylenebismaleimide
251	10	17
263	12	22*
280	16	15
290	17	7
298	18*	2.8
310	16	0.0
320	14	0.0
350	4	0.0

* $\epsilon_{\max}$.

present as free base at that pH. The fraction can be calculated if the pK of the α -amino group is known. Smyth *et al.* (1960) have determined the pK in the adduct to be 8.8. The fractions of free base at pH8, 9 and 10 are therefore 0.138, 0.614 and 0.941 respectively.

Defining k as the rate constant for aminolysis by the free base, and xk as the rate constant for hydrolysis at pH8.0, we have: (1) rate constant for ring breakage by hydrolysis and aminolysis at pH10 = $76 \times 10^{-3} = 100xk + 0.941k$; (2) rate constant for ring breakage by hydrolysis and aminolysis at pH8 = $4.3 \times 10^{-3} = xk + 0.138k$. From these equations x is evaluated as 0.0182, k as 0.0276 s⁻¹ and xk as 5.01×10^{-4} s⁻¹.

The validity of the assumptions on which this analysis is based can be examined by using these values of k and xk to predict the rate constant for ring breakage at pH9.0. This value can then be compared with the observed value of 2.1×10^{-2} s⁻¹. The predicted value is:

$$(0.614 \times 2.76 \times 10^{-2}) + (10 \times 5.01 \times 10^{-4}) \text{ s}^{-1} \\ = 2.2 \times 10^{-2} \text{ s}^{-1}$$

The close similarity of the two values gives grounds for confidence in the validity of this analysis.

The second-order rate constant, k'_2 , for hydrolysis of the second imide ring of the biscysteine adduct of *p*-*NN'*-phenylenebismaleimide is therefore 500 M⁻¹·s⁻¹. This is similar to the values for k'_2 of 530 M⁻¹·s⁻¹ and 460 M⁻¹·s⁻¹ for *p*-*NN'*-phenylenebismaleimide itself. The addition of cysteine to *p*-*NN'*-phenylenebismaleimide does not therefore change the rate of hydrolysis of the imide ring.

The analysis shows that the aminolytic reaction was faster than hydrolysis at pH8.0, about 88% of the ring breakage resulting from this reaction.

Effect of hydrolysis of p-NN'-phenylenebismaleimide on its ability to react with cysteine

From Table 1, it is apparent that there are distinct differences between the spectra of *p-NN'*-phenylenebismaleamic acid and of its biscysteine adduct. Thus if cysteine were able to react with *p*-phenylenebismaleamic acid there should be a change in the absorption spectrum.

When excess cysteine was added after complete hydrolysis of *p-NN'*-phenylenebismaleimide at 25°C and pH 9.0, no change in the A_{263} was detected over a period of 15 min, indicating that cysteine did not react with hydrolysed *p-NN'*-phenylenebismaleimide. In support of this conclusion, it was found that, when excess cysteine was added partway through the hydrolysis of *p-NN'*-phenylenebismaleimide, the spectrum that was finally produced showed an absorbance maximum intermediate between those described above. Furthermore, the earlier that cysteine was added, the lower the wavelength of the absorbance maximum. Similar spectra were produced when less than 1 mol of cysteine/mol of maleimide group was present at the beginning of the reaction.

Discussion

The present study has shown that *p-NN'*-phenylenebismaleimide, like other imides (Hargreaves *et al.*, 1970), is hydrolysed in alkaline conditions. The reaction is kinetically of the second-order, depending on the concentrations of both imide and hydroxyl ion over the pH range 8–10. The hydrolysis of the two imide rings of each *p-NN'*-phenylenebismaleimide molecule can be kinetically distinguished: the second is the slower. Both second-order rate constants (1600 and 500 M⁻¹·s⁻¹ at 25°C) are much higher than the corresponding value for *N*-ethylmaleimide (approx. 30 M⁻¹·s⁻¹; from Gregory, 1955). This difference is similar to that between the second-order hydrolytic rate constants of *N*-phenyl- and *N*-ethylsuccinimides (5.45 and 0.017 M⁻¹·s⁻¹ respectively), where it has been ascribed to the electron-withdrawing properties of the benzene ring (Hargreaves *et al.*, 1970). The hydrolysis of one imide ring of *p-NN'*-phenylenebismaleimide would produce a modification of electron distribution around the benzene ring. This may be the origin of the lower rate of hydrolysis of the second ring.

The maleamic acids produced by hydrolysis of *p-NN'*-phenylenebismaleimide exhibit strong absorbance in the region 250–320 nm, where the parent compound absorbs only weakly. In this respect *p-NN'*-phenylenebismaleimide is similar to *N*-phenylsuccinimide (Herd *et al.*, 1966).

The addition reaction between cysteine and *p-NN'*-phenylenebismaleimide is much more rapid than

hydrolysis of the reagent at pH 8.0, and this hydrolysis prevents the addition reaction; in this respect, too, *p-NN'*-phenylenebismaleimide is similar to *N*-ethylmaleimide (Gregory, 1955). The cysteine adduct of *p-NN'*-phenylenebismaleimide was hydrolysed to the corresponding succinamic acid derivative with rate constants similar to those of *p-NN'*-phenylenebismaleimide. The hydrolysis, like that of *p-NN'*-phenylenebismaleimide, results in a large increase in absorbance in the range 250–300 nm, although the absorbance maximum is shifted from 298 to 263 nm and is more intense (Table 1). Thus the hydrolysis of *p-NN'*-phenylenebismaleimide will change the absorption spectrum of the protein with which the reagent has reacted. The magnitude and position of the change will depend, not only on the number of molecules of *p-NN'*-phenylenebismaleimide bound to each protein molecule, but also on the relative amounts of monofunctionally and bifunctionally attached molecules.

Aminolysis of the cysteine adduct of *p-NN'*-phenylenebismaleimide by the α -amino group of the cysteine is a rapid reaction with a rate constant of $3.8 \times 10^{-3} \text{ s}^{-1}$ compared with $0.5 \times 10^{-3} \text{ s}^{-1}$ for hydrolysis at pH 8.0. Though this aminolytic reaction is feasible only for *N*-terminal cysteine in polypeptides, it shows that the succinimide ring is susceptible to attack by amines, and it is therefore possible that a cross-link could be formed between a cysteine residue and an adjacent lysine residue by their reaction with a single maleimide moiety of *p-NN'*-phenylenebismaleimide. Cross-links of this type have actually been found after the reaction of *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide with bovine serum albumin (Witter & Tuppy, 1960).

The cross-linking of amino acid residues in proteins often proceeds more slowly than the reaction of the reagent with model compounds in solution, either because of steric hindrance to reaction or because of changed ionization of the residues in the local environment on the protein. In addition, the high local concentration of reagent around the site of initial reaction of a bifunctional reagent with protein can induce a significant extent of reaction with a species of amino acid that would be unreactive in dilute solution. In all cases the rate of hydrolysis of the reagent can then become a significant factor in determining the extent of cross-link formation. Such a situation has been encountered in the reaction of *p-NN'*-phenylenebismaleimide with F-actin. Here the reagent reacts quickly at a cysteine residue in every subunit and forms cross-links by a slower, subsequent reaction with a lysine residue on a neighbouring subunit. Hydrolysis of the unreacted maleimide moiety of the actin-bound reagent appears to be the major reaction that limits the formation of cross-links (Knight & Offer, 1978).

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