Biochem. J. (1978) **175**, 1023–1032
Printed in Great Britain

p-NN'-Phenylenebismaleimide, a Specific Cross-Linking Agent for F-Actin

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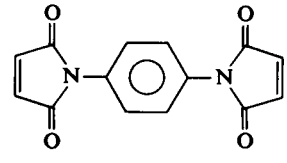
(*Received* 4 *May* 1978)

Covalent cross-links can be inserted between the subunits of F-actin by using *p-NN'*-phenylenebismaleimide. Cross-linking reaches its maximum value when one molecule of reagent has reacted with each actin subunit. *p-NN'*-Phenylenebismaleimide reacts initially with a cysteine residue on one subunit, the slower cross-linking reaction involving a lysine residue on a neighbouring subunit. Hydrolysis of the actin-bound reagent limits the extent of cross-linking. Quantitative analysis of the amounts of cross-linked oligomers seen on polyacrylamide gels containing sodium dodecyl sulphate suggests that neither the binding of the reagent to actin nor the formation of cross-links introduces strain into the structure. The cross-links do not join together different F-actin filaments, and evidence is presented that suggests that the cross-links join subunits of the same long-pitched helix.

Actin plays a central role in muscular contraction. The interaction of actin with myosin generates the force required for the sliding of filaments, and the regulation of the interaction by tropomyosin and troponin is mediated through their action on actin (Weber & Murray, 1973). There has therefore been much interest in the structure of actin, and now that it has been crystallized either alone (Oriol *et al.*, 1977) or as a complex with another protein (Mannherz *et al.*, 1977) the three-dimensional structure of the monomer may be available soon. However, even when this information is available, the way in which the subunits make contacts with each other in the helical polymer (F-actin) will not be known, nor will it be known whether changes in the contact regions occur when actin interacts with myosin and the regulatory proteins.

For these reasons we have studied the effect of inserting covalent cross-links between the subunits of F-actin. Cross-linking reagents are useful tools for investigating the spatial arrangement of multi-subunit proteins (see, e.g., Lad & Hammes, 1974; Wang & Richards, 1974). Not only can they be used to find out what amino acid residues in neighbouring subunits lie close to one another, but the rate of the cross-linking reaction can be used as a sensitive indicator of small conformational changes. Furthermore, cross-linking reagents may be used to constrain polymers in a particular quaternary structure (Benesch *et al.*, 1975) so that it can be decided whether changes at the contact areas between subunits are essential for their activity. We have applied all these approaches to the study of actin. We were particularly concerned to find a reagent that would insert a single cross-link between neighbouring actin subunits with no other modification. In this paper we show that *p-NN'*-phenylenebismaleimide (Kovacic & Hein, 1959) is such a reagent and we describe the preparation and characterization of cross-linked actin.



p-NN'-Phenylenebismaleimide

Abbreviation used: SDS, sodium dodecyl sulphate.

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Experimental

Actin preparation and cross-linking with p-NN'-phenylenebismaleimide

G-actin was prepared by the methods of either Spudich & Watt (1971) or Hitchcock (1973) from an acetone-dried powder of rabbit skeletal muscle (Straub, 1942; Katz & Hall, 1963). The G-actin was polymerized by dialysis overnight into a cross-linking medium, which usually contained 0.1M-KCl, 1.0mM-$MgCl_2$, 0.2mM-ATP and 2.5mM-disodium tetraborate (borax), usually at pH9.5 and 4°C. The F-actin formed, at a concentration of 2mg/ml, was then cross-linked by the addition of 0.01vol. of a 2–6mg/ml solution of *p-NN'*-phenylenebismaleimide

in dimethylformamide. *p-NN'*-Phenylenebismaleimide (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) was recrystallized from dimethylformamide by the addition of 20% (v/v) ethanol. Dimethylformamide was Uvasol grade from Merck A.G., Darmstadt, West Germany. (Dimethylformamide from BDH Chemicals, Poole, Dorset, U.K. was found to render *p-NN'*-phenylenebismaleimide unreactive towards cysteine.) The reaction of *p-NN'*-phenylenebismaleimide with actin was quenched, when required, by a 20–500-fold molar excess of 2-mercaptoethanol over maleimide groups.

Determination of actin concentration

Because *p-NN'*-phenylenebismaleimide modifies the u.v.-absorption spectrum of actin (Knight, 1976), spectrophotometry cannot be used to estimate actin concentrations; instead the biuret method (Gornall *et al.*, 1949) was used, standardized with unmodified G-actin, for which the concentration was determined by using a specific absorption coefficient of 0.63 litre·g^{-1}·cm^{-1} at 290nm (Lehrer & Kerwar, 1972). The molecular weight of actin was taken as 42000 (Collins & Elzinga, 1975).

Amino acid analyses

Hydrolysis and analysis were performed essentially as described by Moore & Stein (1963). Actin (4mg) was hydrolysed in 2ml of 6M-HCl (Aristar grade, BDH) in evacuated tubes at 110°C for 85h. Before the tubes were sealed, the contents were thoroughly degassed by three cycles of freezing and thawing, to maximize yields of *S*-(1,2-dicarboxyethyl)cysteine (Smyth *et al.*, 1964). After drying down under vacuum at 40°C, the hydrolysates were redissolved and analyses performed at 52.5°C on a Beckman 120B amino acid analyser by using a 50cm×0.9cm column of resin 50B, and a flow rate of 40ml/h. The elution buffer was 0.2M-sodium citrate, pH3.25, followed by 0.2M-sodium citrate, pH4.25.

Quantification of the modified amino acids in hydrolysates of actin cross-linked by *p-NN'*-phenylenebismaleimide is inaccurate, because most other amino acids are present in comparatively large amounts and are therefore imprecisely recorded under the loading conditions used to observe the modified species. A ninhydrin colour value of 1.09 relative to aspartic acid was taken for *S*-(1,2-dicarboxyethyl)-cysteine (Smyth *et al.*, 1964), 0.96 relative to leucine for N^{ε}-(1,2-dicarboxyethyl)lysine (Brewer & Riehm, 1967) and 0.85 relative to leucine for glutamic acid. Aspartic acid and glutamic acid respectively were used as references, and 34mol of Asx and 39mol of Glx per mol of actin were assumed (Collins & Elzinga, 1975).

Determination of cysteine content

Thiol groups in actin were determined with 5,5'-dithiobis-(2-nitrobenzoate) (Ellman, 1959), by using 1% SDS at 22°C to denature the protein and expose all its cysteine residues. The absorption coefficient (13.6mM^{-1}·cm^{-1}; Ellman, 1959) of the thionitrobenzoate anion at 412nm was not affected by 1% SDS. For the reaction, 2.4ml of 1.3% SDS, 0.2ml of 0.4M-Tris/0.35M-Tris/HCl/15mM-disodium EDTA and 0.1ml of 20mM-5,5'-dithiobis-(2-nitrobenzoate)/50mM-K_2HPO_4 were mixed in each of two cuvettes and any small difference in A_{412} was recorded. Then 0.5ml of actin (dialysed free of 2-mercaptoethanol) was added to one cuvette, 0.5ml of dialysis medium to the other, and the maximum difference in A_{412} measured. Full colour development required about 5min incubation and was stable for at least 30min.

SDS/polyacrylamide-gel electrophoresis

The method of Weber & Osborn (1969) was used, with the modifications of technique described by Offer *et al.* (1973). The density of bands was measured by using the scanning attachment of the Pye–Unicam SP.1800 spectrophotometer at a wavelength in the range 540–600nm. Relative peak areas were measured by planimetry. The apparent chain weights of the cross-linked oligomers were obtained by calibration with myosin, C-protein and α-actinin, assuming chain weights of 200000, 140000 and 90000 respectively (Offer *et al.*, 1973).

Electron microscopy

Negative staining with unbuffered 1 or 2% uranyl acetate was performed as described by Huxley (1963) by using a carbon support film on copper grids. Grids were examined in a Philips EM200 instrument operated at 80kV.

Results

Extent of the cross-linking reaction

SDS dissociates F-actin completely into its constituent polypeptide chains (mol.wt. 42000), which migrate as a single zone during SDS/polyacrylamide-gel electrophoresis. When F-actin was incubated with *p-NN'*-phenylenebismaleimide at slightly alkaline pH, new zones were present in SDS/polyacrylamide gels of the product, corresponding in mobility to dimers and trimers after short times of reaction and to these and higher oligomers after extended times (Fig. 1). This shows that the reagent introduces covalent links between the subunits of F-actin. However, monomeric actin can still be seen in the SDS/polyacrylamide gels even after long times, so the reaction evidently does not go to completion. The mass fraction of each species in the cross-linked

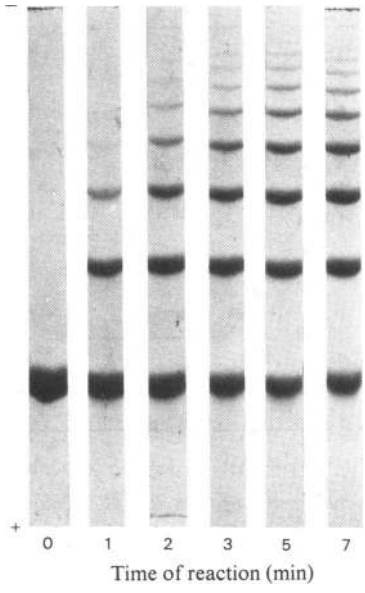


Fig. 1. *Electrophoresis on 4% polyacrylamide/SDS gels showing the time course of cross-linking of F-actin by p-NN'-phenylenebismaleimide*

F-actin at 2mg/ml in 0.1M-KCl/1mM-$MgCl_2$/2.5mM-sodium borate/0.2mM-ATP, pH9.0, at 25°C was made to react with *p-NN'*-phenylenebismaleimide (molar ratio of *P-NN'*-phenylenebismaleimide:actin 2:1) for the times shown. The reaction was quenched with a 150-fold molar excess of 2-mercaptoethanol over maleimide groups. Then 16μg of actin was loaded on each gel.

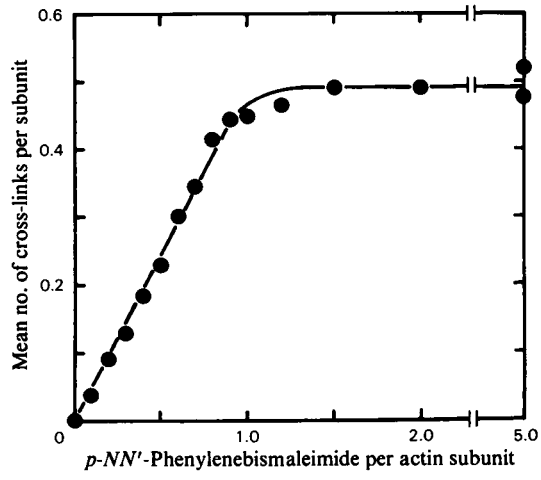


Fig. 2. *Stoicheiometry of the reaction of p-NN'-phenylenebismaleimide with F-actin*

F-actin (2mg/ml) was made to react with a range of concentrations of *p-NN'*-phenylenebismaleimide for 1h in the same solvent as in Fig. 1 but at pH9.1 and 30°C. Relative peak areas from densitometry of SDS/polyacrylamide gels were used to compute the mean number of cross-links per actin subunit.

mixture was obtained by densitometry of the gels. The extent of cross-linking was then estimated as follows: an n-mer has $(n-1)$ cross-links and therefore $(n-1)/n$ cross-links per subunit. The product of the mass fraction of each n-mer in the sample and $(n-1)/n$ then gives the contribution of each species to the cross-linking of the whole sample, so that the mean number of cross-links per actin subunit, p, is given by:

$$p = \sum_{n=1}^{n=\infty} \left(\text{mass fraction } n\text{-mer} \times \frac{(n-1)}{n} \right) \qquad (1)$$

Since F-actin is a very long polymer, p would be 1 for complete cross-linking.

The stoicheiometry of reaction of *p-NN'*-phenylenebismaleimide with F-actin was determined by incubating F-actin with between 0.1 and 5.0 molecules of *p-NN'*-phenylenebismaleimide per actin subunit and analysing the products on 4% polyacrylamide/SDS gels. Fig. 2 shows that with less than one molecule of the reagent per subunit the number of cross-links was proportional to the amount of added reagent, showing that all the added bismaleimide was bound to actin and equally able to form cross-links. The maximum amount of cross-links (0.5 under these conditions) was obtained at about 1 molecule of *p-NN'*-phenylenebismaleimide per subunit, showing that the reagent reacts initially at a single site on each subunit to produce cross-links.

In an effort to produce a product in which all the subunits were cross-linked, the effects of pH and temperature on the extent of cross-linking were examined. Changing the pH had only a small effect on the extent of the reaction. For instance, with excess *p-NN'*-phenylenebismaleimide at 23°C and either pH9.0 or 9.5 the mean number of cross-links per subunit was 0.48. Fig. 3 shows that changing the temperature of the cross-linking reaction had a greater effect; a greater extent of cross-linking was achieved at higher temperatures. However, it has not proved possible to insert more than a mean of 0.5 cross-link per actin subunit by treating F-actin with *p-NN'*-phenylenebismaleimide.

Nature of the cross-links formed

The following experiments show that *p-NN'*-phenylenebismaleimide forms cross-links between subunits of the same filament, and not between different filaments. (i) When F-actin reacted at various protein concentrations from 0.1 to 6.4mg/ml with an excess of *p-NN'*-phenylenebismaleimide, the extent of cross-linking was constant, showing that

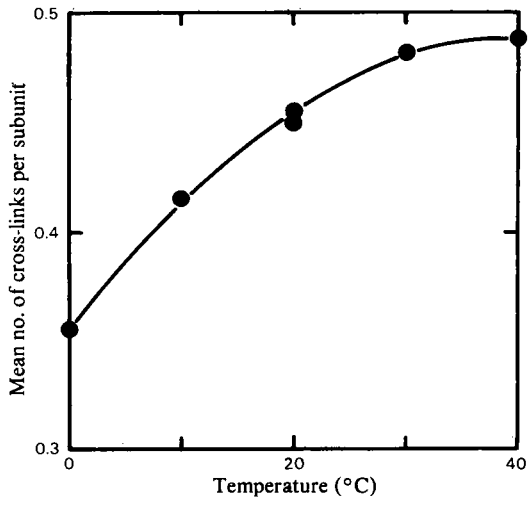


Fig. 3. *Effect of temperature on the extent of cross-linking of F-actin by p-NN'-phenylenebismaleimide*
F-actin (4mg/ml) was made to react with phenylenebismaleimide (molar ratio of *p-NN'*-phenylenebismaleimide:actin 2:1) for up to 5h as described in the Experimental section. The pH of the reaction varied from 9.1 at 40°C to 9.5 at 0°C. Mean numbers of cross-links per actin subunit were calculated from densitometry of 4% polyacrylamide/SDS gels.

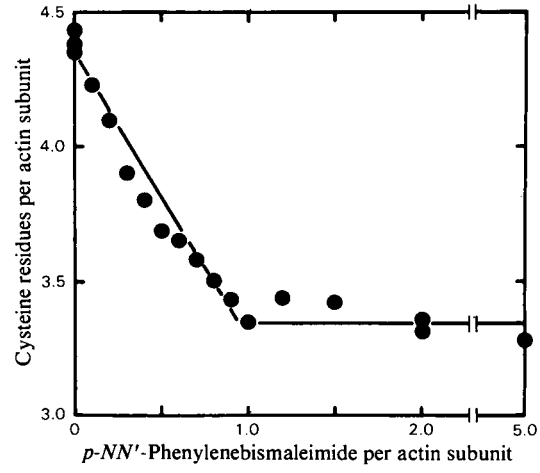


Fig. 4. *Stoicheiometry of reaction of p-NN'-phenylenebismaleimide with cysteine residues in F-actin*
F-actin was made to react with *p-NN'*-phenylenebismaleimide as described in the legend to Fig. 2, and after at least 1h of reaction assayed for cysteine content by using 5,5'-dithiobis-(2-nitrobenzoate) in SDS.

cross-linking did not depend on the proximity of F-actin filaments. (ii) There was no noticeable change in the viscosity of F-actin solutions during cross-linking, even when SDS/polyacrylamide gel electrophoresis showed that about three-quarters of the subunits became involved in cross-linked complexes at a mean of 0.5 link per subunit. If cross-links were indeed formed between F-actin filaments, a very marked change in viscosity would be expected to result. (iii) Electron microscopy gave no evidence of a greater tendency of actin filaments to be associated side-by-side after cross-linking, such as would occur if cross-links formed between filaments.

Since *p-NN'*-phenylenebismaleimide is a maleimide derivative, we would expect it to react with cysteine residues. The extent of reaction of the reagent with the cysteine residues of actin was therefore investigated by using 5,5'-dithiobis-(2-nitrobenzoate) to measure the thiol groups which had not reacted. Fig. 4 shows that the number of available thiol groups decreased until 1 molecule of *p-NN'*-phenylenebismaleimide was added per subunit. At this point 1 thiol group had reacted. No further loss was observed when more reagent was added. When the monofunctional analogue of the reagent, *N*-phenylmaleimide, was used in place of *p-NN'*-phenylenebismaleimide, an identical result was obtained. *p-NN'*-Phenylenebismaleimide did not introduce cross-links into F-actin that had previously reacted with an excess of *N*-phenylmaleimide, showing that both reagents react with the same cysteine residue. Since only one cysteine residue reacted with *p-NN'*-phenylenebismaleimide, cross-links formed by the reaction of excess *p-NN'*-phenylenebismaleimide with F-actin did not involve a second cysteine residue.

To discover the identity of the other amino acid residue involved in cross-linking, amino acid analyses were performed on acid hydrolysates of cross-linked actin. The imide rings of *p-NN'*-phenylenebismaleimide are not stable to acid hydrolysis, and hydrolysis of cross-linked actin would be expected to produce the 1,2-dicarboxyethyl derivatives of the amino acids involved in the cross-linking reaction, together with *p*-phenylenediamine (Smyth *et al.*, 1964). To provide suitable markers, *N*-acetylcysteine and N^{α}-tosyllysine were made to react with *N*-ethylmaleimide as described by Smyth *et al.* (1964) and Brewer & Riehm (1967) respectively, and their acid hydrolysates were then used to establish the elution position from the amino acid analyser of the 1,2-dicarboxyethyl derivatives of cysteine and lysine. In the elution profile of the hydrolysate from cross-linked actin two peaks were seen that were not present in the profile from unmodified actin. These were eluted at the same position as the *S*-(1,2-dicarboxyethyl)-cysteine and N^{ϵ}-(1,2-dicarboxyethyl)lysine standards.

We detected no 1- or 3-N^t-(1,2-dicarboxyethyl)-histidine (Brewer & Riehm, 1967). Two hydrolysates of cross-linked actin gave 0.83 and 0.70mol of *S*-(1,2-dicarboxyethyl)cysteine and 0.65 and 0.75 mol of N^ϵ-(1,2-dicarboxyethyl)lysine per mol of actin, consistent, within the error of determination, with their involvement in the cross-linking reaction. We conclude that *p-NN'*-phenylenebismaleimide forms cross-links between a cysteine and a lysine residue.

Mechanism of cross-linking reaction

In order to understand the mechanism of the cross-linking reaction, the time course of the reaction of the thiol group was compared with that of the production of cross-links (Fig. 5). At pH9.0 and 25°C, reaction of *p-NN'*-phenylenebismaleimide with one cysteine residue on F-actin is complete within 1min and no further loss occurs. Cross-linking, however, is much slower than this. The reaction of the reagent with F-actin can therefore be divided into two steps: a rapid and stoicheiometric reaction with a single cysteine residue on each F-actin subunit, followed by a slower cross-linking reaction involving a lysine residue of an adjacent F-actin subunit.

Since the mean number of cross-links per subunit eventually formed is appreciably less than one, there must be a reaction competing with the reaction with lysine that limits the formation of cross-links. One possibility is suggested by the fact that maleimide derivatives are hydrolysed at an appreciable rate (Gregory, 1955). Knight (1976) has shown that the maleimide rings of *p-NN'*-phenylenebismaleimide are hydrolysed at pH9.0 and 25°C with a half-time of less than 3min, that is within the time-span of the cross-linking reaction. The products of hydrolysis are shown in Scheme 1. This hydrolytic reaction occurs at the same rate whether or not the maleimide ring has already reacted with cysteine. However, once a maleimide ring has been hydrolysed it is incapable of reacting with cysteine and presumably therefore with lysine; that is, reaction with cysteine can precede the hydrolytic reaction, but it cannot follow it. So if, after one of the maleimide rings of *p-NN'*-phenylenebismaleimide reacted with a cysteine residue of actin, the other maleimide ring became hydrolysed, no cross-linking would occur. A simple competition between reaction with lysine and hydrolysis is not, however, sufficient to explain the observed temperature-dependence of cross-linking, in particular the fact that the extent of cross-linking approaches 0.5 cross-link per subunit as the temperature is raised (Fig. 3). A mechanism that does explain this dependence is shown in Scheme 2. In the scheme, A shows the *p-NN'*-phenylenebismaleimide molecule bound through one of its maleimide rings to a cysteine residue of an actin subunit. We assume that the other maleimide ring cannot at this point react

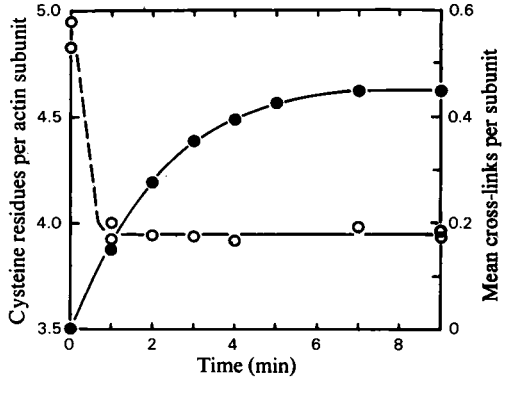
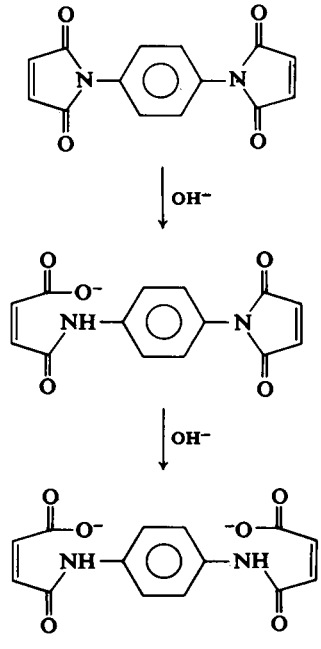
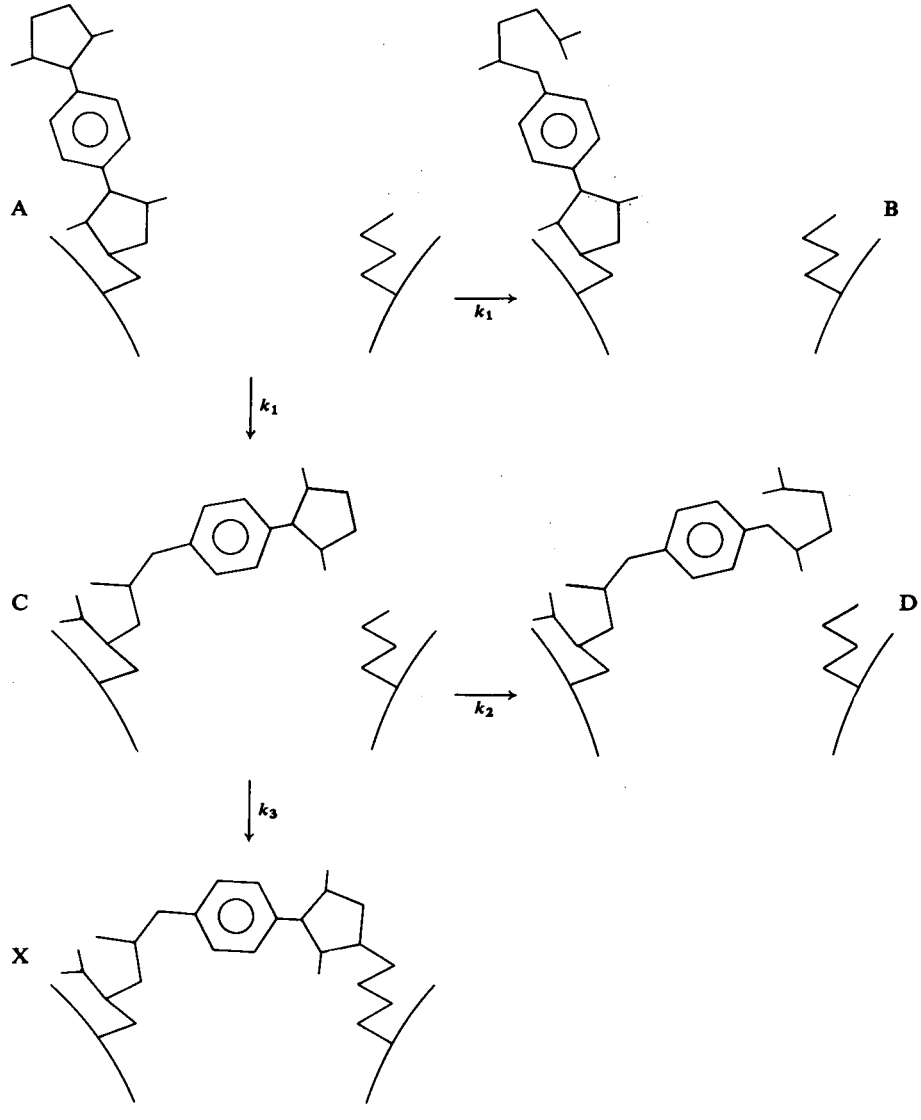


Fig. 5. *Comparison between the time courses of the reaction of p-NN'-phenylenebismaleimide with a cysteine residue in F-actin and the formation of cross-links*

The reaction and quenching were performed as described in the legend to Fig. 1. After dialysis to remove 2-mercaptoethanol, the cysteine content was measured by using 5,5'-dithiobis-(2-nitrobenzoate) in SDS (○), and the mean number of cross-links per actin subunit was determined from densitometry of 4% polyacrylamide/SDS gels (●).



Scheme 1. *Hydrolysis of p-NN'-phenylenebismaleimide to p-NN'-phenylenebismaleamic acid*



Scheme 2. *Postulated mechanism for the cross-linking reaction*

In each diagram the surfaces of two neighbouring actin subunits are represented by the two arcs. *p-NN'*-Phenylenebismaleimide bound through one of its maleimide rings to a cysteine residue is represented by the three rings on the left of each diagram. The lysine residue on the other actin subunit is represented by the zig-zag line.

with the lysine residue of the adjacent actin subunit because *p-NN'*-phenylenebismaleimide is insufficiently flexible. The two imide rings in A can be hydrolysed at equal rates. The product (B), in which the second maleimide ring is hydrolysed, cannot form a cross-link, but because of its increased flexibility the other product (C), in which the substituted ring is hydrolysed, can. The step in the pathway producing the cross-linked product (X) is in competition with an alternative reaction in which the second maleimide ring is hydrolysed to form a non-cross-linked product (D). The branching of the pathway at the first step limits the cross-linking to a maximum of 0.5 per subunit and the competition at the second step is responsible for decreasing the cross-linking below 0.5 depending on the temperature.

The values of the rate constants k_1 and k_2 in the reaction scheme may be calculated from the observed rate of cross-linking. In the Appendix it is shown that according to this reaction scheme a graph of

$\log(p_\infty - p)$ against time should be linear, where p_∞ is the mean number of cross-links per actin subunit at infinite time. The slope of the line should be $-2k_1/2.303$ and the intercept on the ordinate should be $\log [k_3/2(k_2+k_3-2k_1)]$. Fig. 6 shows that the time course of insertion of cross-links at pH9.0 and 25°C fits this expression, and the analysis yields values: $k_1=4.7\times10^{-3}$ s^{-1}, $k_2=7.9\times10^{-3}$ s^{-1} and $k_3=66\times10^{-3}$ s^{-1}.

If this mechanism were correct, the values of k_1 and k_2 should be comparable with the rate constants for the hydrolysis of *p-NN'*-phenylenebismaleimide in solution (Knight, 1976). These are 9×10^{-3} s^{-1} (per ring) for the first ring to be hydrolysed and 5×10^{-3} s^{-1} for the second. We conclude that the limited extent and the rate of cross-linking can be explained by the hydrolytic reactions shown in our suggested kinetic scheme. We cannot, however, rule out the possibility that some intramolecular cross-links are formed.

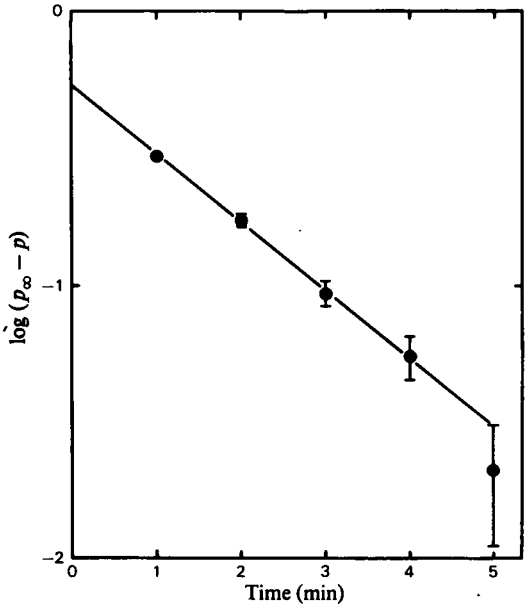


Fig. 6. *Analysis of the kinetics of cross-link formation*
Data are taken from Fig. 5. Error bars are drawn for ±0.005 mean cross-links per actin subunit. For the derivation of the equation used to analyse the data, see the Appendix. The mean number of cross-links per actin subunit after 9min reaction is used for p_∞.

Do the actin subunits react independently?

For studies on the activity of cross-linked actin it is important to know that the cross-linking reaction itself has not changed the structure of the actin. *p-NN'*-Phenylenebismaleimide is a rather hydrophobic molecule, so when it binds to an F-actin subunit it might bury itself in the structure and thus bring about changes in the tertiary structure of the subunit. Such changes could affect the binding of the bismaleimide to neighbouring subunits and would lead to a non-random distribution of *p-NN'*-phenylenebismaleimide along the polymer at substoicheiometric ratios of bismaleimide to actin. Similarly, if the cross-linking reaction between two subunits induced a change in structure (for example, a rotation near the contact regions), then the ability of the subunits to be involved in further cross-linking reactions could be affected. This effect would also lead to a non-random distribution in the amount of each oligomeric species at the end of the cross-linking reaction. The existence of such effects can be examined by comparing the relative amounts of each species (as determined by densitometry of SDS/polyacrylamide gels) with the relative amounts predicted from a random reaction mechanism.

Consider the case when every actin subunit has reacted with *p-NN'*-phenylenebismaleimide. If all subunits are equivalent and independent, then the probability that the bismaleimide bound to a subunit will form a cross-link is numerically equal to the measured mean number of cross-links per actin subunit (p) that are formed overall. The probability that the actin-bound reagent will not cross-link is $(1-p)$. The probability that a run of n subunits containing the particular subunit will form an n-mer is therefore $(1-p)^2\,p^{n-1}$ and there are n ways in which the given subunit can be incorporated into the n-mer. The probability of the given subunit being incorpor-

Table 1. *Comparison of measured mass fractions of oligomers in cross-linked actin with the values predicted for independent reaction of p-NN'-phenylenebismaleimide*
F-actin was cross-linked with excess *p-NN'*-phenylenebismaleimide at pH9.2 and 20°C, and run on a 4% polyacrylamide/SDS gel. The mean number of cross-links per subunit, calculated from the first line of data by using eqn. (1) of the Appendix was 0.454. This was used to calculate the second line of numbers as described in the text.

Species ...	Monomer	Dimer	Trimer	Tetramer	Pentamer	Hexamer	Heptamer	Octamer	Nonamer	Decamer	Undecamer
Measured	0.298	0.268	0.189	0.115	0.0627	0.0340	0.0173	0.0088	0.0044	0.0019	0.0010
Predicted	0.298	0.271	0.184	0.112	0.0633	0.0345	0.0183	0.0095	0.0048	0.0024	0.0012

ated into an *n*-mer, which is numerically equal to the mass fraction of *n*-mer present, is therefore $n(1-p)^2p^{n-1}$. The predicted value for the mass fraction of each oligomer can be compared with that obtained by gel densitometry.

It can be seen intuitively that if there is positive co-operativity, that is second and subsequent cross-links are more easily formed than the first, then clusters of cross-links will be produced in the F-actin, and for a given value of *p*, there will be more monomer and oligomers, and less dimer, observed than predicted.

Table 1 shows that there is very good agreement between the observed and expected amounts of monomeric and cross-linked oligomeric actin when excess *p-NN'*-phenylenebismaleimide reacts with actin. The formation of cross-links between F-actin subunits is therefore a random process. The method was also used to analyse data from F-actin that had reacted with sub-stoicheiometric amounts of *p-NN'*-phenylenebismaleimide, and the distribution of the reagent was also found to be random. These results suggest that the insertion of cross-links does not itself introduce strain into the structure.

Discussion

We have succeeded in using *p-NN'*-phenylenebismaleimide to introduce covalent cross-links between the subunits of F-actin. Maximum cross-linking is achieved with about equimolar *p-NN'*-phenylenebismaleimide and actin, and the only amino acid residues that are modified are those involved in the cross-linking reaction.

A detailed analysis of the reaction of excess *p-NN'*-phenylenebismaleimide with F-actin has shown that one molecule of the reagent initially becomes bound to every actin subunit by rapid reaction with a cysteine residue, the slower cross-linking reaction involving a lysine residue. Peptide analyses of F-actin treated with *N*-ethylmaleimide (Lusty & Fasold, 1969) and with a spin-label derivative of maleimide (Sleigh & Burley, 1973) showed that in both cases cysteine-373 was the sole cysteine residue modified. Since *p-NN'*-phenylenebismaleimide and *N*-phenylmaleimide compete for reaction with a single cysteine residue in F-actin, there is little doubt that *p-NN'*-phenylenebismaleimide, like the other maleimide derivatives, reacts with cysteine-373.

Although *N*-ethylmaleimide can be used as a modifying reagent that is specific for cysteine residues in proteins (Gregory, 1955), there are precedents for the present observation of reaction of a maleimide moiety of *p-NN'*-phenylenebismaleimide with a lysine residue in the cross-linking reaction under mildly alkaline conditions. Smyth *et al.* (1964) showed that *N*-ethylmaleimide reacted with α-amino groups, and Brewer & Riehm (1967) showed that it could react with the ε-amino group of lysine and the imidazole ring of histidine in ribonuclease and lysozyme. Bis-(*N*-maleimidomethyl) ether will cross-link a cysteine residue to a histidine residue in oxyhaemoglobin (Arndt *et al.*, 1971). The close proximity of the actin-bound bismaleimide molecule to a lysine residue no doubt enables it to react at an appreciable rate. Because of the short length of *p-NN'*-phenylenebismaleimide (about 1.2nm), it is unlikely that more than one particular lysine residue in the actin sequence is involved in the formation of cross-links.

F-actin can be considered to be constructed of two strings of subunits wound round each other to form two long-pitched helical strands (Hanson & Lowy, 1963). *p-NN'*-Phenylenebismaleimide might insert cross-links either between these strands or within each one. Since only one species of link is introduced, it cannot do both. A direct determination of the type of cross-link introduced, by observation of depolymerized cross-linked actin in the electron microscope, was inconclusive. There are, however, some indirect lines of evidence which favour the alternative that the cross-links are formed between subunits in the same long-pitch helical strand.

(i) The binding of subfragment 1 or of heavy meromyosin to actin inhibits the formation of actin–actin cross-links (P. Knight & G. Offer, unpublished work). From the work of Moore *et al.* (1970) it is known that subfragment 1 binds on the outer side of each actin subunit and would therefore be expected not to interfere with links formed between neighbouring subunits in different long-pitched helical strands, but might sterically block the formation of links between actin subunits in the same long-pitched helical strand.

(ii) It is also found (Knight, 1976) that tropomyosin and troponin do not affect the formation of cross-links between actin subunits either at low or high Ca^{2+} concentrations. Since tropomyosin lies in the grooves of the F-actin structure (Hanson & Lowy, 1963; O'Brien *et al.*, 1971; Huxley, 1972), it might be expected to affect the formation of cross-links between subunits of actin on different long pitched helices.

We have used F-actin specifically cross-linked by *p-NN'*-phenylenebismaleimide to examine the possibility that changes in the structure of F-actin accompany its interactions with myosin, tropomyosin and troponin. We have also prepared cross-linked dimers from this F-actin and examined their ability to activate myosin adenosine triphosphatase.

We thank Mr. Roger Starr and Miss Ruby Hynes for their help with the amino acid analyses and Dr. P. Bennett, Dr. S. Lovell and Dr. E. J. O'Brien for discussions. P. K. received a training award from the Medical Research Council.

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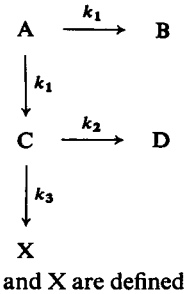
APPENDIX

Kinetics of the Cross-Linking Reaction

Consider the reaction scheme:



where A, B, C, D and X are defined as in the text and their molar concentrations at time t are a, b, c, d and x respectively. All reaction steps are taken to be irreversible. Let a_0 be the initial concentration of A. Application of the Law of Mass Action gives the time-dependence of these concentrations:

$$-\frac{da}{dt} = 2k_1 a \quad (1)$$

$$a = a_0 \exp(-2k_1 t) \quad (2)$$

$$\frac{dc}{dt} = k_1 a - (k_2+k_3)c \quad (3)$$

$$\frac{dx}{dt} = k_3 c \quad (4)$$

Combining eqns. (2) and (3) gives:

$$\frac{dc}{dt} + (k_2+k_3)c = k_1 a_0 \exp(-2k_1 t) \quad (5)$$

The solution of this differential equation is:

$$c = \frac{k_1 a_0}{(k_2+k_3-2k_1)} \{\exp(-2k_1 t) - \exp[-(k_2+k_3)t]\} \quad (6)$$

Hence from eqns. (4) and (6)

$$\frac{dx}{dt} = \frac{k_3 k_1 a_0}{(k_2+k_3-2k_1)} \{\exp(-2k_1 t) - \exp[-(k_2+k_3)t]\} \quad (7)$$

which on integration yields:

$$\frac{x}{a_0} = \frac{k_3}{2(k_2+k_3)} - \frac{k_3 \exp(-2k_1 t)}{2(k_2+k_3-2k_1)} + \frac{k_1 k_3 \exp[-(k_2+k_3)t]}{(k_2+k_3)(k_2+k_3-2k_1)} \quad (8)$$

x/a_0 is the mean number of cross-links per actin subunit (p) at time t and has a value at infinite time (p_∞) of $k_3/2(k_2+k_3)$. Under conditions where p_∞ is close to 0.5, $k_3 \gg k_2$. Since k_1 would be expected to be

of similar magnitude to k_2, it follows that $(k_2+k_3) \gg 2k_1$. Hence at large t the second exponential term in eqn. (8) will be negligible and the production of x will be described by:

$$p = \frac{x}{a_0} = \frac{k_3}{2(k_2+k_3)} - \frac{k_3 \exp(-2k_1 t)}{2(k_2+k_3-2k_1)} \quad (9)$$

Rearranging and taking logarithms we obtain:

$$\log(p_\infty - p) = \log\left[\frac{k_3}{2(k_2+k_3-2k_1)}\right] - \frac{2k_1 t}{2.303} \quad (10)$$

Hence a plot of $\log(p_\infty - p)$ against t should be a straight line with a slope of $-2k_1/2.303$ and an intercept on the ordinate of $\log[k_3/2(k_2+k_3-2k_1)]$. Hence k_1, k_2 and k_3 can be obtained.