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Photochemical cross-linking of protein and DNA in chromatin*

Synthesis and application of a photosensitive cleavable derivative of 9-aminoacridine with two photoprobes connected through a disulphide-containing linker

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A novel cleavable photo-cross-linking reagent, *N*-(2-methoxy-6-azidoacridin-9-yl)-*N'*-(4-azidobenzoyl)cystamine, for analysis of protein–nucleic acid interactions, has been synthesized. The reagent contains two photosensitive groups that can be activated sequentially. The azidoacridinyl moiety is sensitive to u.v. and visible light ($\lambda \leq 450$nm), whereas the azidobenzoyl part needs higher-energy light ($\lambda \leq 350$nm). Furthermore, the disulphide bridge connecting the two photoactive groups can be cleaved by reduction with mercaptans. The reagent is shown to induce cleavable cross-links between all five major histones and DNA in chromatin from Ehrlich ascites cells on activation with long-wavelength u.v. light ($\lambda > 300$nm) at an efficiency of $\sim 3\%$ of the added reagent.

Photochemical cross-linking has been widely used in the study of the interaction between proteins and nucleic acids (Shetlar, 1980; Sperling & Havron, 1977). Most frequently such cross-linking was accomplished with short-wavelength light ($\lambda = 254$nm) in the absence of any added reagent (Shetlar, 1980; Sperling & Havron, 1977; Sato & Hosokawa, 1981; Cao & Sung, 1982). However, in some cases bifunctional cross-linking reagents were used (Maassen, 1979; Fink *et al.*, 1980; Vanin & Ji, 1981). These reagents contain both a thermally and a photochemically active ligand, and in some cases they were furthermore supplied with a cleavable linker (Maassen, 1979; Vanin & Ji, 1981), which permitted the cross-linking to be reversed. The use of such reagents greatly facilitates the analysis of the macromolecules in question.

We have recently developed reagents for the photoaffinity labelling of chromatin, which contain 9-aminoacridine moieties in order to obtain strong reversible binding to DNA by intercalation, and a photoprobe, e.g., an aryl azide group, which reacts irreversibly *in situ* on irradiation with long-wavelength u.v. light (Nielsen, 1981, 1982; Nielsen *et al.*, 1983; Buchardt *et al.*, 1984).

As an extension of this work, we now describe the synthesis and use of a novel type of reagent for photo-cross-linking containing two different photoprobes that can be activated sequentially, and a disulphide-containing linker that is easily cleaved on reduction with mercaptans. In contrast with cross-linking reagents containing one or two thermally activated probes, purely photochemically functioning reagents would allow a very accurate time-resolved kinetic study of the substrates by flash-photolysis activation.

Abbreviations used: Boc, t-butoxycarbonyl; SDS, sodium dodecyl sulphate; f.a.b., fast atom bombardment.

* The present paper is the first of a series of papers on this topic.

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Materials and methods

I.r. spectra were recorded on a Perkin–Elmer 157 spectrometer and ^{1}H n.m.r. spectra on a JEOL FX90Q spectrometer. F.a.b. mass spectra were determined on a Varian MAT 311A instrument using an Ion Tech FAB-11 NF saddle field atom gun with Ar atoms at 8keV. All chemicals for synthesis and analysis were commercially available unless described below.

Synthesis (see Scheme 1)

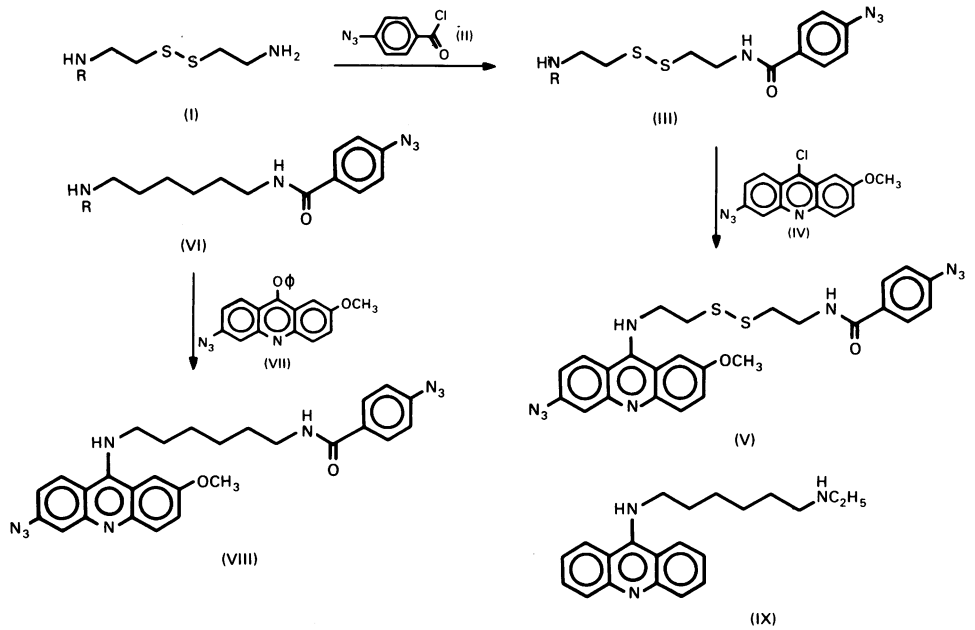
Mono-Boc-protected cystamine (Ia) and mono-Boc-protected hexane-1,6-diamine (Hansen *et al.*, 1982), 4-azidobenzoyl chloride (III) (Griess, 1867; Nielsen & Buchardt, 1982), 2-methoxy-6-azido-9-chloroacridine (Mueller *et al.*, 1981), and 9-[(6-ethylamino)hexylamino]acridine (Hansen *et al.*, 1983) were prepared as described previously.

N-Boc-N'-(4-azidobenzoyl)cystamine (IIIa). To a solution of mono-Boc-protected cystamine hydrochloride (Ib) (0.6g, 2.1mmol) in dichloromethane (25ml) and triethylamine (2ml) was added 4-azidobenzoyl chloride (II) (0.22g, 2.2mmol). The solution was stirred at room temperature for 16h, washed with water (4 × 10ml) and saturated NaCl (1 × 10ml), dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. This gave 0.7g (t.l.c. pure) of a yellow oil (84%) that on recrystallization from dichloromethane/light petroleum (b.p. 35–50°C) gave pure white crystals: m.p. 95–96°C; i.r. (cm^{-1}): 2100, 1685, 1630. Elemental analysis. Calc. for $C_{16}H_{23}N_5O_3S_2,\frac{1}{2}H_2O$: C, 47.27; H, 5.95; N, 17.23; S, 15.77. Found: C, 47.20; H, 5.83; N, 17.12; S, 15.60.

N-(4-Azidobenzoyl)cystamine (IIIb) hydrochloride. Compound IIIa (150mg, 0.37mmol) was dissolved in acetic acid saturated with HCl (5ml). The solution was stirred at room temperature for 15min and then concentrated *in vacuo*. The semi-crystalline product was recrystallized from ethanol/diethyl ether (2:3, v/v) to give 110mg (89%) of the title compound: m.p. 176–177°C (decomp.). Analysis. Calc. for $C_{11}H_{15}N_5OS_2$,HCl: C, 39.57; H, 4.83; N, 20.98; S, 19.21; Cl, 10.62. Found: C, 39.59; H, 4.80; N, 20.87; S, 19.32; Cl, 10.67.

N-(2-Methoxy-6-azidoacridin-9-yl)-N'-(4-azidobenzoyl)cystamine (V). *N*-(4-Azidobenzoyl) cystamine hydrochloride (440mg, 2mmol) and 2-methoxy-6-azido-9-chloroacridine (IV) in phenol (4g) was stirred at 100°C for 5h, after which the mixture was cooled to room temperature and diethyl ether added to precipitate the title compound as a yellow powder (1.2g, 100%). The crude product was recrystallized from ethanol/diethyl ether (1:1, v/v) yielding 0.28g (23%) of pure yellow crystals: m.p. 150–158°C (destr.). i.r. (cm^{-1}): 2100, 1630. ^1H n.m.r: δ 2.9, m, 4*H*, -CH_2-S_2-CH_2-; δ 3.92, s, 3*H*, -OCH_3; δ 4.27, broad signal, 4*H*, -NH-CH_2-; δ 7.1–8.2, m, 10*H*, aromatic protons. Analysis. Calc. for $C_{25}H_{23}N_9O_2S_2 \cdot HCl,H_2O$: C, 50.03; H, 4.20; N, 21.01; S, 10.69; Cl, 6.08. Found: C, 50.40; H, 4.21; N, 18.92; S, 10.48; Cl, 7.06. Mass spectrum (f.a.b.), double focusing. Calcd. for $C_{25}H_{24}N_9O_2S_2$ ($M+1$): 546.1494. Found: 546.1537. Owing to its absorbance of visible light and its high quantum yield for photolysis, it is difficult to avoid some loss of N_2 during handling in connection with the elemental-analysis procedure.

2-Methoxy-6-azido-9-phenoxyacridine (VII). 2-Methoxy-6-azido-9-chloroacridine (1.0g, 3.2mmol) was heated with NaOH (0.20g) in phenol (5g) at



Scheme 1. *Synthesis of reagents*
(I), (III) and (VI): a, R = Boc; b, R = H. In (VII), $\phi = C_6H_5$.

100°C for 90min. The mixture was poured into 0.2M-NaOH (100ml). After 30min the precipitated material was isolated by filtration, and recrystallized from ethanol (0.60g, 58%): m.p. 145–150°C. Analysis. Calc. for $C_{20}H_{14}N_4O_2$: C, 70.16; H, 4.12; N, 16.37. Found: C, 70.33, H, 4.03; N, 15.88.

N-(2-Methoxy-6-azidoacridin-9-yl)-N'-(4-azidobenzoyl)hexanediamine (VIII) hydrochloride. *N*-(4-Azidobenzoyl)hexane-1,6-diamine (Nielsen *et al.*, 1983) (600mg, 2mmol) and 2-methoxy-6-azido-9-phenoxyacridine (350mg, 1mmol) in phenol (2g) was stirred at 100°C for 3.5h, whereupon the mixture was allowed to cool to room temperature and a crude yellow product precipitated by the addition of diethyl ether. Purification by column chromatography (silica-gel 60; Merck 70–230 mesh ASTM; eluted with a gradient of ethanol in ethyl acetate, 0–50%, v/v) afforded a t.l.c.-pure yellow oil, which was converted into its hydrochloride and recrystallized from ethanol/diethyl ether to give the title compound (210mg, 36%): m.p. 156–169°C (destr.). Analysis. Calc. for $C_{27}H_{27}N_9O_2 \cdot HCl,2H_2O$: C, 55.72; H, 5.54; N, 21.66; Cl, 6.09. Found: C, 56.18; H, 4.89; N, 20.03; Cl, 7.28. Owing to its absorbance of visible light, and its high quantum yield for photolysis, it is very difficult to avoid some loss of N_2 during handling. The ^1H n.m.r. (δ 1.42. 6*H*; δ 1.89, 2*H*; δ 3.26, 2*H*; δ 3.8, 2*H*, broad unstructured signals, aliphatic protons; δ 3.95, s, 3*H*, -OCH_3; δ 7.1–8.5, multiplet, 10*H*, aromatic protons) and the i.r. spectra [(cm^{-1}): 2100, 1640, 1620] are in agreement with the assigned structure.

Isolation of chromatin. Chromatin was isolated from mouse Ehrlich-ascites-tumour cells essentially as described by Lerner & Steitz, 1979: 6g of washed cells were lysed in 100ml of 140mM-NaCl/10mM-Tris/HCl(pH7.4)/1.5mM-MgCl/0.5% Nonidet P40 and the nuclei isolated by pelleting (5000*g*, 7min) through a 50ml sucrose cushion [10mM-Tris/HCl (pH7.4)/5mM-$MgCl_2$/1% Nonidet P40/0.8M-sucrose]. The nuclei were lysed in 20ml of 1mM-EDTA (pH8.0)/50μM-phenylmethanesulphonyl fluoride, and the chromatin pelleted (10000*g*, 10min). The chromatin was washed in 1mM-EDTA, pH8.0, and finally allowed to swell in 1mM-EDTA, pH8.0, at a concentration of ~5mg (DNA equivalents)/ml.

Chromatin solubilized by nuclease was prepared by treating nuclei (15mg of DNA equivalents) in 12ml of sucrose/buffer [0.34M-sucrose/60mM-KCl/15mM-NaCl/1mM-$CaCl_2$/10mM-Tris/HCl (pH7.4)/0.5% Nonidet P40] with 200 units of micrococcal nuclease (Boehringer) for 15min at 37°C. The nuclei were then isolated by centrifugation (1000*g*, 5min) and lysed in 5ml of 1mM-EDTA, pH8. The supernatant (10000*g*, 10min) of this lysate, which contained approximately half of the total DNA, was used as solubilized chromatin.

Photo-cross-linking of chromatin. To 2mg of chromatin in 500μl of 1mM-EDTA, pH8.0, was added 100μg of compound V. The mixture was irradiated for 10min at 0°C (while being stirred magnetically) with Pyrex-filtered light from a 200W super-pressure mercury lamp (Osram SP 200; 1.1×10^{17} quanta$\cdot s^{-1} \cdot cm^{-2}$). After irradiation, 50$\mu$l of 4M-NaCl was added and the chromatin was pelleted. This pellet was extracted twice with 500μl of 0.2M-H_2SO_4 (3s of sonication was used with the first extraction), and finally extracted with 50μl of sample buffer [2% (w/v) SDS/5% (v/v) 2-mercaptoethanol/60mM-Tris/HCl (pH6.8)/10% glycerol/Bromophenol Blue marker]. NaOH (0.1M) was used to neutralize the acid from the extractions. This extract was analysed by SDS/polyacrylamide-gel electrophoresis as described by Thomas & Kornberg (1978). Proteins were revealed by Coomassie Blue staining.

Separation of cross-linked chromatin on hydroxyapatite. Nuclease-solubilized chromatin (2mg in 400μl of 1mM-EDTA, pH8.0) was cross-linked with 75μg of compound V as described above. A portion (400μl) of 4M-NaCl/4M-urea was added and the sample was applied to a 3ml column of hydroxyapatite. Non-cross-linked proteins were first eluted by 10ml of 2M-NaCl/2M-urea/50mM-sodium phosphate, pH7.0 (Bloom & Anderson, 1978). Subsequently cross-linked proteins were liberated and eluted in 10ml of 2M-NaCl/2M-urea/0.1M-2-mercaptoethanol. Both fractions were collected and dialysed against 1litre of 0.05% SDS (three changes). The dialysis residue was freeze-dried, redissolved in SDS-gel sample buffer and analysed by SDS/polyacrylamide-gel electrophoresis. The gels were scanned on a Shimadzu CS 930 instrument equipped with a DR-2 data recorder.

Results and discussion

The novel photo-cross-linking reagent (V) consists of an azidoacridine group and an azidobenzoyl group, connected through the disulphide-containing cystamine linker. The synthesis (Scheme 1) was undertaken by treatment of the mono-Boc-protected cystamine (Ia) with 4-azidobenzoyl chloride (II) followed by deprotection with HCl in acetic acid to give compound IIIb, which in turn was treated with 2-methoxy-6-azido-9-chloroacridine (IV) to give compound V. Furthermore, compound VIII was prepared by treatment of *N*-(4-azidobenzoyl)hexane-1,6-diamine hydrochloride (VI) (Nielsen *et al.*, 1983) with 2-methoxy-6-azido-9-phenoxyacridine (VII), and compound IX was prepared as previously described (Hansen *et al.*, 1983).

The new compounds were identified by spectroscopy and by elemental analysis. It was difficult to obtain the correct elemental analyses for compounds V and VIII. This is presumably due to the high photoreactivity, even with visible light; however, the M_r and the elemental composition of the novel reagent, V, were established by f.a.b. and double-focusing mass spectrometry. The sensitivity to ambient light also makes it advisable that solutions of compound V are kept in the dark and handled in dim red light.

On irradiation of an ethanolic solution of compound V with long-wavelength u.v. light ($\lambda > 300$nm) the changes in the absorption spectrum shown in Fig. 1 are observed.

Two photoreactions can be distinguished, i.e., a fast one of the acridine part of the molecule and a slower one attributed to the azidobenzoyl moiety. By choosing the proper wavelengths these can be segregated (Fig. 1). The azidoacridine moiety absorbs strongly at 350–450nm (Mueller *et al.*, 1981) and is strongly photoactive in this region, whereas the azidobenzoyl moiety shows no absorption and no photoreactivity at these wavelengths. Furthermore, the high photoreactivity of the azidoacridine moiety appears to hinder energy transfer to the azidobenzoyl moiety, which has been observed for other compounds where a 4-azidobenzoyl group was linked to a 9-aminoacridine (Nielsen *et al.*, 1983).

Thus it should be possible to photoactivate selectively the acridine end of the cross-linker before photoactivation of the azidobenzoyl end if desired.

In order to test the usefulness of this compound as a reagent for reversible cross-linking of DNA and histones in chromatin, the following experiments were performed. A mixture of chromatin and compound V was irradiated. Loosely bound non-histone chromosomal proteins were subsequently extracted with 0.35M-NaCl, and histones were extracted with 0.2M-H_2SO_4. The resulting chromatin was pelleted and finally extracted with the gel-electrophoresis sample buffer, containing SDS and β-mercaptoethanol (for cleavage of the disulphide bridge), and analysed by SDS/polyacrylamide-gel electrophoresis. The results are shown in Figs. 2 and 3 and Table 1. If no reagent was present or if it had been pre-photolysed, high-M_r protein bands (presumably tightly bound non-histone chromosomal proteins) were observed, together with weak bands for the core histones, H2A, H2B, H3 and H4 (identified on the basis of their migration in the gel; Fig. 2, lanes 2 and 7). If the reagent was present without irradiation, the histone bands became slightly stronger (Fig. 2, lane 6). A control experiment, using the non-photosensitive 9-[6-(ethylamino)hexylamino]acridine (IX) (Fig. 2, lane 9) indicated that this effect is due to the intercalator. However, when chromatin was irradiated in the presence of compound V, the histone bands, including H1, were significantly enhanced (Fig. 2, lane 8), indicating that the histones had been cross-linked to the DNA in the chromatin, and thus were not extractable by 0.2M-H_2SO_4, but readily by the sample buffer, owing to cleavage of the disulphide bridge. (Even if the sample buffer did not contain β-mercaptoethanol, some cleavage was observed; results not shown). As a test for the necessity of the disulphide bridge, similar experiments were performed with compound VIII (Scheme 1), which does not contain such a bridge, and this compound showed very little if any cleavable photocross-linking (Fig. 2, lanes 3 and 5).

The results collected in Table 1 indicate that all of the five major histones are photo-cross-linked to the DNA in chromatin by compound V (cf. lanes 7 and 8) and that the cross-linking is more efficient for histones H3, H2A and H2B than for histones H1 and H4.

Owing to the high background observed for non-cross-linked histones (Table 1; Fig. 2, lanes 2–4, 6–7 and 9) after this extraction method, an alterna-

Table 1. *Photo-cross-linking of histones to DNA in chromatin*

The amount of individual histones was estimated from A_{550} scans of the gel shown in Fig. 2. Roman numerals refer to the compounds depicted in Scheme 1 and described in the text.

Lane	Conditions	Histone ...	Amount (arbitrary units) H1	H2A	H2B	H3	H4
2	No reagent		0	12	11	19	11
3	VIII, no irradiation		0	23	22	35	18
4	VIII, preirradiated		0	17	15	23	15
5	VIII, plus irradiation		5	29	34	50	22
6	V, no irradiation		6	26	28	47	33
7	V, preirradiated		2	15	14	20	15
8	V, plus irradiation		25	74	70	96	32
9	IX, plus irradiation		8	36	31	51	22
	Difference between lanes 8 and 7	...	23	59	56	76	17

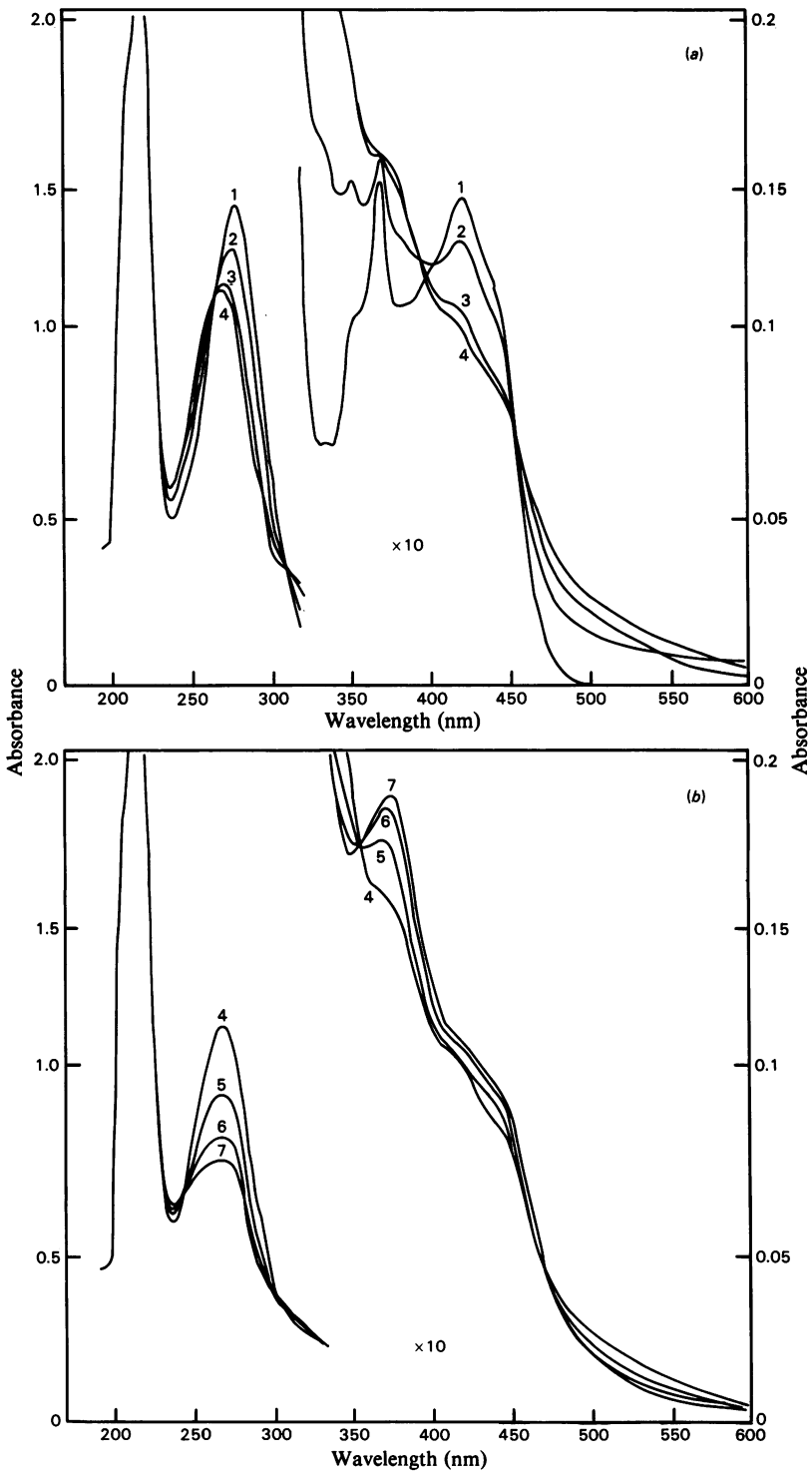


Fig. 1. *Changes of u.v. and visible absorption characteristics of an ethanolic solution (~10 μM) of compound V on irradiation*
The sample was initially irradiated for the following periods using the SP.200 lamp equipped with a WG-375 filter [cut-off 350nm (Nielsen *et al.*, 1983)]: 1–4(*a*): 0, 2, 4, and 6s, and subsequently a Pyrex filter (cut-off ~300nm) was used: 4 (repeated) and 5–7(*b*): 15, 30, and 45s.

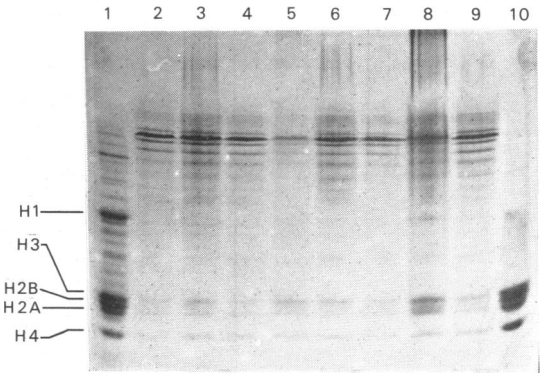


Fig. 2. *SDS/polyacrylamide-gel electrophoresis of 'cross-linked' proteins*

Chromatin (2mg) was treated with the reagents as described in the Materials and methods section and 'cross-linked' proteins were analysed by SDS/polyacrylamide-gel electrophoresis. The samples are: lanes 1 and 10, reference histones; lane 2, no reagents; lane 3, compound VIII, no light; lane 4, compound VIII, pre-irradiated; lane 5, compound VIII, irradiated; lane 6, compound V, no light; lane 7, compound IV, pre-irradiated; lane 8, compound V, irradiated; lane 9, compound IX, irradiated.

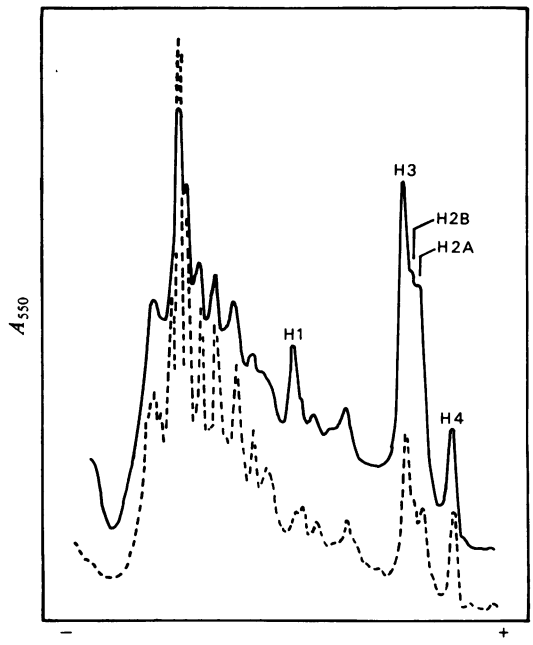


Fig. 3. *Densitometric scans (550nm) of lanes 6 (----) and 8 (——) of the gel shown in Fig. 2*

tive procedure was applied in order to confirm the identity of the cross-linked histones and the cleavibility of the disulphide linker as well as to estimate the efficiency of the photocross-linking reaction. After photo-cross-linking of chromatin with compound V, the chromatin was bound to hydroxyapatite (Bloom & Anderson, 1978) and non-DNA linked proteins were eluted in 2M-NaCl/2M-urea. This fraction consisted mainly of the five major histones (H1, H2A, H2B, H3 and H4) identified by their migration on SDS/polyacrylamide-gel electrophoresis by comparison with standard histones obtained by acid extraction of chromatin (Fig. 4*a*). Subsequently DNA-cross-linked proteins were eluted with 2M-NaCl/2M-urea/0.1M-2-mercaptoethanol and similarly analysed by SDS/polyacrylamide-gel electrophoresis. Without irradiation only very faint protein bands were observed in the gel (Fig. 4*c*), some of which could be histones on the basis of their migration. However, the irradiated samples showed distinct bands migrating as the histones (Fig. 4*b*) and a difference-scanning clearly indicated that all core histones (H2A, H2B, H3 and H4) became cross-linked to the DNA, whereas the reaction with H1 was less pronounced (Fig. 4*d*; distinct bands were clearly seen by visual inspection of the gels).

From the results presented in Table 2 the photo-cross-linking efficiency of reagent V may be estimated. By using the staining intensities of histone bands in the gel as a measure of the amount of histones (with samples assayed for protein by the Lowry method as standards) and a mean M_r of 15000 for the histones, we estimate that ~3% of the reagent molecules have resulted in histone–DNA photo-cross-linking. The overall efficiency seems to be somewhat dependent on the reagent/nucleosome ratio, whereas no significant ratio-dependence in the relative cross-linking of individual histones was observed.

Owing to the intercalative DNA binding of analogous acridines (Georghiou, 1977; Hansen *et al.*, 1983) we suggest that the azidoacridine moiety reacts preferentially with the DNA, whereas the azidobenzoyl part reacts with the histones. We have previously found that the photochemical yield of a similar azidobenzoyl compound with histones is about 35% (Nielsen *et al.*, 1983), whereas an azidomethoxyacridine photoreacts with DNA at 30–50% efficiency (P. E. Nielsen & O. Buchardt, unpublished work). Thus an overall maximum histone–DNA photo-cross-linking efficiency would be estimated at 10–15%, a value agreeing reasonably well with the observed one.

Previously, short-wavelength u.v. irradiation constituted the only means for photochemical cross-linking of proteins to DNA, e.g. in the analysis of chromatin structure (Sperling & Havron, 1977; Mandel *et al.*, 1979; Shetlar, 1980). The presently described photo-cross-linking reagent offers an alternative to this method with at least two advantages. The photoactivation may be

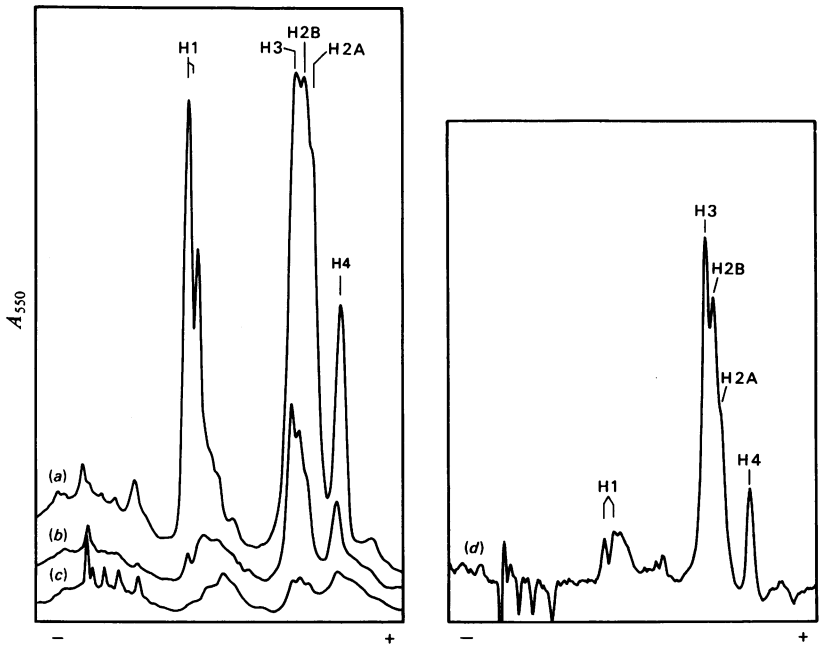


Fig. 4. *SDS/polyacrylamide-gel electrophoresis of 'cross-linked' proteins after separation on hydroxyapatite*
Solubilized chromatin (2mg) was photo-cross-linked with compound V (75μg) and the cross-linked proteins were isolated as described in the Materials and methods section. The densitometric scans of the stained gels are shown. (*a*) Non-irradiated sample; proteins were eluted with 2M-NaCl/2M-urea (1/6 of sample volume). (*b*) Irradiated sample; proteins were eluted with 2M-NaCl/2M-urea/0.1M-2-mercaptoethanol (total sample). (*c*) Non-irradiated sample; proteins were eluted with 2M-NaCl/2M-urea/0.1M-2-mercaptoethanol. (*d*) Differential scan (2× scale expansion) of (*b*) minus (c).

Table 2. *Calculation of photo-cross-linking efficiency of reagent V*
The experiments were performed as described in the legend to Fig. 4.

Amount of reagent V (μg)	Amount of chromatin (mg of DNA equiv.)*	Cross-linked histones† (μg)	Reagent/nucleosome‡ ratio	Yield (cross-links/reagent§) (%)
5	2	6	0.7	3.6
15	2	15	2.0	2.8
25	1	20	6	2.3
75	1	30	16	1.1

* Assuming $\varepsilon_{260} \sim 6600$ litre·mol^{-1}·cm^{-1}.
† Estimated from the staining intensity of the histone bands in an SDS/polyacrylamide gel using histone standards of known protein content (the Lowry protein assay).
‡ Assuming 200 base-pairs/nucleosome.
§ Assuming an M_r for histones of about 15000.

accomplished with generally non-damaging long-wavelength u.v. irradiation, and the cross-linking is easily reversed by reduction, thereby greatly facilitating the characterization of both the proteins and the nucleic acid components involved in the cross-linking.

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