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Reagents for photoaffinity labeling. I. Photobinding efficiency of aryl azido-, diazocyclopentadienyl- and ethyl diazomalonyl-derivatives of 9-aminoacridine

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Summary. A method for the comparison of photoaffinity labeling probes has been developed and tested with model reagents containing 5 different photoprobes attached to 9-acridinylamino groups through hexamethylenoxy or hexamethylenamino linkers. The fluorescence properties of the acridine part of the reagents were employed for detection of the labels. The merits and disadvantages of the different photoprobes are discussed. The photoreaction of the reagents with proteins (bovine serum albumin and histones), RNA (ribosomal), and DNA (calf thymus) were studied in order to compare the efficiency and suitability of aryl azido and diazo photoprobes. By using Pyrex-filtered light ($\lambda > 300$ nm), it was observed that the reagents derived from 4-azidobenzoyl- (**a**), 4-azido-2-nitrophenyl- (**b**), or 2-diazopentadienylcarbonyl- (**c**) are the most efficient, labeling bovine serum albumin in yields of 22%, 9% and 9%, respectively, with relative rates of 0.25:0.06:1. The acridines containing photoprobes **a**, **b** and **c** were shown to function as photoaffinity labeling reagents of the histones in chromatin.

Photoaffinity labeling has by now gained an important position as one of the most versatile tools for the study of active sites and macromolecular interaction in molecular biochemistry[3–6]. Previously aryl azido and diazo compounds were the preferred photoprobes due to their high quantum yields. Furthermore, their photolysis was assumed to lead to nitrenes and carbenes, which could result in very rapid reactions, in

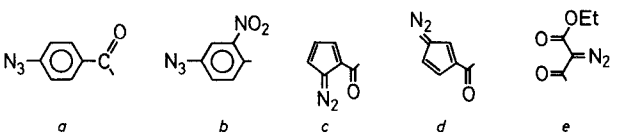
situ. More recently other photoprobes, e.g., diazirines[7] have been employed. However, aryl azides, and to a lesser extent diazo compounds, are still the most versatile and widely used photoprobes.

We have previously employed the technique[8–12], and we have subsequently shown that aryl azido photoaffinity labeling reagents appear to be bound irreversibly to their biological targets upon irradiation via azacycloheptatetraenes and not by nitrene insertion as previously believed[13].

The present work was carried out both as an extension of our general study of the photoaffinity labeling technique, and as a direct consequence of our current interest in employing the technique on specific problems like e.g., chromatin structure, etc. It consists of a comparative study of different photoprobes from a synthetic point of view, and an attempt to develop a method for evaluation of photoprobes.

The reagents examined contain 5 different photoprobes (scheme 1), i.e., the 2 widely used aryl azido probes, **a** and **b**, the previously used diazo probe, **e**, and the potentially interesting diazo probes, **c** and **d**, which had not previously been used. These were attached to a standard carrier, 9-aminoacridine, through a linker consisting of 6 methylene groups and a heteroatom, for the following reasons:

1. The acridine part of the reagents determines their gross physico-chemical properties, while minimizing the influence of the different photoprobes. This and the similarity of the linker are important for reasons of comparison.
2. The fluorescence properties of the acridine part of the molecules serve as a convenient label, eventually after hydrolysis to acridone[12].
3. The 9-aminoacridine combined with the chosen linker results in convenient water solubilities of the reagents.



The presence of the 9-acridine part of the molecule furthermore allows the comparison of the photoprobes in genuine photoaffinity studies of DNA and chromatin due to the DNA affinity exhibited by 9-alkylaminoacridines[14].

A number of targets were considered, and we decided to use a standard protein, bovine serum albumin (BSA), mammalian histones, ribosomal RNA, calf thymus DNA, and mammalian chromatin. These targets are relevant in terms of photobinding efficiency (rates and yields) and results from these can be extrapolated to a wide variety of other systems.

Materials and methods. Bovine serum albumin was purchased from Armour Pharmaceutical Comp. (fraction V) and was used without further purification. Nuclei and chromatin were isolated from Ehrlich ascites tumor cells as previously described[11,12].

Histones were extracted from nuclei of Ehrlich ascites tumor cells. They consisted of the major histone species (H_1, H_{2A}, H_{2B}, H_3 and H_4). RNA was extracted from Ehrlich ascites cells by the hot phenol procedure[15] and consisted mainly of ribosomal RNA (5S, 18S and 28S).

Chemicals for synthesis were standard commercial. Elemental analyses were performed by Mr Preben Hansen and Mrs Kirsten Bidstrup at the Microanalytical Laboratory of the H.C. Ørsted Institute. Melting points were determined on a Büchi apparatus and are uncorrected.

IR-spectra were recorded on a Perkin Elmer model PE 500 spectrometer, UV-spectra on a Pye Unicam SP 1800 UV-spectrophotometer and NMR-spectra on a Varian T60A or a JEOL FX 90 Q spectrograph. HPLC analysis was undertaken as described earlier[13].

Synthesis. Note that all operations with azido or diazo compounds must be undertaken in the dark or with low-intensity yellow light.

4-Azidobenzoyl chloride (**a**-Cl[13,16,17], 4-fluoro-3-nitrophenyl azide (**b**-F[18]), 2-diazocyclopentadienyl-(**c**-OH), 3-diazocyclopentadienylcarboxylic acid (**d**-OH)[19], ethoxycarbonyldiazoacetyl (ethyl diazomalonyl)chloride (**e**-Cl)[20], N-methyl-4-azidobenzamide (**a**-$NHCH_3$)[13], N-methyl-2-nitro-4-azidoaniline (**b**-$NHCH_3$)[21], 9-chloro-, 9-phenoxyacridine (**7**)[22,23], mono Boc-1,6-hexanediamine[24] (Boc = tert-butoxycarbonyl) (now available from Fluka) 9-(6-Boc-aminohexylamine)-, HCl, and 9-(6-aminohexylamino)acridine, 2HCl[25] were prepared by the previously described methods.

*2-Diazocyclopentadienylcarbonyl chloride (**c**-Cl).* 2-Diazocyclopentadienylcarboxylic acid (**2**, 5.0 mM, 0.68 g), sodium carbonate (1 g) and thionyl chloride (3 ml) were mixed and refluxed for 20 min. The excess volatile material was evaporated, in vacuo, and the title compound extracted by ether and the ether evaporated, in vacuo. Yield, 0.79 g, ~100%. No attempts were undertaken to purify the crude material, which was immediately used after its preparation, since storage caused destruction.

*3-Diazocyclopentadienylcarbonyl chloride (**d**-Cl)* was prepared analogously to the method described for **c**-Cl, but at 0 °C, and used immediately upon its preparation.

N-4-Azidobenzol-N'-Boc-1,6-hexanediamine. Mono-Boc-1,6-hexanediamine (0.02 M, 5.1 g) was suspended in pyridine (50 ml) and 4-azidobenzoyl chloride (0.02 M, 3.63 g) was added at once with stirring. This caused a slight heating, and the reaction mixture became homogeneous. The stirring was continued for 20 min after which the reaction mixture was evaporated to dryness, in vacuo, and treated with water. This

caused crystallization, and the crystals were filtered off. The yield was 7.0 g, 97%, based on 4-azidobenzoyl chloride. M.p. 136–137 °C. $C_{18}H_{27}N_5O_3$. Found (calculated): C 59.64 (59.81), H 7.79 (7.53), N 19.32 (19.38).

N-4-Azidobenzoyl-1,6-hexanediamine, HCl. *N*-4-Azidobenzoyl-N′-Boc-1,6-hexanediamine (0.029 M, 10.4 g) was stirred with 1 M HCl in acetic acid (50 ml) for 20 min after which the reaction mixture was evaporated to dryness, in vacuo, and treated with ether. The resulting crystalline material was filtered off. Yield: 8.3 g, 97%. M.p. 191–193 °C. $C_{13}H_{20}ClN_5O$: Found (calculated): C 51.81 (52.43), H 6.96 (6.77), N 23.69 (23.52).

N-2-Nitro-4-azidophenyl-N′-Boc-1,6-hexanediamine. Mono-Boc-1,6-hexanediamine (6.9 mM, 1.5 g) and 3-nitro-4-fluorophenylazide (3.6 mM, 0.65 g) was dissolved in dry dioxane (4 ml) and the reaction mixture was stirred for 2.5 h at room temperature followed by evaporation to dryness, in vacuo, (bath temperature < 60 °C). The resulting material was dissolved in chloroform and purified by gel chromatography on silica gel (30 g) to give 1.35 g (~ 100%) of the title compound. After recrystallization from ether the m.p. was 72–73 °C. $C_{17}H_{26}N_6O_4$. Found (calculated), C 53.95 (53.95), H 6.98 (6.93), N 22.16 (22.21).

N-2-Nitro-4-azidophenyl-1,6-hexanediamine, HCl. *N*-2-Nitro-4-azidophenyl-N′-Boc-1,6-hexanediamine (1.72 mM, 650 mg) was dissolved with stirring in 1 M HCl in acetic acid (10 ml). The dissolution lasted about 10 min. Stirring was continued for another 20 min, after which the reaction mixture was evaporated, in vacuo, treated with ether, and the resulting crystals filtered off to give the title compound. Yield: 400 mg, 74%. After recrystallization from ethanol, m.p. 142–144 °C. $C_{12}H_{19}ClN_6O_2$. Found (calculated) C 45.71 (45.78), H 6.10 (6.08), N 26.50 (26.70).

N-Ethoxycarbonyldiazoacetyl-N′-Boc-1,6-hexanediamine. Mono-Boc-1,6-hexanediamine (**9**, 6 mM, 1.5 g) was suspended with stirring in dry pyridine (15 ml), followed by addition (5 min) of freshly distilled (b.p. 58–72 °C, ~ 0.1 mmHg) ethoxycarbonyldiazoacetyl chloride (**e**-Cl, 5.6 mM, 1.0 g), and the mixture was stirred at room temperature for 0.5 h. The reaction mixture was evaporated to dryness, in vacuo, water was added, and the resulting suspension extracted with ether to give 1.69 g ~ 85% of material which was used without purification for the preparation of **9e**.

9-(6-Hydroxyhexylamino)acridine (8), HCl. 6-Amino-1-hexanol (10 mM, 1.17 g) and 9-chloroacridine (10 mM, 2.13 g) was dissolved in phenol (10 g) at 80 °C, heated to 100 °C for 1 h, cooled to room temperature and treated with ether under stirring. This caused crystallization, and the crystalline material was filtered off and recrystallized from ethanol (with addition of minute amounts of ether). Yield: 2.5 g, 75%. M.p. 178–80 °C. $C_{19}H_{23}ClN_2O$. Found (calculated): C 68.50 (68.97), H 7.13 (7.01), Cl 10.52 (10.74), N 8.37 (8.47).

9-(6-(2-Diazocyclopentadienylcarbonyloxy)hexylamino)acridine, **8c**. a) 9-(6-Hydroxyhexylamino)acridine, HCl (**8**, HCl, 2.5 mM, 0.83 g) was suspended in pyridine (7.5 ml) and poured on crude 2-diazocyclopentadienylcarbonyl chloride (2.5 mM, 0.40 g) at room temperature with stirring. This caused heat development, and the reaction mixture was stirred for 2 h at room temperature, followed by evaporation to dryness, in vacuo. The resulting black oily material was washed with water, extracted with methanol, and passed through silica gel (10 g) by elution with methanol-chloroform. This gave, after evaporation, 1.15 g of a yellow oil, which was dissolved in warm ethanol (about 5 ml). Cooling caused the precipitation of a dark oil which was shown by thin layer chromatography to consist of a complicated mixture, which was discarded. The remaining solution was concentrated, in vacuo, and treated with ether, which caused crystallization. The crystalline material (0.40 g ~ 39%) contained the title compound with some **8**, HCl as impurity. Further purification (200 mg) by column chromatography on silica gel (10 g, eluent ethanol-chloroform, 1:3) allowed the isolation of 70 mg of the title compound, which was uniform by TLC analysis, and which was brought to crystallization by dissolution in ethanol followed by addition of ether. In this way 44 mg of pure **8c**, $3H_2O$ was obtained. b) 9-(6-Hydroxyhexylamino)acridine, HCl (**8**, HCl, 1 mM, 331 mg), 2-diazocyclopentadienylcarboxylic acid (**c**-OH, 1 mM, 136 mg) and 4-toluenesulfonic acid (20 mg) was suspended in dry pyridine (15 ml) followed by the addition of dicyclohexylcarbodiimide (DCC, 1.1 mM, 226 mg), and the reaction mixture was stirred at room temperature, under nitrogen for 45 h. The reaction mixture was filtered, washed with chloroform, and evaporated, in vacuo. The residue was dissolved in chloroform, washed with 5% aqueous sodium hydrogen carbonate and water, dried over anhydrous magnesium sulfate, and evaporated, in vacuo, to give 0.3 g of crude product. This was dissolved in chloroform and chromatographed on silica gel (10 g). Elution with chloroform removed some impure material, and subsequent elution with chloroform-ethanol (3:1) gave about 10% of pure title compound. M.p. (dec > 100 °C). $C_{25}H_{24}N_4O_2$, $3H_2O$. Found (calculated) C 64.48 (64.38), H 5.71 (6.47), N 11.36 (12.01). IR: 1618 cm^{-1}, 2102 cm^{-1}. ^{1}H-NMR (90 MHz, $CDCl_3$, TMS) δ 1.2–1.5 ppm (2 broad signals ~ 8H, aliphatic CH_2), δ 3.4–3.75 ppm (broad signal ~ 4H, NH-CH_2- and OC-O-CH_2), δ 5.9–6.08 ppm (dd, $J_1 \sim J_2 \sim 3$Hz, H-3 from cyclopentadience, 1H), δ 6.75–6.81 ppm (distorted triplet, $J \sim 3$Hz, H-4 or H-5 from cyclopentadiene, 1H), δ 6.96–7.04 ppm (H-5 or H-4 from cyclopentadiene,

1H), δ 7.1–8.25 ppm (characteristic signals from the acridine part of the molecule ~ 8H).

9-(6-(3-Diazocyclopentadienylcarbonyloxy)hexylamino)-acridine, **9d**. a) 9-(6-Hydroxyhexylamino)acridine, HCl (**8**, HCl, 1.05 mM, 350 mg) was treated with 3-diazocyclopentadienylcarbonyl chloride (**d**-Cl, from 1.3 mM, 180 mg of the corresponding acid, **2'**) analogously to the method described for the preparation of **9c**, above. Unfortunately, we never succeeded in preparing **9d** in a satisfactory pure form by this method. b) Analogously with the method described above for **9c; 9d** was prepared in a yield of 20 mg (~ 4%). M.p. (dec > 100 °C). $C_{25}H_{24}N_4O_2$, $4H_2O$. Found (calculated) C 62.72 (61.97), H 6.36 (5.37), N 9.22 (11.57). The IR-spectrum showed the expected diazo-absorption at 2200 cm^{-1} and the ^{1}-H-NMR-spectrum also was in agreement with the assignment. The compound was uniform by TLC.

9-(6-(4-Azidobenzoylamino)hexylamino)acridine (**9a**) *and hydrochloride.* Method a. 9-Phenoxyacridine (2.0 mM, 0.54 g) was dissolved in phenol (2 g) under heating to 80 °C. After this was added N-4-azidobenzoyl-1,6-hexanediamine, HCl (2.0 mM, 0.60 g). The temperature was quickly raised to 120 °C, and the solution kept at this temperature for 1 h. After cooling to room temperature, the resulting oil was treated with ether (3 × 25 ml) which caused crystallization. The crystalline material was dissolved in methanol. Addition of ether gave an oil which crystallized after standing. After one more recrystallization from ethanol-ether a pure sample of **9a**, HCl, H_2O was obtained. Yield: 0.66 g, 67%. M.p. 114–115 °C. $C_{26}H_{27}ClN_6O$, H_2O. Found (calculated): C 63.67 (63.34), H 5.87 (5.92), N 16.74 (17.01). The hydrated free base (**9a**, 1.5 H_2O) was prepared by dissolution of the hydrochloride in ethanol, addition of excess saturated aqueous ammonia followed by water, which caused precipitation. Recrystallization from ethanol-water gave **9a**, 1.5 H_2O, M.p. 80–81 °C. $C_{26}H_{26}N_6O$, 1.5 H_2O. Found (calculated): C 67.36 (67.08), H 6.36 (6.28), N 17.39 (18.04). IR: 1615 cm^{-1}, 2100 cm^{-1}. The ^{1}H-NMR-spectrum showed the expected signals.

Method b. 9-(6-Aminohexylamino)acridine, HCl (0.12 mM, 44 mg) and 4-azidobenzoylchloride (0.12 mM, 22 mg) was dissolved in pyridine (2 ml), and the mixture was stirred for 2 h at room temperature. This was followed by evaporation to dryness, washing with water, and recrystallization of the remaining material to give **9a**, aq, (57%), identified by IR-spectroscopy and comparison with the IR-spectrum from above.

9-(6-(2-Nitro-4-azidophenylamino)hexylamino)acridine, 9b, HCl, H_2O. 9-Phenoxyacridine (1.24 mM, 336 mg) was dissolved in phenol (2 g) at 80 °C and N-2-nitro-4-azidophenyl-hexanediamine, HCl (1.24 mM, 390 mg) was added, whereupon the temperature was raised to 100 °C for 1 h. Cooling to room temperature gave a brown oil which was washed with ether (3 × 10 ml) which caused crystallization. The crystalline material was purified by dissolution in methanol followed by addition of ether and washing with ether to give 0.35 g, 52% of **9b,** HCl, H_2O. M.p. 167–168 °C. $C_{25}H_{28}ClN_7O_3$. Found (calculated): C 59.38 (58.87), H 5.35 (5.53), N 18.44 (19.23), Cl 6.80 (6.95). IR: 1617 cm^{-1}, 2100 cm^{-1}. The ^{1}H NMR-spectrum was in excellent agreement with the assigned structure.

9-(6-(Ethoxycarbonyldiazoacetylamino)hexylamino)-acridine, **9e.** Crude N-ethoxycarbonyldiazoacetyl-N'-Boc-1,6-hexanediamine (~ 4.7 mM, 1.69 g) was dissolved in 1 M HCl in acetic acid (25 ml) and stirred for 25 min, followed by evaporation, in vacuo, of the solvent. (The resulting material crystallized easily, but it was very hygroscopic, and it is preferentially used without purification). The crude material was added, at 80 °C, to a solution of 9-phenoxyacridine (4.7 mM, 1.27 g) in phenol (5 g), and the reaction mixture was heated to 105–110 °C for 1 h. After cooling, the resulting brown solution was washed with ether (3 × 25 ml), dissolved in methanol, and ether was added, which caused the precipitation of a dark oily material. This was chromatographed on silica gel (20 g, eluent, ethanol) to give 550 mg ~ 25% of the title compound as a crystalline material, which was contaminated with some acridine, and which was very hygroscopic. This preparation was used in the labeling experiment. $C_{24}H_{30}Cl_2N_5O_3$. Found (calculated) C 58.40 (56.81), H 6.10 (5.96), N 12.23 (13.80). IR: 1618 cm^{-1}, 1645 cm^{-1} and 2110 cm^{-1}. The ^{1}H-NMR showed the expected signals.

Photolabeling. The irradiations were performed in Pyrex tubes placed in an ice-bath and equipped with a magnetically stirred bar. The light source was an Osram super pressure mercury lamp (SP 200) having a light intensity at the distance used (15 cm) where the experiments were performed of 1.5×10^{17} (quartz), 1.3×10^{17} (pyrex), 1.1×10^{17} (acetone) and 0.8×10^{17} (WG 375) quanta $\times$ $sec^{-1} \times cm^{-2}$. The light intensity was measured by ferric oxalate actinometry. This method can be used only when $\lambda < 450$ nm, thus the intensity resulting when using the Corning 3-72 filter was not determined.

The labeling was performed in the following manner. To BSA (500 µg) in 10 mM Tris-HCl (1000 µl), pH 7.4 was added the acridine (50 µg from a stock solution in dimethyl sulfoxide; 10 mg/ml). The stirred mixture was irradiated, using the appropriate filter and samples (150 µl) were withdrawn at the required times. (Note that solutions of **8c** and **8d** had to be wrapped in aluminium foil when stored to avoid photolysis due to ambient light).

The BSA, histones or RNA were precipitated in ethanol-acetone (1:1, 1 ml) for 2 h at −20 °C, pelleted at 4000 rpm, and washed in ethanol-acetone (1:1,

500 µl). The pellet was dissolved in 5 N NaOH (1000 µl), heated to 90 °C for 1.5 h and finally the fluorescence intensity was measured (λ_{ex}=390 nm, λ_{em}=470 nm)[12]. In order to determine the total amount of added dye, samples (15 µl) of the irradiation mixtures were treated with 5 N NaOH and their fluorescence measured after hydrolysis as described above. Irradiations of various 9-aminoacridines without any photoprobes gave no labeling.

In the photochemical yield determinations (table 1), irradiation took place for 30 min. Photolabeling of nuclei or chromatin was performed as previously described[11,12]. Photobinding to DNA was measured as described[14].

Results and discussion. Chemistry. The synthesis of the photoprobe derivatives is outlined the scheme 2. From this it is seen that monomer cyclopentadiene (**1**) is transformed to a mixture of the acids **2** and **2′**, which are converted to the corresponding diazoacids and separated[19]. The diazoacids are stable at room temperature, in the dark, and their attachment to the reagent was attempted either by converting them, immediately before use, to their unstable chlorides, which reacted with the reagents, or with the aid of DCC. 4-Azidobenzoyl chloride (**a**-Cl) is stable for months when stored dessicated in the dark. It is easy to prepare from anthranilic acid (**4**) as shown in scheme 2[13,16,17]. 4-Fluoro-3-nitrophenylazide (**b**-F) is likewise prepared from the corresponding aniline (**5**) as shown, and it can be stored in the cold[18]. Ethoxycarbonyldiazoacetyl chloride (**e**-Cl) is prepared by addition of phosgene to ethyl diazoacetate (**3**)[20]. The acridine part of the reagents was attached via 9-chloroacridine (**7**, ACCl) or 9-phenoxyacridine (ACO∅), which is available from 2-chlorobenzoic acid (**6**) in 3 easy steps[22,23].

Reagents **9** were prepared by reaction of the mono-Boc-protected hexane-1,6-diamine[24] with the halides of **a**, **b** or **c**, followed by removal of the protective group and reaction with 9-phenoxyacridine; the analogous reactions with the halides of **c** and **d** were unsuccessful in our hands. We succeeded in linking **c** to the 9-aminoacridine moiety by converting 9-chloroacridine to 9-(6-hydroxyhexylamino)acridine (**8**), which reacted with **c**-Cl or **c**-OH and DCC to give **8c**, whereas we did not succeed in obtaining the isomeric **8d** analytically pure by this or any other method. However, **8d** was prepared sufficiently pure for obtaining the warranted labeling results by coupling **d**-OH to **8** with DCC. Compound **9a** was also prepared by first attaching the 9-acridine moiety to hexane-1,6-diamine[25] followed by treatment with **a**-Cl (see Materials and methods). All the reagents (**9a,b,e, 8c,d**) were isolated and used as the corresponding mono- and dihydrochlorides. They were identified on the basis of elemental analysis, IR- and ^{1}H-NMR-spectroscopic properties as well as on the basis of their synthetic origin. Whereas all the precursors were fairly easy to obtain, labeling reagents containing **a**, **b** and **e** were more easy to prepare than those containing **c** and **d**. Some of the yields may appear unsatisfactory for an organic chemist, but only small amounts of reagents are required for this kind of study, and the isolated products from easily manip-

Table 1. Photobinding of the reagents to various substrates[a]

Reagent	Yield (%) BSA[b]	Histones	RNA	DNA
9a	22.3 ± 11.3 (9)	35	40	18 ± 5
9b	9.3 ± 3.2 (9)	17	8	10 ± 1
8c	9.0 ± 1.9 (10)	13	25	19 ± 2
8d	5.0	1.9	8.0	–
9e	0.8 ± 0.7 (3)	0.4	0.25	–

[a] The reagents (50 µg/ml) were mixed with the substrates (500 µg/ml) in 10 mM Tris-HCl, pH 7.4, irradiated for 30 min with Pyrex-filtered light and treated as described in 'Materials and methods'. Nonirradiated blanks were subtracted and the results are expressed as percent of the added dye, measured fluorimetrically. The results for BSA are given with SD and the number of experiments in parenthesis. [b] With either of the filters shown in figure 1.

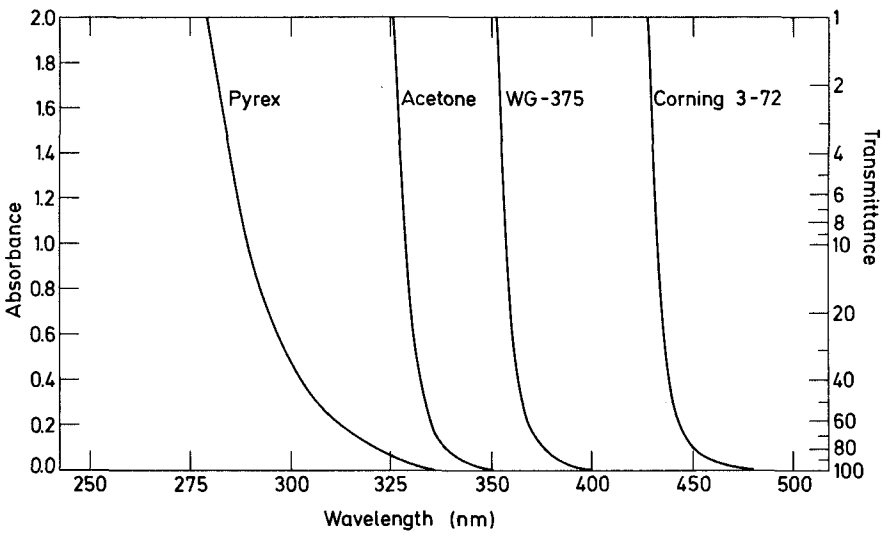


Figure 1. UV-absorption characteristics of the light-filters em ployed.

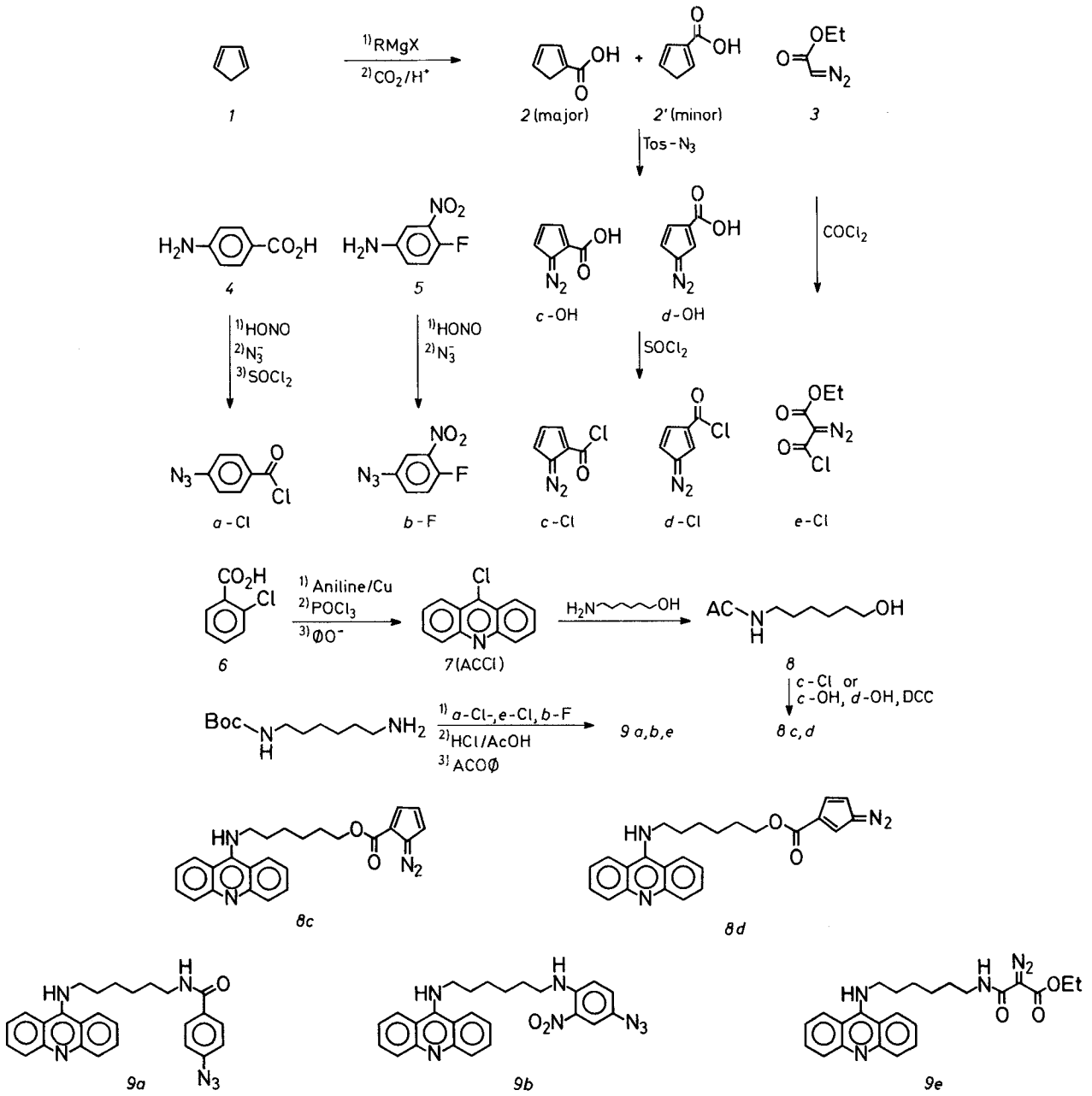
ulated amounts of starting materials are of suitable magnitudes.

Irradiation and labeling. The relative yields of the photobinding of the reagents to protein and RNA (table 1) were determined with various cut-off filters (fig. 1).

No significant dependence of the yield of photobinding on wavelength was observed. Furthermore, the relative rates of photobinding with an identical light-source and various cut-off filters were determined for **9a**, **9b**, and **8c** (fig. 2, table 2). From the rates it can be inferred that compounds **9a** and **8c** react considerably faster than **9b**. Since **8d** was never obtained in the pure state, and since **9e**, on the basis of the photobinding efficiency results (table 1), was considered uninteresting as photoprobe, no rate measurements were undertaken with these compounds.

The photolysis in methanol of simple derivatives of the photoprobes, i.e., **a**-$NHCH_3$, **b**-$NHCH_3$ and **c**-OH, which have only one chromophore, was also undertaken (table 3).

The relative photolysis rates of **a**, **b** and **c**, in the form of their simple derivatives, **a**-$NHCH_3$, **b**-$NHCH_3$ and **c**-OH, exhibit some interesting features (table 3, figs 1, 3 and 4). All show relatively effective photolysis at very short wavelengths (Pyrex, and particularly quartz filtered light). Irradiation with light of such short wavelengths is generally unwarranted in photolabeling experiments due to the absorption of light of biological substrates and subsequent photochemical



reactions. However, irradiation with Pyrex-filtered light has often been employed, and in such cases all three photoprobes are so photolabile that they appear useable as photoprobes. At longer wavelengths the aryl azides (**a** and **b**) exhibit rather poor photoreactivity, whereas the diazoprobe (**c**) retains efficient photolysis (table 3). In the case of **a** this is due to very poor absorption at longer wavelength, whereas in the case of **b** the quantum yield appears to be very low for the 400–500 nm transition; the photochemistry of **c** is due to absorption in its long-wavelength tail.

The photochemical behavior of the reagents **9a**, **9b** and **9c** is particularly interesting. The wavelength dependency of their photobinding rates as compared to those of the parent photoprobes (tables 2 and 3, figs 1, 3 and 4) clearly shows a dramatical improvement at wavelengths (WG 375 and Corning 3-72 filters), where the acridine moiety of the molecule is responsible for almost all (**9a**), most (**8c**) or a substantial (**9b**) part of the light absorption. It was ascertained by control irradiation experiments with 9-(6-aminohexylamino)acridine that the acridine part of the molecule could not be photobound to either proteins or DNA.

There is good evidence to indicate that only singlet excitation of aryl azides will result in effective photobinding to biological substrates, whereas their triplet states mainly undergo hydrogen abstraction[26]. This is presumably also the case for the presently examined reagents and substrates. Thus, the improved photoreactivity of **9b** as compared to the isolated photoprobe is believed to be due either to singlet energy transfer from the acridine part of the molecule to the aryl azido part with subsequent photochemical reaction, or to excitation transfer via a weak interaction

Table 2. Photobinding rates of reagents to BSA[a]

Compound	Rate (% · sec^{-1}) with filter			
	Pyrex	Acetone	WG-375	Corning 3-72
9a, HCl	3.1	2.6	1.9	0.34
9b, 2HCl	0.69	0.23	0.17	0.08
8c	12.5	10.0	6.2	0.21

[a] The rates were estimated from the results in figure 2, by extrapolating to zero time.

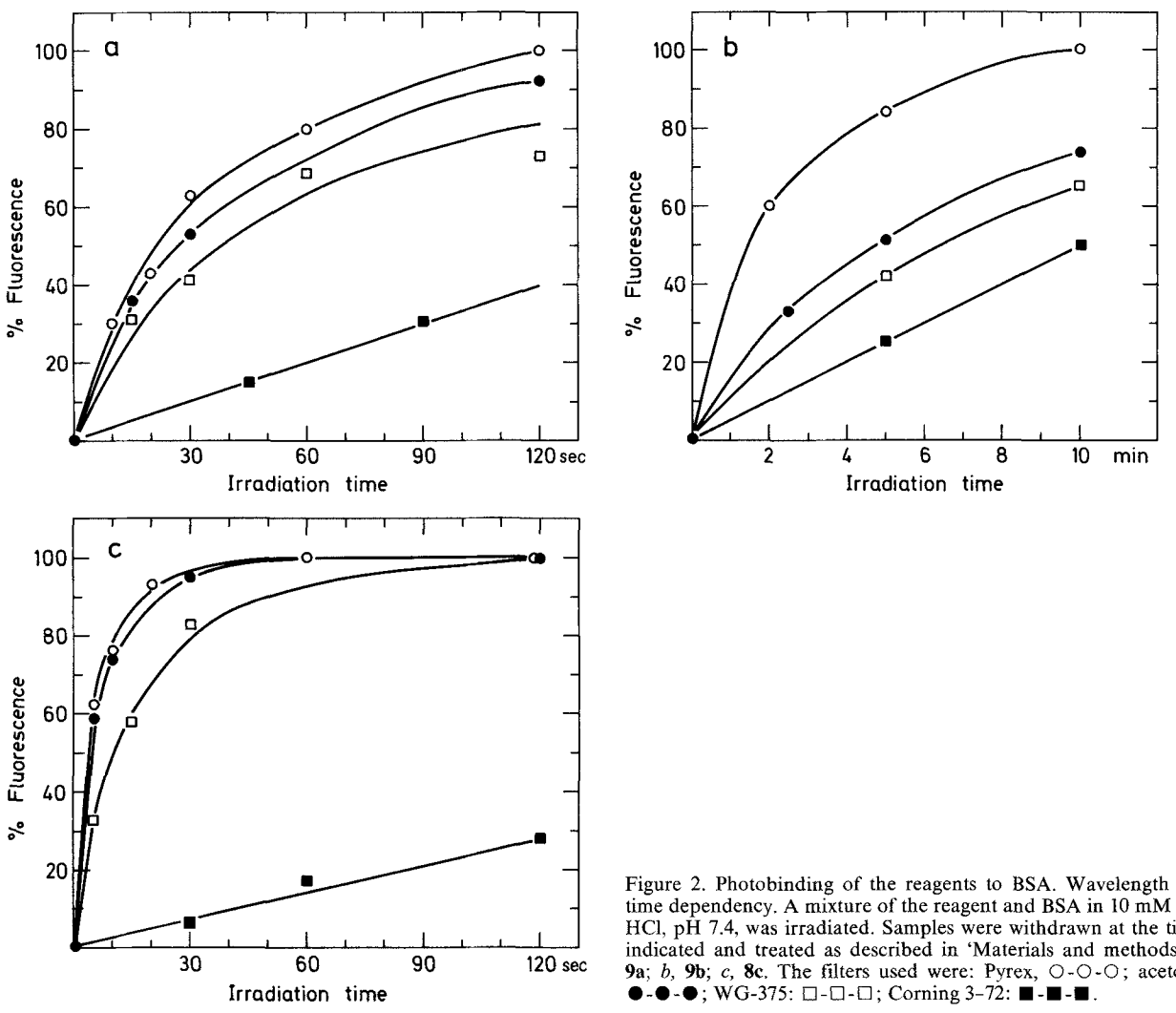


Figure 2. Photobinding of the reagents to BSA. Wavelength and time dependency. A mixture of the reagent and BSA in 10 mM Tris HCl, pH 7.4, was irradiated. Samples were withdrawn at the times indicated and treated as described in 'Materials and methods'. *a*, **9a**; *b*, **9b**; *c*, **8c**. The filters used were: Pyrex, O-O-O; acetone: ●-●-●; WG-375: □-□-□; Corning 3-72: ■-■-■.

excimer[27]. The improved photoreactivity of **9a** as compared to **a**-$NHCH_3$ appears only to be explicable by assuming excitation transfer via a weak interaction excimer, whereas that of **8c** as compared to **c**-OH may be due to either singlet or triplet energy transfer. However, these results show that the action spectrum of a photoprobe may be changed by attaching it to another chromophore, and it may furthermore be inferred that the photochemical behavior of a photoprobe in a reagent may be influenced to various degrees when it is in close contact with the substrate.

The observed differences in photobinding yields (table 1) to various substrates are not straightforward to explain, but they show that **9a**, **9b** and **8c** all contain well suited photoprobes, both in RNA and protein labeling, with **9a** giving the best results in all cases, thereby indicating its excellence as photoprobe. The low yield observed in the photobinding of **9e** is most likely due to rearrangement of the primarily formed carbene to a ketenoid species which is not sufficiently reactive for efficient labeling to take place[28].

Table 3. Photolysis rates of **a**-$NHCH_3$, **b**-$NHCH_3$ and **c**-OH with various cut-off filters

Compound[a]	Rate (% · sec^{-1}) with filter Quartz	Pyrex	Acetone	WG-375	Corning 3-72
a-$NHCH_3$	16	4.5	0.4	0.2	0.1
b-$NHCH_3$	9	6	1.5	0.4	0.1
c-OH[b]	100	40	24	7	0.5

[a] 10^{-4} molar solutions in methanol. The rates were determined by monitoring the change in UV-absorption of the solutions as a function of irradiation time. [b] Compound **b**-$NHCH_3$ showed a very complex photochemical behavior which did not allow the observation of isosbestic points. This is due to secondary photochemistry which showed wavelength dependency. Consequently the rates for this compound were determined by HPLC-analysis.

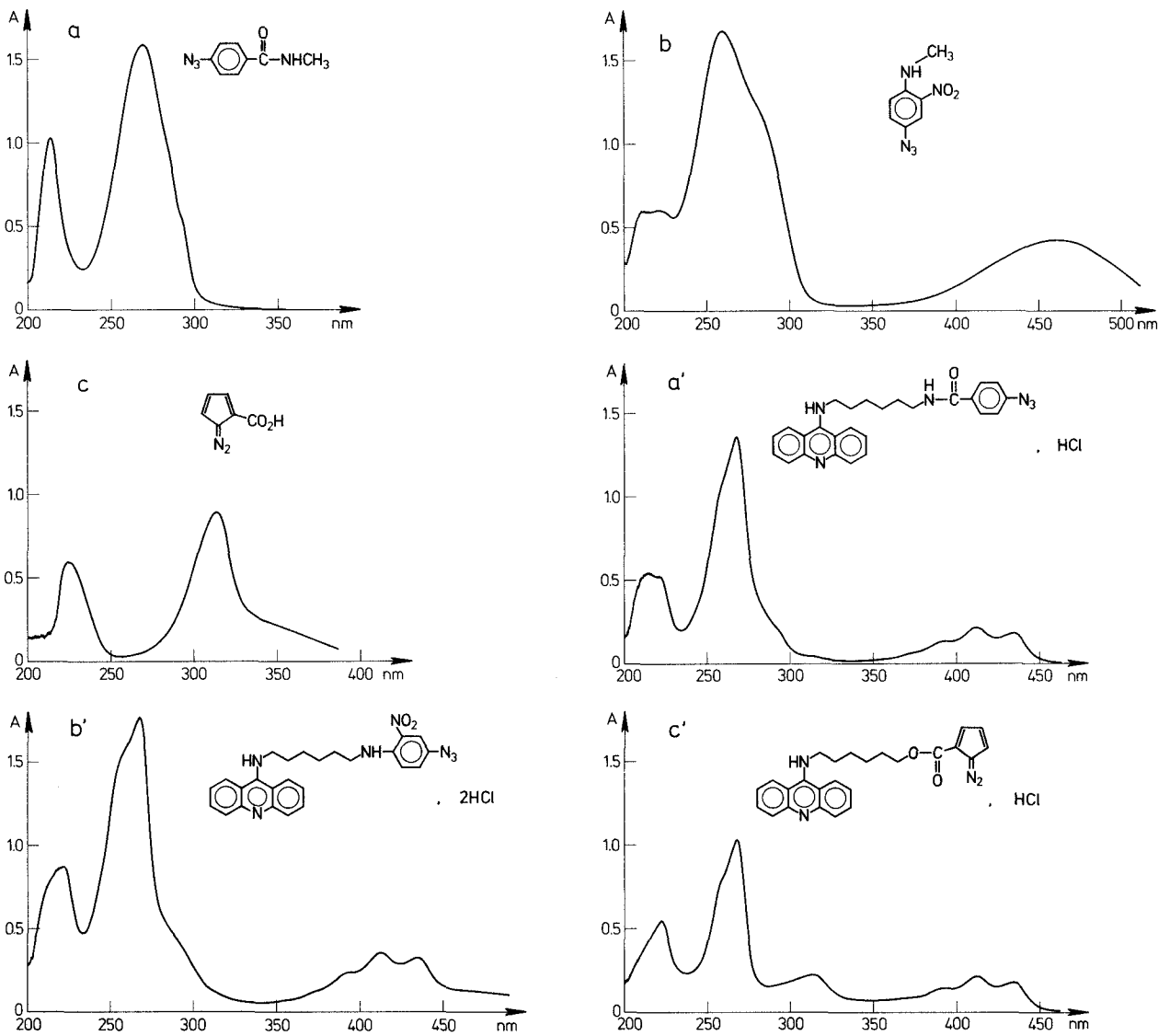


Figure 3. UV-spectra of the photoprobes and reagents. *a* **a**-$NHCH_3$; *b* **b**-$NHCH_3$; *c* **c**-OH; *a'* **9a**, HCl; *b'* **9b**, 2HCl; *c'* **8c**. The spectra were obtained in methanol.

We have previously found that the half-lives of the primary photoproducts of one actual photoaffinity labeling reagent and some model compounds are of the order of msec[13], and the carbenes presumably generated from derivatives of **c** upon irradiation are expected to have even shorter half-lives. Thus sufficiently fast photoreactions for effective in situ binding are expected with the photoprobes **a**–**c**.

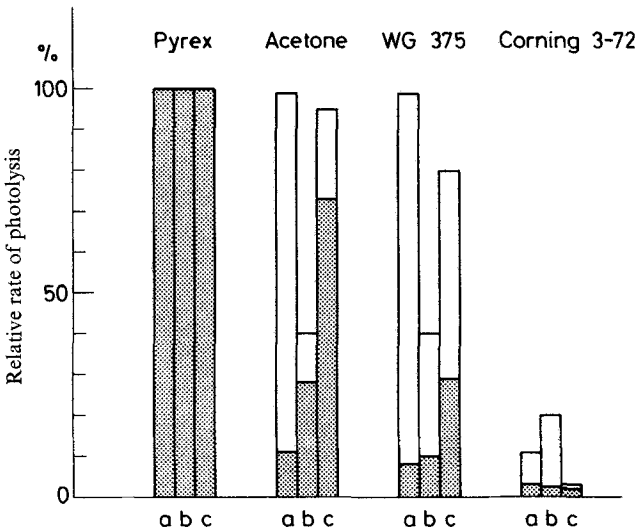


Figure 4. Relative photolysis rates of the photoprobes (shaded bars): **a**-$NHCH_3$, **b**-$NHCH_3$ and **c**-OH, and their acridine derivatives (open bars): **9a**, **9b** and **8c**. The photolysis rates taken from tables 2 and 3 have been normalized to equal light intensity (see 'Materials and methods') using the same factor for WG-375 and Corning 3-72.

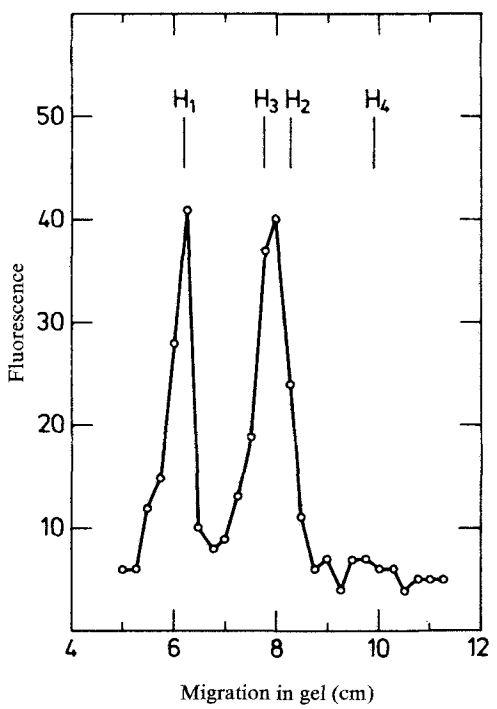


Figure 5. Photoaffinity labeling of chromatin with **9a**. ~ 10 OD_{260} of chromatin in 500 µl 10 mM Tris-HCl (pH 7.4) was photolabeled with 100 µg of **9a**. The histones were extracted and analyzed by polyacrylamide gel electrophoresis[12]. Labeled histones were identified by fluorescence. The fluorescence units are arbitrary.

9-Alkylaminoacridines bind to DNA by intercalation with association constants comparable to that of 9-aminoacridine[14]. As shown in table 1, the reagents **9a**, **9b** and **8c** serve as quite efficient photolabels of calf thymus DNA, and we are presently initiating a study of the base specificity of these reagents.

The DNA affinity of the reagents also allows photoaffinity labeling of histones in chromatin. Our preliminary results show that compound **9a** gives labeling of histones H_1, H_{2A}/H_{2B} and H_3 (fig. 5), quite similar to the results obtained with the related bis-acridine reagents[12]. However, labeling of the histones of chromatin with **9b** or **8c** lead to different labeling pattern (unpublished results), thereby indicating that the outcome of the photoaffinity labeling experiment is dependent on the character of the photoprobe.

Obviously, other than the presently examined qualities of the photoprobes must be taken into account in the construction of a photoaffinity labeling reagent. Both the biochemical properties and the label (fluorescence, spin label, radioactivity) are just as important. However, the present examination is hoped to give a rational background for a choice between photoprobes **a**–**e**, as well as for a comparative evaluation of other potential photoprobes. In particular, the potentials of the diazo photoprobes (**c**, **d**) should be examined, especially with relation to the labeling of membranes, since carbene precursors appear to be superior photolabelers in such systems[7].

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Mammalian skeletal muscle: Long-lasting contractures and potentiated tetani produced by conditioning with weak acid anions[1]

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Summary. Reversible contractures can be induced in slow mammalian muscles by manipulations that probably generate a long-lasting alkalinization of the muscle cell interior. Such contractures reach about ¼ of the tetanic force, P_0, and last 10 times longer than potassium contractures. While in contracture, the muscle fibers have high resting potentials so that they can be electrically stimulated. Tetanic force is then increased and added to that of the contracture so that total force may reach 2 P_0. This level of potentiation has not been reached by any previously-known method.

The procedure for producing slow contractures consists of a 2-step solution change. In the 1st step, thin muscle preparations (consisting of several anatomical bundles) dissected from rat or mouse soleus, rat lumbricalis or human intercostalis muscles, are bathed in a Na propionate solution (140 mM Na, 4 mM K, 2 mM Ca as gluconate, 2 mM Tris (tris(hydroxymethyl)aminomethane), 146 mM propionate, pH 7.2, 19-23 °C) for 10 to 20 min. In the 2nd step, a TrisCl solution (156 mM Tris, 4 mM K, 2 mM Ca, 147 mM Cl, pH 7.2) is applied. In the experiment illustrated in figure 1, force began to develop about 30 sec after the application of the TrisCl solution and reached a maximum at about 6 min (range 3-8 min in 10 tests). The peak force was 24% (range 15-60%) the force produced in a maximal potassium contracture provoked earlier on. Relaxation to ½ the peak force occurred in 23 min (range 18-30 min). Since neither solution produced slow contractures when applied alone and since the contracture appeared only when the sequence of application was Na propionate-TrisCl it seemed that exposure to Na propionate somehow sensitized the muscle to TrisCl.

The fact that slow contractures did not appear on application of TrisCl after a Ringer solution indicated that Na ions had no specific role in sensitizing the muscle. Indeed, about the same conditioning effects were produced with Na and Li, somewhat less with TEA (triethanolamine), about 40% less with K and about 70% less with Tris, all as propionate. The weaker conditioning effectiveness of K and of Tris will be explained later.

The conditioning effect of the propionate anion was quantitatively assessed by measuring the relation between the concentration of propionate and the amplitude of the slow contracture caused by TrisCl. The relation is shown in figure 2. It can be seen that 10 mM propionate was sufficient to produce a noticeable conditioning effect and that about 30 mM propionate was required for the force of the ensuing TrisCl-contracture to reach 50% maximum. The conditioning effect reached a maximum at about 150 mM propionate. In 3 out of 4 muscles, maximum conditioning was reached in less than 15 min. Conditioning was not improved by soaking the muscles in Na propionate for more than 20 min.

Reimmersion of a contracted muscle into a Na propionate-containing solution caused a prompt, complete, and reversible relaxation (fig. 3). Relaxation was incomplete when the concentration of propionate in the relaxing solution was less than 20 mM. The relation between the amount of relaxation and the concentration of propionate is given in figure 4. Relaxing activity was clearly present in the presence of as little as 2 mM propionate and about 50% relaxation was reached with 5 mM propionate. Fur-