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PHOTOAFFINITY LABELING OF THE TETRACYCLINE BINDING SITE OF THE *ESCHERICHIA COLI* RIBOSOME

THE USES OF A HIGH INTENSITY LIGHT SOURCE AND OF RADIOACTIVE SANCYCLINE DERIVATIVES

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Abstract—[^{3}H]Tetracycline (TC) has been shown to photoincorporate into the *Escherichia coli* ribosome. However, the utility of this process for characterizing the TC binding site on the ribosome is diminished by competing side reactions which also lead to incorporation of radioactivity. In this work we first conducted a detailed study of the labeling processes occurring when ribosomes are irradiated in the presence of [^{3}H]TC with a common, rather low intensity, lamp. On the basis of the results of this study we next explored the usefulness for photoaffinity labeling of the TC site of both irradiation with a high-intensity laser and radioactive, functional TC derivatives having different photochemical properties than TC itself. Labeling patterns determined by polyacrylamide gel electrophoretic analysis of ribosomal proteins extracted from photoaffinity-labeled 30S subunits provided strong evidence that these two approaches offer distinct advantages for characterizing the TC binding site.

Over the last several years, we and others have been using the technique of photoaffinity labeling to identify sites of antibiotic binding to the *Escherichia coli* ribosome [1]. This approach has allowed us to identify specific ribosomal proteins into which puromycin [2–4], photolabile puromycin derivatives [5–7], and tetracycline (TC)§ have been photoincorporated [8]. In this latter work we have shown that, in addition to photoincorporation of TC into ribosomal proteins, ultraviolet irradiation of TC in the presence of ribosomes leads to several other processes that reduce the utility of TC as a photoaffinity label. Specifically, (a) on ultraviolet irradiation TC photodecomposes to one or more photoproducts, denoted collectively as ph_1TC; (b) on incubation with ribosomes, ph_1TC incorporates into ribosomal protein in a light-independent manner probably reflecting the electrophilic character of ph_1TC; (c) on ultraviolet irradiation in the presence of ribosomes, ph_1TC shows, in addition, light-dependent incorporation into ribosomal protein; and (d) ph_1TC is, compared with TC, a poor inhibitor of both aminoacyl tRNA binding to ribosomes and poly (U)-directed ribosome-dependent polyphenylalanine synthesis. The net effect of these other processes is to oblige us to factor labeling by ph_1TC out of the observed labeling pattern in order to determine the labeling due to native TC, the more potent antibiotic. Because such factoring is not only a tedious process but also yields results subject to considerable uncertainty, we believe it worthwhile to seek alternative approaches for photoaffinity labeling of the TC inhibitory site on the *E. coli* ribosome.

In this paper we first performed a more detailed study of ribosome labeling by both TC and ph_1TC than was presented previously [8] and then, on the basis of these studies, explored the use of both intense light sources and TC analogues for improved labeling of the TC inhibitory site.

MATERIALS AND METHODS

Non-radioactive TC and TC derivatives. TC and 7-chloro TC were obtained from commercial sources (Sigma and Boehringer). 6-Demethyl-6-deoxy-TC (sancycline) was supplied by Lederle Laboratories and by Drs. N. Belcher and W. Celmer of Chas. Pfizer. 7-Dimethylaminosancycline (minocycline) was provided by Drs. N. Kuch and A. C. Dornbush of Lederle Laboratories. Published procedures were used for the syntheses of 7-bromo-, 7-amino- and 9-aminosancycline [9] and for the synthesis of 9-azidosancycline [10].

Radioactive TC and TC derivatives. [7-^{3}H]TC (1 Ci/mmole) was obtained from New England Nuclear. 7-N$(CH_3)_2$-[^{3}H]minocycline was synthesized by reductive methylation of 7-aminosancycline with Na$B[^3H]_4$ (10 Ci/mmole, New England Nuclear). [^{3}H]Minocycline at a specific radioactivity of 5 Ci/mmole was separated from the starting 7-aminosancycline by thin-layer chromatography on polyamide plates (Analtech) developed in acetonitrile–water (9:1, v/v) (R_f: 7-aminosancycline, 0.67; minocycline, 0.83). The region of the plate comigrating with authentic minocycline was

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§ Non-standard abbreviations used in this text are: PAGE, polyacrylamide gel electrophoresis; ph_1TC, tetracycline photoproduct; poly (U), polyuridylic acid; and TC, tetracycline.

Table 1. Incorporation of [^{3}H]labeled TC and TC derivatives into gel regions on PAGE analysis of ribosomal proteins derived from photoaffinity-labeled 30S subunits

No.	Experimental conditions				Incorporation* (% mole/mole × 10^2)			
	[^{3}H]TC or [^{3}H]TC derivative, concn (μM)	Target, concn (μM)	Light source†	Light fluence (Joules)	Region I	Region II	Region III	Incorporation into Region II / Incorporation into Region III
1	TC, 50	70S, 2.6	1	12	48	47	54	0.87
2	TC, 53	70S, 2.6	2	6	44	103	74	1.39
3	TC, 53	70S, 2.6	2	12	63	169	136	1.24
4	TC, 53	70S, 2.6	2	24	138	259	222	1.17
5	TC, 53	30S, 3.9	1	12	84	183	111	1.65
6	Minocycline, 5.9	30S, 9.8	1	108	6.3	30	25	1.21
7	9-Aminosancycline, 2.2	30S, 3.9	2	12	0.43	0.86	0.34	2.5
8	9-Aminosancycline, 2.2	30S, 3.9	2	24	0.83	1.89	0.78	2.4
9	9-Azidosancycline, 0.55‡	30S, 4.9	2	6	0.66	1.88	0.70	2.7
10	9-Azidosancycline, 0.55‡	30S, 4.9	2	24	0.76	2.78	1.35	2.1

* Region I includes proteins S3, S6 and portions of proteins S4, S5 and S8; Region II includes proteins S7, S10 and portions of proteins S4, S5 and S8; Region III includes proteins S14 and S18. † 1: PCQ 008L lamp; 2: Argon Ion laser.
‡ Incorporation values shown are corrected for small contributions from 9-aminosancycline which contaminated the 9-azidosancycline sample.

extracted with methanol, the precipitated impurities were removed by centrifugation, and the supernatant fraction was lyophilized. This procedure was repeated three times, and the material obtained from the combined extracts was used for the photoaffinity labeling experiments. [7-^{3}H]-9-Aminosancycline (21 Ci/mmole) was prepared by catalytic (Pd on charcoal) reduction of 7-bromo-9 nitrosancycline (a gift from Dr. M. Cava) dissolved in triethylamine: tetrahydrofuran and subjected to a tritium atmosphere [11]. This procedure was carried out under contract with New England Nuclear. Purification of the [7-^{3}H]-9-aminosancycline was achieved using Analtech polyamide plates, using a procedure analogous to that described above for [^{3}H]minocycline purification. [7-^{3}H]-9-Azidosancycline (21 Ci/mmole) was prepared in 45% yield by diazotization of [7-^{3}H]-9-aminosancycline with isoamylnitrite, followed by substitution with sodium azide, in 45% yield. The reaction mixture was resolved by thin-layer chromatography on 0.25 mm silica gel plates, saturated with 0.1 M EDTA, and developed in ethanol–water (70:30, v/v) (R_f: 9-aminosancycline, 0.2; 9-azidosancycline, 0.34). Due to the very limited amounts of the radioactive material available and the inevitable losses encountered on purification, photoaffinity labeling experiments were performed using the reaction mixture. The results obtained therefore represent a contribution from both compounds, as discussed in Table 1.

Preparation of ribosomes, ribosomal subunits and tRNA. 70S Ribosomes were prepared from *E. coli* Q13 bacteria harvested in mid or late log phase, using the modifications of the Traub *et al.* [12] procedure previously described [2]. All operations were performed at 4°. Large-scale preparations of 30S and 50S subunits were obtained by zonal centrifugation in a Beckman Ti 15 rotor essentially as described by Sypherd and Wireman [13]. Small scale preparations of subunits from 70S ribosomes labeled with TC or a TC photoproduct were obtained by ultracentrifugation through a 5–20% sucrose gradient made up in a high salt buffer [50 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 50 mM KCl, 500 mM NaCl], denoted TMKNa, which also contained β-mercaptoethanol as described in Goldman *et al.* [8]. Gradient fractionation and subunit collection were as previously described [2] except that subunits were precipitated by addition of 1 vol. of ethanol and incubation at −20° for 1 hr. [^{14}C]Phe-tRNA^{Phe} was prepared from [^{14}C]Phe (New England Nuclear, 400–450 Ci/mole) and either bulk stripped tRNA (Grand Island Biological) or purified yeast tRNA^{Phe} (Boehringer-Mannheim), and crude factors, as described by Ravel and Shorey [14].

Photoinduced incorporation of tetracyclines into ribosomes. All photolyses were performed in TMK buffer [50 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 50 mM KCl] at ribosome or ribosomal subunit concentrations indicated in Table 1, and followed either procedure A or B. (A) Photolyses were performed at 4° in 4 mm diameter quartz tubes with a UV Products, Inc. PCQ-008L lamp assembly having a maximal output between 340 and 380 nm. The typical duration of a photolysis experiment was 5–90 min. (B) Photolyses were carried out in 1 ml quartz cuvet-

tes (1 cm path length) using an Argon Ion laser with continuous output at 351.1 and 363.8 nm (radiance ratio equal to 1.0). The samples in the laser experiments were precooled on ice for 30 min prior to photolysis. The typical duration of a photolysis experiment was 2–8 sec.

For both procedures A and B, immediately following photolysis (within 10 sec) ribosomes were precipitated with 2 vol. of ethanol–β-mercaptoethanol (9:1), resuspended in TMK buffer containing 1% β-mercaptoethanol, and reprecipitated with 2 vol. of ethanol. For experiments in which 70S ribosomes were the target, the pellets were resuspended in TMKNa buffer containing 1% β-mercaptoethanol for eventual subunit separation and protein extraction. For experiments in which 30S ribosomal subunits were used directly, the above pelleting procedure was repeated five times and the samples were used for protein extraction as described below.

Polyacrylamide gel electrophoresis (PAGE). Protein from labeled 30S subunits was extracted using the Mg^{2+}-acetic acid procedure [15] and precipitated by addition of 5 vol. of acetone and −20° incubation [16]. One-dimensional urea-PAGE analysis of labeled proteins was performed as previously described [2, 8].

Biochemical assays. The growth inhibition activity of the various TC analogues was quantitated by a modification of the turbidometric method of Kersey *et al.* [17]. [^{14}C]Phe-tRNA^{Phe} binding to ribosomes was measured with a filter assay as described previously [18]. Reaction mixtures were dissolved in 1% dimethyl sulfoxide (DMSO) as a precaution to fully solubilize the compounds. This amount of DMSO was shown to have no effect on observed levels of Phe-tRNA^{Phe} binding. Poly(U)-dependent polyphenylalanine synthesis was also performed as previously described [5] but with 1.5% DMSO added to ensure solubility of the drugs.

Spectral measurements. All u.v.-visible spectra were recorded on a double beam Cary 14 spectrophotometer. Infrared spectra were recorded on a Perkin–Elmer 137 spectrometer.

RESULTS AND DISCUSSION

Dependence of the distribution of covalently incorporated radioactivity into ribosomal proteins on photolysis of [^{3}H]TC and Ribosomes on light fluence; Incorporation of both TC and TC-photoproduct. Photolysis of ribosomes and [^{3}H]TC in TMK buffer leads to incorporation of radioactivity into ribosomes that is almost exclusively into ribosomal proteins, predominantly 30S proteins [8, 18]. Here we have used one-dimensional urea-PAGE analysis to study the distribution of radioactivity into 30S proteins as a function of light fluence. The results, presented in Fig. 1, provide clear evidence for a change in labeling pattern as a function of time, as may be seen by comparison of the radioactivities in gel areas centered at gel slices no. 20 and no. 35 at 10 min and at 40 min.

This change arose from the formation of one or more TC photoproducts, ph_1TC, during the photoincorporation process. As both TC and ph_1TC incorporated covalently into 30S proteins, the observed labeling pattern dependence on time of irradiation reflected the decreasing relative contribution from TC photoincorporation and the increasing relative contribution from ph_1TC incorporation.

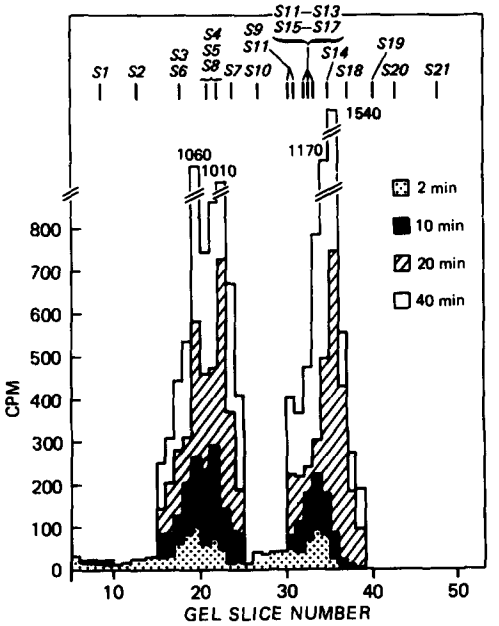

Fig. 1. One-dimensional urea-PAGE analysis of 30S proteins derived from 70S ribosomes irradiated in the presence of [^{3}H]TC for various times. 70S Ribosomes (2.6 μM) were irradiated with [^{3}H]TC (110 μM, 1 Ci/mmole) in TMK buffer at 4° with the PCQ-008L lamp. At the indicated times, samples were removed from the photolysis apparatus and 2 vol. of ethanol–β-mercaptoethanol (9:1, v/v) were added as a quench as soon as possible (<10 sec). All subsequent buffers contained 1% β-mercaptoethanol. The precipitates were collected by centrifugation, resuspended in TMK, and separated into subunits by high salt sucrose density centrifugation. Protein from collected 30S subunits was prepared as described in Materials and Methods. Calibrated protein staining pattern is shown at top.

Scheme 1 summarizes the reactions of interest occurring during photolysis, the evidence for which has been presented elsewhere [8]. By separately determining (a) the overall labeling pattern after irradiation by ribosomes and [^{3}H]TC (Fig. 2a), (b) the rate constant for photodecomposition of TC [reaction (1)], measured by the decrease in TC absorbance at 375 nm as a function of irradiation time, (c) the labeling pattern after irradiation of ribosomes and [^{3}H]ph_1TC (Fig. 2b), and (d) the labeling pattern after dark incubation of ribosomes and [^{3}H]ph_1TC (Fig. 2c), it is possible to calculate the contributions of reactions (2) (Fig. 2d), (3) (Fig. 2e), and (4) (Fig. 2f) to the overall labeling pattern (Fig. 2a), similar to what has been described earlier [8].

$$\text{TC} \xrightarrow{h\nu} \text{Ph}_1\text{TC} \quad (1)$$

$$\text{TC} + \text{Rib} \xrightarrow{h\nu} \text{TC-Rib} \quad (2)$$

$$\text{ph}_1\text{TC} + \text{Rib} \xrightarrow{h\nu} \text{ph}_1\text{TC-Rib} \quad (3)$$

$$\text{ph}_1\text{TC} + \text{Rib} \xrightarrow{\text{dark}} \text{ph}_1\text{TC-Rib} \quad (4)$$

Scheme 1

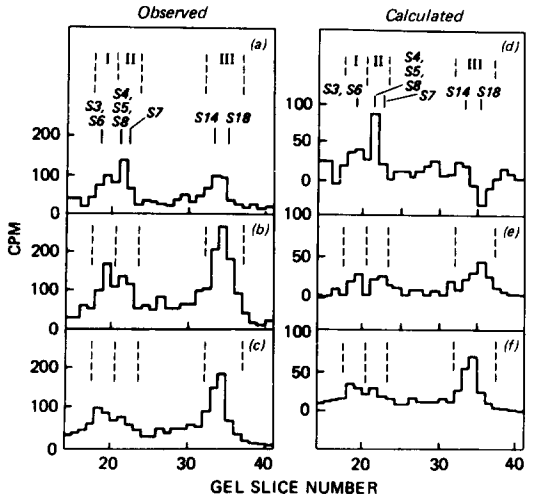

Fig. 2. One-dimensional urea-PAGE analysis of 30S proteins derived from 70S ribosomes irradiated in the presence of [^{3}H]TC or [^{3}H]ph$_1$TC, or incubated in the dark with [^{3}H]ph$_1$TC: Observed and calculated labeling patterns. *Observed patterns:* (a) [^{3}H]TC + hν; (b) [^{3}H]ph$_1$TC + hν; (c) [^{3}H]ph$_1$TC − hν. Experimental conditions: 70S ribosomes (2.6 μM) were incubated with [^{3}H]TC or [^{3}H]ph$_1$TC (50 μM) in TMK buffer at 4° for 10 min with, (a) and (b), or without, (c), concomitant irradiation with the PCQ-008L lamps. Quench and 30S protein isolation procedures were performed as described in the legend to Figure 1. Calculated contributions to observed pattern (a): (d) via reaction (2); (e) via reaction (3); (f) via reaction (4).

Labeling (Fig. 2) via reaction (2) gave dominant incorporation of radioactivity into gel region II (containing proteins S7 and S10 and a portion of proteins S4, S5, and S8), whereas labeling by both reactions (3) and (4) was dominated by incorporation of radioactivity into gel region III (containing proteins S14 and S18). A minor amount of labeling by both TC and ph$_1$TC was also found in gel region I (containing proteins S3 and S6 and the remaining portion of proteins S4, S5 and S8).

The results presented in Figs. 1 and 2 show that the observed labeling pattern had a major contribution from incorporation of native TC only for very low extents of photincorporation. To overcome this difficulty, we did preliminary studies using two different strategies.

Use of an intense light source to increase the fraction of incorporation via native TC. Scheme 1 predicts that, for a given light fluence, the fraction of overall incorporation that arises from reaction (4), the extent of which is time dependent, will decrease as the intensity of the irradiating light source increases. This prediction is borne out by comparison with the labeling pattern obtained when a high-intensity laser is used as the light source (Fig. 3a) in place of the comparatively low-intensity PCQ lamps used above (Fig. 2a). In making this comparison, we used as an index the ratio (incorporation into gel region II)/(incorporation into gel region III) based on the results of Fig. 2 showing that incorporation via reaction (2) took place principally in gel region II whereas incorporation via reaction (4) took place principally in gel region III. The results of several incorporation experiments are collected in Table 1 (No. 2–4). Note that our index decreases with laser dose, reflecting the increased relative importance of incorporation via reaction (3), which also took place principally in gel region III (Fig. 2e). Thus, although high-intensity irradiation improved the situation somewhat, incorporation via reaction (3) still restricted labeling patterns dominated by native TC photoincorporation to

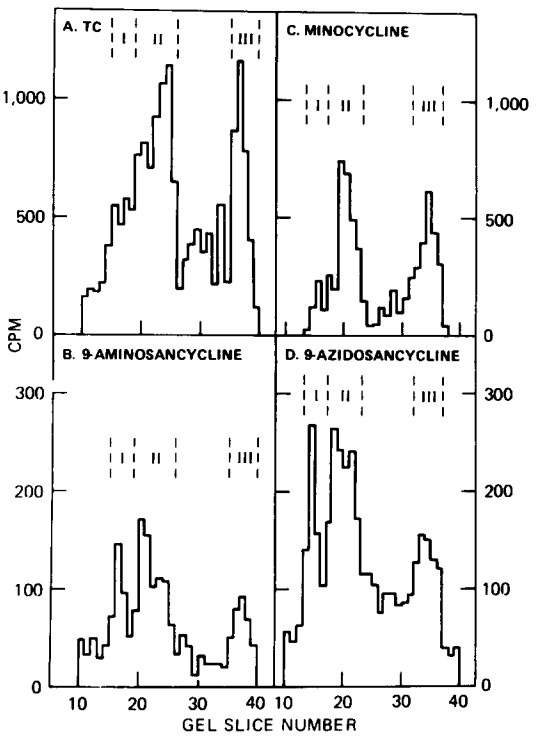

Fig. 3. Urea-PAGE analysis of 30S proteins derived from either 70S ribosomes or 30S subunits irradiated with [^{3}H]TC or tritiated TC derivatives. In all cases, radioactivities are for 5A_{260} units of 30S subunits and are corrected for 100% recovery from the gel. (A) With [^{3}H]TC. Experimental conditions: 70S ribosomes (2.6 μM) were irradiated in the presence of 53 μM [^{3}H]TC with a light dose of 24 Joules of using the continuous wave Argon Ion laser in the presence of 53 μM [^{3}H]TC. At the end of the photolysis, samples were quenched as described for Fig. 1 above. Separation of subunits, preparation of labeled 30S proteins, and urea-PAGE analyses were all performed as described in the legend to Fig. 1. The yield of radioactivity recovered from the gel was 30%. (B) With [^{3}H]-9-aminosancycline. Experimental conditions: Same as A except that 30S subunits (3.9 μM) were used in place of 70S ribosomes, [^{3}H]-9-aminosancycline (2.2 μM) was used in place of [^{3}H]TC, and protein for PAGE analysis was extracted directly from 30S subunits ethanol-precipitated from the photolysis mixture. The light dose was 24 Joules. The yield of radioactivity recovered from the gel was 31%. (C) With [^{3}H]minocycline. Experimental conditions: 30S ribosomal subunits (9.8 μM) were irradiated with PCQ-008L lamps for 90 min (108 Joules) in the presence of 5.9 μM [^{3}H]minocycline. Proteins for PAGE analysis were prepared as in part B. The yield of radioactivity recovered from the gel was 18%. (D) With a mixture of [^{3}H]-9-aminosancycline and [^{3}H]-9-azidosancycline. Experimental conditions: Same as B, except [30S] = 4.9 μM, 9-azidosancycline = 0.55 μM, 9-aminosancycline = 0.65 μM, and the light dose was 24 Joules. The yield of radioactivity recovered from the gel was 40%.

Table 2. Growth inhibition of *E. coli* by, inhibitory activity of ribosomal activity of, and photolability of various tetracyclines

Compound*	Growth inhibition	Polyphenylalanine synthesis		Phenylalanyl-tRNA binding		
	Relative inhibitory activity†	IC_{50} (μM)	Relative inhibitory activity†	IC_{50} (μM)	Relative inhibitory activity†	% Photo-destruction‡
Minocycline	194	6.0	292	2.2	576	7.0§
7-Chlorotetracycline‖						46.9
Tetracycline	100	17.5	100	8.9	100	70.6
7-Aminosancycline	92	15.8	111	14.0	106	5.7
9-Aminosancycline	66	14.4	122	14.0	106	9.9
Sancycline	66	21.5	81	24	59	65.3
7-Bromosancycline	38	44	40	31	55	74.0
9-Azidosancycline	30	38.5	45	37	37	¶

* Sancycline is 6-demethyl-6-deoxytetracycline; minocycline is the trivial name for 7-dimethylaminosancycline.

† Activity relative to tetracycline taken as 100.

‡ The compounds (50 μM) were dissolved in TMK buffer and irradiated at 4° for 1 hr in a 2 ml cuvette with a PCQ-008L lamp assembly (UV Products, Inc.) having a maximal output between 340 and 380 nm. % Photodestruction = $(A_0 - A)/A_0$ where A_0 is the initial absorbance at 375 nm, and A is the absorbance after 1 hr of photlysis.

§ Minocycline photolyzed for 90 min.

‖ 7-Chlorotetracycline was not assayed for inhibitory activity but, on the basis of clinical efficacy and relative inhibition of mammalian protein synthesis [23], it would be expected to be more potent than tetracycline.

¶ See Fig. 4.

rather low overall incorporation levels (<5% of the ribosomes).

Functional and photoincorporation studies with TC derivatives. Another approach to obtaining higher levels of incorporation in the inhibitory TC site than is possible with TC itself is to use TC derivatives as photoaffinity labels that, while they retain the inhibitory activity of TC, are less subject to the complications encountered with TC use. Two classes of such derivatives that could be suitable are those having either lesser or greater photolability than TC. In terms of Scheme I, the desired result for the former class would be that the rate of reaction (1) would be decreased relative to the rate of reaction (2), thereby generating less photoproduct, and less incorporation of photoproduct, during photolysis. The latter class of TC derivatives are those containing pendant photolabile groups with higher quantum yields for photodestruction than TC itself, thus enabling photoincorporation via reaction (2) to proceed much more rapidly than photoincorporation via reaction (3).

Both halo- and amino-derivatives of TC and of sancycline, substituted at the 7- or 9-positions, are known to be effective inhibitors of bacterial growth [9, 10, 19, 20]. Such derivatives were tested for their abilities to inhibit (i) cell growth, as measured by turbidometric assay, (ii) cell-free poly(U)-directed polyphenylalanine synthesis, and (iii) Phe-tRNA binding to ribosomes. They also were tested for their intrinsic photolabilities, as measured by the rate of u.v. irradiation-induced loss of absorbance at 375 nm [8]. The results of these studies are summarized in Table 2. The 7- and 9-amino derivatives of sancycline have clear potential value as photoaffinity labels, having exhibited biological activities comparable to TC but much decreased photolability (the especially high activity of minocycline had been noted earlier by Tritton [21]; a more detailed study of TC derivative photolability is presented in Hasan *et al.* [22]). By contrast, sancycline itself, as well as 7-chlorotetracycline and 7-bromosancycline, appear to be unsuitable. Though active as ribosomal inhibitors, these derivatives had photodecomposition rates similar to that of TC. Also summarized in Table 2 are inhibitory results obtained with 9-azidosancycline. This derivative was 1/2 to 1/3 as active as sancycline and, by virtue of its aromatic azide group, has definite potential as a photoaffinity label containing a pendant photolabile group whose quantum yield for photodestruction is much larger than that of TC itself. This difference in quantum yields is clearly illustrated by the changes observed in the electronic absorption spectrum on irradiation of a solution of 9-azidosancycline (Fig. 4), in which loss of the shoulder at 265 nm, corresponding to photolysis of the azido function, was essentially complete at a light fluence corresponding to only minor loss of the sancycline chromophore at 378 nm.

On the basis of the results presented in Table 2, preliminary photoincorporation studies on isolated 30S subunits were conducted with the limited quantities of [^{3}H]-minocycline, [^{3}H]-9-aminosancycline, and [^{3}H]-9-azidosancycline of high specific radioactivity thus far available. Sample PAGE analyses of photoaffinity labeling experiments conducted with these derivatives are shown in Fig. 3, b-d, and the incorporation levels obtained in these and similar experiments are listed in Table 1. One clear result is the apparent similarity in labeling patterns among

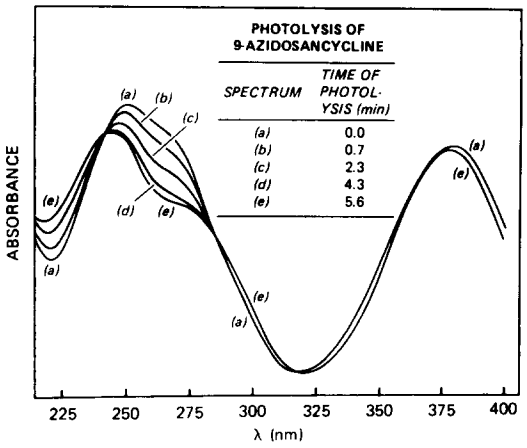

Fig. 4. Changes in the ultraviolet spectrum of 9-azidosancycline as a function of irradiation time. 9-Azidosancycline (~ 50 μM) dissolved in TMK buffer was irradiated with the PCQ-008L lamps for the times indicated.

these three derivatives and TC itself; in particular, each derivative labeled region II to the highest extent, thus paralleling labeling by native TC.

At the light fluences typically used in our work with TC (6–24 Joules), 9-aminosancycline showed a considerably higher fraction of total labeling in region II than did TC itself (compare experiments 7 and 8 with experiments 1–5). We take this result as a preliminary indication that 9-aminosancycline labels principally via a photoexcited state of its native structure, rather than via a photoproduct and, from this standpoint, appears superior to TC as a photoaffinity label. However, the stoichiometric level of incorporation achieved with this derivative was considerably lower than that achieved with TC, even allowing for the large differences in the concentrations of TC and 9-aminosancycline employed. Thus, for example, comparing experiments 3 and 5 with experiment 7, at a similar light fluence a 24-fold greater TC concentration resulted in a 200-fold greater TC incorporation. It is possible that the drawback presented by the apparent low quantum yield for photoincorporation of 9-aminosancycline could be overcome using much higher light fluences than those used in experiments 7 and 8. A first test of this approach is provided by experiment 6, in which [^{3}H]minocycline, another aminosancycline, was used as the photoffinity label. Here, as compared with experiment 8, a 2.7-fold greater concentration of photoaffinity label irradiated with 30S subunits with approximately 4.5 times the light fluence, afforded a 16-fold increase in photoincorporation into region II. On the other hand, labeling of gel region III was relatively high in experiment 6, suggesting that incorporation of aminosancycline photoproduct may become important at very high light fluences.

The photolabeling of 30S subunits with [^{3}H]-9-azidosancycline shows dominant incorporation of radioactivity in gel region II at both light fluences tested (experiments 9 and 10). However, in contrast to the results obtained with 9-aminosancycline, the extent of incorporation increased much less than linearly with light fluence. This is consistent with the notion that labeling was proceeding predominantly via the highly photolabile azide function, which would be expected to be largely destroyed following exposure to a light fluence of 6 Joules, as in experiment 9. What is encouraging about the potential of 9-azidosancycline as a photoaffinity label is that, in addition to its dominant labeling of region II, it also exhibited much higher yields of photoincorporation than did 9-aminosancycline. Thus, comparing experiments 8 and 9, a 4-fold lower concentration of 9-azidosancycline irradiated with 30S subunits with a 4-fold lower light fluence yielded the same amount of region II labeling. Clearly, further photolabeling experiments with this derivative, as well as with other biologically active photolabile derivatives of sancycline such as 7-azidosancycline and 7- and 9-diazosancycline [10] are merited. Such experiments are currently underway.

In conclusion, in this work we show that substitution of an intense laser light source for the lower intensity lamp used formerly in photoaffinity labeling studies of ribosomes by TC increased the fraction of labeling via native TC and decreased the fraction of labeling by TC photoproduct. This result, which presumably reflects a minimization of TC photoproduct incorporation via a light-independent reaction, is fully consistent with results obtained earlier [8]. We also show that TC derivatives that retain inhibitory activity toward ribosomal function and that have favorable photochemical properties are potentially more useful that TC itself as photoaffinity labels for the TC inhibitory site. In particular, as shown using derivatives synthesized in radioactive form, two amino-substituted sancyclines that are less photolabile than TC, and 9-azidosancycline which is much more photolabile than TC, gave photolabeling patterns much like that calculated for native TC. Of the three derivatives tested, 9-azidosancycline appears most promising for further investigation, since it photoincorporated into ribosomes with much higher yield than the amino derivatives. Such higher yields would be useful not only for determining the identities of labeled proteins, the obvious next step in this work, but also for preparing appreciable quantities of labeled subunits. Such labeled subunits, in combination with antibodies toward TC, should allow localization of the site or sites of photoincorporation on the 30S subunit by the technique of immunoelectron microscopy, in direct analogy to our earlier work with puromycin [24–26].

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