Photoincorporation of Tetracycline into *Escherichia coli* Ribosomes. Identification of the Major Proteins Photolabeled by Native Tetracycline and Tetracycline Photoproducts and Implications for the Inhibitory Action of Tetracycline on Protein Synthesis†

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ABSTRACT: Radioactivity is incorporated essentially exclusively into ribosomal protein when [^{3}H]tetracycline is irradiated in the presence of ribosomes. Such incorporation is shown to arise from three different processes: photoincorporation of native tetracycline, photoincorporation of tetracycline photoproduct, and, in the absence of β-mercaptoethanol, light-independent incorporation of tetracycline photoproduct. When both the rate of tetracycline to tetracycline photoproduct conversion and the protein labeling pattern produced by tetracycline photoproduct (utilizing both polyacrylamide gel electrophoresis and specific immunoprecipitation) are determined separately, it is possible to subtract out the contribution of tetracycline photoproduct to the overall labeling pattern obtained on irradiation of tetracycline and ribosomes and thus determine the labeling pattern due to native tetracycline incorporation. In this way we show that protein S7 is the major protein labeled by native tetracycline in both the presence and absence of β-mercaptoethanol. High labeling of proteins S18 and S4, which was reported in a previous study of tetracycline photoincorporation [Goldman, R. A., Cooperman, B. S., Strycharz, W. A., Williams, B. A., & Tritton, T. R. (1980) *FEBS Lett. 118*, 113–118], is shown here to result from the incorporation of tetracycline photoproduct. The significance of the photolabeling of protein S7 by native [^{3}H]tetracycline has been investigated, first, by measuring the effect of nonradioactive tetracycline on [^{3}H]tetracycline photoincorporation and second, by comparing the photoincorporation of [^{3}H]tetracycline with that of its biochemically less active epimer, 4-epitetracycline. Photoincorporation of native [^{3}H]tetracycline into several ribosomal proteins is decreased on addition of nonradioactive tetracycline (the "competition" experiment). The labeling of protein S7 is decreased the most, thus providing evidence for the saturability of such labeling. 4-Epitetracycline photoincorporates into ribosomal protein to a much lower extent than does tetracycline, thus demonstrating the stereospecificity of the photoincorporation process. The same stereospecificity is also found with respect to both inhibition of ribosome-dependent protein synthesis and binding to the 30S subunit. The results of the competition and stereospecificity experiments, taken together with the relative lack of sensitivity of S7 labeling to added β-mercaptoethanol, implicate protein S7 as a component of the high-affinity ribosomal site believed to be directly involved in the inhibitory action of tetracycline. Such placement of S7 suggests a structural model for the mechanism of tetracycline inhibition of aminoacyl-tRNA binding to the A site in which tetracycline binding prevents simultaneous two-site attachment of aminoacyl-tRNA at both the codon–anticodon site and the peptidyltransferase center.

The *Escherichia coli* ribosome is currently the object of study in a number of laboratories, with the ultimate goal being the construction of a model for its mechanism of action. A necessary step in such a process is the localization of functional domains within the ribosome structure. In our own work we have been using photoaffinity labeling with both antibiotics (Cooperman et al., 1979; Cooperman, 1980a,b; Nicholson et al., 1982a,b) and initiation factor 3 (Cooperman et al., 1981) to identify such domains. This approach, combined with immunoelectron microscopy, has led most recently to a localization of a puromycin binding site on the 50S subunit at or close to what is presumptively the peptidyltransferase site (Olson et al., 1982).

Recently this approach has been extended to investigate tetracycline (TC)[1] binding sites on both *E. coli* ribosome (Goldman et al., 1980) and the rat liver ribosome (Reboud et al., 1982). In our previous publication (Goldman et al., 1980) we demonstrated that TC, an inhibitor of tRNA binding to the A site, will photoincorporate into *E. coli* ribosome and identified the major proteins labeled as S18 and S4, and, to a lesser extent, S7, S13, and S14 (Goldman et al., 1980). We here report on results obtained from more detailed examination of the photoincorporation process. These studies have revealed that TC photodecomposes appreciably during a typical photoincorporation experiment. As a result, the covalent incorporation observed an irradiation of TC and ribosomes arises not only from light-dependent incorporation of TC but also from light-dependent and, in the absence of β-mercaptoethanol, light-independent incorporation of TC photoproduct. In this paper we develop a methodology for determining the contribution that native TC makes to the observed labeling pattern and show that the major protein photolabeled by native TC is S7.

TC is known to inhibit aminoacyl-tRNA binding to the ribosome at the so-called A site (Kaji, 1979; Gale et al., 1981), and the current prevailing view (Strel'tsov et al., 1975; Tritton, 1977) is that such inhibition is a consequence of TC binding

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[1] Abbreviations: βME, β-mercaptoethanol; NEM, *N*-ethylmaleimide; TC, tetracycline; E-TC, 4-epitetracycline; TP30, TP50, and TP70, total protein from 30S, 50S, and 70S particles, respectively; $NaDodSO_4$, sodium dodecyl sulfate; Me_2SO, sulfoxide.

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to a unique high-affinity site on the ribosome, rather than to any of the very large number of f low-affinity sites. Therefore, in order to conclude that TC-photolabeled ribosomal proteins are at or near the A site, it is necessary to obtain evidence that photolabeling proceeds from the high-affinity site.

In the current work such evidence is sought via three approaches: first, a comparison of labeling obtained in the presence and absence of β-mercaptoethanol in order to obtain evidence regarding the sensitivity of photolabeling to scavenging from solution; second, a comparison of the labeling obtained by using [^{3}H]TC with that obtained by using 4-[^{3}H]epitetracycline (E-TC), which is much weaker than TC as an inhibitor of protein synthesis (Summ & Christ, 1967), in order to obtain evidence regarding the stereospecificity of photolabeling; third, a study of the effect of added nonradioactive TC on the incorporation of [^{3}H]TC in order to obtain evidence regarding the saturability of photolabeling. In addition, the binding of [^{3}H]TC to ribosomal subunits is reinvestigated and compared with subunit binding of [^{3}H]E-TC.

Experimental Procedures

Materials

Buffers. The following buffers were used: TMK, 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 50 mM KCl; TMKNa, TMK plus 400 mM NaCl; TKM, 50 mM Tris-HCl (pH 7.6), 0.5 mM $MgCl_2$, and 50 mM KCl; A, 1 M aqueous NaH_2PO_4–methanol, 1:2, apparent pH 3.5.

Materials. TC and [7-^{3}H]TC (1 Ci/mmol) were obtained and utilized as previously described (Goldman et al., 1980). E-TC was obtained as a mixture with TC by incubation of TC in buffer A for 16 h at 25 °C as previously described (McCormick et al., 1957). This mixture was resolved by thin-layer chromatography on Kieselguhr G plates [Hoeck, 1972; R_f(E-TC) 0.03; R_f(TC) 0.08]. Appropriate regions of the plate were extracted with absolute ethanol, a 0.5 volume of acetone was added, and the mixture was maintained at 0 °C for 1 h. Precipitated salts and other impurities were removed by centrifugation, and the supernatant was lyophilized. Repetition of the ethanol–acetone precipitation procedure with continually decreasing amounts of solvent for a total of 5 times afforded preparations of E-TC and TC in overall yields (based on initial TC) of 23% and 16%, respectively, whose visible absorption spectra were essentially indistinguishable from that of a standard sample of TC. Concentrations of solutions of TC and E-TC were calculated by using an ϵ_{373} equal to 23 840 (measured in TMK buffer containing 0.1% β-mercaptoethanol). [^{3}H]E-TC was prepared in an analogous fashion.

Ribosomes (70S) were prepared from *E. coli* Q13 bacteria harvested in mid- or late-log phase, using the modifications of the Traub et al. (1971) procedure previously described (Jaynes et al., 1978). All operations were performed at 4 °C. Large-scale preparations of 30S and 50S subunits were obtained by zonal centrifugation in a Beckman Ti15 rotor essentially as described by Sypherd & Wireman (1974). Small-scale preparations of subunits from 70S ribosomes labeled with TC or a TC photoproduct were obtained by ultracentrifugation through a sucrose gradient made up in TMKNa, which in most experiments, except as noted, also contained 0.1% β-mercaptoethanol. Ribosomes (70S) were incubated in TMKNa for 10 min at 37 °C prior to loading in the centrifuge tube. For swinging bucket rotors, a 5–20% gradient was used and the following centrifugation conditions (4 °C) were employed for various quantities of labeled 70S ribosomes applied per tube: 50–150 A_{260} units, SW 27 rotor, 20 000 rpm, 16 h; 10–50 A_{260} units, SW 27.1 rotor, 20 000 rpm, 16 h; 3–10 A_{260} units, SW 50 rotor, 45 000 rpm, 135 min. When vertical rotors were utilized, following Winkelman & Kahan (1979), 70S ribosomes were layered over 100 μL of 7.5% sucrose which had itself been layered over a 15–30% sucrose gradient made up in TMKNa buffer. The following centrifugation conditions (4 °C) were employed: 3–10 A_{260} units/tube, VTi80 rotor, 50 000 rpm, 34 min; 10–100 A_{260} units/tube, VTi50 rotor, 50 000 rpm, 90 min. Gradient fractionation and subunit collection were as previously described (Jaynes et al., 1978) except that subunits were precipitated by addition of 1 volume of ethanol and incubation at −20 °C for 1 h.

[^{14}C]Phe-tRNA^{Phe} was prepared from [^{14}C]Phe (New England Nuclear; 400–450 Ci/mol) and either bulk stripped tRNA (Grand Island Biological) or purified yeast tRNA^{Phe} (Boehringer-Mannheim) and crude factors, as described by Ravel & Shorey (1971).

Methods

Photoinduced Incorporation of TC or TC Photoproducts into Ribosomes. Photolysis experiments were performed in a 4-mm diameter quartz tube with Rayonet RPR 3500-Å lamps as previously described (Cooperman et al., 1975) or a UV Products, Inc. PCQ 008L lamp assembly having a maximal output between 3400 and 3800 Å. Photolyses were performed at 4 °C in TMK buffer at a 70S ribosome concentration of 2.6 μM (100 A_{260}/mL). Immediately following photolysis (within 10 s) ribosomes were precipitated with 2 volumes of ethanol–β-mercaptoethanol (9:1), resuspended in TMK buffer containing 1% β-mercaptoethanol, reprecipitated with 2 volumes of ethanol, and resuspended in TMKNa containing 1% β-mercaptoethanol for eventual subunit separation (see Materials).

Light-Independent Incorporation of TC Photoproducts into Ribosomes. These experiments were performed in an essentially identical manner with that used for the photoinduced incorporations described above, but without irradiation.

Polyacrylamide Gel Electrophoresis and Immunoprecipitation Analysis of Labeled Proteins. Protein from labeled subunits was extracted by using the Mg^{2+}–acetic acid procedure (Hardy et al., 1969) and precipitated by addition of 5 volumes of acetone and −20 °C incubation (Barritault et al., 1976). One- and two-dimensional urea–polyacrylamide gel electrophoresis and one-dimensional $NaDodSO_4$–urea–polyacrylamide gel electrophoresis analyses of labeled proteins were performed as previously described (Nicholson et al., 1982a). Idealized one-dimensional protein staining patterns may be seen in Figures 2 and 3. Specific immunoprecipitation analyses were performed by a sandwich technique (Roberts & Roberts, 1975) as previously described (Grant et al., 1979). All antisera employed showed no cross-reactivity against other 30S proteins, were titered against either total 30S protein or purified proteins, and were used at a concentration at or close to their equivalence points (Lindahl et al., 1977).

Binding Measurements. For measurement of TC (or E-TC) binding, ribosomes or ribosomal subunits and [^{3}H]TC (or [^{3}H]E-TC) were combined in a total volume of 100 μL and centrifuged in a VTi80 rotor at 4 °C essentially as described above, following a protocol for binding experiments suggested by Draper & von Hippel (1979). Following centrifugation, gradients were pumped through a flow cell in a recording spectrophotometer, and 0.4-mL fractions were collected. TC binding stoichiometries to ribosomal subunits were estimated from determinations of A_{260nm} (1 A_{260nm} equals 26 pmol of 70S ribosomes, 39 pmol of 50S subunits, and 78 pmol of 30S subunits) and of radioactivity within fractions corresponding

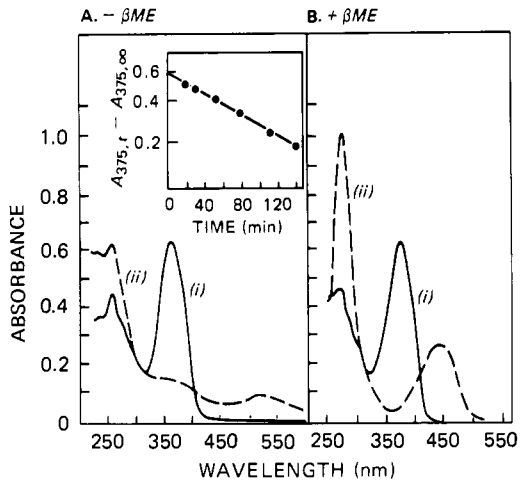

FIGURE 1: Spectral monitoring of TC photolysis. (A) In the absence of 0.1% (v/v) β-mercaptoethanol: (i) initial spectrum; (ii) after full conversion to photoproducts (ph_1TC); (inset) first-order plot of change in A_{375} with time. $A_{375\infty}$ is the limiting value of A_{375} at full photolysis. (B) In the presence of 0.1% (v/v) β-mercaptoethanol: (i) initial spectrum; (ii) after full conversion to photoproducts ($ph_{2s}TC$). Experimental conditions: TC (30 μM) was dissolved in TMK buffer and irradiated at 4 °C in a 2-mL cuvette. For part A (inset), the sample was irradiated by using the RPR-3500-Å lamps. Spectra were taken at the indicated times. For part B, the sample was irradiated with the PCQ-008L lamps.

to the 30S or 50S subunit (see Figure 5). Such determinations were corrected for background, defined as the A_{260nm} units and radioactivities observed within the fractions of interest in control experiments lacking ribosomes or ribosomal subunits. Background values for A_{260nm} units and radioactivities typically fell within 13 ± 4% and 9 ± 1%, respectively, for 30S subunits, and 10 ± 2% and 39 ± 10%, respectively, for 50S subunits, compared with observed experimental values.

Assays of Ribosomal Function. [^{14}C]Phe-tRNA^{Phe} binding to ribosomes was measured with a filter assay as described previously (Goldman et al., 1980). Reaction mixtures were 1% in Me_2SO as a precaution to fully solubilize TC photoproducts. This amount of Me_2SO was shown to have no effect on observed levels of Phe-tRNA^{Phe} binding. Poly(U)-dependent polyphenylalanine synthesis was also performed as described previously (Cooperman et al., 1975), but with 1.5% Me_2SO added to ensure solubility of TC and TC photoproducts.

Results

TC Photodecomposes To Form Photoproducts ph_1TC and $ph_{2s}TC$. Irradiation of a TC solution in TMK buffer equilibrated with air with the 3500-Å RPR lamps to a change in its electronic absorption spectrum in both the presence and absence of β-mercaptoethanol. For both conditions, the major peak at 265 nm increases, the major peak at 375 nm decreases, and a new peak appears, centered at 534 nm in the absence of β-mercaptoethanol and at 435nm in its presence. The rate of this photodecomposition shows apparent first-order kinetics when monitored by the decrease in 375-nm absorbance (Figure 1A, inset). These spectral results provide direct evidence that different photoproducts are formed when TC is irradiated in the presence of oxygen on the one hand or in the presence of β-mercaptoethanol on the other. We may thus define two reactions, 1 and 2s, where it is understood that ph_1TC and

$$TC \xrightarrow{h\nu,\ O_2} ph_1TC \quad (1)$$

$$TC \xrightarrow{h\nu,\ \beta ME} ph_{2s}TC \quad (2s)$$

Table I: Incorporation of TC, ph_1TC, and $ph_{2s}TC$ into Ribosomal Protein[a]

radioactive ligand	expt	added 0.1% βME	irradiation (10 min)	dark incubation time (min)	incorporation (% mol/mol) TP30	TP50
ph_1TC	1	−	−	0 (120)[b]	0.2	0.4
	2	−	−	10	1.18	0.55
	3	−	−	120	11.5	3.8
	4	−	−	120 (+50 mM NEM)[c]	2.7	3.4
	5	−	+	0	1.98	0.71
$ph_{2s}TC$	6	−	−	120	1.4	0.7
	7	+	+	0	0.38	0.35
TC	8	−	−	0	0.03	0.04
	9	−	+	0	1.30	0.45
	10	+	+	0	0.56	0.38

[a] All experiments were conducted in TMK buffer with 50 μM radioactive ligand and 2.6 μM 70S ribosomes. Experiments 1, 3, 4, and 6 reactions were performed at 30 °C, and the remaining experiments were performed at 4 °C. All experiments were quenched by addition of 2 volumes of ethanol–β-mercaptoethanol (9:1). Separation of the precipitated 70S ribosomes into subunits and protein extraction was performed as described under Methods. [b] In experiment 1, the 9:1 quench solution was added to ribosomes prior to ph_1TC addition, and the resulting suspension was incubated with ph_1TC for 2 h. [c] In experiment 4, ph_1TC and *N*-ethylmaleimide were added to 70S ribosomes at the same time.

$ph_{2s}TC$ correspond to all of the photoproducts produced under each experimental condition.[2] We have not as yet structurally characterized these products.

Covalent Incorporation of TC Photoproducts. Since our overall purpose is to identify the major sites of photolabeling by native TC, it is important to determine whether the TC photoproducts described above incorporate into ribosomes and, if so, to evolve a strategy for minimizing such incorporation during the course of a photolabeling experiment. Accordingly, a series of experiments were carried out to examine possible incorporation of TC photoproducts into ribosomes, the results of which are summarized in Table I. These experiments show that ph_1TC undergoes light-independent covalent incorporation (experiment 2), the extent of which increases with time (experiment 3 vs. 2) and is greatly diminished in the presence of the sulfhydryl reagent *N*-ethylmaleimide (experiment 4 vs. 3), a result consistent with the notion that ph_1TC is an electrophile. The higher incorporation seen on irradiation compared with dark incubation (experiment 5 vs. 2) shows that ph_1TC also undergoes light-dependent incorporation. Light-independent incorporation of $ph_{2s}TC$ is much less extensive (experiment 6 vs. 3) so that incorporation seen at limited photolysis times (experiment 7) in the presence of β-mercaptoethanol is predominantly light dependent. It should be noted that the incorporation seen with TC photoproducts is essentially exclusively into ribosomal protein.

One-dimensional urea–polyacrylamide gel electrophoresis analyses of the 30S proteins labeled by ph_1TC in the absence of β-mercaptoethanol and by $ph_{2s}TC$ in its presence (Figure 2a,b) show much lower levels of incorporation for the latter, in accord with the results in Table I, and a simpler labeling pattern. That is, ph_1TC shows major incorporation into gel region I, which includes proteins S3 and S6, into gel region II, which includes proteins S4, S5, S7, and S8, and into gel

[2] Recent examination of solutions of TC photolyzed in the presence of β-mercaptoethanol by high-performance liquid chromatography has revealed the presence of one major and several minor photoproducts (T. Hasan, M. Allen, and B. S. Cooperman, unpublished observations).

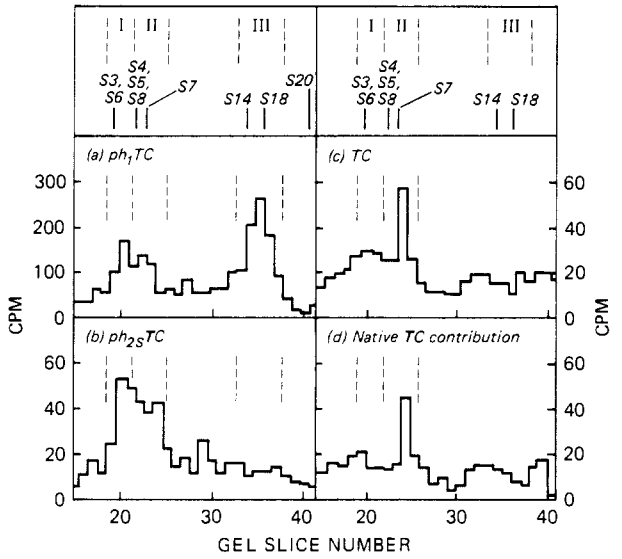

FIGURE 2: One-dimensional urea–polyacrylamide gel electrophoresis analysis of 30S proteins derived from 70S ribosomes irradiated in the presence of [^{3}H]ph$_1$TC, [^{3}H]ph$_{2s}$TC, or [^{3}H]TC. Observed and calculated labeling patterns. Observed patterns: (a) [^{3}H]ph$_1$TC; (b) [^{3}H]ph$_{2s}$TC; (c) [^{3}H]TC. Calculated pattern: (d) native [^{3}H]TC contribution to pattern c. Note change in scale in parts b–d vs. part a. Regions of gel not shown contained negligible radioactivity. Experimental conditions: 70S ribosomes (2.6 μM) were irradiated with [^{3}H]ph$_1$TC (50 μM) in the absence of β-mercaptoethanol or with [^{3}H]ph$_{2s}$TC (50 μM) or [^{3}H]TC (50 μM) in the presence of 0.1% β-mercaptoethanol in TMK buffer at 4 °C for 10 min. Quench and 30S protein isolation procedures were performed as described under Experimental Procedures.

region III, which includes proteins S14 and S18, whereas major ph$_{2s}$Tc incorporation is restricted to gel regions I and II.

Photoincorporation of Native TC into Ribosomes. S7 Is the Major Labeled Protein: Explanation of Previous Results. Photolysis of ribosomes and [^{3}H]TC leads to incorporation of radioactivity into ribosomes (Table I, experiments 8–10) that is almost exclusively into ribosomal protein. Our results on TC photoproducts incorporation lead to the conclusion that carrying out photoincorporation experiments for limited times offers the best chance to being able to determine the labeling pattern of native TC, since as TC is converted to photoproducts the observed labeling pattern will reflect a relatively greater contribution from photoproduct and a relatively lesser one from native TC.

Scheme I summarizes the reactions of interest during photolysis in the presence of β-mercaptoethanol. Since reactions 3s and 4s both lead to incorporation, our strategy for determining incorporation via native TC is to directly determine the contribution of ph$_{2s}$TC incorporation to the observed incorporation and to subtract this contribution from the observed incorporation.

Scheme I: Relevant Reactions in the Presence of β-Mercaptoethanol

$$\text{TC} \xrightarrow{h\nu,\ \beta\text{ME}} \text{ph}_{2s}\text{TC} \qquad (2s)$$

$$\text{TC} + \text{ribosomes} \xrightarrow{h\nu,\ \beta\text{ME}} \text{TC–ribosomes} \qquad (3s)$$

$$\text{ph}_{2s}\text{TC} + \text{ribosomes} \xrightarrow{h\nu,\ \beta\text{ME}} \text{ph}_{2s}\text{TC–ribosomes} \qquad (4s)$$

A one-dimensional urea–polyacrylamide gel electrophoresis analysis of 30S proteins derived from 70S proteins labeled by TC in the presence of β-mercaptoethanol is shown in Figure 2c. The contribution of ph$_{2s}$TC incorporation to this pattern is overestimated by the results presented in Figure 2b since, for the experiment analyzed in Figure 2b, ph$_{2s}$TC is present

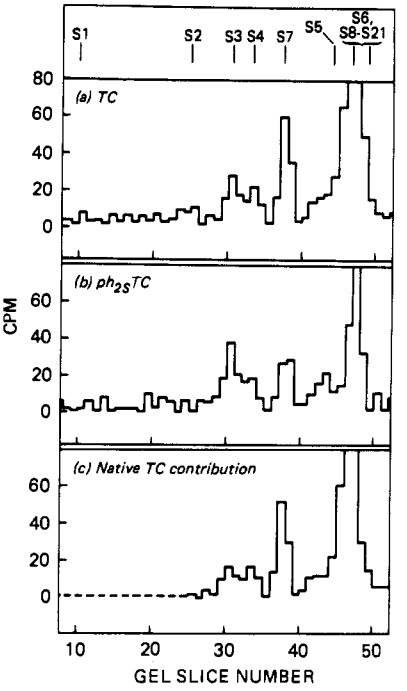

FIGURE 3: One-dimensional NaDodSO$_4$–urea–polyacrylamide gel electrophoresis analysis of 30S protein derived from 70S ribosomes irradiated in the presence of [^{3}H]TC or [^{3}H]ph$_{2s}$TC, and β-mercaptoethanol. Observed and calculated patterns. Observed patterns: (a) [^{3}H]TC; (b) [^{3}H]ph$_{2s}$Tc. Calculated pattern: (c) native [^{3}H]TC contribution to pattern a. Experimental conditions: Same as in Figure 2, parts b and c.

at 50 μM from the beginning, whereas for the experiment analyzed in Figure 2c ph$_{2s}$TC concentration is initially zero. Determination of the appropriate correction factor requires evaluation of the rate constant for reaction 2s and this constant was determined by monitoring A_{375nm} during the photolabeling reaction. For a 10-min photolysis the correction factor was found to be 0.30 (a detailed calculation is presented in the Appendix). Thus, we may calculate a urea–polyacrylamide gel electrophoresis labeling pattern for native TC by a gel slice-by-gel slice subtraction of the pattern for ph$_{2s}$TC labeling in Figure 2b (multiplied by the correction factor 0.30) from the pattern in Figure 2c.[3] This calculated pattern (Figure 2d) shows a single dominant region of labeling in gel region II (centered at gel slice 25), which includes proteins S4, S5, S7, and S8. No other 30S proteins migrate close to this peak of radioactivity. Radioactivities found in regions I and II via native TC and ph$_{2s}$TC incorporation are summarized in Table II (experiments 1 and 2).

In order to identify which of the proteins in gel region II is (are) most highly labeled, we next performed the same kind of subtractive analysis using an NaDodSO$_4$–urea–polyacrylamide gel electrophoresis gel, as seen in Figure 3. Radioactivities incorporated into proteins S3–S5 and S7 via native TC and ph$_{2s}$TC incorporation are also summarized in Table II. It should be noted that the large peak of radioactivity seen in gel slices 46–50 represents the combined labeling not only of the 15S proteins which bunch up at the bottom end of the gel but also of small amounts of noncovalently bound TC and

[3] Our treatment is somewhat oversimplified and thus approximate since it neglects possible secondary effects of TC on TC photoproducts incorporation and vice versa. At the concentrations employed in the experiments in Figure 2, inclusion of such effects would have at most only minor effects on the calculated patterns. However, at much higher concentrations such effects are not always negligible (see Table II, experiments 7 and 8).

Table II: Incorporation of TC, $ph_{2s}TC$, ph_1TC, E-TC, and ph_{2s}-E-TC into Protein Gel Regions or Individual Proteins[a]

expt	radioactive ligand (50 μM)	added 0.1% βME		incorporation (% mol/mol of subunit) urea-PAGE region I	II	III	30S proteins ($NaDodSO_4$-urea-PAGE) S2	S3	S4	S7	S5
1	$ph_{2s}TC$	+		0.120	0.120			0.097	0.047	0.077	0.047
2	TC	+	overall	0.094	0.152		0.042	0.075	0.053	0.125	0.035
			via native TC	0.058	0.116			0.046	0.039	0.102	0.021
			via $ph_{2s}TC$	0.036	0.036			0.029	0.014	0.023	0.014
3	ph_1TC	−		0.41	0.60	1.32		0.24	0.18	0.21	0.21
4	TC	−	overall	0.27	0.44	0.50		0.175	0.117	0.22	0.105
			via native TC	0.117	0.21	0.00		0.083	0.047	0.141	0.027
			via ph_1TC	0.156	0.23	0.50		0.092	0.070	0.078	0.078[b]
5	E-TC	+		0.055	0.059			0.020	0.016	0.022	
6	ph_{2s}E-TC	+		0.110	0.118						

expt	radioactive ligand (50 μM)	added 0.1% βME		rel incorporation[c] on addition of nonradioactive TC urea-PAGE region I	II	III	30S proteins ($NaDodSO_4$-urea-PAGE) S2	S3	S4	S7	S5
7	TC	+	via native TC	0.93	0.56			0.86	0.94	0.67	0.99
			via $ph_{2s}TC$	0.98	2.8			1.04	1.15	2.35	1.33
8	TC	−	via native TC	0.80	0.50			0.72	0.79	0.55	0.94
			via ph_1TC	1.40	1.54	0.97		1.40	1.27	1.85	0.99

[a] Radioactivities reported include corrections of each gel track to 100% yield from a typical observed value of 35 ± 5% (Jaynes et al., 1978; Nicholson et al., 1982a). Such corrections are justified because of the very low incorporation values seen in suitable control experiments (e.g., experiment 8 in Table I). However, since gel yields did not vary much, the only change which would result from not making the correction would be in the absolute amount of incorporation of radioactivity. The relative sizes of the peaks of radioactivity would be unaffected. Experiments were repeated at least 2–3 times, giving fully reproducible calculated labeling patterns. For example, for incorporation via native TC in experiment 4, the labeling order S7 > S3 > S4 > S5 was observed consistently. The reproducibility of the numbers shown, in a relative sense, was ±10%. For urea-polyacrylamide gel electrophoresis (PAGE) results such reproducibility was possible because determining radioactivity in a region, consisting of three to four gel slices, eliminates errors due to imperfect alignment. For the $NaDodSO_4$-urea-polyacrylamide gel electrophoresis (PAGE) results, the proteins S2, S3, S4, S5, and S7 were clearly resolved from one another, and misalignment was not a problem. Absolute values of incorporation showed a day-to-day variation of ±20%, in part due to slight variations in ribosomal preparation and/or light fluence. [b] This value may include some labeling from S9 and S12. [c] Relative incorporation is the ratio of cpm found in the presence of added nonradioactive TC (250 μM) to that found in its absence. The correction factors for calculating $ph_{2s}TC$ and ph_1TC contribution in experiments 7 and 8 are 0.37 and 0.28, respectively.

$ph_{2s}TC$ which have not been removed prior to polyacrylamide gel electrophoresis analysis (see Experimental Procedures). From Figure 3 it is clear that protein S7 is the major protein photolabeled by native TC within the group S3–S5, S7. The identification of S7 is secure, since TC incorporation should have only a minor effect on protein mobility in an $NaDodSO_4$ gel and the S7 peak is very well resolved. The remaining uncertainty concerns protein S8. A two-dimensional polyacrylamide gel electrophoresis analysis of 30S protein labeled by TC is presented in Figure 4. As may be seen, the only major area of radioactivity is found near the group S3, S4, S5, and S7. This result is not inconsistent with the one-dimensional $NaDodSO_4$–urea–polyacrylamide gel electrophoresis results because retarded migration of affinity labeled proteins in two-dimensional polyacrylamide gel electrophoresis analysis has been noted earlier (Czernilofsky et al., 1974; Grant et al., 1979; Goldman et al., 1980). However, from Figure 4 it is clear that protein S8 and the area around it contain little radioactivity. Confirmatory immunoprecipitation evidence for low S8 labeling and high S7 labeling (in the absence of β-mercaptoethanol) is presented below.

Similar one-dimensional polyacrylamide gel electrophoresis analyses were carried out on 30S proteins derived from 70S ribosomes labeled by TC and ph_1TC in the absence of β-mercaptoethanol [see Goldman (1980) for details]. The results, presented in Table II (experiments 3 and 4), yield calculated native TC labeling patterns (region II > region I ≫ region III; S7 > S3, S4, S5) similar to that calculated for native TC labeling in the presence of β-mercaptoethanol (experiment 2).

Because of the greater complexity of the labeling pattern obtained in the absence of β-mercaptoethanol, specific immunoprecipitation of 30S proteins derived from 70S ribosomes labeled by [^{3}H]TC and [^{3}H]ph_1TC was also used to identify the major proteins labeled by native TC and ph_1TC, again by a subtractive analysis. The results are presented in Table III and clearly show that S18, falling within region III, is the major protein labeled by ph_1TC and that S7 is the major protein labeled by TC, in agreement with the one-dimensional polyacrylamide gel electrophoresis results presented in Table II. The precision of the calculated values is not high, thus rendering unreliable the lower percentage values. It is also worth noting that S6 is substantially labeled by both native TC and ph_1TC, that S8 is not labeled at all, and that S18 labeling by native TC is very low.

We previously identified S18 > S4 as the two major proteins photolabeled by TC (Goldman et al., 1980; see Table III). Our current results show that these two proteins are labeled chiefly by ph_1TC [via the light-dependent and light-independent reactions; see Goldman (1980)]. We attribute the prominence of labeled S18 and S4 in our earlier study to experimental protocols used in that work (long photolysis times and absence of β-mercaptoethanol during workup) which favor ph_1TC incorporation.

One-dimensional urea–polyacrylamide gel electrophoresis analyses were also carried out for 50S proteins derived from 70S ribosomes labeled with TC and $ph_{2s}TC$ in the presence of β-mercaptoethanol and with TC and ph_1TC in the absence of β-mercaptoethanol. In neither case was any region labeled by native TC to an extent greater than one-third the labeling seen in region II for 30S protein. Accordingly, further identification of labeled 50S proteins was not pursued.

In summary, S7 is shown to be the major ribosomal protein labeled by native TC in both the presence and absence of

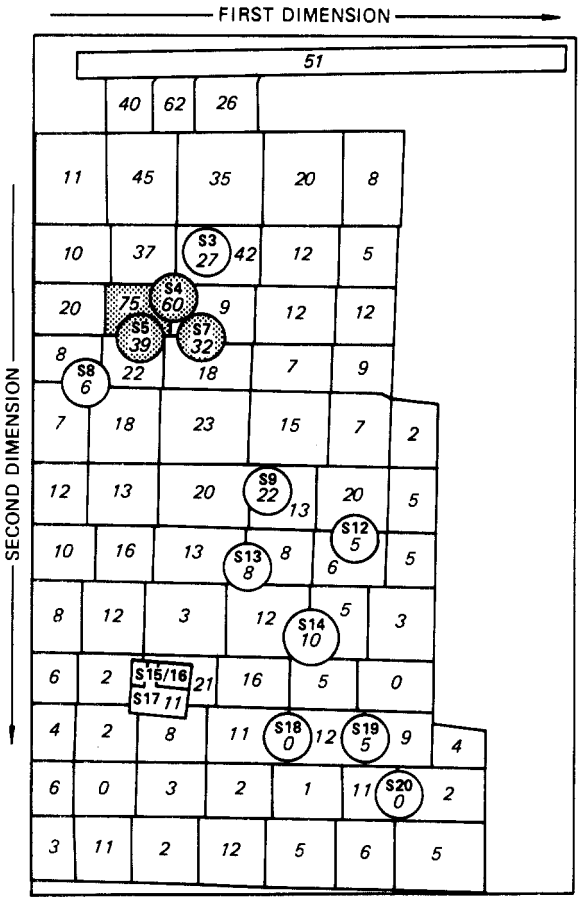

FIGURE 4: Two-dimensional urea–polyacrylamide gel electrophoresis analysis of basic 30S proteins derived from 70S ribosomes irradiated in the presence of [^{3}H]TC and β-mercaptoethanol. Experimental conditions: Same as in Figure 2, part c. Boldface numbers refer to proteins and italicized numbers correspond to radioactivity (cpm). The area of highest radioactivity is stippled.

β-mercaptoethanol, and labeling by ph_1TC is seen to account for the high incorporation of radioactivity into proteins S18 and S4 previously reported (Goldman et al., 1980).

Tests for the Significance of Labeling by Native TC. Having identified S7 as the major protein photolabeled by TC, we next extend our studies to examine the significance of such labeling. In particular, we ask two questions. First, what is the stereospecificity of labeling? Here we compare photoincorporation of TC with that of 4-epitetracycline (E-TC), a considerably weaker inhibitor of ribosomal function (Summ & Christ, 1967, and below). Second, what is the effect of added nonradioactive TC on the incorporation of native [^{3}H]TC? A reduction would be evidence for the saturability of photolabeling.

One-dimensional polyacrylamide gel electrophoresis analyses of labeled 30S proteins derived from either 70S ribosomes or isolated 30S subunits labeled with [^{3}H]E-TC and [^{3}H]ph_{2s}-E-TC (prepared by photolysis of E-TC, in analogy to ph_{2s}TC) are shown in Figure 5, and radioactivities found in subunits or in given gel regions or proteins are listed in Table II (experiments 5 and 6). The $NaDodSO_4$–urea–polyacrylamide gel electrophoresis results clearly show that E-TC incorporates to a lesser extent than TC into each of the resolved 30S proteins, with the relative reduction in S7 labeling being the most marked. The urea–polyacrylamide gel electrophoresis analysis is in accord with this result. Both region I, containing proteins S3 and S6, and region II, containing proteins S4, S5, and S7, and S8, show lower labeling by E-TC, and the reduction is somewhat greater in region II.

Table III: Immunoprecipitation Results for Protein Labeled by [^{3}H]TC in the Absence of β-Mercaptoethanol[a]

antiserum to protein	% radioactivity precipitated above background[b]	calcd contributions to labeling by TC[c] via native TC	via ph_1TC	previous results[d]
S3	7	−1	8	2
S4	6	0	6	23
S6	22	7	15	
S7	21	10	11	11
S8	0	0	0	
S13	9	0	9	9
S14	2	−4	6	12
S18	21	3	18	36
S20	(0)	0	0	

[a] Experimental conditions are as in Table II, experiments 3 and 4. [b] Values reported are for percent labeled 30S protein precipitated. Radioactivity precipitated by antiserum to S20 was taken as zero, since urea–polyacrylamide gel electrophoresis analysis (see Figure 2a) showed no radioactivity migrating close to this protein. [c] The ph_1TC contribution was calculated by multiplying values found in a labeling experiment with ph_1TC by the factor which corrects for ph_1TC formation during the experiment with TC (0.38; see text) and by the ratio of the total incorporation of radioactivity in the experiments with ph_1 TC to that with TC (equal to 1.8). The TC contribution is the difference between the observed percent precipitated and the contribution from ph_1TC. [d] % radioactivity precipitated above background. From Goldman et al. (1980). Experimental conditions: 70S ribosomes (2.6 μM) were irradiated with [^{3}H]TC (50 μM) in TMK buffer at 4 °C for 1 h with the RPR 3500-Å lamps. β-mercaptoethanol was not included in the workup procedure.

In contrast, ph_{2s}-E-TC photoincorporation (experiment 6) proceeds to essentially the same extent and with the same one-dimensional urea–polyacrylamide gel electrophoresis labeling pattern as does ph_{2s}TC photoincorporation (experiment 1). In fact, to a first approximation, essentially all of the incorporation seen with E-TC can be attributed to incorporation via ph_{2s}-E-TC. Thus, the incorporation of native TC into *E. coli* ribosomes, and into 30S proteins in particular, is critically dependent on the stereochemistry at position 4 in the TC structure.

Two additional points need to be made with respect to labeling by E-TC. First, E-TC has apparently the same intrinsic photoreactivity as does TC, as evidenced by the identical changes observed as a function of light flux in the electronic absorption spectrum of a solution of E-TC in TMK buffer containing β-mercaptoethanol as compared with the changes observed for a similar solution of TC (see Figure 1B). Second, our E-TC preparation involves its extraction from Kieselguhr plates, and suitable controls were performed which ruled out the possibility that material extracted from the plates was interfering with photoincorporation.[4]

The effects of added nonradioactive TC (250 μM) on the incorporation of [^{3}H]TC and [^{3}H]ph_{2s}TC into 30S proteins derived from labeled 70S ribosomes in the presence of β-

[4] In one control, parallel photoincorporation experiments showed no significant difference on urea–polyacrylamide gel electrophoresis analysis for TC extracted from a plate as compared with a standard sample of TC. In a second control, the possibility that E-TC, which is extracted from a different region of the Kieselguhr plate than TC, might be contaminated with an interfering substance not found in TC was eliminated by showing that neither nonradioactive TC nor nonradioactive E-TC, each extracted from Kieselguhr plates and each at a concentration of 50 μM, had an appreciable effect on the photoincorporation of standard [^{3}H]TC (50 μM) into 70S ribosomes, as measured by one-dimensional polyacrylamide gel electrophoresis analysis of labeled 30S proteins.

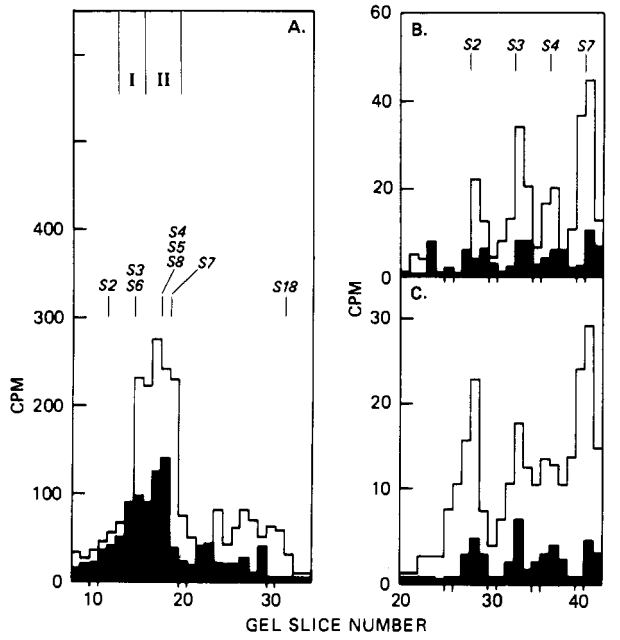

FIGURE 5: One-dimensional polyacrylamide gel electrophoresis analyses of 30S proteins derived from either 70S ribosomes or isolated 30S subunits irradiated in the presence of either [^{3}H]TC or [^{3}H]E-TC. (A) Urea–polyacrylamide gel electrophoresis analyses of 30S proteins derived from 70S ribosomes irradiated with [^{3}H]TC or with [^{3}H]E-TC. Experimental conditions: Same as in Figure 2c for both TC and E-TC. Radioactivities shown are for proteins extracted from 30 A_{260} units of 30S subunits. The average yield of radioactivity recovered from the gel was 40 ± 5%. (B) $NaDodSO_4$–urea–polyacrylamide gel electrophoresis analyses of 30S proteins derived from 70S ribosomes irradiated with [^{3}H]TC or with [^{3}H]E-TC. Experimental conditions: As described in part A. Radioactivities shown are for proteins extracted from 30 A_{260} units of 30S subunits. The yield of radioactivity recovered from the gel was 10%. (c) $NaDodSO_4$–urea–polyacrylamide gel electrophoresis analyses of 30S proteins derived from 30S subunits irradiated with [^{3}H]TC or with [^{3}H]E-TC. Experimental conditions: As described for part A except that 30S subunits (4.6 μM) replace 70S ribosomes. Following the ethanol–β-mercaptoethanol (9:1) quench, the resulting 30S pellet was resuspended in TMK buffer and reprecipitated with ethanol–β-mercaptoethanol (9:1). This washing process was repeated 5 times. Protein was extracted from the washed pellet in the usual manner and subjected to polyacrylamide gel electrophoresis analysis. Radioactivities shown are for 4 A_{260} units of 30S subunits. The yield of radioactivity recovered from the gel was 14%.

mercaptoethanol and of [^{3}H]TC and [^{3}H]ph$_1$TC in its absence were also measured by one-dimensional urea–polyacrylamide gel electrophoresis and $NaDodSO_4$–urea–polyacrylamide gel electrophoresis. By use of a subtractive analysis, as discussed above, the contributions to overall incorporation of native TC photoincorporation, ph$_{2s}$TC photoincorporation, and ph$_1$TC incorporation were determined. The results are presented in Table II in terms of incorporation of radioactivity relative to that found in the absence of added nonradioactive TC (experiments 7 and 8). They show that labeling by native [^{3}H]TC is markedly reduced in gel region II and in protein S7 in both the presence and absence of β-mercaptoethanol. Lesser reductions in region I and S3 and S4 labeling are seen in the absence of β-mercaptoethanol, and these effects are even smaller in the presence of β-mercaptoethanol. In both cases the labeling of S5 is essentially unaffected. In contrast, labeling by both [^{3}H]ph$_{2s}$TC and [^{3}H]ph$_1$TC is generally stimulated by added nonradioactive TC, with the largest effects seen for region II and protein S7 labeling.

Functional Assays of TC, E-TC, and TC Photoproducts. E-TC, ph$_1$TC, and ph$_{2s}$TC were tested for their functionality, relative to TC, in (1) binding to or competing with TC binding

Table IV: TC and E-TC[a] Binding to Ribosomal Subunits As Measured by Cosedimentation

		bound per subunit (mol/mol)			
		30S		50S	
particle[b]	buffer[c]	TC	E-TC	TC	E-TC
70S	TKM	0.062	0.019	⩽0.016[d]	⩽0.005[d]
70S	TMKNa	0.056	0.021	⩽0.016[d]	⩽0.004[d]
30S	TMK	0.092 (1.16)[e]	0.028		
30S	TMK			0.008	0.004

[a] Added to 100 μL of preincubation solution at a concentration of 0.55 μM except as indicated. [b] Added to 100 μL of preincubation solution at a concentration of 1.54 μM. [c] Both in preincubation solution and in sucrose gradient. [d] Upper limit–possibly contaminated with TC or E-TC bound to 30S subunits due to incomplete resolution in sucrose gradients. [e] TC concentration, 20.6 μM.

Table V: Relative Biochemical Activities[a] of TC or TC Photoproducts

concn of TC, E-TC, or TC photoproducts (μM)	poly(U)-dependent poly(Phe) synthesis				Phe-tRNA^{Phe} binding[b]		
	TC	E-TC	ph$_1$TC	ph$_{2s}$TC	TC	ph$_1$TC	ph$_{2s}$TC
3	0.94[c]	1.02			0.90		
10	0.77	0.95	0.88	0.99			
30	0.49	0.68	0.99	0.99	0.78	0.98	0.94
100	0.27	0.42	0.83	0.85	0.55	0.94	0.93
300		0.18	0.60			0.58	0.80

[a] Activity in the absence of TC or TC photoproduct taken as 1.00. [b] Relative activity at saturating TC is 0.40. [c] Values reported are reproducible to ±5–10%.

to ribosomal subunits, (2) inhibiting poly(U)-dependent polyphenylalanine synthesis, and (3) inhibiting poly (U)-dependent Phe-tRNA^{Phe} binding to ribosomes.[5] Typical binding experiments are shown in Figure 6. When [^{3}H]TC is combined with 70S ribosomes in molar excess and the resulting solution is subjected to ultracentrifugation through a sucrose density gradient under conditions leading to dissociation into subunits, the TC binding surviving the gradient is seen to be preferential for 30S subunits over 50S subunits (Figure 6A). A qualitatively similar conclusion is reached from the results of testing the TC binding separately to isolated 30S and 50S subunits (Figure 6B,C). E-TC, though binding to a much lesser extent than TC, shows the same relative specificity toward the 30S subunit. The amounts of TC and E-TC cosedimenting with subunits are listed in Table IV.

Added nonradioactive TC (20 μM) decreases bound radioactive TC by about two-thirds although the overall stoichiometry of TC binding is increased (Table IV, footnote e). By contrast added nonradioactive ph$_1$TC and ph$_{2s}$TC at 20 μM have little effect on radioactive TC binding (data not shown). The poorer binding abilities of E-TC, ph$_1$TC, and ph$_{2s}$TC compared with TC are paralleled by their lower inhibitory activity in assays of ribosomal function (Table V).

Discussion

In this work we seek to use the approach of photoaffinity labeling to identify the ribosomal binding site for TC which

[5] TC extracted from a Kieselguhr plate had indistinguishable activity from a standard TC sample in these functional assays.

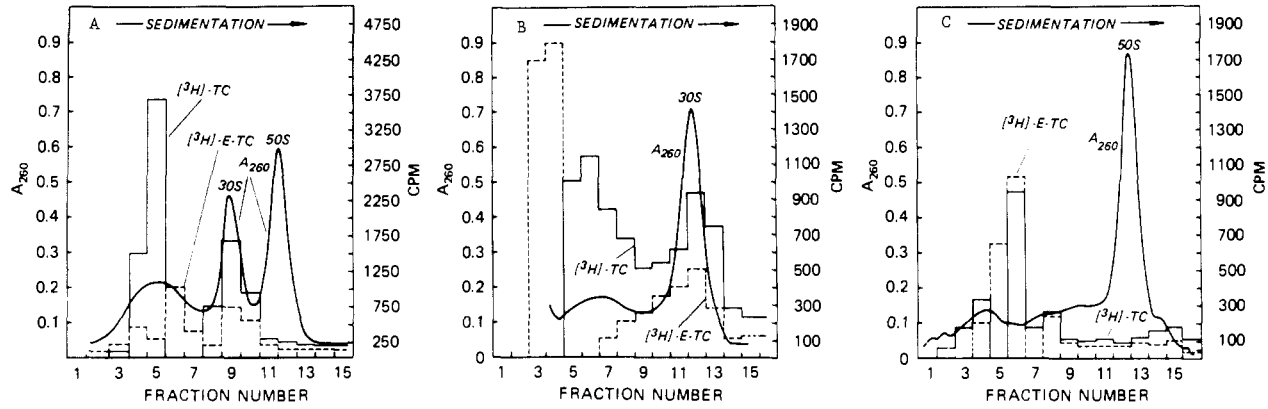

FIGURE 6: Binding of [^{3}H]TC and [^{3}H]E-TC to ribosomal subunits. In these experiments, initial ribosome or subunit concentration in the 100-μL incubate was 1.54 μM, and [^{3}H]TC or [^{3}H]E-TC concentration was 0.55 μM. A VTi80 rotor was used. (A) 70S ribosomes; sucrose gradient buffer TKM; 50 000 rpm, 34 min. (B) 30S subunits; sucrose gradient buffer TMK; 50 000 rpm, 65 min. (C) 50S subunits; sucrose gradient buffer TMK; 50 000 rpm, 40 min. In this figure, the radioactivities presented are corrected for background radioactivity observed in the absence of added ribosomal particles. Such correction has a relatively modest effect (10–40%; see Experimental Procedures) on the magnitudes of the radioactivities migrating with ribosomal subunits but, as expected, decreases to a large extent the radioactivity representing unbound [^{3}H]TC or [^{3}H]E-TC at the top of the gradient. Negative values, which were sometimes observed at the top of the gradient, are shown as zero and are artifactual, representing inexact matching of the fraction volumes of the subunit-containing and background samples. Such inexact matching also accounts for the large positive values seen at the top of the gradient. Typically 90% of the radioactivity is found at the top of the gradient when the data are uncorrected.

is related to its inhibitory function. There are two major problems to be overcome in order for this approach to be successful. The first is to resolve the labeling due to native TC from an overall pattern which includes labeling by TC photoproduct. The second is to demonstrate that labeling by native TC takes place from the functional site.

Above we have shown how a separate study of the labeling pattern obtained with TC photoproducts allows identification of S7 as the major protein labeled by native TC. Our work provides an illustration of the importance in photoaffinity labeling studies of studying possible incorporation by the photoproduct of the labeling reagent (Bayley & Knowles, 1977, Mas et al., 1980; Payne et al., 1980), something which is all too frequently left undone.

With respect to the second problem, the results of several previous studies (Connamacher & Mandel, 1965, 1968; Day, 1966a,b; Maxwell, 1968; Strel'tsov et al., 1975; Tritton, 1977) allows the following conclusions to be drawn regarding TC binding to the bacterial ribosome: (a) there is a single tight TC binding site per 70S ribosome, with a K_D estimated between 1 and 20 μM; (b) there are a large number of weaker sites (perhaps several hundred), having an average K_D of approximately 1 mM, and (c) TC inhibition of template-dependent aminoacyl-tRNA binding is a consequence of tight-site binding. In addition, the inhibitory tight site is thought to be located on the 30S subunit, based on preferential binding of TC to 30S as opposed to 50S subunits at low TC concentration (Connamacher & Mandel, 1965, 1968; Day, 1966a; this work, Table III). The question then is whether S7 labeling proceeds from this functional tight site. The results consistent with such functional site labeling by native TC are the following: (1) A 30S protein, S7, is labeled to the highest extent by the potent ribosomal inhibitor TC. Labeling by the much weaker inhibitors ph_1TC and ph_{2s}TC shows no such specificity for S7 labeling. (2) Similarly, inhibitory activity, 30S subunit binding, and labeling of S7 (as well as of other 30S proteins) show the same stereospecific preference for TC over E-TC. (3) Labeling of S7 is competed for by nonradioactive TC. (4) Labeling of S7 is relatively insensitive to added β-mercaptoethanol.

The latter two points require further comment. From Table II it is clear that S7 labeling by [^{3}H]TC (50 μM) shows the greatest reduction on addition of nonradioactive TC. Such a reduction is evidence that S7 labeling takes place from a ribosomal binding site, since random labeling from solution should show no such reduction. However, the magnitude of the reduction seen (33–45%) cannot be taken as evidence for or against the labeling of S7 from the high-affinity site referred to above. It cannot be taken as evidence for such labeling because the observed reduction is much less than the approximately 80% reduction expected for high-affinity site labeling. This latter figure results from assuming that the high-affinity site is almost or completely saturated by 50 μM [^{3}H]TC, so that for our experiments, in which the extent of photoincorporation is proportional to light fluence, the addition of 250 μM unlabeled TC should lead to a reduction in tritium labeling which approximately parallels the reduction in TC specific radioactivity. The magnitude of the observed reduction of S7 labeling cannot be taken as evidence against high-affinity site labeling, because of the photosensitizing properties of TC (Esparza et al., 1976; Wiebe & Moore, 1977). Elsewhere, for example, we have shown that addition of TC greatly stimulates site-specific photoincorporation of puromycin (Grant et al., 1979), and data presented in Table I show clear TC-induced stimulation of both ph_1TC photoincorporation and ph_{2s}TC photoincorporation into some ribosomal proteins. If added TC also photosensitized [^{3}H]TC photoincorporation, the observed reduction in labeling by [^{3}H]TC on addition of nonradioactive TC would underestimate the reduction which would result from a competition effect alone.

Previous photoaffinity labeling studies have led to the suggestion that added solutes, such as aromatic amines and thiols, can act as scavengers to preferentially decrease labeling of a receptor from solution or from low-affinity sites compared to labeling from a high-affinity site (Ruoho et al., 1973; Maasen & Moller, 1974). Our results (Table II) showing that added β-mercaptoethanol reduces native TC photolabeling of S7 by only 28% whereas native TC photolabeling of region I and S3 labeling are reduced by 50% and 45%, respectively, may thus be taken as evidence that S7 labeling takes place from a relatively high-affinity site.

It is instructive to compare results 1–4 above obtained with S7 with those obtained for S18, the other protein labeled to a major extent in this study. Here we use region III labeling

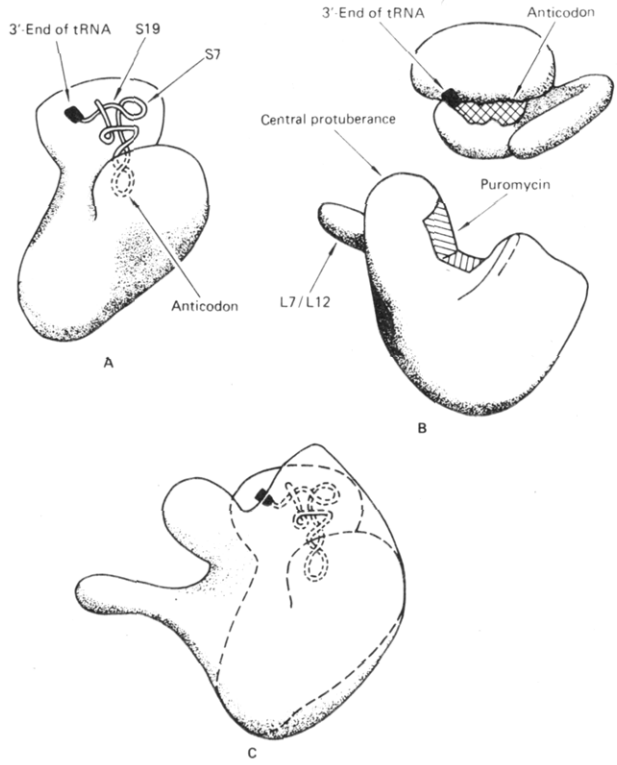

FIGURE 7: Models for aminoacyl-tRNA binding to both 30S subunits and 70S ribosomes. (A) To 30S subunit. The site for the 3′ end of tRNA is identified with the site of puromycin photoincorporation into the 30S subunit (Olson et al., 1980). (B) To 70S ribosome. Exploded view. The 3′ end of tRNA would lie within the site of puromycin photoincorporation into the 50S subunit (Olson et al., 1982). (C) To 70S ribosome. Condensed view. The 50S subunit is shown on top of the 30S subunit. A figure similar to this one has been presented previously (Olson et al., 1982).

(Table II) as an indication of S18 labeling, which is reasonable based on the immunoprecipitation results (Table III). In the absence of β-mercaptoethanol, added TC has no effect on region III labeling by ph_1TC (Table II, experiment 8), and this is true for both the light-dependent and light-independent processes (Goldman, 1980). Also, addition of β-mercaptoethanol totally suppresses region III labeling (Figure 2c). These results, as well as the low activity of ph_1TC in functional assays, lead us to conclude that the apparent specificity of ph_1TC labeling of region III (Table II, experiment 3) is a consequence of the uniquely high chemical reactivity of the Cys-10 position in protein S18 (Yaguchi et al., 1978; Ghosh & Moore, 1979) and does not reflect labeling from a high-affinity site for ph_1TC labeling binding. The phenomenon of high S18 labeling in a variety of affinity labeling studies has been discussed by us elsewhere (Nicholson et al., 1982b).

Although results 1–4 above are only consistency checks and fall short of proving that S7 is labeled from the functional tight site, S7 is clearly *the* ribosomal protein that is most clearly implicated at this site by our work. It is thus of interest to briefly consider what placement of S7 at the functional TC site would imply about the mechanism of TC inhibition of aminoacyl-tRNA binding to the A site of the ribosome. In the discussion that follows we use the Lake (1980) model for the structure of the 30S subunit and, in slightly modified form, of the 70S ribosome (Figure 7).

By its function as an adaptor molecule, two positions of aminoacyl-tRNA are constrained to bind directly to the ribosome, the anticodon triplet at the codon–anticodon site and the 3′ terminus containing the charged amino acid at the peptidyltransferase center. There is a great deal of evidence [summarized in Lake (1980)] that the codon–anticodon site is within the cleft region of the 30S subunit. Furthermore, we have recently shown (Olson et al., 1982) that puromycin, a structural analogue of the 3′ terminus of aminoacyl-tRNA, incorporates into the region of the 50S subunit indicated in Figure 7, thus providing direct evidence for the location of the peptidyltransferase center. These two positions suffice to fix, at least approximately, the orientation of the tRNA molecule on the ribosome surface as indicated in Figure 7. Given the rigidity of the tRNA molecule, a substance which binds to the ribosome anywhere within the tRNA binding locus could, by preventing simultaneous two-site attachment, weaken tRNA binding to the ribosome and disrupt normal functioning of an aminoacyl-tRNA ribosome complex in protein synthesis. This is a plausible mechanism for the inhibitory action of TC and one which is in accord not only with the available evidence suggesting that TC has no direct competitive interaction with either the codon–anticodon site or the peptidyltransferase center (as summarized in Kaji, 1979; Gale et al., 1981) but also with the overlap between the position of S7 on the 30S subunit determined by immunoelectron microscopy (Lake, 1978) and the putative aminoacyl-tRNA binding locus (Figure 7). Additional evidence for such an overlap is provided by the result of Ofengand (1980) that an aryl azide derivative of the 8-(4-thiouridine) base in Phe-$tRNA^{Phe}$ bound in the A site photo-cross-links to S19, a protein shown by immunoelectron microscopy to neighbor S7.

In summary, the results presented in this paper (a) demonstrate that native TC photoincorporates into 70S ribosomes and labels S7 to the highest extent of any ribosomal protein, (b) support the idea, suggested by others previously (Strel'tsov et al., 1975; Tritton, 1977), that TC binding to a unique high-affinity site on the 30S subunit is responsible for TC inhibition of aminoacyl-tRNA binding, (c) show that S7 labeling is site specific and quite possibly takes place from the aforementioned high-affinity site, and (d) lead to formulation of a proposed mechanism for the inhibitory action of TC.

Acknowledgments

The excellent technical assistance of Nora Zuño is gratefully acknowledged.

Appendix: Calculation of Contribution of Labeling via $ph_{2s}TC$ to Overall Labeling

Under the conditions of our experiments, where only a small fraction of ribosomes ($\leq 2\%$) are labeled, the rate of formation of ribosomes labeled by $ph_{2s}TC$ is given by eq A1. Here k_{4s}

$$\frac{d[R - ph_{2s}TC]}{dt} = k_{4s}[R]_0[ph_{2s}TC] \qquad (A1)$$

is the rate constant for $ph_{2s}TC$ incorporation during a photolysis experiment via reaction 4s (see text), and $[R]_0$, the initial ribosome concentration, is considered constant. For experiment 1 in Table II in which labeling is carried out starting with $ph_{2s}TC$, $[ph_{2s}TC]$ may also be considered a constant equal to the initial concentration and $[R - ph_{2s}TC]$ is given by eq A2, where t is the time.

$$[R - ph_{2s}TC] = k_{4s}[R]_0[ph_{2s}TC]_0 t \qquad (A2)$$

For experiment 2 in Table II, $[ph_{2s}TC]$ is forming during the course of photolysis according to eq A3:

$$[ph_{2s}TC] = [TC]_0(1 - e^{-k_{2s}t}) \qquad (A3)$$

where k_{2s} is the rate constant for eq (2s) (see text), the photodestruction of TC. Substituting this value of $[ph_{2s}TC]$ into A1 and integrating gives eq A4. Therefore, the ratio of R

$$[R - ph_{2s}TC] = k_{4s}[R]_0[TC]_0\frac{k_{2s}t - 1 + e^{-k_{2s}t}}{k_{2s}} \quad (A4)$$

$-ph_{2s}TC$ formed starting with TC (experiment 2) as compared with that formed starting with an identical concentration of $ph_{2s}TC$ (experiment 1) is given by eq A5 and is equal to the

$$\frac{[R - ph_{2s}TC]\ (\text{experiment 2})}{[R - ph_{2s}TC]\ (\text{experiment 1})} = \frac{k_{2s}t - 1 + e^{-k_{2s}t}}{k_{2s}t} \quad (A5)$$

correction factor employed in the text (see Results). For TC photolysis in the presence of β-mercaptoethanol, $k_1 = 0.077$ min^{-1}. Thus for a 10-min photolysis the correction factor is equal to 0.30. Similarly, for TC photolysis in the absence of β-mercaptoethanol, the correction factor is given by eq A6:

$$\frac{[R - ph_1TC]\ (\text{experiment 4})}{[R - ph_1TC]\ (\text{experiment 3})} = \frac{k_1t - 1 + e^{-k_1t}}{k_1t} \quad (A6)$$

where k_1 is the rate constant for TC photodestruction via reaction 1. For a 10-min photolysis the correction factor is 0.38.

Registry No. TC, 60-54-8; E-TC, 79-85-6.

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