Howard, G. A., & Traut, R. R. (1974) *Methods Enzymol. 30*, 526.
Jaynes, E. N., Jr., Grant, P. G., Giangrande, G., Wieder, R., & Cooperman, B. S. (1978) *Biochemistry 17*, 561.
Kaltschmidt, E., & Wittmann, H. G. (1970) *Anal. Biochem. 36*, 901.
Kinoshita, J., Tanaka, N., & Umezawa, H. (1970) *J. Antibiot. 23*, 288.
Lee, C. K., & Vince, R. (1978) *J. Med. Chem. 21*, 176.
Lessard, J. L., & Pestka, S. (1972) *J. Biol. Chem. 247*, 6909.
Lindahl, L., Post, L., Zengel, J., Gilbert, S. F., Strycharz, W. A., & Nomura, M. (1977) *J. Biol. Chem. 252*, 7365.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Maxwell, I. H. (1968) *Mol. Pharmacol. 4*, 25.
Miskin, R., & Zamir, A. (1974) *J. Mol. Biol. 87*, 121.
Nathans, D., & Neidle, A. (1963) *Nature (London) 197*, 1076.
Pestka, S. (1970) *Arch. Biochem. Biophys. 136*, 80.
Pestka S. (1974a) *Antimicrob. Agents Chemother. 6*, 474.
Pestka, S. (1974b) *Methods Enzymol. 30*, 462.
Roberts, J. W., & Roberts, C. W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 147.
Strel'tsov, S. A., Kukhanova, M. K., Gurskii, G. V., Kraevskii, A. A., Belyavskaya, I. V., Viktorova, L. S., Treboganov, D. D., & Gottikh, B. P. (1975) *Mol. Biol. (Engl. Transl.) 9*, 729.
Symons, R. H., Harris, R. J., Greenwell, P., Eckermann, D. J., & Vanin, E. F. (1978) in *Bioorganic Chemistry: A Treatise to Supplement Bioorganic Chemistry, An International Journal* (Van Tamelen, E., Ed.) Vol. 4, p 409, Academic Press, New York.
Tada, K., & Trakatellis, A. C. (1970) *Antimicrob. Agents Chemother.*, 227.
Traub, P., Mizushima, S., Lowry, C. V., & Nomura, M. (1971) *Methods Enzymol. 20*, 391.
Tritton, T. R. (1977) *Biochemistry 16*, 4133.
Vazquez, D. (1966) *Biochim. Biophys. Acta 114*, 277.
Vince, R., Almquist, R. G., Ritter, C. L., & Daluge, S. (1975) *Antimicrob. Agents Chemother. 8*, 439.
Zia, H., & Price, J. C. (1976) *J. Pharm. Sci. 65*, 226.

On the Mechanism of Chloramphenicol-Induced Changes in the Photoinduced Affinity Labeling of *Escherichia coli* Ribosomes by Puromycin. Evidence for Puromycin and Chloramphenicol Sites on the 30S Subunit†

P. G. Grant, B. S. Cooperman,* and W. A. Strycharz

ABSTRACT: Chloramphenicol has been shown to cause a major change in the ribosomal protein labeling pattern when *Escherichia coli* ribosomes are photolyzed in the presence of radioactive puromycin. In the absence of chloramphenicol, the major labeled protein is L23, while in its presence S14 becomes the major labeled protein [Grant, P. G., Strycharz, W. A., Jaynes, E. N., Jr., & Cooperman, B. S. (1979) *Biochemistry* (preceding paper in this issue)]. This paper reports a detailed investigation of this change, which has allowed the following conclusions to be drawn. (1) The labeling of S14 by puromycin proceeds from a puromycin binding site. (2) The stimulation of S14 labeling by chloramphenicol requires a specific chloramphenicol binding site. (3) Both of the above binding sites are located on the 30S subunit. (4) The stimulation of S14 labeling occurs as a result of a chloramphenicol-dependent light-induced alteration of the 30S subunit. Overall, our results and those obtained in related studies provide evidence that there are binding sites for both chloramphenicol and puromycin on both the 50S and 30S subunits and that the sites on each of the subunits are close to one another. They also provide a clear demonstration of the importance of examining labeling patterns as a function of light fluence in photoincorporation experiments. The possible significance of the previously unsuspected puromycin and chloramphenicol sites on the 30S subunit is discussed.

We have been investigating the photoinduced affinity labeling of the *Escherichia coli* ribosome by puromycin. In previous work we have shown that L23 is the major protein labeled by puromycin, that S14 is labeled to a secondary extent, and that labeling of these proteins occurs from a puromycin-specific binding site or sites (Cooperman et al., 1975; Jaynes et al., 1978). In the preceding paper (Grant et al., 1979) we extended these studies to measure the effects of other ribosome-directed antibiotics on the labeling process. A dramatic change was noted in the presence of chloramphenicol, in the presence of which S14 becomes the major protein labeled. In this paper we explore this change in detail by addressing the following questions. (a) Does the labeling of S14 proceed from a puromycin binding site? (b) Does the effect of chloramphenicol arise from a specific chloramphenicol–ribosome interaction? (c) Are S14 and L23 labeled from a single puromycin binding site on the 70S ribosome or from separate 30S and 50S sites? (d) Are photoinduced alterations in the ribosome important for the observed change in the labeling pattern?

Experimental Section

Materials. L-*erythro*-2,2-Dichloro-*N*-[β-hydroxy-α-(hydroxymethyl)-*p*-nitrophenyl]acetamide (LECAM)[1] was a gift

† From the Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104 (P.G.G. and B.S.C.), and the Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin 53706 (W.A.S.). *Received December 19, 1978.* This work was supported by research grants from the National Institutes of Health (AM 13212 to B.S.C.) and the National Science Foundation (PCM 76-84278 to B.S.C. and PCM 78-18490 which was administered by M. Nomura at the University of Wisconsin).

0006-2960/79/0418-2154$01.00/0

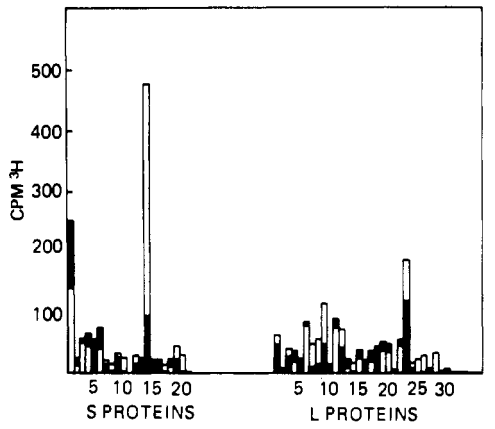

FIGURE 1: Pattern of 70S ribosomal protein labeling at high and low puromycin concentrations in the presence of chloramphenicol. The two-dimensional gels were analyzed as described previously (Grant et al., 1979; Jaynes et al., 1978). The results at low puromycin concentration are the average of seven gels. The results at high puromycin concentration are the average of three gels. The incorporation levels reported for S14 are reproducible to ±20%. Experimental conditions: 100 A_{260} units/mL ribosomes, 0.10 mM chloramphenicol, 20-min photolysis, plus 0.10 mM puromycin (1500 Ci/mol) (□) or 2.1 mM puromycin (65 Ci/mol) (■). In displaying the data the lower value is superimposed on the higher one.

from Dr. M. Rebstock of Parke-Davis. (*p*-Nitrophenyl)acetic acid was purchased from Sigma. [^{14}C]Chloramphenicol (7.94 Ci/mol) was obtained from Amersham. All other materials were obtained as described (Grant et al., 1979).

Methods. Ribosomes were prepared from *E. coli* Q13 as described (Grant et al., 1979). Subunits were prepared from 70S ribosomes labeled with puromycin as previously described (Jaynes et al., 1978). Isolated subunits used directly in photolabeling experiments were prepared by ultracentrifugation through high-salt (0.4 M NaCl) 5–20% sucrose gradients [SW27 rotor, 20000 rpm, 16 h, 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, and 50 mM KCl] (Dondon et al., 1974) and purified by a second gradient centrifugation. No cross contamination of these purified subunits was detected by either sucrose gradient analysis or by one-dimensional polyacrylamide gel electrophoresis (PAGE) analysis of ribosomal proteins derived from them. Photolytic incorporation, using Rayonet RPR-3500 Å lamps (Jaynes et al., 1978), one-dimensional and two-dimensional PAGE, and immunoprecipitation with antisera to purified S14 and L23 (Grant et al., 1979) were performed as previously described. Counts reported in the PAGE experiments are for a counting efficiency of 20%. Ultraviolet absorption spectra were taken on a Cary 15 spectrophotometer.

Results

Puromycin Incorporation into S14 in the Presence of Chloramphenicol Proceeds from a Puromycin Binding Site. The results of two-dimensional PAGE analysis of proteins obtained from ribosomes labeled at low (0.1 mM) and high (2.1 mM) puromycin concentrations, at a constant level of radioactive puromycin, are shown in Figure 1. The large reduction in radioactivity incorporated into S14 at the higher puromycin concentration is evidence that labeling proceeds from a puromycin binding site which is largely saturated at the higher concentration. Failure to obtain such a reduction would have meant that S14 labeling was increasing with puromycin concentration and thus was not proceeding from

[1] Abbreviations used: LECAM, L-*erythro*-2,2-dichloro-*N*-[β-hydroxy-α-(hydroxymethyl)-*p*-nitrophenyl]acetamide; PAGE, polyacrylamide gel electrophoresis.

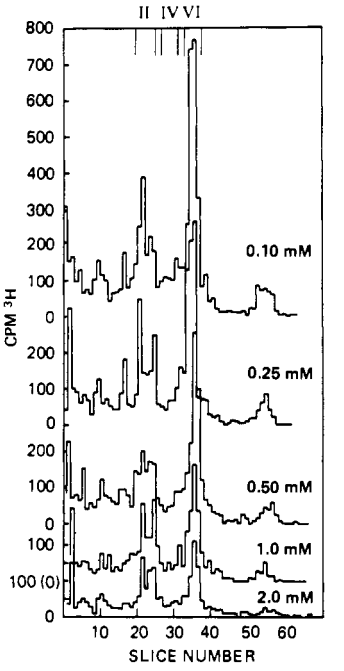

FIGURE 2: One-dimensional polyacrylamide gel pattern of labeled proteins from 30S subunits derived from labeled 70S ribosomes as a function of puromycin concentration. Experimental conditions: 101 A_{260} units/mL ribosomes, 1.0 mM chloramphenicol plus the indicated concentration of puromycin, 20-min photolysis. The specific activities of puromycin were (a) 1515 Ci/mol, (b) 602 Ci/mol, (c) 301 Ci/mol, (d) 150 Ci/mol, and (e) 75 Ci/mol. Reported counts are for protein from 2.5 A_{260} units of 30S particles.

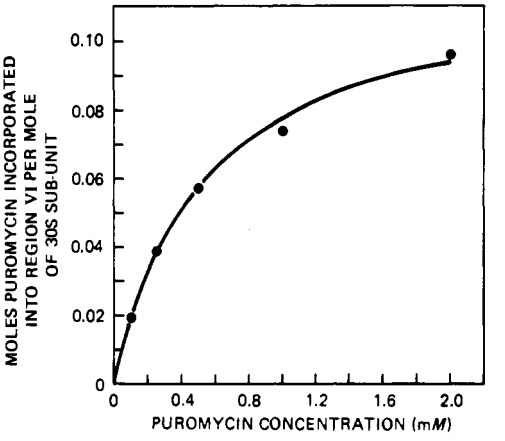

FIGURE 3: Saturation of region VI labeling with increasing puromycin concentration. Data are obtained from the experiment shown in Figure 2 by using the three gel slices in region VI containing the highest radioactivity.

a site saturable below 2.1 mM puromycin. Labeling of some other proteins, such as L9 and L23, is also reduced at 2.1 mM puromycin but to a lesser degree than is seen with S14. Protein S1 is also labeled to a considerable extent, but the labeling is not reduced at 2.1 mM puromycin and so is presumably not specific. One-dimensional PAGE analysis of labeled 30S protein derived from labeled 70S ribosomes as a function of puromycin concentration (Figure 2) provides similar results. Again, the total radioactivity is constant as puromycin concentration is increased. Although the radioactivity incorporated in both regions II and VI decreases with increasing puromycin concentration, the effect is clearly more pronounced for region VI. A plot of puromycin incorporation into region VI as a function of puromycin concentration shows simple saturation behavior (Figure 3), which, when analyzed as described previously (Jaynes et al., 1978), allows estimation

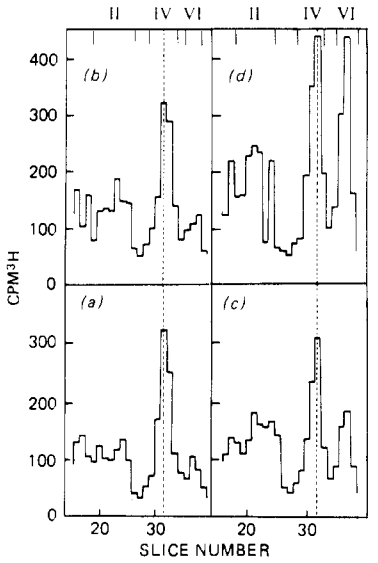

FIGURE 4: One-dimensional polyacrylamide gel pattern of proteins from 70S ribosomes labeled with puromycin in the presence of chloramphenicol and other nitrophenyl compounds. Experimental conditions: 100 A_{260} units/mL ribosomes, 0.10 mM puromycin (1500 Ci/mol), 20-min photolysis. (a) Puromycin alone, (b) plus 0.05 mM (*p*-nitrophenyl)acetic acid, (c) plus 0.05 mM LECAM, (d) plus 0.05 mM chloramphenicol. Reported counts are for protein from 6.45 A_{260} units of 70S ribosomes. The dotted line indicates the position of labeled L23 in the gel.

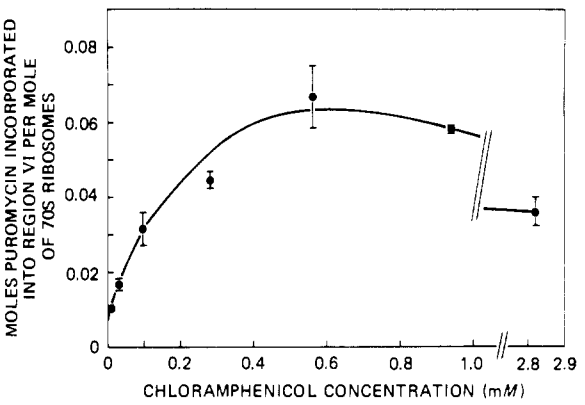

FIGURE 5: Variation in region VI labeling with increasing chloramphenicol concentration. Experimental conditions: 98 A_{260} units/mL ribosomes, 0.10 mM puromycin (750 Ci/mol), indicated concentration of chloroamphenicol, 16-min photolysis.

of a dissociation constant for puromycin of 0.53 ± 0.04 mM. Protein S14 migrates within region VI and is clearly resolved from other 30S proteins (Hardy et al., 1969; Wittmann, 1974). Moreover, the results in Figure 1 clearly show that the proteins migrating on either side of S14 (S11, S12, and S15–S18) are labeled to only minor extents. Thus, to a good approximation, the radioactivity in region VI can be taken to represent S14 labeling. For a dissociation constant of 0.53 mM, one would predict that the observed incorporation of radioactivity into S14 at 2.1 mM puromycin would be 25% of that found at 0.1 mM puromycin, in good agreement with the observed value of 20 ± 10% (Figure 1).

Chloramphenicol Stimulation of Puromycin Photoincorporation into S14 Requires a Specific Chloramphenicol Binding Site. Chloramphenicol-induced stimulation of S14 labeling by puromycin could require a specific chloramphenicol binding site on the ribosome or could arise from a nonspecific photosensitization effect due to the nitrophenyl moiety in the chloramphenicol structure. Strong evidence that the former possibility is correct comes from two kinds of studies utilizing one-dimensional PAGE analysis of labeled 70S protein. First, patterns obtained in the presence of either (*p*-nitrophenyl)-acetic acid or the diastereomer of chloramphenicol, LECAM, which is inactive as a ribosomal antibiotic (Pestka, 1977), show clearly that neither of these compounds is as effective as chloramphenicol in stimulating region VI (S14) labeling (Figure 4). Second, radioactivity found in region VI reaches a saturating value as a function of chloramphenicol concentration, which allows estimation of an apparent K_D value for chloramphenicol of approximately 0.2 mM (Figure 5). Some further comments are in order about these studies. When 70S proteins are analyzed by one-dimensional PAGE, S14 is not completely resolved from two 50S proteins, L20 and L29 (Hardy et al., 1969; Mora et al., 1971; Wittmann, 1974). Thus, equating radioactivity in region VI with S14 labeling is less valid than when 30S proteins alone are analyzed but again, from Figure 1, is still not an unreasonable approximation. Also, although it is true that in the lower concentration range (≤0.3 mM) the major effect of increasing chloramphenicol concentration is to stimulate region VI labeling, as chloramphenicol concentration is increased above this level the labeling of many other proteins is stimulated, so that the PAGE pattern becomes quite complex. In addition, at very high (2.8 mM) chloramphenicol concentration, the absolute level of region VI labeling is reduced. These results may reflect nonspecific effects of chloramphenicol and have prompted us to conduct most of our studies at reasonably low (0.1 mM) chloramphenicol concentration.

Chloramphenicol and Puromycin Binding Sites Involved in Chloramphenicol Stimulation of Puromycin Photoincorporation into S14 Are Located on the 30S Subunit. Puromycin Incorporation into L23 Proceeds from a Site on the 50S Subunit. The experiments described above utilized 70S ribosomes as the target for puromycin photoincorporation. When 30S subunits are the target, PAGE analysis of the labeled proteins gives results similar to those obtained for proteins derived from 30S subunits isolated from labeled 70S ribosomes. In particular, there is a large chloramphenicol stimulation of incorporation into region VI of a one-dimensional gel (Figure 6a,b) to approximately the same level (expressed as incorporation per 30S subunit) as that obtained with 70S ribosomes. A high (2.1 mM) concentration of puromycin specifically blocks region VI labeling (Figure 6c), and LECAM and (*p*-nitrophenyl)acetic acid fail to stimulate it (Figure 6d,e). Two-dimensional PAGE analysis confirmed that the major peak in region VI corresponds to labeled S14.

These results thus provide a clear demonstration that the puromycin and chloramphenicol sites involved in S14 labeling are located on the 30S subunit. They also led us to examine whether puromycin incorporation into L23 in the absence of chloramphenicol (Jaynes et al., 1978) depends on the presence of the 30S subunit. One-dimensional PAGE analysis of the labeled proteins obtained when isolated 50S subunits are the target gives results which are qualitatively very similar to those obtained for proteins derived from 50S subunits isolated from labeled 70S ribosomes (data not shown), although the extent of region IV (corresponding to protein L23) labeling is reduced about twofold. However, a control experiment in which 30S subunits are added back to 50S subunits (under conditions favoring association to form 70S ribosomes) prior to puromycin photoincorporation also shows reduced region IV labeling compared to that obtained with native 70S ribosomes. These results lead us to conclude that the puromycin site leading to L23 labeling is localized entirely on the 50S subunit. The reduction in labeling on isolated 50S subunits is probably

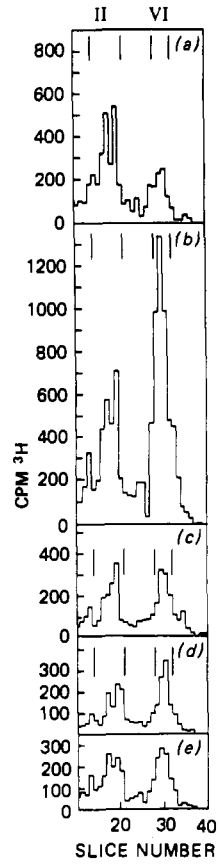

FIGURE 6: One-dimensional polyacrylamide gel pattern of proteins from 30S ribosomal subunits labeled at high and low puromycin concentrations in the presence of chloramphenicol and other nitrophenyl containing compounds. (a) 0.10 mM puromycin, (b) 0.10 mM puromycin plus 0.10 mM chloramphenicol, (c) 2.1 mM puromycin plus 0.10 mM chloramphenicol, (d) 0.10 mM puromycin plus 0.10 mM LECAM, (e) 0.10 mM puromycin plus 0.10 mM (*p*-nitrophenyl)acetic acid. Experimental conditions: 35 A_{260} units/mL 30S ribosomal subunits, 30-min (a) or 20-min (b–e) photolysis. Specific activity of puromycin: (a, b, d, and e) 1500; (c) 64 Ci/mol. The reported counts are for protein from 4.0 A_{260} units of 30S subunits.

attributable to partial denaturation of the 50S subunit during the isolation procedure.

Puromycin Labeling Pattern in the Presence of Chloramphenicol Is Markedly Light Fluence Dependent. Previously we showed that the labeling pattern for puromycin incorporation into 70S ribosomes had only minor dependence on light fluence and in particular that the labeling of the major site of incorporation, protein L23, did not depend on any photoinduced change in either the ribosome or the puromycin (Jaynes et al., 1978). The situation is quite different when photoincorporation is carried out in the presence of chloramphenicol. One-dimensional PAGE gels of labeled 70S proteins as a function of light fluence (which is proportional to the time of irradiation) are shown in Figure 7. As can be seen, the pattern obtained at low fluence (≤5 min) closely resembles that obtained with puromycin alone (region IV dominant, corresponding to L23 labeling), and it is only as light fluence increases that region VI becomes dominant. Two-dimensional analysis (Figure 8) provides confirmatory evidence, showing that even in the presence of chloramphenicol, L23 is the major protein labeled at low light fluence, and it is only as light fluence is increased that S14 becomes the major labeled protein.

The light fluence dependences of S14 and L23 labeling were next investigated in greater detail by using both one-dimensional PAGE and immunoprecipitation analysis. The

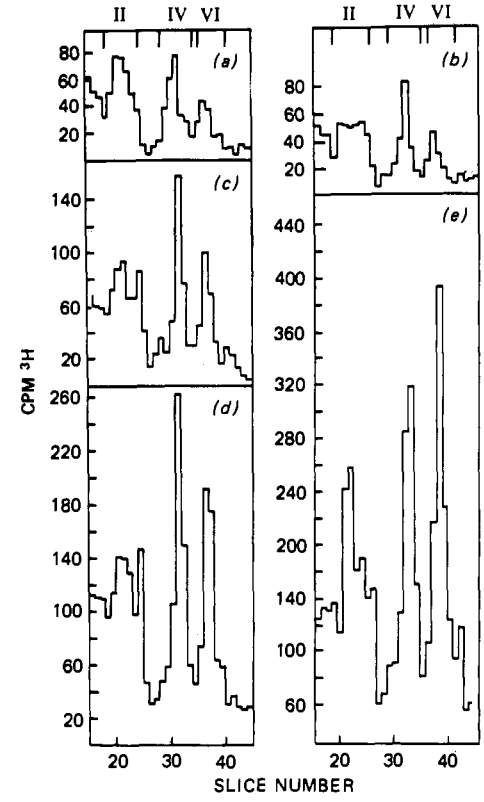

FIGURE 7: Light fluence dependence of one-dimensional polyacrylamide gel patterns of proteins from 70S ribosomes labeled with puromycin in the presence of chloramphenicol. (a) Two-minute photolysis, without chloramphenicol, (b) 2-min photolysis, (c) 5-min photolysis, (d) 10-min photolysis, (e) 20-min photolysis. Experimental conditions: 100 A_{260} units/mL ribosomes, 0.10 mM puromycin (1000 Ci/mol). (a) No added chloramphenicol, (b–e) plus 0.10 mM chloramphenicol. Counts reported are for protein from 10.5 A_{260} units of ribosomes.

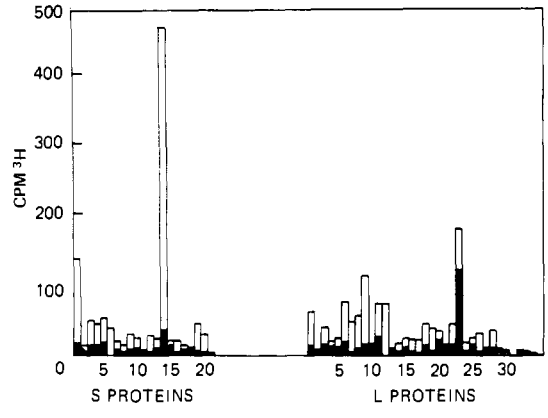

FIGURE 8: Two-dimensional polyacrylamide gel analysis of protein from 70S ribosomes labeled with puromycin in the presence of chloramphenicol at low and high light fluence. Experimental conditions: 100 A_{260} units/mL ribosomes, 0.10 mM puromycin (1500 Ci/mol), 0.10 mM chloramphenicol, photolysis for 5 (■) or 20 min (□). The 5-min photolysis results are the average of two gels; the 20-min results are the average of seven gels. The high and low light fluence experiments were performed with different ribosomal preparations so that comparison of absolute incorporation levels would be subject to considerable error (Grant et al., 1979).

results presented in Figure 7 show an increase in the ratio of S14/L23 labeling as a function of light fluence and imply the existence of a photochemical event in addition to that directly responsible for puromycin incorporation. To determine the nature of this event, the effect of prephotolyzing various incomplete reaction mixtures on the subsequent photoincorporation of radioactive puromycin into S14 was investigated

Table I: Effect of Prephotolysis on Chloramphenicol Stimulation of S14 Labeling

reaction components present during prephotolysis[a]				
30 S	nonradioactive puromycin	chloramphenicol	rel incorporation into S14	rel enhancement over separate prephotolysis of chloramphenicol
(1) −	−	−	(1.00)	
(2) +	−	−	1.00	
(3) −	−	+	0.66	
(4) +	−	+	1.26	1.91
(5) +	+	−	1.08	
(6) +	+	+	1.34	1.88

[a] All completed reaction mixtures contained 35 A_{260} units/mL 30S particles, 0.1 mM puromycin (998 Ci/mol), and 0.1 mM chloramphenicol and were photolyzed for 8 min. Prior to this photolysis, the indicated reaction components were mixed together and prephotolyzed for 10 min. When nonradioactive puromycin was present during prephotolysis, its concentration was 0.082 mM. The final puromycin concentration and specific radioactivity were achieved by adding puromycin at 5700 Ci/mol. Incorporation values into S14 are normalized to the value obtained in the absence of prephotolysis (line 1).

by using isolated 30S subunits as the target and by making use of the facts, noted above, that S14 is clearly resolved from all other 30S proteins by one-dimensional PAGE analysis and that labeled S14 comigrates with native S14. The results are summarized in Table I. Although separate prephotolysis of 30S subunits has little effect on S14 labeling (2 vs. 1) and separate prephotolysis of chloramphenicol leads to decreased labeling (3 vs. 1) (vide infra), prephotolysis of 30S subunits and chloramphenicol together leads to increased S14 labeling (4 vs. 1). Essentially the same results are obtained when prephotolysis is carried out in the presence of nonradioactive puromycin (5 and 6 vs. 1). The increase in S14 labeling due to prephotolysis of 30S subunits and chloramphenicol, 1.9-fold, is properly calculated by comparing experiments 3 and 4 (or 3 and 6), thus correcting for the reduced efficiency of prephotolyzed chloramphenicol in stimulating S14 labeling. By contrast, parallel experiments using one-dimensional PAGE analysis of proteins extracted from 50S subunits isolated from labeled 70S ribosomes showed little change in radioactivity in region IV (L23) as a result of separate prephotolysis of 70S ribosomes and chloramphenicol.

Immunoprecipitation analyses of labeled ribosomal proteins from similar experiments give results which are in good qualitative accord with the PAGE results (Table II). By use of a low light fluence (5 min), puromycin labels L23 to a much greater extent than S14 when chloramphenicol is omitted, and L23 is still the major incorporation site in the presence of chloramphenicol. However, when 70S ribosomes are prephotolyzed in the presence of chloramphenicol, the level of labeled S14 rises dramatically while that of L23 is almost unchanged, so that S14 becomes the major incorporation site.[2]

These results, taken together, show that the increase in the ratio of S14/L23 labeling as a function of light fluence is due to an enhancement of S14 labeling which occurs as the result

Table II: Relative Levels of S14 and L23 Labeling by Immunoprecipitation Analysis

conditions[a]	S14	L23
(1) puromycin alone	0.20	1.0
(2) puromycin plus chloramphenicol	(1.0)	1.9
(3) prephotolysis of 70S and chloramphenicol	5.8	1.9

[a] Photolyses were performed for 5 min with 0.1 mM [^{3}H]puromycin, ±0.1 mM chloramphenicol, 100 A_{260} units/mL 70S ribosomes. In experiment 3, 70S ribosomes and chloramphenicol were combined and prephotolyzed for 20 min, puromycin was then added, and the complete reaction mixture was photolyzed for 5 min. Values are normalized to the level of S14 labeling obtained in the presence of chloramphenicol but in the absence of prephotolysis (line 2).

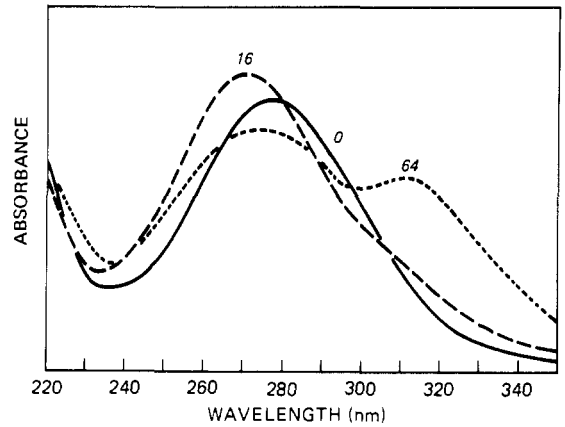

FIGURE 9: Changes in the ultraviolet absorption spectrum of chloramphenicol with increasing light fluence. A 1.0 mM solution of chloramphenicol was photolyzed, aliquots were taken at indicated times, and the spectrum was measured on a Cary 15 spectrophotometer.

of a chloramphenicol-dependent photoinduced change in the 30S subunit. This raises a legitimate question as to the significance of such labeling with respect to the native ribosome structure. We believe such labeling is significant for several reasons. First, even in the absence of chloramphenicol, S14 is the second highest labeled protein (Jaynes et al., 1978), and thus the observed enhancement could require only a very small structural change. Second, the enhancement is only found with chloramphenicol, not with its biologically inactive diastereomer nor with (*p*-nitrophenyl)acetate ion, and thus depends on a specific-site interaction on the 30S subunit rather than on a generalized photoinduced alteration of structure. Third, in separate experiments, we have shown that 70S ribosomes irradiated with the 3500-Å lamps for 20 min lose only minor amounts of peptidyl transferase activity (~10%) and that the extent of this loss is only slightly increased in the presence of 0.1 mM chloramphenicol.[3] Since the peptidyl transferase assay we use requires a functional 30S particle, this result also argues against a generalized structural alteration.

Photodegradation of Chloramphenicol. The decreased efficiency of prephotolyzed chloramphenicol in stimulating S14 labeling prompted us to study the influence of light fluence on the ultraviolet absorption spectrum of chloramphenicol (Figure 9). Two different transitions are clearly visible. The first, which is almost complete after 8 min of irradiation, results

[2] The observed effect of prephotolysis on S14 labeling is more pronounced in the experiments analyzed by immunoprecipitation (Table II) than in those analyzed by PAGE (Table I). This is due at least in part to the fact that a longer prephotolysis and a shorter photolysis were used for the experiments in Table II as compared with those in Table I. However, some of the difference may also be due to the use of 70S ribosomes as the target in one experiment (Table II) and 30S subunits in the other (Table I), as well as inherent differences in the analytical methods themselves (Grant et al., 1979).

[3] A. W. Nicholson and B. S. Cooperman, unpublished experiments. The assay employed measures poly(U)-dependent *N*-acetylphenylalanine transfer from *N*-acetyl-Phe-tRNA^{Phe} to puromycin (Nicholson & Cooperman, 1978).

in a shift in λ_{max} from 277 to 270 nm with a 10% increase in ϵ_{max}. The second, which is incomplete even after 64 min, leads to the appearance of a new absorption band at 310 nm. Thus, the light fluence used in the prephotolysis experiments described above (10–20 min) is sufficient to complete the first transition, which may account for the decreased efficiency of prephotolyzed chloramphenicol in stimulating S14 labeling. The structural changes responsible for the two spectral transitions have not been characterized.

Photoincorporation of Chloramphenicol. Sonenberg et al. (1974) have shown that chloramphenicol photoincorporates into ribosomes. We were interested to see whether such incorporation had any direct influence on puromycin incorporation. When [^{14}C]chloramphenicol (0.1 mM) and 70S ribosomes were photolyzed for 20 min in the presence of [^{3}H]puromycin (0.1 mM) by using essentially the same conditions as those described previously, the stoichiometry of chloramphenicol incorporation was 1.5–2 times larger than that of puromycin. However, the amount of chloramphenicol incorporation was unchanged in the absence of puromycin, and one-dimensional PAGE analysis not only showed no marked peaks but also the ^{14}C radioactivity in region VI of the gel was relatively low. It thus seems highly unlikely that chloramphenicol-dependent puromycin photoincorporation into S14 occurs via a chloramphenicol bridge.

Discussion

Because of their inhibitory effects on peptidyl transferase, an activity clearly localized on the 50S subunit, both puromycin and chloramphenicol have long been known to interact with the 50S subunit of *E. coli* ribosomes. Under some conditions, chloramphenicol has been shown to be a competitive inhibitor toward puromycin in the peptidyl transferase assay (Coutsogeorgopoulus, 1966; Pestka, 1970), leading to the suggestion that both antibiotics bind to a common site on the 50S subunit. Our results, showing the lack of effect of added chloramphenicol on puromycin incorporation into L23 at low light fluences (Table II), argue against this suggestion, in accord with conclusions reached by Vince et al. (1975) from studies on the inhibitory activities toward polyphenylalanine synthesis of a series of chloramphenicol and puromycin analogues. However, the recent finding of Kenney & Traut (1978) that protein L16, which has been strongly implicated as being present at the chloramphenicol binding site by both affinity labeling (Pongs et al., 1973) and reconstitution studies (Nierhaus & Nierhaus, 1973), is cross-linked to protein L23 when 50S ribosomes are treated with 2-iminothiolane may be taken as evidence for physical proximity of the two antibiotic sites on the 50S subunit.

The K_D value for puromycin incorporation into S14 in the presence of chloramphenicol (0.53 ± 0.04 mM) is indistinguishable within experimental error from the K_D value (0.7 ± 0.2 mM) previously measured for puromycin incorporation into L23 in the absence of chloramphenicol (Jaynes et al., 1978). This result initially led us to speculate that L23 and S14 labeling might be proceeding from a single puromycin site on the 70S ribsome located at or close to the interface between the 30S and the 50S subunits. However, the findings that S14 is labeled to a similar extent whether the target is a 70S ribosome or an isolated 30S subunit and that a similar situation is obtained for L23 labeling with 70S vs. isolated 50S subunits are strong evidence that the two puromycin sites are topologically distinct. The similarity in the K_D values must then be attributed to coincidence.

Our photoincorporation results using 30S subunits as the target provide the first strong evidence for the presence of binding sites for both puromycin and chloramphenicol on the isolated 30S subunit. The stimulatory effect of chloramphenicol on S14 labeling is at least indirect evidence for a physical proximity of the two sites, although linkage via an allosteric effect cannot be excluded. Considering puromycin as a structural analogue of the 3′ end of a charged tRNA, the finding of a 30S site for this antibiotic is consistent with previous results showing that both an electrophilic derivative (Girshovich & Bochkareva, 1974) and a photolabile derivative (Girshovich et al., 1974) of *N*-acyl-Phe-tRNA^{Phe} affinity label 30S proteins in poly(U)-dependent reactions when bound to 30S subunits. Furthermore, in the latter study, S14 is the major protein labeled.

Several investigators have found a single tight site for chloramphenicol binding to 70S ribosomes, with a dissociation constant in the range 1–5 μM, and there is good evidence that this site is localized on the 50S subunit. In addition, Lessard & Pestka (1972) have presented equilibrium dialysis evidence for the existence of a second, weaker site having a dissociation constant of approximately 0.2 mM. Other evidence for such a site with approximately the same affinity has also been obtained recently by Le Goffic and his co-workers.[4] The close agreement between these estimates for the dissociation constant for second-site chloramphenicol binding and our estimate for the dissociation constant of the chloramphenicol site responsible for enhanced S14 labeling (Figure 5) supports the conclusion that the second chloramphenicol site is localized on the 30S subunit.

While the functional significance, if any, of a 30S site for puromycin remains to be demonstrated, we would like to briefly consider two possibilities. During protein biosynthesis, aminoacyl-tRNA is brought up to the ribosome as part of a ternary complex with EF-Tu and GTP, but it is only after GTP hydrolysis and EF-Tu release from the ribosome that peptidyl transfer takes place. Thus, prior to EF-Tu release, the 3′ end of aminoacyl-tRNA may not be in the so-called A′ site (defined as that portion of the ribosome responsible for binding the 3′ end of aminoacyl-tRNA during peptide formation). These and other results have led several authors (Johnson et al., 1977; Lake, 1977) to conclude that, in addition to the classical A and P sites for tRNA binding, there is an additional R (for recognition) site to which aminoacyl-tRNA is first bound before it reaches the A site. Lake (1977), in reviewing the evidence concerning ribosomal proteins implicated in tRNA binding, has placed this site on the side of the 30S subunit facing away from the 50S subunit. In his model for the R site, the 3′ end of aminoacyl-tRNA lies in the region of the 30S particle containing S14, as determined by immune electron microscopy. Since puromycin is a structural analogue of the 3′ end of aminoacyl-tRNA, our results, along with those of Girshovich et al. (1974) cited above, can be taken as support for Lake's model. Second, the evidence that both puromycin and the 3′ end of *N*-acyl-Phe-tRNA^{Phe} have specific interactions with the 30S subunit raises the possibility that peptidyl transferase could occur on an isolated 30S subunit, albeit at much lower efficiency than that found on either 50S subunits or 70S ribosomes. The evolutionary implications of such activity would be important and this question is currently being explored.

References

Cooperman, B. S., Jaynes, E. N., Brunswick, D. J., & Luddy, M. A. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2974.

[4] F. Le Goffic, private communication, 1978.

Coutsogeorgopoulos, C. (1966) *Biochim. Biophys. Acta 129*, 214.
Dondon, J., Godefroy-Coburn, T., Graffe, M., & Grunberg-Manago, M. (1974) *FEBS Lett. 45*, 82.
Girshovich, A. S., & Bochkareva, E. S. (1974) *Dokl. Acad. Nauk SSSR 217*, 1201.
Girshovich, A. S., Bochkareva, E. S., Kramorov, V. M., & Ovchinnikov, Y. A. (1974) *FEBS Lett. 45*, 213.
Grant, P. G., Strycharz, W. A., Jaynes, E. N., Jr., & Cooperman, B. S. (1979) *Biochemistry* (preceding paper in this issue).
Hardy, S. J. S., Kurland, C. G., Voynow, P., & Mora, G. (1969) *Biochemistry 8*, 2897.
Jaynes, E. N., Jr., Grant, P. G., Giangrande, G., Wieder, R., & Cooperman, B. S. (1978) *Biochemistry 17*, 561.
Johnson, A. E., Fairclough, R. H., & Cantor, C. R. (1977) in *Nucleic Acid Protein Recognition* (Vogel, H. J., Ed.) p 469, Academic Press, New York.
Kenny, J. W., & Traut, R. R. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1740.
Lake, J. A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1903.
Lessard, J. L., & Pestka, S. (1972) *J. Biol. Chem. 247*, 6909.
Mora, G., Donner, D., Thammana, P., Lutter, L., Kurland, C. G., & Craven, C. R. (1971) *Mol. Gen. Genet. 112*, 229.
Neirhaus, D., & Nierhaus, K. H. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2224.
Nicholson, A. W., & Cooperman, B. S. (1978) *FEBS Lett. 90*, 203.
Pestka, S. (1970) *Arch. Biochem. Biophys. 136*, 80.
Pestka, S. (1977) in *Molecular Mechanisms of Protein Biosynthesis* (Weissbach, H., & Pestka, S., Eds.) p 468, Academic Press, New York.
Pongs, D., Bald, R., & Erdmann, V. A. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2229.
Sonenberg, N., Wilchek, M., & Zamir, A. (1974) *Biochem. Biophys. Res. Commun. 59*, 663.
Vince, R., Almquist, R. G., Ritter, C. L., & Daluge, S. (1975) *Antimicrob. Agents Chemother. 8*, 439.
Wittmann, H. G. (1974) in *Ribosomes, Cold Spring Harbor Monograph Series* (Nomura, M., Tissières, A., & Lengyel, P., Eds.) p 93, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Selective Displacement of Histone H1 from Whole HeLa Nuclei: Effect on Chromatin Structure in Situ as Probed by Micrococcal Nuclease†

George M. Lawson‡ and R. David Cole*

ABSTRACT: In order to study the role of histone H1 in chromatin structure under conditions minimizing chromatin denaturation, a technique was developed to displace this histone from whole nuclei. Isolated HeLa nuclei washed at pH 3.0 selectively released histone H1 without detectable displacement of the core histones H2A, H2B, H3, and H4. Nucleosomal structure remained intact in such washed nuclei as judged by the characteristic electrophoretic banding patterns of double-stranded DNA fragments generated by in situ digestion with micrococcal nuclease and pancreatic DNase I, respectively. The patterns were identical with those produced from nuclei maintained at physiological pH. The kinetics of nucleolytic cleavage of the linkage DNA between nucleosome cores in control nuclei containing histone H1 or in nuclei completely or partially depleted of histone H1 were all apparently first order and in each case could be described with a single rate constant. The overall rate of cleavage was increased by a factor of 2.7 ± 0.2 following quantitative displacement of histone H1. A quantitative inverse relationship between the nuclear content of histone H1 and the nucleolytic sensitivity, observed at various degrees of H1 depletion, suggested that the enhanced rate of nucleolysis was in fact dependent upon the displacement of this histone. Calibration of the sizes of resulting DNA fragments indicated that for equal extents of digestion, the fragment sizes were identical regardless of the degree of histone H1 depletion; the ratio of primary endonucleolytic cleavage of linker DNA to subsequent exonucleolytic cleavage was therefore found to be independent of the nuclear content of histone H1. This technique failed to reveal that histone H1 preferentially protected a significant stretch of linkage DNA against exonucleolytic cleavage. It is suggested that the predominant effect on chromatin structure resulting from the displacement of histone H1 under these conditions involves a loosening or dispersal of an otherwise more compact chromatin superstructure in a way that increases the accessibility of the strands of nucleosomes generally rather than directly exposing a specific site on the linkage DNA.

A current model of chromatin structure, derived primarily from histone cross-linking (Kornberg & Thomas, 1974), nuclease digestion studies (Hewish & Burgoyne, 1973; Noll, 1974a), and electron microscopy (Woodcock, 1973; Olins & Olins, 1974; Van Holde et al., 1974a; Oudet et al., 1975), consists of a linear array of repeating protein complexes, linked and circumscribed by a continuous thread of supercoiled duplex DNA. Each protein complex is comprised of two each of histones H2A, H2B, H3, and H4 and chromosomes associated with approximately 200 base pairs of DNA (Kornberg, 1974) to form a subunit of chromatin that has been denoted a nu body or nucleosome. Studies on the precise location of the lysine-rich histone H1 within the strands of nucleosomes have suggested an association of this histone with the DNA that links nucleosomal cores. This suggestion has

† From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received November 28, 1978.* This work was supported by Contract NOI-CB-43866 from the National Cancer Institute, Grant GMS 20338 from the National Institute of General Medical Sciences, and the Agricultural Experimental Station.
‡ Present address: Department of Cell Biology, Baylor College of Medicine, Texas Medical Center, Houston, TX 77030.

0006-2960/79/0418-2160$01.00/0