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Photoaffinity Labeling of *Escherichia coli* Ribosomes by an Aryl Azide Analogue of Puromycin. On the Identification of the Major Covalently Labeled Ribosomal Proteins and on the Mechanism of Photoincorporation†

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ABSTRACT: *p*-Azido[^{3}H]puromycin [6-(dimethylamino)-9-[3′-deoxy-3′-[(*p*-azido-L-phenylalanyl)amino]-β-D-ribofuranosyl]purine] has been used to photoaffinity label the *Escherichia coli* ribosome. Initial studies with this compound, reported earlier [Nicholson, A. W., & Cooperman, B. S. (1978) *FEBS Lett. 90*, 203–208], indicated a very diffuse labeling pattern with many proteins labeled to significant extents. In the present work, much of this previous apparent labeling is shown to arise from both light-independent noncovalent binding and light-independent incorporation of photolyzed *p*-azidopuromycin with ribosomal protein, and procedures are described for measuring true covalent photoincorporation. When these new procedures are used, *p*-azidopuromycin is shown to photoincorporate into ribosomal protein and RNA. The protein labeling pattern, as determined by both polyacrylamide gel electrophoresis and immunoprecipitation, is quite specific and is essentially unchanged whether 2537 Å or 3500 Å lamps are used. The extent of photoincorporation into proteins falls in the order S18 > L23 > L18/22 > L15 > S7, S14. When β-mercaptoethanol is present during photolysis as a photoaffinity label scavenger, S18 and most other S protein labeling is suppressed, and the order of labeling becomes L23 > L18/22 > L15, S7 > S1. This result suggests that high S18 labeling is not site specific but is due rather to its high chemical reactivity. The specific suppression of S18 labeling by pretreatment of the ribosomes with *N*-ethylmaleimide supports this view. L23 labeling by *p*-azidopuromycin is azide dependent and proceeds by a mechanism which is most probably different from that responsible for photoincorporation of puromycin [Cooperman, B. S., Jaynes, E. N., Brunswick, D. J., & Luddy, M. A. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2974–2978; Jaynes, E. N., Jr., Grant, P. G., Giangrande, G., Wieder, R., & Cooperman, B. S. (1978) *Biochemistry 17*, 561–569] yet under the appropriate conditions both of these compounds label L23 to the highest extent of any ribosomal protein. This constitutes strong evidence for the site specificity of L23 labeling.

Photoaffinity labeling has become an important method for identifying functional sites on the *Escherichia coli* ribosome (Cooperman, 1978, 1980; Kuechler & Ofengand, 1980). We have been pursuing such studies with the antibiotic puromycin (Cooperman et al., 1975; Jaynes et al., 1978; Grant et al., 1979a,b). Puromycin is a substrate for the peptidyltransferase activity of the ribosome, and accordingly, localization of its site of binding on the ribosome should provide direct information on the peptidyltransferase center. In these studies we found that puromycin photoincorporated into ribosomes, that protein L23 was the major labeled protein, and that such labeling was site specific. Because of the uncertainties to which photoaffinity labeling studies are subject (Cooperman, 1976; Bayley & Knowles, 1977; Chowdhry & Westheimer, 1979), it is important to verify and extend results obtained in any one study by altering the labeling process in a deliberate fashion. One such approach is to compare the labeling patterns obtained when different photolabile derivatives of the same parent ligand are photoincorporated into the receptor. Accordingly, we have synthesized *p*-azido[^{3}H]puromycin [6-(dimethylamino)-9-[3′-deoxy-3′-[(*p*-azido-L-phenylalanyl)amino]-β-D-ribofuranosyl]purine], a functionally competent analogue of puromycin (Symons et al., 1978; Krassnigg et al., 1978; Nicholson & Cooperman, 1978; Nicholson et al., 1982), and used it to photoaffinity label the ribosome. A preliminary report of our findings has already appeared (Nicholson & Cooperman, 1978) in which labeling was found to be distributed over a large number of proteins (as judged by one- and two-dimensional polyacrylamide gel electrophoresis), with the most

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highly labeled proteins falling in the order S18 ≈ L11 > S3, S4, S5. Krassnigg et al. (1978) have also reported that *p*-azidopuromycin photoaffinity labels a large number of proteins, although the identities of the labeled proteins they indicate are somewhat different from those listed above.

We now show that much of the protein labeling we previously described [and, we believe, that described by Krassnigg et al. (1978) as well] was in fact due mostly to light-independent interaction of photolyzed *p*-azido[^{3}H]puromycin with ribosomal proteins, much of which is noncovalent in nature. We further report the development of a procedure that suppresses such apparent labeling, thus allowing examination of labeling arising from light-dependent rapid covalent incorporation. This latter labeling is of greatest interest, since the primary virtues of photoaffinity labeling as a technique for defining receptor sites are, first, that it results in formation of covalent bonds with a portion or portions of the site and, second, that it uses an intermediate of high chemical reactivity so as to maximize the chances that covalent reaction will occur at or close to the binding site. The protein labeling observed on application of such a procedure, as determined by both polyacrylamide gel electrophoresis and immunoprecipitation, is much simpler and more specific than that obtained earlier and is examined under a variety of conditions. Most strikingly, when photoincorporation is carried out in the presence of β-mercaptoethanol, L23 is found to be the protein most highly labeled by *p*-azidopuromycin, although, as distinct from labeling studies with puromycin, other 50S proteins are also highly labeled.

Experimental Procedures

Materials

Puromycin was purchased from Sigma. *p*-Azidopuromycin was synthesized according to Nicholson & Cooperman (1978). *p*-Azido[^{3}H]puromycin was synthesized from [^{3}H]puromycin aminonucleoside (Amersham) as described (Nicholson & Cooperman, 1978), except that the *t*-Boc[1] protecting group was removed by treatment with trifluoroacetic acid for 5 min at room temperature. *p*-Azido[^{3}H]puromycin was stored at 4 °C in 5% ethanol, the presence of which increases its stability (Evans, 1976). [^{3}H]Puromycin was either purchased from Amersham or synthesized, using N^{α}-(*t*-Boc)-*O*-methyl-L-tyrosine (Nicholson, 1981) in a manner analogous to that used for *p*-azido[^{3}H]puromycin. Photolyzed *p*-azido[^{3}H]puromycin (φ-*p*-azido[^{3}H]puromycin) was prepared by photolyzing a solution of *p*-azido[^{3}H]puromycin (50 μM) in TMK buffer for either 30 min (3500 Å lamps) or 50 s (2537 Å lamps). These light fluences were sufficient to destroy the azido group but did not significantly affect the (dimethylamino)purine chromophore. TLC analysis (silica gel, ethyl acetate–methanol–water–acetic acid, 25:10:1:1 v/v/v/v) using a Packard radiochromatogram scanner showed a complex mixture of radioactive products. Of these *p*-amino[^{3}H]puromycin, the reduction product of *p*-azido[^{3}H]puromycin, could have contributed only a minor fraction, as judged by using independently synthesized material as a TLC standard.

Ribosomes (70 S) were prepared from *E. coli* Q13 bacteria harvested in mid- or late-log phase, using the modification of the Traub et al. (1971) procedure previously described (Jaynes et al., 1978). Ribosomal subunits were prepared by sucrose gradient centrifugation as described elsewhere (Grant et al., 1979a) using either TMKNa buffer [50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 50 mM KCl, and 400 mM NaCl] or TKM buffer [50 mM Tris-HCl (pH 7.6), 50 mM KCl, and 1 mM $MgCl_2$].

Methods

Photoincorporation of p-Azidopuromycin into Ribosomes and Ribosomal Subunits. Photolyses were performed in TMK buffer [50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 50 mM KCl] at 4 °C as previously described (Jaynes et al., 1978) using Rayonet RPR-2537 Å or RPR-3500 Å lamps. Ribosomes (70S) were thawed and used directly; 50S and 30S subunits were heat activated at 37 °C for 15 min prior to use. Three different procedures were used to measure incorporation into ribosomes and/or ribosomal subunits following photolysis. All operations were performed at 4 °C.

In procedure A, which is now preferred, samples were quenched with 2 volumes of β-mercaptoethanol–ethanol (1:9 v/v) as soon as possible following photolysis (<30 s) to prevent light-independent reaction and centrifuged at 13 000 rpm for 10 min in an SM 24 rotor. The pellets were suspended in TMK buffer and reprecipitated by addition of 2 volumes of the β-mercaptoethanol–ethanol solution. This latter procedure was repeated 3 times. The final pellets were resuspended in TMKNa buffer and centrifuged at 13 000–16 000 rpm for 20–30 min to remove polymeric *p*-azidopuromycin photoproducts. The ribosome supernatant was removed, and 70S incorporation was measured as described (Cooperman et al., 1975). So that subunit incorporation could be measured, 70S ribosomes isolated as above were incubated at 37 °C for 20 min, then layered atop a 15–30% sucrose gradient made up in TMKNa buffer containing 1% β-mercaptoethanol, and centrifuged at 50 000 rpm in a VTi50 rotor for 90 min (4 °C). The gradients were fractionated by using a Beckman flow cell, and the subunit fractions were pooled and precipitated by adding 2 volumes of ethanol, followed by storage at −20 °C for at least 2 h, and then centrifugation at 18 000 rpm for 90 min in an SS34 rotor. The ribosome pellets were resuspended in TMK buffer, and subunit incorporation was measured as described by Cooperman et al. (1975). Protein was extracted by using the method of Hardy et al. (1969) and precipitated from the acetic acid supernatant by acetone precipitation (Barritault et al., 1976). The final protein pellets were resuspended in 8 M urea which was 6 mM in β-mercaptoethanol and stored at −80 °C prior to electrophoresis. Incorporation into ribosomal RNA was determined as previously described (Jaynes et al., 1978).

Procedure B for isolation of 70S ribosomes and ribosomal subunits is essentially identical with procedure A except that β-mercaptoethanol was omitted from all of the isolation steps. In addition, subunits were separated in TMKNa buffer by ultracentrifugation through a 5–20% sucrose gradient, using an SW27 rotor (20 000 rpm, 16 h, 4 °C).

In procedure C for isolation of 70S ribosomes, ribosomes were simply precipitated (4 times in all) by ethanol addition to a ribosome solution made up in TMK buffer. Incorporation into 70S ribosomes, ribosomal protein, and ribosomal RNA was determined as previously described (Cooperman et al., 1975).

Polyacrylamide Gel Electrophoresis. Labeled ribosomal proteins were analyzed by one-dimensional urea–polyacrylamide gel electrophoresis as previously described (Jaynes et al., 1978). Assignment of 50S and 30S protein migrations within the gels followed those of Mora et al. (1971) and Rummel & Noller (1973), respectively. In addition one-di-

[1] Abbreviations: AcPhe, acetylphenylalanine; βME, β-mercaptoethanol; NAPLysPANS, [N^{ϵ}-(2-nitro-4-azidophenyl)-L-lysinyl]puromycin aminonucleoside; NEM, *N*-ethylmaleimide; φ-*p*-azidopuromycin, photolyzed *p*-azidopuromycin; $NaDodSO_4$, sodium dodecyl sulfate; TLC, thin-layer chromatography; *t*-Boc, *tert*-butoxycarbonyl.

Table I: Apparent Incorporation of *p*-Azido[^{3}H]puromycin or Photolyzed *p*-Azido[^{3}H]puromycin into Ribosomes

expt	*p*-azido-[^{3}H]puromycin (μM)	photolyzed *p*-azido-[^{3}H]puromycin (μM)	irradiation in the presence of ribosomes:[a] lamp, time	dark incubation with ribosomes:[a] time	isolation procedure	incorporation per 70S particle (% mol/mol) as measured by EtOH precipitation	incorporation per 70S particle (% mol/mol) as measured by combining subunit incorporation[c]
1	31		2537, 10 min		C	95	
2	31		3500, 10 min		C	56	
3	31				C	10	
4		31			C	50	
5	31		2537, 12 min		B	50	20
6	50		3500, 10 min		B	76	27
7	31				B	2.5	
8	50			10 min	A	0.2	
9	50			24 h	A	1.3	
10	30	20			A	0.5	0.0
11	30	20		10 min	A	9.8	1.8
12	30	20		16 h	A	23	5.2
13	50		2537, 10 min		A	70	41
14	50		3500, 10 min		A	43	20, 25[b]

[a] 70S ribosome concentrations were 1.3 (1, 3, and 4), 2.6 (2, 6, and 8–12), or 3.9 μM (5 and 7). When procedure A was used, time of irradiation or dark incubation is prior to quenching with β-mercaptoethanol. [b] The value of 25% was obtained for subunits separated in TKM (low Mg^{2+}) buffer. [c] Combining incorporation found in 30S and 50S subunits following sucrose gradient centrifugation in TMKNa buffer.

mensional $NaDodSO_4$–urea–polyacrylamide gel electrophoresis was performed according to Thomas et al. (1975) and analyzed similarly. Levels of *p*-azidopuromycin incorporation into gel regions were determined by (1) subtracting the background (defined as the average counts per minute of the least radioactive area of the gel—this region was usually between gel slice numbers 50 and 60 and ranged from 15 to 50 cpm) from the gel slices of interest and then summing the counts per minute across the whole gel and determining the percent recovery of radioactivity, (2) correcting the counts per minute in a gel region to 100% recovery, and (3) converting the counts per minute to picomoles of *p*-azidopuromycin per picomole of (subunit) protein by using the known specific radioactivity of *p*-azido[^{3}H]puromycin and by assuming that picomoles of protein applied to the gel is equivalent to the picomoles of subunits from which the protein was extracted (1 A_{260} unit equals 26 pmol of 70S, 39 pmol of 50S, and 78 pmol of 30S protein). The average yield for all urea–polyacrylamide gels was 42 ± 24%. However, on a given gel slab, reproducibility was much better, averaging 42 ± 12%. In presenting one-dimensional polyacrylamide gel electrophoresis results, counts per minute reported are normalized to an average gel radioactivity yield for purposes of comparison, as indicated. Incorporation values reported in tabular form (Table II) are normalized to 100% recovery.

Two-dimensional polyacrylamide gel electrophoresis analysis was performed by using the Howard & Traut (1974) modification of the method of Kaltschmidt & Wittmann (1970). The protein sample was applied only to the anodic end of the first-dimension gel in order to increase the resolution. Prior to the second-dimension electrophoresis, the pH of the first-dimension gels was changed by incubation in an acetic acid saturated chamber at 50 °C for 45 min, or until an indicator gel containing chlorophenol red showed the change to be mostly complete. Following electrophoresis, the gel was stained and destained (Howard & Traut, 1974). Areas of the gel containing and surrounding stained proteins were excised, and their counts per minute were determined as previously described (Jaynes et al., 1978). The typical recovery of radioactivity from the gels using this procedure was 17%.

Immunoprecipitation analyses were performed by the method of Roberts & Roberts (1975) as described by Grant et al. (1979a).

Protein determination was performed according to the method of Schaffner & Weissmann (1973) on samples isolated from 70S ribosomes or ribosomal subunits. Absolute values were determined by using bovine serum albumin as a standard.

Results

Development of a Method for Measurement of Light-Dependent Covalent Incorporation of p-Azidopuromycin into Ribosomes and Minimizing the Contribution from a Slow Covalent Reaction. Table I lists the measured apparent incorporation of *p*-azido[^{3}H]puromycin or ϕ-*p*-azido[^{3}H]puromycin into ribosomes as a function of both experimental and isolation protocols. In our initial experiments, ribosomes were irradiated in the presence of *p*-azido[^{3}H]puromycin, and incorporation was measured as the radioactivity remaining with ribosomes after four cycles of ethanol precipitation (procedure C). This procedure was based on earlier studies with puromycin (Cooperman et al., 1975; Jaynes et al., 1978) and appeared justified based on the relatively low background values seen in the absence of photolysis (compare experiments 1 and 2 with experiment 3). However, further investigation showed that ϕ-*p*-azido[^{3}H]puromycin gave a substantially higher background (experiment 4). These results led to the development of a procedure (procedure B) in which, following irradiation, samples were first subjected to a low-speed centrifugation to remove polymeric material formed on irradiation of *p*-azidopuromycin, ethanol-precipitated 4 times to remove unphotolyzed *p*-azidopuromycin and ethanol-soluble photoproducts, and then ultracentrifuged in a high-salt or low Mg^{2+} sucrose gradient which separates the ribosome into 30S and 50S subunits. The combined apparent incorporation into the two subunits measured by this procedure is reduced 4–6-fold compared with that measured by procedure C (as seen by comparing the results of experiments 1 and 5 and experiments 2 and 6 and assuming that apparent labeling is approximately proportional to initial *p*-azido[^{3}H]puromycin concentration) and leads to only a very low background value in the absence of photolysis (experiment 7). A final refinement is the addition of β-mercaptoethanol as an electrophile scavenger immediately following photolysis, which prevents any light-independent incorporation during workup (procedure A). Such incorporation is essentially negligible for *p*-azidopuromycin itself, as seen in experiments 8 and 9. However, dark incubation of

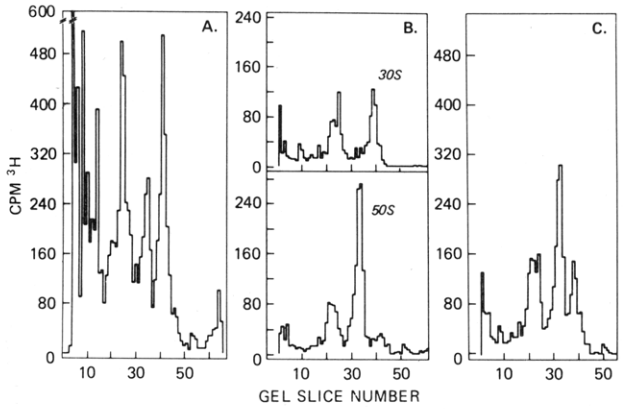

FIGURE 1: One-dimensional urea–polyacrylamide gel electrophoresis analyses of ribosomal proteins extracted from 70S ribosomes irradiated with *p*-azido[^{3}H]puromycin. (A) 70S proteins from labeled ribosomes isolated by procedure C. (B) 50S and 30S proteins from labeled 70S ribosomes isolated by procedure B. (C) 70S protein labeling derived by addition of the two patterns in part B. Experimental conditions: Photolyses were with the 2537 Å lamps for 4 min. Reported counts per minute are for protein extracted from 316 pmol of 70S ribosomes normalized to a gel radioactivity yield of 42%. (A) 70S ribosomes, 1.5 μM; *p*-azido[^{3}H]puromycin, 33 μM, 1350 Ci/mol; gel radioactivity yield 38%. (B) 70S ribosomes, 2.3 μM; *p*-azido[^{3}H]puromycin, 27 μM, 1670 Ci/mol; gel radioactivity yield 42%.

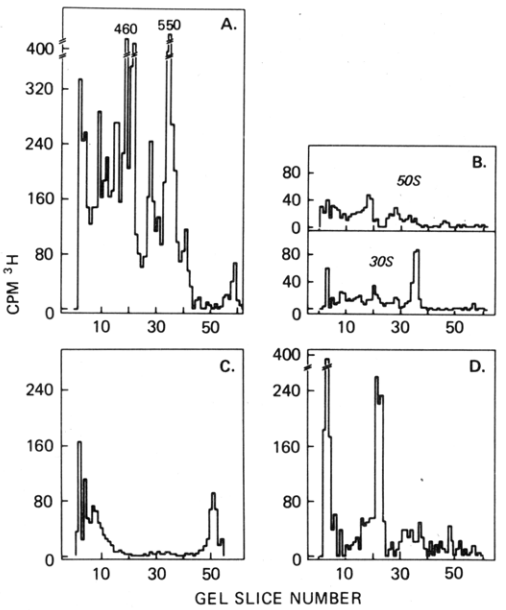

FIGURE 2: One-dimensional urea–polyacrylamide gel electrophoresis analyses. (A) 70S proteins from 70S ribosomes incubated in the dark with photolyzed *p*-azido[^{3}H]puromycin and isolated by procedure C. (B) 50S and 30S proteins from 70S ribosomes incubated with photolyzed *p*-azido[^{3}H]puromycin and isolated by procedure B. (C) Photolyzed *p*-azido[^{3}H]puromycin by itself. (D) Proteins from 50S subunits incubated with photolyzed *p*-azido[^{3}H]puromycin and isolated by procedure C. Experimental conditions: In (A) and (B), 70S ribosomes (final concentration 2.6 μM) were added to photolyzed *p*-azido[^{3}H]puromycin (final concentration 50 μM, 1000 Ci/mol), and proteins were obtained as indicated. Gel radioactivity yields: (A) 31%; (B) 51%. In (C), 1.0 nmol of photolyzed *p*-azido[^{3}H]puromycin (88 Ci/mol) was applied to the gel; radioactivity yield 4%. In (D), 50S subunits (final concentration 2 μM) were added to photolyzed *p*-azido[^{3}H]puromycin (final concentration 25 μM, 944 Ci/mol), and proteins were obtained as indicated. Gel radioactivity yield 19%. In (A), (B), and (D), reported counts per minute are for protein extracted from 316 pmol of 70S ribosomes or subunits. Radioactivities shown are not normalized to a common percent yield.

ribosomes with a mixture of *p*-azidopuromycin and ϕ-*p*-azidopuromycin does lead to a significant time-dependent incorporation (experiments 10–12), suggesting the presence of an electrophilic component of ϕ-*p*-azidopuromycin, possibly an azirine (Staros, 1980). Incorporations measured using procedure A for photolysis with both the 2537 Å and 3500 Å lamps are reported in experiments 13 and 14, respectively. It should be emphasized that, even in the absence of β-mercaptoethanol quench, slow covalent reaction makes only a modest contribution ($\leq$25%) to overall covalent incorporation during a typical procedure B isolation (compare experiments 6 and 13), so that results obtained with procedure B provide a reasonable approximation of incorporation via the fast, light-dependent reaction.

The effect of the isolation procedure on apparent incorporation into ribosomal protein as measured by one-dimensional urea–polyacrylamide gel electrophoresis analysis (Figure 1) parallels that seen for overall incorporation. Thus, proteins extracted from *p*-azido[^{3}H]puromycin-labeled ribosomes isolated by procedure C display three large bands of radioactivity migrating with ribosomal protein (Figure 1A), whereas if procedure B is used, the radioactivity in the 30S and 50S proteins (Figure 1B) add up to give a calculated 70S pattern (Figure 1C) reduced in amount and quite different in distribution from that seen in Figure 1A.

That apparent labeling of ribosomal proteins isolated from a photoincorporation experiment using procedure C mainly reflects light-independent interaction of ϕ-*p*-azido[^{3}H]puromycin with ribosomal protein which does not survive procedure B isolation is clearly shown by the results presented in Figure 2. Thus, although ϕ-*p*-azido[^{3}H]puromycin when analyzed by itself displays no radioactivity migrating in a gel region corresponding to ribosomal protein (Figure 2C), when ϕ-*p*-azido[^{3}H]puromycin is added to 70S ribosomes in the dark and a procedure C isolation is employed, radioactivity migrating with ribosomal protein (Figure 2A) yields a pattern similar to that seen in Figure 1A. Similarly, use of a procedure C isolation of proteins from a dark incubation of 50S subunits and ϕ-*p*-azido[^{3}H]puromycin gives a clear peak of radioactivity on polyacrylamide gel electrophoresis analysis (Figure 2D). By contrast, when ϕ-*p*-azido[^{3}H]puromycin is added to 70S ribosomes in the dark and a procedure B isolation is employed, almost no radioactivity is seen with 50S protein and only a small peak of radioactivity is seen with 30S protein (Figure 2B). A similar result, no radioactivity in the gel, is obtained when ϕ-*p*-azido[^{3}H]puromycin is incubated with 50S subunits in the dark and a procedure B isolation is used (data not shown).

These results, and the very low background for overall incorporation in the absence of photolysis using procedure B (or procedure A, Table I), lead us to conclude that the labeling seen when proteins are isolated from a photoincorporation experiment by procedure B in the normal manner (Figure 1B,C) principally reflects light-dependent photoincorporation of *p*-azido[^{3}H]puromycin. There is in addition a small amount of light-independent incorporation of ϕ-*p*-azido[^{3}H]puromycin into ribosomal protein (Figure 2B) which also survives procedure B isolation (vide infra).

Light-Dependent Covalent Incorporation of p-Azidopuromycin into Ribosomes: Distribution of Label. Having described protocols allowing measurement of light-dependent incorporation of *p*-azidopuromycin into ribosomes (procedure B) and minimizing the extent of slow light-independent incorporation (procedure A), we next present results (Table II) measuring the extent of incorporation into ribosomal subunits, into ribosomal RNA, and into discrete gel regions when proteins extracted from labeled ribosomes are subjected to one-dimensional urea–polyacrylamide gel electrophoresis analysis. Typical 50S and 30S gel patterns are shown in Figure

Table II: Incorporation of *p*-Azidopuromycin and Puromycin into Ribosomal Proteins and RNA[a]

expt	radioactive ligand, mM[b]	addition or comment	lamp	irradiation time (min)	isolation procedure	incorporation of *p*-azidopuromycin (% mol/mol) sub-unit	overall	RNA	gel region I	gel region II
1	AzPur, 0.05		3500	10	A	50	11.4 ± 2.0	5.5 ± 1.0	0.5 ± 0.1	1.9 ± 0.05
						30	8.4 ± 2.0	2.1 ± 0.4	1.0 ± 0.4	1.8 ± 0.07
2	AzPur, 0.05		2537	10	A	50	24.6	12.2	0.8	3.6
						30	16.4	4.3	2.0	3.7
3	AzPur, 0.05	subunit separation in TKM gradient	3500	10	A	50	13.4	4.8	1.3	3.0
						30	11.8	2.6	1.5	3.4
4	AzPur, 0.03; φ-AzPur, 0.02	10-min dark incubation with ribosomes prior to quench			A	50	0.5	0.1	0.14	0.19
						30	1.3	0.1	0.12	0.51
5	AzPur, 0.05	+2 mM βME	3500	10	A	50	6.8 ± 0.2	3.8 ± 0.6	0.19 ± 0.05	1.01 ± 0.08
						30	3.0 ± 0.2	1.5 ± 0.3	0.22 ± 0.01	0.06 ± 0.01
6	AzPur, 0.05	Ribosomes pre-incubated with 1.5 mM NEM[c]	3500	10	A	50	14.6	6.4	0.57	2.4
						30	6.9	2.6	1.19	0.28
7	AzPur, 0.05		3500	2	B	50	3.5	1.7	0.10	0.33
						30	2.9	0.9	0.46	0.40
8	AzPur, 0.05		3500	10	B	50	14.8 ± 1.8	6.4 ± 1.6	0.66 ± 0.14	2.9 ± 0.4
						30	12.2 ± 1.7	3.0 ± 0.7	1.85 ± 0.07	2.1 ± 0.7
9	AzPur, 0.05		3500	30	B	50	23.2	10.4	1.84	4.4
						30	17.3	5.4	2.20	2.5
10	Pur, 0.05		3500	10	B	50	0.3	0.2		0.1
						30	0.1	0.0		
11	Pur, 2.0		3500	10	A	50				0.72
						30				
12	Pur, 2.0	+0.05 mM AzPur	3500	10	A	50				0.81
						30				

[a] In all experiments, 70S ribosomes were the target of photoincorporation, and subunits were separated in a TMKNa sucrose gradient, except as indicated. [b] AzPur, *p*-azidopuromycin; φ-AzPur, photolyzed *p*-azidopuromycin; Pur, puromycin. [c] 30 min, 37 °C, TMK buffer.

3A,B. The two regions containing appreciable radioactivity on analysis of 50S proteins are designated 50S-I and 50S-II, and include proteins L2–L6, L8–L10 and L13–L19, L21–L23, L25, respectively. Similarly, two regions containing appreciable radioactivity on analysis of 30S proteins are designated 30S-I and 30S-II, and include proteins S3–S8 and S14, S18, respectively. It should be noted that no major peaks of radioactivity were observed outside of these regions.

From experiment 1 (Table II), it is clear that incorporation takes place into the protein and RNA portions of both 50S and 30S particles. In the absence of β-mercaptoethanol, incorporation into 50S subunits is slightly favored, with the difference being largely due to a preferred incorporation into 50S RNA as compared with 30S RNA. Gel regions 50S-II and 30S-II contain the highest amounts of radioactive label and, on average, are labeled to approximately the same extents. Changing the light source to 2537 Å lamps (experiment 2) has little effect on the overall distribution of label, although the yield of photoincorporation is increased approximately 2-fold. Somewhat higher apparent yields of photoincorporation, again with little change in the overall labeling pattern, are also seen when subunits are separated on a low Mg^{2+} sucrose gradient (experiment 3) instead of the standard high-salt gradient. This is probably due to incomplete removal of noncovalently bound material (see Discussion) and/or a slightly higher yield of ribosomal protein surviving the gradient. Experiment 4 was designed to provide an estimate of incorporation arising from light-independent reaction of φ-*p*-azidopuromycin with ribosomes when procedure A is employed. From comparison of experiments 1 and 4, such incorporation makes essentially no contribution to RNA labeling and only a minor contribution to labeling of gel regions 50S-II and 30S-I (~10%), but is an important component (~30%) of the labeling of gel regions 50S-I and 30S-II. Somewhat higher yields of incorporation are also obtained, as expected, when procedure B (experiment 8) is used in place of procedure A, with the largest relative increase (~85%) occurring in region 30S-I.

Inclusion of 2 mM β-mercaptoethanol in the reaction mixture undergoing photolysis (experiment 5) results in a large change in both the amount (decreased about 2-fold) and distribution of photoincorporated *p*-azido[^{3}H]puromycin. This change is in no way related to thiol reduction of the azido function, as described by Staros et al. (1978), a process which proceeds on a much longer time scale. For example, at 20 °C, we find a $t_{1/2}$ of 96 min, as measured by loss of the A_{261} absorption maximum, for reduction of *p*-azidopuromycin in TMK buffer which is 5 mM in dithiothreitol (Nicholson & Cooperman, 1978). In the presence of β-mercaptoethanol, labeling of the 50S subunit is more than double that of the 30S subunit, and protein labeling becomes very specific for gel region 50S-II (Figure 3C), accounting for 68% of the total labeling in the four major gel regions (Table II). This contrasts with a figure of 37% for photolysis performed in the absence of β-mercaptoethanol (experiment 1). As may be seen in Figure 3, the increase in specificity is a result of the much smaller decrease in labeling of 50S-II (45%) caused by inclusion of β-mercaptoethanol in the photolysis medium as compared with the very large decreases seen in 30S-II (97%) and 30S-I (78%) labeling and the large decrease seen in 50S-I

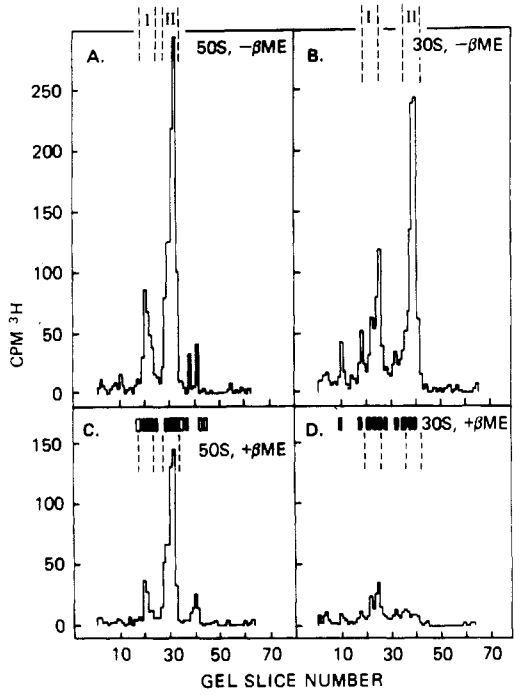

FIGURE 3: One-dimensional urea–polyacrylamide gel electrophoresis analyses of ribosomal proteins derived from 70S ribosomes irradiated with *p*-azido[^{3}H]puromycin in the absence and presence of β-mercaptoethanol. (A) 50S proteins − β-mercaptoethanol. (B) 30S proteins − β-mercaptoethanol. (C) 50S proteins + 2 mM β-mercaptoethanol. (D) 30S proteins + 2 mM β-mercaptoethanol. Gel regions are indicated at the top of each panel. Staining patterns and gel regions are indicated at the top of panels C and D [see also Jaynes et al. (1978)]. Experimental conditions: 70S ribosomes, 2.6 μM; *p*-azido[^{3}H]puromycin, 50 μM, 2500 Ci/mol; photolysis time 10 min; 3500 Å lamps; counts per minute are for protein extracted from 156 pmol of subunits normalized to an average gel radioactivity yield of 26%. Isolation: procedure A. Gel radioactivity yields: (A) 20%; (B) 27%; (C) 26%; (D) 33%.

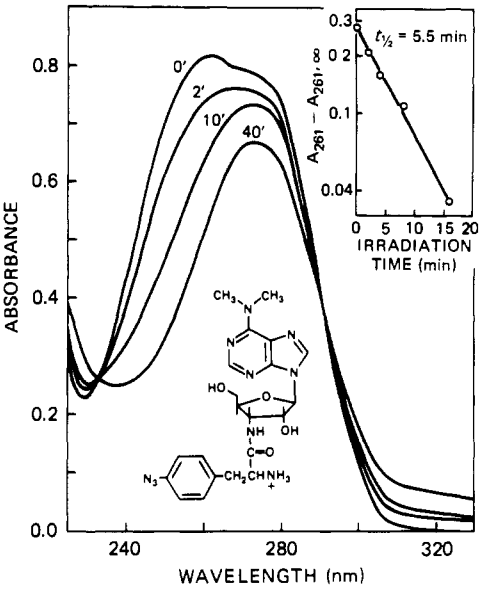

FIGURE 4: Changes in the ultraviolet spectrum of *p*-azidopuromycin as a function of irradiation time. *p*-Azidopuromycin (33 μM) dissolved in TMK buffer was irradiated with the 3500 Å lamps for the times indicated. A $t_{1/2}$ or photolysis was obtained by a first-order plot of A_{261} (inset).

(62%) labeling. Labeling of either 50S or 30S RNA is also decreased to a relatively small extent (31% and 29%, respectively) on inclusion of β-mercaptoethanol and as a result accounts for a larger fraction of total labeling (>50% for each subunit). Brief pretreatment of ribosomes with the sulfhydryl reagent *N*-ethylmaleimide (experiment 6) also leads to a large decrease in 30S-II labeling (84%) but has little effect on the labeling of the other three major gel regions. This result suggests the importance of a sulfhydryl group for labeling of S18 (see Discussion).

As noted above (see Experimental Procedures) values reported in Table II are normalized for 100% recovery of radioactivity applied to the gel. Since the average yield was 42%, the observed values would typically be some 2.5 times lower. We believe the normalized values are the more reliable for two reasons. First, the low background values for total incorporation seen in procedure A (Table I, experiment 12) are evidence that the apparent incorporation at the subunit level is all covalent. Thus, the loss of radioactivity on polyacrylamide gel electrophoresis analysis corresponds to loss of covalently incorporated material. Additional support for this idea is provided by the almost total lack of radioactivity we observe in the acetone–acetic acid supernatant on precipitation of ribosomal protein isolated from subunits (see Experimental Procedures). Second, experiments 1, 5, and 8 (Table II) were performed several (4–7) times, permitting calculation of meaningful average deviations for radioactivities found in gel regions I and II, which are of the order of 30%. If normalized values are not used, considerably more scatter is found, and average deviations are found to be of the order of 60%. It must be emphasized that correcting the gels to 100% recovery has little effect on the qualitative conclusions reached as a result of this study. This is because experiments in which direct comparisons are being made (e.g., experiment 1 vs. 5) were performed at the same time and run on the same slab gel, and variability in gel radioactivity yield from a common slab gel was much less than that between gels run at different times (see, for example, legends to Figures 1 and 3).

Light-Dependent Incorporation of p-Azido[^{3}H]puromycin into Ribosomes Is Azide Dependent. Two experimental approaches were used to demonstrate the azide dependence of light-dependent incorporation of *p*-azido[^{3}H]puromycin. First, the time dependence for photoincorporation was shown to be essentially identical with the time dependence for photolysis of the aryl azide moiety. Figure 4 shows the change in the ultraviolet spectrum of a solution of *p*-azidopuromycin as a function of irradiation time. A first-order plot of ln A_{261} − ln $A_{261,\infty}$ vs. time allows calculation of a $t_{1/2}$ of 5.5 min for destruction of the phenyl azide moiety under conditions paralleling those of a photoincorporation experiment. Approximate half-lives for the photoincorporation of *p*-azidopuromycin into ribosomal subunits or protein gel regions can be obtained from the time-dependence studies presented in Table II (experiments 7–9) and are all approximately the same, averaging 6 ± 1 min, or about the same as the $t_{1/2}$ for aryl azide photolysis. Second, under identical conditions photoincorporation of *p*-azido[^{3}H]puromycin into gel region 50S-II (Table II, experiment 8) was found to proceed to an almost 30-fold greater extent than photoincorporation of [^{3}H]puromycin into this same region (Table II, experiment 10). Thus, although 50S-II is the gel region most heavily photolabeled by native puromycin (Jaynes et al., 1978), *p*-azidopuromycin photoincorporation into 50S-II must proceed in the main via a different photochemistry from that responsible for puromycin photoincorporation. A comparison of experiments 11 and 12 (Table II) also shows that at the concentration used in most of our photoincorporation experiments (0.05 mM), *p*-azidopuromycin exerts no stimulatory effect on native puromycin photoincorporation.

FIGURE 5: Two-dimensional polyacrylamide gel electrophoresis analysis of basic ribosomal proteins extracted from 70S ribosomes irradiated with *p*-azido[^{3}H]puromycin in the absence of β-mercaptoethanol. (A) 50S proteins. (B) 30S proteins. Areas of high radioactivity (>100 cpm) are stippled. Experimental conditions: 70S ribosomes, 2.6 μM; *p*-azido[^{3}H]puromycin, 50 μM, 2500 Ci/mol; photolysis time 10 min; 3500 Å lamps; counts per minute are for protein extracted from 663 pmol of subunits. Isolation: procedure A. Gel radioactivity yields: (A) 18%; (B) 18%. Note that no area corresponding to protein L21 is shown in (A). This protein is close to its isoelectric point at the pH of the first dimension and thus is only sometimes found to migrate toward the anode. A two-dimensional polyacrylamide gel electrophoresis analysis of an essentially identical experiment in which L21 was seen showed little radioactivity comigrating with this protein.

Identification of the Major Proteins Labeled by p-Azidopuromycin. Two-dimensional polyacrylamide gel electrophoresis, specific immunoprecipitation, and one-dimensional $NaDodSO_4$–polyacrylamide gel electrophoresis analyses were performed on proteins extracted from ribosomes photolyzed with *p*-azido[^{3}H]puromycin in order to establish the identities of the major labeled proteins. Only the basic proteins were examined by two-dimensional urea–polyacrylamide gel electrophoresis since it was clear from one-dimensional urea–polyacrylamide gel electrophoresis analysis of total 70S proteins that no acidic proteins were labeled to major extents (Nicholson, 1981) and the resolving power of two-dimensional polyacrylamide gel electrophoresis is doubled when proteins are applied only to the anodic end as opposed to the middle of the first-dimensional gel. Typical two-dimensional gels of 50S proteins and 30S proteins labeled in the absence of β-mercaptoethanol are presented in Figure 5. From Figure 5A it is clear that three major radioactive gel regions are found on analysis of 50S protein comigrating with or near proteins included in gel region 50S-II. These areas are at or above proteins L23, L18, and L15 and are separated from one another by gel regions of low radioactivity. A two-dimensional gel of 50S protein labeled in the presence of β-mercaptoethanol gave qualitatively similar results. The amounts of radioactivity found in these three areas in both experiments are listed in Table III.

Table III: Amounts of Radioactivity (cpm) in Major Labeled Areas on Two-Dimensional Polyacrylamide Gel Electrophoresis Analysis of Photolabeled Proteins[a]

protein area	absence of β-mercaptoethanol during photolysis	presence of 2 mM β-mercaptoethanol during photolysis
"L23"	911	383
"L18"	609	361
"L15"	308	132
"S18"	1253	ND[b]
S7	271	ND

[a] Experimental conditions are as described in the legend to Figure 3. Radioactivity reported is for protein from 702 pmol of subunits. Gel radioactivity yield 18% for both analyses. Radioactivities migrating with or adjacent to proteins L23, L18, L15, and S18 are listed (see text). For S7, only radioactivity comigrating with protein staining is included. [b] ND, not determined.

Previously we showed that puromycin photoincorporation into protein L23 retards migration of this protein in both dimensions on two-dimensional polyacrylamide gel electrophoresis analysis, with the result that puromycin-labeled L23 migrates slightly to the upper left of native L23 (Jaynes et al., 1978; Grant et al., 1979a). From the similarity of *p*-azidopuromycin to puromycin, it is reasonable to expect *p*-azido-

Table IV: Immunoprecipitation Results

experiment no.:	1	2	3	4
irradiation lamps (Å):	3500	3500	3500	2537
presence of 2 mM βME:	–	+	–	–
isolation procedure:	A	A	B	B
antiserum to protein	% labeled 30S or 50S protein precipitated			
L11				2
L13	2 ± 1	7		
L14	−1 ± 1	2	3	
L15	7			
L17	1 ± 0	3	5	4
L18/22	7.5 ± 0.5	13	13	18
L19	1 ± 1	1		1
L23	21 ± 0.5	17	21	29
S3	4		0	5
S4	3 ± 1	7	23	27
S5	5		10	9
S7	11 ± 2	17		
S8	1 ± 1			
S14	9 ± 3		7	17
S18	31 ± 1		23	22
S19			6	12

puromycin photoincorporation to have effects on migration similar to those of puromycin photoincorporation. As a result, two-dimensional polyacrylamide gel electrophoresis results constitute strong evidence that L23 ≥ L18 > L15 are the major 50S proteins labeled by *p*-azidopuromycin in both the absence and presence of β-mercaptoethanol. However, identification of the major labeled proteins by two-dimensional polyacrylamide gel electrophoresis analysis above must be considered tentative, because of the proximity to L23, L18, and L15 on the two-dimensional gel of several other proteins, in particular, L13, L14, L17, and L22 (Figure 5A).

The results of specific immunoprecipitation analysis, summarized in Table IV, allow more conclusive identification of the major labeled proteins. These results are in good qualitative accord with the polyacrylamide gel electrophoresis results presented above, although quantitatively the labeling levels estimated by immunoprecipitation are generally lower than those estimated by polyacrylamide gel electrophoresis analysis. Such a difference was also observed for puromycin photoincorporation (Grant et al., 1979a,b), and possible reasons for it are discussed in this earlier study.

Considering first experiments 1 and 2 (350-nm irradiation, procedure A workup, absence and presence of β-mercaptoethanol, respectively) in Table IV, L23 is clearly shown to be the protein labeled to the highest extent, and either protein L18 or protein L22 is confirmed as the second most highly labeled protein. Unfortunately, the unavailability of an antiserum with single specificity for either of these latter two proteins does not allow a definitive choice to be made between them. There is a minor discrepancy in the amount of L18/L22 (relative to L23) labeling in experiment 1 which is considerably lower by immunoprecipitation analysis that would have been expected on the basis of the two-dimensional polyacrylamide gel electrophoresis results. The reason for this discrepancy is not understood, particularly as the agreement of the immunoprecipitation and two-dimensional polyacrylamide gel electrophoresis results for experiment 2 is excellent. The specific immunoprecipitation results also show that proteins L15 and L13 are labeled to significant, although lesser, extents. For protein L15, this is shown directly in experiment 1. Although a shortage of antiserum to protein L15 prevented analysis of its labeling in experiment 2, significant L15 labeling in the presence of β-mercaptoethanol may be inferred from the low measured levels of L15 and L17 labeling in experiment 2, and the significant labeling in the L15 region on two-dimensional polyacrylamide gel electrophoresis analysis (Table III) of this same sample. Protein L13 labeling accounts for a significant percentage of overall 50S protein labeling only in experiment 2, an indication that photoincorporation into this protein is much more resistant toward the presence of β-mercaptoethanol in the photolysis medium than is overall photoincorporation into 50S protein. Experiments 3 and 4 in Table IV were performed at an earlier stage of this research and employed a method B workup, so that some of the labeling seen should reflect slow covalent reaction of φ-*p*-azidopuromycin with ribosomal protein. As demonstrated above, such reaction is of only minor importance for 50S-II protein labeling, so the results obtained represent further evidence that proteins L23 and L18/L22 are the major proteins photolabeled by *p*-azidopuromycin on irradiation with either the 2537 Å or the 3500 Å lamps.

30S Protein Labeling. The general approach described above for identification of the major labeled 50S proteins was also applied for identification of the major labeled 30S proteins. Considering labeling results obtained in the absence of β-mercaptoethanol first, the one-dimensional urea–polyacrylamide gel electrophoresis results in Figure 3B showed that major 30S protein labeling is found in gel region II, comprising proteins S14 and S18, while a lesser peak of radioactivity was found in region 30S-I, comprising proteins S3–S8. Two-dimensional polyacrylamide gel electrophoresis analysis (Figure 5B) provides fairly convincing evidence that S18 is the major labeled protein. Protein S7 appears to be the most highly labeled protein in the group S3, S4, S5, S7, and S8, and the extent of labeling of protein S14 is unclear, because of the possible overlap with S18 labeling. The immunoprecipitation results (Table IV, experiment 1) confirm that S18 is the major labeled protein and that S7 and S14 are labeled to secondary extents. For labeling performed in the presence of β-mercaptoethanol, the one-dimensional urea–polyacrylamide gel electrophoresis results (Figure 3D) show that none of the 30S proteins is labeled to a major extent compared with 50S proteins. The largest peak of radioactivity seen migrates with protein S7 on this gel, and not far from proteins S4, S5, and S8. Further evidence that S7 is the 30S protein labeled to the highest extent is provided by immunoprecipitation analysis (Table IV, experiment 2), which compares directly S7 and S4 labeling, and by one-dimensional $NaDodSO_4$–urea–polyacrylamide gel electrophoresis (Figure 6). This gel system affords very good resolution of S4, S5, and S7 and clearly shows that S7 is the most highly labeled within this group of proteins. S8 labeling is negligible in the absence of β-mercaptoethanol (Table IV, experiment 1) and is assumed to be negligible in its presence. Protein S1 is also seen to be labeled to a significant extent in Figure 6, although a similar 30S protein sample, when analyzed by urea–polyacrylamide gel electrophoresis (Figure 3D, gel slice 9), showed no label in S1. This variation is most probably due to variable S1 recovery in purified subunits (vide infra).

A comparison of experiments 1 and 3 in Table IV shows that the most dramatic change which occurs on going from a procedure B to a procedure A isolation is the large decrease in protein S4 labeling. This is consistent with the one-dimensional polyacrylamide gel electrophoresis results presented earlier (Table II), since gel region 30S-I, containing S4, shows the greatest relative decrease on comparing labeling obtained with the two procedures.

Table V: Relative Photoincorporation Levels[a]

	Nicholson & Cooperman (1978)	Krassnigg et al. (1978)	present work, procedure A	present work, procedure A
exptl protocols				
βME present during photolysis	−	−	−	+
βME postphotolysis quench	−	−	+	+
subunit separation	−	−	+	+
identification method	PAGE (2-D, 1-D)[d]	PAGE (2-D)	PAGE (2-D, 1-D), IP[b]	PAGE (2-D, 1-D), IP[b]
L proteins labeled				
L2	++	−	−	−
L3	++	−	−	−
L6	+	+++	−	−
L11	+++	+	−	−
L13	+	+++	−	+
L14	+	−	−	−
L15	+	−	+	+
L17	++	−	−	−
L18/22	++	+++	++	+++
L23	+	+	+++	+++
L24	+	−	−	−
L25	−	+++	−	−
L27	++	+	−	−
L29	+	−	−	−
L30	−	+	−	−
S proteins labeled				
S1	−	−	−	+[c]
S3	++	−	−	−
S4	++	+	−	−
S5	++	−	−	−
S7	+	−	+	+
S9	+	−	−	−
S13	+	−	−	−
S14	+	−	+	−
S15	+	++	−	−
S18	+++	−	+++	−

[a] Number of pluses correlates qualitatively with amount of radioactive labeling. For the present work, a dash indicates that incorporation amounted to ≤15–20% of that of the major labeled protein. [b] Immunoprecipitation. [c] See text. [d] PAGE, polyacrylamide gel electrophoresis; 2-D, two-dimensional; 1-D, one-dimensional.

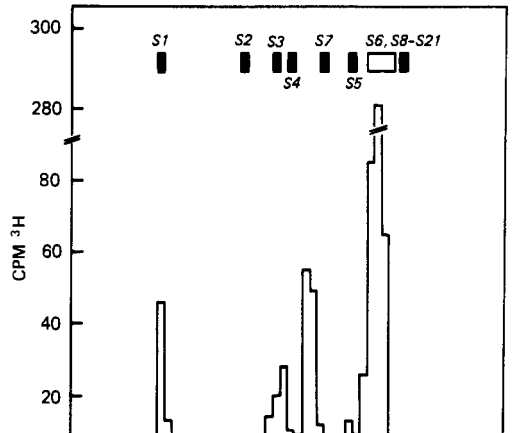

FIGURE 6: One-dimensional $NaDodSO_4$–urea–polyacrylamide gel electrophoresis analysis of 30S proteins derived from 70S ribosomes irradiated in the presence of *p*-azido[^{3}H]puromycin and β-mercaptoethanol. Conditions are similar to those described in Figure 3C,D. Counts per minute reported are for protein extracted from 312 pmol of 30S subunits. Gel radioactivity yield 20%. The radioactivity found with protein S1 was quite variable, reflecting perhaps variable S1 recovery in purified subunits.

S1, L7, and L12 Labeling. Above we have invoked variable S1 recovery in purified subunits. Proteins L7 and L12 are also easily removed during subunit separation and isolation (Cohlberg, 1980). As a result, reliable estimation of *p*-azidopuromycin photoincorporation into these proteins cannot be carried out by using the standard isolation procedure A or B. Instead, we used a modified procedure A isolation to prepare 70S proteins from a photoincorporation experiment performed in the absence of β-mercaptoethanol in which centrifugation through a 10% sucrose cushion made up in TMK buffer was substituted for subunit separation on a sucrose gradient. The 70S proteins were then analyzed by one-dimensional urea–polyacrylamide gel electrophoresis, since in this system S1, L7, and L12, which were present in the 70S protein extract, are fully resolved from each other and from all other ribosomal proteins. Although the modified wash procedure did not completely remove noncovalently bound material from the gel, the amount of radioactivity migrating in the region corresponding to 50S-II was similar to that found on analysis of 50S proteins using a normal procedure A isolation and was much greater than radioactivity migrating with or near S1, L7, or L12 (data not shown). Thus, none of these proteins is labeled to a major extent by *p*-azido[^{3}H]puromycin.

Discussion

In our previous report describing initial photoincorporation experiments with *p*-azido[^{3}H]puromycin (Nicholson & Cooperman, 1978), we tentatively identified S18 and L11 as the two major labeled proteins and listed a large number of other proteins, as summarized in Table V, as being labeled to significant extents. These identifications were based on radioactivity migrating with protein on two-dimensional polyacrylamide gel electrophoresis. At about the same time Krassnigg et al. (1978) presented similar photolabeling experiments with *p*-azido[^{3}H]puromycin, in which once again two-dimensional polyacrylamide gel electrophoresis was used to identify labeled proteins. Their results are also summarized in Table V. Although the two studies show considerable

Table VI: Protein Content of 70S, 50S, and 30S Ribosomes[a]

particle	gradient buffer used for subunit isolation	μg of protein/ A_{260} unit	g of protein/ mmol of particle	
			found	expected[b]
70 S		24.3 ± 1.2	935	870
50 S	TKM	20.6 ± 0.1	528	
	TMKNa	19.3 ± 1.3	495	530
30 S	TKM	25.0 ± 0.1	321	
	TMKNa	23.6 ± 2.0	303	340

[a] See Experimental Procedures. [b] For 50S proteins, expected values were obtained assuming single copies of L1-L6, L9-L11, and L13-L34 and four copies of L7/L12 (L8 is a complex of L10 and L7/L12) (Pettersson et al., 1976). For 30S proteins, single copies of S1-S19 and S21 were assumed (S20 is the same as L26). Molecular weights were estimated from primary sequences (Wittmann et al., 1980) or from $NaDodSO_4$ gel data (Wittmann, 1974). Calculations assume 1 A_{260} unit is equal to 26 pmol of 70S ribosomes, 39 pmol of 50S subunits, and 78 pmol of 30S subunits.

differences regarding the identity of many of the proteins labeled to a significant extent, they are similar in showing a spread of label over a large number of proteins. Moreover, it is possible that some of the differences are only apparent. For example, L6 migrates very close to L11 (Figure 5A), so a radioactive area which was assigned to L11 in our experiment might have been assigned to L6 by Krassnigg et al. In any case, the careful reinvestigation of the labeling process performed in this paper provides cogent evidence that most of the apparent labeling seen previously was not due to rapid, photoincorporation of *p*-azidopuromycin but rather to light-independent interaction with φ-*p*-azidopuromycin. Such interaction may be divided into two kinds. One increases slowly with time, survives gradient separation, and almost certainly results from covalent reaction of an electrophilic component of φ-*p*-azido[^{3}H]puromycin with nucleophilic groups on the ribosome. Such covalent reaction, because it is slow, makes only a minor contribution to observed overall labeling when procedure B is used and is effectively suppressed when procedure A is used. The second type of interaction is lost on going from a procedure C (Figure 2A) to a procedure B (Figure 2B) or procedure A isolation and is most likely noncovalent in nature, since subunit separation suffices for its removal. An alternative explanation for the difference in polyacrylamide gel electrophoresis results on comparing procedures B and C would be that proteins which are fairly rapidly (within 10 min) covalently labeled by φ-*p*-azido[^{3}H]puromycin are removed during subunit separation. This alternative explanation is unlikely on three grounds: First, overall subunit incorporation (Table I) and one-dimensional polyacrylamide gel electrophoresis patterns (see Table II, Figure 3B) for photoincorporation experiments are essentially identical irrespective of whether a rigorous, high-salt (0.4 M NaCl) buffer or a gentler, low Mg^{2+} (1 mM) buffer is used to wash and resolve the subunits. Second, overall protein loss on gradient separation is modest and only slightly greater using high-salt (−15%) rather than low Mg^{2+} (−9%) gradients, as shown in Table VI. Since the loss in light-independent interaction of φ-*p*-azido[^{3}H]puromycin is close to 100%, in order for the alternative explanation to be true, protein labeled with φ-*p*-azido[^{3}H]puromycin would have to be removed with high selectivity on subunit dissociation. Third, our earlier work on puromycin photoincorporation (Jaynes et al., 1978) suggests that such selective removal is not the general rule. Thus, in this latter study a procedure C isolation gave low backgrounds, and the 70S protein labeling pattern was essentially identical with the pattern obtained on addition of 50S and 30S protein labeling.

In contrast to the two previous studies (Nicholson & Cooperman, 1978; Krassnigg et al., 1978), essentially all of the protein labeling observed in the present study when a procedure A isolation is used is due to rapid photoincorporation of *p*-azidopuromycin. A summary of such labeling for photolysis performed in the absence and presence of β-mercaptoethanol is presented in Table V alongside of the results reported in earlier studies. The labeling patterns are clearly more specific in the current studies. The reasons for some of the more obvious differences with our earlier study (Nicholson & Cooperman, 1978) are apparent from work presented under Results. Thus, the major band of radioactivity on electrophoresis of a solution containing φ-*p*-azido[^{3}H]puromycin and 50S protein comigrates with protein L11 (Figure 2D), thus accounting for our earlier report of high L11 labeling by *p*-azidopuromycin. Also, the major labeling of protein S4 seen earlier must have been due at least in part to light-independent incorporation of φ-*p*-azido[^{3}H]puromycin, as shown by the large decrease in S4 labeling on going from a procedure B to a procedure A isolation (Table IV, experiments 1 and 3). This is consistent with the known high relative nucleophilicity of protein S4 (Cooperman, 1980). Similar reasons probably account for the lack of labeling found in several other proteins, using a procedure A isolation, which we previously reported to be labeled to high extents.

When a procedure A isolation is used, the major labeled 50S proteins are L23 > L18/22 > L15 and L23 > L18/22 > L15, L13 in the absence and presence of β-mercaptoethanol, respectively. For 30S proteins, S18 > S7, S14 and S7 > S1 are the major proteins labeled in the absence and presence of β-mercaptoethanol, respectively. It is important to emphasize that because our efforts have been mainly directed at identifying those proteins labeled to the highest extents, proteins labeled to lesser extents have only been carefully studied if they migrate close to the major labeled proteins on gel electrophoresis. Thus, the two-dimensional polyacrylamide gel electrophoresis evidence in Figure 5A,B suggests significant labeling of L3, L5, S4, and S5, but because the amount of radioactivity migrating with these proteins is <15% that migrating with or near L23, labeling of these proteins was not further analyzed by immunoprecipitation.

The noncovalent binding and light-independent covalent incorporation of photoproduct we observe are not unique to *p*-azidopuromycin and ribosomes. Effects similar to those reported here have been noted previously by Bayley & Knowles (1978), using phenyl azide to probe membrane structure, and in photoaffinity labeling studies on α-fetoprotein, using 16-diazoestrone (Payne et al., 1980), on rhodospin, using NAP-taurine (Mas et al., 1980) and on the *E. coli* ribosome, by Maasen & Möller (1978), using a 4-azidosalicyl alcohol derivative of GTP, and by R. A. Goldman et al. (R. A. Goldman, T. Hasan, W. A. Strycharz, and B. S. Cooperman, unpublished results) with tetracycline. It is thus clear that as a general proposition greater attention must be paid to the interaction of photoproduct with receptor than has hitherto been the case in many photoaffinity labeling studies. For example, in preliminary work, we have prepared ^{3}H-labeled [N^{ϵ}-(2-nitro-4-azidophenyl)-L-lysinyl]puromycin aminonucleoside (NAP-LysPANS) (1000 Ci/mol) following the synthesis of Vince et al. (1978) and found that when this compound is photolyzed in the presence of ribosomes, in addition to true incorporation there is a great deal of light-independent and presumably noncovalent binding of photoproduct to ribosomal protein,

although unphotolyzed NAPLysPANS is easily removed. Urea–polyacrylamide gel electrophoresis analysis of proteins extracted from 50S subunits isolated from 70S ribosomes incubated in the dark with photolyzed [^{3}H]NAPLysPANS gives a pattern identical with that seen in Figure 2D. Thus the behavior of NAPLysPANS parallels that of *p*-azidopuromycin. In fact, it appears somewhat harder to remove noncovalently bound photolyzed NAPLysPANS from ribosomal protein than it is to remove noncovalently bound photolyzed *p*-azidopuromycin. Earlier, Vince et al. (1978) had reported that radioactivity from [^{14}C]-*N*-AcPhe-tRNA could be transferred to the protein faction of ribosomes photolyzed in the presence of NAPLysPANS in a poly(uridylic acid)-dependent reaction. This result was taken as evidence that some of the covalently bound photoaffinity label was oriented correctly in the peptidyltransferase center to accept *N*-AcPhe from *N*-AcPhe-tRNA and was thus, by definition, in the A site. Our results with NAPLysPANS and *p*-azidopuromycin suggest an alternative explanation of the Vince et al. (1978) result, whereby the *N*-AcPhe group is merely transferred to noncovalently bound photolyzed NAPLysPANS which acts as a functional analogue of puromycin in much the same manner as does ϕ-*p*-azidopuromycin (Nicholson et al., 1982), and the product thus formed copurifies with ribosomal protein (in the experiment under consideration, protein was isolated from 70S ribosomes by RNase digestion and trichloroacetic acid precipitation).

A major problem in photoaffinity labeling studies is the determination of the extent to which an observed labeling pattern is site specific rather than determined by the chemical reactivity of receptor components (Cooperman, 1976, 1980). Protein L23 is the 50S protein photolabeled to the highest extent by both *p*-azido[^{3}H]puromycin and [^{3}H]puromycin (Cooperman et al., 1975; Jaynes et al., 1978). Since major labeling of the same protein by different photochemistries would only be coincidental if labeling were determined by chemical reactivity alone, these results would provide important support for the site specificity of labeling if it could in fact be shown that each compound incorporates into L23 via a different photochemistry. Experiments presented above (see Results), showing a time dependence for labeling essentially identical with that for azide destruction and a much higher yield of photoincorporation of *p*-azido[^{3}H]puromycin compared to [^{3}H]puromycin, clearly demonstrate the azide dependence of *p*-azido[^{3}H]puromycin photoincorporation. The enhanced L23 labeling is unlikely to be due to a higher affinity of *p*-azidopuromycin for the ribosome as compared with that of puromycin, since they are structurally similar ligands and have comparable K_m values in peptidyltransferase and other assays of ribosomal function (Symons et al., 1978; Krassnigg et al., 1978; Nicholson et al., 1982). It is also unlikely that enhanced labeling is due to intermolecular photosensitization of intrinsic puromycin photoincorporation by the aryl azide group in a manner analogous to that of tetracycline (Grant et al., 1979a) and of flavin mononucleotide (Cooperman et al., 1977), since experiments 11 and 12 in Table II show that added *p*-azidopuromycin has little or no effect on [^{3}H]puromycin photoincorporation into either 70S ribosomes or gel region 50S-II. We therefore conclude either that *p*-azidopuromycin directly labels L23 via a nitrene or that the phenyl azide group acts as an intramolecular photosensitizer of the intrinsic puromycin photoincorporation mechanism. A definitive choice between the two mechanisms would require determination of the covalent bond(s) joining *p*-azidopuromycin to protein L23, about which there is at present no information. However, the lack of intramolecular interaction between the *O*-methyltyrosine and dimethyladenine groups of puromycin found both in its crystal structure (Sundaralingam & Arora, 1969) and in solution studies by NMR (Johnson & Bhacca, 1963) argues against an intramolecular photosensitization mechanism.

Thiol effects on photolabeling can also be used to obtain evidence for true photoaffinity labeling, since by acting as scavengers thiols should preferentially decrease labeling of a receptor from solution or via slowly reacting photogenerated intermediates (Ruoho et al., 1973; Maasen & Möller, 1974). Labeling of lower affinity sites, which will in general exchange ligand more rapidly with solution, might also be preferentially decreased compared with higher affinity site labeling. Thus the dramatic effect of 2 mM β-mercaptoethanol on our labeling results, as summarized in Table IV, supports the notion that *p*-azidopuromycin photolabels the 50S proteins L23, L18/22, and L15 from a higher affinity site or sites, whereas S18 labeling occurs either from solution or from a lower affinity site. The very high extent of S18 labeling seen in the absence of β-mercaptoethanol is then most plausibly attributed to the uniquely high nucleophilic reactivity of the cysteine-10 in this protein (Ewald et al., 1976; Yaguchi et al., 1978; Ghosh & Moore, 1979), a conclusion which is well supported by the strongly selective inhibition of S18 labeling found on preincubation of the ribosomes with the sulfhydryl derivatizing reagent *N*-ethylmaleimide (Table II). Other pieces of evidence for the site specificity of *p*-azidopuromycin photoaffinity labeling, as well as the relationship of the proteins labeled to functional sites on the ribosome, are considered in the following paper (Nicholson et al., 1982).

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