Photoaffinity Labeling of *Escherichia coli* Ribosomes by an Aryl Azide Analogue of Puromycin. Evidence for the Functional Site Specificity of Labeling†

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ABSTRACT: The photoincorporation of *p*-azido[^{3}H]puromycin [6-(dimethylamino)-9-[3′-deoxy-3′-[(*p*-azido-L-phenylalanyl)amino]-β-D-ribofuranosyl]purine] into specific ribosomal proteins and ribosomal RNA [Nicholson, A. W., Hall, C. C., Strycharz, W. A., & Cooperman, B. S. (1982) *Biochemistry* (preceding paper in this issue)] is decreased in the presence of puromycin, thus demonstrating that labeling is site specific. The magnitudes of the decreases in incorporation into the major labeled 50S proteins found on addition of different potential ribosome ligands parallel the abilities of these same ligands to inhibit peptidyltransferase. This result provides evidence that *p*-azidopuromycin photoincorporation into these proteins occurs at the peptidyltransferase center of the 50S subunit, a conclusion supported by other studies of ribosome structure and function. A striking new finding of this work is that puromycin aminonucleoside is a competitive inhibitor of puromycin in peptidyltransferase. The photoincorporation of *p*-azidopuromycin is accompanied by loss of ribosomal function, but photoincorporated *p*-azidopuromycin is not a competent peptidyl acceptor. The significance of these results is discussed. Photolabeling of 30S proteins by *p*-azidopuromycin apparently takes place from sites of lower puromycin affinity than that of the 50S site. The possible relationship of the major proteins labeled, S18, S7, and S14, to tRNA binding is considered.

We have been carrying out photoaffinity labeling experiments on the *Escherichia coli* ribosome with puromycin (Cooperman et al., 1975; Cooperman, 1978, 1980a,b; Jaynes et al., 1978; Grant et al., 1979a,b) and *p*-azidopuromycin [6-(dimethylamino)-9-[3′-deoxy-3′-[(*p*-azido-L-phenylalanyl)amino]-β-D-ribofuranosyl]purine] (Nicholson & Cooperman, 1978) in an effort to identify the components of the peptidyltransferase center in this particle. In the preceding paper (Nicholson et al., 1982) we developed a procedure for eliminating labeling artifacts which had plagued earlier studies with *p*-azidopuromycin and showed that the proteins which were covalently labeled in a rapid, light-dependent process on photolysis of ribosomes and *p*-azidopuromycin were S18 > L23 > L18/22 > L15 ~ S7, S14 for photolyses conducted in the absence of β-mercaptoethanol and L23 ≥ L18/22 > L15, L13, S7, and possibly S1 for photolyses conducted in its presence. Portions of 50S and 30S RNA were also labeled, accounting for about half of the overall labeling. We now extend these studies to examine the functional site specificity of such labeling.

We find a parallelism between the ability of a potential ribosomal ligand to bind to the A′ site, defined as the site of binding of the 3′ end of aminoacyl-tRNA within the peptidyltransferase center (Harris & Symons, 1973), and the ability of a potential ribosomal ligand to inhibit *p*-azidopuromycin incorporation into the major labeled 50S proteins, and on this basis conclude that *p*-azidopuromycin is photoincorporating from the A′ site. However, other approaches designed to test this conclusion give inconclusive results.

While our results clearly show that true photoaffinity labeling is largely directed toward the 50S subunit, some site specificity is also observed for 30S subunit labeling. The possible significance of this labeling is also considered.

Experimental Procedures

Materials

Adenosine, puromycin aminonucleoside (PANS),[1] L-phenylalaninamide and D(+)-2-glucosamine hydrochloride were purchased from Sigma. L-[U-^{14}C]Phenylalanine was obtained from New England Nuclear and poly(U) from Miles. Bulk stripped *E. coli* tRNA was purchased from Grand Island Biologicals or Sigma and purified yeast tRNA^{Phe} from Boehringer. [^{14}C]Phe-tRNA was prepared from both types of tRNA by the method of Ravel & Shorey (1971), using either crude synthetase factors from ribosomal preparations or a more purified synthetase preparation obtained as a generous gift of Dr. J. Ofengand (Roche Institute of Molecular Biology, Nutley, NJ). [^{14}C]Phe-tRNA was stored in 10 mM potassium acetate (pH 5.5) at −80 °C. *N*-Ac[^{14}C]Phe-tRNA was prepared according to Haenni & Chapeville (1966), but the Sephadex G-25 column chromatography was omitted. All other materials were obtained as described in the preceding paper (Nicholson et al., 1982).

Methods

One- and two-dimensional polyacrylamide gel electrophoresis, photolyses, and postphotolysis protein and RNA isolation,

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[1] Abbreviations: AcPhe, acetyl-L-phenylalanine; AcLeu, acetyl-L-leucine; CpA(2′Gly)H, cytidylyl-(3′–5′)-2′-*O*-glycyl-3′-deoxyadenosine; CpA(2′Phe)H, cytidylyl-(3′–5′)-2′-*O*-L-phenylalanyl-3′-deoxyadenosine; CpA(2′H)Gly, cytidylyl-(3′–5′)-2′-deoxy-3′-*O*-glycyladenosine; CpA(2′H)Phe, cytidylyl-(3′–5′)-2′-deoxy-3′-*O*-L-phenylalanyladenosine; Me_2POPOP, 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene; poly(U), poly(uridylic acid); PPO, 2,5-diphenyloxazole; PANS, puromycin aminonucleoside; GlyPANS, glycinylpuromycin aminonucleoside; NAP-LysPANS, [N^{ϵ}-(2-nitro-4-azidophenyl)-L-lysinyl]puromycin aminonucleoside.

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using procedure A or B, were performed as described (Nicholson et al., 1982). Briefly, in procedure A, an ethanol–β-mercaptoethanol quench is added right after photolysis of *p*-azido[^{3}H]puromycin and ribosomes to halt any possible light-independent covalent reaction, and in buffers containing β-mercaptoethanol, samples are subjected to a low-speed centrifugation to remove polymeric *p*-azido[^{3}H]puromycin photoproducts, separated into subunits by ultracentrifugation on a sucrose density gradient in high-salt or low Mg^{2+} buffers, and protein is extracted with 67% acetic acid (Hardy et al., 1969). In procedure B, the quench step is omitted, and the separation buffers do not contain β-mercaptoethanol.

tRNA Binding. Phe-tRNA binding and *N*-AcPhe-tRNA binding were assayed by combining ribosomes, poly(U), and tRNA in buffer at 0 °C as described for each experiment under Results. Following incubation at 37 °C for 10 min, the samples were cooled to 0 °C, and aliquots were filtered onto a Millipore filter (HAWP, 0.45 μM), presoaked in assay buffer. The filter was washed 3 times with 1-mL aliquots of cold assay buffer, dried under a heat lamp, and counted in 2.5 mL of toluene containing 0.5% PPO and 0.01% Me_2POPOP. Percent binding was expressed as the total counts per minute bound on the filter divided by the total counts per minute added and multiplied by 100. A control which lacked poly(U) gave 5% of the binding obtained in its presence.

Peptidyltransferase Assay. Unless otherwise specified, the assay volume was 0.1 mL (25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 200 mM KCl). The ribosome concentration was 10 A_{260} units/mL and was in 5-fold molar excess over *N*-Ac[^{14}C]Phe-tRNA (approximately 5000 cpm of ^{14}C). Poly(U) was at a concentration of 0.12 mg/mL. Assays were prepared at 0 °C and then incubated at 37 °C for 5 min. After the assay solutions were cooled to 0 °C for 1 min, puromycin was added to a final concentration of 0.1 mM. Reactions were quenched by adding 0.5 volume of saturated $MgSO_4$ in 0.4 M potassium acetate–acetic acid (pH 5). A 1.5-mL sample of ethyl acetate was added, the samples were vortexed for 10 s, and the layers were allowed to separate for 15 min. One milliliter of the ethyl acetate layer was removed and counted for radioactivity in 2.5 mL of scintillation cocktail [1:1 toluene (0.5% PPO, 0.01% Me_2POPOP)–Triton X-100]. Percent transfer was expressed as the total counts per minute appearing in the ethyl acetate layer divided by the input counts per minute and multiplied by 100.

Results

(A) Site Specificity of p-Azidopuromycin Photoincorporation into Ribosomes: 50S Protein, 30S Protein, and RNA. (1) 50S Protein. One-dimensional urea–polyacrylamide gel electrophoresis analyses of 50S proteins obtained from 70S ribosomes irradiated with *p*-azido[^{3}H]puromycin in the presence and absence of puromycin (2 mM) are shown in Figure 1. Although it is clear that puromycin markedly reduces the labeling of gel region 50S-II (containing proteins L13–L19, L21–L23, and L25), no direct information is provided regarding the labeling of individual proteins migrating within region 50S-II. Accordingly, the samples were further analyzed by two-dimensional polyacrylamide gel electrophoresis, yielding the results shown in Figure 2.

As can be seen, labeling of each of the major areas of radioactivity, those at or near proteins L23 > L18 > L15 (Nicholson et al., 1982), is markedly reduced by puromycin, and to approximately the same extents (L23 by 64%, L18 by 57%, and L15 by 65%), whereas the labeling of the other proteins is, in general, less affected. Similar results were obtained by immunoprecipitation analysis of the 50S proteins

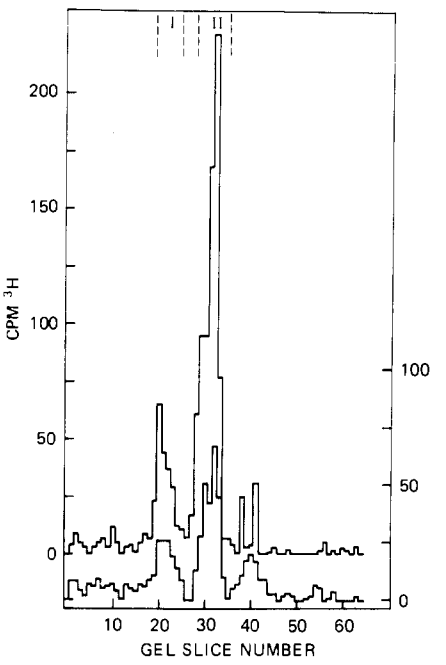

FIGURE 1: One-dimensional urea–polyacrylamide gel electrophoresis analyses of 50S proteins derived from 70S ribosomes irradiated with *p*-azido[^{3}H]puromycin in the absence and presence of puromycin. Upper pattern: −puromycin. Lower pattern: +2 mM puromycin. Experimental conditions: 70S ribosomes, 2.6 μM; *p*-azido[^{3}H]puromycin, 50 μM, 2500 Ci/mol; photolysis time 10 min; 3500 Å lamps at 4 °C in TMK buffer [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 50 mM KCl]. Isolation: procedure A. Counts per minute are for protein extracted from 156 pmol of 50S subunits, normalized to a gel radioactivity yield of 25%. Gel radioactivity yields: (A) 20%; (B) 24%.

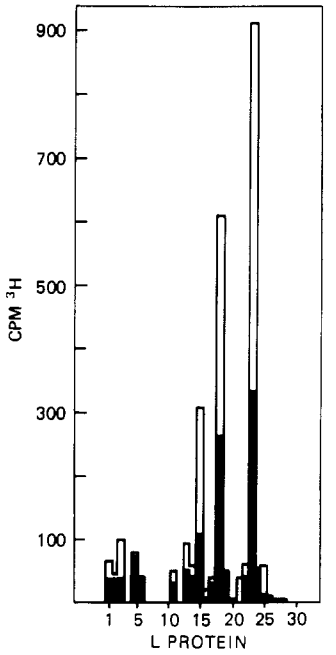

FIGURE 2: Two-dimensional polyacrylamide gel electrophoresis labeling pattern of 50S protein derived from 70S ribosomes irradiated with *p*-azido[^{3}H]puromycin in the absence and presence of puromycin. Conditions are as described in the legend to Figure 1. In displaying the data, lower values are superimposed on higher values: −puromycin (□); +2 mM puromycin (■). Radioactivity reported is for protein from 702 pmol of 50S subunits and a gel radioactivity yield of 18%.

(Table I). In agreement with the two-dimensional polyacrylamide gel electrophoresis results, the percentage of 50S protein radioactivity precipitated by antisera to proteins L23 and L18/L22 falls markedly when puromycin is added (from

Table I: Immunoprecipitation Results[a]

	% labeled subunit protein		% protection by puromycin[b]
	−puromycin	+puromycin	
50S proteins			
L18/22	10 ± 3	3.5 ± 1.5	71 ± 18
L23	21 ± 1	11.5 ± 1.5	69 ± 2
30S proteins			
S7	11	10	27
S14	8 ± 1	8 ± 1	29 ± 7
S18	27 ± 4	24 ± 4	41 ± 3

[a] Experimental conditions are as described in the legend to Figure 1. [b] Calculated from the values listed in the first two columns, as well as the overall decrease in subunit protein labeling on addition of 2 mM puromycin. For 50S protein this decrease was 44% and for 30S protein 31%.

31% to 15%), and the reduction in L23 and L18/L22 labeling can be calculated as 69 ± 2% and 71 ± 18%, respectively.

A comparison of Figure 1 with Figure 2 and Table I shows that the protective effect of puromycin on the labeling by *p*-azidopuromycin of gel region 50S-II is very similar to the protective effect on L23, L18/22, and L15 labeling measured by using either two-dimensional polyacrylamide gel electrophoresis or specific immunoprecipitation. Accordingly, we have used the much more convenient one-dimensional polyacrylamide gel electrophoresis analysis to monitor the labeling of 50S proteins. The results, presented in Table II, lead to the following conclusions: (1) The extent of *p*-azidopuromycin photoincorporation into gel region 50S-II is similar when either 70S ribosomes or 50S subunits are the target (experiments 1 and 2). (2) Puromycin reduces photoincorporation into 50S-II to about the same extent (~60–70%) in the presence and absence of β-mercaptoethanol, using an A or B isolation procedure, and with either 70S ribosomes or 50S subunits as the target (experiments 4 and 5). (3) The protective effect of puromycin cannot be attributed to a nonspecific scavenging of a photogenerated intermediate by the amine functionality in puromycin, since D(+)-2-glucosamine, which has a similar functionality, has no protective effect (experiment 7). (4) PANS, corresponding to one part of the puromycin molecule, protects almost as well as puromycin and more strongly than either L-phenylalaninamide, corresponding to the remaining portion of puromycin, or adenosine (experiments 8–10). Thus the site (or sites) labeled by *p*-azidopuromycin appears (appear) specific for an adenosine-like molecule containing a positive charge. (5) Both Phe-tRNA^{Phe} and poly(uridylic acid) added alone have little protective effect (experiments 11 and 12), but Phe-tRNA^{Phe} added in the presence of poly(uridylic acid) exerts significant protection (experiment 13).

(*2*) *30S Protein.* We next consider 30S protein labeling. In the absence of β-mercaptoethanol, S18 is by far the dominant 30S protein photolabeled by *p*-azidopuromycin (Nicholson et al., 1982), so that changes in S18 labeling can be monitored by measuring radioactivity in the one-dimensional polyacrylamide gel electrophoresis region 30S-II, which contains S18 (Nicholson et al., 1982). As summarized in Table II, S18 is labeled when either the 70S ribosome or the 30S subunit is the target. The extent of S18 labeling is markedly increased (>3-fold) when isolated 30S subunits are labeled (experiments 1 and 3), although the labeling pattern of 30S protein does not change much (Figure 3). PANS (experiment 8) and puromycin (experiments 4 and 6) both decrease 30S-II labeling, but the protective effects (20% and 30%, respectively) are significantly smaller than that found for 50S-II labeling, (45% and 65%, respectively). Immunoprecipitation results

Table II: *p*-Azidopuromycin Photoincorporation into Ribosomes and Ribosomal Subunits: Protection Effects[a]

ribosome fraction			50S-II[d]	50S-II[d]	50S-II[d]	30S-II[e]	30S-I[e]	50S RNA	50S RNA	50S RNA	30S RNA	30S RNA	30S RNA
isolation procedure			A	A	B	A	A	A	A	B	A	A
added β-mercaptoethanol			+	−	−	−	+	+	−	−	+	−
			incorporation (% mol/mol)										
expt	target	addition	relative incorporation[c]										
1	70 S		1.01	1.9	2.9	1.8	0.22	3.8	5.5	6.4	1.5	2.1	
2	50 S			2.4					7.4				
3	30 S					5.7						3.1	
4	70 S	puromycin	0.30	0.32	0.46	0.68	0.55	0.63	0.50	0.51	0.67	0.55	
5	50 S	puromycin		0.50					0.35				
6	30 S	puromycin				0.64						0.48	
7	70 S	2-glucosamine			0.99					1.02			
8	70 S	PANS	0.40	0.67	0.51	0.78	0.77	0.78	0.71	0.77	0.83	0.61	
9	70 S	phenylalaninamide			0.80					1.00			
10	70 S	adenosine	0.83	0.74		1.4	1.4	1.0	0.89		1.2	0.94	
11	70 S	Phe-tRNA^{Phe}		0.96 (0.24)[b]	0.89 (0.32)[b]				1.2	1.4			
12	70 S	poly(U)		0.88					0.97				
13	70 S	Phe-tRNA^{Phe} + poly(U)		0.72 (0.41)[b]	0.76 (0.64)[b]				1.04	1.4			

[a] For experiments 1–10, experimental conditions are as described in the legend to Figure 1; additions are always to a final concentration of 2 mM. For experiments 11–13, 70S ribosomes (2.6 μM), poly(U) (0.12 mg/mL), and ±[^{14}C]Phe-tRNA^{Phe} (10.5 μM, 12 Ci/mol) were incubated for 10 min in MTK buffer [50 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, and 50 mM KCl] at 37 °C and cooled to 0 °C. Aliquots were removed to measure Phe-tRNA^{Phe} binding per ribosome (numbers in parentheses). *p*-Azido[^{3}H]puromycin was added to the remainder of the sample and photolysis carried out in the usual manner. [b] Phe-tRNA^{Phe} bound per ribosome. [c] Incorporation in the absence of added ligand equals 1.00. [d] 50S protein region. [e] 30S protein.

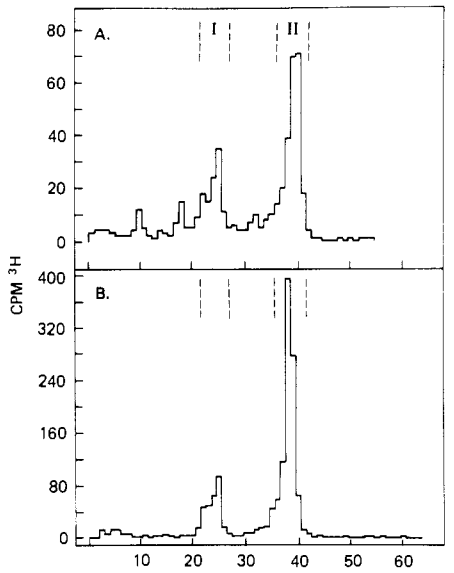

FIGURE 3: One-dimensional urea–polyacrylamide gel electrophoresis analyses of 30S proteins derived from either 70S ribosomes or isolated 30S subunits irradiated with *p*-azido[^{3}H]puromycin. (A) 30S protein from labeled 70S ribosomes. (B) 30S protein from labeled isolated 30S subunits. Experimental conditions: As described in legend to Figure 1 except that in (B) 2.6 μM 30S subunits [isolated on low Mg^{2+} zonal gradient (Eikenberry et al., 1970) and heat activated for 15 min at 37 °C (Zamir et al., 1974) before use] replaces 2.6 μM 70S ribosomes and the specific radioactivity of *p*-azido[^{3}H]puromycin was 2000 Ci/mol. Counts per minute are for protein extracted from 78 pmol of 30S subunits, normalized to a gel radioactivity yield of 20%. Gel radioactivity yields: (A) 20%; (B) 27%.

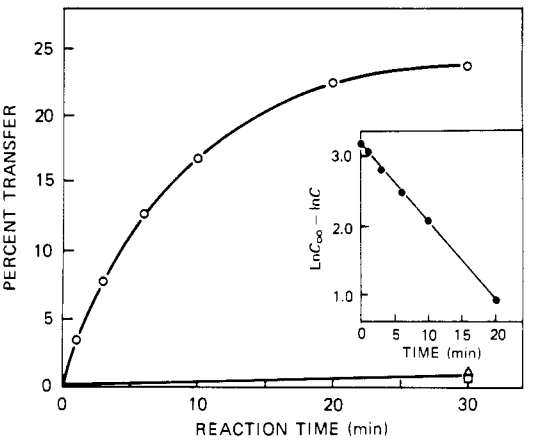

FIGURE 4: Time course for *N*-AcPhe-puromycin formation. In constructing the first-order plot, C_∞ was taken as 25%. For details, see Experimental Procedures. Lower points at 30 min are background controls: (△) −poly(U); (□) −puromycin.

Table III: Kinetic Parameters for Peptidyltransferase Ligands

substrate or inhibitor	K_D (mM)	K_I (mM)	k_2 (min^{-1})
puromycin	0.037		0.088
p-azidopuromycin	0.062		0.035
photolyzed *p*-azidopuromycin	0.033		0.037
PANS		0.47	

(Table I) lead to the same conclusion, showing that not only S18 but also the other proteins of interest, S7 and S14, are less well protected by puromycin (30–40%) than either L18/L22 or L23 (~70%). In the presence of β-mercaptoethanol, S7 is the 30S protein labeled to the greatest extent (Nicholson et al., 1982), and effects on its labeling can be followed by monitoring gel region 30S-I (Table II).[2] Again, both puromycin and PANS are seen to protect 30S-I less well than they protect gel region 50S-II.

(*3*) *RNA.* As demonstrated in the preceding paper (Nicholson et al., 1982), *p*-azidopuromycin photoincorporates into both 50S RNA and 30S RNA in addition to its photoincorporation into ribosomal proteins. Measurements of *p*-azidopuromycin photoincorporation into RNA are shown in Table II. Incorporation into 50S RNA amounts to approximately half of total 50S subunit labeling, when either the 70S ribosome or the 50S subunit is the target (experiments 1 and 2). Puromycin protects against this incorporation to a somewhat smaller extent (~45%) than it does against incorporation into 50S-II (~65%) (experiments 4 and 5). However, the relative order of effectiveness of small molecules in protecting 50S RNA incorporation parallels that found with 50S-II labeling; puromycin protects better than does PANS (experiment 8), whereas 2-glucosamine, L-phenylalaninamide, and adenosine show little, if any, protection (experiments 7, 9, and 10). A difference from the 50S-II results is that there is no apparent protection of 50S RNA labeling in the presence of Phe-tRNA^{Phe} and poly(uridylic acid) (experiment 13). Photoincorporation into 30S RNA amounts to about 30% of total 30S labeling when isolated 30S subunits are labeled (experiment 3), as a result of the large increase of incorporation into protein. Puromycin protection is similar to that seen for 50S RNA (experiments 4 and 6) and is greater than that for PANS (experiment 8) whereas adenosine (experiment 10) shows no protection. Since these results are for RNA as a whole, the protections observed must be considered lower limits for the possible protections at specific sites. The important conclusion is that the extents and structural specificities of the protections observed strongly suggest that at least some of the RNA labeling on both subunits proceeds from puromycin binding sites.

(*B*) *p-Azidopuromycin and Photolyzed p-Azidopuromycin Are Functional Analogues of Puromycin in the Peptidyltransferase Assay; PANS Is a Competitive Inhibitor.* Ribosome-dependent *N*-AcPhe-puromycin formation from *N*-AcPhe-tRNA^{Phe} and puromycin displays apparent first-order kinetics (Figure 4), permitting graphical evaluation of k_{obsd} (inset). If it is assumed that the reaction proceeds according to eq 1 and that puromycin binds reversibly and rapidly to the peptidyltransferase A′ site

$$\text{70S·}N\text{-AcPhe-tRNA·poly(U)} + \text{puromycin} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}}$$

$$\text{70S·}N\text{-AcPhe-tRNA·poly(U)·puromycin} \xrightarrow{k_2}$$

$$\text{70S·tRNA·poly(U)} + N\text{-AcPhe-puromycin} \quad (1)$$

allows expression of $1/k_{obsd}$ as a function of puromycin concentration (eq 2) where $K_D = k_{-1}/k_1$. The kinetics of ribosome-dependent *N*-AcPhe-*p*-azidopuromycin formation may be expressed in a totally analogous manner. Double-reciprocal plots of $1/k_{obsd}$ vs. 1/[puromycin or analogue] are shown in Figure 5, permitting calculation of the k_2 and K_D values presented in Table III. From these values we conclude that

$$\frac{1}{k_{obsd}} = \frac{1}{k_2} + \frac{K_D}{k_2}\left(\frac{1}{[\text{puromycin}]}\right) \quad (2)$$

[2] Protein S1 labeling was found in some experiments to be comparable to S7 labeling (Nicholson et al., 1982), but variability in S1 recovery on subunit separation made a reliable quantitative study of protection effects on its labeling infeasible.

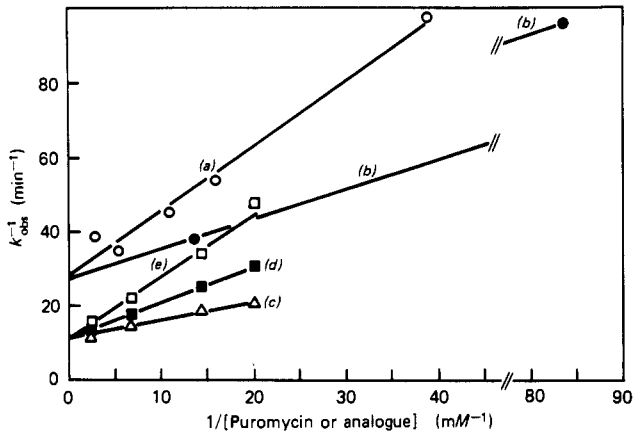

FIGURE 5: Concentration dependence of rates of ribosome-dependent *N*-AcPhe transfer to puromycin, *p*-azidopuromycin, or photolyzed *p*-azidopuromycin. Rates of *N*-AcPhe transfer from *N*-AcPhe-tRNA were measured (a) to *p*-azidopuromycin, (b) to the soluble fraction of photolyzed *p*-azidopuromycin, (c) to puromycin in the absence of PANS, and to puromycin in the presence of (d) 0.7 mM PANS or (e) 1.4 mM PANS. For line b, a solution of *p*-azidopuromycin was photolyzed for sufficient time (Nicholson et al., 1982) to remove the azide function and centrifuged to remove polymeric photoproduct. The concentration of soluble photolyzed *p*-azidopuromycin was determined spectrally, assuming an ϵ_{275} of 20000 M^{-1} cm^{-1}, corresponding to the N^6,N^6-dimethylaminopurine chromophore.

both *p*-azidopuromycin [in agreement with the work of others: Krassnigg et al. (1978) and Symons et al. (1978)] and the soluble fraction of photolyzed *p*-azidopuromycin are reasonably good functional analogues of puromycin.

Our finding that PANS protects region 50S-II labeling by *p*-azidopuromycin almost as well as does puromycin led us to examine the effect of PANS on the rate of *N*-AcPhe-puromycin formation. The results are shown in Figure 5. The data obtained fit eq 3 where K_I is the dissociation constant for

$$\frac{1}{k_{obsd}} = \frac{1}{k_2} + \frac{K_D}{k_2[\text{puromycin}]}\left(1 + \frac{[\text{PANS}]}{K_I}\right) \quad (3)$$

PANS binding to the 70S·*N*-AcPhe-tRNA·poly(U) complex and provide evidence that PANS is a competitive inhibitor of puromycin in this assay. The value calculated for K_I, 0.47 mM, is about 10-fold higher than the K_D value for puromycin.

Several other potential ribosomal ligands were also tested for their abilities to inhibit *N*-AcPhe-puromycin formation at a single puromycin concentration (Table IV). As can be seen, the apparent order of effectiveness is GlyPANS > PANS > adenosine > L-phenylalaninamide. Thus, for the latter three compounds, where our data allow comparisons to be made, the order of effectiveness in decreasing *p*-azidopuromycin photoincorporation into region 50S-II is the same as the order of effectiveness in inhibiting ribosome-dependent *N*-AcPhe-puromycin formation.

As shown in Figure 5, PANS behaves as a pure competitive inhibitor of puromycin in the puromycin reaction. The assumption that the other compounds tested are also pure competitive inhibitors permits calculation of apparent K_I values from the relative velocities summarized in Table IV and eq 4. These values are shown in Table IV, but it must be emphasized that, except for PANS, they represent lower limit values since the other compounds have not been shown to be

$$K_I = \frac{[\text{inhibitor}]}{\left(\frac{[\text{puromycin}]}{K_D} + 1\right)\left(\frac{1}{\text{rel velocity}} - 1\right)} \quad (4)$$

Table IV: Effects of Added Compounds on Peptidyltransferase Activity

additions, mM	puromycin (mM)	rel velocity	app K_I (mM) (lower limit)
		1.00	
PANS, 1.1	0.06	0.66	
PANS, 2.0	0.10	0.32	0.48 ± 0.22
PANS, 5.9	0.06	0.14	
adenosine, 1.1	0.06	0.77	
adenosine, 6.0	0.06	0.33	1.3 ± 0.2
N^6,N^6-dimethyladenosine, 5.8	0.06	0.36	1.2
L-phenylalaninamide, 2.0	0.10	0.89	4.4
GlyPANS, 2.0	0.10	0.25[a]	0.18

[a] Corrected for a small amount of *N*-AcPhe transfer to GlyPANS.

competitive inhibitors. They do, however, clearly show that adenosine and L-phenylalaninamide are bound to the peptidyltransferase center at least 2.5-fold and 10-fold less tightly than PANS, respectively.

(C) p-Azidopuromycin Photoincorporation into Ribosomes Leads to Inactivation of Ribosomal Functions. In the photoincorporation experiments summarized in Tables I and II, the concentration of *p*-azidopuromycin was kept low (50 μM) so as to maximize the specificity of labeling. In the experiment described in Table V, higher levels of *p*-azidopuromycin were employed, the object being to test whether functional activity was lost on *p*-azidopuromycin incorporation. As can be seen, both *N*-AcPhe-tRNA binding and peptidyltransferase activity decrease with increasing *p*-azidopuromycin photoincorporation, with the relative decrease in *N*-AcPhe-tRNA^{Phe} binding being greater than in peptidyltransferase activity. However, it is unclear whether the observed activity decreases arise from an accumulation of several small inactivations resulting from *p*-azidopuromycin incorporation into a number of sites or whether incorporation into a specific site on the ribosome, accounting for only a fraction of total incorporation, completely inactivates the ribosome.

(D) Photoincorporated p-Azidopuromycin Is Inactive as an Acceptor of N-AcPhe from N-AcPhe-tRNA. Two attempts were made to test the acceptor ability of photoincorporated *p*-azidopuromycin toward *N*-AcPhe transfer from ribosome-bound *N*-AcPhe-tRNA^{Phe}. In these experiments *p*-azidopuromycin was photoincorporated to a level of 1.4–1.7 mol per 70S ribosome, the samples were dialyzed to remove the soluble noncovalently bound material, ^{14}C-labeled *N*-AcPhe-tRNA^{Phe} and poly(uridylic acid) were added, and following incubation at 0 °C for 1 h, the reactions were quenched by adding excess puromycin. The subunits were then resolved in a high-salt (0.4 M NaCl) sucrose gradient and the ^{14}C contents of the subunits and protein extracted from the subunits were determined. As background controls, ribosomes separately photolyzed or ribosomes added to separately photolyzed *p*-azidopuromycin were taken through the same procedure. In neither case was significant ^{14}C radioactivity above background seen at either the subunit or subunit protein levels, despite the fact that suitable tests showed that more than half of the *N*-AcPhe-tRNA^{Phe} binding and *N*-AcPhe transfer activities remained following photoincorporation of *p*-azidopuromycin and dialysis, and our experiments were set up to be able to detect transfer from as little as 0.1% or 10% of ribosome-bound *N*-AcPhe-tRNA^{Phe} to ribosomal protein or 50S subunits, respectively. We thus conclude that no major component of covalently bound *p*-azidopuromycin is a competent acceptor in a peptidyltransferase reaction.

Table V: Effects of *p*-Azidopuromycin Photoincorporation on Ribosomal Activities[a]

expt	[*p*-azidopuromycin] (mM)	incorporation (% mol/mol)					activities: % poly(U)-dependent *N*-AcPhe-tRNA^{Phe} binding	activities: % *N*-AcPhe transfer to puromycin	% inactivation: *N*-AcPhe-tRNA^{Phe} binding	% inactivation: peptidyltransferase	% inactivation: overall transfer
		subunit	overall	protein gel regions: RNA	protein gel regions: I	protein gel regions: II					
1							27.0	12.2	0	0	0
2	0.43	50	120	59	17	45	20.4	6.9	24	24	43
		30	110	26	35	42					
3	2.0	50	770	550	103	108	5.4	1.3	80	45	89
		30	550	770	144	88					

[a] Samples were photolyzed in the presence or absence of *p*-azidopuromycin, as indicated, as described in the legend to Figure 1. Following photolysis, ribosomes were precipitated with 2 volumes of ice-cold ethanol (3 times in all) and then centrifuged at low speed to remove polymeric photoproduct. One portion of the sample was used to determine incorporation values, by a continuation of procedure B. The other portion was layered atop a 10% sucrose cushion made up in TMKA buffer [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM KCl, and 1 M NH_4Cl] and centrifuged at 48 000 rpm for 14 h (4 °C). This procedure removes almost all noncovalently bound photolyzed *p*-azidopuromycin. The resulting pellets were resuspended in a buffer [25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 200 mM KCl], and aliquots were removed and tested for poly(U)-dependent *N*-AcPhe-tRNA^{Phe} binding (concentrations in binding assay: ribosomes, 0.26 μM; *N*-AcPhe-tRNA^{Phe}, 0.052 μM; poly(U), 0.12 mg/mL) and ribosome-dependent *N*-AcPhe transfer from *N*-AcPhe-tRNA^{Phe} to puromycin as described (see Experimental Procedures).

Discussion

As we have demonstrated, *p*-azidopuromycin photoincorporates into both the 50S and 30S subunits. Since both the 50S and 30S protein labeling patterns are essentially the same whether the target of labeling is either the 70S ribosome or the isolated 30S and 50S subunits (Table II and Figure 3), we conclude that such labeling proceeds from separate sites on the 30S and 50S subunits.

Considering first 50S labeling, the results presented here and in the preceding paper (Nicholson et al., 1982) provide evidence that *p*-azidopuromycin labels 50S-II proteins, and perhaps some part of 50S RNA, by a common mechanism and from a common binding site corresponding to the A′ site within the peptidyltransferase center. The site specificity of *p*-azidopuromycin labeling of these proteins is shown by the following observations. First, labeling is decreased in the presence of puromycin (Figures 1 and 2; Tables I and II); were labeling nonspecific, puromycin would have no effect. Second, labeling of these proteins is fairly resistant to β-mercaptoethanol added to the photolysis medium in contrast to the marked sensitivity of S18 labeling (Nicholson et al., 1982). Resistance to nucleophilic scavengers is diagnostic of a true photoaffinity labeling process (Ruoho et al., 1973), whereby covalent incorporation takes place upon light-dependent generation of a reactive intermediate at the receptor site at a rate which is fast compared to the ligand dissociation rate. Third, both *p*-azidopuromycin and puromycin (Cooperman et al., 1975; Jaynes et al., 1978) photolabel L23 to high extents, by apparently different photochemical mechanisms (Nicholson et al., 1982). Such a result is characteristic of a labeling process determined by site specificity, rather than by chemical reactivity. Evidence for a common site and common mechanism for labeling of 50S proteins comes from the essentially identical percentage decreases in L23, L18/22, and L15 labeling in the presence of either puromycin (Figure 2) or β-mercaptoethanol (Nicholson et al., 1982). Puromycin and β-mercaptoethanol both decrease total 50S RNA labeling, but to somewhat different extents than for the 50S proteins of interest. However, it is possible that a portion of 50S RNA is labeled from the same site and by the same mechanism as the 50S-II proteins.

p-Azidopuromycin is a good functional analogue of puromycin (Table III), so that it must bind to the A′ site. Evidence that labeling of 50S-II proteins and perhaps 50S RNA labeling occurs from the A′ site comes from the parallelism we observe between the ability of added compounds to inhibit gel region 50S-II and 50S RNA labeling and the functional activity of these compounds in the peptidyltransferase assay. Thus, such labeling is strongly inhibited by both puromycin, a peptidyltransferase substrate, and PANS, a reasonably good peptidyltransferase inhibitor (Table IV), less inhibited by adenosine, a poor peptidyltransferase inhibitor, and hardly inhibited at all by L-phenylalaninamide, a structural analogue of the *O*-methyltyrosyl moiety of puromycin, which hardly inhibits peptidyltransferase (Table IV). In addition, Phe-tRNA^{Phe}, in the presence of poly(U) but not in its absence, is effective in reducing 50S-II labeling (Table II, experiments 11–13), and A-site binding of Phe-tRNA^{Phe} is poly(U) dependent. The major technical difficulty with these latter experiments is that since only partial stoichiometric binding of Phe-tRNA^{Phe} was achieved, only partial protection could be expected. Nevertheless, the reduction in 50S-II labeling, though small, is reproducible and is quantitatively consistent with the idea that Phe-tRNA^{Phe} in the A site blocks 50S-II labeling. Thus, in the absence of EF-Tu, Phe-tRNA^{Phe} binds to ribosomes in approximately equal amounts, labeling to the A and P sites (Tanaka et al., 1972), and the 26 ± 2% protection of 50S-II corresponds to half of the total Phe-tRNA^{Phe} binding, 52 ± 12% (experiment 13). *p*-Azidopuromycin appears to label the 50S subunit from the same site as puromycin itself, based first on the commonality of protein L23 as a major site of photoincorporation and second on the consistency of the magnitude of inhibition of *p*-azidopuromycin labeling by 2 mM puromycin (65–70%; Tables I and II) and our previous estimate of the K_D for puromycin binding to the site from which it photoincorporates into ribosomes (0.7 ± 0.2 mM; Jaynes et al., 1978). Thus, the evidence that puromycin labels L23 from the A′ site (Jaynes et al., 1978; Grant et al., 1979a) can also be taken as evidence for *p*-azidopuromycin labeling from the A′ site.

On the other hand, our attempt to transfer *N*-AcPhe from *N*-AcPhe-tRNA^{Phe} to covalently photoincorporated *p*-azidopuromycin was unsuccessful. Similar experiments have been tried in other studies with puromycin or puromycin-like affinity labels. Thus, Quiggle et al. (1978) were unsuccessful in their attempts to transfer *N*-AcPhe to what was presumably photoincorporated 2′(3′)-*O*-(4-azido-L-phenylalanyl)adenosine. Vince et al. (1978) presented evidence for a successful transfer to presumably covalently incorporated [N^{ϵ}-(2-nitro-4-azido-

phenyl)-L-lysinyl]puromycin aminonucleoside (NAPLys-PANS), but, as we discuss elsewhere (Nicholson et al., 1982), it is possible that transfer was actually to noncovalently bound material. This possibility is underscored by the activity of photolyzed *p*-azidopuromycin in the peptidyltransferase assay (Table III). On a more positive note, Greenwell et al. (1974) were able to show that a small fraction of material covalently incorporated into 23S RNA when ribosomes were incubated with an electrophilic puromycin affinity label could act as an acceptor from *N*-AcLeu pentanucleotide in the fragment assay, although there remain uncertainties regarding this experiment as well (Symons et al., 1978). Previously we showed that photoincorporated puromycin had no acceptor activity (Jaynes et al., 1978) and pointed out that this might have been due to a modification of the puromycin molecule on incorporation which rendered it inactive as a receptor. Since *p*-azidopuromycin presumably incorporates via the photogenerated nitrene (Nicholson et al., 1982), at a position distant from the α-amino group which is the potential *N*-AcPhe acceptor, such an explanation should not be relevant in accounting for the lack of acceptor ability of photoincorporated *p*-azidopuromycin. The most plausible remaining possibilities are either that photoincorporation takes place in an amino acid residue essential for peptidyltransferase catalysis or that the photoincorporated molecule is inappropriately positioned to act as an acceptor. A less stringent criterion for functional site labeling would be the demonstration of a loss of ribosomal activity on *p*-azidopuromycin photoincorporation. Such losses are observed (Table V) but are not clearly the result of any single incorporation process.[3] Studies in progress, which are directed toward assaying the functional activities of ribosomes reconstituted with a single protein labeled stoichimetrically with puromycin or *p*-azidopuromycin, should permit determination of the functional consequences of labeling individual proteins.

Assignment of proteins L23, L18/22, L15, and perhaps L13 as being at or near the peptidyltransferase center is in good accord with related studies on ribosome structure and function. Thus, L18 is labeled by two aryl azide derivatives of peptidyl-tRNA (Hsiung & Cantor, 1974; Hsiung et al., 1974), and the covalently bound peptidyl-tRNAs are active as peptidyl donors in the peptidyltransferase reaction, L15 is labeled by an electrophilic derivative of Met-tRNA^{fMet} (Czernilofsky et al., 1974) and perhaps by an electrophilic derivative of Phe-tRNA^{Phe} (Pellegrini et al., 1974), L13 is labeled by electrophilic derivatives of both chloramphenicol (Stöffler et al., 1980) and lysyl-tRNA (Johnson & Cantor, 1980), and L15 and L18 have both been shown to be proteins essential for peptidyltransferase activity in single omission–reconstitution experiments (Hampl et al., 1981). L23 is nonessential in these latter experiments but apparently lies close to L18 within the 50S subunit on the basis of both immunoelectron microscopy experiments (Stöffler et al., 1980) and cross-linking experiments showing L18 and L23 to be cross-linked to two 50S proteins in common, L31 and L32 (Traut et al., 1980), although no L18–L23 crosslink has been described. In addition,

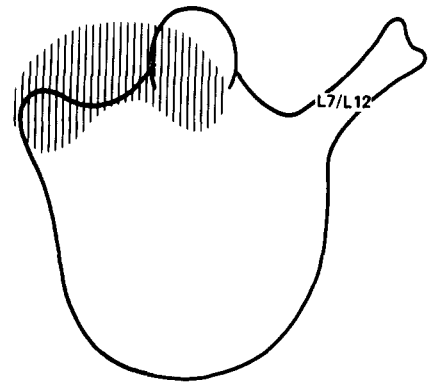

FIGURE 6: Proposed location of the peptidyltransferase center on the 50S subunit. According to the models of Lake & Kahan (1975), Lake (1980), and Lührmann et al. (1981).

L23 cross-links to L5, a protein which is a component of the L5–L18–L25–5S RNA complex, and to L16, another protein which has been implicated as being important for peptidyltransferase (Moore et al., 1975; Baxter & Zahid, 1978; Dohme & Fahnestock, 1979; Hampl et al., 1981). These, and similar arguments presented in more detail elsewhere (Cooperman, 1978, 1980a), lead us to concur with the suggestions of Lake & Kahan (1975) and of Lührmann et al. (1981) for the placement of the peptidyltransferase center within the shaded area indicated in Figure 6, using the Lake model of the 50S subunit structure. More recently, we have shown by immunoelectron microscopy that for a photoincorporation reaction in which 65–70% of overall puromycin labeling of the ribosome is in protein L23, the dominant site of puromycin labeling is essentially identical with the shaded area in Figure 6 (Olson et al., 1982). This result is consistent with a tentative placement of L23 according to Stöffler et al. (1980).

The major 30S proteins labeled by *p*-azidopuromycin are S18 > S7, S14 in the absence of β-mercaptoethanol and S7 and perhaps S1 in its presence. The labeling of these proteins, and of at least a portion of 16S RNA as well, is site specific based on the decrease in labeling observed in the presence of puromycin (Tables I and II; puromycin effects on S1 labeling were not measured).[2] That these decreases are smaller than that seen for 50S protein labeling suggests that the 30S site or sites involved have lower affinity for puromycin than the 50S site discussed above. However, from the similarities in the decreases observed for S protein labeling, it appears that S proteins are labeled from either a single site or from sites having comparable puromycin affinity. Thus, the different sensitivities of 30S protein labeling to added β-mercaptoethanol most likely reflects different mechanisms of labeling; i.e., S18 and S14 appear to be labeled by a relatively long-lived electrophilic photoproduct of *p*-azidopuromycin ("pseudophotoaffinity labeling"; Ruoho et al., 1973), whereas S7 and 16S RNA, like the proteins and RNA at the 50S site, appear to be labeled by a shorter-lived *p*-azidopuromycin photoproduct, presumably a nitrene. The very high labeling of S18 observed in the absence of β-mercaptoethanol is then due mostly to the high chemical reactivity of the Cys-10 residue within protein S18, as discussed in the preceding paper (Nicholson et al., 1982). It is worth emphasizing that S18 is found as *a* or *the* major labeled protein in several affinity labeling studies on ribosomes. Thus, we have found S18 to be the major protein photolabeled in the absence of β-mercaptoethanol by two other puromycin derivatives, (N^{α}-ethyl-2-diazomalonyl)puromycin (Cooperman et al., 1975) and NAPLys-PANS (Nicholson, 1981), by a tetracycline photoproduct (R. A. Goldman, T. Hasan, W. A. Strycharz, and B. S.

[3] In contrast to our results, Symons et al. (1978) detected no inactivation of peptidyltransferase by covalently incorporated *p*-azidopuromycin as revealed by the fragment reaction. This apparent difference might be due to *p*-azidopuromycin incorporation into a site(s) important for the *N*-AcPhe-tRNA transfer reaction, but unimportant for transfer from *N*-AcLeu pentanucleotide in the fragment assay. However, it should be noted that the covalent incorporation reported by Symons et al. (1978) must be an overestimate, given the problem of noncovalent binding (Nicholson et al., 1982), so that it is possible that the true covalent labeling was too low to give rise to measurable irreversible inactivation.

Cooperman, unpublished results), and by (2,4-dinitrophenyl)streptomycin hydrazone.[4] Further, S18 is a major site of incorporation of electrophilic derivatives of Phe-tRNA^{Phe} (Pellegrini et al., 1974), oligonucleotides (Pongs et al., 1976, 1979; Yaguchi et al., 1978), and the antibiotic pleuromutilin (Högenauer et al., 1981). This promiscuity of S18 toward ribosomal affinity labels makes it clear that interpretations of S18 labeling in terms of functional site localization is suspect a priori and should be made with the greatest care.

Although a definitive conclusion would be premature, the available evidence from immunoelectron microscopic (Lake, 1980; Stöffler et al., 1980), cross-linking (Traut et al., 1980), and neutron diffraction (Moore, 1980) studies suggests that none of the three 30S proteins of interest are in close proximity to one another. Thus, there is no clear functional interpretation of the 30S labeling we observe. One possibility we would like to consider is that all three proteins fall within the tRNA binding locus on the 30S subunit. The protection of labeling by puromycin could then be accounted for by assuming that the portions of S7, S14, and S18 labeled by *p*-azidopuromycin bind to adenine or purine-invariant positions in the family of tRNA structures (Singhal & Fallis, 1979). In support of this possibility we note the following: (1) Protein S7 is the major ribosomal protein photolabeled by native tetracycline (R. A. Goldman, T. Hasan, W. A. Strycharz, and B. S. Cooperman, unpublished results), an antibiotic which inhibits aminoacyl-tRNA binding to both 30S subunits and 70S ribosomes. (2) Protein S14 is the major 30S protein photolabeled by puromycin itself (Jaynes et al., 1978; Grant et al., 1979a,b) and by an aryl azide derivative at the 3′ end of tRNA (Girshovich et al., 1974). These results have led us to speculate that S14 forms part of the 30S binding site for the 3′ end of tRNA. Other experiments, summarized elsewhere (Cooperman, 1980a), also show S14 to be important for tRNA binding. (3) Modification of 30S ribosomes with either *N*-ethylmaleimide (Ginzburg et al., 1973) or an electrophilic photoproduct of tetracycline (Goldman et al., 1980) leads to loss of tRNA binding and gives S18 as the major labeled protein in both cases.

Despite the extensive attention that has been devoted toward delineating the structural specificity of the A′ site [for reviews, see Symons et al. (1978) and Krayevsky & Kukhanova (1979)], to our knowledge this is the first time that PANS has been examined as an inhibitor of the peptidyltransferase reaction itself, although it was long ago shown to be inactive as an inhibitor of polypeptide synthesis (Nathans & Neidle, 1963). There is no inconsistency between this earlier result and our present finding that PANS is a reasonably good inhibitor of peptidyltransferase, since inhibition of polypeptide synthesis by puromycin or puromycin analogues tests the ability of the inhibitor to both bind to the peptidyltransferase center and accept a peptidyl group from peptidyl-tRNA, whereas inhibition of peptidyl transfer to puromycin tests only the binding step. Considering puromycin to be made up of two halves, corresponding to N^6,N^6-dimethyladenosine and *O*-methyltyrosinamide, the results presented in Table IV suggest that N^6,N^6-dimethyladenosine (or adenosine alone) is the more powerful determinant for binding to the A′ site. This result is in accord with the known specificity of the A′ site for adenosine as opposed to other nucleosides (Rychlik et al., 1969) and with the only minor differences in inhibitory activities toward ribosome-dependent *N*-AcPhe transfer to puromycin shown by the aminoacyl dinucleoside monophosphates CpA(2′Phe)H as compared with CpA(2′Gly)H and CpA(2′H)Phe as compared with CpA(2′H)Gly (Ringer et al., 1975). In comparing various acyl adenylates, Zemlicka et al. (1975) obtained very strong evidence of the importance of a charged α-ammonium group for binding to the A′ site. Although the ammonium group is at a different position in PANS, its presence presumably accounts for the superiority of PANS over N^6,N^6-dimethyladenosine as an A′ site ligand, as manifested by their relative abilities to inhibit both peptidyltransferase and 50S-II labeling by *p*-azidopuromycin.

[4] M. A. Luddy, E. Plotzker, and B. S. Cooperman, unpublished experiments.

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Dilatometric Studies of the Subtransition in Dipalmitoylphosphatidylcholine†

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ABSTRACT: The phase transition between the newly discovered low-temperature subgel phase and the gel phase of dipalmitoylphosphatidylcholine has been studied by using dilatometry. Equilibrium measurements show that the subtransition upon heating is centered at 13.5 °C, has a dilatometric half-width of 0.6 °C, and comprises a specific volume change of 0.009 mL/g (about one-fourth the size of the main transition). When the gel phase is cooled, the subtransition does not occur until below 5 °C. The rate of formation as a function of incubation temperature for 1 °C < T_I < 6 °C was determined; it is not well fit by quantitative theories based upon homogeneous nucleation. However, some form of nucleation is present since temperature-jump studies show that once the subgel phase has started to form, it continues to grow in the range 6 °C < T_J < 12.8 °C. Thus, the true transition temperature lies between 12.8 and 13.5 °C, but nucleation of the subgel phase is severely retarded above 6 °C, leading to the large hysteresis observed upon cooling.

The polymorphism of fully hydrated phosphatidylcholines has been extensively studied, revealing the existence of a low-temperature gel phase, an intermediate phase, and a high-temperature "melted" phase. Recently, the transition into yet another phase has been reported by Chen et al. (1980) in hydrated phosphatidylcholine bilayers with alkyl chains with 16 (DPPC), 17, or 18 carbon atoms. The new transition, which these authors called the "subtransition", takes the gel phase into a new low-temperature phase, which we will call the subgel phase. This transition was observed to be extremely hysteretic in differential scanning calorimetry (DSC) experiments. When the phase was heated, it occurred between 15 and 19 °C, whereas when the gel phase was cooled, the subgel

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