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Escherichia coli Tryptophan Synthase: Synthesis of Catalytically Competent α Subunit in a Cell-Free System Containing Preacylated tRNAs†

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ABSTRACT: A cell-free protein biosynthesizing system prepared from *Escherichia coli* CF300 was found to synthesize *E. coli* tryptophan synthase α subunit in a time-dependent manner when programmed with pBN69 plasmid DNA. This plasmid contains the *trp* promoter from *Serratia marcescens* adjacent to the coding region of *E. coli* tryptophan synthase α protein [Nichols, B. P., & Yanofsky, C. (1983) *Methods Enzymol. 101*, 155–164]. The synthesized tryptophan synthase α subunit was found to be indistinguishable from authentic α subunit protein when analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and to have the same specific activity for catalyzing the conversion of indole → L-tryptophan by tryptophan synthase β_2 subunit, as well as the conversion of indole + glyceraldehyde 3-phosphate to indole-3-glycerol phosphate. In the absence of exogenously added phenylalanine, admixture of *E. coli* phenylalanyl-tRNA^{Phe} to the protein biosynthesizing system stimulated the production of functional α protein; the analogous result was obtained when valine was replaced by *E. coli* valyl-tRNA^{Val}. The ability of a misacylated tRNA to participate in α protein synthesis in this system was established by the use of *E. coli* phenylalanyl-tRNA^{Val} in the absence of added valine. Protein biosynthesis proceeded normally and gave a product having the approximate molecular weight of tryptophan synthase α subunit; as expected, this polypeptide lacked catalytic activity.

The study of gene expression has been greatly facilitated by the development of cell-free protein biosynthesizing systems in which transcription and translation are coupled (Zubay, 1980; Yang et al., 1980). These coupled systems have varied in complexity from relatively simple S-30 homogenates (Nirenberg & Matthaei, 1961) to more complex systems reconstructed from purified, isolated components (Herrlich & Schweiger, 1974; Yang et al., 1980). Such in vitro protein biosynthesizing systems have been employed to examine the

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expression of a variety of viral and bacterial genes [see, e.g., Bryan et al. (1969), Crawford and Gesteland (1973), O'Farrell and Gold (1973), Pelham and Jackson (1976), and Brown et al. (1983)] as well as the production of specific structural proteins (Zubay, 1973; Yang et al., 1976; Yates et al., 1980). Some studies have also addressed the issue of production of catalytically active proteins for study, e.g., the synthesis of T4 lysozyme (assayed by competence for bacterial cell wall degradation) (Salser et al., 1967; Gesteland et al., 1967, 1969; Coolsma & Haselkorn, 1969; Schweiger & Gold, 1969; Wilhem & Haselkorn, 1971; Beck & Gassen, 1977) and the production of β-galactosidase or fragments thereof (assayed by hydrolysis of chromogenic substrates) (Zubay, 1973; Zubay & Chambers, 1969; Kung et al., 1979).

Recent advances in the preparation of misacylated tRNAs (Heckler et al., 1984a,b) have facilitated a more quantitative assessment of the extent to which the adaptor hypothesis (Crick, 1958; Hoagland, 1959) obtains in a well-defined protein biosynthesizing system (Pezzuto & Hecht, 1980), as well as the range of structural analogues accessible via peptidyltransferase-mediated polypeptide synthesis (Heckler et al., 1983; Roesser et al., 1986). This work has also suggested that polypeptides of intentionally altered structure might be accessible via in vitro protein biosynthesis if an appropriate experimental system could be constituted. Accordingly, we sought a polypeptide of relatively low molecular weight that could be expressed readily in a coupled transcription–translation system and that would carry out some well-defined chemical transformation in a catalytic fashion once biosynthesized.

Reported herein is the use of pBN69 plasmid DNA for the expression of *Escherichia coli* tryptophan synthase α subunit in vitro. Novel features of this study include the exact quantification of α subunit production, the demonstration that several copies of the α protein were produced for each pBN69 DNA molecule employed, and that the derived α subunit was fully active as judged by each of two enzymatic assays. Also illustrated was the stimulation of α protein synthesis by phenylalanyl-tRNA^{Phe} in the absence of added phenylalanine and by valyl-tRNA^{Val} and phenylalanyl-tRNA^{Val} in the absence of added valine.

Materials and Methods

Materials

Bacto-tryptone and Bacto yeast extract were obtained from Difco Laboratories. Chloramphenicol, alumina (type 305), folinic acid, *p*-aminobenzoic acid, NADH, FAD, poly(ethylene glycol) (M_r ~8000), *E. coli* MRE600 tRNA$_f^{Met}$, rabbit muscle pyruvate kinase (unit definition: 1 unit will convert 1.0 μmol of phosphoenolpyruvate to pyruvate per minute at pH 7.6 and 37 °C), and human placental ribonuclease inhibitor (unit definition: 1 unit will cause 50% inhibition of 5 ng of ribonuclease A in a cytidine cyclic 2′,3′-phosphate system) were from Sigma Chemical Co. DEAE-cellulose[1] (DE-23) was from Whatman. NENSORB nucleic acid purification cartridges were obtained from New England Nuclear. [2,3-^{3}H]Valine, [^{3}H]serine, and [2-^{14}C]indole were purchased from ICN Radiochemicals; [2,3-^{3}H]-L-phenylalanine was from Amersham. Unfractionated *E. coli* tRNA was derived from *E. coli* M72, as described (Chinault et al., 1977).

Methods

Isolation of pBN69 Plasmid DNA. *Escherichia coli* strain W3110 Δ (*ton* B − *trp* A 905), harboring plasmid pBN69 (Nichols & Yanofsky, 1983), was grown at 37 °C in enriched medium containing 10 g of Bacto-tryptone, 5 g of Bacto yeast extract, 0.5 g of NaCl, and 2 g of glucose per liter, supplemented with 50 mg of ampicillin. The plasmid DNA was amplified with chloramphenicol and isolated essentially as described by Maniatis et al. (1979) using the lysozyme SDS–alkali procedure of Birnboim and Doly (1979). In a typical preparation, a 1-L culture afforded 2 mg of plasmid DNA after cesium chloride density gradient centrifugation. The isolated DNA was assayed for purity by agarose gel electrophoresis (Berry et al., 1985) and stored at 4 °C at a concentration of 0.5–0.7 mg/mL in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA.

Cell-Free Coupled Transcription–Translation System. All of the components of the protein biosynthesizing system were isolated from *E. coli* strain CF300 (Barbour & Clark, 1970), which was cultured at 37 °C in media containing 25 g of Bacto-tryptone, 12.5 g of Bacto yeast, 1.25 g of NaCl, and 5 g of glucose per liter. The bacteria were harvested at mid-exponential growth by centrifugation.

The S-30 protein fraction was prepared from *E. coli* CF300 after homogenization with alumina A-305 under nitrogen, essentially as described (Zubay, 1973). Crude initiation factors and S-100 supernatant were prepared as described by Traub et al. (1971), which included removal of endogenous DNA by absorption to DEAE-cellulose. Ribosomes were prepared according to the method of Crawford and Gesteland (1973). These components were stored in aliquots at −85 °C.

Protein biosynthesis was carried out in a reaction mixture (40-μL total volume) containing 30 mM Tris–OAc (pH 8.2), 6.8 mM NH_4OAc, 4.5 mM NaOAc, 10 mM KOAc, 34 μM folinic acid, 0.7 μg of NADH, 0.7 μg of FAD, 0.3 μg of *p*-aminobenzoic acid, 0.45 mg of poly(ethylene glycol), 3.5 mM K_2ATP, 0.45 mM CTP, 0.45 mM UTP, 0.45 mM GTP, 17 mM phosphoenolpyruvate, 11 mM $Mg(OAc)_2$, 5 mM $CaCl_2$, 0.3 unit of pyruvate kinase, 1.5 units of human placenta ribonuclease inhibitor, 5 μM *E. coli* tRNA, 40 μM [^{3}H]-phenylalanine (sp act. 10 Ci/mmol), and each of the remaining 19 amino acids at 0.14 mM final concentration. In addition, the reaction mixture contained 0.12 μM *E. coli* RNA polymerase, 0.64 μM ribosomes, 7.6 μg of S-100 protein, 6.4 μg of S-30 protein, and 22 μg of initiation factors. RNA polymerase, ribosomes, initiation factors, S-30 supernatant, and S-100 supernatant were combined at 0 °C immediately prior to initiation of the reaction. Protein synthesis was initiated by the addition of 6.4 μg of pBN69 DNA. The reaction mixture was incubated at 37 °C; aliquots were removed at the indicated times for analysis of protein synthesis and tryptophan synthase α subunit activity. This protein biosynthesizing system was very similar to that described by Yang et al. (1980).

Where preactivated *E. coli* tRNA^{Phe} was employed for protein biosynthesis, activation was carried out as described for phenylalanyl-tRNA^{Phe} (Pezzuto & Hecht, 1980) and phenylalanyl-tRNA^{Val} (Roe et al., 1973). Purification of the tRNAs included a chromatographic step that employed NENSORB nucleic acid purification cartridges. This was accomplished as described (Johnson et al., 1986); washing with 50% methanol effected elution of the preacylated tRNAs from the column. This material was lyophilized and stored at −40 °C. Protein biosynthesis was then carried out as described above, with the preacylated tRNAs added in 1 mM KOAc,

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate; Pipes, 1,4-piperazinediethanesulfonic acid; DEAE, diethylaminoethyl; PAGE, polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

pH 4.5, immediately prior to the initiation of protein biosynthesis with pBN69 DNA.

Assay for Tryptophan Synthase α Protein Activity. Homogeneous *E. coli* tryptophan synthase α subunit was a gift from Dr. C. Robert Matthews, The Pennsylvania State University. Homogeneous *E. coli* tryptophan synthase β_2 subunit was isolated from *E. coli* strain A2/F′A2 cultured at 37 °C in a minimal media (Vogel & Bonner, 1956) supplemented with 18 mM glucose and 27 μM indole. Bacteria were grown to early stationary phase, harvested by centrifugation, and used for the isolation of tryptophan synthase β_2 subunit according to the procedure of Adachi and Miles (1974). The crystalline tryptophan synthase β_2 subunit was dissolved in 0.10 M potassium phosphate, pH 7.8, containing 2 mM pyridoxal phosphate, 2 mM dithiothreitol, 5 mM EDTA, and 50% glycerol at a protein concentration of 1.2 mg/mL. This protein was stored at −20 °C, with no observable loss of activity even after several months.

Assays for tryptophan synthase α subunit activity were carried out at 37 °C in 25 μL (total volume) of 100 mM Tris-HCl, pH 7.8, containing 1.0 mM [^{3}H]-L-serine (sp act. 300 Ci/mol), 0.4 mM indole, 0.32 mM pyridoxal phosphate, 0.15 M NaCl, 4.0 mM EDTA, 1.0 mM dithiothreitol, 0.10 pmol of *E. coli* tryptophan synthase β_2 subunit, and 1 mg/mL bovine serum albumin. Four-microliter reaction aliquots were removed at predetermined time intervals up to 120 min and cospotted on poly(ethylenimine)–cellulose TLC plates with 100 nmol of unlabeled L-tryptophan. Ascending chromatography (development with water) effected separation of [^{3}H]serine and [^{3}H]tryptophan, the latter of which was identified by its fluorescence. The portion of the TLC plate containing [^{3}H]tryptophan was cut out and used directly for determination of radioactivity. Tryptophan synthase α subunit activity was expressed as picomoles of tryptophan synthesized per minute per 5 μL of protein biosynthesis reaction mixture, after subtraction of activity due to tryptophan synthase β_2 subunit alone.

Alternatively, the catalytic activity of the tryptophan synthase α subunit was measured on the basis of the conversion of [2-^{14}C]indole and glyceraldehyde 3-phosphate to [2-^{14}C]-indole-3-glycerol phosphate (Hatanaka et al., 1962). Reactions (50-μL total volume) were carried out in 125 mM potassium phosphate, pH 7.0, containing 1.0 mM [2-^{14}C]indole (9.5 Ci/mol), 4 mM hydroxylamine hydrochloride, 4 mM NaEDTA, 2 mM dithiothreitol, 50 mM fructose 1,6-bisphosphate, 0.2 unit of purified rabbit muscle aldolase, and 25 pmol of purified *E. coli* tryptophan synthase β_2 subunit. Reactions were initiated by the addition of tryptophan synthase α subunit and incubated at 37 °C. Ten-microliter aliquots were removed at predetermined time intervals up to 180 min and quenched by addition to 100 μL of 0.1 NaOH containing a trace of Bromphenol Blue (to aid visualization of the phase boundary). Extraction with five 0.5-mL portions of toluene effected removal of unreacted [2-^{14}C]indole; the amount of [2-^{14}C]-indole-3-glycerol phosphate present in the aqueous phase was then determined by liquid scintillation counting. Activity was expressed as picomoles of indole-3-glycerol phosphate synthesized per minute per 5 μL of reaction mixture. For the generation of standard curves, authentic samples of tryptophan synthase α and β_2 subunits were diluted as necessary in 125 mM potassium phosphate buffer, pH 7.0, containing 2 mM dithiothreitol, 4 mM NaEDTA, and 1.0 mg/mL bovine serum albumin.

Gel Electrophoresis and Fluorography Tritiated proteins were analyzed by SDS–polyacrylamide gel electrophoresis. Slab gels (20 × 16 × 0.1 cm) containing 12% (w/v) acrylamide and 0.32% (w/v) *N,N′*-methylenebis(acrylamide) were prepared according to Laemmli (1970) with a 1.5-cm stacking gel consisting of 4% (w/v) acrylamide and 0.1% *N,N′*-methylenebis(acrylamide). Proteins were electrophoresed at 20 mA for 3.5 h, and the gels were fixed in an aqueous solution containing 10% acetic acid and 50% methanol. The gels were stained with Coomassie blue, destained in an aqueous solution containing 10% acetic acid and 50% methanol, and prepared for fluorography by soaking in a 1.0 M sodium salicylate solution containing 1% glycerol, essentially as described by Chamberlain (1979). The salicylate-impregnated gels were dried onto Whatman 3MM paper under vacuum and used for exposure of Kodak XAR-2 X-ray film at −80 °C for 3–5 days.

RESULTS

The biosynthesis of tryptophan synthase α protein was achieved by the use of pBN69, a pBR322-derived plasmid in which the expression of the gene for tryptophan synthase α subunit is under the control of the *trp* promoter from *Serratia marcescens*. Expression of this plasmid in vitro has resulted in yields of tryptophan synthase α subunit as high as 20% of total cellular protein (Nichols & Yanofsky, 1983). Protein biosynthesis was carried out in a cell-free coupled transcription–translation system similar to that reported by Yang et al. (1980). All components of the system were isolated from *E. coli* strain CF300, a *rec* B mutant shown to be deficient in exonuclease activity.

Tryptophan synthase holoenzyme exists as an $\alpha_2\beta_2$ tetramer that catalyzes the final step in tryptophan biosynthesis (Creighton & Yanofsky, 1966). The overall reaction i is the

$$\text{indole-3-glycerol phosphate} + \text{L-serine} \xrightarrow{\alpha_2\beta_2} \text{L-tryptophan} + \text{glyceraldehyde 3-phosphate} \quad \text{(i)}$$

$$\text{indole-3-glycerol phosphate} \xrightleftharpoons{\alpha} \text{indole} + \text{glyceraldehyde 3-phosphate} \quad \text{(ii)}$$

$$\text{indole} + \text{L-serine} \xrightarrow{\beta_2} \text{L-tryptophan} \quad \text{(iii)}$$

sum of reactions ii and iii that can be catalyzed by the individual α and β_2 subunits. Interestingly, the catalytic activity of β_2 in reaction iii is enhanced 40–50-fold upon association with the α subunit (Wilson & Crawford, 1965), which forms the basis for one of the tryptophan synthase α subunit assays employed here.

Because the original β_2 complementation assays (Crawford & Yanofsky, 1958; Smith & Yanofsky, 1962; Faeder & Hammes, 1970) were neither sufficiently sensitive nor convenient for our purposes, a modified assay was developed. This involved the use of [^{3}H]-L-serine as a substrate (+indole), resulting in the formation of [^{3}H]-L-tryptophan. The latter could be separated conveniently from L-serine on poly(ethylenimine)–cellulose TLC, permitting direct determination of product formation even at low (<0.5%) conversion (data not shown). In order to maintain the sensitivity of the assay system, it was necessary to use β_2 subunit devoid of contaminating α subunit, as even small amounts of the α subunit enhance L-tryptophan production significantly. Accordingly, we employed a (crystalline) β subunit preparation shown to be homogeneous on SDS–polyacrylamide gel electrophoresis (supplementary material Figure 1; see paragraph at end of paper regarding supplementary material). The enhancement of β_2 subunit activity by authentic α protein was determined at each of several concentrations of β_2 subunit. It was found that the assay was maximally sensitive in the region of interest (0.05–0.15 pmol of α subunit) when 0.1 pmol of β_2 subunit

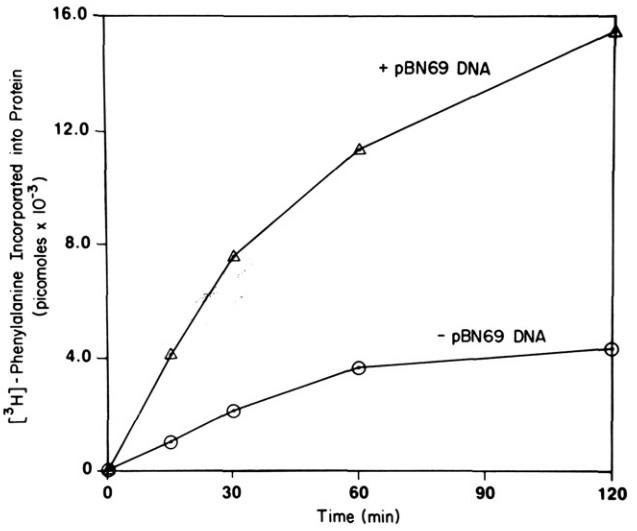

FIGURE 1: Stimulation of protein biosynthesis by pBN69 DNA. Reaction conditions were as described under Materials and Methods, except that the final reaction volume was 166 μL and reactions were initiated with 30 μg of pBN69 DNA. TCA precipitation of protein was carried as described by Mans and Novelli (1961).

was employed. Further, initial velocity measurements in this concentration range indicated that tryptophan synthase activity was linearly proportional to the amount of α subunit employed (supplementary material Figure 2).

A second assay was also developed on the basis of the work of Hatanaka et al. (1962) in which the conversion of [2-^{14}C]indole → [2-^{14}C]indole-3-glycerol phosphate was measured directly. Initial velocity measurements demonstrated that the production of indole-3-glycerol phosphate was proportional to the amount of α subunit employed within the region of interest (0.05–0.20 pmol of α subunit) (supplementary material Figure 3).

The ability of the cell-free system to support protein biosynthesis was assayed initially as incorporation of [^{3}H]-phenylalanine into TCA-precipitable material. As shown in Figure 1, protein synthesis proceeded steadily as a function of time and was stimulated approximately 4-fold by pBN69 DNA; in the presence of the plasmid DNA both the rate and extent of [^{3}H]phenylalanine incorporation were increased. Although not certain, background protein synthesis observed in the absence of pBN69 DNA was probably due to contaminating DNA introduced with the S-30 supernatant fraction.

The molecular weight composition of the polypeptides synthesized under several different conditions was studied next by parallel determinations of TCA-precipitable material formed and behavior on polyacrylamide gels. As illustrated in Figure 2, lane 1, protein synthesis in the absence of pBN69 DNA produced polypeptides of M_r <15 000, presumably reflecting the nature of the contaminating DNA. The addition of pBN69 DNA (lane 2) resulted in the appearance of a new band of M_r ~29 000, i.e., having the properties expected of tryptophan synthase α subunit (Guest & Yanofsky, 1966; Guest et al., 1967). In separate experiments aliquots from the in vitro reactions were admixed with 5 μg of authentic tryptophan synthase α subunit protein prior to polyacrylamide gel electrophoresis. Alignment of the Coomassie blue stained gel with its fluorogram verified that the synthesized polypeptide comigrated with authentic tryptophan synthase α chain.

Also evident from Figure 2 (lane 8) was the dependence of the appearance of putative α subunit on the presence of S-30 supernatant; absent this fraction, little protein biosynthesis was obtained. Appearance of putative α subunit was also shown to be dependent on the presence of RNA polymerase (lane 3)

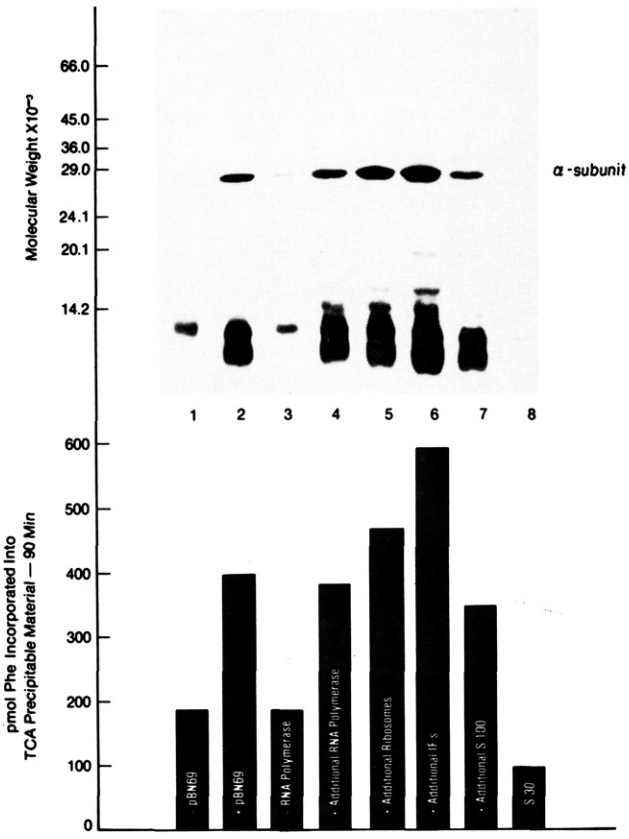

FIGURE 2: Concentration effect of RNA polymerase, ribosomes, initiation factors, S-100 supernatant, and S-30 supernatant on the in vitro synthesis of tryptophan synthase α subunit. Reactions were constituted as described under Materials and Methods. Except as noted below, each (100-μL) reaction mixture contained 0.17 μM RNA polymerase, 0.50 μM *E. coli* ribosomes, 30 μg of S-100 protein, and 45 μg of initiation factors. Reactions were initiated by the addition of 10.2 μg of pBN69 DNA and incubated at 37 °C for 90 min. Fifteen-microliter aliquots of the reaction mixtures were subjected to polyacrylamide gel electrophoresis; tritiated protein bands were visualized by fluorography. Five-microliter aliquots were subjected to TCA precipitation for determination of [^{3}H]phenylalanine incorporation. (Lane 1) No pBN69 DNA; (lane 2) complete system as indicated above; (lane 3) no RNA polymerase; (lane 4) 0.34 μM RNA polymerase; (lane 5) 1.0 μM ribosomes; (lane 6) 90 μg of initiation factors; (lane 7) 60 μg of S-100 protein; (lane 8) no S-30 supernatant.

and to increase in amount upon addition of greater amounts of RNA polymerase, ribosomes, S-100 supernatant, and, especially, initiation factors (lanes 4–7).

In addition to the appearance of a band that comigrated with authentic α subunit, the coupled transcription–translation system was also studied for production of α subunit activity, i.e., for the appearance of a protein capable of enhancing the conversion of indole → L-tryptophan by tryptophan synthase β_2 subunit. As shown in Figure 3, α subunit activity appeared in a time-dependent fashion in a reaction mixture that contained pBN69 DNA, with maximal synthesis occurring after 60 min of incubation. The appearance of α subunit activity was found to correlate with the fluorographic intensity of the band that comigrated with authentic α subunit protein on a polyacrylamide gel (data not shown). A control reaction from which pBN69 DNA was omitted gave no significant amount of α subunit activity (open bars). To test the stability of synthesized α subunit, 3.4 pmol of authentic α subunit was added to an incubation mixture that contained all components normally present during protein biosynthesis, with the exception of pBN69 DNA. As indicated in the figure, no decrease in activity of the authentic α protein was observed.

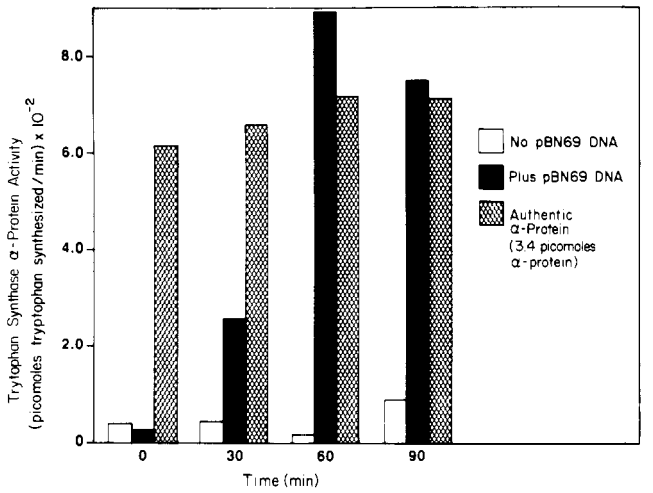

FIGURE 3: In vitro synthesis of tryptophan synthase α protein: stability and time-dependent appearance of enzymatic activity in the coupled transcription–translation system. Conditions employed for the synthesis and assay of tryptophan synthase α subunit are given under Materials and Methods.

Table I: Quantitation of Tryptophan Synthase α Subunit Synthesis and Activity[a]

pBN69 DNA (pmol)	incubation time (h)	α subunit synthesized (pmol)[b]	α subunit/ pBN69 DNA	% activity[c]
	1	*d*		*d*
	4	*d*		*d*
0.05	1	0.08	1.6	99
	4	0.15	3.0	99
0.10	1	0.16	1.6	99
	4	0.26	2.6	91

[a] Carried out essentially as described under Materials and Methods. [b] Determined by quantitation of [^{3}H]phenylalanine incorporated into protein that comigrated with α subunit on SDS–PAGE. [c] Determined relative to authentic α subunit. [d] No significant α subunit detectable.

Also investigated was the specific activity of the synthesized α subunit in comparison with authentic material. An experiment was carried out to produce α subunit via in vitro protein biosynthesis with [^{3}H]phenylalanine of known specific activity. Because the phenylalanine content of the α subunit is known (Guest & Yanofsky, 1966; Guest et al., 1967), quantitation of the radioactivity in the protein band that comigrated with authentic α subunit on a polyacrylamide gel provided a measure of α subunit protein synthesized (Table I). As shown in the table, when compared with authentic α subunit in the β_2 complementation assay, the synthesized protein was found to be essentially fully active. The synthesized tryptophan synthase α protein was also utilized for the conversion of indole to indole-3-glycerol phosphate in the presence of glyceraldehyde 3-phosphate (eq ii, vide supra). As shown in Table II, the synthesized α subunit was also fully active in this assay system.

As indicated in Table I, under the conditions employed 2.6–3.0 α subunit molecules were produced for each pBN69 DNA molecule used in the incubation. The effect of certain experimental parameters on the yield of catalytically competent α subunit was also studied. When the Mg^{2+} concentration employed during protein biosynthesis was lowered from 11 to 5 mM, little incorporation of [^{3}H]phenylalanine into protein or appearance of tryptophan synthase activity was noted (Figure 4). Inclusion of 1 or 5 mM spermidine in addition to 5 mM Mg^{2+} restored much of the polypeptide synthesis observed at higher Mg^{2+} concentration, but not the tryptophan synthase activity. Interestingly, at 7.5 and 9.5 mM Mg^{2+}

Table II: Quantitation of Activity of Synthesized Tryptophan Synthase α Subunit Activity in Two Different Assays[a]

		α subunit synthesized	
pBN69 DNA (pmol)	incubation time (h)	β_2 complementation assay (pmol)	indole-3-glycerol phosphate synthesis (pmol)
none	2	0	−0.04
none[b]	0		0.52
	1		0.42
	2	0.47	0.50
0.15	0		0
	1	0.20	0.31
	2	0.66	0.67

[a] Carried out as described under Materials and Methods. [b] Reaction mixture treated with 0.48 pmol of authentic tryptophan synthase α subunit.

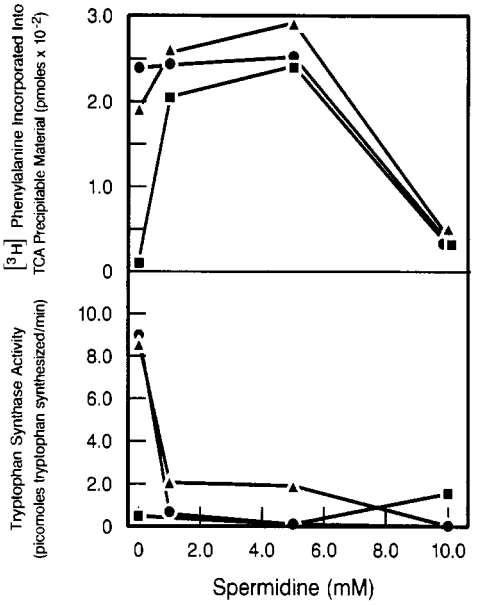

FIGURE 4: Effect of spermidine and Mg^{2+} on in vitro protein biosynthesis. (Upper panel) Incorporation of [^{3}H]phenylalanine into hot TCA precipitable material at 9.5 (●), 7.5 (▲), or 5.0 mM (■) added $MgCl_2$ concentrations in the presence of the indicated amounts of spermidine. (Lower panel) Dependence of tryptophan synthase activity on spermidine and Mg^{2+} concentrations. Incubation mixtures contained 0.98 μg (0.25 pmol) of pBN69 DNA/40-μL reaction. After incubation at 37 °C for 70 min, assays for in vitro protein biosynthesis and tryptophan synthase activity (β_2 complementation assay) were carried out as described under Materials and Methods.

concentration, the addition of 1 or 5 mM spermidine had little effect on polypeptide synthesis per se but virtually eliminated tryptophan synthase activity.

The nucleoside tetraphosphate ppGpp has been reported to enhance the fidelity of translation of proteins (Abraham, 1983; Yarus & Thompson, 1983; Dix & Thompson, 1986), and its effect on the production of catalytically active tryptophan synthase α protein was measured. When employed at concentrations up to 0.5 mM, ppGpp was found to have little effect on incorporation of [^{3}H]phenylalanine into protein or on the activity of tryptophan synthase activity of the α subunit as measured in the β_2 complementation assay (supplementary material Figure 4).

Also investigated was the ability of preactivated tRNAs to support protein biosynthesis in lieu of that tRNA plus its cognate amino acid and aminoacyl-tRNA synthetase. As shown in Figure 5 (panel A), *E. coli* phenylalanyl-tRNA^{Phe} was able to stimulate protein biosynthesis in a system that lacked exogenous phenylalanine; the extent of polypeptide synthesis was monitored by incorporation of added [^{3}H]valine

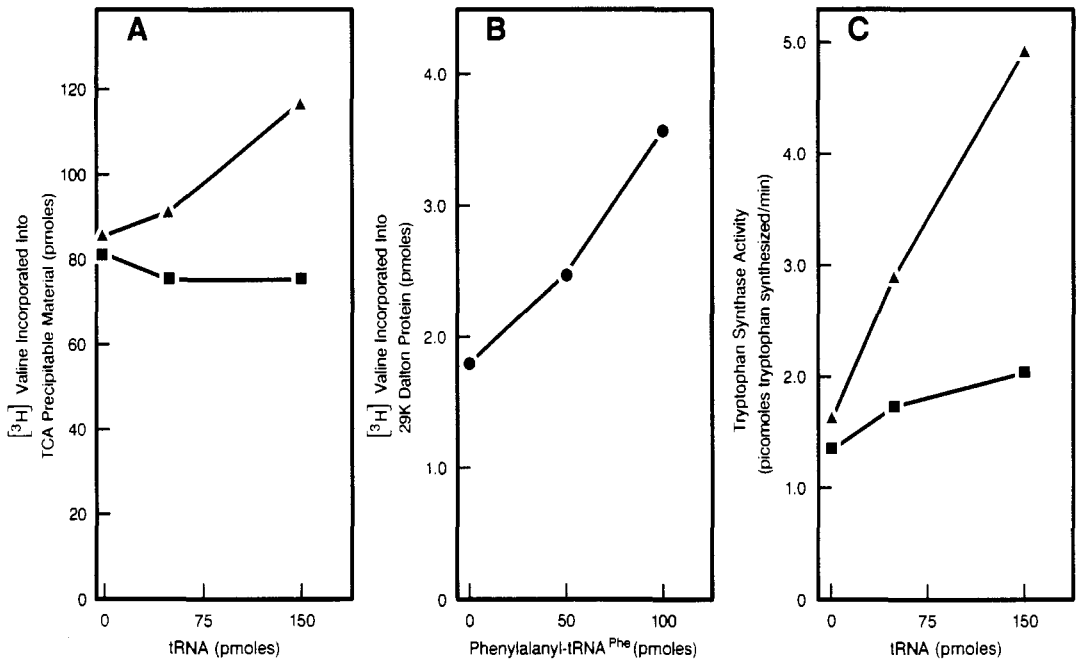

FIGURE 5: Stimulation of tryptophan synthase synthesis by exogenous aminoacylated tRNA. (Panel A) Stimulation of protein biosynthesis was demonstrated in reaction mixtures containing [^{3}H]valine (10^4 Ci/mol), no phenylalanine, and either *E. coli* phenylalanyl-tRNA^{Phe} (▲) or unacylated *E. coli* tRNA^{Phe} (■). The tRNA^{Phe} samples were added immediately prior to initiation of the reactions by addition of 0.98 μg of pBN69 DNA. Following incubation at 37 °C for 45 min, protein biosynthesis was assayed by TCA precipitation of protein (Mans & Novelli, 1961). (Panel B) Stimulation of the synthesis of protein having $M_r \sim 29\,000$ was carried out in 40-μL reaction mixtures containing [^{3}H]valine, no phenylalanine, and 0, 50, or 100 pmol of *E. coli* phenylalanyl-tRNA^{Phe}, as well as 0.98 μg of pBN69 DNA. Following incubation at 37 °C for 70 min, aliquots of the reaction mixtures were analyzed by polyacrylamide gel electrophoresis. Gel slices corresponding to the area comigrating with authentic tryptophan synthase α subunit were excised and solubilized for determination of radioactivity by liquid scintillation counting. (Panel C) Stimulation of tryptophan synthase activity by phenylalanyl-tRNA^{Phe}. Reactions were carried out in the presence of *E. coli* phenylalanyl-tRNA^{Phe} (▲) or tRNA^{Phe} (■) as described in panel A and then analyzed for tryptophan synthase activity (β complementation assay) as described under Materials and Methods.

into TCA-precipitable material. Analysis of the derived polypeptides by polyacrylamide gel electrophoresis (panel B) indicated that the synthesized material had the molecular weight expected for tryptophan synthase α protein. Also studied was the ability of the synthesized protein to stimulate the synthesis of tryptophan in the presence of tryptophan synthase β_2 subunit. As shown in panel C, tryptophan synthase activity was observed in proportion to added phenylalanyl-tRNA^{Phe}.

An analogous experiment was run in the absence of valine, but containing either *E. coli* [^{3}H]phenylalanyl-tRNA^{Val} or [^{3}H]valyl-tRNA^{Val}. As shown in Figure 6, analysis of these reaction mixtures by polyacrylamide gel electrophoresis indicated the formation of radiolabeled polypeptide having the molecular weight of tryptophan synthase α subunit in response to each of the aminoacyl-tRNA^{Val}s (lanes 1 and 3). None of this radiolabeled species was formed in a control experiment that contained added tRNA plus [^{3}H]phenylalanine, but no valine (data not shown). Assay of aliquots of these reaction mixtures for tryptophan synthase activity revealed that putative α protein synthesized in the presence of valyl-tRNA^{Val} facilitated the conversion of indole → tryptophan by tryptophan synthase β_2 subunit. As illustrated in Table III, this material was essentially as active as that derived from an incubation mixture containing [^{3}H]valine plus tRNA. As anticipated, the putative α protein analogue obtained by the use of phenylalanyl-tRNA^{Val} in the protein biosynthesizing system gave no measurable enhancement of tryptophan synthase activity when admixed with authentic β_2 subunit.

Table III: Quantitation of Synthesis and Activity of Putative Tryptophan Synthase α Subunit Prepared in the Presence of Preactivated tRNA^{Val}s[a]

[^{3}H]valine (μM)	[^{3}H]aminoacyl-tRNA^{Val} (μM)	TCA-precipitable material (pmol)	tryptophan synthesized (pmol)[b]
	phenylalanyl-tRNA^{Val} (1.7)	48.3	0
	valyl-tRNA^{Val} (0.9)	57.7	2.86
1.1		56.2	3.40
8.0		135.3	6.24

[a] Carried out as described under Materials and Methods and in the legend to Figure 6. All incubations contained 5.2 μg of pBN69 DNA and 2 mM phenylalanine. [b] No tryptophan synthase α subunit activity was evident in control experiments that lacked DNA.

DISCUSSION

This study was undertaken to demonstrate the feasibility of synthesizing catalytically competent *E. coli* tryptophan synthase α subunit in a cell-free system, and particularly to demonstrate that specific amino acids added to the protein biosynthesizing system in the form of aminoacyl-tRNAs could be transferred to the derived protein. As discussed below, realization of these objectives is essential to the more ambitious goal of producing proteins containing modified amino acids at specific, predetermined sites.

As shown in Figures 1–3, protein biosynthesis occurred only in response to pBN69 DNA. The amount of α subunit increased steadily as a function of time and was also shown to be dependent on the presence of RNA polymerase, ribosomes, and initiation factors (Figures 2 and 3). In numerous experiments, the presence of tryptophan synthase activity was found to correlate both qualitatively and quantitatively with the production of a protein having the molecular weight expected of tryptophan synthase α protein. Further, authentic α protein was shown to be stable in the protein biosynthesizing system employed here.

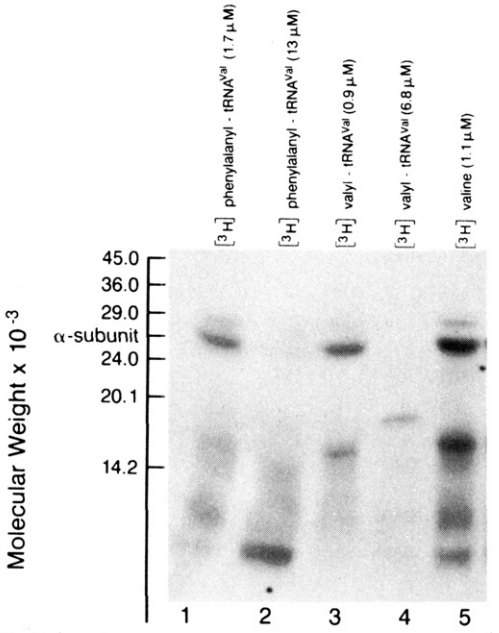

FIGURE 6: Synthesis of tryptophan synthase α subunit in the presence of [^{3}H]phenylalanyl-tRNA^{Val} and [^{3}H]valyl-tRNA^{Val}. Protein biosynthesis was carried out in reaction mixtures containing 5.2 μg of pBN69 DNA and either [^{3}H]phenylalanyl-tRNA^{Val} (lanes 1 and 2) or [^{3}H]valyl-tRNA^{Val} (lanes 3 and 4) but no valine. A control reaction contained [^{3}H]valine but no aminoacylated tRNA (lane 5). The reaction mixtures were incubated at 37 °C for 40 min; aliquots were then analyzed by polyacrylamide gel electrophoresis. The lack of product in lanes 2 and 4 was shown to be due to inhibition of the reaction by contaminants (e.g., phenol) present in the larger amounts of tRNA added to these incubations; the inhibition was subsequently overcome by chromatographic purification of the aminoacyl-tRNAs.

Key features of this study were the careful characterization of α subunit synthesis and of the specific activity of the derived tryptophan synthase α protein. As shown in Table I, synthetic α protein was fully as active as authentic material in facilitating the conversion of indole to L-tryptophan. It has been shown previously that missense mutants of *trp A* can produce altered α subunits catalytically inactive in the conversion of indole-3-glycerol phosphate to indole plus glyceraldehyde 3-phosphate, but capable nonetheless of activating the β_2 subunit for the conversion of indole to tryptophan (Murgola, 1985). Accordingly, the α protein obtained via in vitro protein biosynthesis was also employed for determination of intrinsic α subunit activity, as measured by the conversion of indole + glyceraldehyde 3-phosphate → indole-3-glycerol phosphate. That the synthesized α subunit was also fully active in this assay system is documented in Table II.

Also interesting was the efficiency of the coupled transcription–translation system in producing α subunit. As indicated in Table I, after 4 h of incubation 2.6–3.0 α subunit molecules were synthesized for each pBN69 DNA molecule employed. In other experiments, as many as eight tryptophan synthase α subunit molecules have been synthesized per plasmid DNA without significant diminution in the specific activity of the synthesized protein (data not shown). Thus, while the factor(s) that limit(s) synthesis of α subunit is (are) uncertain at present, the available system does appear to produce functional α subunit in sufficient amounts to permit its use in experiments that employ misacylated tRNAs for intentional alteration of α subunit structure.

Because previous studies have demonstrated that mRNA mistranslation in vitro can greatly exceed that which normally obtains in vivo [Jelenc and Kurland (1979) and references cited therein and Rice et al. (1984) and references cited therein], it seemed important to characterize the effects of parameters shown previously to affect the fidelity of protein biosynthesis on the activity of our synthetic tryptophan synthase α subunit. Polyamines have been reported to improve the fidelity of translation in vitro [Jelenc and Kurland (1979) and references cited therein; Igarashi et al., 1979; Abraham, 1983; Yarus & Thompson, 1983], and the effect of one polyamine on the coupled transcription–translation system utilized here was assayed directly. As illustrated in Figure 4, at each of three concentrations of Mg^{2+}, the addition of spermidine at concentrations up to 5 mM had little effect on the extent of polypeptide synthesis, except where the concentration of Mg^{2+} was suboptimal. Interestingly, the resulting tryptophan synthase activity was virtually eliminated under all conditions that employed spermidine during protein biosynthesis. It may be noted that this finding is not necessarily inconsistent with reports of improved fidelity of translation by polyamines, as the earlier work employed systems that lacked a transcriptional component; the alteration of DNA structure and function by polyamines is well-known (Anderson & Bauer, 1978; Srivenugopal & Morris, 1985; Feuerstein et al., 1986).

The nucleoside tetraphosphate ppGpp has been reported to increase the fidelity of protein biosynthesis (Abraham, 1983; Yarus & Thompson, 1983; Dix & Thompson, 1986). Interestingly, in the present case, concentrations of ppGpp up to 0.5 mM had no significant effect on the biosynthesis of tryptophan synthase α subunit, as measured either by incorporation of [^{3}H]phenylalanine into TCA-precipitable material or by the ability of the derived protein to convert indole → L-tryptophan (supplementary material Figure 4). The lack of effect of ppGpp on the biosynthesis of tryptophan synthase is consistent with earlier observations of selective inhibition of the biosynthesis of certain types of proteins by ppGpp (Lindahl et al., 1976). While no direct measure of the misincorporation of any specific amino acid was made in the present study, the finding that ppGpp had no effect on the activity of the derived tryptophan synthase is consistent with the interpretation that any microheterogeneity in this protein due to utilization of inappropriate aminoacyl-tRNAs during protein biosynthesis was insufficient to reduce enzymatic activity to a measurable extent.

Although it has been demonstrated previously that specific amino acids can be incorporated into polypeptides starting with preacylated tRNAs [see, e.g., Mitra et al. (1977)] and that tRNAs bearing other than their cognate amino acids can be utilized in such experiments (Chapeville et al., 1962; von Ehrenstein et al., 1963; Jacobson, 1966; Johnson et al., 1976; Pezzuto & Hecht, 1980), it has also been noted that some derived peptides were of inappropriate structure (von Ehrenstein et al., 1963; Jacobson, 1966) and that some of the misacylated tRNAs were utilized with reduced efficiency (Johnson et al., 1976; Yarus, 1972). In this case, tryptophan synthase α protein synthesis was clearly stimulated by phenylalanyl-tRNA^{Phe} in the absence of added phenylalanine, as judged by incorporation of another amino acid ([^{3}H]valine) into polypeptide, formation of product having the molecular weight of the expected protein, and appearance of tryptophan synthase α subunit activity (Figure 5). Polypeptide having the same approximate molecular weight as authentic tryptophan synthase α protein was also formed when protein biosynthesis was carried out with [^{3}H]phenylalanyl-tRNA^{Val} or [^{3}H]valyl-tRNA^{Val} but no added valine (Figure 6). As anticipated in the latter experiment, only the polypeptide formed in the presence of valyl-tRNA^{Val} retained catalytic activity. Thus, the ability of a well-defined protein biosynthesizing system to

utilize preacylated tRNAs for the production of catalytically competent tryptophan synthase α subunit was verified, as was the dependence of catalytic activity on the use of preacylated tRNAs bearing the cognate amino acid.

These experiments suggest the feasibility of utilizing misacylated tRNAs for the substitution of amino acids at defined sites in polypeptides. Previous work from this laboratory provides encouragement that certain structurally modified amino acids may act as substrates in such experiments (Heckler et al., 1983; Roesser et al., 1986).

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SUPPLEMENTARY MATERIAL AVAILABLE

Four figures showing SDS–polyacrylamide gel electrophoresis of crystalline *E. coli* tryptophan synthase β_2 subunit prepared from *E. coli* strain A2/F′A2, dependence of tryptophan synthase activity on tryptophan synthase α subunit, initial velocity of tryptophan α subunit catalyzed indole-3-glycerol phosphate formation, and effects of ppGpp on in vitro synthesis of tryptophan synthase α subunit (6 pages). Ordering information is given on any current masthead page.

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A Comparative Monomolecular Film Study of a Straight-Chain Phosphatidylcholine (Dipalmitoylphosphatidylcholine) with Three Isobranched-Chain Phosphatidylcholines (Diisoheptadecanoylphosphatidylcholine, Diisooctadecanoylphosphatidylcholine, and Diisoeicosanoylphosphatidylcholine)†

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ABSTRACT: The surface pressure vs. area per molecule isotherms for monomolecular films of a straight-chain lecithin [dipalmitoylphosphatidylcholine (DPPC)] and three isobranched-chain lecithins [diisoheptadecanoylphosphatidylcholine (DIHPC), diisooctadecanoylphosphatidylcholine (DIOPC), and diisoeicosanoylphosphatidylcholine (DIEPC)] are reported over a wide range of temperatures and surface pressures such that the full range of the liquid expanded/liquid condensed transition is documented in each case from the temperature at which a fully condensed film is observed to that at which a fully expanded film is found. A comparison of two lecithins having the same length for the primary alkane chain (DPPC and DIHPC), on either an absolute or reduced temperature basis, indicated that the isobranched lecithin differed primarily from the straight-chain lecithin in having a more expanded condensed state. This was attributed to impaired packing in the condensed state due to the methyl isobranch. The isobranched lecithin also differed in having a slightly more condensed expanded state. This was ascribed to reduced flexibility in the expanded state due to hindered rotation of the methyl isobranch. Similar conclusions were recently drawn for bimolecular assemblies of isobranched lecithins. A comparison of the three isobranched lecithins at similar reduced temperatures indicated that, while the condensed states are very similar, the expanded states occupied increasing areas per molecule with increasing chain length. Two points that may be of biological significance are that the earlier onset of a liquid expanded (or liquid-crystalline-like) state on insertion of an isobranch could provide a wider temperature range for membrane and cell survivability and that the branched-chain lecithins would appear to provide an improved alternative to unsaturated lecithins in terms of fluidity coupled with reduced oxidation susceptibility in various model membrane experiments.

Kaneda (1977) found that bacteria of the genus *Bacillus* contain isobranched and anteisobranched fatty acids as major fatty acid components of their membrane lipids. Methyl-isobranched and methyl-anteisobranched fatty acids are also found in eight other genera of Gram-positive and in four genera of Gram-negative eubacteria. These findings seem to have provided the impetus for a recent series of investigations (Lewis & McElhaney, 1985; Mantsch et al., 1985; McDonald et al., 1983; Kannenberg et al., 1983; McDonald et al., 1984; Church et al., 1986; C. P. Yang, M. C. Wiener, R. N. A. H. Lewis, R. N. McElhaney, and J. F. Nagle, unpublished results) into the physical properties of phosphatidylcholines (PCs)[1]

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[1] Abbreviations: PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DIHPC, diisoheptadecanoylphosphatidylcholine; DIOPC, diisooctadecanoylphosphatidylcholine; DIEPC, diisoeicosanoylphosphatidylcholine; π, surface pressure; A, molecular area; T_0, extrapolation of π_t vs. T to zero pressure; π_t, surface pressure at onset of LE/LC transition; T_c, monolayer critical temperature; LE, liquid expanded state; LC, liquid condensed state; T_m, main bilayer transition.

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