Stimulation, by Two *Escherichia coli* Supernatant Proteins, of the Initiation of Polypeptide Synthesis†

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ABSTRACT: Two protein factors (A and B) have been partially purified from *Escherichia coli* supernatant which, in combination, are more effective than 0.5 M NH_4Cl in stimulating ribosomes for AcPhe-tRNA and fMet-tRNA binding, for the puromycin reaction, and for incorporating acetylphenylalanine from AcPhe-tRNA into polypeptide. The factors appear to differ from the initiation factors, the elongation factor EF-T, and ribosomal proteins. Some uncertainty exists as to whether factor B is different from EF-G. To maximize the effect of the factors in initiator tRNA binding, we preincubated the ribosomes with the factors and carried out the binding assay for a short period at 15 °C. Maximal stimulation of binding occurred after about a 2-min preincubation at 37 °C. Longer preincubation times were required at 15 °C, and only slight stimulation was observed after preincubation at 0 °C. The extent of stimulation by the factors was not affected when the NH_4Cl concentration was increased from 40 to 500 mM in the preincubation. The presence of both the 30S and 50S ribosomal subunits is required for the enhancement of AcPhe-tRNA binding. Polyphenylalanine synthesis carried out without AcPhe-tRNA is inhibited by the factors. It is suggested that the factors may act by inducing a structural rearrangement of the ribosomes.

The observation that *Escherichia coli* ribosomes are activated by preincubation in a high concentration of salt has provided some insights into ribosome function (Nakamoto and Hamel, 1968; Miskin et al., 1968, 1970; Zamir et al., 1971). The physiological significance of the observation, if any, has remained uncertain, however. The nonphysiological conditions under which the ribosomes become inactive initially in vitro and subsequently active in high salt concentrations do not support any serious speculation as to whether the observation represents a process that may occur in the cell. It is conceivable, nonetheless, because of the structural complexity of the ribosomes and because of the requirement for regular dissociation and association, that the fully assembled ribosomes are not always in the most active conformation during the various stages of protein synthesis, and that they may require activation.

Our recent studies on the enhancement of ribosomal activity in vitro have focused on two proteins (factors A and B) from the *E. coli* supernatant which were purified initially as stimulators of the puromycin reaction (Tsuda and Nakamoto, 1976). The two proteins, which act in combination, are more effective than high salt concentrations in stimulating ribosomes to bind initiator tRNA and to initiate polypeptide synthesis. We report here on the partial purification and properties of factors A and B and compare their effectiveness with that of high salt concentrations in stimulating ribosomal activity.

Experimental Section

Materials. DEAE[1]-cellulose (Whatman DE-32) was obtained from Reeve Angel, *E. coli* B cells from Grain Processing Corp., hydroxylapatite from Clarkson Chemical Co., poly(U) and poly(A,G,U) from Miles Laboratories, and ACS scintillation fluid from Amersham/Searle Corp.

Methods. Preparation of Ac[^{14}C]Phe-tRNA and f[^{3}H]-Met-tRNA was done as described previously (Hamel et al., 1972). Initiation factors, salt-washed ribosomes, and elongation factors EF-G and EF-T were prepared from *E. coli* B as reported previously (Blumberg et al., 1974).

The assay mixture for the puromycin reaction contained, in a final volume of 0.1 mL, 50 mM imidazole (pH 7.4), 10 mM $MgCl_2$, 5 μg of poly(U) or poly(A,G,U), 5 mM 2-mercaptoethanol, 0.2 mM GTP, 0.1 mM puromycin, 20 pmol of Ac[^{14}C]Phe-tRNA or f[^{3}H]Met-tRNA, 55 μg of 70S ribosomes, and sufficient initiation factors to give maximal activity. Incubation was for 5 min at 37 °C. The reaction was terminated by the addition of 1 mL of 0.1 M sodium acetate, pH 5.5. The reaction product was then extracted with 2 mL of ethyl acetate, and the radioactivity of the extract was measured in an Isocap 300 scintillation counter.

The assay conditions for AcPhe-tRNA and fMet-tRNA binding were similar to those described before (Blumberg et al., 1974), except for the substitution of 10^{-5} M aurintricarboxylic acid for dextran sulfate in the stopping buffer. When indicated, 15 μg of 30S ribosomes or 55 μg of salt-washed 70S ribosomes were used. Polyphenylalanine synthesis was carried out as reported previously (Nakamoto and Hamel, 1968).

Purification of Factors A and B

Preparation of Ammonium Sulfate Fraction. A cell-free extract of *E. coli* B, prepared as described before (Hamel et al., 1972), was heated at 55 °C for 10 min with constant stirring, and the resulting heavy precipitate was removed by centrifugation at 4 °C. In a typical preparation, 110 g of ammonium sulfate was added to 455 mL of the supernatant from the heated extract; the precipitate was collected 10 min later by centrifugation and discarded. The supernatant was then made 60% saturated in ammonium sulfate at 4 °C by addition of the solid salt. The precipitate was collected after 15 min by centrifugation and dissolved and dialyzed at room temperature in a buffer solution containing 20 mM imidazole, pH 7.4, and

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[1] Abbreviations used are: DEAE, diethylaminoethyl; GTP, guanosine triphosphate.

TABLE I: Stimulation of the Puromycin Reaction by *E. coli* Supernatant.[a]

	Additions		AcPhe-Puromycin (pmol)	fMet-Puromycin (pmol)
	mRNA	Supernatant		
1.	poly(U)	−	2.0	
2.	poly(U)	+	5.3	
3.	poly(A,G,U)	−		1.8
4.	poly(A,G,U)	+		3.1
5.	poly(A,G,U)	+ (55 °C)		3.4
6.	poly(A,G,U)	+ (75 °C)		2.2

[a] The reactions were carried out as described in the text, with 5 μg of poly(U) or poly(A,G,U). Where indicated, *E. coli* supernatant was heated for 10 min at the specified temperature.

3 mM 2-mercaptoethanol (buffer I). The factors described below were further purified at room temperature.

Chromatography on DEAE-Cellulose. The dialyzed ammonium sulfate fraction (about 50 mL from 455 mL of the supernatant of the heated extract) was applied to a 2.5 × 27 cm DEAE-cellulose column which had been equilibrated with buffer I. The column was then washed with 300 mL of buffer I. Factor A is not adsorbed by the ion exchanger and is recovered during this washing. After the column had been washed further with 200 mL of buffer I containing 150 mM KCl, factor B was eluted with a linear gradient obtained by mixing 300 mL each of buffer I containing 500 and 150 mM KCl. The second factor was eluted at about 0.2 M KCl. Fractions with factor A and B activity were pooled separately and concentrated by dialysis overnight against saturated ammonium sulfate solution. The resulting precipitates were collected by centrifugation and dissolved and dialyzed in buffer I.

Further Purification of Factor A. The factor A preparation from DEAE-cellulose chromatography was layered onto a 2 × 55 cm Sephadex G-100 column which was equilibrated with buffer I and eluted at a flow rate of 5 mL/h. Factor A emerged from the column after the passage of about 100 mL of buffer I. Active fractions were pooled and concentrated by dialysis against buffer I containing 50% glycerol and 80 mM NH_4Cl. The factor has a 280- and 260-nm absorbance ratio of about 1.5 at this stage; it is inactivated by trypsin.

Further Purification of Factor B. The factor B preparation from DEAE-cellulose chromatography was applied to a 2.5 × 10 cm hydroxylapatite column which was equilibrated with 10 mM potassium phosphate, pH 7.0, and 3 mM 2-mercaptoethanol. Elution was carried out stepwise with 200-mL portions of buffer solution containing 3 mM 2-mercaptoethanol and potassium phosphate, pH 7.0, at 20, 30, and 50 mM concentrations. The factor, which was eluted with the solution containing 50 mM potassium phosphate, was concentrated by dialysis against saturated ammonium sulfate solution. The precipitate obtained upon dialysis was collected by centrifugation and dissolved in 2 mL of buffer I. The factor was then further chromatographed on Sephadex G-100 exactly as described for the purification of factor A. Fractions with factor B activity, which emerged from the column after the passage of about 65 mL of buffer I, were pooled and concentrated by dialysis against buffer I with 50% glycerol and 80 mM NH_4Cl. The factor has a 280- to 260-nm absorbance ratio of about 1.3 at this point; like factor A, it is inactivated by trypsin.

Stability of Factors A and B. Although the factors in the crude extract are very stable at 4 and −20 °C, both are unstable at low temperatures after they have been separated by DEAE-cellulose chromatography. Less than 10% of the initial activity remained when the purified factors were stored at −20 °C for 2 weeks. The purified factors, however, are relatively stable at room temperature, retaining almost full activity after 3 weeks. For this reason, their purification was carried out at room temperature, beginning with the dialysis of the ammonium sulfate fraction.

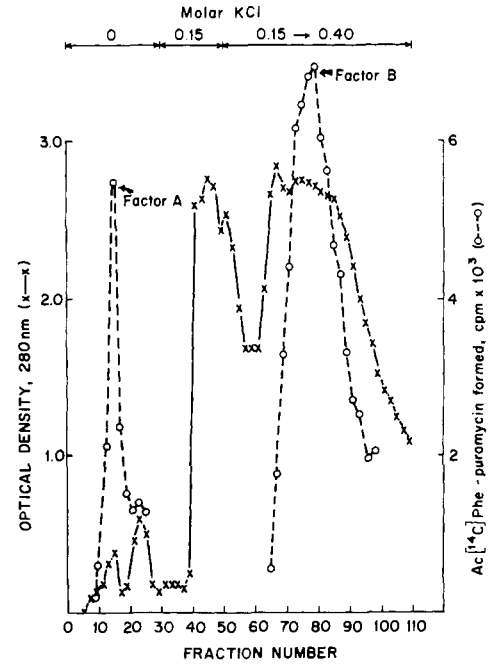

FIGURE 1: Separation of factors A and B by DEAE-cellulose chromatography. Factor A was detected by measurement of AcPhe-puromycin formation in the presence of factor B, and factor B in the presence of factor A. Ten-microliter aliquots of each fraction were assayed in the presence of saturating amounts of the second factor and the values obtained were corrected for a baseline of 2130 cpm with either factor alone. Other components and conditions of the assay were as described in the text.

Results

In the course of conducting kinetic studies on the puromycin reaction in an *E. coli* system with AcPhe-tRNA and fMet-tRNA, we noticed that the reaction was stimulated by ribosome-free supernatant (Table I). The system contained saturating amounts of the three initiation factors. We have previously shown that the puromycin reaction with synthetic RNA was strongly stimulated by the initiation factor IF-3 in the presence of IF-1 and IF-2 (Bernal et al., 1974). The active component in the supernatant was relatively heat stable; it withstood heating for 10 min at 55 °C. It was, however, inactivated by heating for 10 min at 75 °C.

To identify and study the function of the component(s) responsible for the stimulation of the puromycin reaction, we purified the active factor from the supernatant as described under Methods. After purification by DEAE-cellulose chromatography, the stimulatory activity was found in two proteins, each of which was inactive by itself (Figure 1). The protein that was not retained by the ion exchanger has been designated as factor A, and the other, as factor B. The stimulation of AcPhe-puromycin formation by increasing amounts of each of the purified proteins in the presence of saturating amounts of the other is shown in Figure 2.

The effect of the two proteins and of the initiation factors on fMet-puromycin formation with poly(A,G,U) as messenger

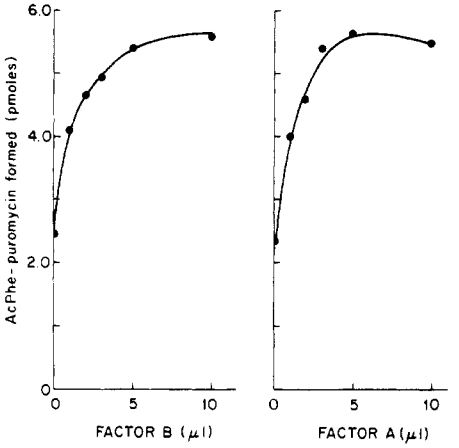

FIGURE 2: Stimulation of AcPhe-puromycin formation by factors A and B. The assays were carried out as described in the text. The effect of factor B was measured in the presence of 3.3 μg of factor A, and that of factor A in the presence of 6.6 μg of factor B. The concentrations of the factor A and B preparations were 0.33 and 0.63 mg/mL, respectively.

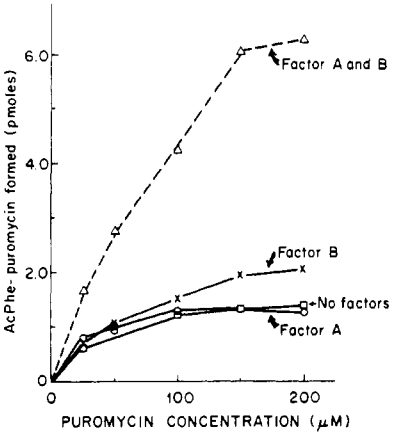

FIGURE 3: Effect of puromycin concentration on AcPhe-puromycin formation. The assays were carried out as described in the text, with 3.3 μg of factor A and 6.3 μg of factor B.

is seen in Table II. The stimulation of the puromycin reaction by the two proteins in the presence of saturating amounts of the three initiation factors, together with the abundance of the two proteins in the supernatant, clearly differentiates them from the initiation factors.

Factors A and B appear to differ from the supernatant factor that has been reported to stimulate the puromycin reaction at low concentraions of the antibiotic (Ganoza and Fox, 1974). Not only are there two separate proteins involved in this case, but the stimulation of the reaction can also be observed in the presence of excess puromycin (Figure 3).

The two proteins are also distinct from the elongation factor EF-T. The latter could not be replaced by the factors in polyphenylalanine synthesis which was carried out without AcPhe-tRNA and without the initiation factors (Table III). Factor B preparations, however, always contained high EF-G activity; therefore, no conclusion can be drawn as to whether the proteins are different unless further purification is done, especially since our best preparation of EF-G also contained high factor B activity. The absence of any stimulation of polyphenylalanine synthesis when the two proteins were added together with the elongation factors EF-G and EF-T, moreover, tends to rule out the possibility that the factors A and B are ribosomal proteins. In fact, inhibition of this reaction by

TABLE II: Effect of Various Factors on fMet-Puromycin Formation.[a]

Omissions	fMet-Puromycin (pmol)
None	5.2
IF-1	2.6
IF-2	1.4
IF-3	1.6
Factor A	2.9
Factor B	2.2

[a] The reaction mixture contained 5 μg of poly(A,G,U) and, where indicated, saturating amounts of initiation factors and of factors A and B. Other conditions of the assay were as described in the text.

TABLE III: Effect of Factors A and B on Polyphenylalanine Synthesis.[a]

	Additions	Phe Incorp. (pmol)
1.	EF-G, EF-T	3.2
2.	EF-G	0.5
3.	EF-G, Factors A and B	0.7
4.	EF-T	0.2
5.	EF-T, Factor A	0.2
6.	EF-T, Factor B	3.1
7.	EF-G, EF-T, Factors A and B	2.6

[a] The assays were carried out as described previously (Hamel and Nakamoto, 1968). The reaction mixture contained, when indicated, 3.3 μg of factor A, 6.3 μg of factor B, 4 μg of EF-T, and 8.3 μg of EF-G. Incubation was carried out for 1 min at 37 °C.

the factors has been observed consistently, and was increased by preincubation of the ribosomes with the factors (data not presented).

To determine how factors A and B stimulate the puromycin reaction, we examined the effect of the proteins on the binding of AcPhe-tRNA to 70S and 30S ribosomes. The ribosomes were preincubated with and without the factors and incubated for 30 s at 15 °C in the binding assay to maximize the effects of the factors. As shown in Table IV, the proteins stimulate the binding of AcPhe-tRNA to the 70S ribosomes. Surprisingly, the two proteins failed to stimulate AcPhe-tRNA binding with only the 30S subunits, or with 30S and 50S subunits preincubated separately with the factors. An inhibition which occurs when subunits only are preincubated together is diminished to the same degree by preincubating the subunits separately with and without the factors. AcPhe-tRNA binding is stimulated to a level greater than that of the 30S subunit alone only by preincubation of both subunits together with the factors.

A relatively short time is required for "activation" of the ribosomes by preincubation with the factors (Figure 4). Near-maximal stimulation is obtained by preincubation of the ribosomes for about 2 min at 37 °C. To reach the same level of stimulation at 15 °C, more than 10 min of preincubation are required. Only a slight activation of the ribosomes occurs when the preincubation is carried out at 0 °C. The two proteins are more effective than 500 mM NH_4Cl in enhancing the activity of the ribosomes. The highest stimulation obtained with the salt is only comparable to that observed with the proteins without any preincubation. The concentration of salt does not appear to be critical for the action of the factors (Figure 5). The

TABLE IV: Effect of Factors A and B on AcPhe-tRNA Binding.[a]

	Additions		AcPhe-tRNA Bound
	Ribosomes	Factors	(pmol)
1.	70S	−	1.7
2.	70S	+	3.1
3.	30S	−	1.5
4.	30S	+	1.6
5.	30S + 50S	−	0.4
6.	30S + 50S separately	−	1.3
7.	30S + 50S separately	+	1.2
8.	30S + 50S	+	2.1

[a] Assay conditions were as described in the text. The ribosomes were preincubated in a solution containing 50 mM imidazole (pH 7.4), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 500 mM NH_4Cl, with and without factors A and B, at 37 °C for 10 min. Fifteen micrograms of 30S ribosomes or 55 μg of 70S ribosomes was used for each reaction. Thirty micrograms of 50S subunits was added where indicated. Incubation was carried out at 15 °C for 30 s.

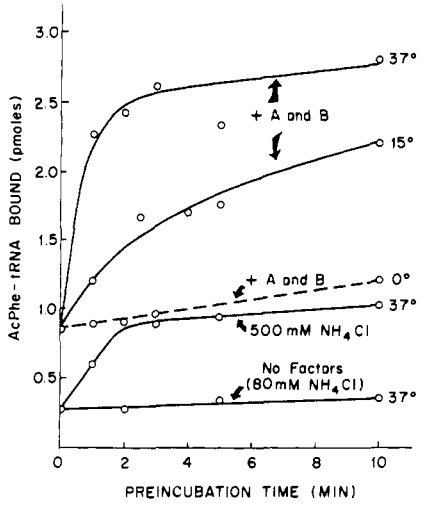

FIGURE 4: Effect of the time of preincubation at different temperatures on the stimulation of AcPhe-tRNA binding by factors A and B. The conditions for the binding assay and for preincubation of the ribosomes were as described in Table IV, except for the variation in the time and temperature of preincubation and the addition of 80 mM NH_4Cl with the factors.

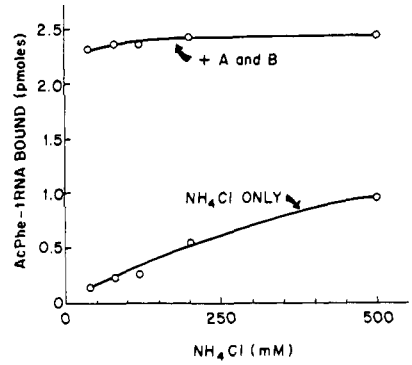

FIGURE 5: Effect on AcPhe-tRNA binding of preincubation of ribosomes with factors A and B in varied concentrations of NH_4Cl. The experimental conditions were as described in Table IV, except for the indicated variation in the NH_4Cl concentration.

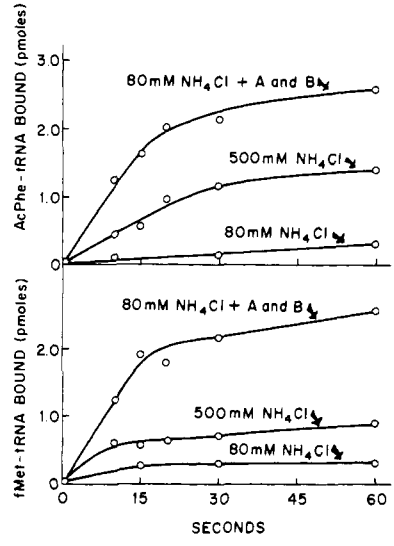

FIGURE 6: Effect of preincubation of ribosomes with factors A and B on the rate of fMet-tRNA and AcPhe-tRNA binding. Experimental conditions were as in Figure 5, except for the variation in the time of incubation for the binding reaction. Five micrograms of poly(A,G,U) were used in each reaction for fMet-tRNA binding.

extent of stimulation remained essentially constant between 40 and 500 mM NH_4Cl; no additive effect of the salt on the activity of the factors was apparent.

The rates of AcPhe-tRNA and fMet-tRNA binding were enhanced by preincubation of the 70S ribosomes with factors A and B (Figure 6). The two proteins were again more effective than 500 mM NH_4Cl in stimulating the ribosomes. The rate of acetylphenylalanine incorporation into polypeptide, i.e., the initiation of polyphenylalanine synthesis with AcPhe-tRNA, was stimulated in a way similar to the binding of AcPhe-tRNA by preincubation of the ribosomes with the factors (Figure 7). This is in contrast to the observed inhibition of polyphenylalanine synthesis by the proteins without AcPhe-tRNA (Table III).

The stimulation of AcPhe-tRNA binding by the factors with 70S ribosomes, but not with 30S subunits alone, as seen earlier (Table IV), suggested that the proteins either induced binding directly to the 70S ribosomes or stimulated binding to the 30S subunits only in the presence of the 50S subunits. To resolve this problem, we made simultaneous measurements of the rate of formation of the 30S and 70S complexes with AcPhe-tRNA by differentiating the two complexes with RNase I, which hydrolyzes AcPhe-tRNA bound to the 30S subunits but not to the 70S ribosomes (Pestka, 1968; Blumberg et al., 1975). One set of duplicate reactions treated with RNase I provided a measure of 70S complex formation, and the difference between this value and that of the second set, not treated with nuclease, provided a measure of 30S complex formation. The results, presented in Figure 8, show that the factors stimulate binding of AcPhe-tRNA to the 70S ribosomes by stimulating binding to the 30S subunits. The rate of 30S complex formation with the factors is greater than the combined rate of formation of 30S and 70S complexes without the factors.

The results presented in Table V summarize the main effects of preincubation of 70S ribosomes with the factors, and of high salt concentration, on AcPhe-tRNA binding and on polyphenylalanine synthesis carried out without AcPhe-tRNA. Preincubation of the ribosomes in 80 mM NH_4Cl did not significantly affect AcPhe-tRNA binding or polyphenylalanine synthesis, but the presence of the proteins during the preincubation enhanced the binding and inhibited the synthesis.

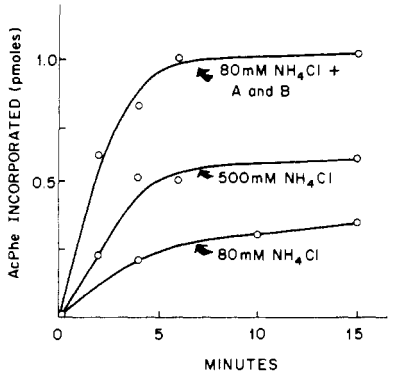

FIGURE 7: Effect of preincubation of ribosomes with factors A and B on AcPhe incorporation. Ribosomes were preincubated as described in Figure 6, and AcPhe incorporation was carried out in a reaction mixture identical to that used for the binding of AcPhe-tRNA, except for the addition of 8.3 μg of EF-G, 4 μg of EF-T, 39.5 of μg of nonradioactive Phe-tRNA, and factors A and B as indicated. Incubation was carried out at 15 °C, and radioactivity incorporated into the polypeptide was measured as before (Bernal et al., 1974).

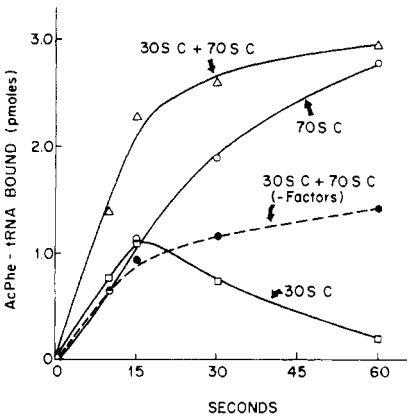

FIGURE 8: Stimulation of the formation of 30S and 70S initiation complex by factors A and B. The ribosomes were preincubated with factors A and B in 80 mM NH_4Cl, as described in Table IV. The method for the binding assay is given in the text. The 30S and 70S initiation complexes (30S C and 70S C) were differentiated with RNase I as reported previously (Blumberg et al., 1975).

TABLE V: Effect of Preincubation of Ribosomes with Factors and NH_4Cl on AcPhe-tRNA Binding and Polyphenylalanine Synthesis.[a]

	Preincubation Conditions	AcPhe-tRNA Bound (pmol)	Phe Incorp. (pmol)
1.	No preincubation (80 mM NH_4Cl)	0.4	4.5
2.	80 mM NH_4Cl	0.4	4.7
3.	80 mM NH_4Cl + Factors	3.7	2.6
4.	500 mM NH_4Cl	1.6	5.1
5.	500 mM NH_4Cl + Factors	4.0	2.5

[a] The assay conditions for polyphenylalanine synthesis were as described in Table III, and the conditions for preincubation of ribosomes and the assay for binding were as in Table IV.

Preincubation of the ribosomes in 500 mM NH_4Cl stimulated both activities. When the factors were included with the high concentration of salt, the effect of the proteins predominated, and AcPhe-tRNA binding was stimulated whereas polyphenylalanine synthesis was inhibited.

Discussion

The studies of Elson and co-workers on the activation of ribosomes preincubated in high salt concentration (Miskin et al., 1968, 1970; Zamir et al., 1971) strongly suggest that salt and an elevated temperature enhance ribosomal activity by inducing some structural rearrangement of the ribosomes. The importance of structural rearrangement has also been underscored in studies on ribosome reconstitution by Nomura and co-workers (Traub and Nomura, 1969); without incubation of the reconstitution mixture at an elevated temperature and in high salt concentration, no active particles are formed.

Factors A and B may enhance the activity of the ribosomes, as has been suggested for salt, by inducing a structural rearrangement of the ribosomes. The same preparations of ribosomes that are activated by salt show enhanced activity when they are preincubated with the two proteins in a process that also requires an elevated temperature. The observation that high salt concentration has no significant effect on the action of the factors (i.e., the stimulatory effects of the salt and of the proteins are not additive) further suggests that the same ribosomes are activated by the factors and by the salt, probably by a similar mechanism. The higher level of activation with the factors may reflect in part the difference, at equilibrium, between ordered and random processes of structural rearrangement.

Another difference between the effect of factors A and B and that of salt on the ribosomes is the inhibition by the factors, and the slight stimulation by the salt, of polyphenylalanine synthesis carried out without AcPhe-tRNA and initiation factors. It is possible that the two proteins function by keeping the ribosomes active and readily dissociable. Since polyphenylalanine synthesis without AcPhe-tRNA is initiated directly by 70S ribosomes, whereas the synthesis with AcPhe-tRNA begins with the 30S subunit (Guthrie and Nomura, 1968; Nakamoto and Hamel, 1968; Blumberg et al., 1975), inhibition of the former reaction and stimulation of the latter by the factors would be expected. The observed alleviation by the factors of the inhibition caused by combining of 30S and 50S subunits is in accord with this interpretation. It is difficult at present, however, to account for the requirement of 50S ribosomes for further activation of the 30S subunit.

The discovery of factors A and B in the supernatant validates the salt-activation studies as meaningful observations of a physiological process. The true nature of the process, however, is unclear, and further studies will have to be conducted. The factors, especially factor B, will have to be purified more extensively. The actual manner in which the factors interact with the ribosomes must be determined. Such studies are currently under way in our laboratory.

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References

Bernal, S., Blumberg, B., and Nakamoto, T. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 774.

Blumberg, B., Bernal, S., and Nakamoto, T. (1974), *Biochemistry 13,* 3307.

Blumberg, B., Nakamoto, T., and Goldberg, I. S. (1975), *Biochemistry 14,* 2889.

Ganoza, C. M., and Fox, J. L. (1974), *J. Biol. Chem. 249,* 1037.

Guthrie, C., and Nomura, M. (1968), *Nature (London) 219,* 232.

Hamel, E., Koka, M., and Nakamoto, T. (1972), *J. Biol. Chem. 247,* 805.
Miskin, R., Zamir, A., and Elson, D. (1968), *Biochem. Biophys. Res. Commun. 33,* 551.
Miskin, R., Zamir, A., and Elson, D. (1970), *J. Mol. Biol. 54,* 355.
Nakamoto, T., and Hamel, E. (1968), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2453.
Pestka, S. (1968), *J. Biol. Chem. 243,* 4038.
Traub, P., and Nomura, M. (1969), *J. Mol. Biol. 40,* 391.
Tsuda, Y., and Nakamoto, T. (1976), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1728.
Zamir, A., Miskin, R., and Elson, D. (1971), *J. Mol. Biol. 60,* 347.

Cross-Complexing Pattern of Plant Histones†

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ABSTRACT: Pea histones H2a, H2b, H3, and H4 have been isolated and their interactions studied by fluorescence anisotropy, light scatter, and circular dichroism. Histones H3 and H4 are almost identical in plants and animals, but plant histones H2a and H2b differ markedly from their mammalian counterparts. Pea H2b has a molecular weight approximately 20% greater than that of calf thymus H2b; the amino acid compositions of the two proteins are different. Calf thymus H2a exists as a single molecular weight species, while pea H2a exists as two species which differ by about 1500 daltons. The larger plant H2a is about 19% greater in molecular weight than calf thymus H2a. The smaller is about 8% greater. Despite these differences between calf and pea histones, the strong interactions between histone pairs H3 and H4, H2b and H4, and H2a and H2b, previously demonstrated for calf histones, also exist for pea histones. There are also weak interactions between pea H2a and H4 and between pea H2b and H3, and an interaction of intermediate strength between H2a and H3. The cross-complexing pattern of the plant histones is therefore the same as that reported for calf thymus histones [D'Anna, J. A., Jr., and Isenberg, I. (1974), *Biochemistry 13,* 4992], despite the dissimilarities of H2a and H2b.

The subunit structure of chromatin has been demonstrated by nuclease digestion and electron microscopy (Olins and Olins, 1973, 1974; Woodcock, 1973; Van Holde et al., 1974; Kornberg, 1974). A general picture has emerged in which two each of the histones H2a, H2b, H3, and H4 associate strongly with one another to form a globular histone core. The DNA is wrapped about the core (Pardon et al., 1975). The subunit structure of chromatin has now been found in a wide variety of organisms (Lohr and Van Holde, 1975; Griffith, 1975; McGhee and Engle, 1975; Gorovsky and Keevert, 1975; Jerzmanowski et al., 1976; Morris, 1976; Noll, 1976; Thomas and Furber, 1976; Nicolaieff et al., 1976).

Two of the histones, H3 and H4, have the most highly conserved primary structures of any proteins known (DeLange et al., 1969; Patthy et al., 1973). The classic comparison between calf thymus H4 and pea H4 demonstrated (DeLange et al., 1969) that these differ by only two conservative replacements.

Although we know the sequence of H3 and H4 from widely divergent species (DeLange et al., 1969; Patthy et al., 1973), such knowledge is not yet available for H2a and H2b. Indeed, H2a and H2b must have changed to a much greater degree throughout evolution. Their molecular weights are much greater in plants than in animals (Sommer and Chalkley, 1974) and, as we point out in this paper, based on amino acid content, H2a and H2b have evolved perhaps more like cytochrome *c* than like the highly conserved H3 and H4 histones.

Before the discovery of the subunit structure of chromatin, histones were defined operationally by their chemical composition, electrophoretic mobility, and solubility characteristics. However, the plant histones H2a and H2b have higher molecular weights and differing amino acid contents from their animal counterparts. These differences prevented a definitive identification of the plant histones by operational criteria (Oliver et al., 1972). This was emphasized by Nadeau et al. (1974) and Brandt and Von Holt (1975), who made no attempt at identifying plant histones H2a and H2b, but rather used the neutral designation "plant histones" (PH1 and PH2) for certain bands seen on gels. Others (Panyim et al., 1970; Spiker and Chalkley, 1971; Spiker and Krishnaswamy, 1973; Sommer and Chalkley, 1974; Spiker, 1975, 1976a,b) speculated on which bands might be H2a and H2b and made tentative identifications based on the pioneering work of Fambrough and Bonner (Fambrough and Bonner, 1966, 1969; Fambrough et al., 1968). More recently, Spiker et al. (1976) suggested that the identification could be made on the basis of electrophoretic mobility in Triton-containing gels, staining properties, and solubility characteristics. As the present paper shows, these suggestions were correct.

Calf thymus histones interact with one another in specific ways (Skandrani et al., 1972; D'Anna and Isenberg, 1973, 1974a,b; Kornberg and Thomas, 1974; Roark et al., 1974; Lewis, 1976; Van Holde and Isenberg, 1975; Isenberg, 1977; Weintraub et al., 1975; Sperling and Bustin, 1975). These interactions are responsible for maintaining the histone core structure. It is therefore important to ask: Will histone–histone interactions occur in organisms evolutionarily distant from vertebrates and, if so, will the interaction pattern be the same as that found for calf thymus histones? We will show here that

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