Partial Reconstitution of Active Ribosomes and 50S Subunits†

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ABSTRACT: *Escherichia coli* ribosomes and their 50S subunits disassembled by LiCl treatment can be reconstituted into structurally completed but inactive particles. However, peptidyltransferase and polyphenylalanine synthesizing activity can be partly recovered by the addition of methanol to the reconstitution system. Furthermore, entirely active ribosomes and 50S subunits are reconstituted when methanol is present during the initial treatment with LiCl to disassemble the ribosomal components. The presence of methanol (10% v/v) during this treatment diminished the release of some proteins but does not affect the separation of the 5S RNA.

Reconstitution of active ribosome subunits of *Escherichia coli* from their resolved components has been achieved in the case of the 30S (Traub and Nomura, 1968) but not in the case of the 50S subunit. However, partial reconstitution of the 50S subunits from their protein-deficient derived particles (cores) and split proteins has been extensively used in studies of the ribosomal structure with fruitful results (Staehelin et al., 1969; Ballesta et al., 1971; Nierhaus and Montejo, 1973). Even in this case, however, the loss of more than 14 to 16 proteins makes the cores unable to reconstitute into active particles.

Treatment with 2*M* LiCl has been used to study the release of the 5S RNA from the 50S subunit (Marcot-Queiroz and Monier, 1967; Yu and Wittman, 1973a,b). This treatment releases about 20 proteins and the 5S RNA which, upon dialysis of the salt, can reconstitute inactive 50S subunits. According to Yu and Wittmann (1973a) it is precisely the loss of the 5S RNA that determines the inability of the resulting cores to reconstitute active particles, since the 5S RNA cannot be reintegrated into the ribosomal structure in the proper position.

Short-chain alcohols, specially methanol and ethanol, modify ribosomal structure by inducing conformational changes which affect ribosomal function (Monro and Marcker, 1967; Monro et al., 1969; Scolnick and Caskey, 1969; Hamel and Nakamoto, 1972; Ballesta and Vazquez, 1972a,b, 1973). We have observed that these alcohols facilitate the reconstitution of LiCl-treated ribosomes and the results obtained are presented in this paper.

Experimental Section

Materials

Ribosomes were prepared from *E. coli* D-10 by grinding with alumina and washing with buffers containing 1 *M* NH_4Cl as previously described (Ballesta and Vazquez, 1972b). Subunits were prepared by sucrose gradient centrifugation in a zonal rotor (Eikenberry et al., 1970). Both particles were stored in liquid nitrogen or unfrozen at −20° in buffers containing 50% glycerol. Labeled aminoacyl-tRNAs were obtained by charging commercial tRNA with[^{3}H]phenylalanine or [^{3}H]leucine (7.83 and 50.5 Ci/mmol, respectively; The Radiochemical Centre, Amersham, England). From the [^{3}H]Leu-tRNA the fragment C-A-C-C-A-Leu-Ac was obtained as described (Monro, 1971). [γ-^{32}P]GTP was prepared according to Glynn and Chappell (1964). Elongation factor G (EF G) was prepared following the method of Parmeggiani et al. (1971). [^{3}H]GDP (5 Ci/mmol) was purchased from The Radiochemical Centre, Amersham, England.

Methods

LiCl Treatment of Ribosomes. The particles were resuspended at a final concentration of either 2.5 or 1 mg/ml in a buffer containing the indicated LiCl concentration and either 2.5 m*M* Tris-HCl (pH 7.4) and 2.5 m*M* $MgCl_2$ in the former case or 10 m*M* Tris-HCl (pH 7.4) and 10 m*M* $MgCl_2$ in the latter. Methanol, when present, was added at 10% (v/v) final concentration. The samples were kept at 4° for 5 hr and then either centrifuged in a Spinco 65 rotor for 4 hr at 65,000 rpm to separate the core particles from the split proteins or dialyzed for 20 hr against buffer I (60 m*M* NH_4Cl, 10 m*M* Tris-HCl (pH 7.4), 10 m*M* $MgCl_2$, and 6 m*M* β-mercaptoethanol), with four changes of the buffer, for reconstitution experiments. After dialysis the reconstituted particles were again pelleted by centrifugation and resuspended in the dialysis buffer at 25 mg/ml. Methanol was added to the dialysis buffers when required during the LiCl treatment.

Release of the 5S RNA by LiCl treatment was determined by Sepharose 6B filtration. The reaction mixtures (650 μl, containing 635 μg of 50S subunits) were treated with 65 μl of 37% formaldehyde and left at room temperature for 20 min; they were then dialyzed for 12 hr against 10 m*M* Tris-HCl (pH 7.8), 10 m*M* $MgCl_2$, and 30 m*M* NH_4Cl, filtered through columns (17 × 0.6 cm) of Sepharose 6B, and eluted with the same buffer. The cores and other high molecular components of the mixture are excluded and the 5S RNA and split proteins are eluted as a small peak of ultraviolet (UV) absorbing material.

Reactivation of the Reconstituted Ribosomes. The reconstitution mixtures were incubated at 50° for the required time at 2 mg/ml final concentration in 20 m*M* Tris-HCl buffer (pH 7.8), containing 350 m*M* NH_4Cl, 20 m*M* $MgCl_2$, and 1 m*M* β-mercaptoethanol. Methanol (20%)

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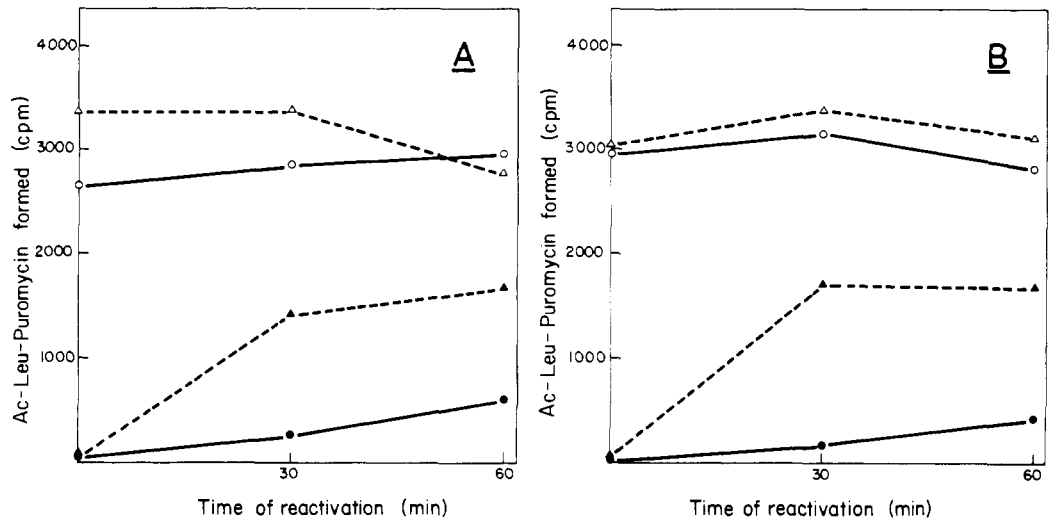

FIGURE 1: Reactivation of the peptidyltransferase activity of 2 *M* LiCl treated ribosomes: (A) ribosomes; (B) 50S ribosomal subunits. Control (open symbols) and treated particles (filled symbols) were incubated in the presence (dashed lines) or in the absence (continuous lines) of 20% methanol as described in the Experimental Section. Aliquots were taken at the indicated times and the peptidyltransferase activity was assayed in the fragment reaction. The reaction mixtures (0.1 ml) contained 1 mg/ml of ribosomal particles, 40 m*M* Tris-HCl (pH 7.4), 20 m*M* $MgCl_2$, 0.27 *M* KCl, 0.175 *M* NH_4Cl, 0.5 m*M* 2-mercaptoethanol, 1 m*M* puromycin, and 0.073 μM C-A-C-C-A-Leu-Ac. The reaction was initiated by addition of 50 μl of methanol.

was added when required. The samples were cooled at 0° after incubation and aliquots directly taken for activity assay. The samples were dialyzed against buffer I for 6 hr when the elimination of the alcohol was required.

All assays for ribosomal activities were according to methods previously described (Ballesta et al., 1974; Modolell and Vazquez, 1973). Two-dimensional electrophoresis of ribosomal proteins on polyacrylamide gels was carried out as described (Kaltschmidt and Wittman, 1970) with proteins extracted by acetic acid (Hardy et al., 1969).

Results

Activity of 2 M LiCl Ribosomes. We confirmed that either 70S ribosomes or 50S ribosomal subunits treated with 2 *M* LiCl and reconstituted by dialysis under the conditions described in the Experimental Section were inactive in polyphenylalanine synthesis, as well as in the fragment reaction assay for peptidyltransferase activity (Figure 1) (Reynier and Monier, 1968; Yu and Wittmann, 1973a). Incubation of these particles at 50° in the ionic conditions described by Maglott and Staehelin (1971) slightly reactivated the peptidyltransferase activity of the ribosome. However, marked stimulation was obtained with 20% methanol or ethanol present during the incubation. Under these conditions up to 60% of the control activity of 50S subunits was recovered (Figure 1).

Effect of Ribosomes and LiCl Concentration. The number of proteins released during the LiCl treatment depends on the salt and ribosome concentrations (Homann and Nierhaus, 1971). We studied the peptidyltransferase activity of 50S subunits treated with different LiCl concentrations, dialyzed, and subsequently incubated for reconstitution at 50° for 30 min (Table I). The results indicate, as expected, that the reconstitution was poorer when the treatment was more drastic, but even after treatment with 2 *M* LiCl and 4 *M* urea, which virtually releases all the proteins of the 50S subunit, significant reactivation of peptidyltransferase was obtained by incubation in the presence of methanol.

Effect of RNase Inhibitors on Reconstitution. A number of workers have described a nucleolytic activity on the 50S subunit responsible for the autodegradation of ribosomes (Szer, 1969; Staehelin et al., 1969; Ceri and Maeba, 1973). Indeed, this RNase activity acting on the ribosomal RNA during LiCl treatment and even during incubation for reconstitution might interfere with the latter. Since Ceri and Maeba (1973) stated that $HgCl_2$ and methanol inhibit the autodegradation of ribosomal RNA, we have studied the effect of the addition of these components during the LiCl treatment, and in the incubation for reconstitution, on the subsequent activity of the treated particles. $HgCl_2$ completely inactivated the LiCl-treated particles at concentrations which did not inhibit ribosome activity. Thus, $HgCl_2$ strongly interferes with reconstitution of the 50S subunits from their derived cores. However, the addition of 10% methanol during the LiCl treatment considerably improved the recovery of ribosome activity. Almost 100 and 80% of the peptidyltransferase activity of LiCl-treated 50S subunits and ribosomes, respectively, could be reconstituted by incubation at 50° for 30 min (Figure 2) plus 20% methanol in the standard conditions. Up to 50% of the peptidyltransferase activity was recovered in a similar system in the absence of methanol.

Especially interesting is the drastic effect of methanol on the phenylalanine polymerizing activity of LiCl-treated ri-

Table I: Reconstitution of the Peptidyltransferase Activity of LiCl-Treated Ribosomes.[a]

Treatment of Ribosomes	Reactivation at 50°	
	+ Methanol (% Control)	− Methanol (% Control)
2 *M* LiCl + 2.5 mg/ml of ribosomes	60	20
2 *M* LiCl + 1 mg/ml of ribosomes	51	18
4 *M* LiCl + 1 mg/ml of ribosomes	40	13
2 *M* LiCl + 4*M* urea + 2.5 mg/ml of ribosomes	30	3

[a] Conditions are as described in the Experimental Section. In the control assays with untreated ribosomes from 6 to 8 pmol of *N*-acetylleucylpuromycin was synthesized.

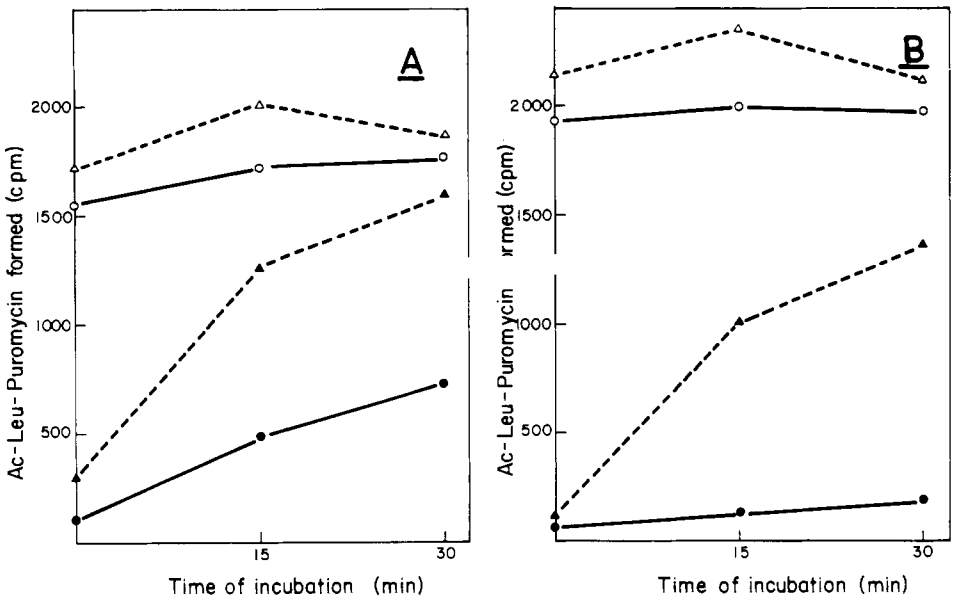

FIGURE 2: Reactivation of peptidyltransferase activity of ribosomes treated with LiCl in the presence of methanol: (A) ribosomes; (B) 50S ribosomal subunits. Controls (open symbols) and treated particles (filled symbols) were reactivated in the presence (dashed lines) or in the absence (continuous lines) of 20% methanol and tested for activity as indicated in Figure 1.

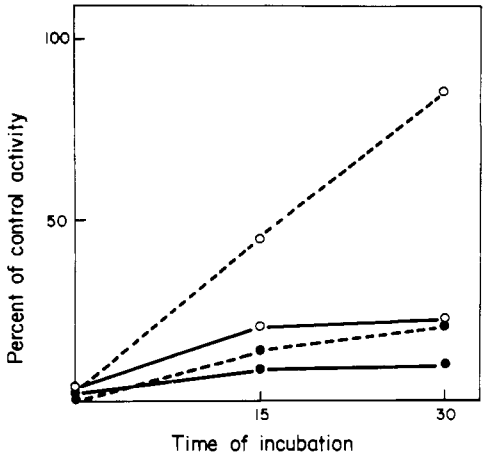

FIGURE 3: Peptidyltransferase activity and polyphenylalanine synthesis by LiCl-treated ribosomes. Treated ribosomes in the presence (open symbols) and in the absence (filled symbols) of 10% methanol were reactivated by incubation at 50° (with no ethanol) and directly tested for polyphenylalanine synthesis (dashed lines) and peptidyltransferase activity (continuous lines). Samples for polymerization assays (0.1 ml) contained 0.25 mg/ml of ribosomes, 60 m*M* Tris-HCl (pH 7.4), 20 m*M* $MgCl_2$, 60 m*M* KCl, 175 m*M* NH_4Cl, 5.5 m*M* 20mercaptoethanol, 0.5 m*M* GTP, 0.077 μM [^{3}H]phenylalanine-tRNA, 0.1 mg/ml of poly(U), and 10 μl of S-100 supernatant from centrifugation at 100,000*g*. Conditions for peptidyltransferase activity were as in Figure 1.

bosomes. 2 *M* LiCl–methanol treated ribosomes recovered almost 100% of their polyphenylalanine synthesizing activity upon incubation at 50°, whereas the same treatment was hardly effective in restoring activity of 2 *M* LiCl treated particles (Figure 3). The peptidyltransferase activity of the same samples, as measured by the fragment reaction assay, is also presented in Figure 3. It will be seen that particles that are very active in phenylalanine polymerization are comparatively less active in the fragment reaction assay. Incubation in the presence of methanol is required for maximal recovery of the peptidyltransferase activity as already noted.

EF G Dependent Activities of LiCl-Treated Ribosomes.

Table II: Reconstitution of EF G Dependent GTPase Activity of LiCl-Treated Ribosomes.[a]

Treatment of 70S Ribosomes	Molecules of GTP Hydrolyzed per Ribosome			
	Reconstitution − Methanol		Reconstitution + Methanol	
	No Incubation	Incubation at 50° for 30 min	No Incubation	Incubation at 50° for 30 min
Experiment 1[b]				
Control	72.3	74.9	71.9	73.0
LiCl	43.0	78.7	75.4	69.1
Experiment 2[c]				
Control	71.1	66.9	89.4	74.7
LiCl	67.4	80.0	107.9	79.4

[a] The tests for EF G dependent GTP hydrolysis were carried out at 30° for 5 min in 50-μl reaction mixtures containing 1 mg/ml of ribosomes, 25 m*M* Tris-HCl buffer (pH 7.4), 15 m*M* $MgCl_2$, 175 m*M* NH_4Cl, 0.5 m*M* 2-mercaptoethanol, 30 μM [γ-^{32}P] GTP, and about 0.01 mg/ml of EF G. [b] LiCl treatment of the ribosomes was carried out in the absence of methanol. [c] LiCl treatment of the ribosomes was carried out in the presence of 10% methanol.

As is shown in Table II the EF 2 dependent GTPase activity is more easily recovered by LiCl-treated particles than the other activities studies. When the LiCl treatment was carried out in the absence of methanol (experiment 1, Table II) the reconstituted particles showed 60% of the control activity even without incubation for reactivation. Furthermore, the recovery was 100% after 30-min incubation at 50° or simply by addition of 20% methanol without further incubation. When methanol was included during the LiCl treatment (experiment 2, Table II) GTPase activity was essentially unaffected. Similar results were obtained for the EF G dependent ribosomal binding of GDP stimulated fusidic acid (Table III). Particles reconstituted from 2 *M* LiCl–methanol treated ribosomes bind the same amount of GDP as the controls when incubated in the absence of methanol

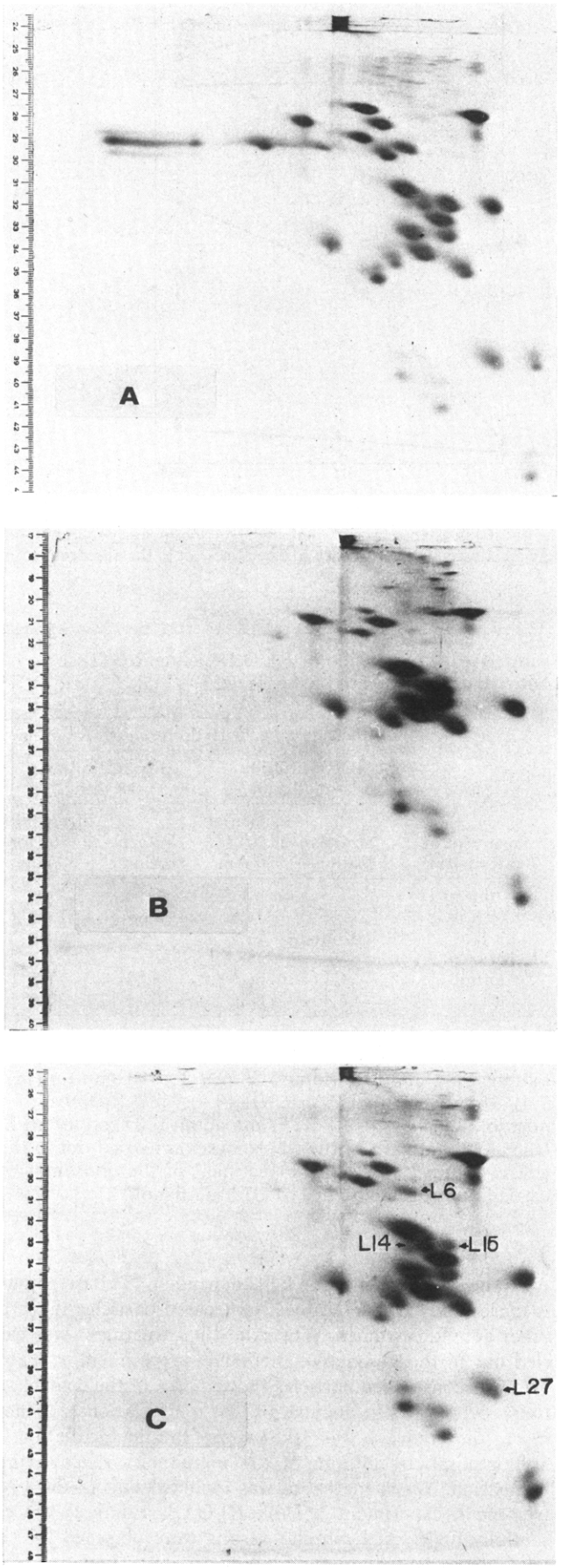

FIGURE 4: Two-dimensional gel electrophoresis of ribosomal proteins extracted from: (A) intact 50S subunits; (B) 2 *M* LiCl treated 50S subunits at 1 mg/ml; and (C) 2 *M* LiCl treated 50S subunits at 1 mg/ml in the presence of 10% methanol.

Table III: EF G Dependent Binding of GDP to Ribosomes in the Presence of Fusidic Acid.

Treatment of 70S Ribosomes	pmol of [^{3}H]GDP Bound			
	Reconstitution – Methanol		Reconstitution + Methanol	
	No Incubation	Incubation at 50° for 30 min	No Incubation	Incubation at 50° for 30 min
Control	0.93	1.29	0.96	0.84
LiCl	1.01	1.43	0.76	0.48

Table IV: Protein Composition of LiCl Core Particles.[a]

Protein	– Methanol	+ Methanol	Protein	– Methanol	+ Methanol
			L17	(+)	+
L1	(±)	(±)	L18	(±)	(+)
L2	+	+	L19	(+)	(+)
L3	(+)	+	L20	+	+
L4	(+)	+	L21	+	+
L5	(±)	(+)	L22	+	+
L6	–	(+)	L23	+	+
L7	–	–	L24	(+)	(+)
L8	–	–	L25	(±)	(±)
L9	(±)	(±)	L27	–	+
L10	–	–	L28	–	–
L11	–	–	L29	(+)	+
L12	–	–	L30	(+)	+
L13	+	+	L31	–	–
L14	(±)	(+)	L32	(±)	(+)
L15	–	(+)	L33	–	–
L16	–	–			

[a] LiCl treatment was carried out at 1 mg/ml of ribosomes. The ribosomal proteins loaded on the gels were extracted from the same amount of particles (3 mg). Two-dimensional electrophoresis in polyacrylamide was carried out following methods described. The patterns of spots obtained were compared with those of proteins separated from the same amount of control untreated 50S subunits. The intensity of each spot is indicated as follows: +, intensity of the spot similar to control 50S proteins; (+), intensity less than control; (±), present in traces; —, no spot detected.

during the reactivation incubation at 50°. The presence of methanol slightly inhibits the binding of the treated and control particles.

Two-Dimensional Polyacrylamide Gel Electrophoresis of Ribosomal Proteins. Proteins in core particles from 50S subunits, obtained by LiCl treatment, were separated by two-dimensional gel electrophoresis (Kaltschmidt and Wittmann, 1970) (Figure 4) and the intensity of the spots was estimated by comparison with the control proteins from the same amount of untreated subunits. The results are summarized in Table IV. The effect of the methanol on the release of proteins during the LiCl treatment seems rather specific and is limited to a few proteins. From the protein patterns of cores from 1 mg/ml of ribosomes, it may be seen that L6, L14, L15, L18, and L27 are present in significant amounts only in the case of the LiCl–methanol particles.

Release of 5S RNA from 50S Subunits after LiCl Treatment. Yu and Wittmann (1973a,b) stated that under the conditions they used for LiCl treatment (10 m*M* $MgCl_2$–10 m*M* Tris-HCl (pH 7.8), less than 1 mg/ml of ri-

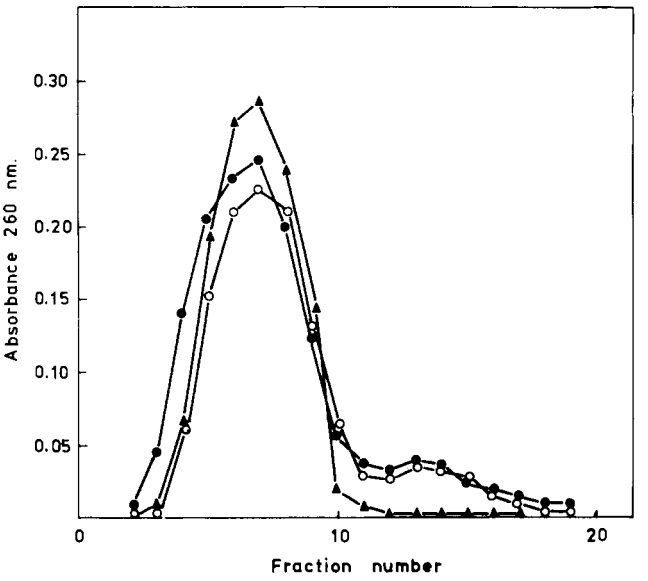

FIGURE 5: Filtration through Sepharose 6B of LiCl-treated 50S subunits fixed with formaldehyde: (▲) control 50S subunits; (●) LiCl 50S subunits treated in the presence of 10% methanol; (○) LiCl 50S subunits.

bosomes) about 85% of the 5S RNA is released from the particles. It was important to ascertain whether the presence of 10% methanol affects this release. For this purpose the cores were separated from released material with the use of Sepharose 6B columns. As shown in Figure 5, the amount of uv-absorbing material released by the LiCl treatment was about the same after LiCl treatment without or with methanol.

Discussion

Our results show that it is possible to reconstitute active ribosomes from 50S subunits which have been partially, or even totally, dissociated by LiCl treatment. For optimal recovery of ribosomal activity methanol must be present both during dissociation and reassociation.

Reconstitution of inactive 50S subunits from LiCl-derived particles has previously been reported; interestingly, the 5S RNA was more accessible to RNase in the reconstituted than in the control 50S subunit (Yu and Wittmann, 1973a). It would appear from this that incubation at 50° in the presence of methanol facilitates correct rearrangement of the ribosomal components. It would be of interest to know whether the 5S RNA is protected from RNase in the reactivated as well as in control 50S subunits. Most of the methanol effect during LiCl treatment may be due to its reported inhibition of the nuclease(s) on the 50S subunit (Ceri and Maeba, 1973). Moreover, the presence of methanol during the LiCl treatment prevents the release of some ribosomal proteins (L6, L14, L15, L18, and L27) without apparently affecting the release of 5S RNA. Thus, the inactivity of 50S subunits reconstituted in the absence of methanol may be due not only to incorrect positioning of the 5S RNA (Yu and Wittmann, 1973a) but also to the absence of certain proteins from the original cores. If so, methanol might facilitate the reconstitution process by preventing the release of these proteins. It is of interest to note that two of the proteins whose release is prevented by methanol (L15 and L27) appear to be located in or near the ribosomal binding site of the 3′-terminal end of aminoacyl-tRNA, i.e. at or near the peptidyltransferase center (Oen et al., 1972; Czernilofsky et al., 1973). There are indications that protein L15 may be part of the peptidyltransferase center (Ballesta et al., 1974). Protein L6 has been implicated in the binding of puromycin and chloramphenicol (Pongs et al., 1974), both inhibitors of peptide bond formation (Monro et al., 1969). Therefore, protein L6 might be also involved in the peptidyltransferase activity.

Yu and Wittmann (1973b) confirmed other reports (Horne and Erdmann, 1972; Gray et al., 1972) showing that proteins L5, L18, and L25 form a complex with 5S RNA and are interdependent for incorporation in the ribosome. Our results are consistent with these findings since the loss of 5S RNA in our case parallels the loss of these three proteins. However, the decrease of protein L18 release in the presence of methanol might be important for reconstitution of active 50S subunits.

It should be emphasized (see Figure 3) that particles with high Phe-tRNA polymerizing activity, and therefore having a functional peptidyltransferase, may be inactive in the fragment reaction. This could be due to the less stable binding of the C-A-C-C-A-Leu-Ac fragment than that of *N*-acetyl-Leu-tRNA to ribosomes (Monro et al., 1968) and accentuation of this difference in the LiCl-treated particles. These results point to the danger of using the fragment reaction as an exclusive for peptidyltransferase activity, especially with ribosomal cores or reconstituted particles. We have in fact observed (unpublished results) that some LiCl ribosomes cores, inactive when tested with C-A-C-C-A-Leu-Ac as substrate in the fragment reaction, were active when *N*-acetyl-Leu-tRNA was used instead.

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Isolation and Partial Characterization of Anglerfish Proglucagon†

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ABSTRACT: Evidence is presented that proglucagon from anglerfish islets is a single chain polypeptide with 78 amino acid residues and that the glucagon portion of it is liberated after tryptic cleavage. The most striking characteristic in the conversion of the anglerfish proglucagon to glucagon is that the cleaved peptide bonds display enormous sensitivity toward trypsin. Thus, conversion of the prohormone to glucagon occurs very rapidly within 3–10 min with a 1:500–1:1000 molar ratio of enzyme to substrate. Further, tryptic cleavage of the anglerfish glucagon requires higher concentrations of trypsin (molar ratio 1:25 enzyme to substrate) and longer incubation time. The behavior of proglucagon and glucagon toward trypsin shows striking similarities with the tryptic conversion of anglerfish proinsulin to insulin.

Several published investigations have dealt with the problem of glucagon biosynthesis. These studies clearly indicated that glucagon is synthesized via a precursor (Rigopoulou et al., 1970; Noe and Bauer, 1971, 1973; Tung and Zerega, 1971; Hellestrom et al., 1972). In a previous brief communication we have described the isolation of anglerfish proglucagon (Trakatellis et al., 1973) and Tager and Steiner (1973) provided the primary structure of the amino terminal 37 residues of bovine/porcine proglucagon. We describe here the methods of isolation of a polypeptide consisting of 78 amino acid residues and which upon trypsin treatment yields glucagon.

Materials and Methods

Extraction of Anglerfish Islets. Anglerfish islets, frozen immediately after excision, were supplied by the New England Biological Associates, Narragansett, R.I. The islets were cleaned from the surrounding tissues and processed in batches of 100–200 islets. In a typical experiment, a suspension of 100 islets in 300 ml of ice-cold 10% trichloroacetic acid was homogenized (Sorvall Omni-Mixer set at maximum speed) for 2 min, and the mixture was centrifuged. The solid residue was suspended in 40 ml of 0.18 *N* HCl in 75% ethanol, allowed to stand for 1 hr, recentrifuged, and the clear supernatant fraction retained. The solid residue was reextracted twice more with 30-ml portions of acidified ethanol as described. All supernatant fractions were combined and concentrated in a rotary evporator to about 20 ml. The concentrate was adjusted to pH 4 with 1 *M* trisodium citrate and extracted with 100 ml of methylene chloride to remove lipids. The aqueous phase was made 10% in trichloroacetic acid and refrigerated overnight. The resulting precipitate was collected by centrifugation, washed successively three times with 40 ml of acetone–ether (1:1, v/v) and 40 ml of ether, and finally dried in vacuo.

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