Davies, G. E., and Stark, G. R. (1970), *Proc. Nat. Acad. Sci. U. S. 66*, 651.
Eikenberry, E. F., Bickle, T. A., Traut, R. R., and Price, C. A. (1970), *Eur. J. Biochem. 12*, 113.
Fakunding, J. L., and Hershey, J. W. B. (1973), *J. Biol. Chem.* (in press).
Howard, G. A., and Traut, R. R. (1973), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 29*, 177.
Kaltschmidt, E., and Wittmann, H. G. (1970), *Anal. Biochem. 36*, 401.
Kurland, C. G., Green, M., Schaup, H. W., Donner, D., Lutter, L. C., and Birge, E. H. (1972), *in* Functional Units in Protein Biosynthesis, Cox, R. A., and Hadjiolov, A. H., Ed., New York, N. Y., Academic, p 75.
Laemmli, V. K. (1970), *Nature (London) 227*, 680.
Lutter, L. C., Zeichhardt, H., and Kurland, C. G. (1972), *Mol. Gen. Genet., 119*, 357.
Mizushima, S., and Nomura, M. (1970), *Nature (London) 226*, 1214.
Perham, R. N., and Thomas, J. O. (1971), *J. Mol. Biol. 62*, 415.
Traut, R. R. (1966), *J. Mol. Biol., 21*, 571.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 224*, 4406.
Wittmann, H. G., and Stöffler, G. (1972), *in* The Mechanism of Protein Synthesis and Its Regulation, Bosch, L., Ed., Amsterdam, North Holland Publishing Co., p 258.
Wold, F. (1972), *Methods Enzymol. 25*, 623.

Rate-Determining Step in the Reconstitution of *Escherichia coli* 30S Ribosomal Subunits†

William A. Held and Masayasu Nomura*

ABSTRACT: Previous studies on the mechanism of reconstitution of 30S subunits of *Escherichia coli* using 16S RNA and unfractionated 30S proteins indicated that there is a rate-limiting, unimolecular reaction which has a high activation energy. In the present study, the 30S proteins required for this temperature-dependent, rate-limiting step have been examined. Reconstitution was done using 16S RNA and a mixture of 21 purified proteins. The proteins were divided into two parts in various ways, and reconstitution was performed in two steps. In the first step, 16S RNA was incubated with a set of proteins at a high temperature (40°) for 30 min. In the second step the remaining proteins were added and the mixture incubated at a low temperature (30°) for 10 min. It was found that the 30S ribosomal proteins S4(P4a), S8(P4b), S7(P5), S16(P9a), and S19(P13) are required in the first heat step to form active 30S particles. S15(P10b) and S17(P9b) are somewhat less essential in this respect, and S11(P7), S18(P12), S9(P8), S5(P4), and S12(P10) are still less essential but must be present during the first heat step for full functional activity. Some 30S proteins which are found in isolated ribosomal intermediate (RI) particles [S20(P14), S13(P10a), and S6(P3b,c)] do not appear to be required. Some proteins which are required for the temperature-dependent step [S5(P4), S12(P10), and possibly S19(P13)] appear to bind very weakly to RI particles at low temperatures; the isolated RI particles are deficient in these proteins. The temperature-dependent step probably involves a substantial conformational change of the RI particles, as indicated by an increase in the sedimentation coefficient of the particles after heating.

Ribosomal subunits (30S) from *Escherichia coli* can be reconstituted from their dissociated molecular components (Traub and Nomura, 1968). *In vitro* reconstitution, although differing in some details from *in vivo* assembly, may provide information relevant to the latter.

Previous studies on the mechanism of the reconstitution of 30S subunits were done using 16S RNA and a mixture of unfractionated total 30S ribosomal proteins (TP30)[1] (Traub and Nomura, 1969a; Traub and Nomura, 1969b). Kinetic studies on the assembly of 30S subunits showed that the rate-limiting unimolecular reaction has a high activation energy (Traub and Nomura, 1969a). When the components of 30S subunits (16S RNA and TP30) are mixed together under the standard reconstitution conditions and incubated at 0° instead of at the higher temperature (usually 40°) required for formation of active subunits, particles are produced which sediment at about 21–22 S in low Mg^{2+} buffer, are deficient in several proteins, and have no functional activity. These particles (called "21S particles" in this paper) are activated when heated at 40° to form particles which are capable of binding the missing proteins at 0° to form functionally active 30S particles (Traub and Nomura, 1969a). The temperature dependence of this activation step suggests that the isolated 21S particles are similar to or identical with the intermediate which undergoes the rate-limiting unimolecular reaction. The following reaction scheme has been proposed.

$$\text{16S RNA} \xrightarrow{+\text{ RI proteins}} \text{RI particles} \xrightarrow{\text{heating}} \text{RI* particles} \xrightarrow{+\text{ S proteins}} \text{30S ribosomes} \quad (1)$$

† From the Institute for Enzyme Research and Departments of Biochemistry and Genetics, University of Wisconsin, Madison, Wisconsin 53706. *Received March 5, 1973*. This is paper No. 1634 from the Laboratory of Genetics and paper XX in the series Structure and Function of Bacterial Ribosomes. Paper XIX in this series is Higo *et al.* (1973). This work was supported in part by the College of Agriculture and Life Sciences, University of Wisconsin, and by grants from the National Institute of General Medical Sciences (GM-15422) and the National Science Foundation (GB-31086X1).

[1] Abbreviations used are: TP30, unfractionated total 30S ribosomal proteins; RI, reconstitution intermediate; RI*, activated reconstitution intermediate; S proteins, 30S ribosomal proteins which do not bind to RI, but bind to RI* during assembly of 30S ribosomal subunits.

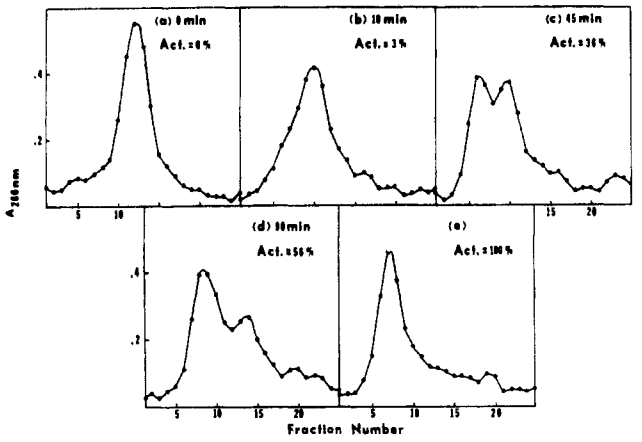

FIGURE 1: Sucrose gradient sedimentation analysis of reconstitution at 30°. 16S RNA and TP30 were mixed in reconstitution buffer and incubated at 30°. Aliquots were taken at the indicated times and cooled in an ice bath. A small sample was taken for assay of activity in poly(U)-directed [^{14}C]phenylalanine incorporation, and the remaining material placed on a 5–20% sucrose gradient in buffer II. Centrifugation was in a Spinco SW40 rotor at 25,000 rpm for 18 hr. Fractions were collected from the bottom of the tube and analyzed for absorbance at 260 nm; (a) 0 min; (b) 10 min, 30°; (c) 45 min, 30°; (d) 90 min, 30°; (e) 60 min, 40°.

"RI proteins" are defined as proteins contained in the proposed intermediate particle ("RI" particle). The rate-determining step is the conversion of RI to RI* (activated RI particles) and involves a structural rearrangement which is strongly temperature dependent. The RI* particles then react with the missing proteins (called "S proteins" in the scheme), to form 30S subunits (Traub and Nomura, 1969a).

In previous studies, the reconstitution of functional 30S particles from isolated 21S particles and the missing proteins was inefficient; this inefficiency was thought to be due to the heterogeneity of the isolated 21S particles (Traub and Nomura, 1969a). It is possible that only a small fraction of the isolated 21S particles represent the actual intermediate particle (RI particle), and that the rest of the particles differ from the RI particle due to instability and loss of some protein components during isolation steps. It is also possible that the presence of some proteins in *isolated* 21S particles may be fortuitous and irrelevant to the rate-determining reaction step in 30S ribosomal assembly.

In this paper, we report the results of further investigations of the temperature-dependent rate-determining step which have led to identification of the protein components which are necessary for, and participate in this reaction step.

Materials and Methods

The following buffers were used: buffer I, 10^{-2} M Tris-HCl (pH 7.4 at 24°)–10^{-2} M $MgCl_2$–3×10^{-2} M NH_4Cl–6×10^{-3} M 2-mercaptoethanol; buffer II, buffer I, except $MgCl_2$ is 3×10^{-4} M; buffer III, 3×10^{-2} M Tris-HCl (pH 7.4 at 24°)–2×10^{-2} M $MgCl_2$–6×10^{-3} M 2-mercaptoethanol; buffer IV, buffer III containing 0.5 M KCl; "reconstitution buffer," buffer III containing 0.33 M KCl.

Crude cell extracts from *E. coli* strain Q13, which lacks RNase I activity (*cf.* Gesteland, 1966), were prepared by grinding 100 g of packed cells with 200 g of alumina. The broken cells were suspended in buffer I containing RNase-free DNase (Worthington) at a concentration of 2 μg/ml. Alumina and cell debris were removed by centrifugation at 15,000 rpm for 20 min in a Sorvall SS34 rotor. Ribosomal subunits were isolated and purified from the crude cell extracts (Traub *et al.*, 1971).

Purification of individual 30S ribosomal proteins has been described (Nomura *et al.*, 1969; Held *et al.*, 1973). The nomenclature used for identifying the 30S ribosomal proteins is that of Wittmann *et al.* (1971). The nomenclature used in our previous publications (*cf.* Nomura *et al.*, 1969) is indicated in parentheses. Protein concentration was measured by the method of Lowry *et al.* (1951), using crystalline bovine serum albumin as a standard.

Reconstitution of 30S subunits from 16S RNA and purified 30S ribosomal proteins was done as follows. Phenol-extracted 16S RNA (Traub *et al.*, 1971) was dialyzed against buffer III. Purified 30S proteins were dialyzed against buffer IV. 16S RNA in buffer III and a mixture of purified 30S proteins in buffer IV were mixed in a volume ratio of 1:2 resulting in a final KCl concentration of 0.33 M. The final concentration of 16S RNA in the reconstitution mixture was between 5 and 10 A_{260} units/ml in a final volume of about 100 μl; 1.7 molar equiv of each protein [calculated on the basis of individual molecular weight as determined by Dzionara *et al.* (1970) and the molecular weight of 16S RNA (Stanley and Bock, 1965)] was added. The reconstitution mixture was then incubated for the appropriate time, usually 30 min at 40° (or as indicated in the text). A more detailed description of reconstitution methods will be published in another paper (Held *et al.*, 1973).

Reconstituted 30S particles were assayed directly (without isolation) for their activity in poly(U)-directed [^{14}C]phenylalanine incorporation (Traub *et al.*, 1971). The assay was modified slightly so that the amount of reconstitution during the assay could be minimized. This was accomplished by changing the specific activity of the [^{14}C]phenylalanine from 10 μCi/μmol to 50 μCi/μmol and reducing the incubation time and temperature to 10 min at 30°. The degree of reconstitution during this assay was less than 10% of that obtained with standard reconstitution conditions.

Two-dimensional polyacrylamide gel electrophoresis was done according to the method of Kaltschmidt and Wittmann (1970). Forty A_{260} units of reconstituted particles were purified by sucrose density gradient sedimentation. Centrifugation was through 5–20% sucrose gradients in reconstitution buffer for 6.5 hr at 27,000 rpm in a Spinco SW27 rotor at 3–4°. The peak fractions were pooled and the particles were pelleted in a Spinco 50 rotor at 50,000 rpm for 14 hr. Recovery was 60–80% of the starting material (as A_{260}). The particles were suspended in buffer I and digested with a mixture of EDTA, 1.5×10^{-2} M, T_1 ribonuclease (Worthington, 1.5 μg/ml), and pancreatic ribonuclease (Worthington, 3.0 μg/ml) at 37° for 30 min. The precipitated ribosomal proteins were dissolved by dialyzing against the sample gel for the first dimension.

Results

Accumulation of 21S Particles during in Vitro Reconstitution at 30°. The sedimentation properties of particles formed during reconstitution were analyzed by sucrose density gradient sedimentation and compared with the increase in functional activity (Figure 1). In order to slow the reconstitution reaction to a measurable convenient rate, the reconstitution was done at 30°. The sample kept at 0° (Figure 1a) showed fairly homogeneous particles sedimenting at 21–22 S (in buffer II). These particles correspond to the "RI particles" studied previously (Traub and Nomura, 1969a; in this paper these particles are called 21S particles, as stated above, to distinguish them from the actual intermediate particles pro-

16S RNA + a mixture of purified prospective RI proteins
↓ Incubation 1: 40° (or temperatures as indicated) for 30 min; [KCl] = 0.33 M
RI* (cool, add remaining proteins and adjust [KCl] to 0.33 M)
↓ Incubation 2: 30° for 10 min
30S
↓ Incubation 3 (assay; poly(U)-directed [^{14}C]phenylalanine incorporation): 30° for 10 min
activity

FIGURE 2: Experimental procedure to identify proteins required for for RI* particle formation.

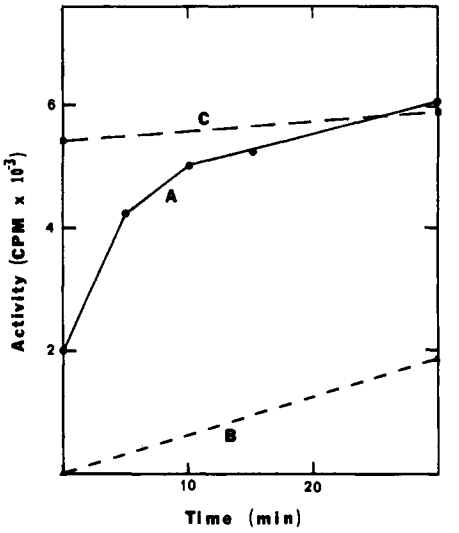

FIGURE 3: Kinetics of conversion of RI* to 30S ribosomal particles at 30°. 16S RNA was incubated with proteins required for RI* formation (groups A + B + C, Table I) at 40° for 30 min. The remaining proteins were added in the cold. The mixture was then incubated at 30° (curve A). Aliquots (0.2 A_{260} unit) were removed at the indicated times, cooled in ice, and later assayed for activity in poly(U)-directed [^{14}C]phenylalanine incorporation. The control particles obtained by incubation at 0° instead of 40° in the first incubation gave 0 cpm at 0 time in the second incubation and 1850 cpm after 30 min at 30° (curve B). Another control particle which had all 30S proteins present during the heating at 40° gave 5405 cpm at 0 time and 5847 cpm after 30 min at 30° (curve C).

posed in eq 1). After 10-min incubation at 30° (Figure 1b), a shoulder in the 30S region was evident. At 45 and 90 min, Figures 1c and 1d, where the degree of reconstitution is 36 and 56%, respectively, two major peaks were observed: one sedimenting at about 21–22 S and another sedimenting at about 30 S. The amount of 30S material increases with the amount of activity. The results indicate that conversion of the 21S particles to 30S limits the overall rate of reaction. However, as mentioned above, isolation and analysis of the protein composition of the 21S particles may not necessarily reveal the protein components of the actual intermediate undergoing the rate-determining step. Therefore, we decided to use a different approach to this question.

Identification of Proteins Required for RI Formation.* As described previously, the reconstitution of 30S subunits can be accomplished using a mixture of 21 purified proteins and 16S RNA (Held *et al.*, 1973; Higo *et al.*, 1973). The kinetics of reconstitution in this system are similar to that in the previous system using unfractionated total 30S ribosomal proteins (Held *et al.*, 1973). An experimental approach utilizing purified individual proteins, was designed to identify those proteins which are required for the presumed rate-limiting unimolecular reaction, RI → RI* (Figure 2).

The 30S ribosomal proteins were divided into two parts in various ways, and reconstitution was performed in two steps. In the first step, 16S RNA was incubated in reconstitution buffer with a chosen set of proteins (prospective RI proteins) at 40° for 30 min to form an inactive intermediate particle (possible RI* particle). The second step was to add the remaining proteins and incubate at 30° for 10 min in reconstitution buffer. Note that when all the 30S ribosomal proteins were included in the first step, reconstitution of 30S subunits was high (Table I, experiment a, tube no. 1). On the other hand, if no protein is included in the first step, and all the proteins are added in the second step, reconstitution (at 30° for 10 min) is very low (about 10% or less relative to the degree attained at 40° for 30 min; *cf.* Held *et al.*, 1973, and Figure 3, curve B).

If all proteins required for the presumed temperature-dependent rearrangement of the intermediate (*i.e.*, RI* formation) were present during the incubation at 40° (step 1), then the particles assayed at step 3 would be as active in poly(U)-directed [^{14}C]phenylalanine incorporation as a control which had all 30S proteins present during the first heating step. If, however, an essential protein was omitted during the heat step but added during the low temperature incubation (step 2), then the activity of the particles should be significantly less than the control with all the proteins present during step 1. Thus, those proteins which must be present during the high temperature incubation (step 1) are the ones which are required for the formation of RI* particles.

The second incubation was done at 30° for 10 min, rather than 0° for several hours used in previous studies (Traub and Nomura, 1969a). During initial experiments, a combination of prospective "RI proteins" was added to 16S RNA, heated at 40° for 30 min, and then cooled to 0°. The remaining proteins were added and the samples incubated 10–60 min at 0°. These particles had only 30–40% activity in the best cases (Figure 3, curve A, time 0) when compared to controls which had all proteins present during the heat step (Figure 3, curve C, time 0). Subsequently, we found that incubation at 30° for a short time (10 min) greatly stimulates the activity of these particles (Figure 3, curve A), whereas particles which had been incubated at 0° during the first incubation show only a slow formation of active particles (Figure 3, curve B). Therefore, the second incubation was done at 30° for 10 min as shown in Figure 2. Thus, we now operationally define RI* particles as particles which do not have activity, but which would become fully active 30S particles upon incubation with the missing proteins at 30° for 10 min.

It is reasonable to expect that proteins found in the isolated 21S particle (see above) would be involved in the heat-dependent formation of RI* particles. However, as discussed above, it appeared to be quite possible that proteins found in very small amounts in 21S particles might also be involved. Therefore, during preliminary experiments we decided to include all proteins which might be possibly involved in RI* formation and attempted to determine the required proteins by omitting unnecessary proteins. The data from many preliminary experiments with various combinations of proteins are not shown. Our final conclusion is that proteins S4(P4a), S8(P4b), S16(P9a), S7(P5), S19(P13), S15(P10b), S17(P9b),

TABLE I: Reconstitution of 30S Ribosomal Particles from RNA and Purified Proteins by the "Two-Step" Incubation Method.[a]

Expt	Tube No.	Proteins Present during Incubation 1	Temp (°C) (Incubn 1)	Proteins Added during Incubation 2	Activity	
					cpm	%
a	1	A + B + C + D + E (complete)	40	0	4797	100.0
	2	A + B + C + D + E (complete)	0	0	562	11.7
	3	A + B	40	C + D + E	1962	40.8
	4	A + B + C	40	D + E	5042	105.2
	5	A + B + D	40	C + E	2548	53.1
	6	A + B + C + D	40	E	5024	104.9
		(Control 30S)		0	5152	
b	1	A + B + C + D + E (complete)	0	0	212	5.4
	2	A + B + C + D + E (complete)	30	0	1340	34.2
	3	A + B + C + D + E (complete)	40	0	3920	100.0
	4	A + B + C	0	D + E	286	7.3
	5	A + B + C	30	D + E	1520	38.8
	6	A + B + C	40	D + E	2920	74.5
	7	A + B + C	40	0	62	1.6
		(Control 30S)		0	5159	

[a] Protein groups are: A, S4(P4a), S8(P4b), S7(P5), and S16(P9a); B, S11(P7), S18(P12), S15(P10b), and S19(P13); C, S5(P4), S9(P8), S17(P9b), S12(P10), and S20(P14); D, S2(P2), S6(P3b,c), and S10(P6); E, S1(P1), S3(P3), S13(P10a), S14(P11), and S21-(P15); 0, no proteins added, but incubation was done according to the procedure shown in Figure 2. Reconstitutions were done as shown in Figure 2 and as described under Materials and Methods; 0.24 A_{260} unit of reconstituted particles was assayed for activity in poly(U)-directed [^{14}C]phenylalanine incorporation.

S11(P7), S18(P12), S9(P8), S5(P4), and S12(P10) are required for the formation of RI* particles. If the mixture of these proteins + S20(P14) (A + B + C in Table I) is incubated with 16S RNA at 40° for 30 min, particles are produced which have no significant activity (Table I, experiment b, tube no. 7), but are able to form active particles comparable to the control standard reconstituted particles upon subsequent incubation at low temperature (30°, 10 min) with the remaining proteins (Table I, experiment a, tube no. 4; experiment b, tube no. 6).

Tables I, II, and III show the data which support our identification of proteins required for the formation of RI* particles. In the experiments shown in Table II, a mixture of the above proteins (A + B + C in Table I) was used as a basal protein mixture. It is clear that, if either S4(P4a), S8(P4b), S16(P9a), S7(P5), or S19(P13) is omitted from the basal protein mixture during the heat step at 40° (but is added subsequent to the heat step), very few active ribosomes are formed. Thus, these proteins are essential for the formation of RI* particles. S15(P10b) and S17(P9b) are somewhat less essential, and S11(P7), S18(P12), S9(P8), S5(P4), and S12(P10) are still less essential but necessary for full activity. S20(P14) does not appear to have an important role in RI* formation even though it binds directly to 16S RNA (Mizushima and Nomura, 1970; Schaup *et al.*, 1970).

In the experiments shown in Table III, each of the additional proteins was added to the above basal protein mixture during the heat step. No significant additional stimulation was observed except a possible slight stimulation by the presence of S1(P1). Both S13(P10a) and S6(P13b,c) are not required for RI* formation (Table III). It was previously observed that S13(P10a) binds, to some extent, directly to 16S RNA under reconstitution conditions (Zimmerman *et al.*, 1972; Mizushima and Nomura, 1970), although others have not observed this binding (Schaup *et al.*, 1971). Thus, three proteins, S20(P14), S13(P10a), and S6(P3b,c), are present in isolated 21S particles (see below), but are not required for RI* formation. Table IV summarizes our conclusions regarding the requirement of proteins for the formation of RI* particles.

Temperature Dependence of RI Formation.* In the experiments described above, the first incubation was done at 40° to form intermediate RI* particles from "16S" RNA and the protein mixture A + B + C. As expected from the previous work (Traub and Nomura, 1969a), the formation of RI* particles is temperature dependent. If the first incubation was done at 0°, very few active 30S particles are formed after the second incubation with the remaining proteins [Table I, experiment b, tube no. 4). Preincubation of the proteins (A + B + C) with 16S RNA at 30° rather than 40° for 30 min, and subsequent mixing with the remaining proteins, produces a significant but low amount of functional 30S particles which is about the same as when one incubates all the proteins at 30° for 30 min (Table I, experiment b, tubes no. 2 and 5).

Kinetics of RI Formation.* If the formation of RI* (as defined in this paper) is the rate-limiting step during reconstitution, then the rate of formation of RI* should be identical with the overall reaction rate. Since RI* particles are not functionally active, the formation of RI* could not be assayed directly. However, the kinetics of RI* formation could be determined by using the two-step reconstitution method outlined in Figure 2. RI proteins were incubated with 16S RNA at 40° in reconstitution buffer (step 1, Figure 2). [As "RI proteins," the proteins in the group A + B + C (*cf.* Table I) were used (Figure 4b); alternatively, A + B + C + S6-(P3b,c) + S13(P10a) were used (Figure 4a). For further discussion, see below.] Aliquots were removed at the indicated time (Figure 4, curve A) and the remaining proteins were added and incubated at 30° for 10 min (step 2, Figure 2). The activity of the reconstituted particles was then assayed. The control reconstitution (Figure 4, curve B) was done in a similar

TABLE II: Reconstitution of 30S Particles from RNA and Purified Proteins by the "Two-Step" Incubation Method; Effect of Omission of Single Proteins from the Standard Protein Mixture during the First Step.[a]

Proteins Present during Incubation 1 (40°)	% Activity		
	Expt 1	Expt 2	Av
A + B + C + D + E (complete)	111.0	140.9	126.0
A + B + C	100.0	100.0	100.0
A + B + C − S4	26.7	12.6	19.7
A + B + C − S8	33.1	20.7	26.9
A + B + C − S16	30.4	23.3	26.9
A + B + C − S7	19.5	14.6	17.1
A + B + C + D + E (complete)	114.1	126.8	120.5
A + B + C	100.0	100.0	100.0
A + B + C − S11	69.4	55.2	62.3
A + B + C − S18	68.7	77.0	72.9
A + B + C − S15	58.8	32.1	45.5
A + B + C − S19	25.9	28.3	27.1
A + B + C + D + E (complete)	100.8	115.8	108.3
A + B + C	100.0	100.0	100.0
A + B + C − S17	51.6	50.7	51.2
A + B + C − S20	90.8	89.5	90.2
A + B + C − S9	63.2	58.4	60.8
A + B + C − S5	65.4	63.0	64.2
A + B + C − S12	75.5	57.5	66.5

[a] Reconstitutions were done as shown in Figure 2 and as described under Materials and Methods. Individual proteins were omitted from the basal protein mixture (A + B + C) during the 40° heat step (column 1) but were added with the remaining proteins (D + E) during the second incubation at 30°; 0.2 A_{260} unit of reconstituted particles was assayed for activity in poly(U)-directed [^{14}C]phenylalanine incorporation. The activity of particles reconstituted with all proteins present during the incubation at 40° (A + B + C + D + E) ranged from 54 to 100% of 30S controls in the various experiments. The composition of protein mixtures A, B, C, D, and E is given in Table I.

TABLE III: Effect of Addition of Single Proteins to Protein Mixture during the First Incubation Step.[a]

Proteins Present during Incubation 1 (40°)	% Activity			
	Expt 1	Expt 2	Expt 3	Av
A + B + C + D + E (complete)	129.5	123.5	156.1	136.4
A + B + C	100.0	100.0	100.0	100.0
A + B + C + S6	100.9	95.1	91.4	95.8
A + B + C + S10	114.1	95.6	105.7	105.1
A + B + C + S2	117.7	83.9	96.6	99.4
A + B + C + D + E (complete)	129.2	140.7	151.4	140.4
A + B + C	100.0	100.0	100.0	100.0
A + B + C + S13	104.2		94.5	99.4
A + B + C + S1	123.4	125.0	110.7	119.7
A + B + C + S14	96.6	124.9	97.7	106.4
A + B + C + S3	88.1	114.5	93.8	98.8
A + B + C + S21	97.8	125.7	89.5	104.3

[a] Reconstitutions were done as shown in Figure 2 and as described under Materials and Methods. Individual proteins were added to the basal protein mixture (A + B + C) during the first incubation step at 40° as indicated in the first column. The remaining proteins (D + E minus the protein which was added during the 40° incubation) were added during the second incubation at 30°; 0.2 A_{260} unit of reconstituted particles was measured for activity in poly(U)-directed [^{14}C]-phenylalanine incorporation. The activity of particles reconstituted with all proteins present during the incubation at 40° (A + B + C + D + E) ranged from 50 to 72% of 30S controls in the various experiments. The composition of protein mixtures A–E is given in Table I.

way except that all proteins were present during the 40° incubation. The particles obtained after the 40° incubation were then incubated at 30° for 10 min so that the total incubation period would be the same as during the two-step reconstitution. Thus, curve B in Figure 4a and b shows the kinetics of reconstitution when all 30S proteins are present during the high temperature incubation (40°). Curve A reflects the kinetics of RI* formation at 40° in the presence of the prospective RI proteins A + B + C + S6(P3b,c) + S13(P10a) in Figure 4a and A + B + C in Figure 4b. The results indicate the kinetics of RI* formation are similar to the overall reconstitution kinetics (compare curves A and B) and that S6(P3b,c) and S13(P10a) do not appear to influence the rate of formation of RI* (compare curve A in Figure 4a and b). These results are consistent with the conclusion that the formation of RI* is the rate-determining step during reconstitution.

Properties of the Intermediate Particles Formed with a Mixture of Purified Proteins. As mentioned in the introductory statement, incubation of 16S RNA and TP30 at 0° produces particles which sediment at 21–22 S. The isolated 21S particles were previously analyzed with respect to their protein composition (Figure 5c and also Traub and Nomura, 1969a; Kaltschmidt, Erdmann, and Nomura, unpublished experiments, cited in Nashimoto *et al.*, 1971). The proteins found are indicated in Table IV. They are somewhat different from those required for the formation of RI* particles in the above experiments.

As mentioned previously, some proteins necessary for conversion of RI to RI* in the proposed scheme may be very weakly bound to the RI and partially lost during isolation of RI particles. Therefore, we analyzed the protein composition of particles formed from 16S RNA and purified proteins required for RI* formation [A + B + C, see Table I, and, in addition, S6(P3b,c) and S13(P10a)], with and without heating at 40°, and compared it with 21S particles formed from 16S RNA and TP30 at 0°. S6(P3b,c) and S13(P10a), as well as S20(P14), were included since they are found in isolated 21S particles.

In the actual experiments, we have found that the incubation of 16S RNA with a mixture of the proteins [A + B + C + S6(P3b,c) + S13(P10a)], or with TP30, in the reconstitution buffer at 0° forms some aggregates when examined by sedimentation analysis in the same buffer. However, incubation at 30° for 5 min reduced such aggregation. Thus, the particles purified and analyzed are (a) those incubated at 30° for 5 min (RI) and (b) those incubated at 40° for 30 min (RI*). The purification was done by sedimentation on 5–20% sucrose density gradients in reconstitution buffer. The peak

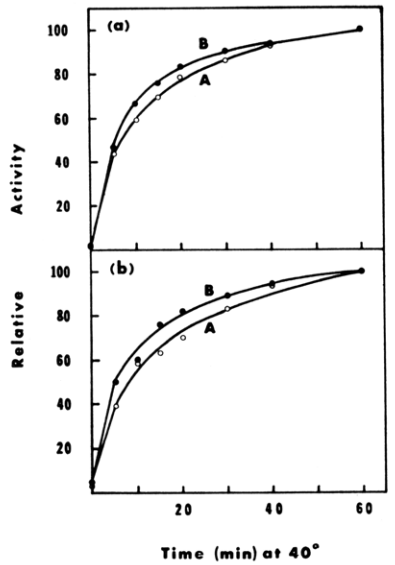

FIGURE 4: Kinetics of RI* formation. 16S RNA was incubated at 40° with a mixture of all the purified 30S proteins (curve B in both a and b or with the proteins required for the formation of RI* (curve A in both a and b). In Figure 4a, the proteins used for the formation of RI* were groups A + B + C + S6(P3b,c) + S13(P10a); in Figure 4b, only groups A + B + C were used (see Table I). Thus, curve B is is identical in both a and b, and curve A in Figure 4a differs from 4b only in that S6(P3b,c) and S13(P10a) were present during the 40° incubation. At the indicated time, aliquots were removed and the remaining proteins were added in the cold (curve A only) and incubated at 30° for 10 min (curves A and B) to activate the particles (see Figure 3). Aliquots (0.2 A_{260} unit) were then assayed for activity in poly(U)-directed [^{14}C]phenylalanine incorporation. The activity was normalized to 100% at 60 min: (a) for curve A (○), 100% = 5202 cpm; for curve B (●), 100% = 6182 cpm; (b) curve A (○), 100% = 4738 cpm; for curve B (●), 100% = 5714 cpm.

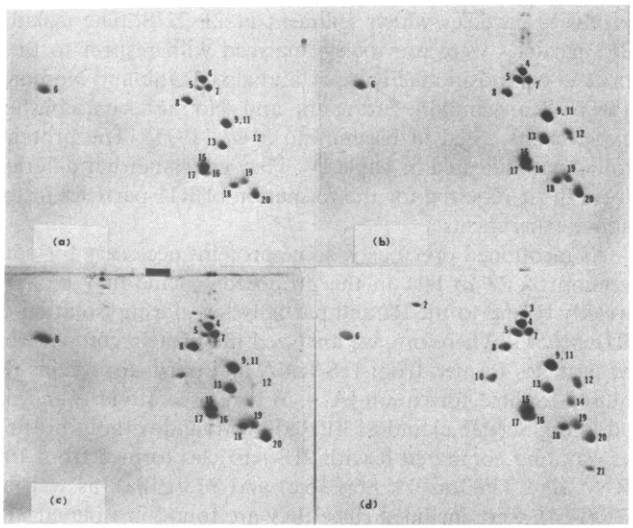

FIGURE 5: Two-dimensional electrophoresis of 30S proteins from RI, RI*, and ΣSi particles. (a) RI, particles prepared from a mixture of 16S RNA and purified proteins A + B + C (and S6(P3b,c) and S13(P10a)) (*cf.* Table I) incubated at 30° for 5 min; (b) RI*, particles prepared in the same way as RI, but incubated at 40° for 30 min; (c) RI-TP30, particles prepared from a mixture of 16S RNA and TP30 (unfractionated total 30S proteins) incubated at 30° for 5 min; (d) ΣSi, particles prepared from a mixture of 16S RNA and all of the purified proteins (A + B + C + D + E in Table I) incubated at 40° for 30 min. Particles were prepared as described in the text. The total protein applied to each gel was extracted by RNase digestion (see Materials and Methods) from the following A_{260} units of particles: (a) RI, 28; (b) RI*, 34; (c) RI-TP30, 30; (d) ΣSi, 50.

TABLE IV: Comparison of Proteins Required for RI* Formation with Those Found in the Isolated *in Vitro* and *in Vivo* 21S Particles.

Proteins	Required for RI* Formation[a]	*In Vitro* 21S Particles[b]	*In Vivo* 21S Particles[c]
S4(P4a)	++	++	+
S8(P4b)	++	++	++
S7(P5)	++	++	+
S16(P9a)	++	++	+
S19(P13)	++	+	+
S17(P9b)	+	++	+
S15(P10b)	+	++	++
S5(P4)	±	−	−
S11(P7)	±	(++)[d]	−
S9(P8)	±	(++)[d]	±
S12(P10)	±	−	−
S18(P12)	±	++	±
S20(P14)	−	++	+
S13(P10a)	−	++	+
S6(P3b,c)	−	++	+
S1(P1)	−	−	−
S2(P2)	−	−	−
S3(P3)	−	−	−
S10(P6)	−	−	−
S14(P11)	−	−	−
S21(P15)	−	−	−

[a] Extent to which each protein is required for the first step in the two-step reconstitution process (see the text): ++, strongly required; +, moderately required; ±, weakly required; −, not required. [b] Protein composition of the isolated RI particles (*in vitro* 21S particles): ++, present; +, present in reduced amounts; −, absent or almost absent (Erdmann, Kaltschmidt, and Nomura, unpublished experiments; Traub and Nomura, 1969a; and this paper). [c] Protein composition of 21S particles accumulated by some cold-sensitive mutants: ++, present in amounts comparable to those in 30S subunits; +, present in reduced amounts; ±, found only in some preparations; −, not detected (Nashimoto *et al.*, 1971). [d] Both S11(P7) and S9(P8) are probably present, but because of poor resolution of these two proteins in the polyacrylamide gel electrophoresis, we cannot make a definite conclusion as to which one (or whether both) is present.

fractions containing the particles were pooled, and their protein composition was analyzed by two-dimensional polyacrylamide electrophoresis.

The results (Figure 5a) show that some proteins, S5(P4) and S12(P10), are present in RI particles (prepared by incubation at 30°, 5 min) only in small amounts. S19(P13) may also be somewhat reduced. The RI* particles formed by heating at 40°, on the other hand, contain all of the proteins added (Figure 5b). It is clear that some proteins are necessary for the formation of RI* particles, but do not bind strongly at lower temperatures. On the other hand, certain other proteins, S6(P3b,c), S13(P10a), and S20(P14), bind to the particles in the reconstitution mixture without heating (Figure 5a and c and Table IV), even though they are not required for the formation of the RI* particles. Two-dimensional electrophoretic analysis of proteins from RI and RI* particles which were prepared in the same way, except for the omission of both

S6(P3b,c) and S13(P10a), gave similar results, except that S18(P12) was present only in very small amounts (data not shown). This is consistent with the previous finding that S6(P3b,c) stimulates the binding of S18(P12) (Mizushima and Nomura, 1970).

21S particles made from 16S RNA and TP30 (Figure 5c) are essentially identical with those made from purified proteins, except for small amounts of other 30S proteins, presumably due to some reconstitution of 30S particles during incubation at 30° for 5 min. The short incubation at 30° may also account for the relatively larger amount of S19(P13) found in RI particles (Figure 5a and c) than was found previously (Traub and Nomura, 1969a; Kaltschmidt, Erdmann, and Nomura, unpublished experiments, cited in Nashimoto *et al.*, 1971).

The particles formed from 16S RNA and a mixture of the purified proteins (A + B + C), as described above, were also analyzed for their sedimentation coefficients in buffer II (Figure 6). It was found that unheated particles show a fairly homogeneous peak sedimenting at about 21–22 S (Figure 6a). The RI* particle obtained after heating at 40° sediments at about 25–26 S (Figure 6b). Addition of the remaining proteins to the RI* particle results in formation of a particle which sediments close to 30S (Figure 6c). The significant difference in sedimentation coefficients indicates that the RI* particles obtained as described have a more compact structure than the particles formed from the same incubation mixture without heating. Sedimentation analysis of these particles in the reconstitution buffer (higher Mg^{2+} and KCl concentrations than buffer II) gave somewhat different results. As mentioned before, particles produced without heating form aggregates which sediment in a broad peak at 23–36 S. Heating for a short time at low temperature (30° for 10 min) appears to reduce the aggregation, giving a fairly homogeneous particle sedimenting at about 25 S. Heating the incubation mixture at 40° for 30 min results in particles sedimenting at 28 S, and addition of the remaining proteins results in particles sedimenting at 30 S (data not shown).

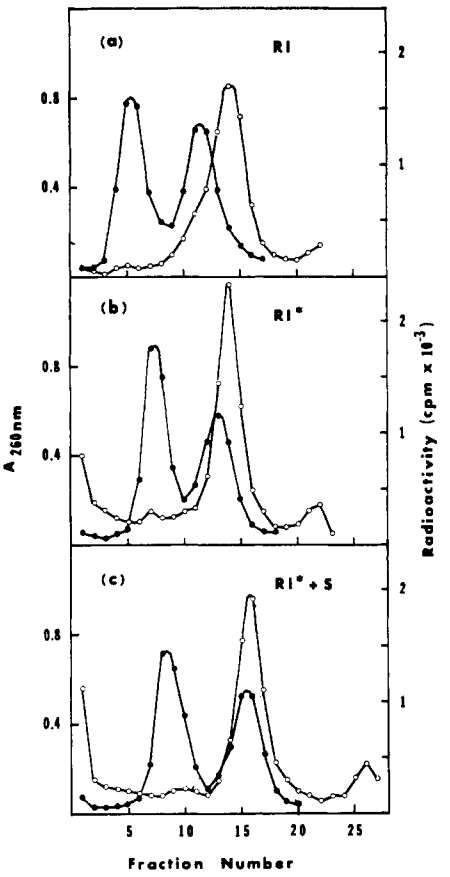

FIGURE 6: Sedimentation analysis of RI, RI*, and RI* + S particles from purified proteins. Particles were prepared as shown in Figure 2 by mixing 16S RNA with proteins A + B + C + S6(P3b,c) + S13(P10a) (*cf.* Table I) at (a) 0° for 30 min (RI); (b) 40° for 30 min (RI*); or (c) 40° for 30 min, cooling, and adding the remaining proteins and incubating at 30° for 10 min (RI* + S). Sedimentation was in 5–20% sucrose in buffer II at 33,500 rpm for 4 hr in a Spinco SW 39 rotor. Fractions were collected from the bottom of the tube and analyzed for absorbance at 260 nm (○) and for radioactivity. [^{14}C]Uracil-labeled MRE600 ribosomes were used as markers (●).

Discussion

The experiments described in the present paper support the mechanism of the *in vitro* assembly of 30S subunits proposed previously (Traub and Nomura, 1969a; eq 1). The reconstitution of 30S subunits from 16S RNA and purified proteins can be performed in two steps. The first step is to incubate 16S RNA and about 12 purified proteins (*cf.* Table IV) at 40° to form an intermediate particle which has no activity. This reaction is slow and requires incubation at high temperature (40°). The second step is addition of the remaining proteins (about nine); it proceeds rapidly at lower temperatures, forming functionally active 30S subunits. As shown in the previous paper (Traub and Nomura, 1969a), the overall reaction follows first-order kinetics, and therefore, the rate-determining step must be a unimolecular reaction. We now tentatively conclude that the *in vitro* reaction proceeds as follows (eq 2–5). In addition to the proteins we have found to be required for the formation of RI* particles (Table IV), we have included S13(P10a), S20(P14), and S6(P3b,c) as proteins participating in the first step because these proteins are found in the 21S particles isolated from the reconstitution mixture without heating. The proposed RI particles which undergo the rate-determining unimolecular reaction (reaction 3) may contain all of those proteins described in reaction 2, but are assumed to be unstable and lose some proteins [especially S5(P4), S12(P10), and possibly S19(P13)] during isolation (reaction 5). Some discrepancy between our analytical data on the *in vitro* 21S particles (Traub and Nomura, 1969a; present paper) and those of Homann and Nierhaus (1971) could be explained by differences in the conditions used for isolation and purification of the *in vitro* 21S particles.

$$16S\ RNA + \begin{bmatrix} S4, S8, S7, S15, S13, S20 \\ S16, S17, S9, S19, S5, S18 \\ S6, S11, S12 \end{bmatrix} \longrightarrow RI\ particles \quad (2)$$

$$RI\ particles \xrightarrow{heating} RI^*\ particles \quad (3)$$

$$RI^*\ particles + \begin{bmatrix} S14, S10, S3 \\ S21, S2, (S1) \end{bmatrix} \longrightarrow 30S\ particles \quad (4)$$

$$RI\ particles \rightleftharpoons 21S\ particles + [S12, S5, S19] \quad (5)$$

Because of the presumed instability of RI particles, it is difficult to study reaction 3 as a separate reaction. However, the results of the present studies strongly support the conclusion that this is the rate-determining step, reflecting the kinetics of the overall assembly reaction studied previously. The increase in S value of the particles after heating in the experiments shown in Figure 6 indicates a large configurational change in the particle during reaction 3.

Construction of the assembly map in our previous studies (Mizushima and Nomura, 1970; other experiments cited in Nashimoto *et al.*, 1971; see Figure 7) was done using methods

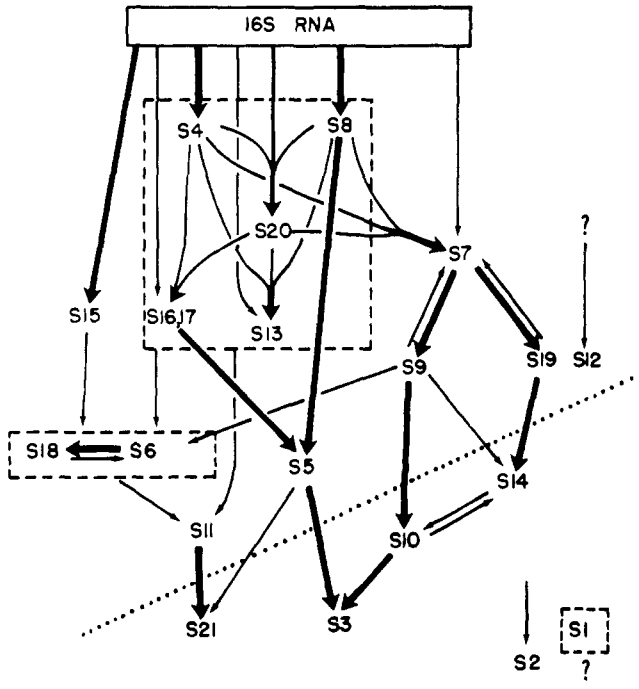

FIGURE 7: Assembly map of the 30S subunit (Mizushima and Nomura, 1970; Nomura, 1970). An arrow from one protein to a second indicates that the binding of the second depends upon the presence of the first. Darker arrows indicate stronger dependence. Proteins connected by arrows to 16S RNA bind to it in the absence of any other protein. Proteins above the dotted line are either those required for RI* formation or are present in the isolated 21S particles. Incorporation of the proteins below the dotted lines into the reconstituted particles is dependent on the presumed conformational change RI → RI*. [In previous studies pertaining to construction of the assembly map, S16 and S17 were used as a mixture (Mizushima and Nomura, 1970). S17, but not S16, binds directly to 16S RNA, although the specificity of this binding has not been proved (Schaup *et al.*, 1970; Ballou, B., Mizushima, S., and Nomura, M., unpublished experiments). As a mixture, both S17 and S16 bind to 16S RNA (Mizushima, S., Kaltschmidt, E., and Nomura, M., unpublished experiments).]

quite different from the present studies. All of the "RI proteins" identified in the present experiments are in the early part of the assembly map. A dotted line on the map as shown in Figure 7 indicates the rate-determining step inferred from the present experiments. Again, it should be noted that S20(P14), S13(P10a), and S6(P3b,c) are included in the RI particle, but do not play any important role in the rate-limiting reaction. It does appear clear that incorporation of other proteins (below the dotted line) into the reconstituted 30S particles is dependent on the presumed conformational change of the intermediate particles.

In the present experiments, it has been found that several proteins have only a moderate or weak influence on the formation of RI* particles (Table IV). It is probable that there are several different routes for assembly of 30S ribosomal subunits. Differences in free energy of activation among these several alternative routes may be rather small. Supporting this general conclusion is the striking fact that 30S-like particles with nearly full activity are slowly produced even in the absence of S16(P9a) and S17(P9b), both of which play major roles in the assembly process (Held, N., and Nomura, M., manuscript in preparation); see also Nomura, 1973). It has not been determined whether such assembly "flexibility" exists *in vivo*.

In vivo, certain cold-sensitive, ribosome assembly-defective mutants accumulate particles which sediment at 21 S and are thought to be a precursor to the 30S subunits or related to them. The protein composition of the purified 21S particles was analyzed (Nashimoto *et al.*, 1971; see Table IV), and found to be similar to that of the *in vitro* 21S particles. It is quite likely that the proteins required for the formation of RI* particles *in vitro* also participate in a rate-limiting step *in vivo*, and that particles similar to the *in vitro* RI particles postulated in this paper accumulate in the mutants at lower temperatures. However, particles accumulated *in vivo* may be unstable and lose some proteins during isolation. This could account for the fact that the isolated *in vivo* 21S particles contain only small amounts of S9(P8) and S18(P12), and lack S5(P4), S11(P7), and S12(P10) more or less entirely. Small differences in the protein composition between the *in vivo* and the *in vitro* 21S particles could also be explained by differences in the RNAs contained in these particles or in conditions encountered during isolation of the particles.

S5(P4) has a moderate role in the formation of RI* particles (Table II). This result may provide an explanation for the accumulation of 21S particles at lower temperatures by some spectinomycin-resistant mutants which have a mutational alteration in S5(P4) (Nashimoto *et al.*, 1971). Thus, the presence of the altered S5(P4) in the intermediate particles may interfere with the temperature-dependent, rate-determining step analogous to that in the *in vitro* reaction (reaction 3); the interference may be deterimental to cells at lower temperatures but not at higher temperatures. Other possibilities have been discussed previously (Nashimoto *et al.*, 1971).

Finally, it should be noted that particles (RI* particles) produced by incubating 16S RNA and a mixture of purified RI proteins at 40° sediment at 25–26 S in buffer II and are distinct from both 21S particles and the complete 30S subunits (Figure 6). Although the conversion of RI* particles to 30S subunits is reasonably fast at lower temperatures relative to the formation of RI* particles *in vitro*, it may still become a rate-determining step under certain conditions. It is well known that particles sedimenting at about 26 S (in ionic conditions similar to buffer II) can be detected by pulse labeling normal cells (Mangiarotti *et al.*, 1968; see also reviews, Osawa, 1968; Nomura, 1970). There are also some cold-sensitive mutants which accumulate 26S particles at 20° without accumulating 21S particles (Nashimoto and Nomura, 1970). These *in vivo* 26S particles have not been characterized with respect to their protein composition. It should be informative to analyze their protein composition and compare it to the RI* particles studied in the present paper.

Acknowledgment

We thank Mrs. C. Nelson for her excellent technical assistance and Dr. L. Kahan for his critical reading of the manuscript and valuable discussions throughout the course of this work.

References

Dzionara, M., Kaltschmidt, E., and Wittmann, H. G. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1909.

Gesteland, R. F. (1966), *J. Mol. Biol. 16*, 67.

Held, W., Mizushima, S., and Nomura, M. (1973), *J. Biol. Chem.* (in press).

Higo, K., Held, W., Kahan, L., and Nomura, M. (1973), *Proc. Nat. Acad. Sci. U. S. 70*, 944.

Homann, H. E., and Nierhaus, K. H. (1971), *Eur. J. Biochem. 20*, 249.

Kaltschmidt, E., and Wittmann, H. G. (1970), *Anal. Biochem. 36*, 401.
Lowry, O. H., Rosebrough, N. J., Farr, L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Mangiarotti, G., Apirion, D., Schlessinger, D., and Silengo, L. (1968), *Biochemistry 7*, 456.
Mizushima, S., and Nomura, M. (1970), *Nature (London) 226*, 1214.
Nashimoto, H., Held, W., Kaltschmidt, E., and Nomura, M. (1971), *J. Mol. Biol. 62*, 121.
Nashimoto, H., and Nomura, M. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1440.
Nomura, M. (1970), *Bacteriol. Rev. 34*, 228.
Nomura, M. (1973), *Science 179*, 864.
Nomura, M., Mizushima, S., Ozaki, M., Traub, P., and Lowry, C. V. (1969), *Cold Spring Harbor Symp. Quant. Biol. 34*, 49.
Osawa, S. (1968), *Annu. Rev. Biochem. 37*, 109.
Schaup, H. W., Green, M., and Kurland, C. G. (1970), *Mol. Gen. Genet. 109*, 193.
Schaup, H. W., Green, M., and Kurland, C. G. (1971), *Mol. Gen. Genet. 112*, 1.
Stanley, W. M., and Bock, R. M. (1965), *Biochemistry 4*, 1302.
Traub, P., Mizushima, S., Lowry, C. V., and Nomura, M. (1971), *Methods Enzymol. 20C*, 391.
Traub, P., and Nomura, M. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 577.
Traub, P., and Nomura, M. (1969a), *J. Mol. Biol. 40*, 391.
Traub, P., and Nomura, M. (1969b), *Cold Spring Harbor Symp. Quant. Biol. 34*, 63.
Wittmann, H. G., Stöffler, G., Hindennach, I., Kurland, C. G., Linkover, T., Randall-Hazelbauer, L., Birge, E. A., Nomura, M., Kaltschmidt, E., Mizushima, S., Traut, R. R., and Bickle, T. A. (1971), *Mol. Gen. Genet. 111*, 327.

Topography of Mouse 2.5S Nerve Growth Factor. Reactivity of Tyrosine and Tryptophan†

William A. Frazier,‡ Ruth A. Hogue-Angeletti, Randolph Sherman, and Ralph A. Bradshaw*,§

ABSTRACT: The chemical reactivity and degree of solvent exposure of the two tyrosine and three tryptophan residues of mouse 2.5S nerve growth factor (NGF) have been investigated. Spectrophotometric titrations and reaction with tetranitromethane indicate that both tyrosines are solvent available to a limited extent and that neither is required for activity. While the two tyrosines appear equivalent, the tryptophans exhibit a spectrum of reactivity. The reaction of these residues with *N*-bromosuccinimide is a kinetically ordered process. The most rapidly reacting residue is also solvent available by the criterion of *N*-methylnicotinamide chloride titration and is not required for activity. Reaction of the intermediate residue destroys both biological and immunological activity, while oxidation of the third residue leads to dissociation of the 2.5S dimer. Reaction of native NGF with dimethyl-(2-hydroxy-5-nitrobenzyl)sulfonium bromide leads to modification of Trp-21 and Trp-99 and leaves Trp-76 unmodified. Modification of the rapidly oxidized residue with *N*-bromosuccinimide followed by reaction with dimethyl-(2-hydroxy-5-nitrobenzyl)sulfonium bromide in 6 M guanidine hydrochloride reveals the modification of Trp-99 and Trp-76, identifying Trp-21 as the solvent available residue. Computer protein modeling studies show that this chemical description of the topography of the tyrosine and tryptophan residues of NGF is in extremely good agreement with topography of the corresponding residues in the three-dimensional structure of insulin. Thus the observed similarities in the function and mechanism of action of NGF and insulin may result from regions of similar primary structure dictating related three-dimensional conformation.

Nerve growth factor (NGF)[1] has been shown to be similar in primary structure to insulin and proinsulin (Frazier *et al.*, 1972). This relationship, presumably the result of evolution from a common precursor, is supported by similarities in the function and mechanism of action of the two proteins. For example, both NGF and insulin may be described as positive pleiotypic activators (Hershko *et al.*, 1971; Frazier *et al.*, 1972) and both have been suggested to exert this effect through a receptor on the surface membrane of their respective target cells (Cuatrecasas, 1969; Bradshaw *et al.*, 1972). While insulin affects many different cell types, the pleiotypic effects of NGF are limited to the differentiation and maintenance of adrenergic neurons, primarily those of the sympathetic nervous system (Levi-Montalcini and Angeletti, 1968). In addition, the conservation of a disulfide bridge in NGF, whose half-cystines represent identical residues in the sequence alignment of NGF with proinsulin (Frazier *et al.*, 1972), suggest that the functional similarities of NGF and insulin may reflect regions of similar three-dimensional structures as well.

Since the three-dimensional structure of insulin has been

† From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Missouri 63110. *Received March 19, 1973.* Supported by a research grant from the U. S. Public Health Service NS 10229.

‡ U. S. Health Service predoctoral trainee.

§ U. S. Public Health Service Research Career Development Awardee AM 23968.

[1] Abbreviations used are: NGF, nerve growth factor; $C(NO_2)_4$, tetranitromethane; SucNBr, *N*-bromosuccinimide; NMN, *N*-methylnicotinamide chloride; $(HO)(NO_2)Bzl$, hydroxynitrobenzyl-; *N*-Ac-Trp-NH_2, *N*-acetyl-L-tryptophanamide.